THE CONTRIBUTIONS OF Cav2.1 ALTERNATIVE SPLICING AND CALCIUM-DEPENDENT MODULATION TO CONGENITAL MIGRAINE

by

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ABSTRACT

$Ca_v2.1$ calcium (Ca^{2+}) channels are expressed throughout the mammalian central nervous system where they mediate P/Q-type Ca^{2+} currents essential for neurotransmitter release at most fast synapses. In humans, naturally occurring mutations in the CACNA1A gene encoding $Ca_v2.1$ are associated with several severe congenital disorders including familial hemiplegic migraine type 1 (FHM-1).

Alternative splicing of the $Ca_v2.1$ transcript generates multiple functionally distinct channel variants with unique spatial and temporal expression patterns. Yet, whether different $Ca_v2.1$ splice variants have distinct responses to FHM-1 missense mutations that relate to the localized, episodic nature of the FHM-1 phenotype has not been explored. Using recombinant $Ca_v2.1$ channels, we systematically compared the biophysical effects of three FHM-1 mutations in two prevalent $Ca_v2.1$ splice variants. All three FHM-1 mutations caused differential effects on voltage-dependent and kinetic properties when expressed in the short carboxyl terminus variant ($Ca_v2.1$ Δ47) compared to the long variant ($Ca_v2.1$ +47). Our findings provide important insight concerning the role of $Ca_v2.1$ alternative splicing and the pathophysiology of FHM-1.

Ca^{2+}-dependent facilitation (CDF) of $Ca_v2.1$ channels is a powerful means of channel control proposed to play a role in short-term facilitation of synaptic release during repetitive action potentials (APs). However, empirical evidence to support CDF of $Ca_v2.1$ as a relevant mechanism of synaptic facilitation in the CNS is limited. As such, short-term facilitation of synaptic release is generally attributed to enhanced vesicle release due to residual Ca^{2+} binding to sensor proteins that directly mediate vesicle fusion and transmitter release. However, we found that two FHM-1 mutations occluded CDF of $Ca_v2.1$ in both recombinant and native systems and cause a corresponding attenuation in short-term synaptic facilitation at the cerebellar parallel fibre to Purkinje synapse. This is the first evidence that presynaptic Ca^{2+} at this fast central synapse also enhances Ca^{2+} influx through $Ca_v2.1$ by means of CDF and acts as an additional required mechanism for short-term plasticity. Thus, the data supports the notion that CDF of $Ca_v2.1$ underlies key aspects of short-term plasticity in the CNS and provides the first evidence that FHM-1 mutations directly affect $Ca_v2.1$ CDF.

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LIST OF ABBREVIATIONS

Ca^{2+}: calcium ions

Na^{+}: sodium ions

APs: action potentials

Sr^{2+}: strontium ions

Ba^{2+}: barium ions

VGCC: voltage-gated Ca^{2+} channel

LVA: low-voltage-activated

HVA: high-voltage-activated

DHPs: dihydropyridines

N: asparagine

P: proline

K^{+}: potassium

pS: picosiemens

EC: excitation-contraction

SA: sinoatrial

AV: atrioventricular

SNc: substantia nigra pars compacta

NMJ: neuromuscular junction

CNS: central nervous system

PNS: peripheral nervous system

SNARE: soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors

VD: voltage-dependent

VI: voltage-independent

KO: knock-out

mRNA: messenger RNA

C-terminus: carboxyl terminus

pre-mRNA: precursor mRNA

N-terminus: amino terminus

EA2: Episodic Ataxia type 2

SCA6: Spinocerebellar Ataxia type 6

FHM-1: Familial Hemiplegic Migraine Type 1

LEMS: Lambert-Eaton Myasthenic Syndrome

tg: Tottering

tg^{la}: Leaner

tg^{rol}: Rolling Nagoya

HEK: human embryonic kidney

SCLC: small-cell lung carcinoma

CSD: cortical spreading depression

H^+: hydrogen ions

NO: nitric oxide

TNC: trigeminal nucleus caudalis

VPM: ventral posterior medial

PAG: periaqueductal gray region

CaM: calmodulin

apoCaM: Ca^{2+} free form of the CaM molecule

IQ: isolucine-glutamine

CBD: CaM binding domain

CDF: Ca^{2+}-dependent facilitation

CDI: Ca^{2+}-dependent inactivation

NSCaTE: N-terminal spatial Ca^{2+} transforming element

P_o: open-channel probability

APWs: action potential waveforms

CaS: Ca^{2+} sensors

SCG: superior cervical ganglion

EPSPs: excitatory post-synaptic potentials

PF: parallel fibre

PC: Purkinje cell

MFs: mossy fibers

GCs: granule cells

DCN: deep cerebellar nuclei

CFs: climbing fibres

LTP: long-term potentiation

LTD: long-term depression

EPSCs: excitatory post-synaptic currents

EGTA-AM: ethylene glycol tetraacetic acid bound to acetoxymethyl ester

τ_{act}: kinetics of activation

τ_{inact}: kinetics of inactivation

PPF: paired-pulse facilitation

PPR: paired-pulse ratio

fEPSPs: field excitatory post-synaptic potentials

nM: nanomolar

μM: micromolar

mM: millimolar

mV: millivolt

μs: microseconds

ms: milliseconds

s: seconds

mg: milligrams

μg: micrograms

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DEDICATION

To Jesus,

my beautiful wife Ronee,

and my amazing children Katelynn and Jacob.

CO-AUTHORSHIP STATEMENT

I designed all experiments and analyzed all experimental results with input and feedback from other researchers. I performed all experiments except for the two-photon microscopy experiments in chapter 3 which were performed by Ravi Rungta, and a few whole cell recordings of recombinant $Ca_v2.1$ channels in chapters 2 and 3 were performed by Esperanza Garcia and RT-PCR reactions in chapter 2 were performed by Laurence David. The FHM-1 knock-in mice used in chapter 3 were kindly provided by Dr. Arn van den Maagdenberg. I wrote the entire manuscripts for chapters 2 and 3, with subsequent editing from Terry Snutch and other authors. I also wrote the entire review article shown in Appendix 1 excluding the introduction section, with subsequent editing by Terry Snutch. Figures 1.6 and 4.1 in this thesis were created by Esperanza Garcia.

1 INTRODUCTION

1.1 Overview of voltage-gated calcium channels

1.1.1 Calcium channel discovery

For some North Americans today, the year 1953 holds relevance in the inauguration of such things as Ian Fleming's first James Bond novel Casino Royale, the release of Walt Disney's Peter Pan, Hugh Hefner's first issue of Playboy Magazine or perhaps the first successful climb to the summit of Mount Everest by Edmund Hillary and Tenzing Norgay. Others may remember the significant political moments of 1953, such as the stroke and death of the Soviet Communist leader Joseph Stalin, the end of the Korean War, the coronation of Queen Elizabeth II, or the dawn of the Cold War following the announcement by both the United States and Russia that they had developed hydrogen bombs. Scientists, on the other hand, might be reminded of such epic discoveries as Jonas Salk's polio vaccine (Salk, 1953), Eugene Aserinsky and Nathaniel Kleitman's discovery of REM sleep (Aserinsky and Kleitman, 1953), or possibly the monumental publication of the double helix structure of DNA by Francis Crick and James Watson (Watson and Crick, 1953). Yet for a small group of scientists, 1953 holds its significance in a classical discovery that would forever change research in the field of electrically excitable cells.

In an article published in the Journal of Physiology in 1953, Paul Fatt and Bernard Katz made the unexpected finding that electrical responses persist in crustacean muscle fibres in sodium (Na^+)-free media (Fatt and Katz, 1953). This was an unexpected because up to that point the "Na^+ theory" of electrical excitability of cells had prevailed. The Na^+ theory stated that action potentials (APs) arose as a result of regenerative processes depending on the permeability of cell surface membranes to Na^+ (Hodgkin and Huxley, 1952). Fatt and Katz were therefore cautious in concluding in their paper that "the mechanism of the action potential, and the species of ions involved in the movement of charge across the membrane, remain a puzzling problem…", and in addition to other potential explanations, suggested "…influx of calcium or magnesium, or outflux of some internal anion may be responsible for

transport of charge". Their conclusions lead to further exploration in 1957 by Alan Hodgkin and Richard Keynes which showed an important role of calcium (Ca^{2+}) in neurosecretion. A year later, Paul Fatt and Bernard Ginsborg showed that full-blown APs in crustacean muscle can be generated in the absence of Na^{+} leading them to confidently conclude that the movement of divalents such as strontium (Sr^{2+}), barium (Ba^{2+}) or even Ca^{2+} could support APs across cell membranes (Fatt and Ginsborg, 1958). Thus had begun a new pursuit for the identity of ions involved in the AP and neurosecretion (for full review of Ca^{2+} channel history see (Tsien and Barrett, 2005; Dolphin, 2006)).

1.1.2 There are multiple types of VGCCs in neurons

By the end of the 1950's, it was recognized that voltage-dependent Ca^{2+} conductances are an important component in excitable cells, and during the 1960's Susumu Hagiwara verified the presence of Ca^{2+} conductances in numerous invertebrate tissues. Reference to "a Ca^{2+} channel" in the singular form however, reveals the original perception that all measured Ca^{2+} currents were through a single type of voltage-gated Ca^{2+} channel (VGCC) and "…that Ca^{2+} channels everywhere are basically the same" (Brown et al., 1982). The view of a single VGCC persisted into the early 1980s, despite the fact that as early as the mid 1970's Hagiwara used voltage clamp experiments to show that in starfish eggs there are at least two distinct types of VGCCs which he termed type I and type II (Hagiwara et al., 1975). Subsequently, through efforts of a number of research groups including Llinas and Yarom, Carbone and Lux, Matteson and Armstrong and Bean, it was generally agreed that VGCCs are present in many systems and cell types and separable into two basic categories, the low voltage-activated (LVA) channels and high voltage-activated (HVA) channels (Carbone and Lux, 1984) depending upon the membrane potentials at which they first open. LVA VGCCs open in response to small changes from the resting membrane potential, whereas HVA channels are activated by stronger depolarizations. LVA channels have other distinguishing properties including a small single channel conductance (~ 5-12 picosiemens, pS), overlapping activation and inactivation ranges, rapid activation and inactivation kinetics, slow deactivation (closing) although a poorly defined pharmacology. Contrastingly, HVA VGCCs generally

possess larger conductances (~ 15-25 pS), variable inactivation kinetics, faster deactivation, and a well-defined pharmacology (reviewed in (Snutch et al., 2005)).

In 1985, classification of VGCCs was further refined. Richard Tsien's lab characterized properties of VGCCs in cell bodies of chick sensory neurons and found, based on single channel slope conductances and activation and inactivation properties, that VGCCs can be further classified into three categories. Tsien's lab termed the LVA channels T-type because they generate *t*iny unitary Ba^{2+} currents, give rise to a *t*ransient average current, have slow deactivation and resemble those previously described in heart. They divided the HVA channels into either L-type or N-type. L-type were named based on their *l*arge unitary conductance to Ba^{2+}, *l*ong-lasting Ba^{2+} current and resemblance to those previously described in the heart. The N-type were named based on the fact that they have similar characteristics to VGCCs previously described in *n*eurons and because they have an intermediate conductance to Ba^{2+} and activate at higher voltages and thus are *n*either L- nor T-type.

Identification and classification of VGCCs up to the mid 1980s had been primarily based on the biophysical properties of channels. Starting in the mid 1980's, however, Richard Tsien, Rodolfo Llinas, Michael Adams, Peter Hess and Bruce Bean began using VGCC pharmacological antagonists to further distinguish HVA VGCCs. The first major class of antagonists utilized were the 1,4-dihydropyridines (DHPs) which specifically and potently block L-type VGCCs (Hess et al., 1984). The peptide toxin ω-conotoxin-GVIA from marine snail venom was found to potently and specifically block N-type channels (McCleskey et al., 1987; Hirning et al., 1988; Plummer et al., 1989). Use of these toxins by Llinas and collaborators led to the discovery of a non-inactivating, DHP and ω-conotoxin-GVIA-insensitive Ca^{2+} current in the cell bodies of cerebellar Purkinje neurons that is instead blocked by extract from venom of the American funnel-web spider. They termed the newly identified channel P-type because of its dominance in *P*urkinje neurons (Llinas et al., 1989; Llinas et al., 1992). In 1992 the lab of Michael Adams determined that the peptide ω-agatoxin IVA isolated from the venom of the American funnel web spider is the active component that blocks P-type Ca^{2+} currents (Mintz et al., 1992b).

In 1995, armed with channel specific blockers nimodipine (a DHP), ω-conotoxin GVIA and ω-agatoxin IVA, Andy Randell from the Tsien lab set out to identify the contribution of L-type, N-type and P-type currents, respectively, in rat cerebellar granule cells (Randall and Tsien, 1995). In so doing, he identified two Ca^{2+} current types not previously identified in neurons but similar to VGCCs cloned from mammalian brain and characterized in exogenous mammalian expression systems by the labs of Shosaku Numa and Terry Snutch (cloning of VGCCs is discussed below). The first of these, although resembling HVA P-type currents described in Purkinje neurons, has reduced sensitivity to ω-agatoxin IVA and faster inactivation kinetics and was thus thought to be distinct from the P-type channel and was termed the Q-type VGCC. The second distinct current identified exhibited an activation threshold between HVA and LVA channels, a very rapid decay rate and insensitivity to high concentrations of nimodipine, ω-conotoxin GVIA and ω-agatoxin IVA. Since this current was the *r*esidual current remaining after the application of the three known VGCC blockers it was designated the R-type VGCCs (Randall and Tsien, 1995).

Thus, by the mid 1990's it had been demonstrated by measuring Ca^{2+} currents in different tissues that VGCCs are separable into T-type, L-type, N-type, P-type, Q-type and R-type based on biophysical and pharmacological properties. However, during the late 1980's and early 1990s, identification of the protein composition and DNA sequences of distinct VGCCs greatly enhanced understanding of channel structure and lead to new ways of channel classification and naming.

1.1.3 Current understanding of VGCC structure, classification and nomenclature

The protein composition of VGCCs was determined through work by Bill Catterall, Kevin Campbell, Franz Hoffman and others (Takahashi et al., 1987; Ellis et al., 1988; Ruth et al., 1989; Jay et al., 1990). The DHP sensitive (L-type) VGCC complex was first purified from rabbit skeletal muscle and it was determined that this HVA VGCC is a large multimeric protein complex containing an equal stoichiometric ratio of a α_1 pore-forming subunit and auxiliary subunits α_2, δ, β and perhaps γ (Takahashi et al., 1987) (Fig. 1.1). It is now clear that the α_1 pore-forming subunit of all VGCCs is the largest

(ranging between 190-250 kDa) and contains most of the molecular components necessary to produce a functional channel, including the pore which allows ion permeation and selectivity, voltage sensors which allow the channel to respond to membrane depolarization, activation gate and the intrinsic inactivation machinery (Hille, 2001). Functional differences in pharmacological sensitivities and basic biophysical properties such as ion conductance, threshold of activation and kinetics of inactivation between the VGCCs result primarily from variations in the primary structure of the channel. The auxiliary subunits have important roles in channel surface expression and modulation. The α_2 and δ subunits are encoded by a single gene whose polypeptide product is post-translationally cleaved to yield an extracellular α_2 product and a transmembrane δ product linked by disulfide bonds. The $\alpha_2\delta$ subunit enhances channel expression (De Jongh et al., 1990; Jay et al., 1991). β subunits bind the channel on the intracellular linker between domains II and III and enhance expression and modulate voltage and kinetic properties (Pragnell et al., 1994) (reviewed in (Dolphin, 2003a)). Although the transmembrane γ subunit was originally shown to associate with the skeletal-muscle L-type VGCC, its necessity and functional role for this and other VGCCs remains uncertain (Freise et al., 2000). More recently the γ subunit family has been shown rather to be a part of a group of transmembrane AMPA receptor regulatory proteins (Tomita et al., 2003) (for full review of Ca_v auxiliary subunits and their modulatory properties see (Dolphin, 2009)).

Purification of LVA T-type channels has yet to be accomplished. However, from molecular cloning it is known that mammalian T-type channels share only 20 to 30% amino acid sequence homology with mammalian HVA channels and that T-type channels lack entire structural motifs conserved within the HVA channels such as a β auxiliary subunit binding site in the I-II linker and an EF hand Ca^{2+} binding motif in the carboxyl terminus (C-terminus). Thus, the association of typical HVA auxiliary subunits with native T-type channels is unlikely to have a significant role, although a minor role is possible (Dolphin et al., 1999; Dubel et al., 2004).

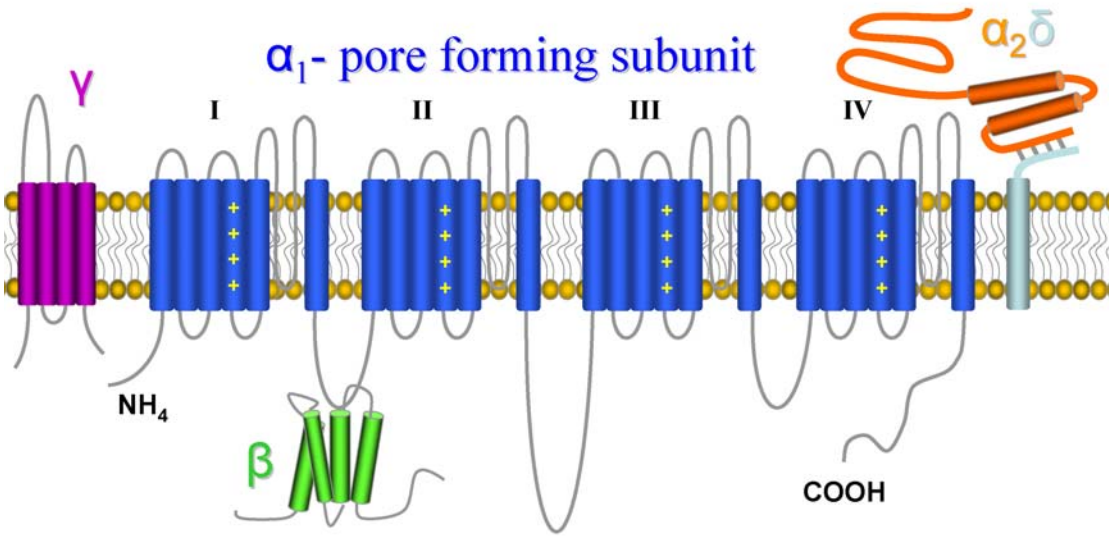


Figure 1.1: HVA VGCCs

HVA VGCCs are large multimeric proteins containing a main α_1 pore-forming subunit (blue) which includes four homologous domains (I-IV) each composed of six transmembrane segments and a pore forming loop between segments 5 and 6. The 4th transmembrane segments have four regularly-spaced, positively charged amino acids (yellow +) which form the voltage sensor. The N-terminus, C-terminus and inter-domain linkers are all on the cytoplasmic side of the membrane and are key sites for channel modulation. Auxiliary subunits include the α_2 and δ subunits (orange and light blue, respectively) (whose polypeptide product is post-translationally cleaved to yield an extracellular α_2 product and a transmembrane δ product linked by disulfide bonds), β subunits (green), which bind the α_1 pore-forming subunit on the intracellular linker between domains II and III and perhaps the transmembrane γ subunit (purple).

Knowledge of the individual subunits composing a VGCC opened the possibility for molecular cloning and reconstitution of recombinant VGCCs. The first VGCC cloned was the DHP-sensitive L-type channel from skeletal and heart muscle by the lab of Shosaku Numa (Tanabe et al., 1987) followed by the demonstration that the mammalian brain expresses multiple distinct VGCCs by Terry Snutch (Snutch et al., 1990). In 1991, a VGCC resembling the P-type channel was cloned from mammalian brain both by the labs of Snutch and Numa (Mori et al., 1991; Starr et al., 1991). Within a year the

Snutch lab also cloned the neuronal N-type (Dubel et al., 1992) and that which was later called the R-type channel (Soong et al., 1993). The last cloned VGCCs where the T-type channels by the lab of Ed Perez-Reyes (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999c) and the Snutch lab (McRory et al., 2001). Cloning made it possible to express VGCCs in recombinant systems and to more thoroughly characterize their basic properties than was previously possible in native systems.

In terms of VGCC discovery and classification, one of the most notable discoveries following the cloning of the channels was made by the Snutch lab in 1999. They showed that P-type and Q-type currents are actually conducted through the same channel, rather than through distinct channels as originally believed. The P- and Q-type currents result from alternative splicing of a single gene transcript in which the inclusion or exclusion of two residues (asparagine (N) and proline (P)) in the S3-S4 linker of domain IV determines the distinct pharmacological sensitivities, P-type (-NP; high ω-agatoxin IVA sensitivity) and Q-type (+NP; low ω-agatoxin IVA sensitivity) (Bourinet et al., 1999). From that point onward it has been generally agreed that P and Q-type currents are conducted through different splice variants of the same channel.

Molecular cloning allowed researchers to identify the genes that encode for VGCCs and powerfully shaped the classification of channels. Beyond the five categories of L-type, N-type, P/Q-type, R-type and T-type based on activation thresholds and pharmacological sensitivities of Ca^{2+} currents measured in native systems established by the end of the 1990s, it is now known that there are at least ten genes that encode distinct α_1 pore-forming subunits in the VGCC family in mammals. Sequence analysis of the ten genes has been used to create a systematic organization based on structural and evolutionary relationship. Figure 1.2 shows the phylogeny of the ten mammalian VGCC α_1 pore-forming subunits based on sequence similarities between conserved transmembrane and pore forming domains. The channels are classified into three distinct branches: 1) L-type, 2) P/Q-type, N-type and R-type and 3) T-type. The α_1 subunits are more than 70% identical within a family, but less than 40% between families. All three families have a single representative in the *C. elegans* genome which suggests their divergence was phylogenetically ancient (Ertel et al., 2000).

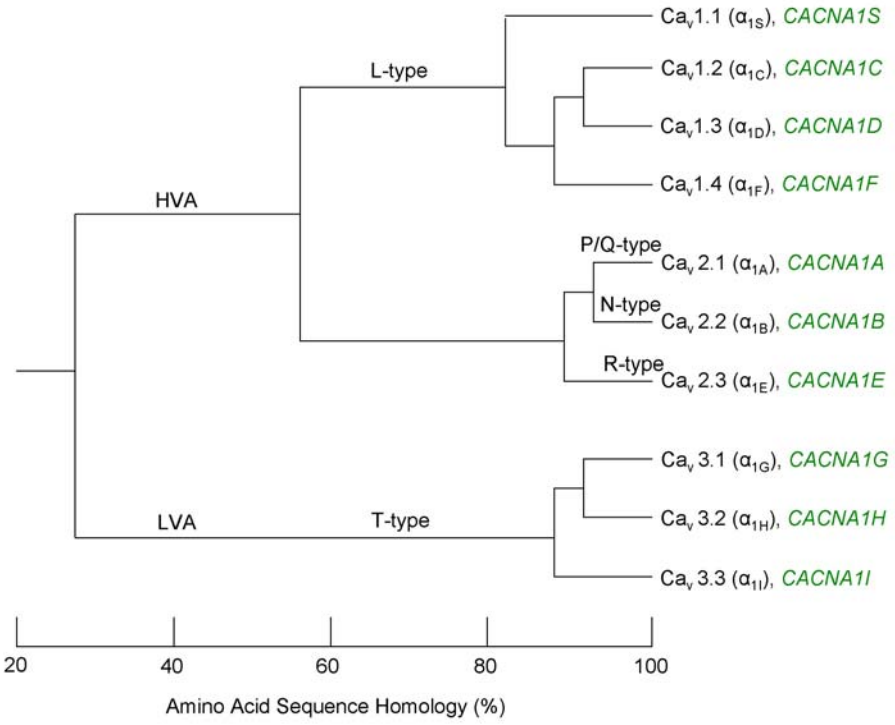


Figure 1.2: Phylogeny of VGCCs

Phylogenetic relationships between primary sequences of cloned mammalian VGCC α_1 pore-forming subunits, including the membrane spanning segments and pore-forming loops. Both the Ca_v and alphabetical (parentheses) nomenclatures are presented for each channel. Gene names for each channel are shown in green.

Identification of VGCCs by different labs over the years led to numerous ways of naming the channels. Initially an attempt was made to create a unifying naming system based on an alphabetical nomenclature, but this proved limited in its ability to reflect the structural homology within families and had the potential for overlap between names (Birnbaumer et al., 1994). Nonetheless, the alphabetical nomenclature is sometimes still used today and is reflected in the gene names. In 2000, a group of leaders in the field devised a systematic naming system of VGCCs based on the 10 α_1 pore-forming subunits. The naming system reflects the functional, structural and evolutionary relationships of the channels and is coordinated with nomenclature used for other voltage-gated channels such as potassium

(K^+) and Na^+ channels (Ertel et al., 2000). The naming system adopted at that time was approved by the Nomenclature Committee of the International Union of Pharmacology and is the preferred use today. The system uses the symbol for the primary permeating ion (Ca) with the main physiological regulator (voltage) indicated as a subscript (Ca_v). A numeric identifier between 1 and 3 is used to identify the α_1 pore-forming subunit gene family (Ca_v1: L-type; Ca_v2: P/Q-type, N-type and R-type; Ca_v3: T-type), and a second numeric identifier after the decimal point to identify the order of discovery of the subunit within the family (figure 1.2). It is now conventional to refer to each VGCC by the Ca_v nomenclature of the α_1 pore-forming subunit and to refer to the currents elicited by the channel as L-type, P/Q-type N-type, R-type or T-type (Catterall et al., 2005). This is the convention used in the remainder of this thesis.

The auxiliary subunits often associated with VGCCs in mammals have a similar naming system that reflects the gene family and the specific splice-variant. To date there have been four β subunit genes cloned (β_{1-4}), four $\alpha_2\delta$ subunits ($\alpha_2\delta_{1-4}$), and seven γ subunits (γ_{2-8}) (Ertel et al., 2000; Dolphin, 2009).

1.1.4 Expression, properties and modulation of VGCCs

Over the past 57 years, the biophysical and modulatory properties of all ten VGCCs have been extensively explored using both recombinant channels in various expression systems and endogenous channels in many *in vivo* preparations. The following section is a brief survey of the key properties and tissue expression of each of the ten VGCCs.

The Ca_v1 family:

Ca_v1 channels are the primary trigger for excitation-contraction (EC) coupling in cardiac, skeletal and smooth muscles (Bean, 1989). They are also found in most central and peripheral neurons where they in part control Ca^{2+}-dependent gene expression, as well as in endocrine cells and many types of non-excitable cells where they contribute to a variety of processes including exocytotic release. The Ca_v1 channels conduct L-type Ca^{2+} currents and are potently blocked by DHPs (Glossmann and Striessnig, 1988; Xu and Lipscombe, 2001; Koschak et al., 2003; McRory et al., 2004), have large single

channel conductance in Ba^{2+} (25 pS), activate between -60 and -10 mV and have a slow rate of inactivation (τ > 500 ms) (Hille, 2001).

$Ca_v1.1$ channels are important in striated muscle cells for coupling membrane depolarization to the release of Ca^{2+} from cytoplasmic stores and triggering EC coupling (Rios and Brum, 1987). In this instance, depolarizing changes in membrane potential cause a conformational change in $Ca_v1.1$ and without a requirement for direct Ca^{2+} influx induce an allosteric interaction with the sarcoplasmic reticulum (SR) ryanodine receptor (RyR1) ultimately inducing Ca^{2+} release and muscle contraction (Tanabe et al., 1990; Rios et al., 1992; Flucher and Franzini-Armstrong, 1996; Kugler et al., 2004). Related to this role as a voltage sensor for the RyR1, several loss-of-function missense mutations in the CACNA1S gene encoding for $Ca_v1.1$ have been implicated in two muscle disorders, hypokalemic periodic paralysis and malignant hyperthermia susceptibility (reviewed in (Adams and Snutch, 2007), Appendix 1).

The $Ca_v1.2$ channel is widely expressed in heart, brain (neurons, preferentially somatodendritic), smooth muscle, pituitary, gastrointestinal systems, lungs, immune system and testes (Ertel et al., 2000; Splawski et al., 2004). These channels have prominent roles in EC-coupling in cardiac and smooth muscle and AP propagation in sinoatrial (SA) and atrioventricular (AV) node, hormone secretion in endocrine cells and synaptic plasticity in neurons (Hell et al., 1993; Bokvist et al., 1995; Striessnig, 1999; Catterall, 2000; Schulla et al., 2003; Sinnegger-Brauns et al., 2004). Unlike EC coupling between $Ca_v1.1$ and RyR1 receptors in skeletal muscle, EC coupling in cardiac tissue requires Ca^{2+} influx through $Ca_v1.2$ in order to activate the RyR2 in the SR and release Ca^{2+} from internal stores to initiate muscle contraction (Meissner, 1994). Two gain-of-function missense mutations in the sixth transmembrane segment of domain I of the $Ca_v1.2$ channel have recently been associated with a severe arrhythmic disorder, Timothy syndrome (reviewed in (Adams and Snutch, 2007), Appendix 1).

$Ca_v1.3$ channels are the least sensitive to DHPs and are primarily found in photoreceptors, cochlear hair cells, endocrine cells (pancreatic cells, pituitary, adrenal chromaffin cells and pinealocytes), some in atrial muscle and SA and AV nodes in heart, vascular smooth muscle and neurons (preferentially

located on cell bodies and proximal dendrites) (Hell et al., 1993; Chik et al., 1997; Takimoto et al., 1997; Platzer et al., 2000; Garcia-Palomero et al., 2001; Mangoni et al., 2003; Michna et al., 2003). Of the HVA channels, $Ca_v1.3$ channels activate at relatively negative membrane potentials and are often referred to as "low-voltage-activated" L-type channels. The $Ca_v1.3$ channels play important physiological roles concerning neurotransmitter release in sensory cells and hormone secretion to affect mood behavior, the control of AV node conductance and cardiac rhythm, and also play a key role in the autonomous activity of adult substantia nigra pars compacta (SNc) dopaminergic neurons (Platzer et al., 2000; Mangoni et al., 2003; Sinnegger-Brauns et al., 2004; Chan et al., 2007).

$Ca_v1.4$ channels have important roles in neurotransmitter release in retinal photoreceptors and bipolar cells as well as in spinal cord (Tachibana et al., 1993; Nachman-Clewner et al., 1999). Photoreceptor neurotransmission is atypical in that photoreceptor cells do not fire action potentials, but rather have continuous graded membrane potentials. Also, photoreceptors are tonically depolarized in the absence of a light stimulus resulting in continuous glutamate release and subsequently hyperpolarize in response to light stimuli (Wu, 1994). Considering their critical role in vision, it is perhaps not surprising that both loss-of-function and gain-of-function mutations in the CACNA1F gene encoding $Ca_v1.4$ in humans are implicated in the vision related diseases incomplete X-linked congenital stationary night blindness and X-linked cone-rod dystrophy (reviewed in (Adams and Snutch, 2007), Appendix 1). The $Ca_v1.4$ channels are also expressed in immune cells and involved in T-lymphocyte activation (Bech-Hansen et al., 1998; Strom et al., 1998; Naylor et al., 2000; Firth et al., 2001; Ball et al., 2002; McRory et al., 2004).

The Ca_v2 family:

The Ca_v2 family of channels is the primary conduit of Ca^{2+} entry at presynaptic terminals and required for neurotransmitter release in both the central nervous system (CNS) and peripheral nervous system (PNS). Their single channel conductances in Ba^{2+} range between 10 and 20 pS, they can activate anywhere between -120 (mostly R-type) and -30 mV and they possess an intermediate inactivation rate (τ = 50-80 ms) (Catterall et al., 2005).

$Ca_v2.1$ channels conduct P/Q-type Ca^{2+} currents and are blocked by ω-agatoxin-IVA and ω-conotoxin MVIIC (Hillyard et al., 1992; Mintz et al., 1992a). $Ca_v2.1$ channels are ubiquitously expressed in neurons throughout the CNS and PNS (presynaptic terminals, dendrites and some cell bodies) as well as in heart, pancreas (β-cells) and pituitary. $Ca_v2.1$ channels play a primary role in neurotransmitter release in CNS and the neuromuscular junction (NMJ) and are known to contribute to excitation-secretion coupling in pancreas (Hillman et al., 1991; Mori et al., 1991; Starr et al., 1991; Mintz et al., 1992a; Ousley and Froehner, 1994; Randall and Tsien, 1995; Westenbroek et al., 1995; Day et al., 1997; Horvath et al., 1998; Ishikawa et al., 2005). Considering the central role of $Ca_v2.1$ channels in neurotransmission, it is perhaps not surprising that mutations in the CACNA1A gene encoding $Ca_v2.1$ are associated with several severe human disorders (discussed in a subsequent section).

$Ca_v2.2$ channels conduct N-type Ca^{2+} currents and are potently blocked by ω-conotoxin GVIA, ω-conotoxin MVIIA and MVIIC (McCleskey et al., 1987; Hillyard et al., 1992). They are expressed in neurons (presynaptic terminals, dendrites and cell bodies) and contribute to neurotransmitter release in CNS and sympathetic neurons, the sympathetic regulation of the circulatory system and play an important role in sensation and transmission of pain (Westenbroek et al., 1992; Dunlap et al., 1995; Ino et al., 2001; Mori et al., 2002; Beuckmann et al., 2003).

$Ca_v2.3$ channels conduct R-type Ca^{2+} currents, are resistant to block by ω-agatoxin-IVA, ω-conotoxin GVIA, ω-conotoxin MVIIA and MVIIC and under some conditions are blocked by SNX-482 (blocks recombinant channels, but is only partially effective at blocking native R-type currents) (Newcomb et al., 1998; Tottene et al., 2000). $Ca_v2.3$ channels are primarily expressed in neurons (dendrites, cell bodies and some presynaptic terminals), heart, testes and pituitary where they have roles in neurotransmitter release, repetitive firing, long-term potentiation and post-tetanic potentiation (Randall and Tsien, 1995; Tottene et al., 1996; Dietrich et al., 2003; Jing et al., 2005; Pereverzev et al., 2005).

The Ca_v3 family:

The Ca_v3 family of channels conducts T-type Ca^{2+} currents. These channels diverged early in VGCC evolution and differ significantly in primary sequence from HVA channels. Although there is

some evidence for an interaction of Ca_v3 α1-subunits with VGCC auxiliary subunits (Dolphin et al., 1999; Dubel et al., 2004; Lin et al., 2008), recombinant Ca_v3 channels display properties similar to those of native T-type currents in the absence of auxiliary subunits and it is not yet clear what roles auxiliary subunits contribute towards Ca_v3 functional properties.

Ca_v3 channels are blocked by the scorpion peptide kurtoxin from *Parabuthus transvaalicus* and $Ca_v3.1$ and 3.2 channels are blocked by the small organic antiepileptic ethosuximide (Chuang et al., 1998; Lee et al., 1999b; Gomora et al., 2001). Ca_v3 channels have variable sensitivities to nickel (Ni^{2+}), $Ca_v3.2$ is highly sensitive to Ni^{2+} with an IC_{50} of 12 μM compared to IC_{50}'s of 250 μM and 216 μM for $Ca_v3.1$ and $Ca_v3.3$, respectively (Lee et al., 1999b). Ca_v3 channels have relatively small single channel conductances (8-10pS), activate at membrane potentials between -90 and -60 mV and have fast inactivation time constants (τ=20-50 ms) (Hille, 2001). The acute voltage sensitivity of Ca_v3 channels plays a pivotal role in regulating cellular excitability and oscillatory behaviours.

$Ca_v3.1$ channels are predominantly expressed in neurons (soma and dendrites), heart (SA node), ovary and placenta, and have roles in burst firing associated with thalamic oscillations and cardiac pacemaking (Perez-Reyes et al., 1998; Craig et al., 1999; Talley et al., 1999; Monteil et al., 2000a; Perez-Reyes, 2003; Ono and Iijima, 2005). In the cerebellum, mGluR1-mediated potentiation of $Ca_v3.1$ T-type currents may promote synapse-specific Ca^{2+} signalling in response to bursts of excitatory inputs (Hildebrand et al., 2009).

$Ca_v3.2$ channels are widely expressed in the juvenile and adult hippocampus, cerebellum, pons/medulla, striatum, thalamus/hypothalamus, olfactory bulb, heart (SA node), and cortex (Talley et al., 1999; McRory et al., 2001; Perez-Reyes, 2003; McKay et al., 2006). They are also important for burst firing and oscillatory behavior and have key roles in smooth muscle contraction, proliferation, aldosterone secretion and cortisol secretion (Chuang et al., 1998; Cribbs et al., 1998; Lee et al., 1999b; Talley et al., 1999; Bohn et al., 2000; Gomora et al., 2001; Hansen et al., 2001; Schrier et al., 2001). In the periphery, $Ca_v3.2$ channels are found in the primary nociceptor pathway and have been shown to contribute both to acute and chronic nociceptive behaviours (reviewed in (Snutch and David, 2006)). In

the last few years, researchers have uncovered several point mutations in the CACNA1H gene encoding $Ca_v3.2$ in patients with idiopathic generalized epilepsy (IGE) and childhood absence epilepsy (CAE), some of which have gain-of-function effects on channel function (reviewed in (Adams and Snutch, 2007), Appendix 1).

$Ca_v3.3$ channels are expressed primarily in neurons and have key roles in thalamic oscillations (Perez-Reyes et al., 1998; Talley et al., 1999; Monteil et al., 2000b; Perez-Reyes, 2003). Whereas $Ca_v3.1$ and $Ca_v3.2$ have similar activation and inactivation kinetics, $Ca_v3.3$ activation and inactivation kinetics are much slower. The $Ca_v3.3$ channels also have faster deactivation kinetics as well as more hyperpolarized voltage dependence of activation and inactivation compared with the other two Ca_v3 channels (McRory et al., 2001). Gαq/11-coupled GPCRs exhibit a strong inhibitory effect on $Ca_v3.3$ T-type Ca^{2+} currents, but have either no effect on or increase $Ca_v3.1$ and $Ca_v3.2$ peak current amplitudes (Hildebrand et al., 2007).

1.2 The $Ca_v2.1$ VGCC

The CACNA1A gene in mammals has 47 exons and encodes the 2,200-2,400 amino acid pore-forming α_1 subunit ($Ca_v2.1$; α_{1A}). In exogenous systems, in order to recapitulate native P/Q-type currents $Ca_v2.1$ channels must be associated with both β and $\alpha_2\delta$ auxiliary subunits. Endogenous $Ca_v2.1$ channels are likely associated with any one of four β subunits (β_{1-4}) and any one of four $\alpha_2\delta$ subunits ($\alpha_2\delta_{1-4}$). The different β subunits enhance trafficking of channels to the plasma membrane and can modulate activation threshold and kinetics of inactivation with varying degrees of effectiveness (Stea et al., 1994; Walker and De Waard, 1998) (reviewed in (Arikkath and Campbell, 2003; Richards et al., 2004; Dolphin, 2009)). β subunits bind via their guanylate kinase-like domain to the α interaction domain within the I–II linker of $Ca_v2.1$ channels with different affinities (Pragnell et al., 1994; De Waard and Campbell, 1995). The $\alpha_2\delta$ subunits promote trafficking of $Ca_v2.1$ channels by an unknown mechanism. The $\alpha_2\delta$ subunit binds via a Von Willebrand factor-A domain (Canti et al., 2005; Davies et al., 2006) to one or more unidentified extracellular domains of the $Ca_v2.1$ channel. Which of the various auxiliary subunits are complexed with

endogenous $Ca_v2.1$ channels in a particular cell type likely depends on both the expression of the subunits and their affinity for $Ca_v2.1$.

$Ca_v2.1$ channels are generally concentrated to presynaptic terminals but are also detected on dendritic spines and shafts (Hillman et al., 1991; Westenbroek et al., 1995). $Ca_v2.1$ (and $Ca_v2.2$ and $Ca_v2.3$ to a lesser degree) channels have been shown to be necessary for the release of neurotransmitters from presynaptic terminals at most fast synapses in spinal cord and brain stem, cerebellum, hippocampus, calyx of Held and cortex (Hillyard et al., 1992; Turner et al., 1992; Takahashi and Momiyama, 1993; Turner et al., 1993; Castillo et al., 1994; Regehr and Mintz, 1994; Wheeler et al., 1994; Regehr and Atluri, 1995). Ca^{2+} influx through Ca_v2 channels initiates neurotransmitter release by triggering vesicle fusion with the plasma membrane by interacting with the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) protein complex composed of syntaxin, SNAP-25 and VAMP/synatobrevin (reviewed in (Catterall, 2000; Evans and Zamponi, 2006; Kisilevsky and Zamponi, 2008)). The function of the SNARE complex in vesicle fusion is regulated by numerous proteins including the Ca^{2+}-binding protein synaptotagmin which acts as a Ca^{2+} sensor. It is believed that the physical interaction of the Ca_v2 channel with the SNARE complex and synaptotagmin enables a tight structural association and functional coupling of Ca^{2+} entry with vesicle fusion (Catterall, 2000; Mochida et al., 2003b). $Ca_v2.1$ channels contain a specific synaptic protein-protein interaction region called the *synprint* site located within the intracellular loop linking domains II and III (Sheng et al., 1994). The *synprint* site of $Ca_v2.1$ interacts with several proteins in the SNARE complex and can either function to modulate target proteins or receive modulation from other proteins (Sheng et al., 1994; Charvin et al., 1997; Sheng et al., 1997; Wiser et al., 1997).

The regulation of $Ca_v2.1$ channels exerts a crucial influence on presynaptic Ca^{2+} influx and the precise tuning of neurotransmitter release. In addition to modulation by SNARE proteins, $Ca_v2.1$ channels are also acutely regulated in a variety of ways by effector molecules acting via protein phosphorylation through second messenger-activated kinase pathways (reviewed in (Catterall, 2000; Snutch et al., 2005; Evans and Zamponi, 2006)). For example, PKA increases $Ca_v2.1$ channel activity

indirectly by interfering with phosphatidylinositol-4,5-bisphosphate mediated regulation which normally produces voltage-dependent current inhibition (Fournier et al., 1993; Fukuda et al., 1996; Huang et al., 1998; Kaneko et al., 1998; Wu et al., 2002). Also, activation of certain G-protein coupled receptors is a well known means of potent and fast inhibition of both $Ca_v2.1$ and $Ca_v2.2$ channels affecting both voltage-dependent (VD) and voltage-independent (VI) mechanisms (Ikeda and Schofield, 1989; Lipscombe et al., 1989; Mintz and Bean, 1993; Kisilevsky and Zamponi, 2008). G-protein mediated VD inhibition is thought to involve a complex interplay between multiple cytoplasmic regions of $Ca_v2.1$ including the I-II linker and N-terminus (Dolphin, 2003b; Agler et al., 2005), and possibly the C-terminus, to enhance Gβγ binding affinity (Li et al., 2004). Furthermore, modulation of $Ca_v2.1$ by Ca^{2+}-calmodulin-dependent protein kinase II interacting with the C-terminus of the channel slows channel inactivation (Jiang et al., 2008). $Ca_v2.1$ channel activity can also be indirectly regulated through phosphorylation of auxiliary subunits (reviewed in (Dolphin, 2009)).

The ablation of P/Q-type currents by genetic deletion of $Ca_v2.1$ in mice ($Ca_v2.1$ knock-out; KO) results in numerous neurological dysfunctions including absence seizures characterized by 3-5 Hz cortical spike-wave discharges, ataxia and dystonia beginning around post-natal day 10 and progressing with age, and selective degeneration of the cerebellum (particularly the anterior vermis) in older mice. If unaided, $Ca_v2.1$ homozygous KO mice typically do not survive past weaning (Jun et al., 1999; Fletcher et al., 2001; Song et al., 2004; Todorov et al., 2006). Examination of pain-related behavioral responses of $Ca_v2.1$ KO mice also reveals that $Ca_v2.1$ plays a role in inhibiting pain signalling during non-injurious noxious thermal stimuli, although on the other hand is necessary for the conduction of pain signalling in inflammatory and neuropathic pain states (Luvisetto et al., 2006).

The $Ca_v2.1$ properties and characteristics described to this point have largely been general. However, there are many variants of $Ca_v2.1$ that arise as a result of alternative splicing of the CACNA1A transcript and that produce unique channels with distinct spatial and temporal expression patterns, biophysical properties and pharmacological sensitivities (Bourinet et al., 1999; Soong et al., 2002; Timmermann et al., 2002; Chaudhuri et al., 2004; Chang et al., 2007). Alternative splicing of $Ca_v2.1$ is a

critical means to achieve channel diversity relevant to a variety of functional roles. The following sections explore alternative splicing and its relevance to $Ca_v2.1$ physiology and pathophysiology.

1.3 Alternative splicing

1.3.1 Alternative splicing overview for VGCCs

In the early 1980s, researchers predicted that about 5% of genes in higher eukaryotes are alternatively spliced (Sharp, 1994). Sequencing of the human genome, however, revealed only approximately 32,000 genes which is far less than the previously predicted 150,000 (Pennisi, 2000). The disparity fueled a revolution in the minds of many scientists regarding biological complexity. It is now believed that the number of human messenger RNA (mRNA) forms is much higher than the number of genes and that alternative splicing plays a central role in the production of biological complexity. Recent estimates are that between 75 and 94% of all human genes produce primary transcripts which undergo alternative splicing to produce structurally and functionally distinct protein variants (Johnson et al., 2003; Wang et al., 2008).

Alternative splicing of VGCCs is a critical component of their functional diversity. As described, there have been ten genes identified encoding the $Ca_v\alpha_1$ subunits, yet this number is insufficient to account for the functional diversity amongst the native Ca^{2+} currents and their roles in Ca^{2+}-dependent signalling in different brain regions and cellular compartments. While there may only be ten Ca_v subunit genes, it is predicted that there are perhaps 1000 times as many splice variants, likely with their own unique functional properties and spatial and temporal expression patterns (Lipscombe et al., 2002; Vigues et al., 2002; Emerick et al., 2006; Gray et al., 2007).

It has been shown for a variety of VGCCs that alternative splicing occurs within key functional domains of the channels. For example, alternative splicing within the S3-S4 extracellular loops is known to occur in $Ca_v2.1$ (Bourinet et al., 1999), $Ca_v2.2$ (Lin et al., 1997; Stea et al., 1999) and Ca_v1 channels (Barry et al., 1995) and is thought to generally influence voltage sensitivity of the channel because of the

close proximity to the S4 voltage sensors which must move upon depolarization (Bezanilla, 2002). The intracellular loop connecting homologous domains II and III (II-III linker) in VGCCs is a key site of channel modulation, important in membrane targeting and in some channels links to downstream effector proteins required for neurotransmitter release (Catterall, 2000). Alternative splicing within the II-III linker has been reported for $Ca_v2.2$ (Pan and Lipscombe, 2000), $Ca_v2.1$ (Soong et al., 2002), $Ca_v2.3$ (Pereverzev et al., 2002) and Ca_v3 channels (Mittman et al., 1999a; Chemin et al., 2001). Splicing in the II-III linker in $Ca_v2.2$ and $Ca_v2.1$ channels significantly affects channel targeting to the plasma membrane, causes differential interaction with β subunits and alters current-voltage relationships (Scott et al., 1996; Pan and Lipscombe, 2000; Lipscombe et al., 2002; Rajapaksha et al., 2008). Another key region of alternative splicing is the C-terminus. The C-terminus makes up about one-third of VGCCs and is prominent in determining functional diversity. This region contains the sites for several regulatory elements such as the binding of Ca^{2+}, calmodulin and G-proteins (Catterall, 2000) and is also important in channel targeting to the plasma membrane (Maximov and Bezprozvanny, 2002). There are at least two C-terminus variants of different lengths for most VGCCs and the variants show distinct Ca^{2+}-dependent modulation, G-protein modulation, changes in the voltage-dependence, kinetics of inactivation and current amplitude (Hell et al., 1994; Zhuchenko et al., 1997; Bourinet et al., 1999; Mittman et al., 1999a; Mittman et al., 1999b; Krovetz et al., 2000; Lu et al., 2001; Sandoz et al., 2001; Murbartian et al., 2002; Pereverzev et al., 2002; Soong et al., 2002).

1.3.2 Mechanisms of alternative splicing

In the process of generating mature, stable mRNA that can be directly translated into protein, non-coding regions corresponding to introns must be removed from precursor mRNA (pre-mRNA) by the process of splicing. A spliceosome protein complex recognizes introns by signature dinucleotide sequences within intron/exon boundaries (Sharp and Burge, 1997; Wu and Krainer, 1999; Modrek and Lee, 2002; Singh, 2002). Splicing out sequences at any position in the pre-mRNA depends on the

combination of both the nucleotide sequences in key positions, as well as the spliceosome composition available within the cells (Grabowski and Black, 2001).

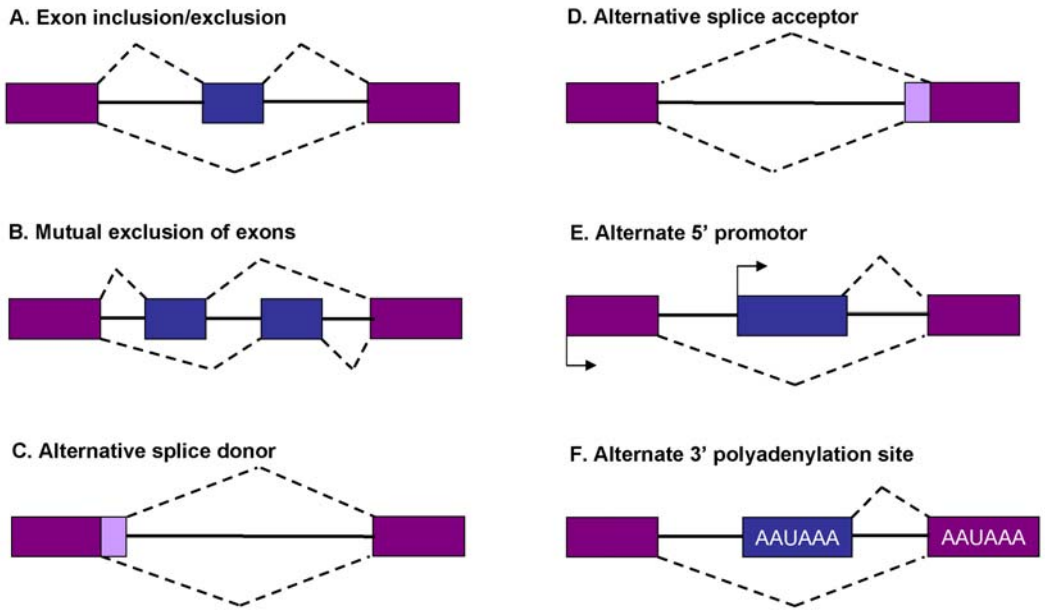


Figure 1.3: Alternative splicing in VGCCs

There are six types of alternative splicing that occurs for VGCC pre-mRNA transcripts, A-F. Exons are shown in dark purple, alternate exons in blue and segments of exons included or excluded as a result of alternative splice donor and acceptor sites are shown in light purple. Small bent arrows indicate promoter start sites.

