CACNA1A mutations with clinical relevance in migraine affect $Ca_V2.1$ channel regulation by G proteins and SNAREs

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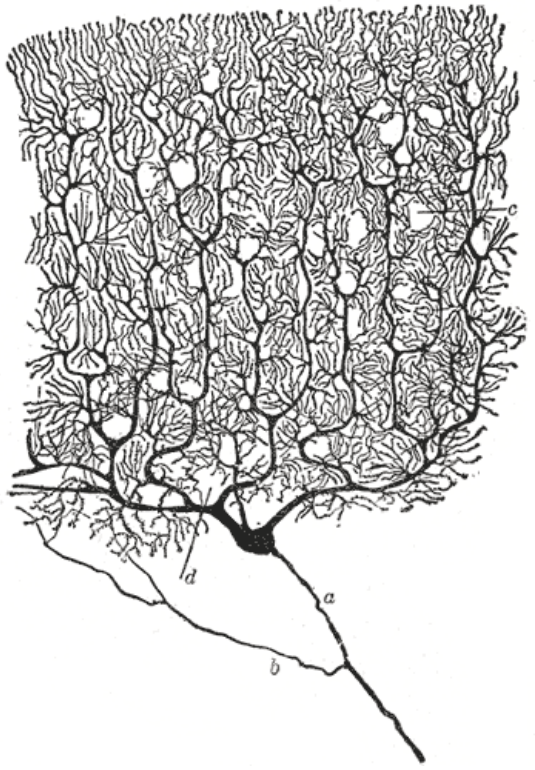

A mi madre,
al meu pare
y a Miguel.

En record del
besavi Salvador
i de l' avi Pere.

"¡Como el entomólogo a la caza de mariposas de vistosos matices, mi atención perseguía, en el vergel de la substancia gris, células de formas delicadas y elegantes, las misteriosas mariposas del alma, cuyo batir de alas quién sabe si esclarecerá algún día el secreto de la vida mental!"

Santiago Ramón y Cajal (1852-1934).

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Son muchas las personas que han aportado su conocimiento e ideas y ofrecido su respaldo durante el desarrollo de mi tesis. Gracias a todos por haberme hecho crecer como científica y como persona, haber contribuido a realizar este sueño y compartido algunas tristezas pero también muchas alegrías.

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ABSTRACT

Familial hemiplegic migraine (FHM)-causing mutations in the gene encoding the P/Q Ca^{2+} channel α_{1A} subunit (*CACNA1A*) normally locate in the pore or voltage sensor regions and involve gain-of-channel function. We have studied the functional consequences of two new α_{1A} mutations found in migraine patients. Mutation Y1245C (which is first missense mutation found in a subject affected with childhood periodic syndromes that evolved into hemplegic migraine and the sole mutation described in any S1 segment of α_{1A} subunit) produced an overall gain of channel function by favouring its activation and lessening $G\beta\gamma$ subunit-dependent channel inhibition (even in response to physiological stimuli). FHM non-causative A454T mutation located in α_{1A} I-II loop and associated with attenuated migraine aura symptoms was found to alter $Ca_V\beta$ and SNARE modulation of channel slow inactivation and drastically reduce channel-dependent secretory efficiency. Altogether, our results underscore S1 structural role in α_{1A} voltage sensor function and unveil the importance of I-II loop in the functional interplay between P/Q channels and SNARE proteins. Lastly, we propose that mutations in *CACNA1A* gene not only cause FHM but also may modify migraine phenotype.

RESUM

Mutacions causants de migranya hemiplègica familiar (MHF) en el gen que codifica per a la subunitat α_{1A} del canal de Ca^{2+} de tipus P/Q (*CACNA1A*) afecten les regions del porus o del sensor de voltatge i produeixen un guany de funció del canal. Hem analitzat funcionalment dues noves mutacions de la subunitat α_{1A} identificades en pacients migranyosos. La mutació Y1245C (la primera mutació amb canvi de sentit identificada en un pacient afectat de síndromes periòdiques infantils que evolucionaren a migranya hemiplègica i l'única trobada en un segment S1 de la subunitat α_{1A}) produeix un guany de funció global del canal afavorint la seva activació i disminuint la inhibició per subunitats $G\beta\gamma$ (fins i tot en resposta a estímuls fisiològics). La mutació A454T ubicada al llaç I-II de la subunitat α_{1A}, que no causa MHF però s'associa a una atenuació dels símptomes de l'aura, altera la regulació de la inactivació lenta per subunitats $Ca_V\beta$ i proteïnes SNARE, observant-se també una reducció dràstica de l'eficiència secretora depenent dels canals mutants. En conjunt, els nostres resultats subratllen el paper estructural del segment S1 en la funció del sensor de voltatge de la subunitat α_{1A} i mostren la importància del llaç intracel·lular I-II en la interacció funcional entre el canal P/Q i les proteïnes SNARE. Finalment, proposem que les mutacions en el gen *CACNA1A*, a més de causar MHF, poden modificar el fenotip migranyós.