There are six types of alternative splicing thus far identified for VGCCs (Figure 1.3). One described type is the inclusion or exclusion of an entire exon (Fig. 1.3A). There are constitutively expressed exons that are essential for basic protein function and structure, and alternatively expressed exons that generally modify protein function and fine-tune the protein for specific cellular tasks. The inclusion or exclusion of alternative exons in the final mRNA transcript is determined by a number of factors including tissue type, cell type, developmental stage, metabolic state of the cell and gender (Black, 1998; Grabowski, 1998; Xie and Black, 2001).

A second type of splicing observed for VGCCs is the mutual exclusion of a series of homologous exons whereby only one of the exons will be selected and included in the final mRNA transcript (Fig. 1.3B).

A third and fourth form of alternative splicing occur at alternative 5' donor and 3' acceptor slice sites. Splicing at an alternative intron sequence near the 5' intron end can cause elongation or shortening of the preceding exon (alternative splice donor) (Fig. 1.3C), and splicing at an alternative intron sequence near the 3' end can cause elongation or shortening of the following exon (alternative splice acceptor) (Fig. 1.3D).

The remaining forms of splicing involve the use of alternative 5' promoters (Fig. 1.3E) and 3' polyadenylation/cleavage sites (Fig. 1.3F), respectively. The use of alternative promoters may join additional exons to the 5' end of the protein product, whereas the composition and length of 3' untranslated regions can modify mRNA stability and alter targeting to specific regions of the cell (Modrek and Lee, 2002).

An additional, albeit less common, means of channel variation arises through RNA editing which may include nucleoside modifications such as cytosine to uracil and adenosine to inosine deaminations, or non-templated nucleotide additions and insertions (Smith et al., 1998; Tsunemi et al., 2002) (for full review of VGCC alternative splicing see (Lipscombe and Castiglioni, 2004)).

1.3.3 Alternative splicing of the $Ca_v2.1$ VGCC

Fitting with the diverse roles of $Ca_v2.1$ channels described in detail above, their functional properties can be fine-tuned on a short time scale by gating modulation through channel phosphorylation (Zamponi et al., 1997), G-protein interaction (Colecraft et al., 2001), and assembly with various auxiliary subunits (Stea et al., 1994; Dunlap et al., 1995; Patil et al., 1998). However, even as early as the first studies to isolate full-length $Ca_v2.1$ cDNA from mammals, it was already evident that there are multiple variants predicted to result from alternative splicing of the CACNA1A gene (Mori et al., 1991; Starr et

al., 1991). In these original studies, both groups independently identified multiple versions of the $Ca_v2.1$ channel with sequence diversity primarily in the intracellular loop between domains II and III and in the C-terminus. Since these original reports, at least seven loci of alternative splicing in the $Ca_v2.1$ channel have been identified in neuronal tissues from mammals, many of which have functionally distinct biophysical and modulatory properties (Fig. 1.4).

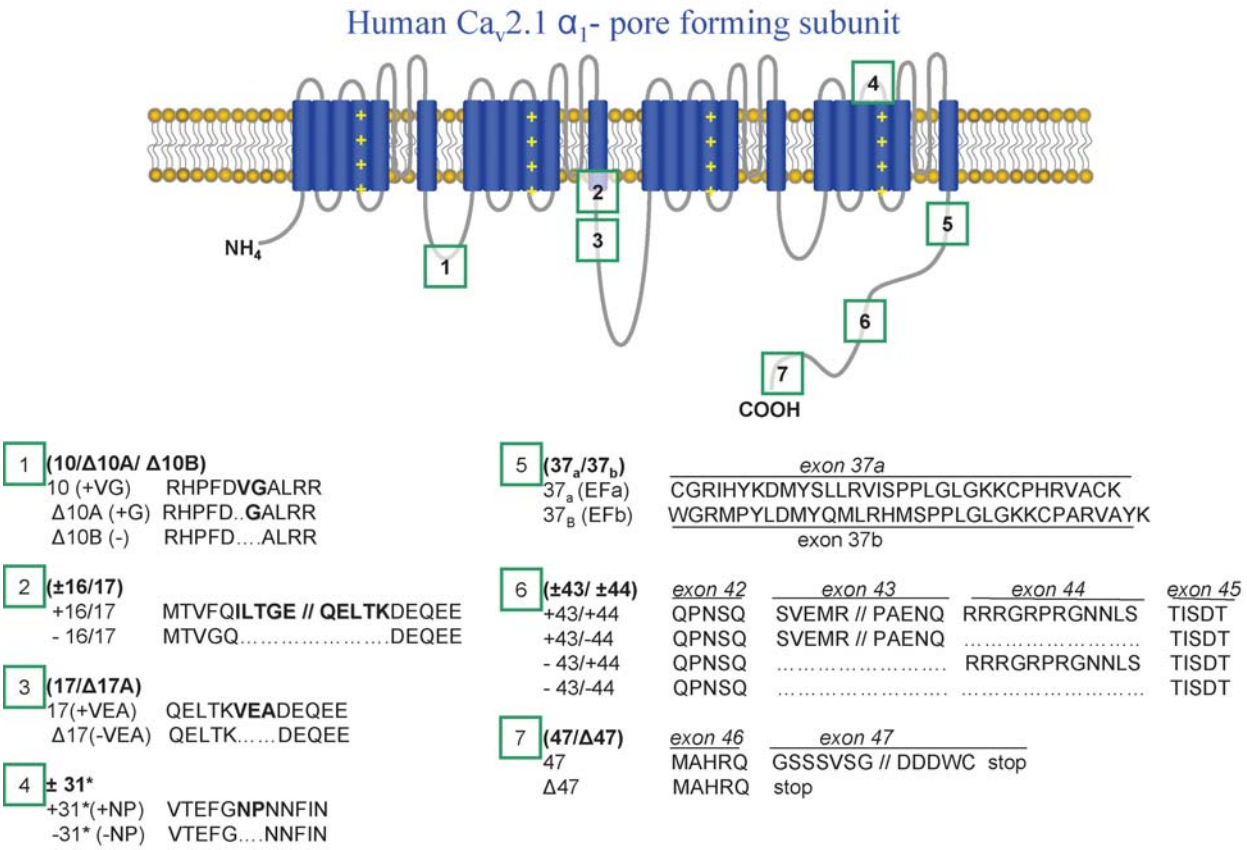


Figure 1.4: Human $Ca_v2.1$ splice variants

Seven known locations of alternative splicing in human $Ca_v2.1$ channels. Top, the location of each of the seven splice sites on the $Ca_v2.1$ channel are indicated by green squares. Bottom, all possible amino acid changes resulting from alternative splicing at the seven loci are shown. Bold lettering indicates amino acids involved in splicing. Dotted lines indicate where amino acids have been spliced out. Slanted lines indicate a break in the amino acid sequence. Adapted from (Soong et al., 2002)

Starting from the amino terminus (N-terminus), the first known region of alternative splicing occurs at the beginning of exon 10 within the intracellular loop connecting domains I-II (10/Δ10A/ Δ10B) (Fig. 1.4, #1) and has been identified in human (Soong et al., 2002) and rodent (Bourinet et al., 1999; Tsunemi et al., 2002; Kanumilli et al., 2006; Richards et al., 2007). There can be an insertion of a valine and glycine (+VG; 10), insertion of glycine alone (+G; Δ10A), or no insertion of either amino acid (- ; Δ10B). These alternative splice variants are a result of alternative splice acceptors within the 5' region of exon 10 (Fig. 1.3D), wherein the two insertions use a non-canonical GT/TG donor-acceptor site and the Δ10B variant results from conventional splicing using a GT/AG donor-acceptor site. In human

cerebellar RNA, the percentage of (10) was identified to be 17% with the Δ10A and Δ10B containing variants together making up the remaining 83% of transcripts (Soong et al., 2002). In rats and mice, both variants are present in cerebellum, although in single Purkinje neurons all $Ca_v2.1$ transcripts lacked the valine insertion (Tsunemi et al., 2002; Kanumilli et al., 2006; Richards et al., 2007). In the $Ca_v2.1$ rat homologue, the valine insertion (10) was shown to both enhance G-protein inhibition and up-regulate channel activity via PKC, as well as to decrease inactivation kinetics (Bourinet et al., 1999).

A second region of splicing occurs within the IIS6 region of the channel where exons 16 and 17 can either be included (+16/17) or excluded (-16/17) in the final mRNA product (Fig. 1.4, #2), identified in human brain tissue (Soong et al., 2002). Both exons are either included or excluded together and follow a customary exon inclusion/exclusion splicing event using a conical GT/AG donor-acceptor site pair (Fig. 1.3A). Notably, the exclusion of the two exons has only been observed during transcript scanning of $Ca_v2.1$ channel fragments and both exons were found included in 100% of full-length cDNA clones analyzed from human cerebellum samples (Soong et al., 2002).

A third alternative splice region is near the end of exon 17 in the II-III intracellular loop and results in either the insertion (+VEA; 17) or exclusion (-VEA; Δ17A) of a tripeptide repeat (Fig. 1.4, #3), identified in human brain (Hans et al., 1999a; Soong et al., 2002). The tripeptide insertion/exclusion is due to the use of alternative splice donor sites at the 5' end of intron 17 (Fig. 1.3C). In cDNA clones obtained from human cerebellar samples only 0.4% possessed the VEA insertion (Soong et al., 2002). The functional consequences of the tripeptide insertion on $Ca_v2.1$ biophysical properties or channel modulation have not been reported.

A fourth region of alternative splicing is the only site of alternative splicing that occurs in a segment constituting an extracellular portion of the channel (±31*) (Fig. 1.4, #4) and has been identified in human brain (Hans et al., 1999a; Soong et al., 2002), human spinal cord (Krovetz et al., 2000) and rodent brain (Bourinet et al., 1999; Toru et al., 2000). The insertion (+NP; +31*) or exclusion (-NP; -31*) of a dipeptide segment occurs within the IVS3-IVS4 extracellular loop. Interestingly, the six nucleotide sequence encoding for NP is a small exon (exon 31*) lying within intron 31 and flanked by

canonical GT/AG acceptor-donor sites. The ±31* variants are thus predicted to result from exon inclusion/exclusion (Fig. 1.3A). In 1999 the Snutch lab determined that inclusion/exclusion of the dipeptide NP segment in rats determines $Ca_v2.1$ sensitivity to ω-Aga IVA (Bourinet et al., 1999). It is now generally agreed that –NP corresponds to native P-type currents with a high sensitivity to ω-Aga IVA (IC_{50} in the low nanomolar (nM) range), whereas +NP corresponds to Q-type currents with a lower sensitivity to ω-Aga IVA (IC_{50} ~ 90 nM). Ninety-five percent of cDNA clones isolated from human whole cerebellum possess the NP insertion (Soong et al., 2002). In mice, the –NP variant was found almost exclusively in single Purkinje neurons and the +NP variant in granule cells (Toru et al., 2000; Tsunemi et al., 2002; Kanumilli et al., 2006; Richards et al., 2007).

Within the proximal end of the C-terminus is a fifth location of alternative splicing involving the use of mutually exclusive exons 37_a and 37_b ($37_a/37_b$) (Fig. 1.4, #5) identified in human brain (Zhuchenko et al., 1997; Soong et al., 2002), human spinal cord (Krovetz et al., 2000) and rat brain (Bourinet et al., 1999). The exons 37_a and 37_b are mutually exclusive exons with canonical GT/AG acceptor-donor sites (Fig. 1.3B). The exons encode two versions of an EF-hand motif that contains conventional Ca^{2+} binding sites important in the Ca^{2+}-dependent regulation of $Ca_v2.1$ channels (Kretsinger, 1976; Bourinet et al., 1999; Chaudhuri et al., 2004). The two EF-hand variants alone appear to determine Ca^{2+}-dependent modulatory properties of $Ca_v2.1$ with incredible selectivity. The EF_a variant confers the ability of channels to undergo forms of Ca^{2+}-dependent modulation selective to local Ca^{2+} concentrations, whereas the EF_b variant is locked in a normal gating mode (Chaudhuri et al., 2004; Chaudhuri et al., 2007). There is approximately equal expression of the 37_a (40.5%) and 37_b (59.5%) variants in mammalian cerebellum, hippocampus and cerebral cortex. Contrastingly, there is a large preferential expression of EF_b in the amygdala and predominant expression of EF_a in the substantia nigra and thalamus (Soong et al., 2002; Chaudhuri et al., 2004). In rodent cerebellum, Purkinje neurons express almost exclusively EF_a-containing channels (Kanumilli et al., 2006; Richards et al., 2007). Furthermore, there are critical changes in the expression pattern of the two variants during development and a clear gender bias in rodent and human brain (Chaudhuri et al., 2005; Chang et al., 2007).

A sixth location of alternative splicing identified in human brain and spinal cord involves exons 43 and 44 which lie within the C-terminus near the Ca^{2+} binding domain (±43/±44) (Fig. 1.4, #6) (Zhuchenko et al., 1997; Krovetz et al., 2000; Soong et al., 2002). Exons 43 and 44 can be either included or excluded in all four combinations and result from two exon inclusion/exclusion splicing events that use canonical GT/AG acceptor-donor sites (Fig. 1.3A). The presence of both exons was identified in 90% of cDNA clones isolated from human cerebellum, whereas 6% had +43/-44, 2% had -43/+44 and 2% lacked both exons (Soong et al., 2002). Similar findings were obtained from mouse cerebellum and single cerebellar Purkinje neurons (Kanumilli et al., 2006). On the other hand, the proportion of the various combinations varied substantially in other areas of the human brain including the amygdala, cerebral cortex, hippocampus, hypothalamus, thalamus and substantia nigra. In general, the proportion of transcripts containing the +43/-44 combination equaled those containing +43/+44, and the proportion containing neither exon was approximately 20-25% (Soong et al., 2002). The inclusion/exclusion of these exons has been reported to either decrease voltage-dependent inactivation of $Ca_v2.1$ (Krovetz et al., 2000) or to have no effect (Soong et al., 2002), discrepancies likely resulting from coexpression with different β subunits (Patil et al., 1998).

The seventh locus of alternative splicing for $Ca_v2.1$ channels is in the distal portion of the C-terminus (47/Δ47)(Fig. 1.4, #7) and has been identified in human brain (Zhuchenko et al., 1997; Hans et al., 1999a; Soong et al., 2002), human spinal cord (Krovetz et al., 2000) as well as in mouse Purkinje neurons (Toru et al., 2000; Tsunemi et al., 2002; Kanumilli et al., 2006; Richards et al., 2007). The 47 and Δ47 splice variants result from the use of alternate canonical acceptor sites (Fig. 1.3D). The use of an acceptor site immediately 5' of exon 47 results in a pentanucleotide insertion (GGCAG) and frame shift that allows the full translation of exon 47. Alternatively, the use of an acceptor site precisely at the exon 47 boundary yields an in-frame stop codon at the very beginning of exon 47. In whole human cerebellum, 65% of transcripts have the pentanucleotide insertion and full translation of exon 47 (47) (Soong et al., 2002), whereas isolated human Purkinje and granule cells have approximately equal amounts of the 47 and Δ47 $Ca_v2.1$ variants (Tsunemi et al., 2008). Studies in mice showed similar findings for whole cerebellum, although for isolated Purkinje neurons all $Ca_v2.1$ variants contained the

pentanucleotide insertion (Kanumilli et al., 2006). Functional differences in the basic channel properties of the 47 and Δ47 $Ca_v2.1$ variant channels have not been reported.

Two additional $Ca_v2.1$ variants recently identified in rat neuroendocrine cells from the supraoptic nucleus of the hypothalamus have large, 200-300 base pair deletions in the II-III cytoplasmic loop region containing the synprint site. The deletion variants are predicted to have multiple consequences including altered coupling to synaptic release machinery, altered subcellular targeting and altered channel function (Rajapaksha et al., 2008).

Taken together, alternative splicing is an important mechanism necessary in achieving the functional diversity of the $Ca_v2.1$ channel. However, the relevance of $Ca_v2.1$ alternative splicing in the context of human diseases associated with the $Ca_v2.1$ channel has not been investigated. The next section introduces the diseases associated with $Ca_v2.1$ channels and raises critical questions regarding a role for $Ca_v2.1$ alternative splicing in disease pathophysiology.

1.4 $Ca_v2.1$ Ca^{2+} channelopathies

1.4.1 General $Ca_v2.1$ channelopathies

Owing to their diverse physiological roles, it is perhaps not surprising that disruption of VGCC function has been implicated in numerous severe human pathologies. Diseases associated with VGCCs are referred to as Ca^{2+} channelopathies and to date there have been ten human channelopathies associated with five of the ten VGCC subunit genes (Appendix 1 for complete review of VGCC Ca^{2+} channelopathies).

Of the three Ca_v2 α_1 subunits, the only one known to be associated with mammalian genetic disorders is the $Ca_v2.1$ channel. In humans, mutations in the CACNA1A gene encoding $Ca_v2.1$ are associated with three congenital, autosomal dominantly inherited neurological disorders: episodic ataxia type 2 (EA2), spinocerebellar ataxia type 6 (SCA6) and familial hemiplegic migraine type 1 (FHM-1). In mice, $Ca_v2.1$ genetic disorders include tottering (*tg*), leaner (tg^{la}), rolling nagoya (tg^{rol}) and rocker. In

addition to the defined mutations in the $Ca_v2.1$ channel causing disease, an apparent autoimmune attack on $Ca_v2.1$ channels is associated with Lambert-Eaton Myasthenic Syndrome (LEMS). I will first provide a brief overview of each of the human and mouse diseases associated with $Ca_v2.1$ channels and then a more in-depth discussion of FHM-1 as it pertains to this thesis.

EA2 patients experience spontaneous episodes of ataxia (poor muscle coordination) that last for hours to days. In between attacks, patients often experience gaze-evoked or down-beat nystagmus (rapid, involuntary eye oscillations). Approximately 50% of patients also experience migraine-like symptoms and cerebellar atrophy is common (Lorenzon and Beam, 2000). Attacks are often initiated by emotional stress, exercise or alcohol. Most patients respond well to treatment with acetazolamide (reviewed in (Jen et al., 2004)). EA2 is genetically variable and has been associated with $Ca_v2.1$ missense, truncation and alternative splice site mutations.

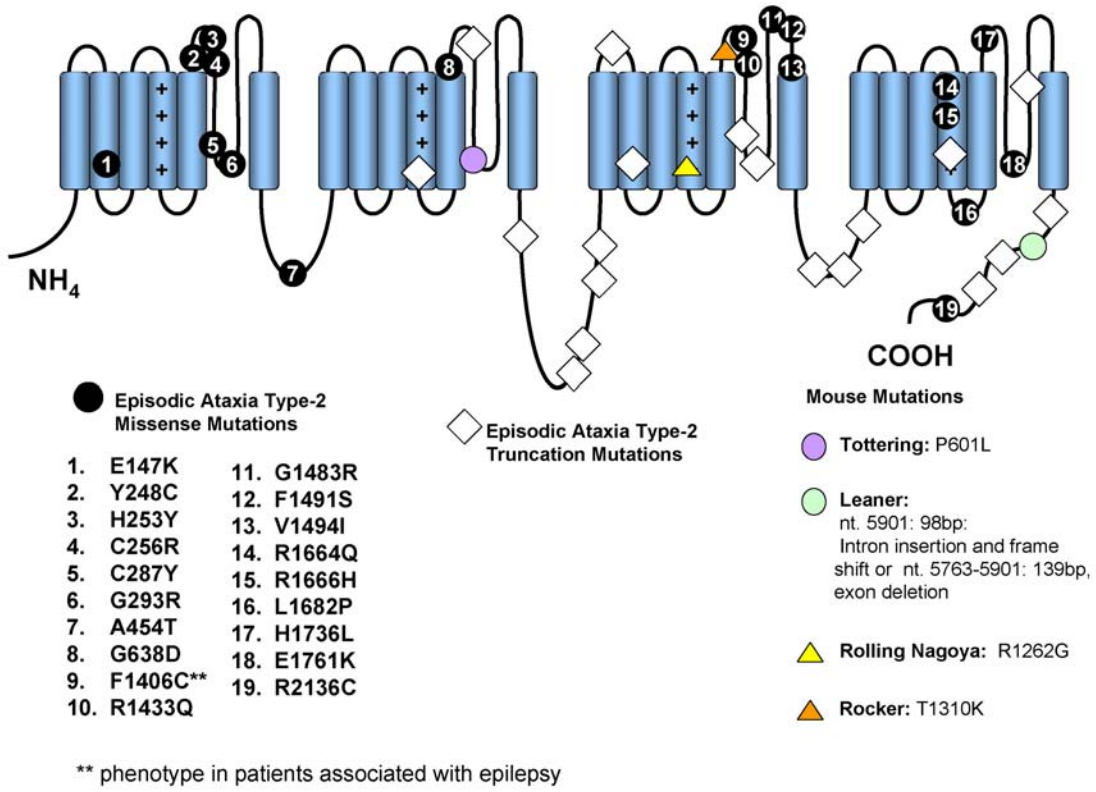


Figure 1.5: $Ca_v2.1$ mutations implicated in EA2 and Epileptic and Ataxic Mouse Models

Location of missense mutations (black circles) and truncation mutations (white diamonds) in the human $Ca_v2.1$ channel associated with Episodic Ataxia Type-2 (EA2). Also shown are mutations in the $Ca_v2.1$ mouse homolog associated with tottering (tg; purple circle), leaner (tg^{la}; green circle), rolling nagoya (tg^{rol}; yellow triangle) and rocker (orange triangle) phenotypes.

To date, more than 40 individual mutations in the CACNA1A gene have been associated with EA2. The EA2 genetic alterations are distributed throughout the channel, with a large number of missense and premature truncations identified within the pore forming P-loops (Fig. 1.5). Overall, the structure-function findings to date are relatively consistent across experimental conditions. A number of both truncation and missense mutations consistently show a reduction in current density, proposed to be a result of fewer channels being properly folded and reaching the plasma membrane (Imbrici et al., 2004; Wan et al., 2005; Jeng et al., 2006; Pietrobon, 2010). The three EA2 missense mutations G293R, C287Y, H1736L and a deletion mutation, nt 4778-4780, all demonstrate a net reduction of available channels due to a depolarizing shift in the voltage of half-maximum activation (V_{50act}), an increased rate

of inactivation and a reduced rate of recovery from inactivation (Wappl et al., 2002; Spacey et al., 2004; Wan et al., 2005).

The EA2 phenotype in humans is paralleled by the epilepsy and ataxia mouse models *tg*, tg^{la}, tg^{rol} and rocker (Fig. 1.5) which all contain $Ca_v2.1$ genetic mutations and show varying degrees of epilepsy and ataxia. The *tg*, tg^{la} and tg^{rol} mutations have similar biophysical effects as those for EA2, namely decreased current densities and depolarizing shifts in V_{50act} (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998; Mori et al., 2000). *In vivo* studies in the animal models show that the reduced channel function seen in the heterologous systems translates to decreased neurotransmitter release in neocortex and at the parallel fibre to Purkinje synapses (Ayata et al., 2000; Matsushita et al., 2002) (for review on $Ca_v2.1$ models see (Pietrobon, 2005, 2010)).

SCA6 is characterized by progressive cerebellar atrophy resulting in progressive gait ataxia, incoordination, nystagmus, proprioceptive sensory loss and dysarthria (Zhuchenko et al., 1997). Zhuchenko and coworkers found that patients with SCA6 possess a polyglutamine (CAG) expansion in exon 47, making SCA6 a member of the group of neurodegenerative disorders containing CAG repeats that includes Huntington's disease amongst others. Whereas unaffected people tend to have CAG repeats numbering between 4 and 16 in CACNA1A, patients with SCA6 have expansions of greater than 21 CAG repeats. The length of the expansion appears directly correlated with age of onset, e.g., greater CAG expansion is associated with early age of disease onset (Ishikawa et al., 1997). The CAG repeat expansion is associated with severe cerebellar Purkinje cell loss, moderate granule cell and dentate nucleus neuronal loss, and mild neuronal loss in the inferior olive (Zhuchenko et al., 1997). The mechanisms involved concerning SCA6 mutations in the $Ca_v2.1$ channel and neuronal death have not been completely resolved. However, biophysical analyses of polyglutamine expansions in the $Ca_v2.1$ channel expressed in heterologous systems show a range of effects on voltage and time-dependent properties with a strong dependence on both auxiliary subunit and α_1 subunit splice-variant composition (Restituito et al., 2000; Toru et al., 2000). It has been generally accepted that the polyglutamine stretches exert toxic effects by forming aggregates. Analysis of cerebellar tissue from SCA6 patients revealed

perinuclear aggregates in Purkinje cells, and transfection of polyglutamine expanded $Ca_v2.1$ channel cDNA into human embryonic kidney (HEK) 293 cells demonstrated that cell death is likely due to the perinuclear aggregates (Ishikawa et al., 1999). An interesting recent report showed that the C-terminus of the $Ca_v2.1$ channel is cleaved and translocated to the nucleus under wild-type conditions, but when the polyglutamine expansion is extended to greater than 33 repeats the nuclear translocated channel segment somehow induces cell death (Kordasiewicz et al., 2006). In contrast, it has also been speculated that the loss-of-function effects on $Ca_v2.1$ channel function may result in reduced intracellular Ca^{2+} concentration and that the reduced Ca^{2+} alone can induce Purkinje neuron apoptosis and cerebellar atrophy (Matsuyama et al., 1999). Overall, it appears that neuronal loss observed in SCA6 brains is likely due to a combination of altered channel properties resulting in abnormal intracellular Ca^{2+} concentrations and perinuclear and/or nuclear channel protein aggregates ultimately causing cell death.

LEMS is a neuromuscular transmission disorder characterized by reduced acetylcholine quantal release and is associated with small-cell lung carcinoma (SCLC) in approximately 60% of patients (Lang et al., 1983). $Ca_v2.1$ channels are implicated in LEMS, although unlike FHM-1, EA2 and SCA6, LEMS is not a true channelopathy as it does not result from defined mutations in the channel. Rather the sera from LEMS patients contain auto-antibodies against VGCCs, with an apparent preferential targeting of $Ca_v2.1$ at the neuromuscular junction (Lennon et al., 1995; Pinto et al., 2002). Clinical features of LEMS includes skeletal muscle weakness in proximal and trunk muscles, with the most severe effects observed in lower limbs (for review see (Flink and Atchison, 2003)). Auto-antibodies are thought to be initiated in response to the SCLC tumour (O'Neill et al., 1988) and, via targeting $Ca_v2.1$ channels at the neuromuscular junctions, reduce channel availability for neurotransmission (Lennon et al., 1995). It has been shown that the LEMS auto-antibodies do not alter channel voltage or kinetic properties, but instead act in an all-or-none fashion, likely eliminating available channels from the population (Grassi et al., 1994; Magnelli et al., 1996). Drugs that prolong the duration of APs and enhance intracellular Ca^{2+} levels, such as 4-aminopyridine and 3,4-diaminopyridine, offer symptomatic relief in some LEMS patients, however, the side effects from treatment can often be severe (Flink and Atchison, 2003).

1.4.2 Introduction to FHM

Migraine is a severe neurological condition that affects approximately 15% of the North American and Western European populations (Lipton et al., 1994; Lipton et al., 2001). Migraine headaches are characterized by recurring unilateral headache that are often accompanied with nausea, phonophobia and/or photophobia. The headaches can occur in isolation, or in approximately 20% of sufferers, the migraine headache can be preceded by or concurrent with an aura. Auras are a subjective sensation most often associated with vision, although other sensory auras can occur (reviewed in (Goadsby et al., 2002)). The complicated genetics and physiology of migraine has slowed the development of adequate treatments and our understanding of underlying disease mechanisms (Montagna, 2004).

FHM is a rare autosomal dominant subtype of migraine with aura, and other than a characteristic hemiplegia, has similar clinical features to typical migraine with aura and may share some pathogenetic mechanisms (Thomsen et al., 2002; Thomsen et al., 2003; Thomsen and Olesen, 2004). Due to its relatively simple genetics, FHM has become a popular research model and has lead to relevant hypotheses for the pathophysiology underlying some aspects of typical migraine (Montagna, 2004).

About 50% of FHM patients have a mutation in the CACNA1A gene (Ophoff et al., 1996) (FHM type 1; FHM-1), whereas the other approximately 50% have mutations in either the ATP1A2 Na^+/K^+-ATPase gene (FHM-2) (De Fusco et al., 2003) or the SCN1A sodium channel gene (FHM-3) (Dichgans et al., 2005). In FHM-1, the aura typically precedes the headache pain and manifests as an obligatory motor aura in combination with one or more other symptoms including visual disturbance, dysphasia (difficulty with speech) and/or sensory loss (usually numbness or paraesthesias of an extremity or the face). The characteristic motor aura most frequently manifests as hemiplegia in both the upper and lower extremities. In the majority of FHM-1 cases, the headache pain directly follows the aura phase of the migraine attack. The headache pain can last from less than 30 minutes to greater than 72 hours, with the mean duration being approximately 24 hours. In addition to these common symptoms, some FHM-1

patients also show permanent cerebellar symptoms that may include progressive cerebellar ataxia, and/or nystagmus, with cerebellar atrophy in some cases (for an extensive review of FHM-1 features and statistics see (Thomsen et al., 2002)).

1.4.3 Mechanisms of migraine pathophysiology

It is generally accepted that both typical migraine and FHM-1 migraine attacks start in the brain (Lauritzen, 1994; Charles, 2009; Goadsby et al., 2009; Levy et al., 2009; Olesen et al., 2009). Based on evidence from neuroimaging studies in human migraine patients (Bowyer et al., 2001; Hadjikhani et al., 2001) and other animal studies, it is apparent that the aura phase of migraine results from cortical spreading depression (CSD). CSD is a transient wave of neuronal hyperexcitability that begins at a focal point and slowly progresses over the cortex (2-6 mm/min), followed by a long neuronal depression lasting minutes (reviewed in (Lauritzen, 1994)). The headache pain component of migraine is thought to involve the meningeal nociceptors and brainstem. Pain sensitivity within the skull is primarily restricted to meningeal blood vessels, and in conscious patients, electrical stimulation of the dura can cause headache pain through activation of branches of the ophthalmic division of the trigeminal nerve afferents that innervate the meningeal blood vessels (Penfield and McNaughton, 1940; Ray and Wolff, 1940) (reviewed in (Pietrobon and Striessnig, 2003; Pietrobon, 2005)). An important link between CSD and headache pain was made by two separate animal studies that showed CSD can cause activation of meningeal trigeminovascular afferents and evoke a series of alterations in the meninges and brainstem that are consistent with the activation of trigeminal nociceptive pathways (Bolay et al., 2002; Moskowitz et al., 2004). Thus at the macroscopic level, the current model of migraine is that CSD underlies the aura phase of migraine and activates the trigeminal pain pathway resulting in headache pain (Fig. 1.6), although a mechanism has not been proposed for the cerebellar dysfunction observed in some FHM-1 patients.

At the tissue and cellular levels, during CSD in rat cortex (Fig. 1.6; 1) there is a substantial change in the composition of glutamate, K^+, hydrogen ions (H^+), nitric oxide (NO), arachidonic acid and

prostaglandins in the extracellular fluid (Somjen, 2002). These molecules activate and/or sensitize the meningeal trigeminovascular afferents either directly or via perivascular inflammation (Wei et al., 1992; Strassman et al., 1996) (Fig. 1.6; 2). Activation of the trigeminovascular afferents causes the release of vasoactive neuropeptides in their peripheral nerve endings which cause vasodilation of meningeal vessels and secretion of proinflammatory substances into the dura (Goadsby and Duckworth, 1987; Markowitz et al., 1988; Goadsby and Edvinsson, 1993; Williamson et al., 1997). In addition, activation of trigeminovascular afferents leads to a subsequent activation of second-order neurons comprising the trigeminal nucleus caudalis (TNC) and the upper divisions of the cervical spinal cord (Fig. 1.6; 3,4). From there, two distinct pathways are activated. First, activation of the superior salivatory nucleus sends impulses that cause further vasodilation of the meningeal blood vessels (Fig. 1.6; 5) and further activation of the trigeminal nerve creating a positive feedback cycle. Second, TNC activation leads to stimulation of trigeminal efferents that project to the ventral posterior medial (VPM) nucleus of the thalamus and periaqueductal gray region (PAG). The VPM sends projections that activate pain centers in the cortex (Fig. 1.6; 6). A self-sustained central sensitization or disinhibitory sensitization of second-order trigeminovascular neurons involving sensory innervation from periorbital skin and neck muscles, descending inhibitory pathway from the PAG (Knight et al., 2002) and facilitatory pathways from the medulla (Urban et al., 2005) likely play key roles in maintaining the severe prolonged pain of migraine headache (for comprehensive review of migraine mechanisms see (Pietrobon and Striessnig, 2003; Moskowitz et al., 2004; Burstein and Jakubowski, 2005; Edvinsson and Uddman, 2005; Pietrobon, 2005; Sanchez-Del-Rio et al., 2006; Goadsby et al., 2009)).

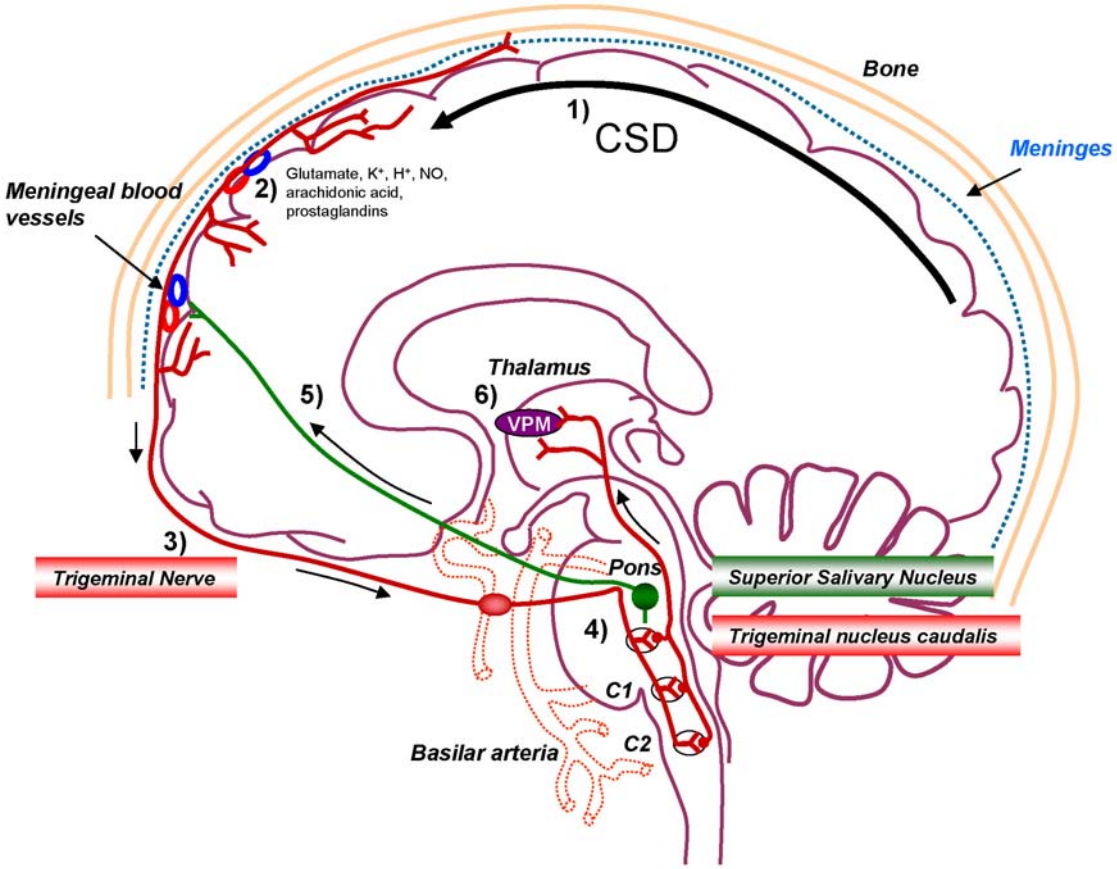


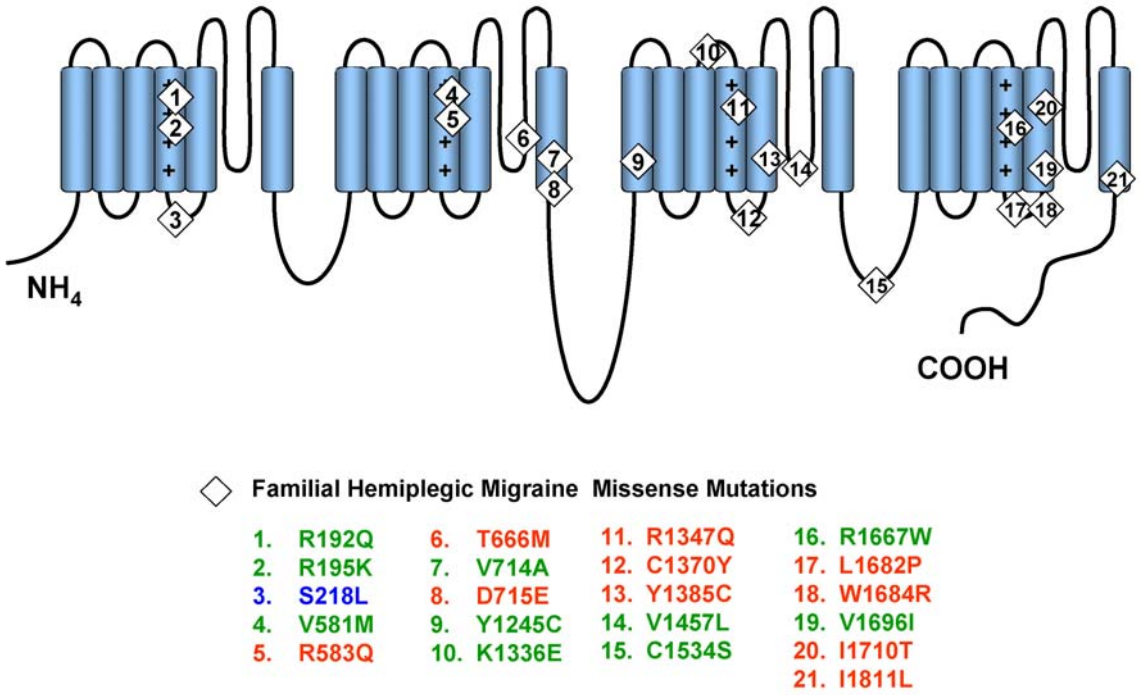
Figure 1.6: Pathophysiological mechanisms of migraine with aura.

1) Cortical spreading depression (CSD) begins at a focal point and propagates as a wave across the cortex. **2)** As the wave of neuronal hyperexcitability moves, excess glutamate, K^+ ions, H^+ ions, NO, arachidonic acid and prostaglandins build up in the extracellular space surrounding cortical neurons. **3)** As these ions diffuse, they depolarize meningeal trigeminovascular afferents (trigeminal nerve). **4)** Activation of the trigeminal nerve leads to activation of the superior salivatory nucleus which in turn sends projections that cause vasodilation of the meningeal blood vessels **(5)**. Dilation of the blood vessels leads to further activation of the trigeminal nerve, creating a positive feedback cycle. **6)** Activation of the trigeminal nucleus caudalis also leads to stimulation of trigeminal efferents that project to the ventral posterior medial (VPM) nucleus of the thalamus. The VPM sends projections that activate pain centers in the cortex.

1.4.4 The role of $Ca_v2.1$ channels in molecular mechanisms of FHM-1

$Ca_v2.1$ channels are highly expressed within areas associated with FHM-1 pathophysiology. They predominate in most neurons in the cerebral cortex (Westenbroek et al., 1995; Timmermann et al., 2002) and are the dominant mediators of glutamate release in cortical neurons (Turner et al., 1992; Qian and Noebels, 2001). $Ca_v2.1$ channels account for about 40% of the whole cell Ca^{2+} current in trigeminal ganglion neurons (Borgland et al., 2001; Xiao et al., 2008; Davies and North, 2009) and contribute to the release of vasoactive neuropeptides from perivascular terminals of meningeal nociceptors (Akerman et al., 2003). $Ca_v2.1$ channels are also involved in the descending inhibitory pathway from the PAG (Knight et al., 2002) and facilitatory pathways from the medulla (Urban et al., 2005), which regulate trigeminal pain transmission. $Ca_v2.1$ channels are also involved in inhibitory control of the TNC by inputs from the dura (Ebersberger et al., 2004). Furthermore, $Ca_v2.1$ channels are highly expressed in cerebellum in both Purkinje and granule cells (Randall and Tsien, 1995) and play predominant roles in both excitatory and inhibitory neurotransmission in cerebellum (Mintz et al., 1995; Iwasaki et al., 2000; Stephens et al., 2001).

To date, there have been 21 mutations in the CACNA1A gene identified in patients with FHM-1 (Fig. 1.7). The mutations all produce amino acid substitutions in conserved functional domains including the S4 voltage sensors and flanking regions, and the pore forming P-loops and S6 transmembrane segments. Analyses of 13 FHM-1 mutations in heterologous expression systems and two FHM-1 knock-in mouse models show a trend in FHM-1 mutation effects that begin to explain the molecular mechanisms behind at least the aura phase of migraine.



◇ Familial Hemiplegic Migraine Missense Mutations

1. R192Q	6. T666M	11. R1347Q	16. R1667W
2. R195K	7. V714A	12. C1370Y	17. L1682P
3. S218L	8. D715E	13. Y1385C	18. W1684R
4. V581M	9. Y1245C	14. V1457L	19. V1696I
5. R583Q	10. K1336E	15. C1534S	20. I1710T
			21. I1811L

Figure 1.7: Missense mutations in $Ca_v2.1$ associated with FHM-1

There are 21 reported missense mutations in $Ca_v2.1$ channels identified in patients with FHM-1. Top, shows the location of mutations throughout conserved functional domains of the $Ca_v2.1$ channel. Bottom, lists the locations and changes in amino acids. The colours indicate the phenotype associated with the mutation in patients: green = pure FHM-1, red = FHM-1 with cerebellar signs, and blue = severe FHM-1, cerebellar signs, diffuse encephalopathy and sometimes coma.

In recombinant human $Ca_v2.1$ channels, the introduction of mutations associated with both pure FHM-1 and FHM-1 with cerebellar signs show a hyperpolarizing shift in V_{50act} (Kraus et al., 1998; Hans et al., 1999b; Melliti et al., 2003; Mullner et al., 2004), indicating the majority of mutant channels are available for opening at lower membrane potentials. Similarly, several mutations cause an increase in open-channel probability and single channel conductance (Hans et al., 1999b; Tottene et al., 2002; Tottene et al., 2005). All of these effects are predicted gain-of-function phenotypes with the potential to increase Ca^{2+} influx at lower membrane potentials (for full review of results from recombinant studies see (Adams and Snutch, 2007), Appendix 1)

The impact of FHM-1 mutations on endogenous P/Q-type currents and neurotransmission in the cortex has been determined directly using two FHM-1 knock-in mouse strains. One of the FHM-1

strains contains the R192Q mutation which in human patients is associated with a mild form of pure FHM-1 (migraine without cerebellar signs) (Ophoff et al., 1996; Ducros et al., 2001), whereas the other strain contains the S218L mutation which in human patients causes severe hemiplegic migraine and may include progressive cerebellar ataxia and atrophy, epileptic seizures, coma or stupor, and severe (sometimes fatal) cerebral oedema triggered by minor head trauma (Fitzsimons and Wolfenden, 1985; Kors et al., 2001; Chan et al., 2008). Neither homozygous nor heterozygous R192Q mice show any overt phenotype, however, homozygous S218L mice exhibit the main clinical features identified in humans, including mild permanent cerebellar ataxia, spontaneous attacks of hemiparesis and/or generalized seizures and can include brain oedema after mild head impact (van den Maagdenberg et al., 2004; van den Maagdenberg et al., 2010). Studies using the mouse strains have provided compelling evidence that the gain-of-function effects of the FHM-1 mutations measured in recombinant systems are recapitulated in cortical neurons from the knock-in mice. The mutations have been shown to enhance excitatory neurotransmission in the FHM-1 mice due to increased Ca^{2+} influx during APs and increased glutamate release at pyramidal cell synapses, predicted to both initiate and perpetuate components of a positive feedback cycle that leads to CSD in FHM-1 (Tottene et al., 2009). In support, *in vivo* studies in FHM-1 mice show a lower threshold for stimulation of CSD and an increased velocity of propagation across the cortex (van den Maagdenberg et al., 2004; Gherardini et al., 2006; van den Maagdenberg et al., 2010).

1.4.5 A key area in future FHM-1 research

In some regards, there is compelling evidence from both the heterologous systems and knock-in mice to support the hypothesis that FHM-1 mutations increase channel availability, enhance Ca^{2+} influx and increase neurotransmitter release and thereby cause greater susceptibility to CSD causing aura. To date however, no data have been presented to explain the headache pain phase or cerebellar dysfunction associated with FHM-1. Furthermore, the FHM-1 phenotype is both highly localized to specific brain regions and episodic in nature, yet the $Ca_v2.1$ channels are expressed at nearly all fast synapses in the

CNS and PNS, and during most stages of development. Why aren't all regions that express $Ca_v2.1$ channels affected by the FHM-1 mutations? Also, there is often considerable discrepancy in how the FHM-1 mutations affect recombinant $Ca_v2.1$ channels when tested by different research groups. Over the past ten years several labs have investigated the effects of FHM-1 mutations on the biophysical properties of recombinant $Ca_v2.1$ channels expressed in *Xenopus* oocytes and mammalian expression systems. Although many findings agree that FHM-1 mutations result in gain-of-function effects, some results vary considerably between research groups, some even contradictory. For example, some researchers find that the R192Q mutation increases $Ca_v2.1$ whole cell current density (Hans et al., 1999b; van den Maagdenberg et al., 2004) while others find a decrease in whole cell current density (Tottene et al., 2002; Cao and Tsien, 2005). In addition, while one lab found that the most prevalent FHM-1 mutation (T666M) shifts the voltage dependence of activation to more hyperpolarized potentials (Kraus et al., 1998), another group reported no change in voltage-dependent properties (Cao and Tsien, 2005). Also of note, mutations found in patients with similar clinical phenotypes have reported opposing effects on the biophysical properties of $Ca_v2.1$ channels. For example, K1336E and V714A have both been associated with pure FHM-1 (without cerebellar signs) but cause either increased (Mullner et al., 2004) or decreased (Kraus et al., 1998) current decay in response to 1 Hz square pulse repetitive stimulations. These types of disparities create significant challenges in interpreting results obtained from different researchers as well as in correlating findings between recombinant and native $Ca_v2.1$ channels.

These discrepancies and how FHM-1 mutations might only affect some $Ca_v2.1$ channels in specific brain regions under certain conditions remain largely unexplored. Different expression systems (Tottene et al., 2002) or association with different auxiliary subunits (Mullner et al., 2004) may account for a portion of the disparity and might contribute to the localized, episodic nature of the FHM-1 phenotype. There are other possibilities, however, that have not been explored. Critically, whether the various known $Ca_v2.1$ splice variants respond differently to the presence of FHM-1 missense mutations, and whether this contributes to spatial and temporal aspects of the disease phenotype has not been explored.

Additional questions regarding FHM-1 that have not been previously explored include whether mutations associated with FHM-1 alter $Ca_v2.1$ Ca^{2+}-dependent modulation and whether any effects on channel modulation might alter synaptic signalling mechanisms.

1.5 Ca^{2+}-dependent modulation

1.5.1 Introduction to Ca^{2+}-dependent modulation of VGCCs

In addition to various forms of protein phosphorylation and G-protein modulation, HVA VGCCs are powerfully modulated by Ca^{2+} itself. Ca^{2+} ions entering through channels bind various Ca^{2+} sensors and modulate channel activity under certain conditions (Meyers et al., 1998; Lee et al., 2002; Haeseleer et al., 2004; Zhou et al., 2004; Few et al., 2005; Lautermilch et al., 2005). The most robust and well understood forms of Ca^{2+}-dependent modulation of Ca_v1 and Ca_v2 channels are mediated by the ubiquitous Ca^{2+} sensing protein calmodulin (CaM) (reviewed in (Halling et al., 2005)).

CaM consists of an N-terminal and C-terminal lobe connected by a central α helix. Each lobe contains two Ca^{2+}-binding E-F hand motifs, which when occupied by Ca^{2+}, expose hydrophobic pockets that modulate the activity of target proteins (Halling et al., 2005). A single Ca^{2+}-free form of the CaM molecule (apoCaM) is constitutively complexed with the C-terminus of HVA VGCCs at an isolucine-glutamine (IQ) domain, and interacts with a second downstream site called the CaM binding domain (CBD). Ca^{2+} binding to the C-terminal or N-terminal lobes of CaM, can each cause distinct forms of channel regulation (DeMaria et al., 2001; Pitt et al., 2001; Erickson et al., 2003; Lee et al., 2003; Liang et al., 2003; Mori et al., 2004). Ca^{2+}-dependent facilitation (CDF) is an augmentation of channel opening in response to a series of depolarizations. Ca^{2+}-dependent inactivation (CDI) is an enhancement of channel closing that occurs during prolonged depolarizations (a process distinct from VDI which progresses at a slower rate and persists when Ba^{2+} is the permeant ion). Although only $Ca_v2.1$ channels undergo CDF, all Ca_v1 and Ca_v2 channels undergo CDI.