PREFACE

Migraine is one the most prevalent neurological disorders affecting around 12% of the general population. However, the molecular and cellular origins of migraine are among the most enigmatic in neuroscience. Most agree that migraine susceptibility is inherited and that its clinical manifestation is strongly modulated by both internal and external factors. In recent years we have advanced considerably in our understanding of the mechanisms participating in the generation of the migraine symptoms or the migraine attack, although many open questions remain unresolved. We have learned that the trigeminovascular system (TGVS) is responsible for the pain itself and that cortical spreading depression (CSD), a wave of neuronal excitability throughout the brain cortex, underlies the aura symptoms reported by many migraineurs. Still a matter of debate is whether the CSD is also responsible for the triggering of the neurovascular inflammation and the subsequent pain. Nevertheless, the identification of the first causative genes for a mendelian subtype of migraine with aura, i.e. familial hemiplegic migraine (FHM), pointed to an alteration in the stimulus-secretion coupling at the neuronal synapses. Gain of function mutations in the pore forming α_1 subunit of $Ca_V2.1$ (P/Q-type) calcium channel (CACNA1A) were identified in families suffering from FHM.

$Ca_V2.1$ channel is a key player in the control of excitatory neurotransmitter release in the central nervous system. It mediates Ca^{2+} entry at the synapse following the arrival of an action potential, thereby triggering the fusion of the neurotransmitter-containing vesicles with the plasma membrane and the release of their content to the synapse cleft. The fact that gain of function mutations in $Ca_V2.1$ channel were linked to FHM suggested that in those patients excitatory synapses were also facilitated, which in turn would explain the generation of the CSD. Once again, despite our substantial advance in the understanding of the mechanism related to FHM, several questions related to both the molecular mechanisms and the clinical phenotype arose. For example, are there any genes capable of modifying the clinical phenotype? This question

relates to the fact that different patients carrying the same migraine causative mutation within a family do not present the same clinical phenotype. Other questions relate to the molecular mechanism that generates the gain of function at the protein level. Most reported mutations in $Ca_V2.1$ are located to the pore or the voltage sensor regions of the channel, thereby affecting the core properties of the channel. But, are there mutations affecting other regions of the channel that mediate its interaction with regulatory proteins or even the secretory machinery itself? This Thesis is an attempt to cast light on at least some of these still unresolved questions.

ABBREVIATIONS

ABP = AID-binding pocket

AID = α_1-interacting domain

AP = action potential

APW = action potential-like waveforms

ATP1A2/ATP1A2 = gene encoding/α_2 subunit of the Na^+, K^+ ATPase

BBB = blood brain barrier

BF = blood flow

BID = β-interaction domain

BOLD = blood oxygenation level-dependent

BoNtC1 = Botulinum neurotoxin type C1

BPT = benign paroxysmal torticollis

BPV = benign paroxysmal vertigo

CACNA1A/CACNA1A= gene encoding/pore-forming α_1 subunit of voltage-gated $Ca_V2.1$ or P/Q-type Ca^{2+} channels