CDI regulation of Ca_v1 and Ca_v2 channels by CaM exhibits exquisite modes of spatial Ca^{2+} sensitivity. The N-terminal lobe of CaM responds to a global rise in intracellular Ca^{2+} levels and causes CDI of the Ca_v2 channel family. Alternatively, the C-terminal lobe of CaM responds to a local rise in Ca^{2+} levels within microdomains surrounding the channel pore and causes CDI of the Ca_v1 channel family (Liang et al., 2003; Chaudhuri et al., 2007). The difference in spatial selectivity of CaM when associated with Ca_v1 and Ca_v2 channels is due, at least in part, to an N-terminal spatial Ca^{2+} transforming element (NSCaTE). NSCaTE is a 13 amino acid sequence located between the first 80 and 100 amino acids in the proximal N-terminus of Ca_v1 channels that interacts with CaM molecules bound to the C-terminus of the channel. Ca_v2 channels on the other hand lack the NSCaTE motif (Dick et al., 2008). CDI is not a an all-or-nothing process as some Ca_v1 channels contain a C-terminal CDI-inhibiting module (Singh et al., 2006; Singh et al., 2008) that can act as an enzymatic competitive inhibitor that retunes channel affinity for apoCaM and alters the magnitude of CDI (Liu et al., 2010).

Critical roles of CDI of Ca_v1 and Ca_v2 channels in biological processes are becoming more apparent. For example, Ca^{2+}/CaM association with Ca_v1 channels is necessary for activation of the Ras/mitogen-activated protein kinase pathway, which conveys local Ca^{2+} signals from Ca_v1 channels to the nucleus to regulate gene transcription (Dolmetsch et al., 2001). Also, Ca^{2+}/CaM regulation of Ca_v1 channels is essential for moment-to-moment control of heart rate by controlling the duration of APs. Disruption of CaM binding to Ca_v1 channels causes a 4- to 5-fold prolongation of the cardiac AP (Alseikhan et al., 2002). In addition, roles for $Ca_v1.3$ channel CDI in the pathophysiology of human diseases such as Parkinson's disease, Alzheimers and/or schizophrenia have been proposed (Liu et al., 2010).

1.5.2 CDF and CDI of $Ca_v2.1$ channels

To date, $Ca_v2.1$ channels are the only VGCCs that exhibit dual regulation mediated by CaM. When associated with $Ca_v2.1$ channels, the N-terminal lobe of CaM responds to a global rise in the intracellular Ca^{2+} level and induces CDI of $Ca_v2.1$. The C-terminal lobe of CaM on the other hand,

responds to a local rise in Ca^{2+} levels within microdomains surrounding the pore of the channel and causes CDF. Both recombinant and endogenous $Ca_v2.1$ channels have been shown to possess robust forms of CDF and CDI (Lee et al., 1999a; Lee et al., 2000; DeMaria et al., 2001; Lee et al., 2003; Chaudhuri et al., 2005; Kreiner et al., 2010).

Human recombinant $Ca_v2.1$ channels transiently expressed in HEK cells (along with auxiliary subunits β_{2a} and $\alpha_2\delta$) is a well characterized system and allows for the clear isolation and measurement of CDF and CDI (illustrated in Figure 1.8A). With a single depolarization to +5 mV from a holding potential of -90 mV, $Ca_v2.1$ channels initially open into a normal mode of gating characterized by a relative low steady-state open probability (P_o) and elicit a relatively small Ca^{2+} current (Fig. 1.8A; **1**, left panel). As Ca^{2+} enters through the channel, two Ca^{2+} ions bind the C-terminal lobe of preassociated apoCaM and subsequent Ca^{2+} currents through the channel are enhanced (i.e. CDF) (Fig. 1.8A; **2**, left panel) (Lee et al., 1999a; DeMaria et al., 2001; Lee et al., 2003). The Ca^{2+}/CaM complex is thought to selectively induce a conformational change in the channel structure that favors channel opening. Facilitated $Ca_v2.1$ channels have a "facilitated mode of gating" featuring a genuine enhancement of $Ca_v2.1$ steady-state P_o (Fig. 1.8C; left two panels) (Chaudhuri et al., 2007). During paired depolarizations, channels become facilitated during the first depolarization as Ca^{2+} enters, and during the second depolarization, $Ca_v2.1$ channels are already facilitated and open immediately with a facilitated mode of gating that elicits maximum Ca^{2+} currents (Fig. 1.8A; **2,** middle panel). When Ba^{2+} is the permeant ion, channels remain in the low P_o state and exhibit the "unfacilitated" or "normal" gating mode and do not transition to the facilitated state regardless of prepulse potential (Fig. 1.8A; right panel), confirming CDF is a true Ca^{2+}-dependent process (Chaudhuri et al., 2007). Strong CDI of $Ca_v2.1$ is evident during prolonged depolarizations, in which whole cell Ca^{2+} currents decay much faster relative to Ba^{2+} currents (Fig. 1.8B). As global Ca^{2+} levels rise, two Ca^{2+} ions bind the N-terminal lobe of resident CaM molecules and induce additional conformational change that inactivates channels (Fig. 1.8 C; right panel) (Chaudhuri et al., 2007).

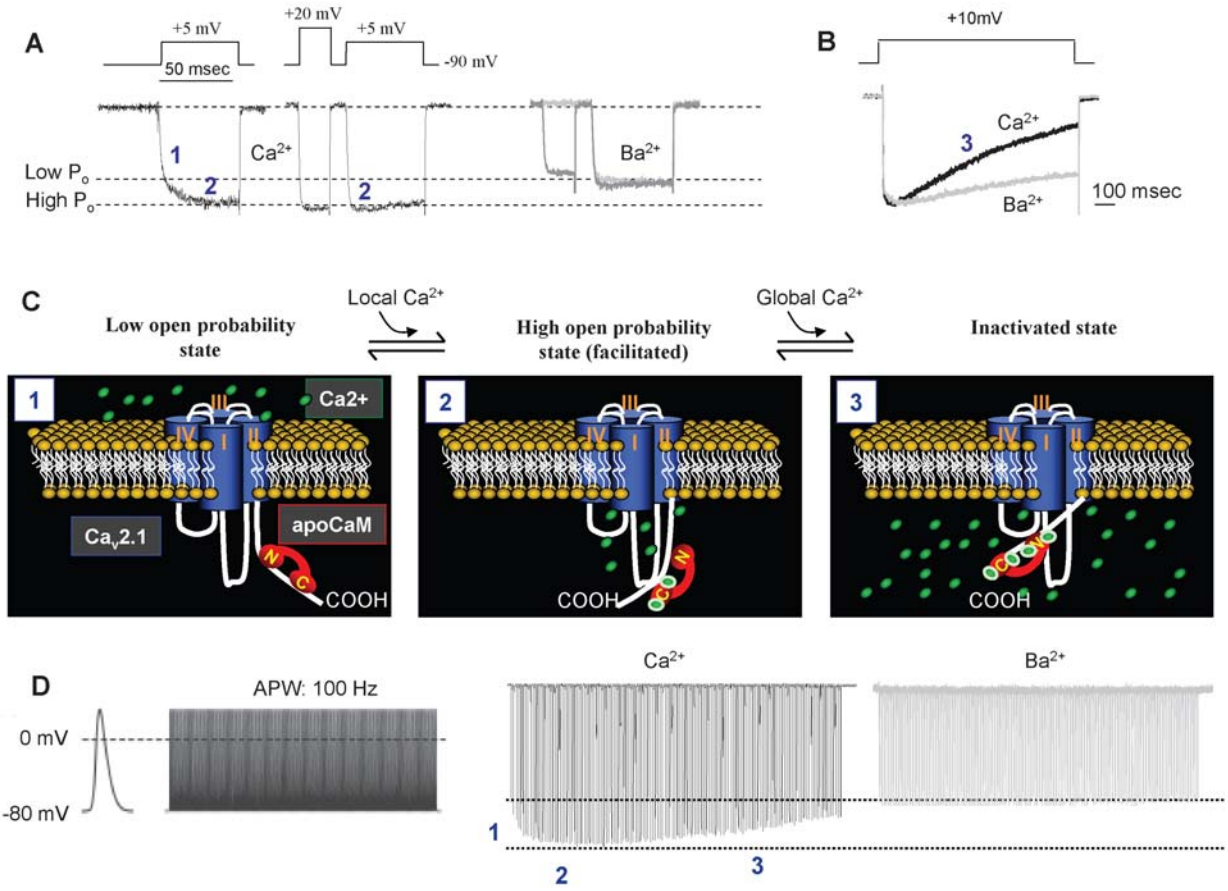


Figure 1.8: CDF and CDI of $Ca_v2.1$ channels

A. Left; when Ca^{2+} is used as the charge carrier, wild-type $Ca_v2.1$ channels respond to a +5 mV square test pulse with an initial rapid activation (1) followed by a slow phase of current increase as Ca^{2+} enters through the channel, due to CDF (2). Middle; on the other hand, when preceded by a +20 mV prepulse, channels are already facilitated at the beginning of the test pulse and activate rapidly with a maximum current response (2). Right; when Ba^{2+} is used as the charge carrier, channels open to the low current level and remain there. **B.** During a 1 second +10 mV test pulse, Ca^{2+} currents inactivate faster than Ba^{2+} currents due to CDI (DeMaria et al., 2001). **C.** Mechanisms that underlie the two phenomena in **A** and **B**. Left panel; without a prepulse wild-type channels open with a normal gating mode (1) then transition to a facilitated mode of gating as Ca^{2+} enters and binds the C-lobe of CaM constitutively bound to the C-terminus of the channel (2) (CDF) (middle panel). Right panel; a rise in global Ca^{2+} levels through many channels, causes Ca^{2+} to bind the N-lobe of CaM which induces further conformational change and channel inactivation (Chaudhuri et al., 2007). **D.** 100 Hz APW derived from APs recorded in the calyx of Held (Borst and Sakmann, 1998; Patil et al., 1998) (left panel). Right two panels show representative traces when using either Ca^{2+} or Ba^{2+} as the charge carrier. All currents were obtained from transiently transfected human recombinant $Ca_v2.1$ channels in HEK cells.

Although square voltage pulses allow maximum resolution of both CDF and CDI, the physiological relevance of CDF and CDI is more evident under simple action potential waveforms (APWs) (Fig. 1.8D). As evident, the dual feedback regulation of $Ca_v2.1$ channels by CaM causes activity-dependent changes of Ca^{2+} currents during repetitive APs, first by enhancing the Ca^{2+} transients during initial APs (Fig. 1.8D; **1** and **2**) and then reducing Ca^{2+} transients during later APs (Fig. 1.8D; **3**). These powerful forms of rapid regulation have the potential to strongly influence Ca^{2+} dynamics in presynaptic terminals expressing $Ca_v2.1$ channels in the CNS.

1.5.3 Ca^{2+}-dependent modulation of $Ca_v2.1$ channels in synaptic plasticity

Short and long forms of synaptic plasticity including facilitation and/or depression of synaptic response during repetitive APs affect the dynamics and strength of neural circuits and are essential for the encoding, processing and storage of information in the CNS. It has been known for many years that activity-dependent dynamics of Ca^{2+} signalling within presynaptic terminals is a central determinant of short-term synaptic plasticity (Katz and Miledi, 1968). The earliest empirical evidence showed that presynaptic Ca^{2+} currents themselves do not change during repetitive stimulation at the squid giant axon synapse (Charlton et al., 1982). This established the notion that mechanisms of synaptic plasticity at most fast synapses in the CNS are likely downstream of Ca^{2+} entry and rather associated with molecular complexes directly involved in vesicle fusion (termed the "residual Ca^{2+} hypothesis"). The residual Ca^{2+} hypothesis has been the most widely accepted mechanism of short-term synaptic plasticity for the past thirty years. It states that facilitation of synaptic responses during repetitive APs is due to the accumulation of intracellular Ca^{2+} in presynaptic terminals, and that build-up of residual Ca^{2+} enhances binding to Ca^{2+} sensor proteins (CaS) (such as synaptotagmin) which directly mediate vesicle fusion and transmitter release. Conversely, decay in synaptic responses during repetitive APs is believed to primarily be due to the depletion of the readily available pool of neurotransmitter containing vesicles (reviewed in (Zucker and Regehr, 2002)). However, this traditional explanation of short-term synaptic plasticity is being challenged by recent findings that residual Ca^{2+} can act on other CaSs independent of

the release machinery (Blatow et al., 2003; Felmy et al., 2003; Muller et al., 2007), and further, that modulation of presynaptic Ca^{2+} currents themselves can in fact be a means to achieve short-term synaptic plasticity at a CNS synapse.

The calyx of Held is a large presynaptic terminal in the mammalian CNS which synapses onto cell bodies of principal neurons in the medial nucleus of the trapezoid body. Whole-cell voltage clamp recordings on the calyx of Held in mice show that an activity- and Ca^{2+}-dependent enhancement of presynaptic Ca^{2+} currents accounts for about 40% of the total facilitation of synaptic response during repetitive APs (Forsythe, 1994; Borst and Sakmann, 1998; Cuttle et al., 1998; Forsythe et al., 1998; Inchauspe et al., 2004; Xu and Wu, 2005; Muller et al., 2008). There is also some evidence that inactivation of presynaptic currents mediates short-term synaptic depression under some conditions (Xu and Wu, 2005). Although both $Ca_v2.1$ and $Ca_v2.2$ channels are expressed presynaptically at this synapse, facilitation of presynaptic Ca^{2+} currents and the corresponding changes in synaptic facilitation are dependent solely on $Ca_v2.1$ channels (Inchauspe et al., 2004; Ishikawa et al., 2005; Xu and Wu, 2005; Inchauspe et al., 2007). At the mechanistic level, it has been proposed that these processes in the calyx of Held are mediated by the neuronal Ca^{2+} sensor NCS-1 (Tsujimoto et al., 2002), although some descrepencies remain (Rousset et al., 2003). Clarity regarding the mechanism of $Ca_v2.1$ channel modulation and whether it represents a general mechanism of synaptic plasticity at other CNS synapses remains to be established.

In 2008, work from Catterall and Mochida provided the first evidence in a model system that CDF and CDI of $Ca_v2.1$ is mediated through CaS such as CaM that bind the IQ-like domain and contributes to both the induction of short-term synaptic facilitation and rapid synaptic depression (Mochida et al., 2008). The model system consisted of recombinant $Ca_v2.1$ channels transfected into cultured superior cervical ganglion (SCG) neurons. SCG neurons do not express endogenous $Ca_v2.1$ channels and when cultured *in vitro* form fast cholinergic synapses (Mochida et al., 2003a; Mochida et al., 2003b). Mochida et al. showed that after transfection of cultured SCG neurons with wild-type $Ca_v2.1$ channels, both robust CDF and CDI of Ca^{2+} currents could be measured in the presynaptic neurons.

Further, a corresponding synaptic facilitation and depression, respectively, could be detected in excitatory post-synaptic potentials (EPSPs) evoked from pairs of synaptically connected SCG neurons in which only the presynaptic neuron was transfected with $Ca_v2.1$ channels. In contrast, when $Ca_v2.1$ channels harboring mutations in the IQ-like and CBD binding domains that prevent CaS binding were transfected into the SCG neurons, CDF and CDI of presynaptic currents was not detected. Furthermore, paired-pulse facilitation (PPF) and facilitation and depression of EPSPs in response to trains of APs were reduced significantly (Mochida et al., 2008). The results provide compelling evidence for a model in which CaM or other CaS proteins respond to residual Ca^{2+} and mediate Ca^{2+}-dependent modulation of $Ca_v2.1$ channels by binding to the IQ-like motif in the C-terminus of the channel and induce short-term synaptic plasticity under repetitive APs. However, it remains to be described whether this mechanism described in the model system applies to intact fast synapses in the CNS.

1.5.4 Key areas concerning future research on $Ca_v2.1$ modulation and synaptic plasticity

The centrality of $Ca_v2.1$ in mediating presynaptic Ca^{2+} influx at nearly all fast presynaptic terminals in the CNS suggests such forms of CDF and CDI of $Ca_v2.1$ may be widespread mechanisms of short-term synaptic plasticity. Interestingly, some FHM-1 missense mutations result in an overall gain-of-function $Ca_v2.1$ channel phenotype as a result of enhanced P_o (Hans et al., 1999b; Tottene et al., 2002) and as described, CaM mediated CDF of $Ca_v2.1$ renders channels toward a "facilitated mode of gating" due to an enhancement of $Ca_v2.1$ P_o. Although the effects of FHM-1 mutations on CDF and CDI of $Ca_v2.1$ have not been reported, the fact that CDF/CDI and FHM-1 mutations both alter $Ca_v2.1$ channel gating suggests FHM-1 mutations may also interfere with the normal Ca^{2+}-dependent regulation of $Ca_v2.1$ channels. Further, if these forms of CDF and CDI of $Ca_v2.1$ are required components of synaptic plasticity, FHM-1 mutations might directly affect synaptic efficacy and have significant implications concerning disease pathophysiology.

A prototypical CNS synapse where $Ca_v2.1$ CDF may play an important role in synaptic plasticity and also be important in FHM-1 pathophysiology (at least in severe forms of FHM-1 associated with

cerebellar signs), is the parallel fibre (PF) to Purkinje cell (PC) synapse in the cerebellar cortex. The PF-PC synapse is a well-characterized central synapse that displays robust presynaptic forms of short-term facilitation via both paired-pulse and AP trains and is known to rely predominantly on $Ca_v2.1$ channels for neurotransmitter release (Mintz et al., 1995; Randall and Tsien, 1995). Also, facilitation at this synapse is mediated by CaSs similar to those reported to mediate $Ca_v2.1$ CDF (Atluri and Regehr, 1996; Lee et al., 1999a; Lee et al., 2000; DeMaria et al., 2001; Lee et al., 2002; Tsujimoto et al., 2002; Lee et al., 2003; Chaudhuri et al., 2004; Chaudhuri et al., 2005; Few et al., 2005; Chaudhuri et al., 2007).

1.6 $Ca_v2.1$ and synaptic plasticity at the PF-PC synapse

1.6.1 Brief cerebellum overview

The cerebellum plays a central role in motor control, reflex adaptation and motor learning. The structure of the cerebellum in mammals is highly organized and composed of repeated modules consisting of three layers of well-defined cell types (molecular, Purkinje and granular layers) (Fig. 1.9 A) (for full review of cerebellar anatomy and circuitry see (Voogd and Glickstein, 1998; Ito, 2000, 2002; Boyden et al., 2004)). In brief, inputs to the cerebellum derive from various regions of the CNS and PNS through precerebellar nuclei axons called mossy fibers (MFs). MFs branch extensively and create large presynaptic terminals (glomeruli) that form excitatory glutamatergic synaptic connections with dendrites of hundreds of granule cells (GCs) within the granule layer of the cerebellar cortex (Fig. 1.9 B). GCs axons ascend to the molecular layer where they bifurcate to form the PFs, which extend 2-3 mm in either direction along the transverse plane making many *en passant* synapses on the dendritic spines of tens of thousands of PCs (the PF– PC synapse) (Fig. 1.9B). Each PC receives as many as 60,000 to 175,000 PF inputs (Napper and Harvey, 1988). PCs have large cell bodies that reside in the Purkinje cell layer and have extensive apical dendritic arbourization in the parasaggital plane that protrude into the molecular layer. This elaborate dendritic tree is the largest and most highly branched of all neurons in the CNS, yet is confined within the saggital plane and at a right angle to the PFs. PC cells also receive excitatory input from climbing fibres (CFs) that originate in the inferior olive and medulla oblongata. Only a single

CF contacts each PC, however the CF wraps around the PC and can form upwards of 26,000 synapses along dendritic spines (Nieto-Bona et al., 1997). Powerful inhibitory inputs from basket cells are made onto cell bodies of PCs and inhibitory input to PC dendrites is mediated by stellate cells that receive input from PFs. The apical dendrites of Golgi cells lie within the molecular layer and receive input from the PFs and then provide inhibitory feedback to the GCs (Ito, 2000, 2002). The PCs are responsible for the sole output of the cerebellar cortex. PC axons project through the granule cell layer and white matter to make inhibitory GABAergic connections with neurons of the deep cerebellar nuclei (DCN).

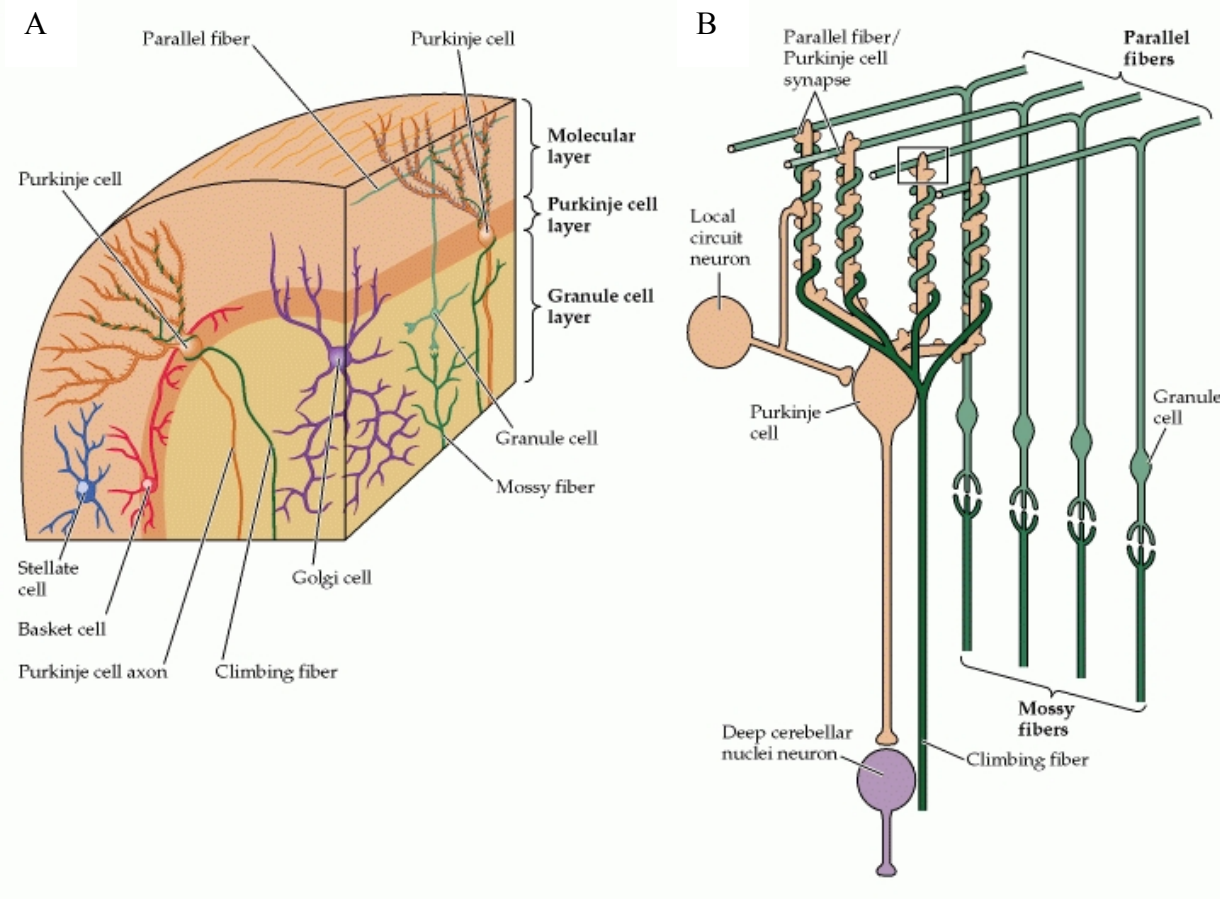


Figure 1.9: Main microcircuitry in the cerebellar cortex

A) Three-dimensional illustration of the cerebellar cortex. PC somas (brown) form the Purkinje cell layer, while extensive dendritic arbourizations of the PCs lie within the parasagittal plane and form the molecular layer. The soma of GCs (light green) form the granule cell layer. Axons of GCs ascend through the Purkinje cell layer to the molecular layer, bifurcate and run within the transverse plane of the cerebellum as PFs. There are several GABAergic interneurons, including stellate cells (blue), basket cells (red), and golgi cells (purple) found in the cerebellar cortex that form synaptic connections with various cellular elements. **B)** MFs (dark green) from various brain regions form excitatory glutamatergic synaptic connections with dendrites of hundreds of GCs. Each PF (light green) forms many *en passant* glutamatergic excitatory inputs onto the distal dendritic spines of many PCs. CFs (deep green) project directly from the inferior olive of the brainstem and form glutamatergic excitatory inputs onto PCs. Each CF forms thousands of synaptic connections with the proximal dendrites of an individual PC. The axons of PCs projects to DCN (purple) via inhibitory GABAergic synaptic connections and form the sole output of the cerebellum. DCN neurons relay signals to the motor cortex via the thalamus. Adapted with permission from Purves et al., 2001, *Neuroscience 2/e*, 416-417.

1.6.2 Synaptic plasticity at the PF-PC synapse: a role for CDF of $Ca_v2.1$?

The geometry and spatial equilibration of Ca^{2+} in the PF presynaptic terminals (or boutons) are ideal for measuring the role of Ca^{2+} dynamics on a tens-of-milliseconds time scale using Ca^{2+}-sensitive fluorescent dyes while simultaneously measuring excitatory post-synaptic currents (EPSCs) in PCs (Mintz et al., 1995; Atluri and Regehr, 1996). Such measurements have provided substantial information as to mechanisms of synaptic plasticity at this synapse. Short-term synaptic facilitation is robust under conditions of both paired-pulse and short AP trains at the PF-PC synapse and is derived from presynaptic mechanisms (Schulz et al., 1994; Goto et al., 2006). Although modulation of presynaptic Ca^{2+} currents via adenosine A1, $GABA_B$ and cannabinoids receptors can reduce synaptic strength at this synapse, synaptic facilitation is driven by Ca^{2+}-dependent processes in PF boutons (Dittman and Regehr, 1996; Kreitzer and Regehr, 2000).

Though the precise Ca^{2+}-dependent mechanisms that drive facilitation have not been fully elucidated, several properties of this terminal suggest CDF of $Ca_v2.1$ channels may be an important player. For one, although a significant portion of facilitation can be explained by traditional mechanisms described by the residual Ca^{2+} hypothesis, some facilitation persists in the absence of residual Ca^{2+}. This has been demonstrated through experiments in which presynaptic terminals were treated with high (100 μM) concentrations of the Ca^{2+} chelator ethylene glycol tetraacetic acid bound to acetoxymethyl ester (EGTA-AM) to make it hydrophobic and amenable for uptake across cell membranes in live cells. In these experiments, EGTA-AM sped the decay of free intracellular Ca^{2+} to only a brief impulse lasting a few milliseconds. During paired pulses, facilitation of the second pulse was still present with interpulse intervals between 40 and 50 milliseconds (Atluri and Regehr, 1996). The authors concluded that there are at least two mechanisms of facilitation at the PF-PC synapse, one driven by residual Ca^{2+} in the traditional sense, and another driven by CaSs with high Ca^{2+} affinity that can detect modest, transient levels of Ca^{2+} likely near the pore of presynaptic VGCCs. Of note, it is known that CaM and other CaS proteins are both present in PFs and sensitive to moderate levels of Ca^{2+} (Ullrich et al., 1994; Li et al., 1995).

While GCs contain $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ channels (Randall and Tsien, 1995), $Ca_v2.1$ is responsible for about 60% of Ca^{2+} influx in PF terminals during APs and is most effective at triggering transmitter release. As such, $Ca_v2.1$ channels are responsible for nearly 93% of the synaptic response at the PF-PC synapse (Mintz et al., 1995) and even small changes in the Ca^{2+} influx through $Ca_v2.1$ channels at these terminals have profound effects on the post-synaptic response. In fact, there is a steep correlation between Ca^{2+} influx in PF boutons and transmitter release such that the power law of synaptic transmission at this synapse can range between 2 and 4 (Mintz et al., 1995; Sabatini and Regehr, 1997). Thus, the enhancement and broadening of APs predicted by CaM mediated CDF of $Ca_v2.1$ (Chaudhuri et al., 2007) could have important implications for the robust short-term facilitation observed at this synapse.

While the PF-PC synapse is a prototypical synapse in the CNS, and the available evidence indicates $Ca_v2.1$ CDF may play an important role in synaptic plasticity, whether this is in fact an important regulatory mechanism at this synapse has not been experimentally shown.

1.7 Thesis hypotheses and objectives

1.7.1 Hypothesis 1

The $Ca_v2.1$ channel plays a central role in neurotransmitter release at nearly all fast synapses in the CNS, yet not all $Ca_v2.1$ channels are the same. As a result of alternative splicing, there are potentially thousands of functionally distinct $Ca_v2.1$ splice variants that are differentially expressed within brain regions, cell types and subcellular compartments and at different developmental stages. Critically, the $Ca_v2.1$ channel is associated with several autosomal dominant human diseases including FHM-1, yet whether the FHM-1 mutations differentially affect $Ca_v2.1$ splice variants has not been tested. In this thesis I specifically hypothesize:

That $Ca_v2.1$ splice variants respond differentially to FHM-1 mutations and contribute to both the episodic nature of the disease phenotype and the localization of the disease to specific brain regions and cell types.

1.7.2 Hypothesis 2

Ca^{2+}-dependent modulation of $Ca_v2.1$ channels is a robust form of channel modulation, however, whether mutations in the $Ca_v2.1$ channel associated with human disease alter CDF or CDI has not been reported. Additionally, CDF and CDI of $Ca_v2.1$ are predicted to play critical roles in short-term synaptic plasticity in the CNS. A direct demonstration of CDF or CDI of $Ca_v2.1$ channels as important determinants of short-term synaptic plasticity at an intact central synapse has not been demonstrated. In this thesis I specifically hypothesize:

That FHM-1 mutations alter Ca^{2+}-dependent modulation of $Ca_v2.1$ channels, and further, that the changes in channel modulation affect synaptic plasticity.

1.7.3 Thesis objectives

The following scientific objectives were formulated to address the above hypotheses:

1. To use molecular genetic tools in combination with whole-cell electrophysiological analyses to compare the effects of several FHM-1 mutations on voltage-dependent and kinetic properties of two common human $Ca_v2.1$ splice variants expressed in HEK cells.
2. To analyse the effects of FHM-1 mutations on CDF and CDI of human recombinant $Ca_v2.1$ channels expressed in HEK cells.
3. To verify effects on CDF or CDI utilizing two FHM-1 knock-in mouse strains expressing endogenous levels of $Ca_v2.1$ channels containing FHM-1 mutations.
4. To examine whether effects of FHM-1 mutations on CDF or CDI in recombinant and endogenous $Ca_v2.1$ channels affect short-term synaptic plasticity by measuring excitatory post-synaptic potentials in PCs during evoked APs in PFs in both wild-type and knock-in mice

cerebellar slices. Also, to compare Ca^{2+} dynamics through the $Ca_v2.1$ channels in PF boutons from wild-type and FHM-1 knock-in mice using two-photon microscopy.

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2 $Ca_v2.1$ Ca^{2+} CHANNEL ALTERNATIVE SPLICING AFFECTS THE FUNCTIONAL IMPACT OF FAMILIAL HEMIPLEGIC MIGRAINE MUTATIONS: IMPLICATIONS FOR Ca^{2+} CHANNELOPATHIES*

2.1 Introduction

VGCCs are important in many normal physiological processes including muscle contraction, neurotransmitter release, regulation of Ca^{2+}-dependent enzymes and gene expression (reviewed in (Catterall, 2000)). It is therefore perhaps not surprising that naturally occurring mutations in Ca^{2+} channel genes have been implicated in a number of severe human diseases. Since the first mutation in a Ca^{2+} channel was identified (Fontaine et al., 1994; Ptacek et al., 1994), over 150 individual mutations have now been reported in five of the ten genes encoding Ca^{2+} channel pore forming α_1-subunits (Ca_v) and are associated with nine distinguishable disorders ("Ca^{2+} channelopathies"). Over the past decade, studies using recombinant channels in various expression systems have shown many of these mutations have significant positive or negative effects on channel gating and/or expression levels, while others result in non-functional channels or have dominant negative effects (reviewed in (Adams and Snutch, 2007)). It is noteworthy that the effects of mutations on channel function have thus far only been tested in a small subset of known Ca^{2+} channel variants and a direct comparison of how mutations affect channel alternative splice variants has been largely unexplored.

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It has been predicted that the ten genes encoding Ca_v subunits have the potential to generate thousands of functionally distinct splice variants (Lipscombe and Castiglioni, 2004; Emerick et al., 2006). Indeed, isolation and characterization of some variants has shown that alternative splicing can be a means to obtain specialized Ca^{2+} channel function and to optimize Ca^{2+} signalling regionally, temporally and under altered environmental conditions (reviewed in (Lipscombe et al., 2002; Lipscombe and Castiglioni, 2004; Gray et al., 2007)). It is evident that mutations directly at a splice-site or in an alternate exon can have effects on pre-mRNA splicing and/or affect a subset of splice variants expressing alternate exons (Zhuchenko et al., 1997; Splawski et al., 2004; Splawski et al., 2005; Graves et al., 2008). However, the majority of identified mutations associated with Ca^{2+} channelopathies are missense mutations in coding sequences other than splice-sites and alternate exons (reviewed in (Adams and Snutch, 2007)). Whether channel splice variants have different functional responses to disease-causing missense mutations has not been explored. We hypothesized that point mutations associated with Ca^{2+} channelopathies might have splice-variant specific effects with important implications for both understanding disease pathophysiology and also towards interpreting results obtained from heterologous studies using recombinant channels.

Familial Hemiplegic Migraine (FHM) is an autosomal dominant subtype of migraine characterized by an aura of hemiplegia that is associated with at least one other aura symptom such as hemianopsia, hemisensory deficit, or aphasia (Ducros et al., 2001; Thomsen et al., 2002). Approximately 20 missense mutations associated with FHM have been identified in the CACNA1A gene (Ophoff et al., 1996) (called FHM-1) which encodes the α_1 subunit ($Ca_v2.1$; α_{1A}) that conducts P/Q-type Ca^{2+} currents. $Ca_v2.1$ channels are abundantly expressed throughout mammalian brain and spinal cord where they mediate Ca^{2+} influx essential for neurotransmitter release, Ca^{2+}-mediated second messenger signalling and Ca^{2+}-dependent gene transcription (Starr et al., 1991; Takahashi and Momiyama, 1993; Westenbroek et al., 1995; Bourinet et al., 1999; Sutton et al., 1999). The functional consequences of FHM-1 mutations on $Ca_v2.1$ channel properties have been investigated in heterologous *Xenopus* oocyte and mammalian expression systems, and more recently in neurons and whole brains of FHM-1 mutant R192Q and S218L knock-in mice (Hans et al., 1999b; Kraus et al., 2000; Tottene et al., 2002; Mullner et al., 2004; van den Maagdenberg et al., 2004; Cao and Tsien, 2005; Tottene et al., 2005; Tottene A., 2005). Biophysical analysis of FHM-1 effects on

$Ca_v2.1$ channels is controversial as both loss-of-function and gain-of-function effects have been reported, as well as no effect (Kraus et al., 1998; Hans et al., 1999b; Kraus et al., 2000; Tottene et al., 2002; Barrett et al., 2005; Cao and Tsien, 2005). Yet despite the noted discrepancies, in both heterologous and knock-in mice systems there is a general demonstrated trend for FHM-1 mutations to exhibit gain-of-function properties: increased channel availability and increased Ca^{2+} influx at lower membrane potentials resulting in a greater susceptibility to the CSD thought to be the underlying mechanism of aura (Kraus et al., 1998; Hans et al., 1999b; Melliti et al., 2003; Mullner et al., 2004; van den Maagdenberg et al., 2004; Gherardini L., 2006).

There are seven identified alternatively spliced sites within the $Ca_v2.1$ subunit gene and the various splice variants exhibit distinct biophysical characteristics, Ca^{2+}-dependent properties, pharmacological sensitivities and subtype-specific temporal and regional localizations in human brain (Bourinet et al., 1999; Soong et al., 2002; Timmermann et al., 2002; Chaudhuri et al., 2004; Chang et al., 2007). However, it is not known whether the functional impact of FHM-1 mutations is similar amongst the different $Ca_v2.1$ splice variants or whether alternative splicing contributes to the spatial and temporal nature of the FHM-1 phenotype. The carboxyl terminus of $Ca_v2.1$ channels is known to affect several physiological processes and alternative splicing in this region confers functional changes in channel properties (Bourinet et al., 1999; Maximov et al., 1999; Krovetz et al., 2000; Restituito et al., 2000; Soong et al., 2002; Chaudhuri et al., 2004). The most substantial changes induced by alternative splicing in the C-terminus of $Ca_v2.1$ channels results from the use of an alternative three prime acceptor site in the intron upstream of the last exon, exon 47 (Mori et al., 1991; Hans et al., 1999a; Krovetz et al., 2000; Soong et al., 2002). Alternative splicing at exon 47 introduces a frame-shift resulting in a stop codon at the beginning of exon 47. As a result, $Ca_v2.1$ channels can be of either the short form (isoform 1; $Ca_v2.1$ (Δ47)) or the long (isoform 2; $Ca_v2.1$ (+47)). The voltage-dependent and kinetic properties of the $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) splice variants and their relative contributions concerning FHM-1 mutations has not been explored.

In the present study we compared the biophysical properties of wild-type $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) channel splice variants and also explored the effects of three FHM-1 mutations introduced into the

two variants. We investigated two mutations, K1336E and R192Q, that are associated with an FHM-1 phenotype of pure hemiplegia and migraine without any other neurological symptoms (Ophoff et al., 1996; Ducros et al., 2001). We further investigated the S218L FHM-1 mutation which is associated with a severe clinical phenotype wherein typical FHM-1 attacks induced by minor head trauma are often followed by a delayed cerebral oedema, fever, stupor and sometimes coma (fatal in one reported instance) (Fitzsimons and Wolfenden, 1985; Kors et al., 2001; Chan et al., 2008). We find that the two $Ca_v2.1$ channel carboxyl tail splice variants exhibit functionally distinct properties and also that the three FHM-1 mutations have differential splice-dependent effects on voltage-dependent and kinetic properties. We discuss the potential importance of the splice-variant differential effects in the context of FHM-1 pathophysiology as well as the implications for other Ca^{2+} channelopathies.

2.2 Results

2.2.1 $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) variants are expressed in human cortex

Mutations in the $Ca_v2.1$ channel underlie FHM-1 and the current consensus is that initiation of migraine attacks is in the cortex; however the expression of splice variants has not yet been described in the human cortex. In order to determine whether the $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) variants are expressed in human cortex we utilized RT-PCR to amplify a ~1.1Kb carboxyl terminal fragment of $Ca_v2.1$ from adult human cortex RNA using oligonucleotide primers that recognize both carboxyl alternatively spliced variants in a non-biased manner. The PCR products were subsequently re-amplified using splice-variant specific primers. Figure 2.1 shows that the $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) splice variants are both expressed in human cortex. To determine their relative proportions, the human cortical $Ca_v2.1$ carboxyl terminal PCR products were sub-cloned and individual cDNAs analyzed using splice-variant specific primers and direct DNA sequencing. From the 53 cDNA clones analyzed we determined that the $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) splice variants were present in whole cortex in relative proportions of 79% and 21%, respectively.

All subsequent biophysical analyses were performed using human long $Ca_v2.1$ (+47) and short $Ca_v2.1$ (Δ47) splice-variant cDNA clones with either wild-type or FHM-1 mutant K1336E, R192Q or S218L changes introduced (see Fig. 2.1A for the location of the FHM-1 mutations).

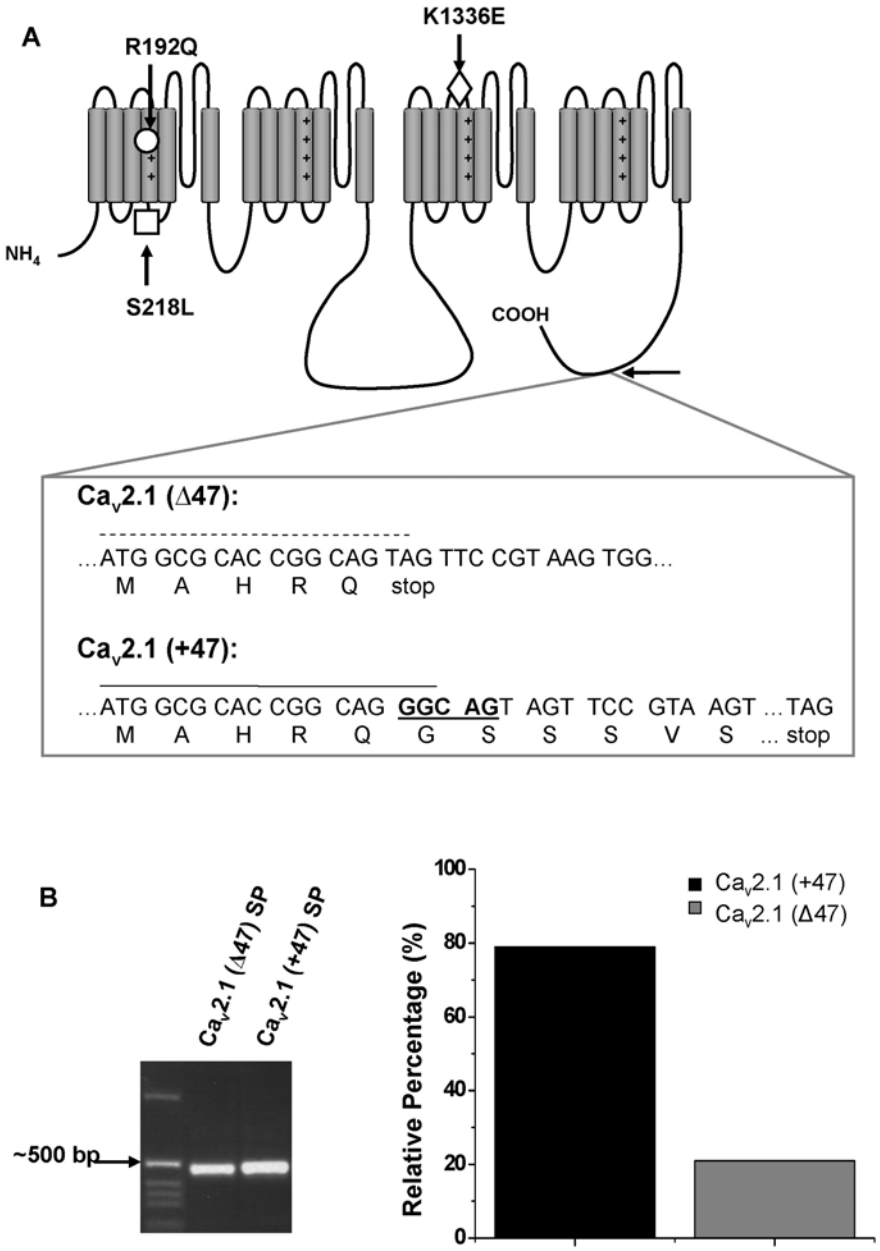

Figure 2.1: **Human $Ca_v2.1$ Ca^{2+} channel topology and splice-variant expression in human cortex.** **A**, schematic showing the location of the three FHM-1 mutations and the carboxyl terminal splice site in the human $Ca_v2.1$ channel. In the box below the channel diagram are partial sequences of the $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) variants at the exon 46/ exon 47 boundary. The pentanucleotide insertion is shown in bold for the $Ca_v2.1$ (+47) variant. **B**, the last ~ 1 Kb of the $Ca_v2.1$ carboxyl terminus was amplified from human cortical RNA and purified. Subsequently, splice-variant specific forward primers designed to exclusively bind either $Ca_v2.1$ (Δ47) or $Ca_v2.1$ (+47) transcripts were used in PCR reactions to generate an ~500bp fragment from the purified carboxyl fragments; $Ca_v2.1$ (Δ47)-SP and $Ca_v2.1$ (+47)-SP, respectively (dotted line is above the sequence that $Ca_v2.1$ (Δ47)-SP binds and the solid line is above the sequence that $Ca_v2.1$ (+47)-SP binds in A. Both splice-variant specific primers generated the expected product from the carboxyl PCR fragment of the $Ca_v2.1$ cDNA obtained from human cortex, verifying both $Ca_v2.1$ (Δ47) or $Ca_v2.1$ (+47) are present in human cortex. Products were verified by direct DNA sequencing and determined to be in relative proportions of 79% $Ca_v2.1$ (+47) and 21% $Ca_v2.1$ (Δ47) (bar graph); for protocol details see Experimental Procedures.

2.2.2 FHM-1 mutations exhibit differential effects on the voltage-dependent properties of $Ca_v2.1$ splice variants

Whole cell current analysis of transiently transfected cells showed that the wild-type $Ca_v2.1$ (Δ47) and wild-type $Ca_v2.1$ (+47) variants possess similar membrane potentials at which half the channels are activated (V_{50act} = -14.02 ± 1.49, and -15.08 ± 1.20, respectively) and similar membrane potentials at which half of the channels are inactivated ($V_{50inact}$ = -58.20 ± 2.04, and -62.07 ± 1.87, respectively; see Table 2.1 and Fig. 2.2). The K1336E, R192Q and S218L mutations have been previously reported to cause a hyperpolarizing shift in the current-voltage relationship relative to wild-type $Ca_v2.1$ channels (Melliti et al., 2003; Mullner et al., 2004; Cao and Tsien, 2005; Tottene et al., 2005; Weiss et al., 2008). Examining the FHM-1 mutations in the $Ca_v2.1$ +47 and Δ47 carboxyl tail splice variants we found differential effects. The K1336E $Ca_v2.1$ (+47) and R192Q $Ca_v2.1$ (+47) channels both exhibited a small but significant shift in V_{50act} relative to wild-type $Ca_v2.1$ (+47) channels (-21.53 ± 1.35 and -19.11 ± 1.11 vs. -15.08 ± 1.20, respectively; $p < 0.05$; ANOVA), while the S218L mutation had no significant affect on V_{50act} of $Ca_v2.1$ (+47) channels relative to wild-type (Table 1 and Fig. 2.2). In contrast, all three FHM-1 mutations caused large significant hyperpolarizing shifts in V_{50act} when expressed in $Ca_v2.1$ (Δ47) variant channels ($p < 0.001$; ANOVA; Table 1 and Fig. 2.2). Similar differential splice-dependent effects of the FHM-1 mutations were apparent in examining $V_{50inact}$. Figure 2.2 shows, that the R192Q and S218L mutations resulted in large (~15 – 17 mV) hyperpolarizing shifts in $V_{50inact}$ in the $Ca_v2.1$ (Δ47) variant relative to wild-type $Ca_v2.1$ (Δ47) channels ($p < 0.001$; ANOVA), while these same two FHM-1 mutations had a smaller effects on $Ca_v2.1$ (+47) variant channels ($p < 0.05$; ANOVA; Table 1). Interestingly, the K1336E mutation did not cause a significant change in $V_{50inact}$ in either splice-variant. Also, all three mutations had significantly different effects on kinetics of activation and inactivation as well as steepness of the curves for activation and inactivation (Table 2.1). Overall, these data indicate that the impact of individual FHM-1 mutations on $Ca_v2.1$ channel gating properties is differentially affected by the nature of the splice-variant background in which the mutation is expressed.

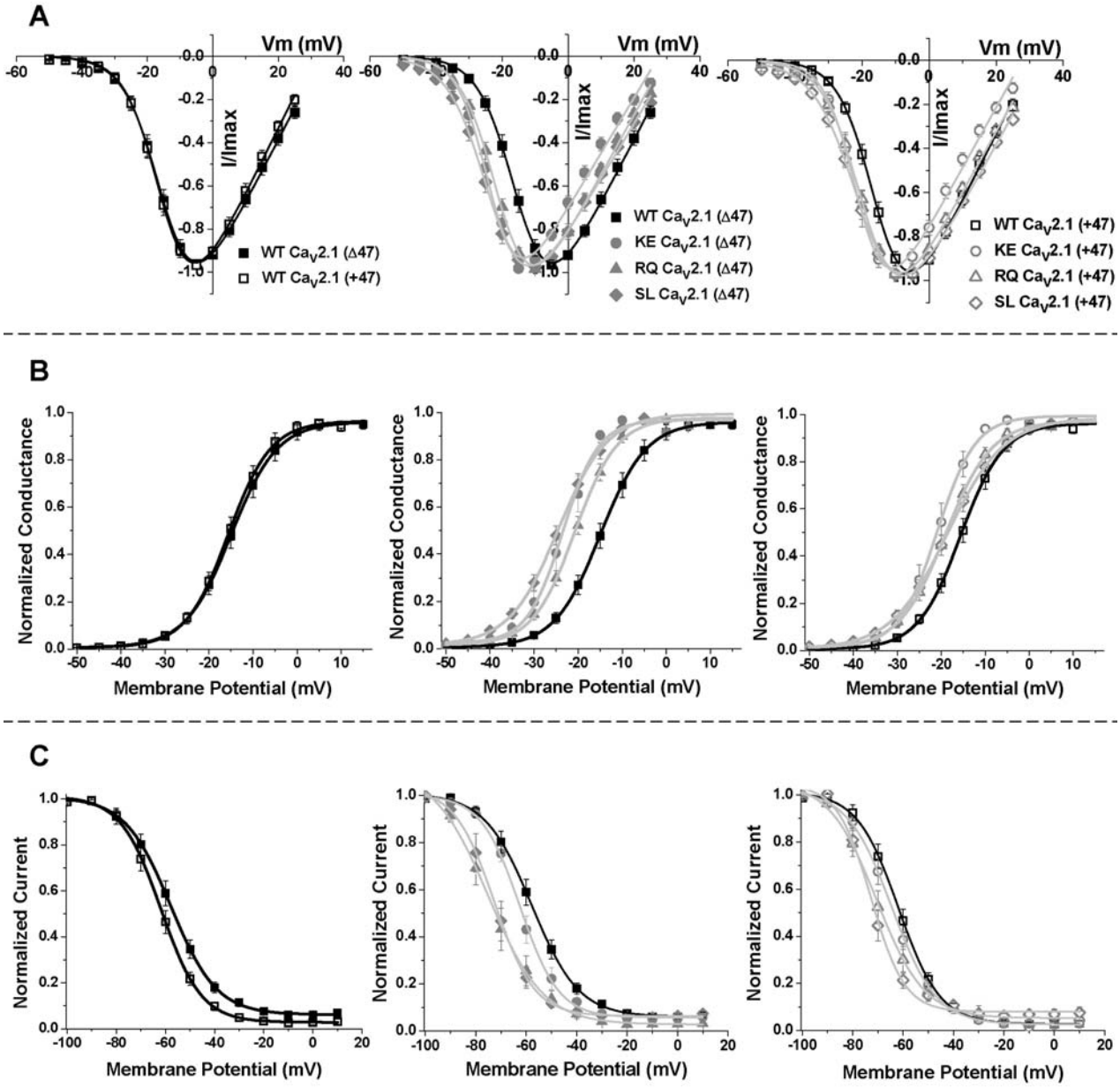

Figure 2.2: FHM-1 mutations differentially affect voltage-dependent properties of $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) variants.