$[Ca^{2+}]$ = concentration of Ca^{2+} ions

$Ca_V X$= voltage-gated Ca^{2+} channel

$Ca_V\beta$ = voltage-gated Ca^{2+} channel beta subunit

CGRP = calcitonin gene-related peptide

CNS = central nervous system

CPS = childhood periodic syndromes

CSD = cortical spreading depression

DHP = dihydropyridine

DTT = dithiothreitol

EPSP = excitatory postsynaptic potential

fMRI = functional magnetic resonance imaging

FHM = familial hemiplegic migraine

FS = fast-spiking

GK = guanylate kinase

GPCR = G protein-coupled receptor

HVA = high voltage activated

KI = knockin

LVA = low voltage activated

MA = migraine with aura

MAGUK = membrane-associated guanylate kinases

MIDAS = metal-ion-dependent adhesion site

MO = migraine without aura

MPC = mouse pheochromocytoma

NO = nitric oxide

PAG = periaqueductal grey region

PECT or PET = photon emission computed tomography

PPF = paired-pulse facilitation

SCGN = superior cervical ganglion neuron

SCNA1A/SCNA1A = gene encoding/pore-forming α_1 subunit of $Na_V1.1$ channels

SH3 = Src homology 3

SHM = sporadic hemiplegic migraine

SM = Sec1/Munc 18-like

SNARE = soluble N-sensitive factor attachment receptor

SP = substance P

SSN = superior salivatory nucleus

Synprint = *syn*aptic *pr*otein *int*eraction

TBA = tetrabutylammonium

TGVS = trigeminovascular system

TNC = trigeminal nucleus pars caudalis

VWA = Von Willebrand factor A

WT = wild-type

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I. INTRODUCTION

1. VOLTAGE-GATED CALCIUM CHANNELS

1.1. Importance of Electrical-to-Chemical Transduction

Calcium (Ca^{2+}) ion is a ubiquitous universal intracellular messenger of electrical signalling implicated in most if not all cellular processes. Increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) require a tight spatiotemporal control for proper functioning of most cells[1], and aberrant Ca^{2+} homeostasis can rapidly result in cell death[2]. Basically, changes in $[Ca^{2+}]_i$ in cells are due to activation of Ca^{2+} entry pathways in the plasma membrane or by activation of release Ca^{2+} channels in the intracellular stores, and countered by transporters acting as Ca^{2+} pumps[1]. Voltage-gated Ca^{2+} channels are a preeminent class of large heteromeric plasma membrane proteins providing Ca^{2+} influx in response to changes in membrane potential[3-5]. They are members of a gene superfamily of transmembrane ion channel proteins that includes voltage-gated potassium (K^+) and sodium (Na^+) channels[6].

Ca^{2+} entry through voltage-gated Ca^{2+} initiates and controls a host of intracellular events such as muscle contraction, hormone secretion, synaptic transmission, and gene expression[2]. Accordingly, voltage-gated Ca^{2+} channel activity is essential to couple electrical signals in the cell surface to chemical messages in excitable cells, leading to the nearly universal rule that their presence is precisely what defines an excitable cell[7].

Mutations of genes encoding voltage-gated Ca^{2+} channels identified in humans, mice and other organisms have been implicated in the aetiology of a diverse group of paroxystic (or episodic) neurological and muscular diseases commonly known as channelopathies. In particular, mutations in *CACNA1A*, the gene encoding the α_{1A} subunit of P/Q-type Ca^{2+}

channels, are associated to familial hemiplegic migraine and some forms of ataxia. The study of the functional consequences of these mutations, in addition to the formerly vast knowledge about Ca^{2+} channels obtained by the work of numerous investigators in the past few decades, not only has allowed the unravelling of the nature of these complex pathologies but has taught us many lessons about the physiology of the nervous system.

Over the length of this introduction, I shall invite you to take part of an enthralling trip that starts from the obscure -yet intriguing- times of Ca^{2+} channel discovery, crosses the confines of our current understanding about Ca^{2+} channel structure and regulation by some of its faithful partners, and ends aiming to picture the most we have learned from P/Q-type Ca^{2+} channels about migraine.

1.2. First Steps in Ca^{2+} Channel Discovery

Despite the pivotal role of Ca^{2+} channels in so many cellular processes, as well as the near-ubiquitous presence of different types of Ca^{2+} channels in excitable cells, they were surprisingly latecomers to cellular electrophysiology (the early work on Ca^{2+} channel discovery has been reviewed in[3,5,8]).

In the early studies of neural circuits in the squid giant axon, excitability became rightfully synonymous with the sodium (Na^+) channel, on the minds of Nobel Prize winners Alan Hodgkin, Andrew Huxley, and Bernard Katz (Figure 1A). In 1953, soon after the “sodium theory” of the action potential (AP) was established, Paul Fatt and Bernard Katz found, accidentally, an exception while studying neuromuscular transmission in crustaceans. When they left Na^+ out of their bathing medium (e.g.

replaced by tetrabutylammonium (TBA)) the muscle still generated APs[9]. However, they were quite circumspect about concluding that Ca^{2+} was the charge carrier: "The mechanism of the AP, and the species of ions involved in the movement of charge across the membrane, remain a puzzling problem […] (i) it may be that TBA remains adsorbed to the fibre surface, but is mobilized during excitation and temporarily transferred into the cell interior; (ii) alternatively, influx of calcium or magnesium, or outflux of some internal anion may be responsible of transport of charge."

The mystery of this new form of excitability was considerably unveiled by Fatt and Ginsborg (1958) who were able to record AP mediated by different divalent ions in crustacean muscle leading to a cautious but clear statement that the movement of Ca^{2+} itself could support APs across the membrane[10]. Another important contributor to the birth of the field of Ca^{2+} channels was Susumu Hagiwara (Figure 1B) who carried out an in-depth characterization of Ca^{2+} conductances in various invertebrate tissues. He is well-known for his studies on "Ca^{2+} spikes" in barnacle giant muscle fibers[11], and also because he provided the first evidences of the existence of various Ca^{2+} channel types in many invertebrate preparations like starfish eggs[12].

Work on mammalian tissues began later in parallel with the invertebrate work. Ca^{2+} APs and subsequently Ca^{2+} currents were identified first in mammalian skeletal and cardiac muscle, and then in all excitable cells. Worth noting are the first voltage-clamp recordings of Ca^{2+} currents in cardiac myocytes performed by Herald Reuter in 1967 (Figure 1C) who was one of the pioneers in establishing the essential role of Ca^{2+} channels in cardiac function[13].

The use of pharmacological approaches has represented a key step to understand the function and to identifying the existence of multiple subtypes of Ca^{2+} currents. A clear example was the application of verapamil or nifedipine drugs, belonging to phenylalkylamines and 1,4-dihydropyridines (DHPs) class of molecules respectively, as "calcium antagonists" (i.e. any drug that blocked excitation-contraction) in the mid-1960s by Albert Fleckenstein (Figure 1D) and others (reviewed in[14]). The various class of "calcium antagonists" were found to block in fact specific Ca^{2+} currents with differential selectivity in cardiac and smooth muscle, and this forms the basis of their well-known therapeutic role in a number of cardiovascular syndromes.

These drugs also proved to be invaluable for the isolation of purified voltage-gated Ca^{2+} channels, an essential step on the path of the cloning era.

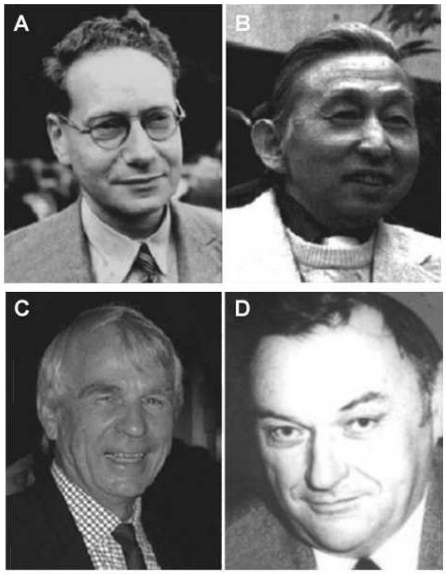

Figure 1. **Some major figures in the early Ca^{2+} channels' era.** A) Sir Bernard Katz. B) Susumu Hagiwara. C) Harald Reuter. D) Albertch Flekenstein. Adapted from[8]

1.3. Voltage-gated Ca^{2+} Current Types

Multiple Ca^{2+} currents in different cell types have been identified following physiological and pharmacological criteria and an alphabetical nomenclature has evolved for the distinct classes of Ca^{2+} currents[4,15].

In the early 1980s, voltage-gated Ca^{2+} currents were distinguished between low- and high-voltage activated (LVA and HVA) through several electrophysiological studies mostly in biological systems less obscure than starfish eggs.

In cardiac, smooth, and skeletal muscle, the major Ca^{2+} currents were defined by high voltage of activation, slow voltage-dependent inactivation, and specific inhibition by DHPs, phenylalkylamines and benzothiazepines[16]. These Ca^{2+} currents were designated as L-type, as they were *l*ong-lasting and showed *l*arge unitary conductance when barium (Ba^{2+}) was the charge carrier[17].