A, shows the comparison between current-voltage relationships (IV-curves) for wild-type (WT; black squares), FHM-1 mutant K1336E (KE; grey circles), R192Q (RQ; grey triangles), and S218L (SL; grey diamond) in both the short $Ca_v2.1$ (Δ47) (filled symbols) and long $Ca_v2.1$ (+47) (open symbols) C-terminus splice variants. IV-curves for all constructs were determined from currents evoked during 90 ms square pulse depolarizations shown between -50 mV and +20 mV from a holding potential of -90 mV. **B**, conductance values were calculated from IV curves to obtain activation curves. **C**, steady-state inactivation curves were generated using a standard protocol in which 5s prepulse holdings of -100 to +10 mV were elicited prior to the 80 ms, 0 mV test pulse from a holding of -120 mV. Normalized current evoked during the test pulse is plotted vs. prepulse membrane potential. For complete statistics see Table 1 and for details of protocols see Experimental Procedures.

	V_{50} Activation (mV)	k Activation	τ_{act} (ms)	V_{50} Inactivation (mV)	k Inactivation
$Ca_V2.1$ (Δ47) Wild-type	-14.02 ± 1.49 (n = 16)	4.82 ± 0.27	1.42 ± 0.09	-58.20 ± 2.04 (n = 21)	7.03 ± 0.29
$Ca_V2.1$ (Δ47) K1336E	-24.12 ± 1.33 # (n = 10)	2.80 ± 0.20 #	1.16 ± 0.21	-62.27 ± 1.81 (n = 7)	7.23 ± 0.45
$Ca_V2.1$ (Δ47) R192Q	-20.84 ± 0.94 # (n = 16)	4.30 ± 0.19	1.31 ± 0.07	-73.41 ± 3.15 # (n = 11)	7.58 ± 0.57
$Ca_V2.1$ (Δ47) S218L	-24.10 ± 1.15 # (n = 9)	5.53 ± 0.39	0.97 ± 0.11 *	-75.07 ± 3.96 # (n = 10)	6.34 ± 0.62
$Ca_V2.1$ (+47) Wild-type	-15.08 ± 1.20 (n = 16)	4.33 ± 0.24	1.28 ± 0.07	-62.07 ± 1.87 (n = 16)	6.85 ± 0.38
$Ca_V2.1$ (+47) K1336E	-21.53 ± 1.35 * (n = 9)	3.10 ± 0.18*	1.33 ± 0.10	-65.02 ± 1.87 (n = 8)	8.39 ± 0.78
$Ca_V2.1$ (+47) R192Q	-19.11 ± 1.11 * (n = 16)	4.79 ± 0.26	1.43 ± 0.10	-70.27 ± 2.36 * (n = 16)	7.19 ± 0.47
$Ca_V2.1$ (+47) S218L	-18.08 ± 1.28 (n = 11)	6.26 ± 0.31 #	1.06 ± 0.08 *	-72.54 ± 1.70 * (n = 9)	5.92 ± 0.37

* $p<0.05$, #$p<0.001$

Table 2.1: Mean values for voltage-dependent activation and inactivation parameters.

The voltage at which half of the channels are in the activated state (V_{50act}) and inactivated state ($V_{50inact}$), and the steepness of the curves for activation (k_{act}) and inactivation (k_{inact}) were obtained by fitting the data with the Boltzmann equation for the indicated number of cells in parentheses. The kinetics of activation (τ_{act}) were obtained by fitting the maximum current trace from the IV curves with a single exponential. Asterisks (*) and number signs (#) indicate significant difference relative to wild-type with p-values less than either 0.05 or 0.001 (one-way ANOVA), respectively. N.A. = not applicable.

2.2.3 FHM-1 mutations exhibit differential effects on recovery from inactivation of $Ca_v2.1$ splice variants

Analysis of wild-type $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) variants showed different rates of recovery from inactivation for these $Ca_v2.1$ channel splice variants. Wild-type $Ca_v2.1$ (Δ47) channels exhibit faster rates of recovery (τ_1 = 0.669 ± 0.108 ms and τ_2 = 3.08 ± 0.614 ms) than the $Ca_v2.1$ (+47) variant channels (τ_1 = 0.760 ± 0.212 ms and τ_2 = 3.39 ± 0.835 ms). As a result, $Ca_v2.1$ (Δ47) channels show a significantly higher percentage of channels recovered at 7.5 s after inactivation relative to $Ca_v2.1$ (+47) channels (89.0 ± 1.8 vs. 77.3 ± 3.8 %, respectively; $p<0.05$; ANOVA) (Fig. 2.3 and Table 2.2).

Examining the effects of FHM-1 mutations in the $Ca_v2.1$ (Δ47) background, Figure 2.3 shows that the K1336E $Ca_v2.1$ (Δ47) and R192Q $Ca_v2.1$ (Δ47) variants exhibit a significant decrease in current recovered at 7.5 s relative to wild-type $Ca_v2.1$ (Δ47) channels (70.4 ± 3.7% and 83.3 ± 2.3% vs. 89.0 ± 1.8 %, respectively; $p<0.05$; ANOVA). Contrastingly, K1336E $Ca_v2.1$ (+47) and R192Q $Ca_v2.1$ (+47) channels showed increases in recovery relative to wild-type $Ca_v2.1$ (+47) channels (86.8 ± 1.7 and 87.0 ± 2.0 vs. 77.3 ± 3.8 %, respectively; $p<0.05$; ANOVA) (Fig. 2.3 and Table 2.2). The S218L mutation was found to increase the rate of recovery in both splice variants, however, only the S218L $Ca_v2.1$ (+47) channels showed a significant increase in recovery relative to wild-type $Ca_v2.1$ (+47) channels at 7.5 s (94.7 ± 1.5 vs. 77.3 ± 3.8 %, respectively; $p<0.001$; ANOVA) (Fig. 2.3 and Table 2.2).

Overall, in agreement with previous reports (Mullner et al., 2004; Tottene et al., 2005), we observed that both the K1336E and S218L mutations can cause significant changes to recovery from inactivation and show for the first time that the R192Q mutation also changes recovery from inactivation. Importantly, we also show that the quantitative effects of the FHM-1 mutations on channel function are dependant upon the nature of the $Ca_v2.1$ splice-variant. We also note that the differential effects of the mutations resulted in significant changes to the functional distinction observed between the two wild-type channel variants; that is, while the wild-type $Ca_v2.1$ (Δ47) channel variant recovered significantly faster than the wild-type $Ca_v2.1$ (+47) channel variant, the K1336E $Ca_v2.1$ (Δ47) channels recovered significantly slower than K1336E $Ca_v2.1$ (+47) channels. In addition, the R192Q and S218L mutations altered recovery

such that the R192Q $Ca_v2.1$ (Δ47) and R192Q $Ca_v2.1$ (+47) channels and S218L $Ca_v2.1$ (Δ47) and S218L $Ca_v2.1$ (+47) channels were not functionally distinct in this parameter (Fig. 2.3 and Table 2.2).

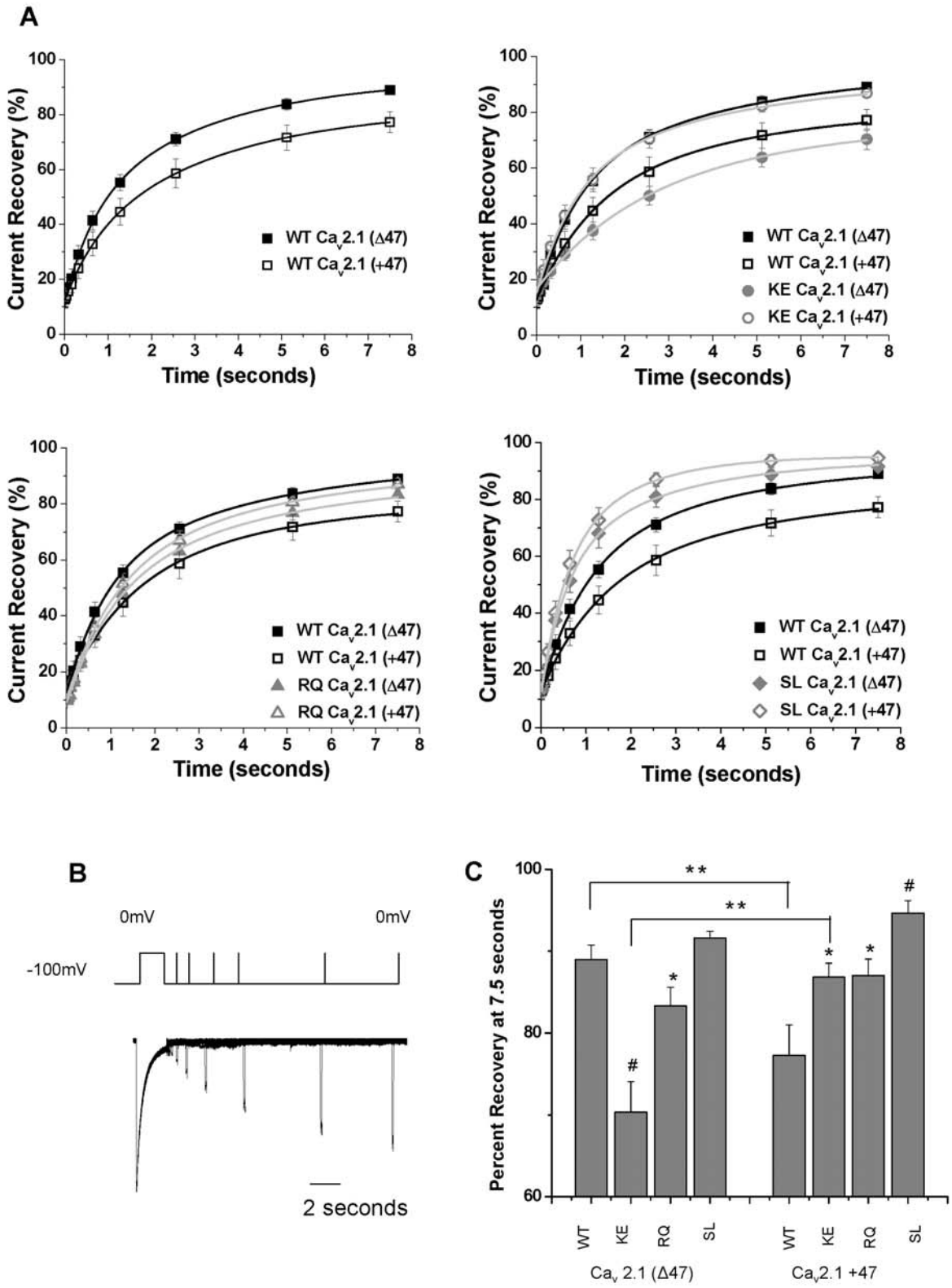
A
Current Recovery (%)
Time (seconds)
WT Ca$_v$2.1 (Δ47)
WT Ca$_v$2.1 (+47)
KE Ca$_v$2.1 (Δ47)
KE Ca$_v$2.1 (+47)
RQ Ca$_v$2.1 (Δ47)
RQ Ca$_v$2.1 (+47)
SL Ca$_v$2.1 (Δ47)
SL Ca$_v$2.1 (+47)
B
0mV
0mV
-100mV
2 seconds
C
Percent Recovery at 7.5 seconds
WT
KE
RQ
SL
Ca$_v$ 2.1 (Δ47)
Ca$_v$2.1 +47

Figure 2.3: Wild-type and FHM-1 mutant $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) variants exhibit different rates of recovery from inactivation.

A, graphs show percentage of the current recovered vs. time given to recover for all wild-type and FHM-1 mutated constructs. Recovery from inactivation was examined for wild-type (WT; black squares), FHM-1 mutant K1336E (KE; grey circles), R192Q (RQ; grey triangles), and S218L (SL; grey diamond) in both the short $Ca_v2.1$ (Δ47) (filled symbols) and long $Ca_v2.1$ (+47) (open symbols) C-terminus splice variants. **B**, shows a representative trace (capacitive currents removed for clarity) and the two pulse protocol used. The protocol consists of a 2 second, 0 mV prepulse followed by a 50 ms, 0 mV test pulse elicited after inter-pulse intervals between 10 ms and 7.5 s. Time constants were determined by fitting the average values for percent recovery with a single exponential or double exponential (values shown in Table 2). **C**, bar graph shows percent recovery at 7.5 s for all wild-type and FHM-1 mutant clones studied. Single asterisks and number signs indicate significant difference between mutant and wild-type of the same variant with p-values less than either 0.05 or 0.001 (one-way ANOVA), respectively. Double asterisks indicate significant difference between the Δ47 and +47 variants containing the same sequence (i.e. wild-type or mutant) with p-value less than 0.05 (one-way ANOVA).

	τ_1 (fast) (ms)	τ_2 (slow) (ms)	% recovery at 7.5 s
$Ca_V2.1$ (Δ47) Wild-type	0.669 ± 0.108	3.08 ± 0.614	89.0 ± 1.8 (n = 5)
$Ca_V2.1$ (Δ47) K1336E	2.89 ± 0.12	N.A.	70.4 ± 3.7 # (n = 8)
$Ca_V2.1$ (Δ47) R192Q	0.918 ± 0.188	4.97 ± 2.65	83.3 ± 2.3 * (n = 7)
$Ca_V2.1$ (Δ47) S218L	0.617 ± 0.095	2.71 ± 1.18	91.6 ± 0.8 (n = 5)
$Ca_V2.1$ (+47) Wild-type	0.760 ± 0.212	3.39 ± 0.835	77.3 ± 3.8 ** (n = 7)
$Ca_V2.1$ (+47) K1336E	0.776 ± 0.149	4.34 ± 2.31	86.8 ± 1.7 * (n = 5)
$Ca_V2.1$ (+47) R192Q	1.08 ± 0.200	5.78 ± 4.96	87.0 ± 2.0 * (n = 6)
$Ca_V2.1$ (+47) S218L	0.500 ± 0.119	1.71 ± 0.593	94.7 ± 1.5 # (n = 5)

* $p<0.05$, #$p<0.001$

Table 2.2: Time constant values and recovery from inactivation.

Time constants were determined by fitting the average percent recovery with a double exponential for all constructs except the $Ca_v2.1$ (Δ47) K1336E which was best fit with a single exponential. Percent recovery is a measure of the percentage of current evoked during the test pulse, given at 7.5 s after the prepulse, relative to the maximum current evoked during the prepulse. Asterisks and number signs indicate significant difference between mutant and wild-type of the same variant with p-values less than either 0.05 or 0.001 (one-way ANOVA), respectively. Double asterisks indicate significant difference between the $Ca_v2.1$ Δ47 and +47 variants containing the same sequence (i.e. wild-type or mutant) with p-value less than 0.05 (one-way ANOVA). Number of cells recorded for each clone is indicated in parenthesis. N.A. = not applicable.

2.2.4 FHM-1 mutations exhibit differential effects on inactivation of $Ca_v2.1$ splice variants during tonic depolarization

Wild-type $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) variants exhibit functional differences with regard to accumulation of inactivation during short (3.5 ms) pulses to +5 mV applied at a frequency of 25 Hz (Fig. 2.4). While $Ca_v2.1$ (Δ47) variant channels showed 95 ± 1.4 % of current remaining at the end of 25 pulses, $Ca_v2.1$ (+47) variant channels had 90 ± 0.9 % ($p < 0.05$; ANOVA) (Fig. 2.4C).

All three FHM-1 mutations examined significantly altered accumulation of inactivation (Fig. 2.4A); however, again the effects were contingent on the nature of the $Ca_v2.1$ variant in which mutations were expressed. The K1336E $Ca_v2.1$ (Δ47) and K1336E $Ca_v2.1$ (+47) channels had a significant increase in accumulation of inactivation and thus a lower percent of current remaining at the end of 25 pulses relative to wild-type $Ca_v2.1$ (Δ47) and wild-type $Ca_v2.1$ (+47) (88 ± 2.2 % and 84 ± 1.7 % vs. 95 ± 1.4 and 90 ± 0.9 %, respectively; $p < 0.05$; ANOVA) (Fig. 2.4C). The changes were such that the K1336E $Ca_v2.1$ (+47) and K1336E $Ca_v2.1$ (Δ47) variants had similar current remaining at the end of the repetitive stimulation and thus lacked the clear functional distinction observed between the wild-type channel variants.

In the context of the $Ca_v2.1$ (Δ47) variant background the R192Q mutation caused a significant increase in accumulation of inactivation relative to wild-type $Ca_v2.1$ (Δ47) (current remaining at the end of the 25 pulses = 90 ± 1.6% vs. 95 ± 1.4 %: $p < 0.05$; ANOVA). In contrast, R192Q $Ca_v2.1$ (+47) channels were similar to wild-type $Ca_v2.1$ (+47) channels (92 ± 1.3% vs. 90 ± 0.9 %) (Fig. 2.4C). As a result, similar to K1336E channels, the R192Q $Ca_v2.1$ (Δ47) and R192Q $Ca_v2.1$ (+47) channel variants lacked the clear functional distinction observed between the wild-type variants for this property.

The S218L mutation showed a large and significant increase in accumulation of inactivation and thus a lower percent of current remaining at the end of 25 pulses relative to both the wild-type $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) channel variants (81 ± 2.5% and 77 ± 1.7 % vs. 95 ± 1.4 and 90 ± 0.9 %, respectively; $p < 0.05$; ANOVA). Similar to that for R192Q and K1336E, a further overall effect of the

S218L mutation is to decrease the relative difference in current remaining observed between wild-type $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) variant channels.

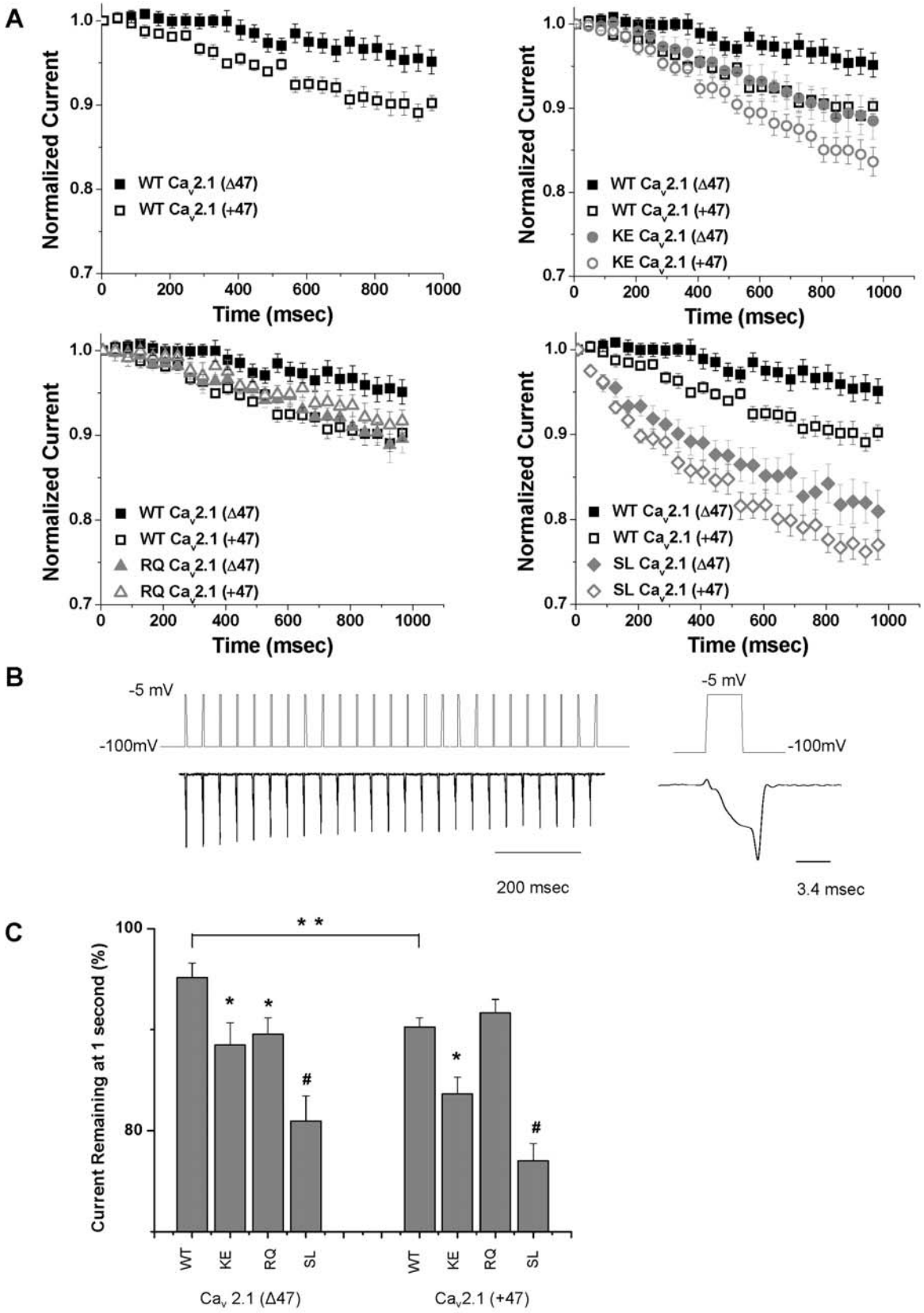
A
Normalized Current
Time (msec)
WT Ca$_v$2.1 (Δ47)
WT Ca$_v$2.1 (+47)
KE Ca$_v$2.1 (Δ47)
KE Ca$_v$2.1 (+47)
RQ Ca$_v$2.1 (Δ47)
RQ Ca$_v$2.1 (+47)
SL Ca$_v$2.1 (Δ47)
SL Ca$_v$2.1 (+47)
B
-5 mV
-100mV
200 msec
3.4 msec
C
Current Remaining at 1 second (%)
WT
KE
RQ
SL
Ca$_v$ 2.1 (Δ47)
Ca$_v$2.1 (+47)

Figure 2.4: Wild-type and FHM-1 mutant $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) variants exhibit different current decay during 25 Hz tonic depolarizations.

A, graphs show normalized current remaining vs. time after initial onset of depolarizations. Current decay was measured for wild-type (WT; black squares), FHM-1 mutant K1336E (KE; grey circles), R192Q (RQ; grey triangles), and S218L (SL; grey diamond) in both the short $Ca_v2.1$ (Δ47) (filled symbols) and long $Ca_v2.1$ (+47) (open symbols) C-terminus splice variants. **B**, to investigate current decay during repetitive stimulations we used 25 square pulses from a holding of -100 mV to a depolarizing potential of -5 mV for a duration of 3.4 ms. The test pulses were given at a rate of 25 Hz. Representative current trace and pulse protocol indicated at bottom (capacitive currents were compensated using a P/4 protocol), with single current response enlarged. **C**, bar graph shows the percent of current remaining at the end of 25 pulses for each clone. Single asterisks and number signs indicate significant difference between mutant and wild-type of the same variant with p-values less than either 0.05 or 0.001 (one-way ANOVA), respectively. Double asterisks indicate significant difference between the Δ47 and +47 variants containing the same sequence (i.e. wild-type or mutant) with p-value less than 0.05 (one-way ANOVA). Number of cells recorded for WT $Ca_v2.1$ (Δ47) (n = 14), WT $Ca_v2.1$ (+47) (n = 13), KE $Ca_v2.1$ (Δ47) (n = 15), KE $Ca_v2.1$ (+47) (n = 18), RQ $Ca_v2.1$ (Δ47) (n = 17), RQ $Ca_v2.1$ (+47) (n = 15), SL $Ca_v2.1$ (Δ47) (n =15), SL $Ca_v2.1$ (+47) (n = 14).

2.2.5 FHM-1 mutations exhibit differential effects on inactivation of $Ca_v2.1$ splice variants during bursts of depolarization

In addition to tonic depolarizations, neurons experience various frequencies of burst firing in which brief periods of tonic firing are interspersed with silent periods as the membrane potential drops below threshold (McCormick et al., 1985; Brumberg, 2002; Womack and Khodakhah, 2002; Fernandez et al., 2007; Shin et al., 2007). During the tonic firing periods $Ca_v2.1$ channels will inactivate and during silent periods they will have the opportunity to recover from inactivation. Based upon the above noted splice-variant changes in accumulation of inactivation and recovery from inactivation, we predicted that bursts of depolarization would also differentially affect wild-type and FHM-1 mutated $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) variant channels.

Figure 2.5 shows that wild-type $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) variants exhibit significant differences in the amount of current remaining at the end of five 25 Hz bursts given at 3.5 Hz. Current through the wild-type $Ca_v2.1$ (+47) variant decayed to 73 ± 3.4% by the end of the fifth burst while current through the wild-type $Ca_v2.1$ (Δ47) variant decayed to 88 ± 3.6% ($p < 0.05$; ANOVA) (Fig. 2.5C). The increased inactivation during the depolarizations and the slower recovery from inactivation of wild-type $Ca_v2.1$ (+47) variants discussed above likely contributed to the overall 15% decrease in current relative to the wild-type $Ca_v2.1$ (Δ47) variant.

Figure 2.5 shows that during burst firing the K1336E mutation in the $Ca_v2.1$ (Δ47) variant background results in an overall lower percentage of current remaining at the end of five bursts (70 ± 4.9 % vs. 88 ± 3.6%, respectively; $p < 0.05$; ANOVA) (Fig. 2.5C), likely resulting from the increased accumulation of inactivation and slowed recovery from inactivation of K1336E $Ca_v2.1$ (Δ47) channels relative to wild-type $Ca_v2.1$ (Δ47) (see Figs. 2.3 and 2.4). Contrastingly, the K1336E mutation in the $Ca_v2.1$ (+47) variant background did not show significant current decay relative to wild-type, likely due to the fact that although the K1336E $Ca_v2.1$ (+47) variant channels exhibit a small increase in accumulation of inactivation during tonic stimulation (Fig. 2.4), they also possess an increased rate of recovery from

inactivation (Fig. 2.3A). We note that unlike wild-type channel variants, the K1336E $Ca_v2.1$ (+47) and K1336E $Ca_v2.1$ (Δ47) variants did not differ significantly relative to one another

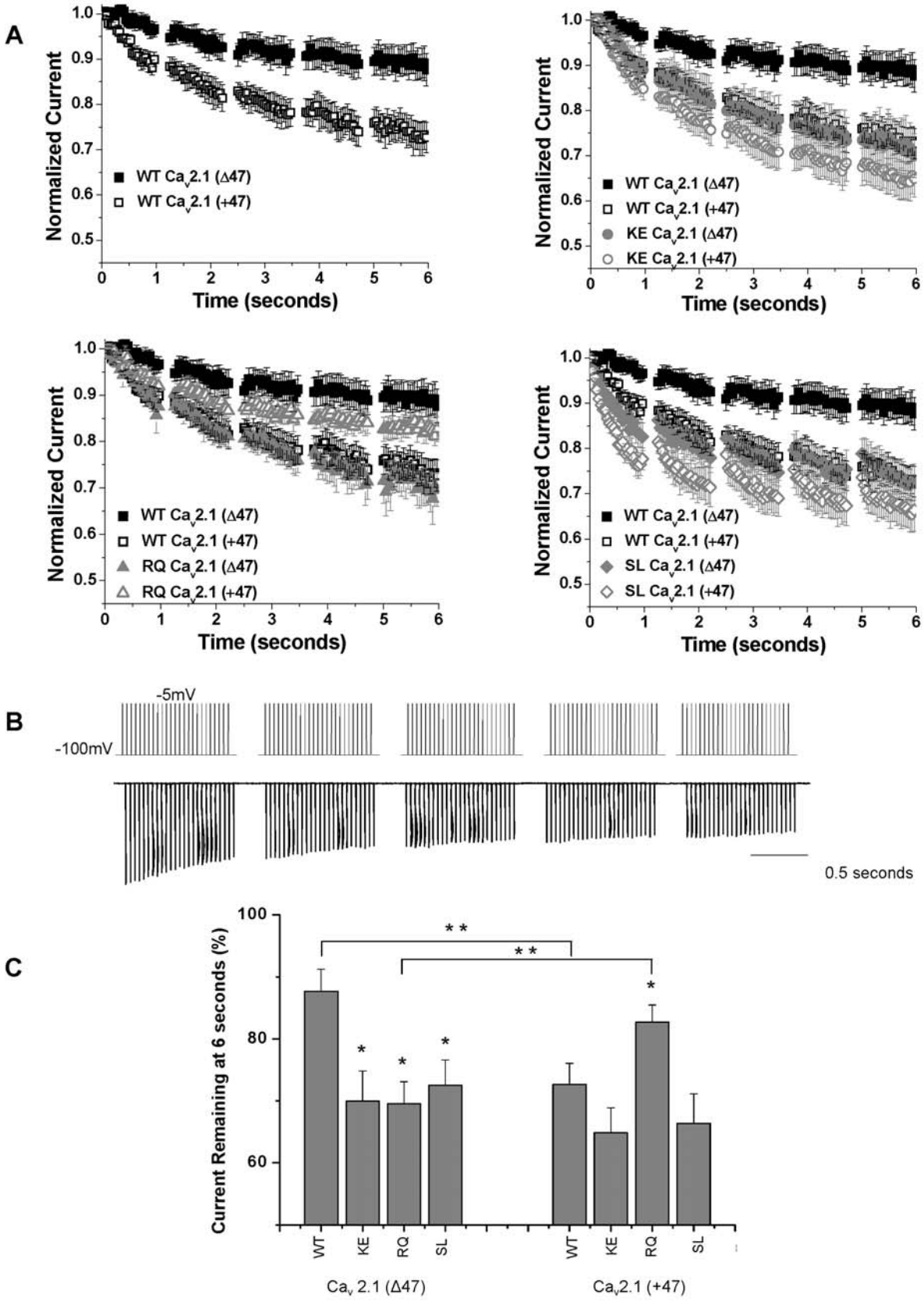
A
Normalized Current
Time (seconds)
WT Cav2.1 (Δ47)
WT Cav2.1 (+47)
KE Cav2.1 (Δ47)
KE Cav2.1 (+47)
RQ Cav2.1 (Δ47)
RQ Cav2.1 (+47)
SL Cav2.1 (Δ47)
SL Cav2.1 (+47)
B
-5mV
-100mV
0.5 seconds
C
Current Remaining at 6 seconds (%)
WT
KE
RQ
SL
Cav 2.1 (Δ47)
Cav2.1 (+47)

Figure 2.5: Wild-type and FHM-1 mutant $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) variants exhibit different current decay during bursts of depolarizations.

A, graphs show normalized current remaining vs. time after initial onset of depolarizations. Current decay was measured for wild-type (WT; black squares), FHM-1 mutant K1336E (KE; grey circles), R192Q (RQ; grey triangles), and S218L (SL; grey diamond) in both the short $Ca_v2.1$ (Δ47) (filled symbols) and long $Ca_v2.1$ (+47) (open symbols) C-terminus splice variants. **B**, to investigate current decay during bursts of repetitive stimulations we used five bursts of 25 square pulses to -5 mV for 3.4 ms from a holding of -100 mV; bursts given at 290 ms intervals (3.5 burst firing). Each burst contained 25 pulses at a rate of 25 Hz. Representative current trace indicated at bottom (capacitive currents were compensated using a P/4 protocol). **C**, bar graph shows the percent of current remaining at the end of 6 s for each clone. Single asterisks and number signs indicate significant difference between mutant and wild-type of the same variant with p-values less than either 0.05 or 0.001 (one-way ANOVA), respectively. Double asterisks indicate significant difference between the $Ca_v2.1$ Δ47 and +47 variants containing the same sequence (i.e. wild-type or mutant) with p-value less than 0.05 (one-way ANOVA). Number of cells recorded for WT $Ca_v2.1$ (Δ47) (n = 8), WT $Ca_v2.1$ (+47) (n = 7), KE $Ca_v2.1$ (Δ47) (n = 8), KE $Ca_v2.1$ (+47) (n = 10), RQ $Ca_v2.1$ (Δ47) (n = 9), RQ $Ca_v2.1$ (+47) (n = 7), SL $Ca_v2.1$ (Δ47) (n =8), SL $Ca_v2.1$ (+47) (n = 8).

Similar to that for the K1336E mutation in the Δ47 background, examination of burst firing effects on the R192Q mutation showed an increase in current decay during burst firing relative to wild-type $Ca_v2.1$ (Δ47) channels (70 ± 3.6% vs. 88 ± 3.6% current remaining, respectively; $p < 0.05$; ANOVA) (Fig. 2.5C). In contrast, the R192Q mutation in the +47 background resulted in a higher degree of current remaining compared to wild-type $Ca_v2.1$ (+47) channels (83 ± 2.7% vs. 73 ± 3.4% current remaining, respectively; $p < 0.05$; ANOVA). This may reflect the fact that R192Q $Ca_v2.1$ (+47) channels exhibit reduced inactivation during tonic firing and increased recovery from inactivation (see Figs. 2.3 and 2.4). We note that an overall effect concerning current decay during burst firing is for R192Q $Ca_v2.1$ (+47) channels to behave more similar to wild-type Δ47 channels and for R192Q $Ca_v2.1$ (Δ47) channels to behave more similar to the wild-type +47 variant.

Similar to that for the K1336E mutation, the S218L mutation only caused a significant current decay during the burst firing in the $Ca_v2.1$ (Δ47) variant background (73 ± 4.0% vs. 88 ± 3.6% current remaining at the end of five bursts, respectively; $p < 0.05$; ANOVA). Although the S218L mutation increased accumulation of inactivation substantially in both splice variants (see Fig. 2.4), S218L $Ca_v2.1$ (+47) channels had a larger increase in the rate of recovery from inactivation (Fig. 2.3) which likely slowed overall accumulation of inactivation during the burst firing. The S218L $Ca_v2.1$ (+47) and S218L $Ca_v2.1$ (Δ47) variants did not differ significantly relative to one another.

2.3 Discussion

2.3.1 FHM-1 mutations differentially affect biophysical properties of $Ca_v2.1$ splice variants

We report here that FHM-1 missense mutations confer differential effects on the biophysical properties of the $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) channel splice variants. Although the current-voltage relationships and steady-state properties of the two wild-type $Ca_v2.1$ splice variants are similar, all three FHM-1 mutations exhibited a greater hyperpolarizing shift when expressed in the $Ca_v2.1$ (Δ47) variant compared to the $Ca_v2.1$ (+47) variant (Fig. 2.2). In addition, we show for the first time that wild-type

$Ca_v2.1$ (Δ47) and wild-type $Ca_v2.1$ (+47) variants have both different kinetics of recovery from inactivation and accumulation of inactivation during tonic depolarization that are likely relevant to the differential response of channel variants during burst of depolarization (Figs. 2.3-2.5). It is known that $Ca_v2.1$ channels in different states possess alternative modes of gating that are reflected in biophysical properties at the whole cell current level (Fellin et al., 2004; Luvisetto et al., 2004). Furthermore, it has been shown that alternative splicing in the EF-hand region of the $Ca_v2.1$ carboxyl terminus can shift gating modes (Chaudhuri et al., 2007). It is therefore possible that wild-type $Ca_v2.1$ (Δ47) and wild-type $Ca_v2.1$ (+47) variants also have distinct gating modes that respond differently to FHM-1 mutations which are localized to voltage sensor regions (e.g. R192Q, S218L, K1336E). Detailed single channel analyses would be required to fully explore this hypothesis.

2.3.2 Differential effects of FHM-1 mutations on $Ca_v2.1$ splice variants may contribute to localized phenotype

Our findings provide the first suggestion for a potential role of $Ca_v2.1$ channel alternative splicing in FHM-1 pathophysiology and raise the notion that even though $Ca_v2.1$ channels are widely expressed in the central and peripheral nervous systems, point mutations can have greater or lesser functional affects on specific splice variants. Although the mechanism of FHM-1 pathophysiology is not completely resolved, the current opinion is that the migraine usually initiates with aura due to cortical spreading depression (CSD) which leads to headache pain through activation of the trigeminovascular pain pathway (Bolay et al., 2002; Pietrobon, 2005). In this regard, specific $Ca_v2.1$ variants within the cortex may have important roles in the onset of migraine attacks.

We show that the $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) variants are both expressed in whole human cortex (Fig. 2.1B), and that the three FHM-1 mutations all cause a greater hyperpolarizing shift in the voltage-dependence of activation in $Ca_v2.1$ (Δ47) channels relative to that for $Ca_v2.1$ (+47) variant channels. A hyperpolarizing shift in $Ca_v2.1$ channel activation has been suggested as an underlying mechanism of increased susceptibility to CSD and the initiation of migraine (van den Maagdenberg et al.,

2004; Pietrobon, 2007). CSD begins within small domains of the cortex and propagates outward from a focal point. Our data supports the notion that $Ca_v2.1$ splice variants with greater sensitivity to hyperpolarizing shifts in the voltage-dependence of activation (e.g., $Ca_v2.1$ (Δ47)) could result in cortical regions with greater susceptibility to CSD and migraine initiation. Conversely, the effects of FHM-1 mutations on other $Ca_v2.1$ splice variants (e.g., $Ca_v2.1$ (+47)), expressed elsewhere in the cortex or other brain regions may be below the threshold to initiate CSD and/or other pathological effects. Future exploration of the exact regional and cellular distributions of these and other $Ca_v2.1$ splice variants within the cortex and throughout the human brain using *in situ* hybridization and/or RT-PCR analyses will be necessary to fully understand the role of $Ca_v2.1$ splice variants in FHM-1 pathology.

2.3.3 Differential effects of FHM-1 mutations on $Ca_v2.1$ splice variants under different conditions may contribute to episodic nature of the phenotype

Our results examining tonic and burst firing patterns also suggests the possibility of differential effects of FHM-1 mutations on $Ca_v2.1$ channel splice variants under different firing conditions. This is most clearly seen with the S218L mutation in the $Ca_v2.1$ (+47) variant; during tonic depolarization current decay is significantly faster relative to wild-type, yet during burst firing the S218L $Ca_v2.1$ (+47) variant has similar current decay to wild-type channels after five bursts, likely due to rapid recovery from inactivation (see Figs. 2.4 and 2.5). On the other hand, in $Ca_v2.1$ (Δ47) variant channels the S218L mutation has significant effects on current decay under both tonic and burst firing conditions. Interestingly, certain initiating factors of FHM-1 attacks such as emotional stress (Ducros et al., 2001) are known to alter neuronal firing patterns in the brain (Weiss and Simson, 1988; McEwen, 2007). Although the exact firing conditions directly associated with precipitating factors of migraine are unknown, the episodic nature of the FHM-1 phenotype may in part be associated with changes in neuronal firing pattern and/or frequency that could be relevant to specific $Ca_v2.1$ splice variants expressed in localized brain regions.

2.3.4 The differential effects of mutations on Ca^{2+} channel function is likely multifaceted and important in all Ca^{2+} channelopathies

It is likely that there exists a complex relationship between channel missense mutations and disease mechanism. While we show alternative splicing at a single $Ca_v2.1$ splice-site can determine the functional impact of FHM-1 mutations, we recognize that across the entire brain many additional factors are likely to be involved in ultimately defining disease pathophysiology. These likely include the expression of multiple splice $Ca_v2.1$ variants with distinct combinations of alternative splicing as well as the interaction with different auxiliary subunits (Mullner et al., 2004) and other structural and regulatory proteins. Nonetheless, our results demonstrate the relevance of alternative splicing as an important factor in considering underlying disease molecular mechanisms and also the need for a comprehensive understanding of the splice-variant profile of $Ca_v2.1$ channels across brain regions and developmental stages as they might relate to FHM-1 pathology.

While in the present chapter we show that individual FHM-1 mutations can have differential effects on the biophysical properties of the short and long $Ca_v2.1$ channel splice variants, we predict this phenomenon is likely relevant to both other FHM-1 mutations and $Ca_v2.1$ variants and also to other types of Ca_v channels and Ca^{2+} channelopathies. Understanding the differential effects of channelopathy mutations on ion channel splice variants is likely to be important for interpreting results obtained in both heterologous and native systems, as well as for making inferences concerning disease mechanisms and phenotypes. Mutations in the $Ca_v1.1$ L-type channel are associated with hypokalemic periodic paralysis, the $Ca_v1.2$ L-type with Timothy syndrome, the $Ca_v1.4$ L-type with incomplete X-linked congenital stationary night blindness and X-linked cone-rod dystrophy, and the $Ca_v3.2$ T-type with idiopathic generalized epilepsy and autism spectrum disorder (reviewed in (Adams and Snutch, 2007)). Similar to FHM-1, many of these disorders exhibit phenotypes with episodic and/or developmentally specific attributes localized to a subset of regions or tissues that express the respective channels, and like $Ca_v2.1$, these channels also undergo alternative splicing that generates functionally distinct channel variants (reviewed in (Lipscombe et al., 2002; Lipscombe and Castiglioni, 2004)). The identification of specific

Ca_v splice variants involved in disease pathophysiology may also provide the opportunity for targeted therapeutic approaches. For example, while the $Ca_v2.2$ N-type channels have a central role in nociceptive signalling, distinct $Ca_v2.2$ splice variants are involved in the transmission of specific types of pain and has led to new strategies for splice-variant-specific targeting in pain therapy (Altier et al., 2007).

2.4 Experimental Procedures

2.4.1 Site-directed mutagenesis

Standard PCR-based in vitro mutagenesis was performed using the Pfu Turbo DNA Polymerase (Stratagene, La Jolla, CA), 10 mM dNTPs (Invitrogen) and paired forward and reverse mutagenesis primers (Zoller and Smith, 1984). The human $Ca_v2.1$ long (+47) (isoform 2) (NCBI accession number NM_023035.1) cloned in pcDNA 3.1 Zeo (+) was used as the source for the generation of the wild-type short human $Ca_v2.1$ (Δ47) (isoform 1) cDNA (the other known six splice sites are: Δ10A, 16+/17+, -VEA, -NP, EFa, 43+/44+). Paired forward and reverse primers were designed to adhere to the C-terminus of the $Ca_v2.1$ isoform 2 at the exon 46/47 boundary nucleotide number 6784 and removed the GGCAG pentanucleotide sequence creating the premature stop in exon 47 ($Ca_v2.1$ (Δ47)). Both $Ca_v2.1$ splice-variant cDNAs were used in site-directed mutagenesis reactions to generate human $Ca_v2.1$ K1336E, R192Q and S218L mutants in the short and long variants; paired forward and reverse primers were designed to convert codon 1336 from AAA to GAA, codon 192 from CGG to CAG, and codon 218 from TCG to TTA. The integrity of all constructs generated through site-directed mutagenesis were verified by direct DNA sequencing.

2.4.2 Cell culture and transfection

Human embryonic kidney (HEK 293) cells were grown in standard Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (heat inactivated) and 50 U/ml penicillin-50 μg/ml streptomycin. Cells were incubated at 37°C in a humidified incubator with 95% atmosphere and 5% CO_2

and grown to 8-15% confluency for transfection. HEK 293 cells were transiently transfected with either wild-type human $Ca_v2.1$ (Δ47) or $Ca_v2.1$ (+47), or mutant K1336E $Ca_v2.1$ (Δ47), R192Q $Ca_v2.1$ (Δ47), S218L $Ca_v2.1$ (Δ47) or K1336E $Ca_v2.1$ (+47), R192Q $Ca_v2.1$ (+47), S218L $Ca_v2.1$ (+47) in combination with Ca^{2+} channel auxiliary subunits β4, $\alpha_2\delta_1$, and the CD8 marker plasmid in a 1:1:1:0.25 molar ratio using Lipofectamine (Invitrogen, La Jolla, CA). To ensure accurate comparisons, transfections were performed at the same time and electrophysiological recordings alternated within the same day for all channel types.

2.4.3 Electrophysiological recordings

On the second day after transfection, macroscopic Ba^{2+} currents where recorded at room temperature using the whole-cell patch-clamp technique (Hamill et al., 1981). The internal pipette solution used contained 105 mM CsCl, 25 mM TEACl, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES and 5 mM ATP (pH 7.2 with CsOH); external: 5 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM TEACl, 10 mM glucose and 87.5 mM CsCl (pH 7.4 with TEAOH). Patch pipettes (borosilicate glass BF150-86-10; Sutter Instrument Company, Novato, CA) were made using a horizontal puller (P-87; Sutter Instruments Company) and fire polished using a microforge (Narishige, Tokyo, Japan), with resistances typically of 3 to 5 MΩ when containing internal solution. External solution bath was connected to ground with a 3M KCl agar bridge. Whole cell currents were recorded and filtered at 2-5 kHz bandwidth using an Axopatch 200A amplifier and monitored and stored on a personal computer running pClamp software package version 9. Sampling frequencies were between 2 and 10 kHz. Recordings were analyzed using Clampfit 9 and figures, fittings and statistics (ANOVA) were made using the software program Origin version 7.5 (OriginLab Corp., Northampton, MA).

2.4.4 Recording protocols and data analysis

Current-voltage relationships were determined by measured currents obtained using a series of 90 millisecond depolarization pulses applied from a holding potential of -90 mV to membrane potentials from -50 mV to +45 mV, increasing by 5 mV increments. Current-voltage relationships were fitted, and IV curves generated, using a modified Boltzmann equation: $I= (G_{max}*(V_m-E_r))/ (1+\exp ((V_m-V_{50})/k))$, where Gmax is the maximum slope conductance, V_m is the test potential, E_r is the extrapolated reversal potential, V_{50} is the half-activation potential, and k reflects the slope of the activation curve. Activation curves were constructed by calculating conductance from the IV curves and plotting the normalized conductance as a function of the membrane potential. The data were fit with the Boltzmann equation: $G/Gmax = A2 + (A1-A2)/ (1 + \exp ((V_m-V_{50})/k))$, where A1 is minimum normalized conductance, A2 is maximum normalized conductance, V_m is the test potential, V_{50} is the half-activation potential, and k reflects the slope of the activation curve (goodness of fit had R^2 values ≥ 0.998).

Voltage-dependence of inactivation was analyzed using depolarizations to 0 mV for 80 ms following 5 s prepulses ranging from -100 to +10 mV at 10 mV increments (holding potential of -120 mV). Steady state inactivation curves were constructed by plotting the maximum normalized current during the test pulse as a function of the prepulse potential. The data were fit with the Boltzmann equation: $I/Imax = A2 + (A1-A2)/ (1 + \exp ((V_m-V_{50})/k))$, where A1 is minimum normalized current, A2 is the maximum normalized current, V_m is the test potential, V_{50} is the half-inactivation potential, and k reflects the slope of the inactivation curve (goodness of fit had R^2 values ≥ 0.998).

The kinetics of activation (τ_{act}) were determined from currents obtained from the IV protocol. Current traces were fit with a standard single exponential equation: $I = A*\exp (-t/\tau)$, where A is the amplitude of the current, and τ is the time constant.

Recovery from inactivation was determined using a double-pulse protocol. The first depolarization was to 0 mV for 2 s (the prepulse), followed by a return to the holding potential of -100 mV for variable lengths between 10 ms and 7.5 s. At the end of the variable repolarization period, a second 0 mV (the test pulse) was given for 50 ms. The time interval between sweeps was a total of 1 minute to

ensure maximum recovery between sweeps. All traces were normalized to the maximum current during the prepulse for each sweep. The peak current from the test pulse was plotted as a percentage of maximum prepulse current vs. repolarization time. Average traces were fit with either a single or double exponential equation (goodness of fit had R^2 values ≥ 0.998).

Current decay during a tonic depolarization was examined using a 25 Hz train of 25 square pulses from a holding of -100 mV to a depolarizing potential of -5 mV for 3.4 ms. Current decay curves were generated by plotting normalized maximum current during the test pulses as a function of the time of pulse onset. Current decay during bursts of depolarization was examined using square pulses to -5 mV for 3.4 ms from a holding of -100 mV. Five bursts were given with 290 ms intervals (3.5 Hz burst firing). Each burst contained 25 pulses at a rate of 25 Hz. Current decay curves were generated by plotting normalized maximum current during the test pulses as a function of the time of pulse onset.

2.4.5 RT-PCR of $Ca_v2.1$ carboxyl-terminal region from human cortex RNA

Prior to reverse transcription, 1 µg total RNA from human cortex (Clontech; 636561) was treated with 1X DNase I reaction buffer and 1 unit DNase I (Invitrogen) in a final volume adjusted to 10 µL using sterile DEPC-treated H_2O. Following a 15 minute incubation period at room temperature, the reaction was inactivated by adding 1 µL of 25 mM EDTA and heating at 65ºC for 10 minutes. cDNA synthesis was performed using Superscript II Reverse Transcriptase (Invitrogen) following manufacturer's instructions with slight modification. A ~1.1Kb nucleotide fragment of the carboxyl end of the $Ca_v2.1$ channel was amplified from the human cortex cDNA using standard PCR. The reaction mixture consisted of 3% DMSO, 1X Phusion enzyme buffer, 0.4 pmol/µL of forward and reverse primers, 0.2 mM dNTPs, 1 µL of cortex cDNA, and 1 unit of Phusion enzyme in a final volume of 25 µL. The forward primer ($^{5'}$GGCACATGGAGTCCGGAACA $^{3'}$) corresponds to nucleotide position 6130 and the reverse primer ($^{5'}$GGTAGTAGCCATGGTGCC$^{3'}$) to nucleotide position 7211 of the human $Ca_v2.1$ α_1 subunit (NCBI accession number NM_023035.1). The cycling profile included an initial activation step of 98^{o}C for 30s followed by 30 cycles of 98^{o}C for 30s, 65^{o}C for 30s, 72^{o}C for 2.5 minutes and a final extension period of

72°C for 10 minutes. As positive controls, the same PCR reaction was performed on human $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) cDNA plasmids to demonstrate unbiased amplification. PCR reactions using primers for tubulin were used to verify the success of RT reactions. As negative controls, identical PCR reactions without template and containing no RT were performed. To analyze individual $Ca_v2.1$ amplified carboxyl terminal transcripts, PCR products were ligated into pGEMT-Easy (Promega) and then transformed into competent XL-1 *E.coli* bacterial cells. Bacteria containing PCR inserts were identified by blue-white screening and collected and subject to $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) specific PCR reactions. The reverse primer ($^{5'}$GGTAGTAGCCATGGTGCC$^{3'}$) was used for both $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47) specific PCR reactions. Forward primer ($^{5'}$ATGGCGCACCGGCAGTA$^{3'}$) and ($^{5'}$CATGGCGCACCGGCAGGG$^{3'}$) were designed to specifically amplify $Ca_v2.1$ (Δ47) and $Ca_v2.1$ (+47), respectively. All PCR products were run on a 1% agarose gel. In determining the percentage of each variants, only colonies positive for $Ca_v2.1$ (Δ47) and negative for $Ca_v2.1$ (+47), and visa versa, were included and ambiguous results discarded. Direct DNA sequence determination of several representative clones confirmed both the veracity of the splice-variant specific PCR reactions and the identity of the cloned PCR products.

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3 CONTRIBUTION OF Ca^{2+}-DEPENDENT FACILITATION TO SHORT-TERM SYNAPTIC PLASTICITY REVEALED BY CONGENITAL MIGRAINE MUTATIONS IN THE $Ca_v2.1$ Ca^{2+} CHANNEL*

3.1 Introduction

Short-term facilitation of synaptic release has historically been attributed to enhanced vesicle release resulting from the accumulation of intracellular Ca^{2+} in presynaptic terminals during repetitive APs, in which the build-up of residual Ca^{2+} enhances binding to CaS proteins that directly mediate vesicle fusion and transmitter release (Zucker and Regehr, 2002). However, there is some evidence that residual Ca^{2+} can interact with other CaSs independent of the release machinery to enhance presynaptic Ca^{2+} currents and contribute towards synaptic facilitation, although the exact underlying mechanism remains uncertain (Forsythe, 1994; Borst and Sakmann, 1998; Cuttle et al., 1998; Forsythe et al., 1998; Tsujimoto et al., 2002; Rousset et al., 2003; Inchauspe et al., 2004; Muller et al., 2008). In a recent study using a model system, Mochida and collaborators demonstrated that CDF and CDI of $Ca_v2.1$ channels via CaS protein interaction with the IQ-like and CBD domains in the $Ca_v2.1$ C-terminus can be a mechanism to induce short-term synaptic facilitation and rapid synaptic depression, respectively (Mochida et al., 2008). Whether similar forms of CDF and/or CDI of $Ca_v2.1$ channels represent crucial mechanisms of synaptic plasticity at intact central synapses has not been reported.

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In the current study, we explored the effects of FHM-1 missense mutations on CDF and CDI of the $Ca_v2.1$ channel. FHM-1 is a congenital, autosomal dominant form of migraine with aura, typified by hemiparesis and linked to missense mutations in the CACNA1A gene encoding the $Ca_v2.1$ α_1-subunit that conducts P/Q-type Ca^{2+} currents (Ophoff et al., 1996). Biophysically, FHM-1 missense mutations result in an overall gain-of-function $Ca_v2.1$ channel phenotype as a result of an underlying shift in channel gating allowing increased Ca^{2+} influx at lower membrane potentials (Hans et al., 1999; Tottene et al., 2002). CDF and CDI are robust forms of $Ca_v2.1$ channel modulation mediated by calmodulin and other Ca^{2+} sensor proteins which interact with the $Ca_v2.1$ carboxyl terminus in bipartite regulatory processes; CDF is mediated by a local rise in Ca^{2+} and CDI through a global rise in Ca^{2+} (Lee et al., 1999; Lee et al., 2000; DeMaria et al., 2001; Lee et al., 2002; Tsujimoto et al., 2002; Lee et al., 2003; Chaudhuri et al., 2004; Chaudhuri et al., 2005; Few et al., 2005; Chaudhuri et al., 2007). The underlying mechanisms of CaM mediated CDF is also attributed to changes in channel gating (Chaudhuri et al., 2007) and it was of interest to examine whether FHM-1 mutations affect these important modulatory properties of $Ca_v2.1$ channels and to explore physiological implications using transgenic models.

We found that FHM-1 gain-of-function missense mutations significantly occlude CDF in both recombinant and native systems and correlate with a reduction in short-term synaptic facilitation. Collectively, the data provide the first evidence that FHM-1 mutations directly affect the Ca^{2+}-dependent regulation of $Ca_v2.1$ channels and support the notion that selective Ca^{2+}-dependent regulation of presynaptic $Ca_v2.1$ channels may underlie several key aspects of short-term plasticity at the PF-PC synapse in cerebellum.

3.2 Results

3.2.1 FHM-1 mutations occlude CDF and CDI of recombinant human $Ca_v2.1$ channels

CaM mediated CDF and CDI of $Ca_v2.1$ channels have been well characterized using human recombinant $Ca_v2.1$ channels expressed in HEK cells (along with auxiliary subunits β_{2a} and $\alpha_2\delta_1$) and

allows for clear isolation and measurement of CDF and CDI (Lee et al., 1999; DeMaria et al., 2001). Consistent with previous findings, human recombinant wild-type $Ca_v2.1$ channels transiently expressed in HEK cells showed typical CDF with prepulse dependent relative facilitation when Ca^{2+} was used as the charge carrier (Fig. 3.1A, black squares) (all clones are the $Ca_v2.1$ (Δ47) splice-variant). Currents elicited by a test pulse showed a rapid initial activation with a normal gating mode and then slowly transitioned to a facilitated mode as Ca^{2+} entered (Fig. 3.1E, grey traces). In contrast, an applied prepulse evoked Ca^{2+} entry that rendered channels in the facilitated mode of gating such that currents evoked by an ensuing test pulse lacked the slower component and displayed instead a fast activation rate (Fig. 3.1E, black traces) (facilitated mode being an enhancement of P_o over the basal level in the normal gating mode (Chaudhuri et al., 2007)). When Ba^{2+} was used as the charge carrier, channels opened directly into the normal gating mode and did not transition to the facilitated state, regardless of the prepulse potential (Fig. 3.1A; grey symbols). A measure of "pure" CDF was calculated by subtracting relative facilitation determined using Ca^{2+} as the charge carrier from relative facilitation when using Ba^{2+} (Fig. 3.1A; denoted *g*) (Chaudhuri et al., 2004). The FHM-1 mutations, R192Q and S218L, represent distinct ends of the clinical spectrum with patients possessing the R192Q alteration exhibiting a mild, pure FHM-1 without other neurological symptoms (Ophoff et al., 1996; Ducros et al., 2001), whereas patients with the S218L mutation have an associated severe clinical migraine phenotype most often associated with ataxia or cerebellar symptoms (Fitzsimons and Wolfenden, 1985; Kors et al., 2001; Chan et al., 2008). Figure 3.1 shows that channels containing either R192Q (Fig. 3.1A; triangles) or S218L (Fig. 3.1A; diamonds) mutations precluded CDF across multiple prepulse potentials, and overall significantly reduced pure CDF relative to wild-type channels (Fig. 3.1B).

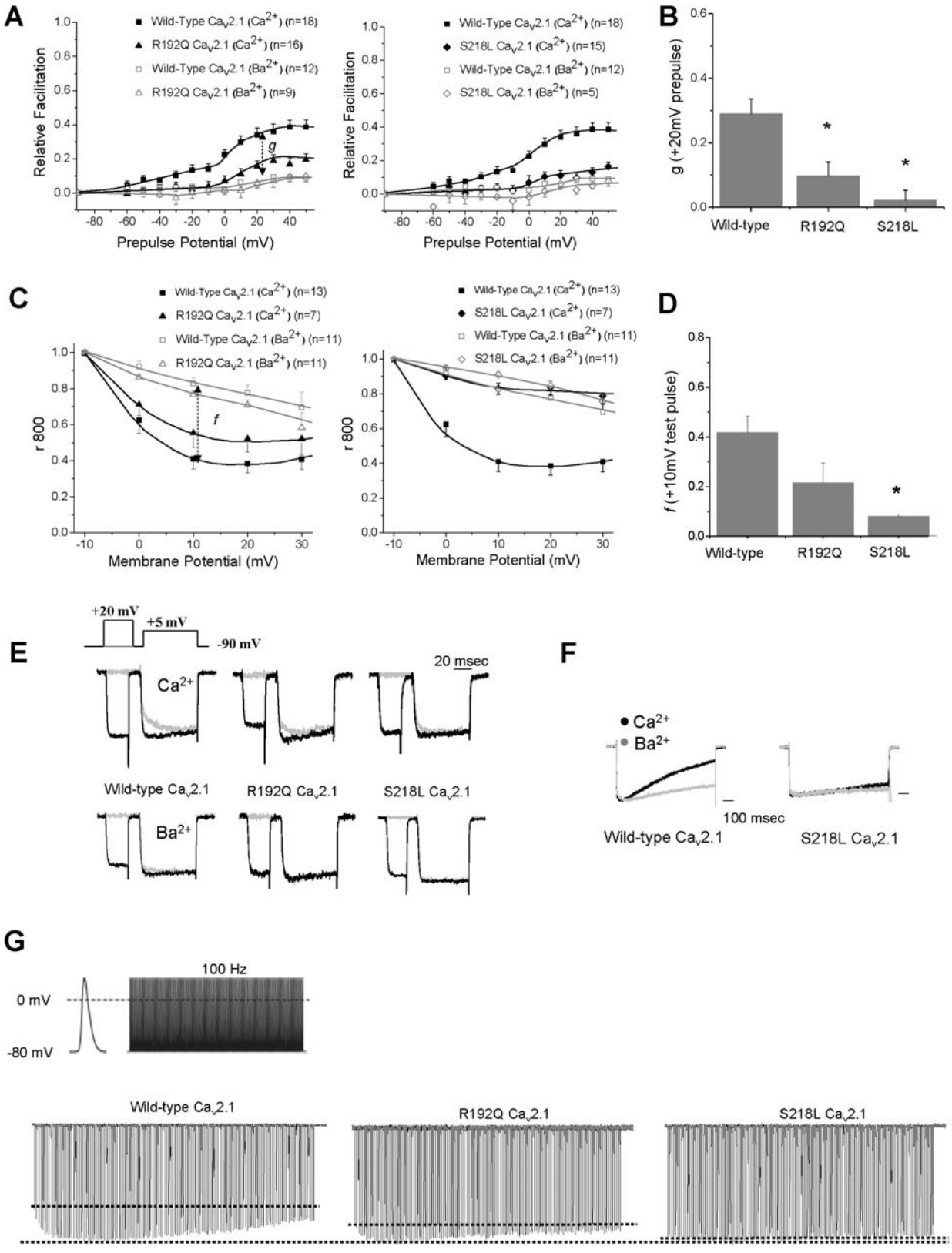
A
Wild-Type $Ca_V2.1$ (Ca^{2+}) (n=18)
R192Q $Ca_V2.1$ (Ca^{2+}) (n=16)
Wild-Type $Ca_V2.1$ (Ba^{2+}) (n=12)
R192Q $Ca_V2.1$ (Ba^{2+}) (n=9)
Relative Facilitation
Prepulse Potential (mV)
g
S218L $Ca_V2.1$ (Ca^{2+}) (n=15)
S218L $Ca_V2.1$ (Ba^{2+}) (n=5)
B
g (+20mV prepulse)
Wild-type
R192Q
S218L
C
r 800
Membrane Potential (mV)
f
D
f (+10mV test pulse)
E
+20 mV
+5 mV
-90 mV
20 msec
Ca^{2+}
Ba^{2+}
Wild-type $Ca_V2.1$
R192Q $Ca_V2.1$
S218L $Ca_V2.1$
F
100 msec
G
100 Hz
0 mV
-80 mV

Figure 3.1: The R192Q and S218L FHM-1 mutations occlude Ca^{2+}-dependent modulation of human recombinant $Ca_v2.1$ channels.

A, Using the paired-pulse protocol previously described (Chaudhuri et al., 2004), we show both the R192Q and S218L FHM-1 mutations reduced CDF across several prepulse potentials shown as relative facilitation vs. prepulse potential; results are means ± s.e.m. **B,** Pure CDF (the difference between relative facilitation obtained using 5 mM Ca^{2+} as the charge carrier minus relative facilitation obtained using 5 mM Ba^{2+}; *g*) (Chaudhuri et al., 2004) following a +20 mV prepulse compared to wild-type (*g* = 0.290±0.046) was significantly reduced by the R192Q (*g* = 0.097±0.042) and S218L (g = 0.023±0.031) mutations. **C,** Using 1 s square test-pulses between -10 and +30 mV, we show current remaining at 800 ms (r800) across several prepulse potentials was reduced by both FHM-1 mutations; results are means ± s.e.m. **D,** Comparison of pure CDI (the difference between r800 values obtained using 5 mM Ca^{2+} as the charge carrier minus r800 obtained using 5 mM Ba^{2+}; *f*) shows that relative to wild-type (*f* = 0.418±0.067), the reduction by R192Q was modest (*f* = 0.214±0.082) although S218L caused a significant reduction (*f* = 0.083±0.038). **E,** Paired-pulse protocol used, and representative traces for peak CDF obtained following a +20 mV prepulse for wild-type, R192Q and S218L $Ca_v2.1$ channels using Ca^{2+} (top) and Ba^{2+} (bottom) as charge carrier; traces normalized to the end of the test pulse. **F,** Representative traces in which traces are normalized to the peak of a +10 mV test pulse. **G,** APW used and representative traces for wild-type, R192Q and S218L $Ca_v2.1$ channels. First hashed line represents the level of Ca^{2+} response during the first action potential, and the second line represents the peak (maximum facilitation). 100 Hz APW used was previously derived from action potentials recorded in the calyx of Held (Borst and Sakmann, 1998; Patil et al., 1998). *n* refers to the number of cells recorded. All statistics were performed using a one-way ANOVA. Asterix denotes $p<0.05$.

The effect of FHM-1 mutations on CDI of exogenous $Ca_v2.1$ channels was tested using a 1 s test pulse to varying potentials in Ca^{2+} and Ba^{2+}. Wild-type $Ca_v2.1$ channels showed a typical CDI characterized by faster inactivation when Ca^{2+} was used as the charge carrier (Fig. 3.1C; black squares) relative to Ba^{2+} (Fig. 3.1C; grey, open squares) (Fig. 3.1F; left panel) (DeMaria et al., 2001; Chaudhuri et al., 2005). A measure of pure CDI was determined by subtracting currents obtained using Ca^{2+} as the charge carrier from currents obtained using Ba^{2+} (Fig. 3.1C; denoted *f*, and Fig. 3.1D) (Chaudhuri et al., 2004). Although the R192Q mutation modestly reduced CDI relative to that of wild-type (Fig. 3.1C; triangles), the more severe S218L mutation (Fig. 3.1C; diamonds) completely removed the ability of channels to undergo CDI (Fig. 3.1D and F, right panel). Importantly, because of its reliance on global Ca^{2+} levels (unlike that for CDF), CDI depends on $Ca_v2.1$ current density (Soong et al., 2002). While the R192Q mutation has little affect on current density, the S218L mutation causes a large reduction in current density (Tottene et al., 2005) (and see Fig. 3.2 for similar findings). As such, at the mechanistic level the reduced ability of mutant channels to undergo CDI is likely, at least in part, a result of reduced current density.

While rectangular depolarizations allow for optimal biophysical resolution of CDF and CDI (Fig. 3.1), it was also important to test the effects of FHM-1 mutations under conditions more resembling neuronal firing, such as APWs (DeMaria et al., 2001). In response to application of a 100 Hz APW, wild-type $Ca_v2.1$ channels displayed typical CDF and CDI that shaped the Ca^{2+} currents; CDF caused an initial facilitation of Ca^{2+} currents during the first few APs, and CDI caused a cumulative reduction as the APWs continued to be applied (Fig. 3.1G) (Chaudhuri et al., 2004; Chaudhuri et al., 2007). The FHM-1 R192Q and S218L mutations both strongly suppressed the dynamics of the response to APWs, consistent with their effects on CDF and CDI described above (Fig. 3.1G) (see Fig. 3.2 for average responses and responses obtained using Ba^{2+} as the charge carrier).

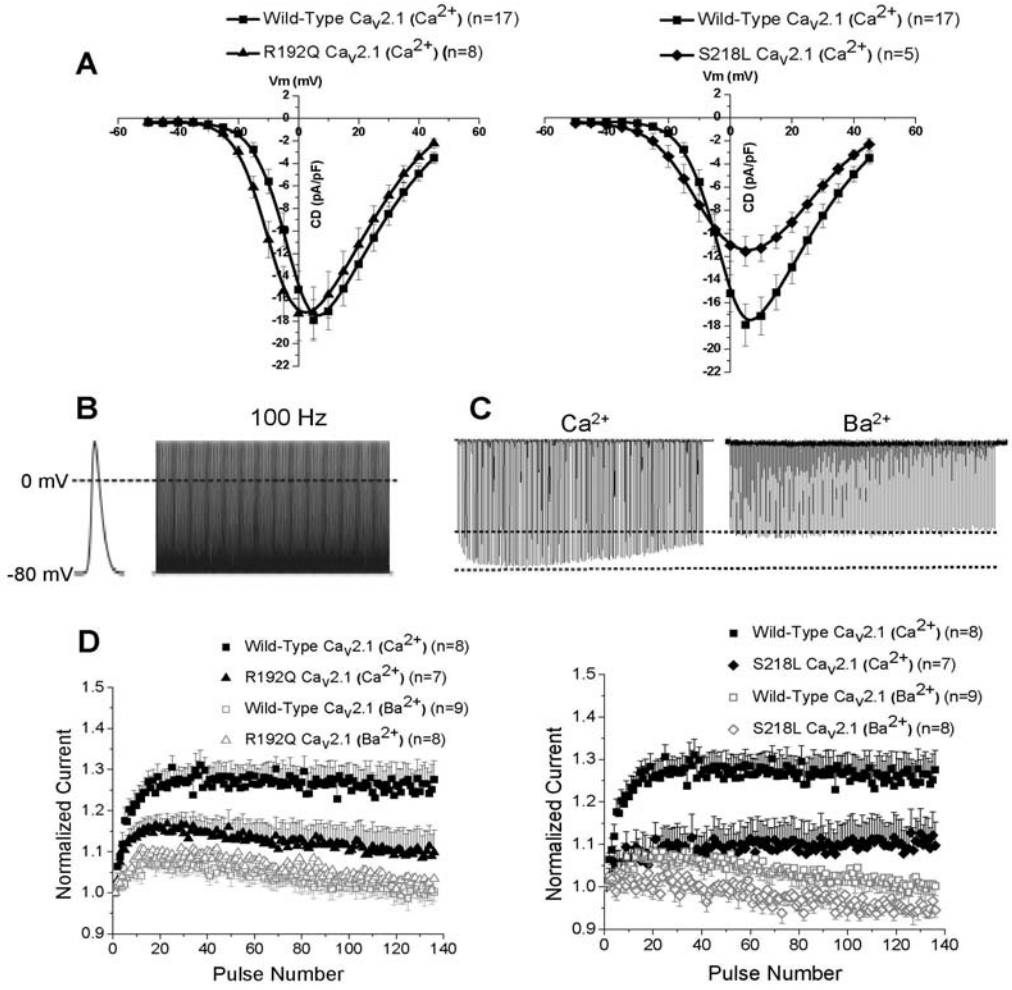

Figure 3.2: The effects of the R192Q and S218L mutations on current density and on CDF and CDI during 100 Hz APWs in Ca^{2+} and Ba^{2+}

A, Current density (CD) for wild-type, R192Q and S218L human recombinant $Ca_v2.1$ channels. **B,** 100 Hz APW derived from APs recorded in the calyx of Held (Borst and Sakmann, 1998; Patil et al., 1998). **C,** Representative traces of transiently transfected human recombinant $Ca_v2.1$ channel responses when Ca^{2+} or Ba^{2+} were used as the charge carrier. **D,** Average responses obtained for wild-type, R192Q and S218L $Ca_v2.1$ channels. *n* refers to the number of cells recorded.

As the biophysical effects of FHM-1 mutations can be affected by a number of factors including $Ca_v2.1$ splice-variation (Adams et al., 2009), β subunit coexpression (Mullner et al., 2004) and the nature of the expression system (Kraus et al., 1998; Hans et al., 1999), it was important to test the FHM-1 mutations in the context of endogenous $Ca_v2.1$ channel modulation. In this regard, we measured P/Q-type currents through endogenous $Ca_v2.1$ channels in acutely dissociated cerebellar PCs from wild-type and R192Q and S218L knock-in mice (van den Maagdenberg et al., 2004; Eikermann-Haerter et al., 2009; Tottene et al., 2009).

3.2.2 FHM-1 mutations occlude CDF of native P/Q-type currents in cerebellar PCs

P/Q-type currents are robustly expressed in dissociated cerebellar PCs (see refs (Mintz et al., 1992a; Mintz et al., 1992b) and Fig. 3.3C) and recapitulate the key features of CDF currents observed for human recombinant $Ca_v2.1$ channels, while CDI is highly variable (Chaudhuri et al., 2005) (and our own unpublished observations). We examined CDF of endogenous P/Q-type currents in acutely dissociated cerebellar PCs from wild-type and homozygous R192Q and S218L knock-in mouse strains (van den Maagdenberg et al., 2004; Eikermann-Haerter et al., 2009; Tottene et al., 2009). P/Q-type currents in PCs from wild-type mice showed similar CDF to currents through wild-type human recombinant $Ca_v2.1$ channels (Fig. 3.3). In contrast, P/Q-type currents from dissociated PCs from R192Q and S218L knock-in mice exhibited reduced CDF both across multiple prepulse potentials (Fig. 3.3A) and during APWs (Fig. 3.3D); with the more severe S218L mutation resulting in a statistically significant reduction in pure CDF (Fig. 3.3B). Taken together, the findings from both recombinant and endogenous $Ca_v2.1$ channels support the notion that the FHM-1 R192Q and S218L mutations reduce or even preclude CDF of $Ca_v2.1$ channels. The effects on $Ca_v2.1$ CDF predict that FHM-1 mutations alter $Ca_v2.1$ channel-dependent functions as they relate to synaptic signalling.

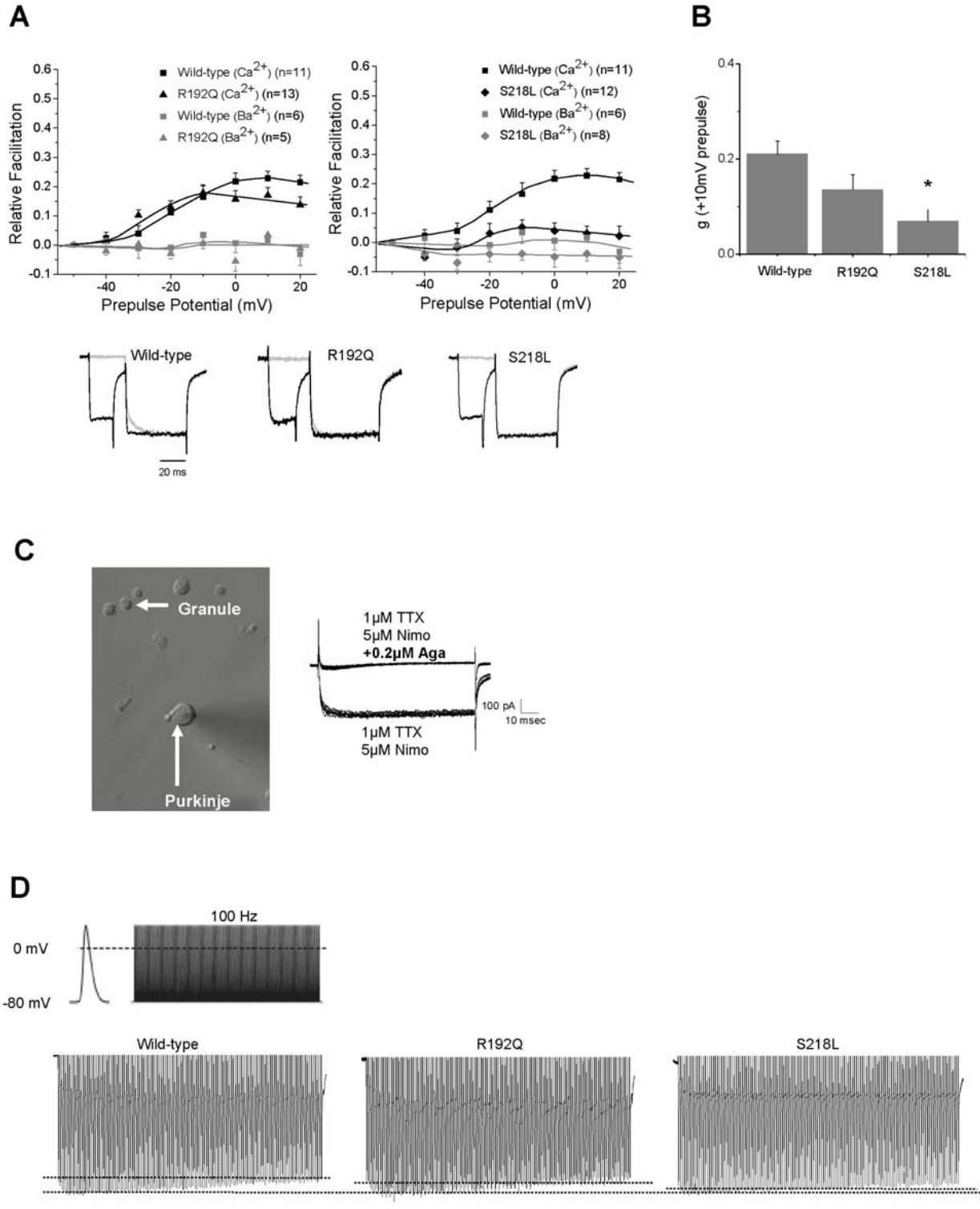
A
Wild-type (Ca2+) (n=11)
R192Q (Ca2+) (n=13)
Wild-type (Ba2+) (n=6)
R192Q (Ba2+) (n=5)
Relative Facilitation
Prepulse Potential (mV)
Wild-type (Ca2+) (n=11)
S218L (Ca2+) (n=12)
Wild-type (Ba2+) (n=6)
S218L (Ba2+) (n=8)
Relative Facilitation
Prepulse Potential (mV)
Wild-type
R192Q
S218L
20 ms
B
g (+10mV prepulse)
Wild-type
R192Q
S218L
*
C
Granule
Purkinje
1μM TTX
5μM Nimo
+0.2μM Aga
100 pA
10 msec
1μM TTX
5μM Nimo
D
100 Hz
0 mV
-80 mV
Wild-type
R192Q
S218L

Figure 3.3: CDF is similarly occluded in endogenous P/Q-type currents in acutely dissociated PCs from $Ca_v2.1$ R192Q and S218L knock-in mice.

A, Both the R192Q and S218L mutations reduced CDF across several prepulse potentials; results are means ± s.e.m (representative traces below). **B,** Pure CDF (*g*) relative to wild-type ($g = 0.210 \pm 0.028$) was not significantly reduced by R192Q ($g = 0.136 \pm 0.03$) while the S218L mutation resulted in a significant reduction ($g = 0.0686 \pm 0.035$). **C,** P/Q-type currents were isolated from freshly dissociated cerebellar PCs from 15-25 day old mice and identified by their characteristic large size and tear-shaped morphology. Exemplar trace below shows that the pharmacologically isolated P/Q-type currents were completely blocked with 0.2 μM ω-Aga-IVA. **D,** Action potential waveform used and representative traces for wild-type, R192Q and S218L $Ca_v2.1$ channels. First hashed line represents the level of Ca^{2+} response during the first action potential, and the second line represents the peak (maximum facilitation). *n* refers to the number of cells recorded. All statistics were performed using a one-way ANOVA. Asterix denotes $p<0.05$.

Neither a contribution of $Ca_v2.1$ CDF towards synaptic plasticity in cerebellum nor demonstration as to whether FHM-1 mutations might affect fast synaptic cerebellar signalling has been reported. The PF-PC synapse is a well-characterized central synapse that displays robust presynaptic forms of short-term facilitation via both paired-pulse and AP trains, relies predominantly on $Ca_v2.1$ channels for neurotransmitter release (Mintz et al., 1995; Randall and Tsien, 1995) and facilitation at this synapse is mediated by CaSs similar to those reported to mediate $Ca_v2.1$ CDF in recombinant and native systems (Atluri and Regehr, 1996; Lee et al., 1999; Lee et al., 2000; DeMaria et al., 2001; Lee et al., 2002; Tsujimoto et al., 2002; Lee et al., 2003; Chaudhuri et al., 2004; Chaudhuri et al., 2005; Few et al., 2005; Chaudhuri et al., 2007). While the specific modulators of facilitation at the presynaptic terminals of PFs have not been defined, we hypothesized that CDF of $Ca_v2.1$ channels is an important means of short-term synaptic facilitation and further, that FHM-1 mutations affecting CDF would have a corresponding effect on synaptic plasticity.

3.2.3 FHM-1 mutations occlude short-term synaptic facilitation at the PF-PC synapse

Synaptic transmission at the PF-PC synapse was measured using extracellular field recordings in transverse slices from wild-type and homozygous R192Q and S218L knock-in mice. A typical facilitation response was evoked by paired, 180 μs stimulations of PFs (50 ms interpulse interval), in which the second EPSP was facilitated relative to the first (termed paired-pulse facilitation: PPF) (Mintz et al., 1995) (Fig. 3.4A; left, representative trace). Of note, the paired pulse ratio (size of the second pulse relative to the first; PPR) was significantly reduced by both the R192Q and S218L mutations (Fig. 3.4A; bar graph), and in a manner quantitatively consistent with the reduction in $Ca_v2.1$ CDF in exogenous and native systems (compare with Fig. 3.1B and Fig. 3.3B). Following five, 180 μs stimulations at 20 Hz, similar results were observed in that both the R192Q and S218L mutations significantly decreased the successive EPSPs relative to the second pulse (Fig. 3.4B). These results support the notion that CDF of $Ca_v2.1$ channels is a contributing and necessary component of synaptic plasticity in presynaptic terminals of PFs. An unresolved question is whether FHM-1 mutant presynaptic

channels are reluctant to facilitate and have a reduced Ca^{2+} influx and decreased transmitter release, or whether they predominate within a facilitated state with enhanced P_o that increases presynaptic Ca^{2+} influx and transmitter release.

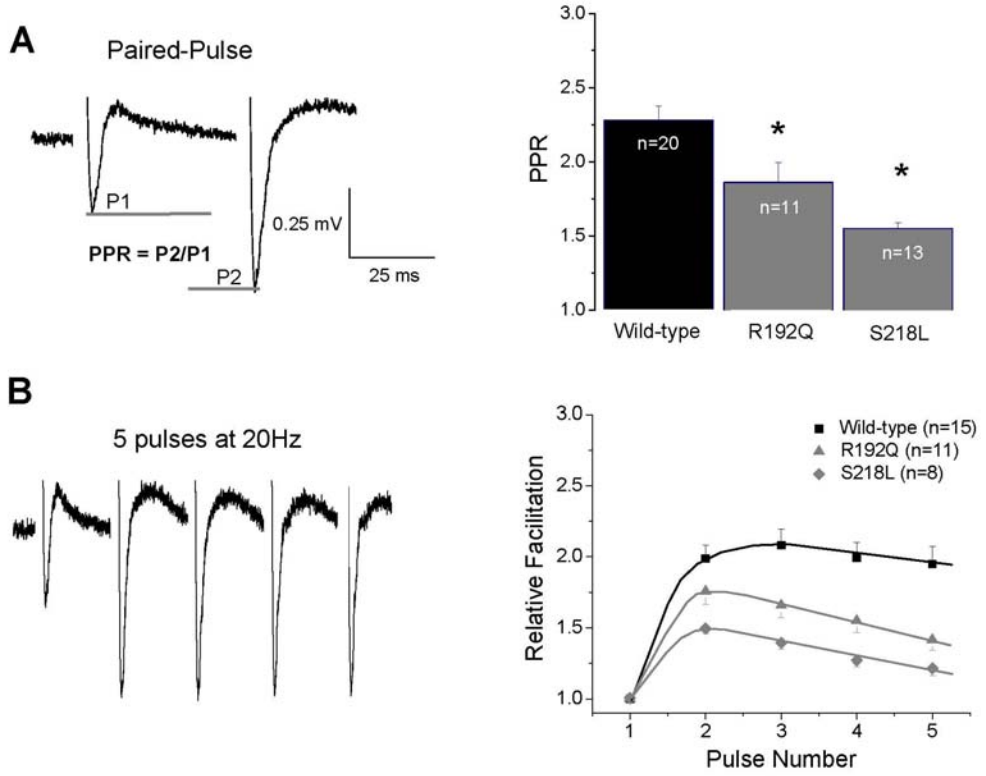

Figure 3.4: Occlusion of $Ca_v2.1$ CDF results in a comparable attenuation in PPF at the PF-PC synapse.

A, Left, exemplar field recording of synaptic responses from the PC layer evoked by two extracellular stimuli of ~15V, 180 μs delivered at a 50 ms interval to PFs in the molecular layer (wild-type mouse). The paired-pulse ratio (PPR) is a quantification of facilitation obtained by dividing response two by response one. Right, PPR was significantly reduced relative to wild-type (2.36±0.096) by both the R192Q (1.86±0.13) and S218L mutations (1.60±0.075); results are means ± s.e.m. **B,** Left, exemplar field recording of synaptic responses from the PC layer evoked by five extracellular stimuli of ~15V, 180 μs delivered at 20 Hz (wild-type mouse). Right, relative facilitation was measured by dividing the peak response from each stimulus by the response obtained from the first stimulus, plotted vs. pulse number; results are means ± s.e.m. Both mutations reduced relative facilitation during 5 pulses at 20 Hz. *n* refers to the number of slices recorded (from 8 wild-type, 4 R192Q and 5 S218L mice). All statistics were performed using a one-way ANOVA. Asterix denotes $p<0.05$.

Several lines of evidence suggest that the FHM-1 mutant channels favour a facilitated state that precludes further CDF. For one, the facilitated gating mode of $Ca_v2.1$ channels induced by CaM mediated CDF is an increase in P_o predicted to both enhance and elongate AP induced Ca^{2+} currents through the channels (Chaudhuri et al., 2007), and critically, both the R192Q and S218L mutations can render channels in a state of increased P_o in some systems (Hans et al., 1999; Tottene et al., 2005) similar to that described for facilitated channels (Chaudhuri et al., 2007).

Furthermore, the kinetics of mutant channels suggest a predominant gating mode consistent with a facilitated state (Fig. 3.5). Without a prepulse, wild-type channels open with a normal gating mode and slowly transition to a facilitated mode of gating as Ca^{2+} enters (Fig. 3.5A; -1- and -2-). When paired with a prepulse, channels open rapidly with the facilitated mode of gating (Fig 3.5A; -3-). Conversely, in the presence of an FHM-1 mutation, channels appear to activate rapidly to the maximum current level regardless of prepulse (Fig 3.5B; -3-). These support a kinetic model whereby FHM-1 mutations favour a functional state with enhanced P_o that shifts the equilibrium of channels toward a gating mode that is the same as (or analogous to) the one achieved during CDF (Fig 3.5A and B; lower panels).

Lastly, the results in Figure 3.4B show that compared to transmission measured in wild-type animals, synapses from both the R192Q and S218L mice exhibit decreased PPF and accelerated decay of EPSPs with successive stimulations. Both effects can be explained by mutant channels being in a facilitated state whereby greater Ca^{2+} influx through facilitated presynaptic $Ca_v2.1$ channels during initial APs results in vesicle depletion and thus a decrease in successive synaptic responses.

Taken together, we predicted that the observed changes in synaptic plasticity at the PF-PC synapse result from a larger initial Ca^{2+} influx through basally facilitated mutant channels relative to unfacilitated wild-type channels in PF boutons.

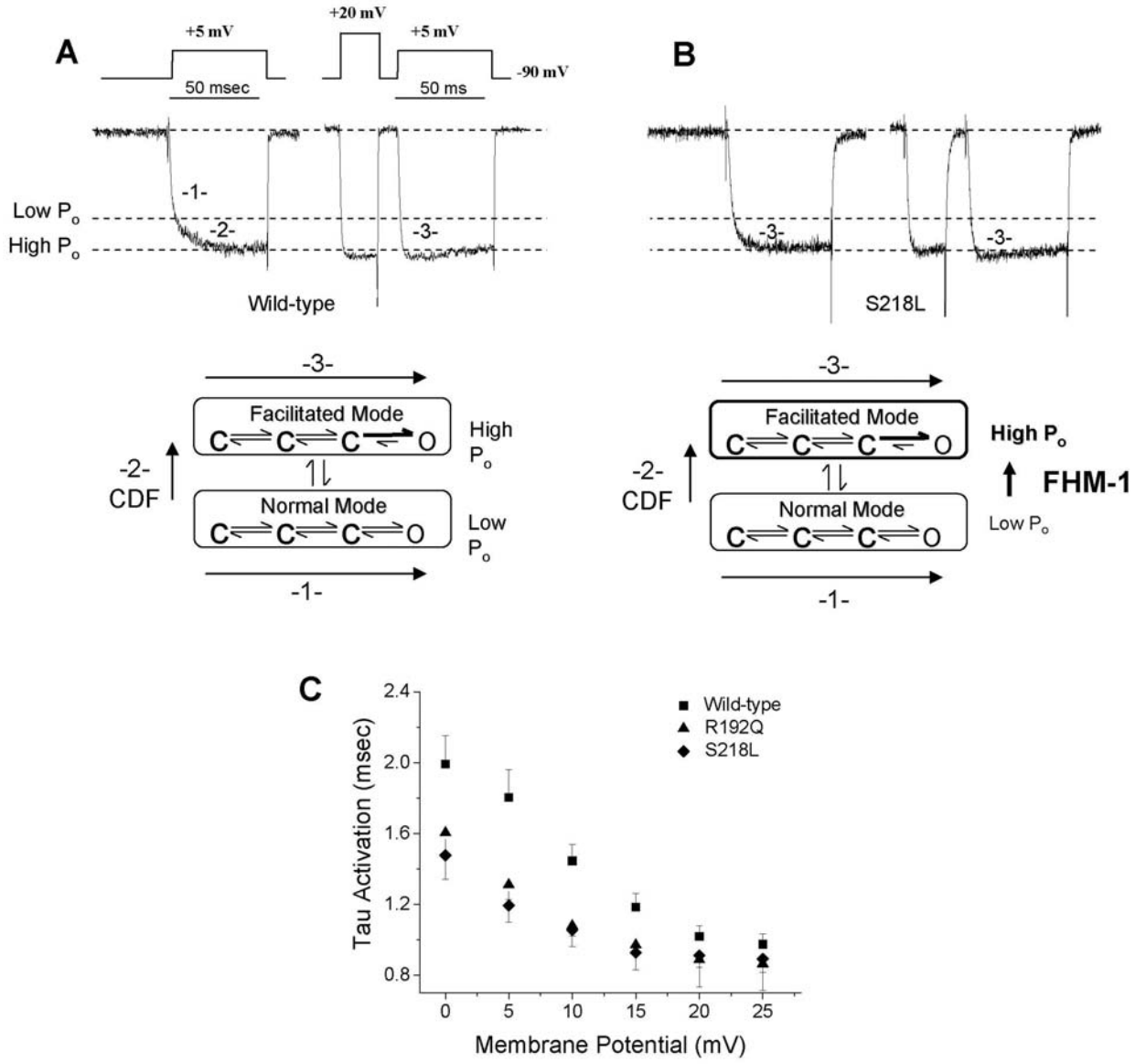
A
+5 mV
+20 mV
+5 mV
-90 mV
50 msec
50 ms
-1-
-2-
-3-
Low P$_o$
High P$_o$
Wild-type
B
-3-
-3-
S218L
-3-
Facilitated Mode
C⇌C⇌C→O
High P$_o$
-2-
CDF
Normal Mode
C⇌C⇌C⇌O
Low P$_o$
-1-
-3-
Facilitated Mode
C⇌C⇌C→O
High P$_o$
-2-
CDF
FHM-1
Normal Mode
C⇌C⇌C⇌O
Low P$_o$
-1-
C
Tau Activation (msec)
2.4
2.0
1.6
1.2
0.8
0
5
10
15
20
25
Membrane Potential (mV)
Wild-type
R192Q
S218L

Figure 3.5: Kinetics of macroscopic currents of mutant channels are consistent with a preferentially facilitated gating mode.

A, upper panel; when Ca^{2+} is used as the charge carrier, wild-type $Ca_v2.1$ channels respond to a +5 mV square test pulse with an initial rapid activation (-1-) followed by a slow phase of current increase as Ca^{2+} enters through the channel (-2-). On the other hand, when preceded by a +20 mV prepulse, wild-type channels activate rapidly with a maximum current response (-3-) (also see ref (DeMaria et al., 2001)). Lower panel; mechanistically this means that without a prepulse wild-type channels open with a normal gating mode (-1-) and slowly transition to a facilitated mode of gating as Ca^{2+} enters (-2-)(i.e. CDF). As Ca^{2+} enters it binds CaM and the Ca^{2+}/CaM complex enhances P_o and shifts the equilibrium of channels to a facilitated mode of gating (see refs (DeMaria et al., 2001; Chaudhuri et al., 2007) for model and single channel analysis showing the mechanism is a true enhancement in P_o). Following a prepulse, channels open rapidly with the facilitated mode of gating (-3-). **B,** upper panel; conversely, in the presence of the S218L mutation, channels appear to activate rapidly to the maximum current level (-3-) regardless of prepulse (similar results obtained for the R192Q mutation; data not shown). Lower panel; a kinetic model whereby FHM-1 mutations cause an enhancement of P_o that shifts the channel equilibrium toward a gating mode that resembles the one achieved during CDF. At the macroscopic level, enhanced P_o of $Ca_v2.1$ will result in faster activation kinetics. **C,** In fact, we observe that both the S218L and R192Q mutations cause an increase in $Ca_v2.1$ activation kinetics across several membrane potentials measured at the macroscopic level during 90 ms square pulses from 0 to +25 mV, from a holding of -90 mV. All representative traces and kinetics of activation recordings were measured using human recombinant channels expressed in HEK cells. The tau activation was determined by fitting current traces with a single exponential.

3.2.4 FHM-1 mutant $Ca_v2.1$ channels appear to be in a basally facilitated state in PF boutons

The geometry and spatial equilibration of Ca^{2+} in the PF boutons is ideal for measuring the role of Ca^{2+} dynamics on a tens-of-millisecond time scale using Ca^{2+}-sensitive fluorescent dyes (Mintz et al., 1995; Atluri and Regehr, 1996). To this end, we evoked a train of 5 PF responses (50 μs pulse durations at 20 Hz) in either wild-type or homozygous S218L mice and simultaneously monitored the fluorescent response of the Ca^{2+} indicator Rhod-2 in presynaptic boutons using two-photon microscopy in line scan mode. We chose to look at the S218L mice because of the consistently larger effect on CDF. Figure 3.6A shows an example of unstimulated (top) and stimulated (bottom) PFs in which the presynaptic boutons were detected as relatively bright regions (indicated by hashed line in stimulated image). Ca^{2+} influx in boutons from S218L mice (Fig. 3.6B; grey diamonds) was clearly enhanced during the train of 5 PF responses compared to those in boutons of wild-type mice (Fig. 3.6B; black squares). Additionally, the amplitudes of Ca^{2+} transients in the presynaptic boutons in S218L mice (Fig. 3.6C; pulse 1) were similar to those expected from Ca^{2+} currents through facilitated $Ca_v2.1$ channels (Fig. 3.6D). The results represent a true gain in $Ca_v2.1$ channel function as there was no apparent compensation by other Ca^{2+} channel subtypes at these boutons in the S218L mice (Fig. 3.7).

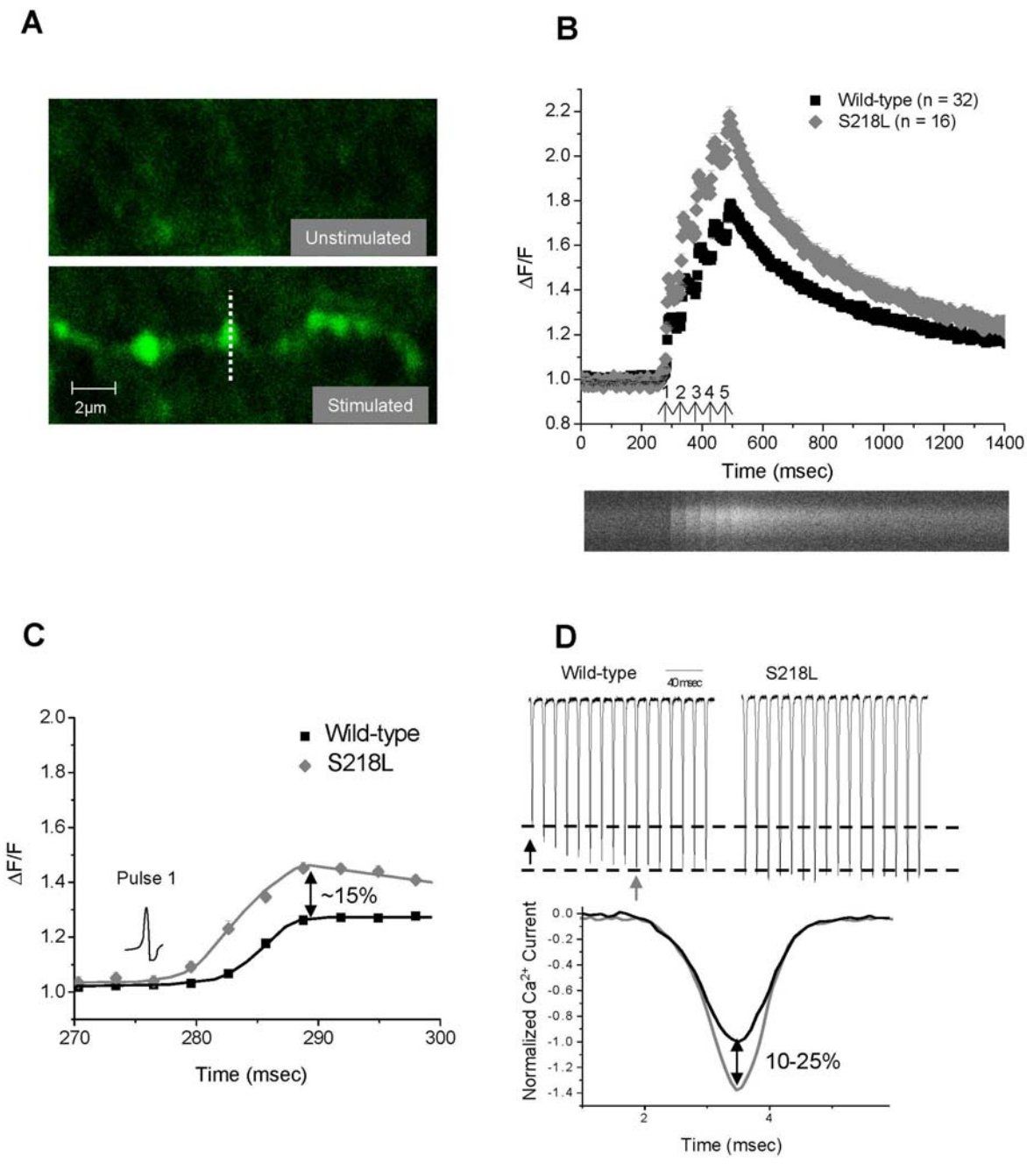
A
Unstimulated
2μm
Stimulated
B
Wild-type (n = 32)
S218L (n = 16)
ΔF/F
1 2 3 4 5
Time (msec)
C
Wild-type
S218L
ΔF/F
Pulse 1
~15%
Time (msec)
D
Wild-type
40msec
S218L
Normalized Ca2+ Current
10-25%
Time (msec)

Figure 3.6: The kinetics of Ca^{2+} influx in S218L presynaptic terminals indicate facilitated $Ca_v2.1$ channels.

A, PFs were stimulated with a silver wire electrode and two photon imaging was used to measure Ca^{2+} transients in presynaptic terminals of the PFs; post-synaptic activity was blocked with 100 μM MCPG, 20 μM CNQX and 50 μM APV. Line scans were used to measure individual terminals (representative terminal indicated by hashed line). Four sets of five 50 μs stimuli at 20 Hz were given and the Ca^{2+} transients were averaged for each terminal. **B,** The average Ca^{2+} influx at PF terminals (normalized to the background fluorescence) is enhanced in S218L mice relative to wild-type littermates; results are means ± s.e.m (arrows represent the five stimuli). A representative average Ca^{2+} transient response measured for one terminal in linescan mode is shown in the lower panel. **C,** An expanded scale of **B** to show Ca^{2+} transient elicited during the first AP. Ca^{2+} transients in mutant terminals are larger in magnitude than wild-type terminals (enhanced ~15% relative to wild-type), as expected if mutant channels are in a basally facilitated gating mode. Stimulus is shown by AP illustration. *n* refers to the number of presynaptic terminals recorded (from 5 wild-type and 5 S218L mice). **D,** Expanded view of CDF of recombinant channels during APW from Fig. 3.1 Ca^{2+} currents through wild-type recombinant $Ca_v2.1$ channels increases as Ca^{2+} influx causes CDF (Left panel), whereas recombinant $Ca_v2.1$ channels containing the S218L mutation have a maximal Ca^{2+} response regardless of APs (Right panel). Bottom panel, Ca^{2+} currents through facilitated P/Q-type channels (grey trace) are larger than through unfacilitated channels (black trace) during evoked APs (10-25% increase in Ca^{2+} current amplitude was the range obtained from recombinant $Ca_v2.1$ channels in HEK cells (Fig. 3.1) and endogenous P/Q-type currents in PCs (Fig. 3.3). The unfacilitated trace is the Ca^{2+} response of a wild-type channel obtained during the first AP, and the facilitated trace is the Ca^{2+} response obtained during the 10^{th} AP (indicated by arrows in top left panel).