In neurons, new components of the HVA Ca^{2+} current showed clear differences from L-type. On the one hand, there was a conductance that required much more negative potentials to be activated, inactivated rapidly, deactivated slowly, had a small single-channel conductance, and was insensitive to DHPs[18,19]. These type of currents were designated LVA Ca^{2+} currents[18] or T-type for their *t*ransient kinetics and *t*iny unitary Ba^{2+} currents[19] and were also found in heart cells[20]. On the other hand, whole-cell voltage-clamp and single-channel recordings revealed additional Ca^{2+} currents which were largely specific to *n*eurons, had an i*n*termediate conductance to Ba^{2+}, voltage-dependence and rate of inactivation indicating that they were *n*either T- or L-type, and thus were named N-type[19] (Fig 2A). N-type currents were found to be insensitive to organic L-

type channel antagonists but blocked specifically by the toxin derived from marine snail venom ω-conotoxin-GVIA (ω-CTx-GVIA)[19,21].

Further studies with the invaluable help of other peptide toxins revealed three additional Ca^{2+} currents: 1) P-type Ca^{2+} currents, first recorded in *P*urkinje neurons[22] and exquisitely sensitive to the funnel web spider toxin peptide ω-agatoxin-IVA (ω-Aga-IVA)[23], 2) Q-type Ca^{2+} currents, identified in cerebellar granule cells and also sensitive to ω-Aga-IVA[24], and 3) R-type Ca^{2+} currents, also found in cerebellar granule neurons where they remained as a *r*esidual current *r*esistant to the combined application of nimodipine (a type of DHP), ω-CTx-GVIA and ω-Aga-IVA[24] (Figure 2B).

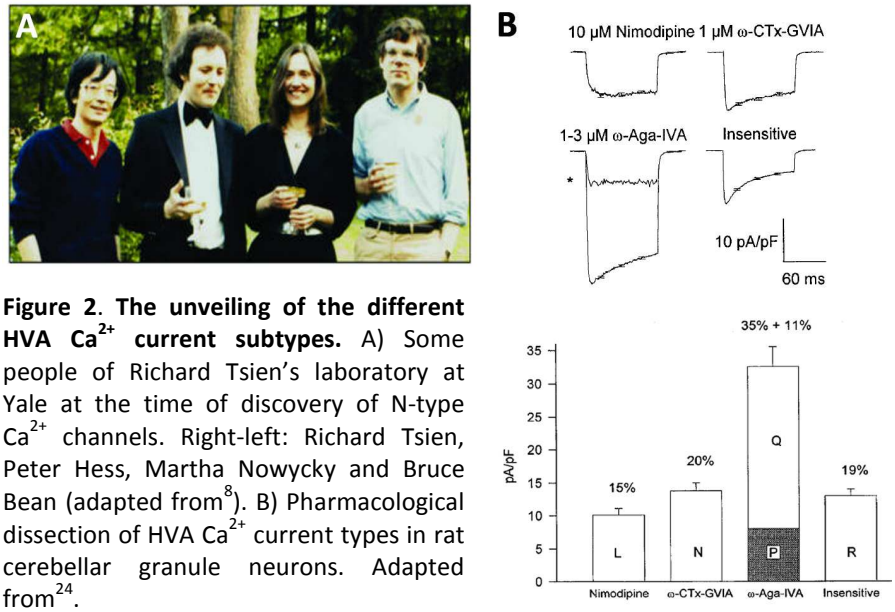

Figure 2. The unveiling of the different HVA Ca^{2+} current subtypes. A) Some people of Richard Tsien's laboratory at Yale at the time of discovery of N-type Ca^{2+} channels. Right-left: Richard Tsien, Peter Hess, Martha Nowycky and Bruce Bean (adapted from[8]). B) Pharmacological dissection of HVA Ca^{2+} current types in rat cerebellar granule neurons. Adapted from[24].

The first full-length Ca^{2+} channel gene to be cloned from brain tissue[25,26] encoded α_{1A} subunit. Because the messenger for this Ca^{2+} channel was

abundant in cerebellum some researchers thought that it belonged to P-type channel[5].

However, when α_{1A} was expressed in *Xenopus* oocytes, Ca^{2+} currents diverged greatly from native P-type ones and rather resembled those from Q-type[5,24]. Several lines of evidence have been needed to convincingly establish that both P- and Q-type currents arise from α_{1A}[5]. Thus, the currently accepted terminology of "P/Q-type" channel is indeed more appropriate, as the initial distinctions between P and Q components mostly accounted for splice variation in α_{1A} gene[27].

1.4. Structure of Voltage-gated Ca^{2+} Channels and Regulation by Auxiliary Subunits

The thrilling race towards voltage-gated Ca^{2+} channel cloning era has involved an enormous work from important laboratories together with the inestimable contribution of DHP drugs and neurotoxins, key molecular tools in the initial steps of Ca^{2+} channel purification.