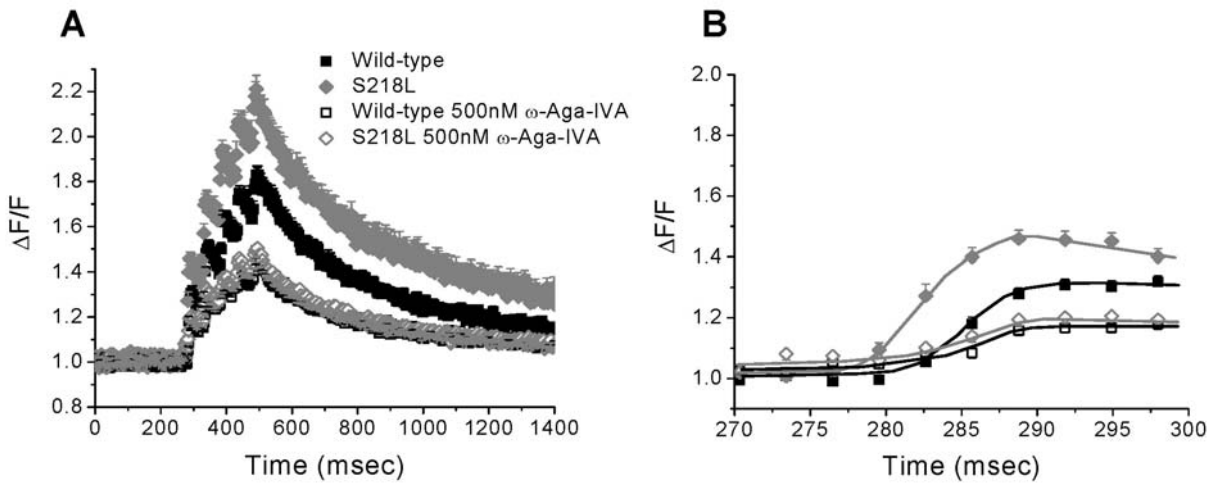

Figure 3.7: Ca^{2+} transients in wild-type and S218L mice are identical in the presence of 500 nM ω-Aga-IVA.

A, PFs were stimulated with a silver wire electrode and two photon imaging was used to measure Ca^{2+} transients in presynaptic terminals of the PFs. Line scans were used to measure individual terminals. Four sets of five 50 μs stimuli at 20 Hz were given and the Ca^{2+} transients measured. Plots are of average Ca^{2+} influx at PF terminals (normalized to the background fluorescence); results are means ± s.e.m. **B,** is an expanded scale of **A**. to show Ca^{2+} transient elicited during the first of the five pulses. In the presence of 500 nM concentration of the potent $Ca_v2.1$ channel specific blocker ω-Aga-IVA (saturating concentration for these terminals (Mintz et al., 1995)), the wild-type (open black squares) and S218L (open grey diamonds) terminals had identical presynaptic Ca^{2+} transients. These results demonstrate that the gain-of-function effect of the S218L mutations is a true gain of $Ca_v2.1$ channel function and not channel compensation by N-type channels known to be present at this terminal (Mintz et al., 1995). Blocked terminals where measured in slices incubated for 40 minutes in ACSF containing 500 nM ω-Aga-IVA at room temperature with continuous perfusion of 95% O_2. Slices from the same animal were incubated in parallel in ACSF without ω-Aga-IVA and measured on the same day. Experiments were performed on 23 and 20 terminals, from 2 animals each for wild-type and S218L, respectively.

3.3 Discussion

3.3.1 A role for $Ca_v2.1$ CDF in short-term synaptic plasticity at the PF-PC synapse

It has been known for some time that there exist at least two mechanisms of facilitation at the PF-PC synapse: a well understood mechanism described by the residual Ca^{2+} hypothesis and an incompletely resolved mechanism driven by a CaS with high Ca^{2+} affinity that can detect modest, transient levels of Ca^{2+} likely near the pore of presynaptic Ca^{2+} channels (Atluri and Regehr, 1996). The exact mechanisms and molecular players involved in the latter form of facilitation have not been previously reported, although a role for high affinity CaSs such as CaM has been predicted (Ullrich et al., 1994; Li et al., 1995; Atluri and Regehr, 1996). In this chapter, we showed that the R192Q and S218L FHM-1 mutations occlude CaM mediated CDF of both human recombinant $Ca_v2.1$ channels (see Fig. 3.1) and endogenous $Ca_v2.1$ channels in dissociated mouse cerebellar Purkinje neurons (see Fig. 3.3). Further, homologous mutations introduced into mice by genetic knock-in resulted in a corresponding attenuation in short-term synaptic facilitation at the PF-PC synapse (see Fig. 3.4). The evidence supports the hypothesis that, in addition to the traditional view that residual Ca^{2+} enhances vesicle fusion by binding to CaSs involved directly in vesicle release (Zucker and Regehr, 2002), an initial Ca^{2+} influx into PF boutons induces CDF of $Ca_v2.1$ channels and acts to enhance Ca^{2+} influx during subsequent action potentials resulting in synaptic facilitation at this central synapse.

3.3.2 Facilitated $Ca_v2.1$ channels may increase susceptibility to CSD and aura

Our findings may also provide insight into the molecular mechanisms of FHM-1 pathophysiology. We find that FHM-1 mutations likely render $Ca_v2.1$ channels in a basally facilitated state and that the facilitated channels result in a larger Ca^{2+} influx during APs in PF boutons relative to wild-type channels (see Fig. 3.6). Of note, in previous experiments using single channel recordings, some FHM-1 missense mutations (including R192Q and S218L) were shown to cause an overall gain-of-function $Ca_v2.1$ channel phenotype as a result of enhanced P_o (Hans et al., 1999; Tottene et al., 2002).

Likewise, the mechanism that underlies CDF of $Ca_v2.1$ channels has been determined to be a Ca^{2+}/CaM mediated transition of channels to a functional state with an enhanced $Ca_v2.1$ P_o and facilitated mode of gating (Chaudhuri et al., 2007). As such, at a mechanistic level, we predict that the R192Q and S218L mutations increase the likelihood of channels in a functional state with enhanced P_o that is the same as (or analogous to) the facilitated state resulting from CDF and precludes further CDF-mediated facilitation. In addition to directly affecting synaptic efficacy, this gain-of-function state is predicted to have important implications for migraine pathophysiology. For example, increased Ca^{2+} influx during APs and increased glutamate release at pyramidal cell synapses are believed to be the underlying cause of both the lower threshold for stimulation of CSD and the increased velocity of propagation of CSD observed in FHM-1 knock-in mice. CSD is believed to be the underlying mechanism of aura which precedes headache in some patients and likely triggers headache pain (van den Maagdenberg et al., 2004; Gherardini et al., 2006; Tottene et al., 2009; van den Maagdenberg et al., 2010).

3.3.3 Impairment of synaptic efficacy may underlie cerebellar ataxia in FHM-1

We provide the first evidence that FHM-1 mutations alter neurotransmission in the cerebellum. There has not been any explanation for the cerebellar ataxia sometimes associated with the FHM-1 phenotypes. The cerebellum plays a central role in motor control, and alteration in neurotransmission at the PF-PC synapse has been correlated with aberrant motor phenotypes in other conditions involving ataxia (Zhou et al., 2003; Schmitt et al., 2009). Here we showed evidence that the R192Q and S218L mutations alter short-term synaptic plasticity at the PF-PC synapse by attenuating CDF of $Ca_v2.1$. We hypothesize that the significant effects on $Ca_v2.1$ CDF by the S218L mutation are sufficient to alter synaptic plasticity to an extent that ultimately leads to cerebellar motor deficits including ataxia under conditions associated with initiating factors of FHM-1 attacks.

3.3.4 Beyond the PF-PC synapse

In summary, the data presented in this chapter provide evidence that $Ca_v2.1$ CDF plays a critical role in synaptic plasticity at the PF-PC synapse and provides the first direct correlation between Ca^{2+}-dependent regulation of $Ca_v2.1$ and human disease. Considering that $Ca_v2.1$ channels are the predominant Ca^{2+} channel underlying synaptic transmission at most other fast synapses in the mammalian CNS, and the PF-PC synapse is prototypical of other CNS synapses (Westenbroek et al., 1995; Evans and Zamponi, 2006), our findings support the notion that $Ca_v2.1$ CDF is an unrecognized but significant contributor to short-term synaptic facilitation at fast CNS synapses. The disruption of $Ca_v2.1$ CDF may be an important factor in FHM-1 pathophysiology, although the precise effects of the FHM-1 mutations at other CNS synapses may vary depending on other factors including the nature of the $Ca_v2.1$ splice variants expressed within the synapse (Soong et al., 2002; Chaudhuri et al., 2004; Chaudhuri et al., 2005; Adams et al., 2009) as well as $Ca_v2.1$ interactions with auxiliary subunits (Mullner et al., 2004) and the expression of CaS proteins (Tsujimoto et al., 2002).

3.4 Experimental Procedures

3.4.1 Cell culture and transfection

HEK 293 cells were grown in standard Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (heat inactivated) and 50 U/ml penicillin-50 ug/ml streptomycin. Cells were incubated at 37^{o}C in a humidified incubator with 95% air and 5% CO_2 and grown to 10-20% confluency for transfection. HEK 293 cells were transiently transfected with either wild-type, R192Q or S218L human $Ca_v2.1$ alpha subunits in combination with Ca^{2+} channel $\beta 2$, $\alpha_2\delta_1$, and CD8 in a 1:1:1:0.25 molar ratio using Lipofectamine (Invitrogen, La Jolla, CA). To ensure accurate comparisons, transfections were performed at the same time and electrophysiological recordings alternated within the same day for all channel types. All clones used in the present chapter were generated as previously described (all clones are the $Ca_v2.1$ ($\Delta 47$) splice-variant) (Adams et al., 2009).

3.4.2 Dissociated PCs

Mouse cerebellar PCs were enzymatically isolated using dissociation techniques previously described (Raman et al., 1997; Swensen and Bean, 2003, 2005). Mice between postnatal days 15-25 were anesthetized using isoflurane (Baxter Corporation, Mississauga, ON), and decapitated according to protocols approved by the University of British Columbia animal care committee. Cerebellum vermis were dissected out and cut into 1 mm cubes in ice cold dissociation solution containing (in mM): 82 Na_2SO_4, 30 K_2SO_4, 10 HEPES, 10 glucose, 5 $MgCl_2$ and 0.001% Phenol Red, buffered to pH 7.4 with NaOH. Cerebellar cubes were transferred to 10 mL of room-temperature dissociation solution containing 3 mg/mL of protease XXIII (Sigma, St. Louis, MO) and incubated for 8-10 minutes at 32^{o}C. After incubation, tissue was transferred to ice-cold dissociation solution containing 1 mg/mL trypsin inhibitor and 1 mg/mL bovine serum albumin (Sigma, St. Louis, MO) and maintained on ice until trituration. Tissue was triturated with a series of three fire-polished Pasteur pipette to dissociate individual cells, and PCs were identified by their large diameter and pear-shaped appearance attributed to the stump of the dendritic tree.

3.4.3 Electrophysiological recording conditions in HEK and dissociated PCs

Recombinant human $Ca_v2.1$ currents in HEK cells were recorded the second day after transfection, whereas mouse endogenous $Ca_v2.1$ currents in acutely dissociated PCs were recorded between 30 minutes and 4 hours after dissociation. In both cases, macroscopic Ba^{2+} and Ca^{2+} currents were recorded at 19-23^{o}C using the whole-cell patch-clamp technique (Hamill et al., 1981). The internal pipette solution contained 135 mM Cs-$MeSO_3$, 5 mM $CsCl_2$, 0.5 mM EGTA, 1 mM $MgCl_2$, 4 mM MgATP, and 10 mM HEPES (pH 7.4); external: 140 mM TEA-$MeSO_3$, 10 mM HEPES, and 5 mM $CaCl_2$ or $BaCl_2$ (pH 7.3). Patch pipettes (borosilicate glass BF150-86-10; Sutter Instrument Company, Novato, CA) were made using a horizontal puller (P-87; Sutter Instruments Company), fire polished using a microforge (Narishinge, Tokyo, Japan), and had resistances typically of 3 to 7 MΩ when

containing internal solution. External solution bath was connected to ground with a 3M KCl agar bridge. Whole cell currents were recorded and filtered at 2-5 kHz bandwidth using an Axopatch 200A amplifier monitored and stored on a personal computer running pClamp software package version 9. Recordings were analyzed using Clampfit 9 and figures, fittings and statistics (ANOVA) were made using the software program Origin version 7.5 (OriginLab Corp., Northampton, MA).

3.4.4 Protocols and data analysis for CDF and CDI

CDF was analyzed using a two-pulse protocol as previously described (Chaudhuri et al., 2004). In short, 50 ms test pulses to +5 mV were given subsequent to no prepulse or prepulses ranging from -90 mV to +100 mV. All traces were normalized to the current at the end of the test pulse. We integrated the current trace for the test pulse obtained with a prepulse and subtracted that integral from the integral for the test pulse obtained without a prepulse in order to obtain the variable delta Q. To obtain the value of relative facilitation (RF) for all channels, we divided delta Q by the time constant obtained from fitting a single exponential to the trace obtained by subtracting the normalized test pulse current obtained without a prepulse from the normalized test pulse current obtained with a +20 mV prepulse from wild-type channels (see supplemental material in (Chaudhuri et al., 2004)). Relative facilitation is plotted vs. prepulse membrane potential. To index pure CDF we measured the difference in the relative facilitation values obtained in Ca^{2+} vs. Ba^{2+} with a prepulse potential of +20 mV. CDF was measured in PCs using both the rectangular two pulse protocol and the APW described above, except the holding potentials were changed form -90 mV to -60 mV to eliminate any contribution of T-type Ca^{2+} currents that may have been present in some cells (Chaudhuri et al., 2005).

CDI was measured using a square pulse protocol including a 1 s test depolarization ranging from -10 mV to +30 mV, in 10 mV increments, from a holding of -90 mV (-60 mV holding in PCs). The fraction of current remaining at 800 ms after the initiation of the test pulse, r800, was plotted as a function of test pulse potential. To index pure CDI we measured the difference in the r800 values obtained in Ca^{2+} vs. Ba^{2+} with a test potential of +10 mV.

3.4.5 Cerebellar slices

For both extracellular field recording and two-photon imaging experiments, mice between postnatal days 25-35 were anesthetized using isoflurane (Baxter Corporation, Mississauga, ON) and decapitated according to protocols approved by the University of British Columbia animal care committee. The Cerebellum was rapidly removed and placed in ice-cold dissection solution containing (in mM): 87 NaCl, 25 $NaHCO_3$, 25 D-glucose, 75 sucrose, 2.5 KCl, 2 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$. Cerebellum was cut into 300 μm transverse slices using a vibrating tissue slicer (Vibratome, St. Louis, MO) and maintained for 1-6 hours at room temperature in artificial cerebral spinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 26 $NaHCO_3$, 2.5 $CaCl_2$, 10 Glucose, 1.4 NaH_2PO_4 and aerated with 95% O_2 / 5% CO_2. For experiments slices were at 22–24 °C and perfused at ~2 ml/min.

3.4.6 Extracellular Field Recordings

fEPSPs were evoked by orthodromic stimulation of the PFs in the molecular layer of the cerebellar vermis using a bipolar tungsten stimulating electrode. Glass micropipettes filled with aCSF (resistance, 1–3M) were used to measure PC fEPSPs. PPF was measured by invoking two extracellular stimuli of ~15V, 180 μs delivered at 50 ms intervals and the PPR quantified by dividing response two by response one. Similarly, synaptic responses were also evoked by five extracellular stimuli of ~15V, 180 μs delivered at 20 Hz and relative facilitation measured by dividing the peak response from each stimulus by the response obtained from the first stimulus.

fEPSP signals were amplified 1000 times with an AC amplifier (AM Systems, Sequim, WA) , bandpass filtered at 1 kHz, digitized at 10 kHz using a Digidata 1322A interface board (Molecular Devices, Foster City, CA), and transferred to a computer for analysis. Data were analyzed using Clampfit 9.0 (Molecular Devices, Foster City, CA).

3.4.7 Two-photon microscopy: Ca^{2+} Imaging

Neurons in cerebellar slices (300 μm) were bulk loaded with Rhod-2 AM (Invitrogen) using a modified Chremophor loading technique adapted from (Trevelyan et al., 2006). Slices were pre-incubated in 3 mL ACSF and 8 uL Chremophor EL solution (0.5% in DMSO) at 34ºC for 5 mins. 50 μg Rhod-2 AM mixed with 8 μL DMSO and 2 μL pluronic F-127 solution (10% in DMSO) was then added, and slices were allowed to incubate for an additional 45minutes. Slices were then allowed to recover at room temperature for at least 30 min. This resulted in robust loading of PFs in addition to staining of other cell types in the tissue. In order to isolate pre-synaptic Ca^{2+} signals, post-synaptic and glial signals were blocked with a cocktail of glutamate receptor blockers; 20 μM CNQX, 50 μM DL-APV, 100 μM MCPG (all purchased from Tocris).

We performed imaging with a two-photon laser scanning microscope (Zeiss LSM510-Axioskop-2 with a 40X/1.0 numerical aperture objective lens) directly coupled to a 10 W Chameleon ultrafast laser (Coherent). PFs were stimulated with a silver wire electrode and two-photon imaging used to measure resulting Ca^{2+} transients in presynaptic terminals of the PFs. Line scans were performed at 325 Hz, and individual terminal responses were measured in response to four sets of five 50 μs duration stimuli applied at 20 Hz. Images were obtained from between 40 and 80 μm deep into the slice. Rhod-2 was excited at 835 nm (~3 mW after the objective) and fluorescence detected with a PMT after passing through a 605-nm (55-nm band-pass) emission filter. Images were collected and analyzed using Zeiss LSM software.

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4 DISCUSSION

4.1 Overall significance and strengths

4.1.1 FHM-1 mutations differentially affect recombinant $Ca_v2.1$ splice variants

The FHM-1 phenotype is localized to specific brain regions including the cortex, meningeal nociceptors, trigeminal nuclei and sometimes cerebellum, yet, $Ca_v2.1$ channels are expressed at nearly all fast synapses in the CNS and PNS. Further, the disease phenotype is episodic, although $Ca_v2.1$ channels are constitutively expressed in CNS and PNS during most stages of development. So why aren't all $Ca_v2.1$ channels affected all of the time? Alternative splicing of the CACNA1A gene has the potential to produce numerous functionally distinct $Ca_v2.1$ splice variants that are differentially expressed regionally within the nervous system in discrete cell types and subcellular compartments and at different developmental stages. The first major objective of this thesis was to explore whether $Ca_v2.1$ splice variants respond differently to FHM-1 mutations and to perhaps contribute toward understanding the localized and episodic nature of FHM-1.

Previous studies exploring FHM-1 effects on $Ca_v2.1$ channels have used either a single recombinant $Ca_v2.1$ channel variant expressed in *Xenopus* oocytes or HEK cells, or endogenous $Ca_v2.1$ channels in neurons which contain numerous $Ca_v2.1$ splice variants associated with various auxiliary subunits (Tsunemi et al., 2002; Kanumilli et al., 2006; Richards et al., 2007). Whether the effects of FHM-1 mutations are contingent on the $Ca_v2.1$ splice-variant has not been discernible. In Chapter 2, we utilized an experimental design that allowed us to specifically compare how two human recombinant $Ca_v2.1$ splice variants expressed in a standardized system (HEK cells co-expressing the same VGCC auxiliary subunits) respond to three different FHM-1 mutations. We found that all three FHM-1 mutations induced significant changes in channel basic biophysical properties and that the effects were in fact contingent upon the nature of the $Ca_v2.1$ splice-variant.

The three FHM-1 mutations all caused greater gain-of-function effects regarding channel activation when expressed in $Ca_v2.1$ (Δ47) channels relative to $Ca_v2.1$ (+47) variant channels; mutant

$Ca_v2.1$ (Δ47) channels activate at lower membrane potentials relative to mutant $Ca_v2.1$ (+47) channels (Fig 2.2 and Table 2.1). Such gain-of-function effects in $Ca_v2.1$ channels are predicted to increase Ca^{2+} influx during APs and enhance susceptibility to CSD and the initiation of migraine (van den Maagdenberg et al., 2004; Pietrobon, 2007; Tottene et al., 2009). The results in Chapter 2 support the hypothesis that $Ca_v2.1$ splice variants (e.g., $Ca_v2.1$ (Δ47)) with greater sensitivity to these and other FHM-1 mediated gain-of-function effects might result in cortical regions (or cell types) with a greater susceptibility to CSD and/or migraine initiation under some conditions, while the effects of FHM-1 mutations on other $Ca_v2.1$ splice variants (e.g., $Ca_v2.1$ (+47)) expressed elsewhere may be below the threshold to initiate CSD and/or other pathological effects.

Further, we found evidence that FHM-1 mutations differentially affect $Ca_v2.1$ channel splice variants under different firing conditions. For example, during tonic depolarizations the R192Q mutation had no effect on the $Ca_v2.1$ (+47) channel, yet during bursts of depolarizations the mutation caused a significant enhancement in channel activity relative to wild-type. On the other hand, when expressed in the $Ca_v2.1$ (Δ47) variant, the R192Q mutation decreased channel activity during both tonic and bursts of depolarizations relative to wild-type (Figs 2.4 and 2.5). Interestingly, neuronal firing patterns in the brain can be altered by events such as emotional stress (Weiss and Simson, 1988; McEwen, 2007), a common initiating factor of FHM-1 attacks (Ducros et al., 2001). As such, although the exact firing conditions directly associated with the precipitating factors of migraine are unknown, our results support the conclusion that the episodic nature of the FHM-1 phenotype may, in part, be associated with changes in neuronal firing patterns and/or frequency that affect certain $Ca_v2.1$ splice variants localized to specific brain regions.

4.1.2 FHM-1 mutations occlude Ca^{2+}-dependent modulation of $Ca_v2.1$ and synaptic plasticity

CDF and CDI are robust forms of $Ca_v2.1$ Ca^{2+}-dependent modulation, yet whether they are altered by FHM-1 mutations has not been reported. Furthermore, $Ca_v2.1$ CDF and CDI are predicted to

have important roles in neurotransmission and synaptic plasticity (Tsujimoto et al., 2002; Chaudhuri et al., 2005; Mochida et al., 2008), however their relevance within intact neuronal circuits is uncertain. The second major objective of this thesis was to determine whether CDF and CDI of $Ca_v2.1$ are altered by FHM-1 mutations, and to explore any physiological implications toward normal synaptic efficacy and also the pathophysiology associated with migraine using transgenic mouse models.

In Chapter 3, we first measured CDF and CDI of recombinant $Ca_v2.1$ channels transiently expressed in HEK cells (along with auxiliary subunits β_{2a} and $\alpha_2\delta_1$). This recombinant system is well characterized (Lee et al., 1999; Lee et al., 2000; DeMaria et al., 2001; Chaudhuri et al., 2005; Kreiner et al., 2010) and allowed us to obtain clear isolation and measurement of CaM mediated CDF and CDI for both wild-type and R192Q and S218L $Ca_v2.1$ channels. We further tested the FHM-1 mutations in the context of endogenous $Ca_v2.1$ channel modulation by measuring P/Q-type currents in acutely dissociated cerebellar PCs from wild-type, R192Q and S218L knock-in mice (van den Maagdenberg et al., 2004; Eikermann-Haerter et al., 2009; Tottene et al., 2009). The use of both recombinant and endogenous experimental paradigms provided strong corroborative evidence that the R192Q and S218L FHM-1 mutations occlude CDF of $Ca_v2.1$ channels. These findings are the first evidence that mutations associated with human disease alter $Ca_v2.1$ Ca^{2+}-dependent modulation.

A correlation between such $Ca_v2.1$ CDF mechanisms and short-term synaptic plasticity had previously been achieved using recombinant $Ca_v2.1$ channels expressed in a model system (Mochida et al., 2008). In order to establish the relevance of this $Ca_v2.1$ CDF in synaptic plasticity at an intact central synapse, we compared fEPSPs at the PF-PC synapse in wild-type and R192Q and S218L mice. We found that the occlusion of $Ca_v2.1$ CDF by the mutations corresponded with an attenuation in short-term synaptic plasticity at the PF-PC synapse. This is the first direct evidence that initial Ca^{2+} influx at these central presynaptic terminals induces CDF of $Ca_v2.1$ channels and acts as a means to enhance Ca^{2+} influx during subsequent action potentials to achieve synaptic facilitation.

In Chapter 3 we also used two-photon microscopy to measure Ca^{2+} transients during APs at PF boutons in wild-type and FHM-1 mice. With this high-resolution technique, we obtained evidence that

FHM-1 mutations render $Ca_v2.1$ channels toward a basally facilitated state, and that the facilitated channels allow larger Ca^{2+} influx during evoked APs in presynaptic terminals that rely predominantly on $Ca_v2.1$ channels. The findings suggest that a similar mechanism of gain-of-function may contribute to the observed increased Ca^{2+} influx during APs and increased glutamate release at pyramidal cell synapses believed to cause both lower threshold for stimulation of CSD and increased velocity of propagation of CSD across the cortex (van den Maagdenberg et al., 2004; Gherardini et al., 2006; Tottene et al., 2009; van den Maagdenberg et al., 2010).

Lastly, the findings outlined in Chapter 3 provide initial evidence for a possible mechanism underlying cerebellar dysfunction in severe FHM-1 phenotypes. The S218L mutation is associated with a phenotype that includes episodes of cerebellar ataxia. Our data show that the S218L mutation enhances presynaptic Ca^{2+} influx in PF boutons and synaptic efficacy at the PF-PC synapse is significantly compromised. The cerebellum plays a central role in motor control and alteration in neurotransmission at the PF-PC synapse has been correlated with aberrant motor phenotypes in other conditions involving ataxia (Zhou et al., 2003; Schmitt et al., 2009).

4.2 FHM-1 mutations differentially affect $Ca_v2.1$ splice variants: a role in the localized, episodic nature of the FHM-1 phenotype

4.2.1 Working hypothesis

Over the years a number of studies have shown that alternative splicing of $Ca_v2.1$ and other Ca_v channels can be integral to the localized phenotype of human disease, although critically, in ways distinct from that described in Chapter 2 (reviewed in (Adams and Snutch, 2007; Liao et al., 2009)). The most apparent is the use of alternative exons by Ca_v channels (see Chapter 1, section 1.3.3). If a disease-causing mutation occurs within an alternative exon that is only expressed in a subset of tissues and/or cells, then the phenotype is localized and the severity of the disease is correlated with the exon expression level. For example, in the L-type $Ca_v1.2$ channel, two *de novo* mutations in one of two

mutually exclusive exons (exon 8 and 8a) have been associated with Timothy syndrome (TS) (Splawski et al., 2005)(and reviewed in (Adams and Snutch, 2007); Appendix 1). Exons 8 and 8a are preferentially expressed in smooth muscle and cardiac muscle, respectively (Welling et al., 1997; Liao et al., 2005). A TS mutation in exon 8 is associated with a mild phenotype and patient longevity is not compromised (Splawski et al., 2004), whereas a mutation in exon 8a is associated with severe cardiac arrhythmia and patient death between two and three years of age (Splawski et al., 2005).

Similarly, three mutations causing premature stop codons in the mutually exclusive exon 37a of $Ca_v2.1$ were identified in several EA2 patients. Exons 37a and 37b are used mutually exclusively in humans and rodent (Bourinet et al., 1999; Soong et al., 2002; Kanumilli et al., 2006). 37a is preferentially expressed in cerebellar cortex, predominantly in Purkinje neurons (Bourinet et al., 1999), and EA2 mutations in exon 37a thus correlate well with cerebellar dysfunction as the primary EA2 phenotype (Ophoff et al., 1996). Also, in humans there is a developmental switch from 37a to 37b in the fourth decade of life (Chang et al., 2007a), which may contribute to the age-dependent decrease in frequency and severity of attacks in some EA2 patients (Jen et al., 2007).

In another example, it has long been understood that expansion of the CAG trinucleotide repeat in exon 47 of $Ca_v2.1$ causes SCA6 (Zhuchenko et al., 1997). Importantly, the trinucleotide expansion has strong effects on voltage and time-dependent properties of the $Ca_v2.1$ (+47) splice-variant containing exon 47, whereas the $Ca_v2.1$ (Δ47) variant lacking exon 47 is unaffected (Restituito et al., 2000; Toru et al., 2000). Interestingly, recent analysis of human cerebellum revealed that the expression profile of $Ca_v2.1$ variants is altered in patients with the SCA6 CAG expansion. $Ca_v2.1$ (+47) is found nearly exclusively in PCs from SCA6 patients, whereas in normal cerebellum $Ca_v2.1$ (+47) and $Ca_v2.1$ (Δ47) are expressed in equal proportion (Tsunemi et al., 2008). The authors argue that the higher levels of $Ca_v2.1$ (+47) in PCs in SCA6 patients causes specific PC degeneration and cerebellar ataxia, perhaps due to $Ca_v2.1$ (+47) C-terminal fragments containing the SCA6 CAG expansion forming cytoplasmic and nuclear aggregates (Ishiguro et al., 2010).

There are other examples of Ca_v mutations that have been identified in alternative exons or within conventional splice-sites at intron-exon boundaries that are predicted to contribute to the localization of disease, however, the majority of identified mutations associated with Ca^{2+} channelopathies are missense mutations in coding sequences other than splice-sites and alternate exons (reviewed in (Adams & Snutch, 2007); Appendix 1). Our results presented in Chapter 2 are therefore important evidence toward what may prove a more common mechanism underlying the localized episodic phenotype associated with the majority of Ca^{2+} channelopathies.

The work in Chapter 2 has proven particularly relevant toward the interpretation of recent results obtained from FHM-1 studies. Two research groups explored synaptic transmission in cortical neuron cultures and cortical slices from FHM-1 knock-in mice in order to gain insight into the mechanisms underlying the observed lower threshold for stimulation of CSD depression and increased velocity of propagation across the cortex (van den Maagdenberg et al., 2004; Gherardini et al., 2006; van den Maagdenberg et al., 2010). In the cortex, layer 2/3 pyramidal cells have glutamatergic synapses onto fast spiking (FS) inhibitory interneurons which in turn make GABAergic inhibitory synapses onto pyramidal cells. Using paired recordings in acute somatosensory cortical slices, it was shown that there is an increased probability of glutamate release from 2/3 pyramidal cells in R192Q mice relative to wild-type, but surprisingly, no change in GABAergic transmission at FS synapses, despite the fact that $Ca_v2.1$ channels are the predominant VGCCs in both types of terminals (Tottene et al., 2009; Inchauspe et al., 2010). The authors hypothesize that pyramidal cells and FS interneurons normally coordinate a balance between excitation and inhibition during cortical activity, and that a compromise in the balance by FHM-1 mutations under some conditions (i.e. migraine triggers that alter firing patterns and/or frequencies in the cortex) could cause an increase in extracellular K^+ above the threshold for CSD initiation (Pietrobon, 2010b, a).

Based on our results, it is possible that the differential effects of the R192Q mutation on the two cortical neuron types is due to the expression of different $Ca_v2.1$ splice variants. For example, we showed that the R192Q mutation caused a greater hyperpolarizing shift in the activation threshold when

expressed in the $Ca_v2.1$ (Δ47) variant relative to $Ca_v2.1$ (+47) (Fig. 2.2); perhaps 2/3 pyramidal neurons express $Ca_v2.1$ (Δ47) and FS neurons $Ca_v2.1$ (+47). Further, under a particular frequency of tonic depolarization, the R192Q mutation had a loss-of-function effect in one $Ca_v2.1$ variant and no detectable effect in the other variant (Fig. 2.4). Also, during certain burst firing conditions, the R192Q mutation had a loss-of-function effect in one $Ca_v2.1$ variant and a gain-of-function effect in another (Fig. 2.5). These differing, or even opposing, effects by the R192Q mutation are potential ways in which the mutation could disrupt the balance between cortical excitation-inhibition in favor of excitation under certain firing conditions if pyramidal and FS neurons in fact express different $Ca_v2.1$ splice variants.

Similar differential effects by FHM-1 mutations may be relevant to migraine pathophysiology within the trigeminovascular pain pathway where $Ca_v2.1$ channels are integral for both excitatory and inhibitory neurotransmission (see Chapter 1, section 1.4.4 for details on FHM-1 mechanism). $Ca_v2.1$ channels are known to be involved in facilitating pain through their role in the release of both vasoactive neuropeptides from perivascular terminals of meningeal nociceptors (Hong et al., 1999; Akerman et al., 2003) and glutamate from trigeminal ganglion neurons (Xiao et al., 2008). On the other hand, $Ca_v2.1$ channels are also involved in suppressing pain through their role in transmitter release from inhibitory projections from the dura and PAG onto the TNC (Knight et al., 2002; Ebersberger et al., 2004). It is possible that differential or opposing effects of FHM-1 mutations on $Ca_v2.1$ splice variants expressed in these excitatory and inhibitory neurons disrupt the balance between excitation and inhibition within the neuronal network of the trigeminovascular system. For example, gain-of-function in excitatory pathways and loss-of-function in inhibitory pathways could ultimately enhance neuronal activity in the trigeminal pain pathway leading to sensitization and migraine pain (Pietrobon and Striessnig, 2003; Moskowitz et al., 2004; Burstein and Jakubowski, 2005; Edvinsson and Uddman, 2005; Pietrobon, 2005; Sanchez-Del-Rio et al., 2006; Goadsby et al., 2009)).

Similar to FHM-1, many other Ca^{2+} channelopathies exhibit episodic and/or developmentally specific phenotypes localized to a subset of regions or tissues, and like $Ca_v2.1$, other Ca_v channels also undergo alternative splicing that generates functionally distinct channel variants (reviewed in

(Lipscombe et al., 2002; Lipscombe and Castiglioni, 2004)). I hypothesize that the differential effects of FHM-1 mutations on $Ca_v2.1$ splice variants represents a central mechanism relevant in the localized and episodic phenotype associated with all Ca^{2+} channelopathies. In support, recent work performed in the Snutch lab showed that a missense mutation in $Ca_v3.2$ associated with a rat model of absence epilepsy differentially affected two distinct thalamic $Ca_v3.2$ T-type channel splice variants (Powell et al., 2009). Specifically, alternative splicing in exon 25 determined the effect of the mutation on recovery from inactivation and charge movement during high-frequency bursts.

4.2.2 Possible limitations

The use of human recombinant $Ca_v2.1$ channels in HEK cells provided a highly controlled environment to specifically address how alternative splicing at a single locus can determine the functional consequences of FHM-1 mutations. Although these experiments offer a powerful proof of principle, in reality, neurons in the brain likely express numerous $Ca_v2.1$ channels consisting of various combinations of splicing at the 7 known splice-sites (Soong et al., 2002; Tsunemi et al., 2002; Chaudhuri et al., 2004; Chaudhuri et al., 2005; Kanumilli et al., 2006; Richards et al., 2007). As such, the overall functional effects of a given FHM-1 mutation in a particular neuron will likely depend on both the combination of $Ca_v2.1$ channel splicing at the seven loci, as well as the relative proportion of each $Ca_v2.1$ variant. In future experiments it will be crucial to identify full-length alternative splice variants from brain regions and specific cell types implicated in FHM-1 pathophysiology.

Another potential limitation in the HEK cell-based recombinant studies is that we only examined the auxiliary subunits β_4 and $\alpha_2\delta_1$ coexpressed with $Ca_v2.1$. Endogenous $Ca_v2.1$ channels in neurons can be associated with various β ($\beta_{1\text{-}4}$) and $\alpha_2\delta$ subunits ($\alpha_2\delta_{1\text{-}4}$) which can both modify $Ca_v2.1$ channel biophysical properties (reviewed in (Dolphin, 2009)) and alter how the channels respond to FHM-1 mutations (Mullner et al., 2004). As such, the effects of FHM-1 mutations on $Ca_v2.1$ splice variants within neurons may also be contingent upon the auxiliary subunit isoforms expressed and their relative proportions. For example, the differential effects induced by the R192Q mutation in cortical 2/3

pyramidal neurons and FS interneurons may be independent of $Ca_v2.1$ alternative splicing and rather due to differential expression of β-subunits, or some combination of both. For instance, the K1336E, W1684R and V1696I FHM-1 mutations all show varying functional effects on a single recombinant $Ca_v2.1$ variant channel when coexpressed with either β_1, β_3 or β_4. Most notably, the V1696I mutation causes a significant hyperpolarizing shift in the voltage dependence of activation of $Ca_v2.1$ when coexpressed with β_1, but has no effect when coexpressed with either β_3 or β_4 (Mullner et al., 2004). If FS interneurons express either β_3 or β_4, and pyramidal neurons express β_1, FHM-1 mutations may have significant gain-of-function affects on pyramidal neurons with little effect on FS interneurons and ultimately lead to enhanced glutamate release and increased extracellular K^+ above the threshold for CSD initiation. All four β-subunit isoforms are expressed in mammalian cortex (Schlick et al.), although isoforms expressed specifically in 2/3 pyramidal and FS neurons has yet to be determined.

4.2.3 Future directions

The identity and functional characterization of full-length $Ca_v2.1$ splice variants and auxiliary subunits expressed in layer 2/3 pyramidal cells, FS interneurons, meningeal nociceptors, trigeminal nuclei and cerebellar granule and Purkinje neurons will be necessary before a full appreciation of the role of $Ca_v2.1$ alternative splicing in the localized and episodic nature of the FHM-1 phenotype can be realized. The first step would be to utilize single cell quantitative RT-PCR to amplify full-length $Ca_v2.1$ mRNA transcripts from these neurons in FHM-1 KI mouse models and wild-type littermates; it would be necessary to look at splice variants in both WT and mutant mice as mutations may alter the expression of $Ca_v2.1$ variants (Tsunemi et al., 2008). It would also be relevant to perform similar $Ca_v2.1$ splice-variant analysis in brain regions of the KI mice that are known to have no involvement in FHM-1 pathology for comparison (for example hippocampus, thalamus and amygdala) (Pietrobon, 2005). $Ca_v2.1$ splice variants identified as unique to brain regions associated with FHM-1 pathology could be investigated further as variants specifically involved in the disease. Experimental procedures outlined in Chapters 2 and 3 in addition to experiments using APWs relevant to neurons implicated in FHM-1 could be

effectively used to establish any differential effects of FHM-1 mutations in the context of the disease variants vs. variants from unaffected brain regions.

4.3 A role for $Ca_v2.1$ CDF in synaptic plasticity

4.3.1 Working hypothesis

In one aspect of Chapter 3 we provide evidence that $Ca_v2.1$ CDF is a required component of short-term synaptic facilitation at the PF-PC synapse. The proportion of facilitation attributed to $Ca_v2.1$ CDF at this synapse can be deduced from experiments in which presynaptic terminals were treated with high (100 μM) concentrations of EGTA-AM (Atluri and Regehr, 1996a). In these experiments, EGTA-AM sped the decay of intracellular Ca^{2+} to the extent of removing the component of synaptic facilitation dependent upon residual Ca^{2+} in the traditional sense. The remaining "residual-Ca^{2+}-independent" form of synaptic facilitation exhibited a short intrinsic time constant, rapid kinetics and high Ca^{2+} affinity all similar to that described for CaM mediated $Ca_v2.1$ CDF (Atluri and Regehr, 1996b; Lee et al., 2000; DeMaria et al., 2001). During paired APs (100 Hz) in absence of EGTA-AM, both forms of facilitation were present and produced 125% facilitation of the second post-synaptic response relative to the first, but in the presence of 100 μM EGTA-AM only 25% facilitation of the post-synaptic response remained (Atluri and Regehr, 1996b). Taken together, this suggests that $Ca_v2.1$ CDF likely accounts for approximately 25% of the facilitation observed at the PF-PC synapse during paired APs.

The coupling between Ca^{2+} influx through $Ca_v2.1$ channels and neurotransmitter release within PF boutons, and the intrinsic properties of $Ca_v2.1$ CDF observed in recombinant and endogenous channels both support a value of around 25% synaptic facilitation mediated by $Ca_v2.1$ CDF. Although PF presynaptic terminals express $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ channels (Randall and Tsien, 1995), $Ca_v2.1$ channels are responsible for approximately 60% of presynaptic Ca^{2+} influx during APs, are the most effective VGCC at triggering transmitter release from PF terminals, and are thus responsible for nearly 93% of the synaptic response (Mintz et al., 1995). There is a strong correlation between Ca^{2+} influx in

PF boutons and transmitter release such that the relationship between Ca^{2+} influx through $Ca_v2.1$ and transmitter release is well approximated by a power law, $n \approx 2.3$ (release = $k(Ca^{2+}_{influx})^n$; k is a constant) (Mintz et al., 1995; Sabatini and Regehr, 1997). Thus, even the relatively small enhancement and broadening of APs predicted to result from $Ca_v2.1$ CDF (Chaudhuri et al., 2007) could significantly contribute to short-term facilitation at this synapse. For example, in Figure 3.6 panel D, the peak Ca^{2+} currents through recombinant $Ca_v2.1$ channels increase during repetitive APs (100 Hz) such that the peak current elicited by a second AP is facilitated by ~10% relative to the first (or ~5% facilitation in $Ca_v2.1$ channels in dissociated mouse Purkinje neurons (Fig. 3.3 D)). With a power law relationship between Ca^{2+} influx and neurotransmission of ~2.3, a 5 to 10% increase in the maximum $Ca_v2.1$ Ca^{2+} influx due to CDF within PF terminals during paired APs could account for ~12 to 25% facilitation in the post-synaptic response at the PF-PC synapse. In support, in a model system $Ca_v2.1$ CDF was shown to account for approximately 30% of synaptic facilitation during paired APs (Mochida et al., 2008). As such, I hypothesize that within PF presynaptic terminals residual Ca^{2+} can control Ca^{2+} influx through $Ca_v2.1$ during an action potential to shape the local Ca^{2+} transient at the active zone and cause between 12 and 25% facilitation during paired APs and likely more during trains of APs through the process of $Ca_v2.1$ CDF.

In addition to an important role in synaptic plasticity, our results in Chapter 3 indicate that $Ca_v2.1$ CDF is likely involved in the pathophysiology of FHM-1. Disruption of $Ca_v2.1$ CDF and synaptic efficacy at the PF-PC synapse may explain the cerebellar ataxia often associated with FHM-1. PCs provide the sole output of cerebellar cortex and are integral in providing signals required for motor planning, execution and coordination (Voogd and Glickstein, 1998; Ito, 2000, 2002; Boyden et al., 2004) (Chapter 1, section 1.6.1). The signals from PCs are encoded in their rate of firing and pattern of activity. PCs have regular intrinsic pacemaking that is shaped by various inhibitory and excitatory inputs onto PCs, and fine-tuning (increase or decrease) of the inter-spike interval from that of intrinsic pacemaking conveys information to the DCN relevant to motor coordination (Ebner, 1998). As such, the significant increase in presynaptic Ca^{2+} influx and presumably enhancement in glutamate release from PF induced by the S218L FHM-1 mutation (Fig. 3.6) may alter the precise balance of excitatory and

inhibitory inputs onto PC under some conditions. In so doing, the fine-tuning of PCs firing and information conveyed to DCN may be compromised sufficiently to lead to cerebellar motor deficits including ataxia. In fact, other mutations in the $Ca_v2.1$ channel that increase, decrease and/or alter the dynamics of excitatory inputs to PC have been correlated with aberrant motor phenotypes in several other animal models involving episodic ataxia. For example, the *tg* and tg^{rol} mice are established animal models of cerebellar ataxia caused by point mutations in the mouse cacna1a gene encoding $Ca_v2.1$. Similar to the severe form of FHM-1 associated with the S218L mutation, the *tg* and tg^{rol} phenotypes include episodic attacks of cerebellar ataxia. The cerebellar motor deficits in these mice have been attributed to decreased excitatory transmission at the PF-PC synapse (Matsushita et al., 2002; Zhou et al., 2003). Although opposite in effect to the enhanced PF-PC transmission in S218L mice, the end result is disruption in the precise balance of excitatory and inhibitory inputs onto PCs that likely alters the fine-tuning of PC intrinsic firing and information processing.

Considering that $Ca_v2.1$ channels are the predominant VGCC underlying synaptic transmission at most fast synapses in the mammalian CNS and that the PF-PC synapse is prototypical of other CNS synapses (Westenbroek et al., 1995; Evans and Zamponi, 2006), I predict that $Ca_v2.1$ channel CDF is a largely unrecognized but significant contributor to short-term synaptic facilitation at other fast synapses in the CNS (Fig. 4.1; proposed model). At terminals expressing $Ca_v2.1$, Ca^{2+} influx during a single AP likely binds both CaS molecules directly associated with vesicle fusion (such as synaptotagmin) and CaS molecules that cause CDF of $Ca_v2.1$ (such as CaM) (Fig. 4.1A-C). As a result, during subsequent APs, $Ca_v2.1$ channels mediate a greater influx of Ca^{2+} that combines with residual Ca^{2+} and causes a larger synaptic response (Fig. 4.1D-F). However, not all $Ca_v2.1$ splice variants exhibit the same level of CDF and synapses with different $Ca_v2.1$ splice variants may have a different reliance on $Ca_v2.1$ CDF. For example, the use of the EF_a and EF_b alternative exons and the Δ47 and +47 variants in the C-terminus of $Ca_v2.1$ affects the magnitude of CaM mediated $Ca_v2.1$ CDF (Chaudhuri et al., 2004). $Ca_v2.1$ channels containing the EF_a exon exhibit robust CDF (in combination with either Δ47 or +47 variants), whereas $Ca_v2.1$ channels containing the EF_b exon exhibit CDF only when in combination with the Δ47 variant. There is approximately equal expression of the 37_a (40.5%) and 37_b (59.5%) variants in mammalian

cerebellum, hippocampus and cerebral cortex, a large preferential expression of EF_b in amygdala, and predominant expression of EF_a in the substantia nigra and thalamus (Soong et al., 2002; Chaudhuri et al., 2004). It is unknown what the expression of each variant is within specific cell types in these various brain regions (Kanumilli et al., 2006; Richards et al., 2007). An additional consideration is that there are profound changes in the expression pattern of the splice variants during development within various brain regions and also a clear gender bias in rodent and human brain (Chaudhuri et al., 2005; Chang et al., 2007b). In addition to $Ca_v2.1$ alternative splicing, coexpression with various beta subunits, AP frequency and the types of CaS proteins expressed within terminals likely determine the magnitude of $Ca_v2.1$ CDF and its contribution to synaptic facilitation in central synapses.

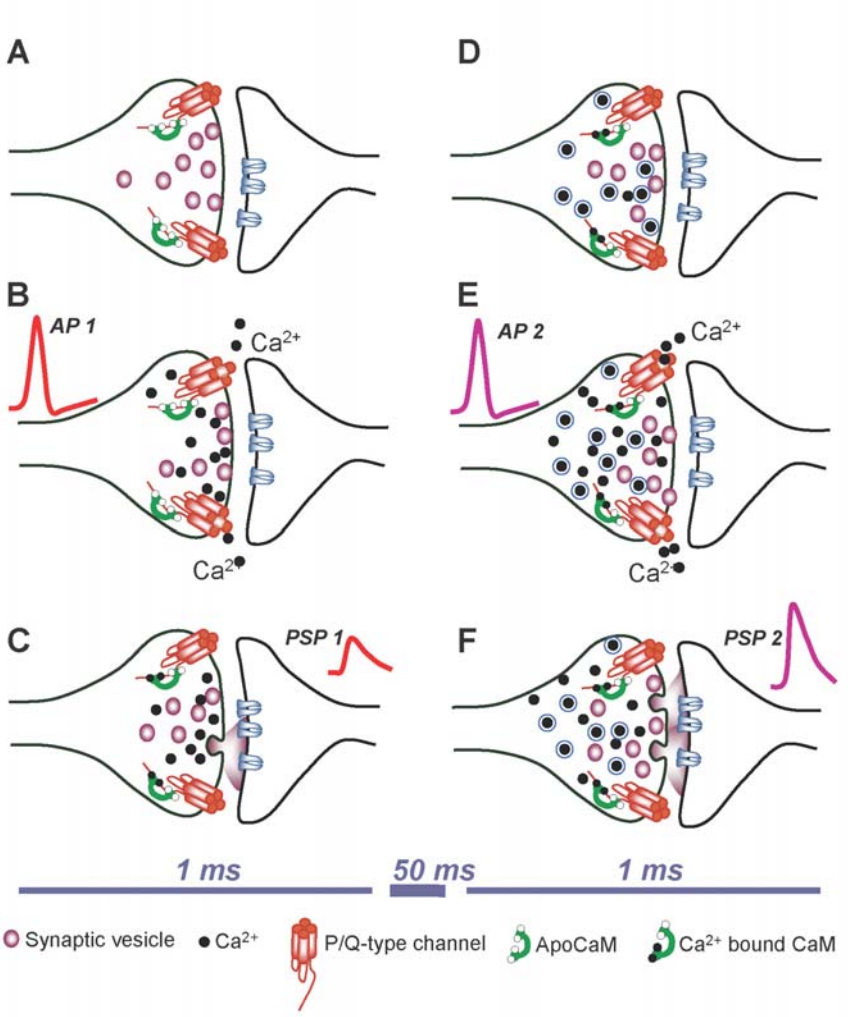

Figure 4.1: Schematic model outlining the role of CDF of $Ca_v2.1$ channels as a key component of short-term synaptic facilitation.

(A) A resident CaS (such as calmodulin; CaM) is bound to the $Ca_v2.1$ channel in a Ca^{2+} free form (ApoCaM). (B) During an action potential (AP1), Ca^{2+} enters synaptic terminals primarily through $Ca_v2.1$ channels. (C) Ca^{2+} ions bind to CaS molecules (such as synaptotagmin) which are directly associated with vesicle fusion and transmitter release functions. Transmitter release causes a modest post-synaptic potential (PSP 1). In addition, according to our model, Ca^{2+} ions near the pore of the channel also bind to a CaS such as ApoCaM causing a conformational change in the $Ca_v2.1$ channel rendering it in a facilitated state; a state of higher open-channel probability. (D) Tens of milliseconds later, some residual Ca^{2+} (Ca^{2+} with blue rings) remains in the terminal and $Ca_v2.1$ channels are in a facilitated state. (E) Upon arrival of a second AP at the terminal (AP 2), facilitated $Ca_v2.1$ channels mediate a greater influx of Ca^{2+} which combines with the residual Ca^{2+}. (F) The combination of residual Ca^{2+} and the greater Ca^{2+} influx through facilitated $Ca_v2.1$ channels result in more vesicles fusing with the membrane and greater transmitter release which is detected as a larger PSP (PSP 2).

If $Ca_v2.1$ CDF is relevant to other CNS synapses besides the PF-PC synapse, then this phenomenon may also be important in FHM-1 pathophysiology beyond cerebellar dysfunction. For example, disruption in the balance between excitation and inhibition within neuronal networks in the cerebral cortex and trigeminovascular pain pathways likely contribute to lower threshold for CSD and sensitization and migraine pain, respectively (see discussion in section 4.2.1). By rendering channels in a constitutively facilitated state, FHM-1 mutations may disrupt the balance between excitation and inhibition. Of note however, preliminary evidence examining the –NP and +NP $Ca_v2.1$ splice variants indicates that the effect of FHM-1 mutations on $Ca_v2.1$ CDF can be splice-variant dependent (Fig 4.2). As such, only cells expressing certain $Ca_v2.1$ variants will likely be affected. I hypothesize that a combination of the differential effects of FHM-1 mutations on CDF of specific $Ca_v2.1$ splice variants combined with differences in the basal level of $Ca_v2.1$ CDF at various CNS synapses will determine the role of CDF toward FHM-1 mediated pathophysiology.

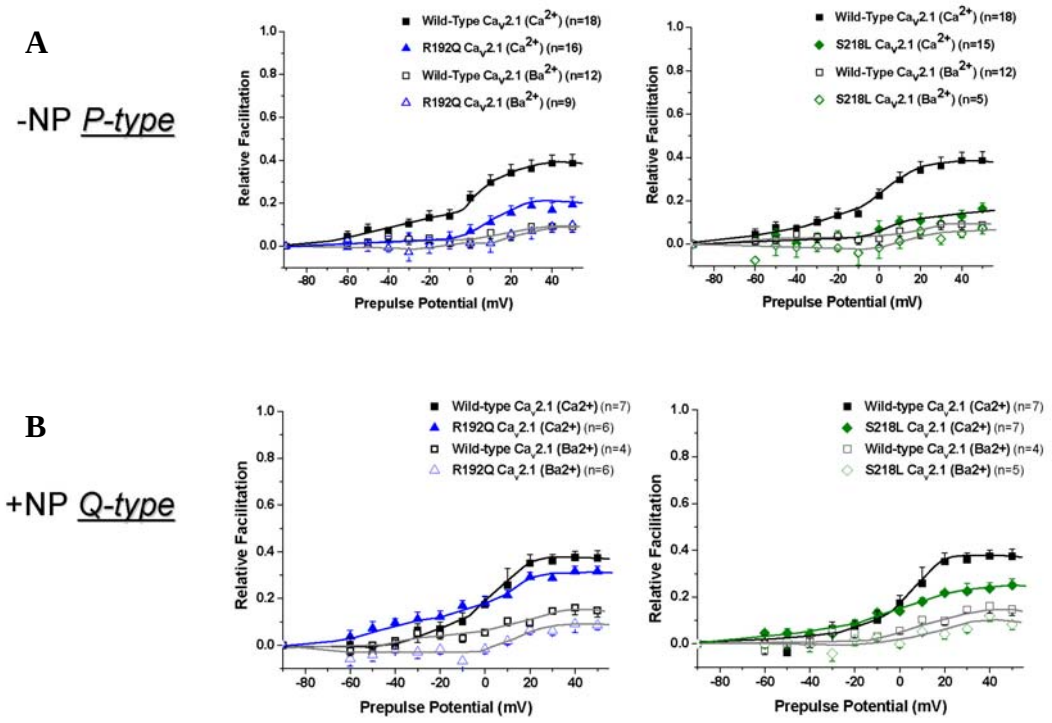

Figure 4.2: R192Q and S218L FHM-1 mutant effects on $Ca_v2.1$ CDF are splice-variant dependent.

Using the paired-pulse protocol as described (Chaudhuri et al., 2004), both the R192Q and S218L FHM-1 mutations are found to reduce CDF across several prepulse potentials shown as relative facilitation vs. prepulse potential (results are means ± s.e.m). Both FHM-1 mutations tested have a differential effect on CDF of two predominant human $Ca_v2.1$ splice variants. Both mutations have a larger effect on CDF when expressed in the $Ca_v2.1$ (–NP) variant, **A**, relative to when expressed in the $Ca_v2.1$ (+NP) variant, **B**. Constructs are human $Ca_v2.1$ (Δ47) transiently transfected with β_2 and $\alpha_2\delta$-1 in HEK 293 cells. The effect of the mutations on CDF was similar in both the $Ca_v2.1$ (Δ47) and $Ca_v2.1$(+47) variants (data not shown).

4.3.2 Possible limitations

In Chapter 3 we provided empirical evidence that FHM-1 mutations disrupt $Ca_v2.1$ CDF and cause a corresponding change in short-term synaptic facilitation at the PF-PC synapse. We discuss several lines of evidence from other CNS synapses, a model system and properties of the PF-PC synapse that support the conclusion that $Ca_v2.1$ CDF is likely an important mechanism of short-term facilitation at the PF-PC synapse. However, we have not yet shown that $Ca_v2.1$ CDF exists under paired APs in wild-type terminals. It would be ideal to demonstrate under paired APs in wild-type animals that Ca^{2+}

influx through $Ca_v2.1$ channels in PF boutons is facilitated 5-10% during the second AP. However, there are several practical and technical challenges. For one, $Ca_v2.1$ channels account for ~60% of Ca^{2+} influx in PF boutons whereas the remainder is mostly through $Ca_v2.2$ and some $Ca_v2.3$ channels (Mintz et al., 1995). Although $Ca_v2.2$ and $Ca_v2.3$ channels do not undergo CDF, they do undergo various forms of VD facilitation (Liang et al., 2003) and to properly isolate $Ca_v2.1$ CDF it would be necessary to block these channels. Due to the small size of mouse PF terminals, with only 60% of the Ca^{2+} signal remaining the Ca^{2+} signal elicited relative to background fluorescence would be very low and a 5-10% change above the residual Ca^{2+} signal would likely not be resolved. In fact, when describing the two components of facilitation involved within the PF boutons, Atluri and Regehr recognized that the smaller "residual-Ca^{2+}-independent" component could not be adequately resolved using Ca^{2+}-sensitive fluorescent indicators (Atluri and Regehr, 1996b). The direct measurement of $Ca_v2.1$ CDF in presynaptic terminals will require higher resolution techniques.

An additional area of potential concern is that both the R192Q and S218L mutations cause a hyperpolarizing shift in the current-voltage relationship under square-pulse depolarizing voltage steps (Chapter 2, and other authors cited elsewhere). It could be argued that the shift to more negative activation voltages is responsible for the increased Ca^{2+} influx at PF terminals in S218L mice; assuming that the kinetics of presynaptic Ca^{2+} currents through $Ca_v2.1$ channels can be modeled by Hodgkin-Huxley equations, a shift to more negative activation voltages theoretically could generate larger Ca^{2+} currents during APs (Borst and Sakmann, 1999). In fact, a hyperpolarizing shift by the R192Q mutation appears to be responsible for increased Ca^{2+} influx during APs in pyramidal neurons in cortical slices from R192Q knock-in mice (Tottene et al., 2009; Inchauspe et al., 2010). However, at the calyx of Held in the same R192Q knock-in mice, even though the mutation caused a similar 6 mV hyperpolarizing shift in the $Ca_v2.1$ current-voltage relationship under square-pulse depolarizing voltage steps, the shift did not alter presynaptic Ca^{2+} influx through $Ca_v2.1$ channels during normal evoked APs (Inchauspe et al., 2010). It was determined that the difference in effects between pyramidal cells synapses and the calyx of Held synapse in the R192Q mice were due to AP duration. During short 1 ms AP durations typical of the calyx of Held, a hyperpolarizing shift in the activation voltage was insufficient to alter presynaptic

Ca^{2+} influx. On the other hand, when the AP duration was prolonged by 1-2 ms to match those typical of cortical pyramidal neurons, the shift in the activation by the R192Q mutation was sufficient to cause greater presynaptic Ca^{2+} influx (Inchauspe et al., 2010). Importantly, the AP duration at PF terminals closely resembles the normal, 1 ms APs at the calyx of Held (Sabatini and Regehr, 1996, 1997). In this regard, a shift to more negative activation voltages observed under square pulse depolarizations does not likely translate to an increase in Ca^{2+} influx during the normal, short APs at PF terminals. In contrast, $Ca_v2.1$ channels in the facilitated state have an enhanced Ca^{2+} influx during APs with durations of 1 ms or less (our results in Chapter 3 as well as (Chaudhuri et al., 2004; Chaudhuri et al., 2005; Chaudhuri et al., 2007; Kreiner et al., 2010)). Overall, if the S218L mutation renders channels into a facilitated state resembling that achieved during CDF (as proposed in Chapter 3, see discussion in section 3.2.3), an enhancement of Ca^{2+} influx during APs at PF boutons in S218L mice relative to wild-type would be expected.

A further potential limitation relates to our hypothesis that $Ca_v2.1$ CDF is a likely mechanism of short-term synaptic facilitation at other central synapses and that changes in $Ca_v2.1$ CDF by FHM-1 mutations likely alters synaptic efficacy at these synapses. In the large calyx of Held, Ca^{2+}-dependent enhancement of presynaptic Ca^{2+} currents through $Ca_v2.1$ accounts for upwards of 40% of the total facilitation of synaptic response during repetitive APs (Forsythe, 1994; Borst and Sakmann, 1998; Cuttle et al., 1998; Forsythe et al., 1998; Inchauspe et al., 2004; Xu and Wu, 2005; Muller et al., 2008), however, as mentioned, Ca^{2+} influx through $Ca_v2.1$ channels at the calyx of Held is unchanged in R192Q knock-in mice under conditions of normal, short AP firing (Inchauspe et al., 2010). Furthermore, the authors showed that facilitation of synaptic transmission was not altered in the R192Q mice relative to wild-type under these same conditions. This indicates that perhaps $Ca_v2.1$ CDF is fundamentally different in terms of the mechanism of facilitation at this particular synapse. In fact, while there is some evidence that the CaS NCS-1 may mediate facilitation of P/Q-type currents at this synapse (Tsujimoto et al., 2002), a role for CaM is uncertain (Sakaba and Neher, 2001; Xu and Wu, 2005; Nakamura et al., 2008). Further, it is possible that CaM mediated $Ca_v2.1$ CDF is a minimal component of facilitation due to the expression of $Ca_v2.1$ variants that do not exhibit this type of $Ca_v2.1$ CDF (e.g. $Ca_v2.1$ $EF_b/\Delta47$).

As such, the R192Q may not affect facilitation at the calyx of Held due to different mechanims of $Ca_v2.1$ facilitation at this synapse or perhaps due to the expression of $Ca_v2.1$ splice variants that have CDF unaffected by the R192Q mutation (such as the $Ca_v2.1$ –NP variant; Fig. 4.2). In future studies it will be interesting to see whether the more severe effects of the S218L mutation on $Ca_v2.1$ CDF alter facilitation at the calyx of Held.

4.3.3 Future directions

An important future study will be to identify the CaSs responsible for mediation of $Ca_v2.1$ CDF in PF terminals. CaM is a primary candidate as its kinetic properties as a CaS correlate with the temporal profile of facilitation at this synapse (Atluri and Regehr, 1996b). The involvement of CaM in short-term facilitation of PF-PC synapses would be best determined by recording EPSCs in PCs during paired stimuli of PFs in transverse slices of the cerebellar vermis. During paired stimuli of PF, PC EPSCs show robust PPF (Atluri and Regehr, 1996b). Recordings at wild-type synapses could be compared in the presence and absence of a potent CaM blocker such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) (Tanaka and Hidaka, 1980). If CaM is the dominant mediator of $Ca_v2.1$ CDF in presynaptic terminals of parallel fibres, then in the presence of W7, PPF of EPSCs would be expected to be reduced by approximately 25%.

Another future study relevant to the work presented here would be to explore the effect of the R192Q and S218L mutations on LTP. LTP is a use-dependent form of synaptic plasticity that has long been considered to play a critical role in motor learning (Teyler and Discenna, 1984). There is evidence that LTP at the PF-PC synapse involves presynaptic processes that overlap with those underlying PPF (Schulz et al., 1995; Salin et al., 1996). Since both the R192Q and S218L mutations reduce PPF at this synapse (Fig 3.4), if the underlying mechanisms of PPF and LTP overlap, then LTP may also be altered in these mice. Furthermore, LTP at the PF-PC synapse is typically induced by eliciting high-frequency stimulations consisting of 10 trains (100 Hz stimulations given for 50 ms) delivered at 200 ms intervals for a total of 50 stimulations over 2 s (Schulz et al., 1994). The periods of AP firing required to induce

LTP are long enough that CDI of $Ca_v2.1$ may also be relevant. Strong CDI of $Ca_v2.1$ is evident during prolonged APs that last beyond 400 ms and in Chapter 3 we show CDI of $Ca_v2.1$ channels is significantly reduced by the S218L mutation (but not R192Q) during a 1 second train of APs delivered at 100 Hz (Fig. 3.1 and 3.2). As such, if $Ca_v2.1$ CDI is also relevant to synaptic plasticity at the PF-PC synapses under conditions of prolonged AP firing as predicted by a model system (Mochida et al., 2008), then additional changes may be observed in LTP at PF-PC in S218L mice compared to R192Q and wild-type mice. LTP in wild-type and mutant KI mice at the PF-PC synapse can be measured using extracellular field recordings in transverse cerebellar slices. EPSPs can be evoked by orthodromic stimulation of the parallel fibres in the molecular layer of the cerebellar vermis. Understanding the effects of FHM-1 mutations on LTP may provide valuable information regarding the role of cerebellar development and motor coordination in ataxia. Further, elucidating the role of $Ca_v2.1$ channels in LTP would be an important advancement toward understanding the basic molecular mechanisms of long-term synaptic plasticity and motor learning in the cerebellum.

4.4 Conclusions

4.4.1 General Conclusions

Overall, we have shown that $Ca_v2.1$ splice variants respond differently to FHM-1 mutations. We showed that a single point mutation can cause significant gain-of-function effects in one $Ca_v2.1$ variant and have little or no effect (or even loss-of-function effects) in another $Ca_v2.1$ variant. We also showed that under different firing conditions, $Ca_v2.1$ variants respond differently to FHM-1 mutations. I conclude that the phenomenon of differential effects of missense mutations on Ca_v splice variants is a major underlying theme in the localized and episodic nature of FHM-1 and other Ca^{2+} channelopathies.

Furthermore, we showed for the first time that FHM-1 mutations alter CaM mediated $Ca_v2.1$ CDF. We showed that the occlusion of $Ca_v2.1$ CDF by two FHM-1 mutations results in a corresponding attenuation in short-term synaptic plasticity at an intact CNS synapse. As such, I conclude that $Ca_v2.1$

CDF is an important mechanism of short-term synaptic plasticity at the PF-PC synapse, and perhaps other CNS synapses. Furthermore, significant changes in PF-PC synaptic transmission by some FHM-1 mutations likely alter the precise balance between excitatory and inhibitory inputs onto PCs (and thus the fine-tuning of PC intrinsic pacemaking), and as a result, lead to cerebellar motor deficits including ataxia under some conditions.

4.4.2 Relevance to treatment of human disease

Migraine headaches affect approximately 15% of the Western population (Stewart et al., 1992; Lipton and Stewart, 1994; Lipton et al., 2001; Henry et al., 2002). However, the complicated genetics and physiology of migraine have slowed both the development of adequate treatments and our understanding of underlying disease mechanisms (Montagna, 2004). FHM-1 however, is an autosomal-dominant inherited subtype of migraine with similar clinical features to typical migraine, and has served as a useful model to study pathogenic mechanisms of common forms of migraine (Ducros et al., 2001; Thomsen et al., 2002). As such, understanding the mechanisms of FHM-1 pathophysiology provides information concerning the disease mechanisms that can hopefully be used to develop more effective migraine treatments. The involvement of the $Ca_v2.1$ channel in migraine pathology make it a potential therapeutic target, although to date its ubiquitous expression and function in the CNS and PNS have led to concerns for the high risk of undesired side-effects. Our work suggests that targeting specific $Ca_v2.1$ splice variants directly involved in migraine pathology could minimize undesired effects. For example, we demonstrate above that CDF of $Ca_v2.1$ (-NP) and $Ca_v2.1$ (+NP) variants are differentially affected by FHM-1 mutations (Fig. 4.2). These same channel variants show discrete pharmacological sensitivities to peptide toxins that differ by more than 100 fold (Mintz et al., 1992; Wheeler et al., 1994). Taken together, this suggests splice variants differentially affected by FHM-1 mutations may also be differentially targeted pharmacologically as a therapeutic approach. This type of targeted approach to disease treatment could be a successful means of obtaining optimal positive results with minimal adverse reactions. In fact, targeting specific $Ca_v2.2$ channel splice variants has become a leading strategy in

chronic pain therapy even though $Ca_v2.2$ channels are also ubiquitously expressed in the CNS (Snutch, 2005; Altier et al., 2007; Swayne and Bourinet, 2008).

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APPENDIX 1: Ca^{2+} CHANNELOPATHIES: VOLTAGE-GATED Ca^{2+} CHANNELS*

CHAPTER 8

CALCIUM CHANNELOPATHIES: VOLTAGE-GATED CALCIUM CHANNELS

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Abstract: Since the initial identification of native calcium currents, significant progress has been made towards our understanding of the molecular and cellular contributions of voltage-gated calcium channels in multiple physiological processes. Moreover, we are beginning to comprehend their pathophysiological roles through both naturally occurring channelopathies in humans and mice and through targeted gene deletions. The data illustrate that small perturbations in voltage-gated calcium channel function induced by genetic alterations can affect a wide variety of mammalian developmental, physiological and behavioral functions. At least in those instances wherein the channelopathies can be attributed to gain-of-function mechanisms, the data point towards new therapeutic strategies for developing highly selective calcium channel antagonists

Keywords: calcium channel, L-type, P/Q-type, T-type, α_1 subunit, β subunit, $\alpha_2\delta$ subunit, γ subunit, familial hemiplegic migraine, episodic ataxia type 2, spinocerebellar ataxia type 6, Lambert-Eaton myasthenic syndrome, incomplete X-linked congenital stationary night blindness, X-linked cone-rod dystrophy, hypokalemic periodic paralysis, malignant hyperthermia susceptibility, Timothy syndrome, idiopathic generalized epilepsy, autism spectrum disorders, lethargic, ducky, stargazin

1. INTRODUCTION

Voltage-gated calcium channels are found in all excitable and many non-excitable cells where they contribute to numerous physiological processes including triggering muscle contraction and neurotransmitter release, regulating calcium-dependent enzymes and gene expression, mediating repetitive firing patterns and pacemaker

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activities, and developmentally controlling both neurite outgrowth and growth cone migration (reviewed in (Catterall, 2000)). As intracellular calcium concentrations must be finely controlled temporally and spatially, disruption of normal calcium channel activity can be highly detrimental physiologically. Biophysical analyses have generally categorized voltage-gated calcium channels into low-voltage activated (LVA) and high-voltage activated (HVA) subtypes, depending upon the membrane potentials at which they first open; LVA (also known as T-type) calcium channels open in response to small changes from the resting membrane potential whereas HVA channels are activated by stronger depolarizations. LVA channels have other distinguishing properties including a small single channel conductance (~5–12 picosiemens, pS), overlapping activation and inactivation ranges, rapid activation and inactivation kinetics, slow deactivation (closing) although a poorly defined pharmacology. Contrastingly, HVA calcium channels generally possess larger conductances (~15–25 pS), variable inactivation kinetics, faster deactivation, and have a well-defined pharmacology. Multiple types of distinct native HVA calcium currents have been categorized on the basis of single channel conductance, voltage-dependent, kinetic and pharmacological characteristics (called (L-, N-, P/Q- and R-types).

Biochemical studies have established that HVA calcium channels are multi-subunit protein complexes. The major α_1 subunit (~175–260 kDa) forms the channel proper and contains both the voltage-sensing mechanism and the calcium-selective pore, and is also the target for most pharmacological agents and second-messenger-dependent modulatory interactions. Mammalian genomes contain seven α_1 subunit genes encoding the HVA calcium channel family; $Ca_v1.1(\alpha_{1S})$, $Ca_v1.2$ (α_{1C}), $Ca_v1.3(\alpha_{1D})$ and $Ca_v1.4(\alpha_{1F})$ all encode distinct L-type calcium channels, $Ca_v2.1(\alpha_{1A})$ encodes P/Q-type channels, $Ca_v2.2(\alpha_{1B})$ encodes the N-type channel, while $Ca_v2.3(\alpha_{1E})$ encodes the R-type channel (reviewed in (Snutch et al., 2005)). There are also three genes encoding distinct T-type channel α_1 subunits; $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$. The various HVA α_1 subunits interact with single β and $\alpha2\delta$ subunit proteins which serve to modulate a variety of calcium channel properties including regulation by second messenger pathways, channel processing and trafficking, and biophysical characteristics. In mammals there are four known genes encoding β subunits ($\beta1$–$\beta4$) and four genes encoding $\alpha_2\delta$ subunits ($\alpha_2\delta1$ – $\alpha_2\delta4$). In the skeletal muscle L-type calcium channel complex there is also an associated forth subunit, γ, whose various members have been shown to also regulate calcium channel properties (Wei et al., 1991; Letts et al., 1998; Klugbauer et al., 2002). The γ subunit family of proteins is also known to be part of a group of AMPA receptor regulatory proteins (called TARPs) and thus they may not be specific to calcium channel complexes *in vivo* (Tomita et al., 2003). While the auxiliary β, $\alpha2\delta$ and γ subunits can also modulate T-type α_1 subunit properties in some exogenous systems, there is as of yet no biochemical data supporting the notion that T-type calcium channels are a multi-subunit complex.

To date, human channelopathies have been associated with five of the ten calcium channel $\alpha_1$subunit genes and two of the auxiliary subunit genes. Given

their widespread expression and contributions in both neuronal and muscle physiologies, mutations in calcium channel genes tend to manifest as severe phenotypes. Also described have been a number of mouse mutations in both α_1 subunit and the auxiliary β, $\alpha 2\delta$ and γ subunits that are deserving of mention as they provide further important clues as to physiological contributions of calcium channel complexes in whole animals.

2. P/Q-TYPE ($Ca_V2.1/\alpha_{1A}$) CALCIUM CHANNELS; CACNA1A GENE

P/Q-type calcium channels are one of the most abundantly expressed calcium channel subtypes in the mammalian nervous system (Bourinet et al., 1999; Jun et al., 1999). As well as being expressed on many cell bodies and dendrites, they are highly localized at presynaptic terminals throughout the brain and spinal cord where they mediate calcium influx essential for neurotransmitter release (Takahashi and Momiyama, 1993). They also contribute to the precise modulation of intracellular calcium levels important in second messenger signaling and calcium-dependent gene transcription (Sutton et al., 1999). Considering the widespread expression and essential neurophysiological roles, it is perhaps not unexpected that mutations in the CACNA1A gene encoding the P/Q-type channel α_1 subunit (Cav2.1) cause several severe channelopathies. CACNA1A mutations are associated with human neurological disorders such as Familial Hemiplegic Migraine (FHM1), Episodic Ataxia type 2 (EA2) and Spinocerebellar Ataxia type 6 (SCA6), and mice disorders tottering, leaner, rolling nagoya and rocker (Table 1 human, Table 2 mice). In addition to the defined mutations in the $Ca_v2.1$ calcium channel causing disease, an autoimmune attack on $Ca_v2.1$ channels is associated with Lambert-Eaton Myasthenic Syndrome (LEMS).

2.1. Familial Hemiplegic Migraine (FHM1)

Migraine is a severe neurological condition that affects approximately 11% of the North American and Western European populations. Migraine headaches can occur in isolation or, in approximately 20% of migraine sufferers, the migraine headache can be preceded by, or concurrent with, an aura (a subjective sensation; reviewed in (Goadsby et al., 2002)) FHM1 is a rare autosomal dominant subtype of migraine with aura, and other than its characteristic hemiplegia, has similar clinical features to typical migraine with aura. Based on neuroimaging and animal studies, it appears the aura phase of migraine is due to cortical spreading depression (CSD); CSD is a transient wave of neuronal hyperexcitability that begins at a focal point and slowly progresses over the cortex, followed by a long neuronal depression (Lauritzen, 1994). In FHM1, the aura manifests as a motor aura in combination with one or more visual, sensory and/or aphasic auras; the characteristic motor aura most frequently manifests as hemiplegia in both the upper and lower extremities and may or may not be associated with transient or permanent cerebellar

Table 1. CACNA1A ($Ca_v2.1$, α_{1A}): Functional results for mutations associated with FHM = familial hemiplegic migraine, EA2 = episodic ataxia type 2, SCA6 = spinocerebellar ataxia type 6, PCA = progressive cerebellar ataxia (although addition mutations have been associated with these disorders, only those with reported functional data are listed)

Mutation and Disease	Functional Analyses
R192Q- Pure FHM	• hyperpolarized shift in V_{50act} (Melliti et al., 2003) • hyperpolarized shift in $V_{50inact}$, reduced excitatory and inhibitory neurotransmission, reduced Ca^{2+} influx during action potential waveform (APW) (Cao and Tsien, 2005) • no change in current voltage relationship (Kraus et al., 1998; Cao and Tsien, 2005) • increased open probability (Hans et al., 1999) • increased current density (Hans et al., 1999; Tottene et al., 2002; van den Maagdenberg et al., 2004) • reduced current density (Tottene et al., 2002; Cao and Tsien, 2005) • increased single channel conductance (Tottene et al., 2002) • reduces G-protein inhibition (Melliti et al., 2003) • reduced threshold and increased velocity of cortical spreading depression (CSD) in knock-in mice. (van den Maagdenberg et al., 2004) • increased transmitter release at neuromuscular junction of knock-in mouse (Kaja et al., 2005)
T666M- FHM with cerebellar signs	• hyperpolarized shift in V_{50act}, increased rate of inactivation, increased current decay in response to 1Hz train pulse, reduced rate of recovery from inactivation (Kraus et al., 1998; Hans et al., 1999); no change in current voltage relationship, reduced Ca^{2+} influx during action potential waveform (APW), reduced excitatory and inhibitory neurotransmission (Cao and Tsien, 2005) • increased open probability (Tottene et al., 2002) • no change in open probability, reduced unitary conductance (Hans et al., 1999) • reduced current density (Hans et al., 1999; Tottene et al., 2002; Barrett et al., 2005; Cao and Tsien, 2005) • increased single channel conductance (Tottene et al., 2002) • reduction in gating current (Barrett et al., 2005)
V714A- Pure FHM	• hyperpolarized shift in V_{50act} (Kraus et al., 1998; Hans et al., 1999) • increased rates of inactivation and recovery from inactivation (Kraus et al., 1998; Hans et al., 1999) • increased open probability, decreased single channel conductance (Hans et al., 1999) • reduced current density (Hans et al., 1999; Tottene et al., 2002) • increased single channel conductance (Tottene et al., 2002) • decreased current decay in response to 1Hz train pulse (Kraus et al., 1998)
I1811L- FHM with cerebellar signs	• hyperpolarized shift in V_{50act} (Kraus et al., 1998; Hans et al., 1999) • no change in current voltage relationship (Cao and Tsien, 2005) • increase rate of recovery from inactivation (Kraus et al., 1998; Hans et al., 1999) • increased open probability (Hans et al., 1999)

	• reduced current density (Hans et al., 1999; Tottene et al., 2002) • increased single channel conductance (Tottene et al., 2002) • decreased current decay in response to 1Hz train pulse (Kraus et al., 1998)
K1336E- Pure FHM	• hyperpolarized shift in V_{50act} and V_{50inac}, increased current decay in response to 1Hz train pulse, decreased inactivation time constant (Mullner et al., 2004) (most effects are β subunit dependent)
V1457L- Mild FHM	• hyperpolarized shift in V_{50act} (Kraus et al., 2000; Tottene et al., 2002) • slowed rate of inactivation and reduce rate of recovery from inactivation (Kraus et al., 2000) • increased open probability, decreased unitary conductance, reduced current density (Tottene et al., 2002)
V1696I- Pure FHM	• slowed rate of inactivation, reduced rate of recovery from inactivation, increased current decay in response to 1Hz train pulse (Mullner et al., 2004) (most effects are β subunit dependent)
W1684R- FHM with cerebellar signs	• hyperpolarized shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, reduced recovery from inactivation, increased current decay in response to 1Hz train pulse (Mullner et al., 2004) (most effects are β subunit dependent)
R583Q- FHM/Ataxia	• hyperpolarized shift in V_{50act} and $V_{50inact}$, reduced rate of recovery from inactivation, decreased current decay in response to 1Hz train pulse (Kraus et al., 2000)
D715E- FHM/PCA	• hyperpolarized shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, increased rate of inactivation, decreased current decay in response to 1Hz train pulse (Kraus et al., 2000)
S218L- FHM with coma, following minor head injury	• hyperpolarized shift in V_{50act}, increased rate of inactivation initially, followed by a large reduction in the rate of inactivation, increased rate of recovery from inactivation, increased open probability, increased current density at low voltages, and decreased current density at high voltages, increased current decay in response to short train pulses but a decrease in current decay in response to long train pulses (Tottene et al., 2005a)
E147K- EA2	• reduced current density, possibly due to less channels reaching the membrane, reduced rate of inactivation (Imbrici et al., 2004)
F1406C- EA2	• reduction in Ba^{2+} current (Jen et al., 2001) • dominant negative effect – dramatic reduction in Ba^{2+} current (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)
F1491S- EA2	• channel activity completely abolished (Guida et al., 2001)
R1547X , R1549X- EA2	• dramatic reduction in Ba^{2+} currents (Jen et al., 2001) • dominant negative effect – dramatic reduction in Ba^{2+} current (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)

(*Continued*)

Table 1. (*Continued*)

Mutation and Disease	Functional Analyses
E1761K- EA2	• dominant negative effect - dramatic reduction in Ba^{2+} current (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)
G293R- EA2	• no significant shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, reduced recovery rate from inactivation (Wan et al., 2005) • depolarized shift in V_{50act}, no change in recovery rate from inactivation, increased rate of inactivation, decreased mean open time, decreased current density (Wappl et al., 2002); likely due to abnormalities in protein folding and trafficking to the plasma membrane (temperature dependent) (Wan et al., 2005) • increased current decay in response to 1Hz train pulse (Wappl et al., 2002; Wan et al., 2005)
C287Y- EA2	• depolarized shift in V_{50act}, hyperpolarized shift in $V_{50inact}$, decreased current density, likely due to protein abnormalities in protein folding and trafficking to the plasma membrane (temperature dependent; occurs at 37°C) (Wan et al., 2005)
H1736L- EA2	• depolarized shift in V_{50act}, increased rate of inactivation, decreased current decay in response to 1Hz train pulse, increased recovery rate from inactivation, decreased current density (Spacey et al., 2004)
R1279X, R1281X- EA2	• reduction in Ba^{2+} current, dominant negative effect (more pronounced effect in 47 than Δ47) (Jeng et al., 2006)
R1820X Generalized epilepsy and episodic and progressive ataxia	• dominant negative effect – dramatic reduction in Ba^{2+} current (Jouvenceau et al., 2001)
nt.4778-4780 : ctt deletion, deletion of A1593 and Y1594- EA2	• complete loss of channel activity in tsA-201 cells; in Xenopus oocytes depolarized shift in V_{50act}, increased rate of inactivation, increased current decay in response to 1Hz train pulse, reduced recovery rate from inactivation (Wappl et al., 2002)
polyQ expansion, 23 repeats -SCA6	• increased channel density (Piedras-Renteria et al., 2001) • no change in voltage-dependent or time-dependent properties (Piedras-Renteria et al., 2001)
polyQ expansion, 24 repeats (-NP) - SCA6	• hyperpolarized shift in $V_{50inact}$ (Toru et al., 2000) • no change in the $V_{50inact}$ (Matsuyama et al., 1999)
polyQ expansion, 27 repeats - SCA6	• increased channel density, no change in voltage-dependent or time-dependent properties (Piedras-Renteria et al., 2001)
polyQ expansion, 28 repeats (-NP)- SCA6	• hyperpolarized shift in $V_{50inact}$ (Toru et al., 2000)

polyQ expansion, 28 repeats (+NP) - SCA6	• depolarized shift in $V_{50inact}$ (Toru et al., 2000)
polyQ expansion, 30 repeats- SCA6	• hyperpolarized shift in $V_{50inact}$ (Matsuyama et al., 1999); hyperpolarized shift in $V_{50inact}$ when expressed with beta 4 subunit, but not with beta 2 or beta 3, increased inactivation time constant when expressed with beta 4 subunit, but not beta 2, and impaired G-protein regulation others (Restituito et al., 2000)
polyQ expansion, 33 repeats - SCA6	• cytotoxic (Kordasiewicz et al., 2006)
PolyQ expansion, 40 repeats - SCA6	• hyperpolarized shift in $V_{50inact}$ (Matsuyama et al., 1999)
polyQ expansion, 28 repeats - SCA6	• perinuclear aggregates of P/Q-type calcium channel in Purkinje cells of SCA6 patients (Ishikawa et al., 1999) • P/Q-type channels with CAG expansion transfected in HEK293 cells results in perinuclear aggregates and cell death (Ishikawa et al., 1999)
polyQ expansion, 72 repeats - SCA6	• increased channel density, no change in voltage-dependent or time-dependent properties (Piedras-Renteria et al., 2001)

signs such as ataxia or nystagmus. In the majority of cases, the headache pain directly follows the aura phase of the migraine attack and is thought to involve the trigeminovascular system (reviewed in (Pietrobon, 2005a)). The headache pain can last from less than 30 minutes to greater than 72 hours, with the mean duration being approximately 24 hours (for an extensive review of FHM1 features and statistics see (Thomsen et al., 2002)). About 50% of FHM1 patients have a mutation in the CACNA1A calcium channel gene (Ophoff et al., 1996) (FHM1), whereas the other approximately 50% have mutations in either the Na(+)/K(+)-ATPase gene (De Fusco et al., 2003) (ATP1A2;FHM2) or the sodium channel gene (Dichgans et al., 2005) (SCN1A; FHM3).

FHM1 is the most extensively studied calcium channelopathy. Since the first mutations in the CACNA1A gene were discovered in 1996 (Ophoff et al., 1996), 17 in total have now been detected (Figure 1). The 17 mutations appear to be localized primarily to the S4 voltage sensors and flanking regions. Over the past ten years, several groups have investigated the pathophysiological effects of FHM1 mutations on biophysical properties of the $Ca_v2.1$ calcium channel using electrophysiological analysis in *Xenopus* oocytes and mammalian expression systems (Table 1). Overall, the results vary considerably, some even contradictory, leaving our understanding of the precise correlation between channel function and disease mechanism unresolved. For example, whereas some results indicate the R192Q mutation increases $Ca_v2.1$ whole cell current density (Hans et al., 1999; van den Maagdenberg et al., 2004), results obtained by other groups indicate the same mutation decreases whole cell current density (Tottene et al., 2002; Cao and Tsien, 2005). In addition, one group found that the most prevalent FHM1 mutation T666M shifts the voltage dependence

Table 2. cacna1a ($Ca_v2.1$, α_{1A}): Functional results for mutations associated with tottering, leaner, rolling nagoya and rocker

Mutation and Phenotype	Functional Analyses
P601L- Tottering (*tg*)	• reduced current density (Wakamori et al., 1998) • no change in current density in dissociated Purkinje neurons (Dove et al., 1998) • decreased P/Q-type channel contribution at hippocampal Schaffer collateral synapses of *tg* mice (Qian and Noebels, 2000; Zhou et al., 2003) • decreased neurotransmitter release at parallel fiber-Purkinje cell synapse of *tg* mice (Matsushita et al., 2002) • no change in neurotransmitter release at parallel fiber-Purkinje cell synapse of tg mice (Qian and Noebels, 2000; Zhou et al., 2003) • decreased glutamate and GABA release in neocortex (Ayata et al., 2000) • increased threshold and decreased duration for CSD (Ayata et al., 2000) • alteration in gene transcription (Cicale et al., 2002) • increased G-protein-dependent modulation at the hippocampal Schaffer collateral synapse of *tg* mice (Zhou et al., 2003)
nt. 5901: 98bp, Intron insertion and frame shift or nt. 5763–5901: 139bp, exon deletion- Leaner (tg^{la})	• reduced current density (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998) • depolarized shifts in V_{50act} and $V_{50inact}$ (Wakamori et al., 1998) • no change in voltage dependent properties but reduced open probability (Dove et al., 1998) • decreased glutamate release in neocortex and increased threshold, decreased duration and reduced rate of CSD (Ayata et al., 2000) • increased apoptotic cell death in cerebellar granule cells in P20 leaner mice (Lau et al., 2004)
R1262G- Rolling Nagoya (tg^{rol})	• depolarized shift in V_{50act}, reduced current density, decreased neurotransmitter release at parallel fiber-Purkinje cell synapse of tg^{rol} mice (Matsushita et al., 2002); increased apoptotic cell death in cerebellar granule cells leaner mice (Suh et al., 2002)
T1310K- Rocker	• no electrophysiology data

of activation to more hyperpolarized potentials (Kraus et al., 1998), while another group found no change in the voltage dependent properties of the T666M mutant $Ca_v2.1$ channels relative to wild-type (Cao and Tsien, 2005). Mutations found in patients with similar clinical phenotypes often have reported opposing effects on the biophysical properties of $Ca_v2.1$ channels; e.g., K1336E and V714A have both been associated with pure FHM1 (without cerebellar signs) but cause reported increased (Mullner et al., 2004) or decreased (Kraus et al., 1998) current decay in response to 1Hz square pulse repetitive stimulations. Although different expression systems (Tottene et al., 2002), auxiliary subunits (Mullner et al., 2004), α_1 subunit splice variants (Adams et al., 2006) and experimental conditions likely account for a portion of the disparity

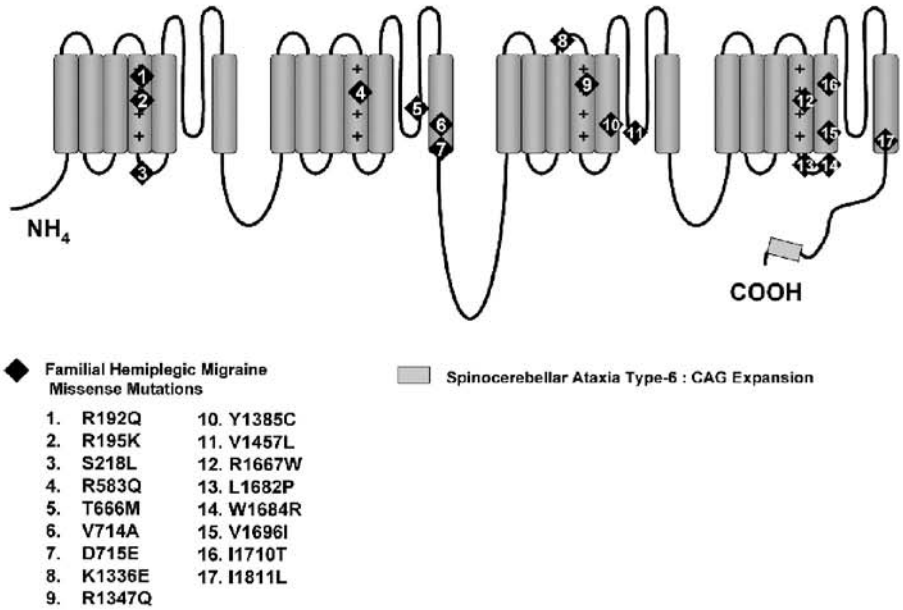

Figure 1. Mutations in the human $Ca_v2.1$ (P/Q-type) voltage-gated calcium channel associated with Familial Hemiplegic Migraine (FHM) and Spinocerebellar Ataxia Type-6 (SCA6)

in results between research groups, it is clear that FHM1 mutation pathophysiology is multivariable and complex.

Interestingly, data from recently generated knock-in mice carrying the R192Q (van den Maagdenberg et al., 2004) and S218L (Gherardini et al., 2006) FHM1 mutations, in conjunction with heterologous expression system analyses, are beginning to show trends in FHM1 mutation effects that may begin to explain the pathology behind the aura phase of migraine. In the heterologous system, several mutations associated with both pure FHM1 as well as FHM1 with cerebellar signs show a hyperpolarizing shift in V_{50act} (Kraus et al., 1998; Hans et al., 1999; Melliti et al., 2003; Mullner et al., 2004) (Table 1), indicating the majority of mutant channels in the expression system are available for opening at lower membrane potentials. Similarly, several mutations studied using single channel recordings show increases in open probability and single channel conductance (Hans et al., 1999; Tottene et al., 2002). All of these affects are predicted gain-of-function phenotypes with the potential to increase calcium influx at lower membrane potentials. *In vivo* work on R192Q and S218L knock-in mice shows a lower threshold for stimulation of CSD depression and increased velocity of propagation across the cortex in intact animals, consistent with gain-of-function effects (van den Maagdenberg et al., 2004; Gherardini et al., 2006). In neuromuscular junction preparations from R192Q knock-in mice, there is increased neurotransmitter release suggesting that FHM1 mutations may directly alter neurotransmitter release at

critical synapses and could therefore contribute to CSD susceptibility (Kaja et al., 2005). Although some data suggests that cultured neurons from Cav2.1 gene knock-out mice and transfected with the R192Q mutation reduce P/Q-type channel contribution to neurotransmitter release without changing overall synaptic strength due to a compensation by N-type calcium channels, *in vivo* evidence from R192Q knock-in mice confirms the neuromuscular junction result in that $Ca_v2.1$ channel-dependent glutamate release at cortical synapses is significantly increased (Tottene et al., 2005b).

Overall, evidence for the pathology of the aura phase of migraine from both the heterologous systems and knock-in mice largely supports the hypothesis that FHM1 mutations increase channel availability and that calcium influx at lower membrane potentials results in increased neurotransmitter release and susceptibility to neuronal firing resulting in CSD causing aura. However, little data has been presented to explain the headache pain phase of migraine. Although one study did show that CSD can activate trigeminovascular afferents and evoke meningeal and brainstem activity consistent with headache pain (Bolay et al., 2002), little is known about the direct involvement, if any, of $Ca_v2.1$ channels in the trigeminal pain pathway itself in the 80% of migraines where aura and CSD are not involved. Some evidence supports the notion that $Ca_v2.1$ P/Q-type channels are important in pain modulation of the trigeminal pain pathway via the periaquaductal gray (Knight et al., 2002; Knight et al., 2003), however further research is required to determine the exact roles of P/Q-type channels and FHM1 mutations in the trigeminal pain pathway relevant to migraine headache.

2.2. Episodic Ataxia Type 2 (EA2)

EA2, similar to FHM1, is an autosomal dominant disorder associated with mutations in the CACNA1A gene, but is clinically quite distinct. EA2 patients experience spontaneous episodes of ataxia (poor muscle coordination) that last for hours to days. In between attacks, patients often experience gaze-evoked or down-beat nystagmus (rapid, involuntary eye oscillations). Approximately 50% of patients experience migraine-like symptoms, and cerebellar atrophy is common (Lorenzon and Beam, 2000). Attacks are often initiated by emotional stress, exercise, or alcohol. Most patients respond well to treatment with acetazolamide (reviewed in (Jen et al., 2004)). EA2 is genetically variable and has been associated with missense, truncation and alternative splice site mutations.

To date, more than 40 individual mutations in the CACNA1A gene have been found to be associated with EA2, although compared to FHM1, significantly less is known concerning the biophysical implications of EA2 mutations on $Ca_v2.1$ properties. EA2 genetic alterations are distributed throughout the channel, with a large number of missense and premature truncations identified within the pore forming p-loops (Figure 2). Generally, the structure-function findings are relatively consistent across experimental conditions (Table 1). Both truncations and missense mutations consistently show a reduction in current density, proposed to be a result

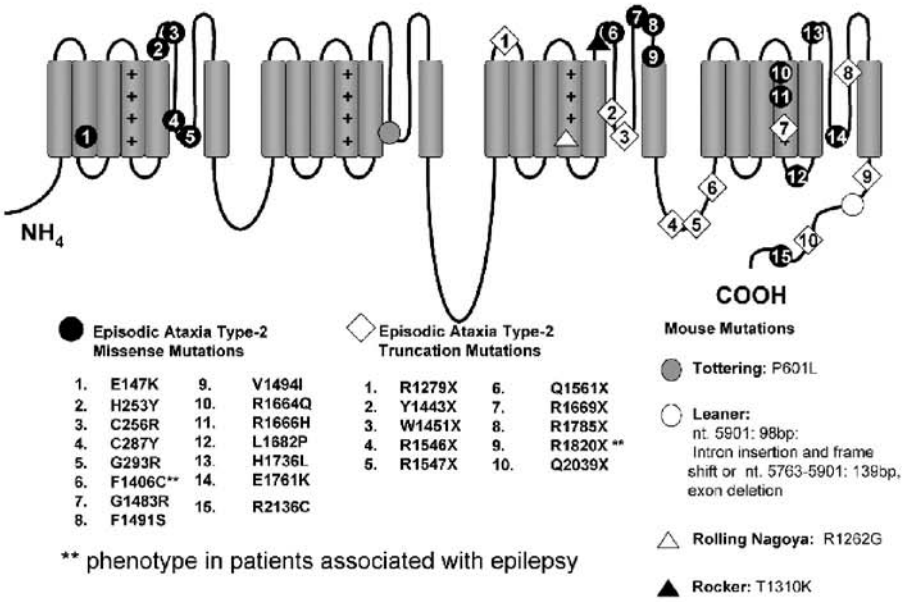

Figure 2. Mutations in the human $Ca_v2.1$ (P/Q-type) voltage-gated calcium channel associated with Episodic Ataxia Type-2 (EA2); mutations in the $Ca_v2.1$ mouse homolog associated with tottering (*tg*), leaner (tg^{la}), rolling nagoya (tg^{rol}) and rocker phenotypes

of fewer channels being properly folded and reaching the membrane (Imbrici et al., 2004; Wan et al., 2005; Jeng et al., 2006). The three EA2 missense mutations G293R, C287Y, H1736L and the deletion mutation nt 4778–4780 are consistent in demonstrating a net reduction of available channels due to a depolarizing shift in V_{50act}, increased rate of inactivation, and a reduced rate of recovery from inactivation (Wappl et al., 2002; Spacey et al., 2004; Wan et al., 2005) (Table 1). Whether it is through reduced trafficking of channels to the membrane or changes in gating properties, all EA2 mutations investigated thus far show a net reduction in $Ca_v2.1$-mediated currents, likely resulting in reduced transmitter release at critical synapses. Overall, unlike the general gain-of-function mutation effects observed for FHM1 mutations, EA2 mutations appear by and large to cause loss-of-channel function phenotypes.

The EA2 data is largely in agreement with results obtained from the epilepsy and ataxia mouse models tottering (*tg*), leaner (tg^{la}), rolling nagoya (tg^{rol}), and rocker (Figure 2). *tg*, tg^{la}, tg^{rol} and rocker all contain $Ca_v2.1$ gene mutations and show varying degrees of epilepsy and ataxia. Heterologous expression of mutations associated with *tg*, tg^{la}, tg^{rol} show similar biophysical properties to those for EA2; decreased current densities and depolarizing shifts in V_{50act} (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998; Mori et al., 2000). *In vivo* studies in the animal models confirms that the reduced channel function seen in the heterologous systems translates to decreased neurotransmitter release in neocortex, dissociated

cerebellar Purkinje cells and parallel fibre – Purkinje synapses (Ayata et al., 2000; Matsushita et al., 2002) (for review on $Ca_v2.1$ models see (Pietrobon, 2005b)). Collectively, these data suggest that EA2 mutations in $Ca_v2.1$ channels likely alter neurotransmitter release in whole animals; although definitive *in vivo* analyses remain to be described.