Based on the biochemical purification of the L-type channel from skeletal muscle[28], members of the HVA Ca^{2+} channels are composed of four subunits: the pore-forming α_1 subunit, and the auxiliary $\alpha_2\delta$, β and γ subunits [4] (see Figure 3, adapted from[29]). Conversely, there is not significant evidence supporting the association of LVA Ca^{2+} channels to auxiliary subunits[30].

Auxiliary subunits have to meet the following criteria to be considered as such: (1) existence in purified channel complexes (2) direct interaction with α_1 (3) capability to directly modulate the biophysical properties

and/or trafficking of the α_1 subunits and (4) stable stoichiometric association with the α_1 subunit[31].

1.4.1. The Pore-forming α_1 Subunit

The α_1 subunit, the principal determinant for Ca^{2+} channel function, is the largest (190-200 kDa) subunit, and it incorporates the conduction pore, the voltage sensor and gating apparatus, and most of the known sites for channel regulation by second messengers, drugs and toxins[32]. The α_1 subunit of voltage-gated Ca^{2+} channels is composed of about 2000 amino acid residues organized in four homologous domains (I-IV). Each domain consists of six transmembrane α helices (S1-S6) and a membrane associated pore (P) loop between S5 and S6.

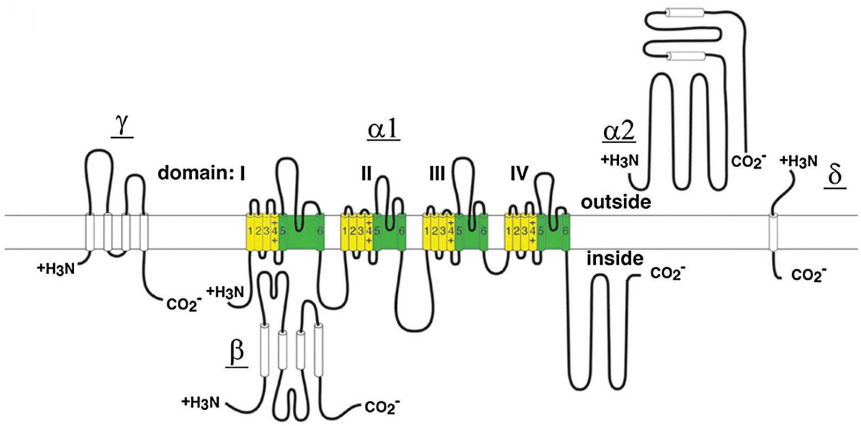

Figure 3. Subunit composition and structure of a HVA Ca^{2+} channels. Predicted helices are depicted as cylinders. The lengths of lines correspond approximately to the lengths of the polypeptide segments represented. The voltage sensor module is illustrated in yellow and the pore-forming module in green.

Intensive structure and function studies of the related pore-forming subunits of Na^+, Ca^{2+}, and K^+ channels have led to identification of their principal functional domains[6]. The S1 through S4 serves as the voltage sensor module whereas transmembrane S5 and S6 in each domain and the P loop between them form the pore module (Figure 3, yellow and green, respectively). The N- and C-terminal regions as well as the large intracellular loops between α_1 domains serve as a signalling platform for channel gating regulation.

Table 1: Voltage-gated Ca^{2+} channel classification. Nomenclature of α_1 subunits, genes encoding Ca_V channels, main tissue localization, type of current, pharmacology and principal functions are described.

Activation	α_1	Ca_V	Gene	Tissue	Ligand	Type	Function
HVA	S	1.1	CACNA1S	Skeletal muscle	DHP	L	Excitation-contraction coupling (= A)
	C	1.2	CACNA1C	Cardiac, smooth muscle, elsewhere			(A) + hormone release (= B) + transcription regulation (= C) + synaptic integration
	D	1.3	CACNA1D	Neuronal/secretory cells, heart, elsewhere			(A) + (B) + (C) + synaptic regulation + cardiac peacemaking + hearing + neurotransmitter release from sensory cells
	F	1.4	CACNA1F	Retina, sensory neurons			Neurotransmitter release from photoreceptors
	A	2.1	CACNA1A	Neuronal, elsewhere	ω-Aga-IVA	P/Q	Neurotransmitter release + dendritic Ca^{2+} transients + hormone release
	B	2.1	CACNA1B	Neuronal	ω-CTx-GVIA	N	
	E	2.3	CACNA1E	Neuronal, elsewhere	SNX 482	R	Repetitive firing + dendritic Ca^{2+} transients
LVA	G	3.1	CACNA1G	Brain, elsewhere		T	Peacemaker + repetitive firing
	H	3.2	CACNA1H	Heart, elsewhere			
	I	3.3	CACNA1I	Brain, elsewhere			

In total, 10 different α_1 subunit genes have been cloned and their function has been characterized by expression in mammalian cells or *Xenopus* oocytes[4] (see Table 1). These subunits were originally designated alphabetically from A to I while more recent nomenclature

has divided voltage-gated Ca^{2+} channels into three structurally and functionally related families: Ca_V1, Ca_V2 and Ca_V3[33] (see Table 1, redrawn from[29,33,34]).

1.4.2. Auxiliary Ca^{2+} Channel Subunits

- $\alpha_2\delta$ Subunits

This subunit is encoded by a single gene, whose polypeptide product is post-translationally cleaved to form a highly-glycosylated extracellular 143 KDa α_2 and transmembrane 27 KDa δ subunit linked by a disulfide bridge[35]. There are 4 genes that code for the $\alpha_2\delta$ family (i.e. $\alpha_2\delta$-1-4) each of which can produce several alternatively spliced isoforms.

Although initial studies suggested that $\alpha_2\delta$ subunit has a transmembrane segment[36], strong evidence now supports that in fact $\alpha_2\delta$ subunits are glycosylphosphatidylinositol (GPI)-anchored rather that transmembrane subunits[37] (see Figure 4A, adapted from[38]).

The $\alpha_2\delta$ subunits have been shown to increase HVA currents about threefold probably by enhancing their forward trafficking to the plasma-membrane[36]. Moreover, they can also influence the biophysical properties of some Ca^{2+} channels, including inactivation kinetics and voltage-dependence[35].

The mechanism(s) involved in $\alpha_2\delta$ modulation of Ca_V1 and Ca_V2 channel trafficking and gating is still not completely understood. It was first proposed that $\alpha_2\delta$ subunits may exert those functions via binding to extracellular regions of α_1 Domain III[36,39]. More recently, it has been demonstrated that the metal-ion-dependent adhesion site (MIDAS) in

the Von Willebrand Factor A (VWA)[1] domain of $\alpha_2\delta$ subunits is required for the correct trafficking of α_1 subunit to the plasma membrane[40] (see Figure 4B, adapted from[38]).

The $\alpha_2\delta$ subunits are expressed in different tissues including heart, brain (e.g. $\alpha_2\delta$-1 and $\alpha_2\delta$-2 expression is particularly high in the cerebellum), and skeletal muscle, among others, being $\alpha_2\delta$-4 the only subunit absent in brain[35].

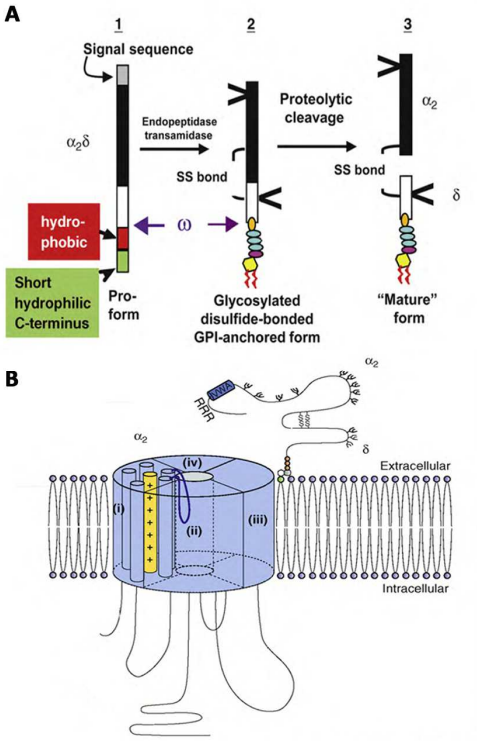

Figure 4. A. Potential sequence of processing and maturation of $\alpha_2\delta$ subunits. N-terminal signal sequence (gray), C-terminal hydrophobic sequence (red), α_2 sequence (black), and δ sequence (white) are depicted. The ϖ-amino acid to which the GPI anchor is attached is indicated in purple. The GPI-anchor itself consists of ethanolamine (orange), three mannose rings (blue), glucosamine (pink) and inositol (yellow), attached to fatty acid side chains. **B. Diagram of calcium channel heteromeric structure.** The potential structure of GPI-anchored $\alpha_2\delta$ subunit is shown.