2.3. Spinocerebellar Ataxia Type 6 (SCA6)

A third autosomal dominant disorder associated with mutations in the $Ca_v2.1$ calcium channel is SCA6. SCA6 is characterized by progressive cerebellar atrophy resulting in progressive gait ataxia, incoordination, nystagmus, proprioceptive sensory loss and dysarthia (Zhuchenko et al., 1997). Zhuchenko and coworkers found that patients with SCA6 have a polyglutamine (CAG) expansion in exon 47 (Figure 1); making SCA6 a member in the group of neurodegenerative disorders containing CAG repeats, which includes Huntington's disease amongst others. Whereas unaffected people tend to have CAG repeats numbering between 4 and 16 in CACNA1A, patients with SCA6 have expansions of greater than 21 CAG repeats. The length of the expansion appears directly correlated with age of onset, e.g., greater CAG expansion is associated with early age of disease onset (Ishikawa et al., 1997). The CAG repeat expansion is associated with severe cerebellar Purkinje cell loss, moderate granule cell and dentate nucleus neuronal loss, and mild neuronal loss in the inferior olive (Zhuchenko et al., 1997). The mechanisms involved between SCA6 mutations in the $Ca_v2.1$ channel and neuronal death have not been completely resolved. Biophysical analyses of polyglutamine expansions in the $Ca_v2.1$ calcium channel show a range of effects on voltage and time dependent properties with a strong dependence on both auxiliary subunit and α_1 subunit splice variant composition when expressed in heterologous systems (Table 1) (Restituito et al., 2000; Toru et al., 2000). It has been speculated that the alterations in channel function may result in changes in intracellular calcium concentrations and consequently induced cell death. However, analysis of cerebellar tissue from SCA6 patients reveals perinuclear aggregates in Purkinje cells, and transfection of polyglutamine expanded $Ca_v2.1$ channel cDNA in HEK293 cells suggests that cell death is likely due to the perinuclear aggregates (Ishikawa et al., 1999). A interesting recent report showed that the carboxyl terminus of the $Ca_v2.1$ channel is cleaved and translocated to the nucleus under wild-type conditions, but when the polyglutamine expansion is extended to greater than 33 repeats the nuclear translocated channel somehow induces cell death (Kordasiewicz et al., 2006). Overall, it appears that neuronal loss observed in SCA6 brains is likely due to a combination of altered channel properties resulting in abnormal intracellular calcium concentrations and perinuclear and/or nuclear channel protein aggregates ultimately resulting in cell death.

Although substantial progress has been made towards understanding the cellular mechanisms behind the channelopathies associated with the $Ca_v2.1$ channel, important questions remain. First, all three disorders described above exhibit temporal-related phenotypes, e.g., FHM1 and EA2 are episodic and SCA6 is

progressive; what is responsible for these phenotypes being periodic or progressive? Second, the $Ca_v2.1$ channel is ubiquitously expressed in the mammalian brain (Bourinet et al., 1999; Jun et al., 1999), yet mutations result in highly localized phenotypes. Are these phenomena due to changes in the isoform expression of the α_1 subunit, auxiliary subunit composition or perhaps due to other protein interactions or environmental conditions? Some initial studies are beginning to address these questions (Toru et al., 2000; Mullner et al., 2004; Adams et al., 2006; Jeng et al., 2006)).

2.4. Lambert-Eaton Myasthenic Syndrome (LEMS)

LEMS is a neuromuscular transmission disorder characterized by reduced acetylcholine quantal release and is associated with small-cell lung carcinoma (SCLC) in approximately 60% of patients (Lang et al., 1983). P/Q-type channels are implicated in LEMS, although unlike FHM1, EA2 and SCA6, LEMS is not a true channelopathy as it does not result from defined mutations in the channel. Rather the sera from LEMS patients contains auto-antibodies against voltage-dependent calcium channels, with an apparent preferential targeting of $Ca_v2.1$ calcium channels at the neuromuscular junction (Lennon et al., 1995; Pinto et al., 2002). Clinical features of LEMS includes skeletal muscle weakness in proximal and trunk muscles, with the most severe effects observed in lower limbs (for review see (Flink and Atchison, 2003)). Auto-antibodies are thought to be initiated in response to the SCLC tumor (O'Neill et al., 1988) and via targeting $Ca_v2.1$ channels at the neuromuscular junctions reduce channel availability for neurotransmission (Lennon et al., 1995). It has been shown that the LEMS auto-antibodies do not alter channel voltage or kinetic properties, but instead act in an all or none fashion, likely eliminating available channels from the population (Grassi et al., 1994; Magnelli et al., 1996). Drugs that prolong the duration of action potentials and enhance intracellular calcium levels, such as 4-aminopyridine and 3,4-diaminopyridine, offer symptomatic relief in some LEMS patients; however, side effects can often be severe (Flink and Atchison, 2003).

3. L-TYPE ($Ca_V1.4/\alpha_{1F}$) CALCIUM CHANNELS; CACNA1F GENE

L-type calcium channels are the primary trigger for excitation-contraction (EC) coupling in cardiac, skeletal, and smooth muscles (Bean, 1989). They are also found in most central and peripheral neurons where they in part control calcium-dependent gene expression, as well as in endocrine cells and many types of non-excitable cells where they contribute to a variety of processes including exocytotic release. Unlike most synapses in the brain and spinal cord that rely on P/Q- and N-type calcium channels for neurotransmitter release, (Wheeler et al., 1994), the presynaptic terminals in photoreceptor cells rely on the $Ca_v1.4$ (α_{1F}) L-type calcium channel for mediating glutamate release (Tachibana et al., 1993; Nachman-Clewner et al., 1999). Photoreceptor neurotransmission is atypical; first,

photoreceptor cells do not fire action potentials, but rather have continuous graded membrane potentials; and secondly, most photoreceptors are tonically depolarized in the absence of a light stimulus resulting in continuous glutamate release and subsequently hyperpolarize in response to light stimuli (Wu, 1994). Considering its critical role in vision, it is perhaps not surprising that mutations in the CACNA1F gene are implicated in the vision related channelopathies incomplete X-linked congenital stationary night blindness (IXLCSNB) and X-linked cone-rod dystrophy (CORDX).

3.1. Incomplete X-linked Congenital Stationary Night Blindness (IXLCSNB)

Congenital stationary night blindness begins in childhood and includes a series of non-progressive disorders of varying hereditary patterns including an autosomal dominant (adCSNB), autosomal recessive (arCSNB) and the X-linked (XLCSNB). Based on electroretinogram (ERG) readings, XLCSNB can be subdivided into complete and incomplete forms based on either no rod function or low level rod-mediated function, respectively (Miyake et al., 1986). More than 60 mutations have been identified in the CACNA1F gene in patients with incomplete X-linked congenital stationary night blindness (IXLCSNB). Missense and truncation mutations have been identified in nearly all structural domains of the channel without predominance of any particular region (Figure 3). Unlike the autosomal dominantly inherited channelopathies associated with the P/Q-type channel, XLCSNB follows an X-linked recessive pattern of inheritance (Berger et al., 1995). IXLCSNB is characterized by variable degrees of night blindness, reduced visual acuity, myopia or hyperopia (short and long sightedness, respectively), and nystagmus. Physiologically, the eye fundus is normal, however, ERG recordings indicate rod-related function is significantly reduced and cone function is below normal (Tremblay et al., 1995). Although it is beginning to gain acceptance that the visual impairments are a result of reduced neurotransmission efficiency between photoreceptors and second-order neurons as a result of mutations in the $Ca_v1.4$ channel, experimental results are not yet conclusive as to the precise molecular mechanisms involved.

Similar to that for the analyses of FHM1 mutations in heterologous systems, biophysical investigation of mutations associated with IXLCSNB in heterologous systems have led to variable results; ranging from no reported effects on biophysical properties to contradicting gain-of-function and loss-of-function effects (Table 3). McRory and coworkers found that three IXLCSNB mutations, G674D, L1364H and A928D, did not alter measurable L-type channel biophysical properties when expressed in HEK293 cells (McRory et al., 2004). Hoda and colleagues found a complete loss of channel expression for IXLCSNB mutation S229P, a loss-of-function effect for mutation L1068P due to reduced channel opening, an increased rate of inactivation and reduced rate of recovery from inactivation, and a gain-of-function effect for the G369D mutation due to a hyperpolarizing shift in

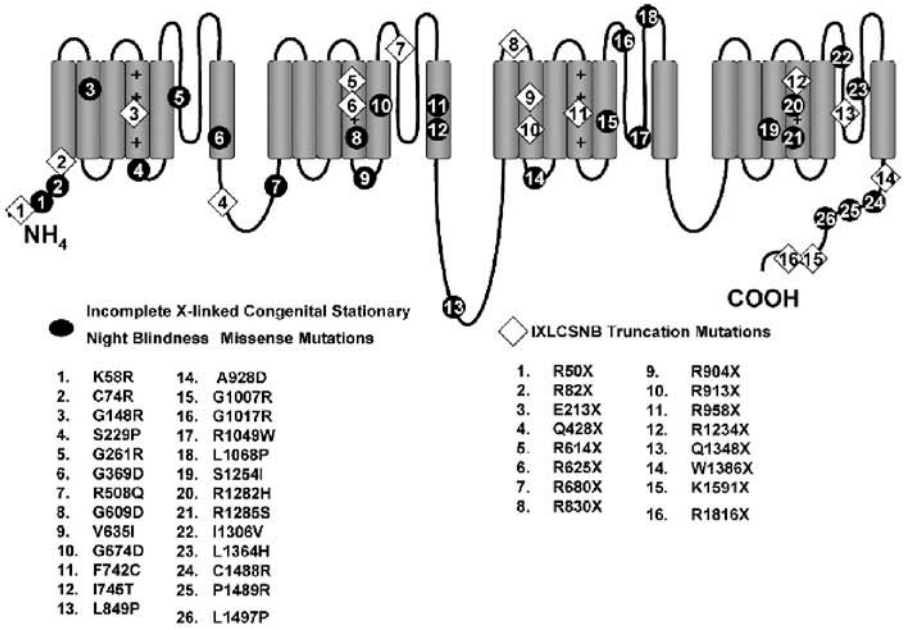

Figure 3. Mutations in the human $Ca_v1.4$ (L-type) voltage-gated calcium channel associated with Incomplete X-linked Congenital Stationary Night Blindness (IXLCSNB)

V_{50act}, reduced rate of inactivation, and a loss of calcium dependent inactivation (Hoda et al., 2005). Contrastingly, McRory and coworkers found a loss-of-function effect due to a depolarizing shift in the V_{50act} for the G369D mutation (McRory et al., 2004). Furthermore, Hemara-Wahanui et al found IXLCSNB mutation I745T caused a gain of function effect by shifting the V_{50act} to more hyperpolarized potentials and reducing the rate of inactivation (Hemara-Wahanui et al., 2005). Collectively, the biophysical data from IXLCSNB mutations thus far does not give a clear indication as to how the distinct point mutations might result in abnormal neurotransmission in photoreceptors. However, a recent investigation by Hoda et al. revealed that mutations R508Q and L1364H affect expression density in a temperature dependent manner (similar to EA2 mutation analysis (Wan et al., 2005)); showing no change at subphysiological temperatures of 29–30 °C but exhibiting reduced protein expression at physiological temperatures (Hoda et al., 2006). The authors speculate that the temperature dependence may be a critical factor in accurately determining the mutation effects in the physiologically relevant environment. Although a plausible hypothesis, similar to that for the FHM1 mutational analyses, the discrepancies in results for IXLCSNB may also be due to channel subunit composition, exogenous expression system, α_1 subunit splice variation and/or experimental conditions including temperature.

Table 3. CACNA1F ($Ca_v1.4$, α_{1F}): Functional results for mutations associated with IXLCSNB = incomplete X-linked congenital stationary night blindness and X-linked cone-rode dystrophy (although addition mutations have been associated with IXLCSNB, only those with functional data completed are listed)

Mutation and Disease	Functional Analyses
S229P- IXLCSNB	• complete loss of channel activity (Hoda et al., 2005)
G369D- IXLCSNB	• hyperpolarized shift in V_{50act}, decreased rate of inactivation, removed calcium-dependent inactivation (Hoda et al., 2005); increased rate of voltage-dependent activation and depolarized shift in V_{50act} (McRory et al., 2004)
R508Q- IXLCSNB	• reduced channel expression (temperature dependent; occurs at 37°C) (Hoda et al., 2006)
G674D- IXLCSNB	• no change in channel properties (McRory et al., 2004)
I745T- IXLCSNB	• hyperpolarized shift in V_{50act} and reduced rate of inactivation (Hemara-Wahanui et al., 2005)
A928D- IXLCSNB	• no change in channel properties (McRory et al., 2004)
L1068P- IXLCSNB	• decreased channel opening, increased rate of inactivation and reduced rate of recovery from inactivation (Hoda et al., 2005)
L1364H- IXLCSNB	• reduced current density, reduced channel expression (temperature dependent), increased rate of inactivation, increased rate of recovery from inactivation (Hoda et al., 2006)
W1386X,	• no change in channel properties (McRory et al., 2004)
W1451X,	• complete loss of protein expression (Hoda et al., 2005)
W1459X, W1440X- IXLCSNB	
IVS28-1, **gcgtc** to **tgg** conversion, aberrant splicing- CORDX	• no electrophysiology data (Jalkanen et al., 2006)

3.2. X-linked Cone-Rod Dystrophy (CORDX)

CORDX usually begins within the first two decades of life and is progressive. Clinical features of the disorder include: reduced visual acuity, poor colour vision, fundus abnormalities, central scotomas in the visual field, photophobia, myopia and low b-waves in ERG recordings (Jacobson et al., 1989; Brown et al., 2000). A single

mutation has been recently found in a CORDX family (Table 3). The mutation is in the splice acceptor site of intron 28 in the CACNA1F gene and is predicted to cause either a premature stop codon in the mRNA transcript, or variable size deletions of the transcript. The mutation co-segregated completely with the disease phenotype in the CORDX family studied (Jalkanen et al., 2006). As of yet, no biophysical analysis has been reported for this mutation.

4. L-TYPE ($Ca_V1.1/\alpha_{1S}$) CALCIUM CHANNELS; CACNA1S GENE

$Ca_v1.1$ (α_{1S}) L-type calcium channels are important in striated muscle cells for coupling membrane depolarization to the release of calcium from cytoplasmic stores and triggering EC coupling (Rios and Brum, 1987). In this instance, depolarizing changes in membrane potential cause a conformational change in $Ca_v1.1$ and without a requirement for calcium influx, induce an allosteric interaction with the sarcoplasmic reticulum (SR) ryanodine receptor (RyR1) ultimately inducing calcium release and muscle contraction (Tanabe et al., 1990; Flucher and Franzini-Armstrong, 1996; Kugler et al., 2004). Related to this role as a voltage sensor for the RyR1, $Ca_v1.1$ has been implicated in two muscle disorders: hypokalemic periodic paralysis (HypoPP) and malignant hyperthermia susceptibility (MHS).

4.1. Hypokalemic Periodic Paralysis (HypoPP)

HypoPP is an autosomal dominant disorder characterized by periodic muscle weakness in association with low serum potassium levels. Disease progression is variable, however, typical progression includes onset during adolescence and symptoms decreasing by fifty or sixty years of age (Morrill et al., 1998). Experiments performed on biopsied intercostal muscle fibres from three patients with HypoPP have revealed that muscle fibres have a reduced excitability and increased sodium conductance, both of which are exacerbated in reduced extracellular potassium concentrations (Rudel et al., 1984). The effects are due to long-lasting depolarizations leading to sodium channel inactivation and loss of membrane excitability (Jurkat-Rott et al., 2000b; Ruff, 2000). Although mutations in both voltage-gated sodium and potassium channels contribute to HypoPP, five missense mutations in the CACNA1S gene encoding for the $Ca_v1.1$ channel have been associated with HypoPP. Four of the five mutations are located in the voltage S4 voltage sensor domains of the $Ca_v1.1$ L-type channel (Figure 4).

Biophysical investigations of mutant $Ca_v1.1$ channels in a variety of heterologous and native systems have also given varying results, some contradictory (Table 4). Although contested, a dominant theme appears to be a reduced current density and reduced rate of activation in mutant channels relative to wild-type (Sipos et al., 1995; Morrill and Cannon, 1999). Both effects are loss of function and could result in less calcium influx into the muscle cells during depolarizations. In

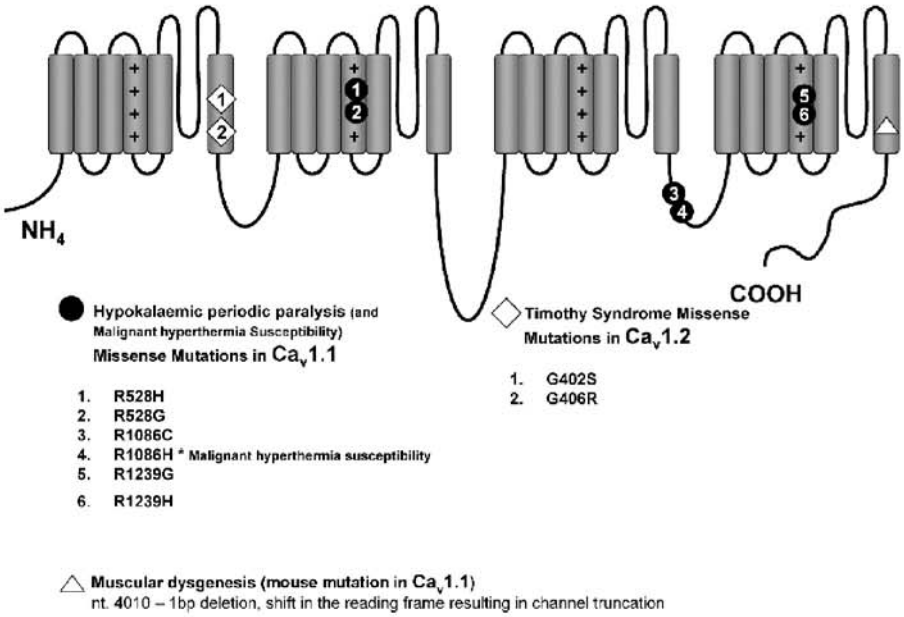

Figure 4. Mutations in the human $Ca_v1.1$ and $Ca_v1.2$ (L-type) voltage-gated calcium channels associated with Hypokalaemic Periodic Paralysis (HypoPP) and Timothy Syndrome (TS), respectively. Also shown is the nucleotide deletion in the mouse $Ca_v1.1$ homolog associated with Muscular Dysgenesis

attempts to correlate the biophysical data from mutant $Ca_v1.1$ with the pathophysiological data, Morill and Cannon hypothesize the reduced calcium influx through $Ca_v1.1$ affects membrane potential through impairment in coupling to a calcium sensitive channel, such as the calcium activated potassium channel that has direct control over the resting potential of the cell. Alternatively, they suggest reduced calcium influx through mutant $Ca_v1.1$ channels throughout development may alter expression and trafficking of channels important in establishing and maintaining resting membrane potential; a hypothesis substantiated by reports that the dominant potassium channel in muscle cells (ATP-sensitive potassium channel) has reduced function in fibres from HypoPP patients and shows irreversible changes in gating properties in response to alterations in calcium concentrations (Morrill and Cannon, 1999; Tricarico et al., 1999). It is equally likely, however, that the pathophysiology of HypoPP is independent of calcium flow through the $Ca_v1.1$ channel, but is instead associated with an uncoupling of EC between the $Ca_v1.1$ channel and the RyR1, resulting in a reduced calcium release from internal stores as seen in the similar muscular dysgenesis disorder in mice (Table 5). To test the possibility of a $Ca_v1.1$ mutation effect on EC, Jurkat-Rott and coworkers compared calcium release from the SR in myotubes from HypoPP patients with the R528H mutation and myotubes from healthy individuals using fura-2 microfluorimetry. Results indicate no detectable difference in the time course or the magnitude of

Table 4. CACNA1S ($Ca_v1.1$, α_{1S}): Functional results for mutations associated with HypoPP = Hypokalemic periodic paralysis and MHS = Malignant hyperthermia susceptibility (only those HypoPP mutations with functional data are listed)

Mutation and Disease	Functional Analyses
R528H- HypoPP	• hyperpolarized shift in V_{50act} (Lerche et al., 1996; Morrill and Cannon, 1999) • no change in V_{50act} (Sipos et al., 1995; Lapie et al., 1996; Morrill et al., 1998) • hyperpolarized shift in $V_{50inact}$ (Sipos et al., 1995; Lerche et al., 1996) • no change in $V_{50inact}$ (Lapie et al., 1996; Jurkat-Rott et al., 1998; Morrill et al., 1998; Morrill and Cannon, 1999) • reduced rate of activation (Morrill et al., 1998; Morrill and Cannon, 1999) • decreased current density (Lapie et al., 1996; Lerche et al., 1996; Morrill et al., 1998; Morrill and Cannon, 1999) • no change in current density (Sipos et al., 1995; Jurkat-Rott et al., 1998) • no change in calcium release from internal stores (Jurkat-Rott et al., 1998)
R1239G-HypoPP	• depolarized shift in V_{50act}, reduced rate of activation, decreased current density (Morrill and Cannon, 1999)
R1239H-HypoPP	• depolarized shift in V_{50act}, reduced rate of activation (Morrill and Cannon, 1999) • decreased current density (Sipos et al., 1995; Morrill and Cannon, 1999) • no change in gating properties (Sipos et al., 1995)

calcium release from SR in response to membrane depolarizations between HypoPP patients and controls, thus it is likely that the pathological effects are too subtle to detect under the experimental conditions used (Jurkat-Rott et al., 1998). Overall, the mechanism by which mutations found in HypoPP patients leads to reduced muscle excitability is yet to be determined.

Table 5. cacna1s ($Ca_v1.1$, α_{1S}): Functional results for mutations associated with muscular dysgenesis

Mutation and Disease	Functional Analyses
nt. 4010 – 1bp deletion, shift in the reading frame resulting in channel truncation Muscular dysgenesis	• membrane depolarization uncoupled from intracellular calcium release (Powell and Fambrough, 1973; Klaus et al., 1983)

4.2. Malignant Hyperthermia Susceptibility (MHS)

MHS is an autosomal dominantly inherited disorder characterized by a predisposition in otherwise healthy individuals for muscle hypermetabolism in response to exposure to volatile anesthetics or depolarizing muscle relaxants. Upon exposure to the precipitating agents, calcium is release from SR in myotubules via the RyR1, resulting in elevated skeletal muscle contraction, glycogenolysis, cell metabolism and subsequent increased heat and lactic acid acidosis. Varying numbers and degrees of symptoms can follow, including: tachycardia, rhabdomyolysis with subsequent creatine kinase elevation, potential cardiac arrhythmia or arrest and possible renal failure. Approximately 70% of patients would die without immediate treatment; early administration of the SR calcium release inhibitor dantrolene drastically reduces mortality (for review see (Striessnig et al., 2004) and (Jurkat-Rott et al., 2000a)). Although many mutations in the RyR1 gene have been associated with MHS (Jurkat-Rott et al., 2000a), a R1086H mutation in the CACNA1S gene has been found in two families with MHS (Figure 4). No biophysical data is available yet and the connection between the mutation and the pathophysiology is still elusive. However, if common CACNA1S mutations are found to be associated with MHS, routine screening prior to exposure to volatile anesthetics and depolarizing muscle relaxants could reduce risks of attacks.

5. L-TYPE ($Ca_V1.2/\alpha_{1C}$) CALCIUM CHANNELS; CACNA1C GENE

The $Ca_v1.2$ L-type channel is widely expressed in heart, brain, smooth muscle, pituitary, gastrointestinal systems lungs, immune system and testis (Ertel et al., 2000; Splawski et al., 2004). In cardiac tissue and lung smooth muscle, the $Ca_v1.2$ channel is involved in EC coupling (Du et al., 2006). However, unlike EC coupling between $Ca_v1.1$ and RyR1 receptors in skeletal muscle, EC coupling in cardiac tissue requires calcium influx through $Ca_v1.2$ in order to activate the RyR2 in the SR and release calcium from internal calcium stores to initiate muscle contraction (Meissner, 1994). Two mutations in the sixth transmembrane segment of domain I of the $Ca_v1.2$ channel have recently been associated with a severe arrhythmic disorder, Timothy syndrome (TS) (Figure 4).

5.1. Timothy Syndrome (TS)

TS is a multisystem disorder, with characteristic functional and developmental abnormalities in several organ systems including heart, skin, eyes, teeth, immune system and the brain; consistent with the ubiquitous expression of the $Ca_v1.2$ channel. In a study of seventeen children with TS, it was established that while arrhythmias are the most serious element of the disorder, patients have additional elements including congenital heart disease, dysmorphic facial features, developmental and cognitive delays, immune deficiency, intermittent hypoglycemia and hypothermia; and four of the seventeen children studied with TS met the criteria

Table 6. CACNA1C ($Ca_v1.2$, α_{1C}): Functional results for mutations associated with TS = Timothy Syndrome and implicated in ASD = autism spectrum disorder

Mutation and Disease	Functional Analyses
G402S- TS	• near complete loss of voltage-dependent channel inactivation (Splawski et al., 2005)
G406R, G436R- TS and implicated in ASD	• near complete loss of voltage-dependent channel inactivation (Splawski et al., 2005) • increased mode 2 gating (Erxleben et al., 2006)

for autism. Greater than 50% of TS patients die between two and three years of age (Splawski et al., 2004). In a study of 13 patients with TS, mutation analysis of a specific $Ca_v1.2$ splice variant (found in ~20% of cardiac $Ca_v1.2$ transcripts) revealed a *de novo* missense mutation in exon 8A in all 13 patients (Splawski et al., 2004). A subsequent study on two patients with severe TS (TS2) revealed two *de novo* missense mutations in exon 8 of the dominant $Ca_v1.2$ splice variant found in heart and brain (Splawski et al., 2005). All patients had a heterozygous genotype suggesting the mutations are dominant.

Functional analysis of all mutations shows a near complete loss of voltage-dependent inactivation (Splawski et al., 2004; Splawski et al., 2005) and increased mode 2 gating (CaMKII dependent channel gating characterized by prolonged channel open time) (Erxleben et al., 2006), both effects predicted to prolong calcium influx (Table 6). Simulation analysis indicates that the prolonged calcium current can cause significant cardiac action potential prolongation and prolonged QT intervals (Q-T interval is the time for electrical activation and inactivation of the ventricles). The prolonged QT interval can result in abnormal secondary depolarizations, arrhythmia and sudden death (Splawski et al., 2004; Splawski et al., 2005). It is conceivable that prolonged calcium influx through the mutant $Ca_v1.2$ channels in TS patients could result in the additional functional and developmental abnormalities considering the requirement for precise regulation of internal calcium concentrations for normal cell signaling and gene transcription during development; however substantiating evidence is lacking.

6. T-TYPE ($CA_V3.2/\alpha_{1H}$) CALCIUM CHANNELS; CACNA1H GENE

T-type calcium channels play critical roles in shaping the electrical and plastic properties of neurons and are also implicated in hormone secretion, differentiation and muscle development (Huguenard, 1996; Perez-Reyes, 2003). In thalamic reticular and relay neurons, T-type channels contribute to rhythmic rebound burst firing and spindle waves associated with slow-wave sleep. T-type channels also play crucial roles in dendritic integration and calcium-mediated spiking in hippocampal pyramidal cells, and in synaptic release at olfactory dendrodendritic

synapses (Tsakiridou et al., 1995; Kim et al., 2001; Anderson et al., 2005). Of the three genes encoding low voltage-activated calcium channels ($Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$), the $Ca_v3.2$ isoform is thus far the only T-type channel known associated with channelopathies. $Ca_v3.2$ channels are widely expressed in the juvenile and adult hippocampus, cerebellum, pons/medulla, striatum, thalamus/hypothalamus, olfactory bulb, and cortex (Talley et al., 1999; McRory et al., 2001; Perez-Reyes, 2003; McKay et al., 2006). Due to their unique biophysical properties, $Ca_v3.2$ channels are predicted to be important in generating short burst firing potentials in the cortex and thalamus during slow wave sleep, however their specific contributions are only beginning to be revealed (Chemin et al., 2002). In the periphery, $Ca_v3.2$ channels are found in the primary nociceptor pathway and have been shown to contribute to both acute and chronic nociceptive behaviors (Snutch and David, 2006).

6.1. Idiopathic Generalized Epilepsy (IGE)

The incidence of epilepsy is variable between age and gender, but occurrence is approximately 1.6% in the general population. IGE is a classification of general epilepsies including juvenile myoclonic and juvenile absence epilepsies (JME and JAE, respectively) and childhood absence epilepsy (CAE) which represent approximately 40% of all epilepsies. Absence seizures are the most characteristic general epilepsies and include short lasting seizures accompanied by sudden impairment of consciousness and a sudden synchronous 3 to 6 Hz spike-wave discharge (SWD) that propagates bilaterally over many cortical areas (Sander, 1996). Based on experiments on the rat genetic model for absence epilepsy, (WAG/Rij), initiation of the neuronal synchronization is thought to occur in the cortex with subsequent generation of an oscillatory cortico-thalamocortical loop network which sustains the synchronous SWD for the duration of the seizure (Meeren et al., 2002). In the last few years researchers have uncovered several point mutations in the CACNA1H gene in patients with CAE and IGE, implicating a pathogenic role of the $Ca_v3.2$ channel in these disorders. The majority of mutations in the CACNA1H channel for both CAE and IGE are densely localized in the $Ca_v3.2$ intracellular loop between domain I and II (Figure 5).

The IGEs are non-Mendelian "complex" idiopathic genetic disorders (Robinson and Gardiner, 2000), therefore, mutations in a single gene, such as the CACNA1H, may not always contribute substantially to the pathophysiology of IGE patients. However, biophysical analyses of CACNA1H mutations associated with IGEs indicate channel properties are in fact altered in many cases (Table 7), suggesting at least a contribution to the pathophysiology. Similar to that for some of the other channelopathy mutations described previously, biophysical analysis of some CAE and IGE mutations in the $Ca_v3.2$ have produced a variety of results, some contradictory. The discrepancies may be due to the various expression systems and conditions utilized by different groups, however even mutations from patients with similar clinical phenotypes have very different, even opposite, affects on

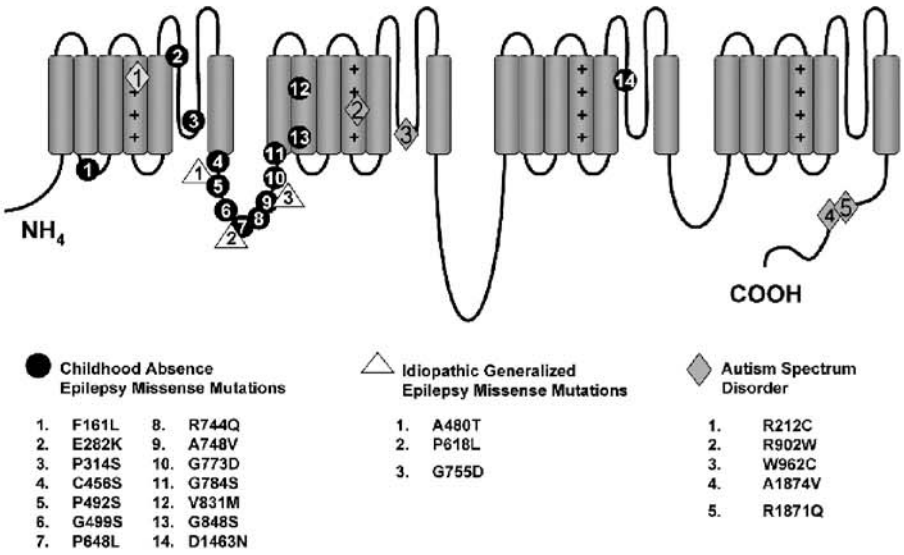

Figure 5. Mutations in the human $Ca_v3.2$ (T-type) voltage-gated calcium channel associated with Childhood Absence Epilepsy (CAE), Idiopathic Generalized Epilepsy (IGE) and Autism Spectrum Disorder (ASD)

Table 7. CACNA1H ($Ca_v3.2$, α_{1H}): Functional results for mutations associated with CAE = childhood absence epilepsy, IGE = Idiopathic generalized epilepsy and ASD = Autism Spectrum Disorder (only those mutations with functional data are listed)

Mutation and Disease	Functional Analyses
F161L- CAE	• hyperpolarized shift in V_{50act} (Khosravani et al., 2004); no change in V_{50act}, depolarized shift in $V_{50inact}$, increased rate of inactivation (Vitko et al., 2005)
E282K- CAE	• hyperpolarized shift in V_{50act} (Khosravani et al., 2004) • no change in channel properties (Vitko et al., 2005)
C456S- CAE	• no change in channel properties (Khosravani et al., 2004); hyperpolarized shift in V_{50act} and increased rate of activation (Vitko et al., 2005)
G499S- CAE	• reduced rate of inactivation (Vitko et al., 2005) • no change in channel properties (Peloquin et al., 2006)
P648L- CAE	• depolarized shift in $V_{50inact}$, reduced rate of inactivation and reduced rate of closed state inactivation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)

(Continued)

Table 7. (Continued)

Mutation and Disease	Functional Analyses
R744Q- CAE	• no change in channel properties (Vitko et al., 2005; Peloquin et al., 2006)
A748V- CAE	• increased rate of activation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)
G773D- CAE	• increased current density (Wang et al., 2006); depolarized shift in V_{50act} and depolarized shift in $V_{50inact}$, reduced rates of activation and deactivation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)
G784S- CAE	• decreased rate of activation and increased rate of closed state inactivation (Vitko et al., 2005); no change in channel properties (Peloquin et al., 2006)
V831M- CAE	• depolarized shift in $V_{50inact}$, reduced rates of activation and inactivation (Khosravani et al., 2004; Vitko et al., 2005); reduced rate of deactivation and increased recovery rate from inactivation (Vitko et al., 2005); no change in recovery rate from inactivation (Khosravani et al., 2004)
G848S- CAE	• reduced rate of deactivation (Vitko et al., 2005); reduced rates of activation and inactivation (Peloquin et al., 2006)
D1463N- CAE	• no change in channel properties (Khosravani et al., 2004) • increased rate of activation (Vitko et al., 2005)
A480T- IGE	• No change in biophysical properties (Khosravani et al., 2005)
P618L- IGE	• increased rates of activation and inactivation (Khosravani et al., 2005)
G755D- IGE	• increased rate of inactivation (Khosravani et al., 2005)
R212C- ASD	• depolarized shift in V_{50act}, reduced rate of inactivation, increased rate of deactivation, reduced current density (Splawski et al., 2006),
R902W- ASD	• depolarized shift in V_{50act}, depolarized shift in $V_{50inact}$, reduced rate of activation, reduced rate of inactivation, increased rate of deactivation, reduced current density (Splawski et al., 2006)
W962C- ASD	• reduced current density (Splawski et al., 2006)
A1874V/R1871Q- ASD	• depolarized shift in V_{50act}, depolarized shift in $V_{50inact}$, reduced rate of activation, reduced rate of inactivation (Splawski et al., 2006)

biophysical properties when analyzed under the same experimental conditions; see analysis data from C456S and G773D (Table 7) (Vitko et al., 2005). However, in many mutations gain-of-function effects are demonstrated by hyperpolarizing shifts in V_{50act}, depolarizing shifts in $V_{50inact}$, increased rates of activation, decreased rates of inactivation, decreased rates of deactivation and increased current densities (Table 7). The gain-of-function effects are predicted to result in more available channels at lower membrane potentials, increased calcium influx, and likely to result in neuronal hyperexcitability and increased spike and wave discharge under certain conditions. Additional studies using native systems in combination with the heterologous data will likely contribute significantly to our understanding of the roles of $Ca_v3.2$ channels in epilepsy. The somewhat mild and various effects of $Ca_v3.2$ mutations are consistent with the polygenic, non-Mendelian inheritance of the IGEs and suggests that interactions with other proteins may come into consideration.

6.2. Autism Spectrum Disorders (ASDs)

The prevalence of ASDs has risen drastically over the last twenty years to a staggering 1 in 166. ASDs include prototypic autistic disorder, Asperger syndrome and pervasive developmental disorder-not otherwise specified (PDD-NOS). Autistic disorder is characterized by deficits in communication, abnormal social interactions and restrictive and/or repetitive behaviours and/or interests with considerable variation in the severity of each phenotype; Asperger syndrome does not typically include intelligence or communication deficits, and PDD-NOS does not include repetitive behaviours or communication deficits. All three disorders often occur within the same family, suggesting they are not genetically distinct (for review see (DiCicco-Bloom et al., 2006)). As diverse as the phenotypes associated with ASDs is the associated diversity in the pathological findings in brains of patients with ASD. Some consistent findings indicate histological abnormalities in limbic structures including the hippocampus and amygdale, abnormal brain size including enlargement in cortex and cerebellum, and reduced tissue volume in cerebellar vermis (Courchesne et al., 2001; Kaufmann et al., 2003; Matsuo et al., 2003; Hazlett et al., 2005; Sadamatsu et al., 2006). Postmortem neuropathological data indicate a dramatic loss of cerebellar Purkinje cells (Ritvo et al., 1986). fMRI imaging studies indicate that skill deficits in ASD patients are accompanied by reduced function in brain regions attributed to the specific skills (for a full review of pathology of ASD see (DiCicco-Bloom et al., 2006). Family studies show a strong polygenic genetic dependence for ASD with complex gene-gene and gene-environmental interactions (Szatmari, 1999).

Four missense mutations have been identified in the CACNA1H gene encoding the $Ca_v3.2$ T-type calcium channel in six families from a sample of 461 individuals with ASD (none present in 480 ethically matched controls; (Splawski et al., 2006)). Two of the mutations are in the S4 voltage sensor regions, one is in the pore forming p-loop and the other is found in the carboxyl terminus (Figure 5). Biophysical analysis of the four missense mutations predicts an overall decrease in calcium

entry (Table 7). Although unconfirmed by experimental data at this time, it is possible that the biophysical effects of the mutations translate into altered neuronal development and communication considering the tight regulation of internal calcium levels necessary for normal cell signaling and gene expression (Splawski et al., 2006). It is important to note that of the 461 ASD patients screened, only 6 carried the CACNA1H gene missense mutations. In addition, three families with a child having a CACNA1H missense mutation had an additional affected child without a CACNA1H mutation and one sibling and several parents contained one of the mutations but were not obviously affected. These data are consistent with the polygenic inheritance pattern of ASD, and indicate a potential, but likely small, role for the $Ca_v3.2$ channel in overall ASD susceptibility.

7. CALCIUM CHANNEL AUXILIARY SUBUNITS

In most instances, HVA calcium channel α_1 subunits appear to be associated with auxiliary β and $\alpha_2\delta$-subunits in order to fully reconstitute most native biophysical and modulatory characteristics (De Waard et al., 1996). The skeletal muscle $Ca_v1.1$ L-type channel also co-purifies with a $\gamma1$ subunit although it remains unknown how generally this particular subunit is associated with voltage-gated calcium channel complexes. In addition to the ten genes coding for α_1 subunits, mammals possess four genes encoding for β-subunits (β_1-β_4), four $\alpha_2\delta$-subunit genes ($\alpha_2\delta1$-$\alpha_2\delta4$) and eight γ subunits genes ($\gamma1$-$\gamma8$; (Snutch et al, 2005)). To date, two human calcium channel auxiliary subunits have been associated with disease; 1) linkage analysis has indicated mutations in the $\alpha_2\delta1$ auxiliary subunit may play a role in malignant hyperthermia susceptibility (discussed previously in association with the $Ca_v1.1$ L-type channel), although no mutations have yet been described in the CACNL2A gene that encodes for the $\alpha_2\delta1$ subunit (Iles et al., 1994) and 2) truncation and missense mutations in the CACNB4 gene (encoding the β_4 subunit) are associated with families with juvenile myoclonic epilepsy, generalized epilepsy/praxis-induced seizures and episodic ataxia (Escayg et al., 2000). As the β_4 subunit is known to interact directly with the $Ca_v2.1$ channel α_1 subunit (Walker and De Waard, 1998; Walker et al., 1999) Escayg and coworkers co-transfected the mutant β_4 subunit with $Ca_v2.1$ into *Xenopus* oocytes and examined channel biophysical properties. The results indicate that both truncation and missense mutations in the β_4 subunit increase current density while the truncation mutation selectively alters channel gating (Table 8). This association between mutations in the β modulatory subunit and $Ca_v2.1$ channel functional alterations and epilepsy and ataxia, supports the evidence described previously indicating a substantial role for the $Ca_v2.1$ P/Q-type channel in these disorders.

Additional substantiating evidence that calcium channel auxiliary subunits might play pivotal roles in disease processes comes from the study of mouse models. Five murine diseases with similar phenotypes to $Ca_v2.1$ P/Q-type channel mutant-associated diseases have been linked to calcium channel auxiliary subunits (Tables 9–11).

Table 8. CACNB4 (β_4): Functional results for mutations associated with JME = juvenile myoclonic epilepsy, GE = generalized epilepsy and EA = episodic ataxia

Mutation and Disease	Functional Analyses
R482X- JME	• increased current density and rate of inactivation (Escayg et al., 2000)
C104F- GE and EA	• increased current density (Escayg et al., 2000)

Table 9. Cchb4 (β_4): Functional results for mutations associated with lh = lethargic

Mutation and Phenotype	Functional Analyses
4 bp insertion, aberrant splicing:deletion of α_1 subunit binding site	• no affect on calcium channel ratio or Ca^{2+} dependent neurotransmitter release in the hippocampal Schaffer collateral synapse in *lh* mice (Qian and Noebels, 2000). • complete loss of β_4 expression in forebrain and cerebellum, associated with decreased $Ca_v2.2$ expression and increased β_{1b} expression; no change in β_2 or β_3 in lethargic brain (McEnery et al., 1998) • increased $Ca_v2.1$ and $Ca_v2.2$ association with β_{1-3}, without any change to protein expression in immunolocalization assay in lethargic brain (Burgess et al., 1999)

Table 10. Cacna2D2 ($\alpha_2\delta2$): Functional results for mutations associated with *du/du*^{2J}= ducky and ent = entla.

Mutation and Phenotype	Functional Analyses
Aberrant splicing resulting in altered mRNA sequence and protein truncation-*du*	• reduced current density, no change in single channel conductance (Barclay et al., 2001; Brodbeck et al., 2002); abnormal cytoarchitecture in Purkinje cells (Brodbeck et al., 2002)
tg – deletion in exon 9, resulting in protein truncation-*du*^{2J}	• no electrophysiological data
39 aa duplication near the N terminus, no protein truncation-*ent*	• reduced current density and decreased gabapentin binding (Brill et al., 2004)

7.1. Lethargic Mouse (Cchb4: β_4)

A deletion in the mouse β_4 gene (Cchb4) has been associated with the lethargic (*lh*) phenotype (Burgess et al., 1997). Lethargic mice display ataxia, lethargic behaviours and focal motor seizures; a similar phenotype to that associated with

Table 11. Cacng2 (γ_2): Functional results for mutations associated with stargazer

Mutation and Disease	Functional Analyses
Transposon insertion in intron 2: loss of transcription	• presence of wt stargazin (γ_2) causes a hyperpolarized shift in $V_{50inact}$ and enhanced current amplitude for $Ca_v2.1$ channels, in mutant stargazin the lack of transcription ultimately would result in a depolarizing shift in $V_{50inact}$(Letts et al., 1998; Kang et al., 2001) • no change in stimulating CSD (Ayata et al., 2000)

human β_4 mutations. Functional studies on hippocampal Schaffer collateral synapses in *lh* mice, although not in complete agreement, indicate that the β_4 deletion results in a shift in β subunit expression and association with $Ca_v2.1$ P/Q-type and $Ca_v2.2$ N-type channels from predominantly β_4 to the β_1, β_2 and β_3 subunits (Table 9). It is likely that this change in β subunit isoform interactions with α_1 subunits causes an overall reduction in channel function (Patil et al., 1998), leading to the observed ataxic and epileptic phenotypes.

7.2. Ducky/Entla Mice (Cacna2d2: $\alpha_2\delta2$)

Several genetic mutations, an aberrant splice, a deletion mutation and an amino terminal duplication in the calcium channel auxiliary subunit Cacna2d2 gene (encoding the $\alpha_2\delta2$ subunit) result in protein truncations associated with the allelic mutants ducky (*du*) and ducky2 (du^{2J}) and an elongated protein associated with *entla* (*ent*), respectively (Barclay et al., 2001; Brill et al., 2004). The ducky phenotypes are characterized by an ataxic gait, paroxysmal dyskinesia and pathological findings of abnormal cerebellar, medulla, and spinal cord development (Meier, 1968; Barclay et al., 2001). The *ent* phenotype is characterized by ataxia, paroxysmal dyskinesia and absence epilepsy without any gross anatomical abnormalities (Brill et al., 2004). The $\alpha_2\delta1$, $\alpha_2\delta2$, $\alpha_2\delta3$ and $\alpha_2\delta4$ subunits have previously been shown to be important modulators of several α_1/β-subunit combinations (Walker and De Waard, 1998; Barclay et al., 2001). *In vitro* analysis using whole cell and single cell recordings of heterozygous, homozygous and wild-type *du* mice in acutely dissociated Purkinje cells demonstrates a significant reduction in $Ca_v2.1$ channel current density in homozygous *du/du* mice, without changing single channel conductance (Table 10) (Barclay et al., 2001; Brodbeck et al., 2002). *In vitro* analysis of acutely dissociated Purkinje cells from heterozygous, homozygous and wild-type *ent* mice demonstrates a significant reduction in current density of $Ca_v2.1$ channels in homozygous *ent/ent* mice and also reduced gabapentin binding. An overall loss-of-function effect on $Ca_v2.1$ P/Q-type channels appears to be a consistent effect of the *du/du* and *ent/ent* mutations, possibly due to interference of the mutant $\alpha_2\delta2$ in contributing to channel function, trafficking and/or assembly.

7.3. Stargazin Mouse (Cacng2: γ_2)

An insertion in the mouse Cacng2 gene, which encodes the γ_2 auxiliary subunit stargazin, has been implicated in the stargazer phenotype. Stargazer mice are characterized by head-tossing, ataxic gait and electrocorticograph readings typical of absence seizures. The γ_2 subunit was found to be ubiquitously expressed in brain synaptic membranes, with altered expression in the stargazer mouse due to an intronic insertion (Letts et al., 1998). Functional studies in BHK cells and *Xenopus* oocytes indicates that co-expression of the wild-type γ_2 with the $Ca_v2.1$ α_1 subunit (and β_{1a}/ or β_3 and $\alpha_2\delta$) demonstrates a modulatory role of γ_2 on P/Q-type channels by shifting the V_{50inac} in the hyperpolarized direction and in reducing current density (Letts et al., 1998) (Table 11). It has been therefore suggested that the altered expression of the stargazin γ_2 may functionally affect P/Q-type channels, potentially leading to the observed seizure susceptibility (Letts et al., 1998). However, studies on stargazin mice examined for the threshold for initiating cortical spreading depression found no significant change in mutant mice relative to wild-type and substantiating evidence for the exact contribution of stargazin concerning the epileptic phenotype remains to be defined (Ayata et al., 2000).

8. CONCLUSIONS

Structure-function analyses involving the introduction of genetic abnormalities into cloned voltage-gated calcium channels has provided important clues concerning gain-of-function and loss-of-function properties and may help lead to new therapeutic pathways. It should however be noted, that the data from exogenous expression studies can sometimes be contradictory and the more labor intensive approach of genetic knock-in will likely be required to resolve some of the mechanistic issues related to disease phenotype. Additionally, we have just touched the surface concerning the more obvious and robust phenotypes and there likely remains much to discover at multiple levels. For example, critical to the phenotypic aspects of human channelopathies, the various calcium channel α_1 and auxiliary subunits exhibit distinct temporal and spatial expression patterns, yet we know little concerning the exact correlation between channel expression-related processes and disease progression and phenotype. Additionally, there is a demonstrated preferential association between individual calcium channel α_1 subunit isoforms expressed in particular tissues and specific subtypes of β subunits (e.g., in skeletal muscle $Ca_v1.1$ with β_{1a}, in cardiac muscle $Ca_v1.2$ with β_{2c}, and in the cerebellum the cell type-specific expression of $Ca_v2.1$ with either β_4 or β_{2a}), yet we have few clues as to the physiological significance as this relates to channelopathies. Also of further importance, and this issues likely relates to all channelopathies, is to resolve the apparent disconnect between widespread ion channel expression patterns and the often highly localized disease phenotypes (e.g., pan-neuronal $Ca_v2.1$ expression yet mutations manifesting as cerebellar ataxias and congenital migraine headache). As alternative splicing can greatly diversify ion channel function and also provide for

the precise localization of unique molecular isoforms, the contribution of splicing has the potential of greatly affecting the phenotypic expression of channelopathies in general. To date, this area has largely been understudied and the analysis of both the effects of individual splice-variants on specific genetic mutations, together with the developmental and spatial correlation of splice variant expression patterns may provide new and important insights into the clinical manifestation of channelopathies.

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APPENDIX 2: OTHER PHD PUBLICATIONS

Besides my above publication that related directly to Cav2.1 alternative splicing and modulation, I also have contributed significantly to another publication during my PhD studies (see below). I helped design and performed all PCR and Genescan experiments and participated in the writing of the paper by Spacey *et al*. (2006). This study revealed a second gene locus for the autosomal dominantly inherited Paroxysmal nonkinesigenic dyskinesia (PNKD). These results are the first to suggest that there are at least two different genes responsible for PNKD, implying its genetic heterogeneity.

S.D. Spacey, MD; **P.J. Adams**, BSc; P.C.P. Lam; L.A. Materek, BSc; A.J. Stoessl, MD; T.P. Snutch, PhD; and G.-Y.R. Hsiung, MD. (2006). Genetic heterogeneity in paroxysmal nonkinesigenic dyskinesia**.** Neurology. 2006 May 23;66(10):1588-90.

APPENDIX 3: UBC RESEARCH CERTIFICATES OF APPROVAL

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0036

Investigator or Course Director: Terry P. Snutch

Department: Michael Smith Laboratories

Animals:

Mice FHM Cacna1a R192Q KI -NEO 144
Mice FHM Cacna1a S218L KI -NEO 108

Start Date: **January 1, 2007** **Approval Date:** **July 13, 2009**

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Structure & function of neuronal calcium channels

Funding Agency: Canada Research Chairs
Funding Title: Canada Research Chair in Biotechnology and Genomics-neurobiology for Dr. Terrance Snutch

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A07-0053

Investigator or Course Director: Terry P. Snutch

Department: Michael Smith Laboratories

Animals:

Mice FHM1 Cacna1a S218L KI -NEO 240
Mice FHM1 Cacna1a R192Q KI -NEO 192

Approval Date: May 15, 2009

Funding Sources:

Funding Agency:	Michael Smith Foundation for Health Research
Funding Title:	Functional characterization of low-voltage activated T-type calcium channels in cerebellar slices of wild-type and P/Q-type calcium channel mutant mice
Funding Agency:	Canada Research Chairs
Funding Title:	Canada Research Chair in Biotechnology and Genomics-neurobiology for Dr. Terrance Snutch
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Structure & function of neuronal calcium channels
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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