The loss of $\alpha_2\delta$ subunits, as in the naturally occurring $\alpha_2\delta$-2 knockout (KO) strains of epileptic and ataxic mice, *ducky* and *ducky*^{2J} [41] results in a

[1] VWA domain is also present in integrins and is often involved in binding extracellular matrix proteins. The MIDAS motif contained in VWA confers divalent metal (usually Mg^{2+})-dependent binding to the ligand.

reduction of Ca^{2+} currents in Purkinje neurons, and has marked effects on Purkinje cell function[42].

Furthermore, gabapentinoid drugs, used in the treatment of epilepsy, neuropathic pain and migraine, have been shown to bind $\alpha_2\delta$-1 and $\alpha_2\delta$-2 and impair their trafficking. As a consequence, both $\alpha_2\delta$ and $Ca_V2.1$ Ca^{2+} channel cell-surface expression is disrupted[43].

- γ Subunits

The γ subunit impact on Ca_V2 channel family has not been as extensively studied as the other auxiliary subunits probably due to the muscle-preferential expression of γ_1, isolated from the purification of skeletal L-type Ca^{2+} channels[28], and the absence of neuronal γ subunit in the initial purification of $Ca_V2.2$[44]. However, a more recent genetic study revealed the existence of an additional γ_2 subunit isoform from brain whose lack of expression is responsible for the epileptic and ataxic phenotype of the stargazer mouse[45]. Subsequently, six new γ subunits (also called stargazing-like proteins) have been identified and all eight have been categorized into two subgroups: skeletal γ (γ_1 and γ_6) and neuronal γ ($\gamma_{2\text{-}5}$ and $\gamma_{7\text{-}8}$)[46].

All γ subunits ($\gamma_{1\text{-}8}$) are ~32 KDa proteins characterized by four predicted transmembrane domains, intracellular N- and C-termini, and the first extracellular loop that includes an N-glycosilation site, and a pair of cystein residues that may form a disulfide link. Particularly, neuronal γ subunits display a longer C-terminus that contains PDZ[2] binding motifs[47] that could be important for physical and/or functional association of

[2] PDZ domains are protein-interaction domains that are often found in multi-domain scaffolding proteins.

these γ subunits with the Ca^{2+} channels and other proteins, such as AMPA receptors[48]. Alternatively, the N-linked glycosilation site or several potential sites for serine or threonine phosphorylation contained in γ subunits may have an important role in the folding of γ subunits, and/or in the targeting of proteins interacting with the γ subunit[46].

Neuronal γ subunits are expressed in diverse areas of the central nervous system (CNS), particularly in cerebellum and cerebral cortex[46].

A relatively small number of functional assays compared with that of other auxiliary subunits have been performed with neuronal γ subunits and the results concerning functional effects of γ subunits on neuronal Ca_V channels remain controversial[49-51]. Thus, although neuronal γ subunits may have important functions both in physiological and pathophysiological conditions in the CNS, more evidence is needed in order to consider γ subunits as "true" auxiliary subunits of Ca_V2 channel members.

- β Subunits

Voltage-gated Ca^{2+} channel β ($Ca_V\beta$) subunits are intracellular essential components of voltage-gated Ca^{2+} channels that profoundly affect multiple α_1 subunit properties such as voltage-dependent activation, inactivation rates, G-protein modulation, drug sensitivity, cell surface expression, etc. [52,53]. However, among all $Ca_V\beta$ actions I will focus on their impact in Ca_V channel trafficking and regulation of voltage-dependence of activation and inactivation. The impact of $Ca_V\beta$ on Ca_V channel regulation by G-proteins as well as the interplay of the different molecular determinants of inactivation in voltage-gated Ca^{2+} channels

based on the current data and how they could be linked into a general model of Ca_V channel inactivation will be also discussed during this introduction.

Four β subunit genes have been cloned (β_{1-4}), each encoding a number of splice variants[52]. Comparison of the $Ca_V\beta$ subunit sequence variants has led to the description of five domains (D1-D5), based on sequence similarities, with D2 and D4 being highly conserved among the four β subunits and domains D1 (corresponding to protein N-terminus) , 3 and 5 (corresponding to protein C-terminus) showing exon varibility[53].

All $Ca_V\beta$ subunits potentiate HVA Ca^{2+} channel currents and hyperpolarize the voltage-dependence of activation. However, the mechanism by which $Ca_V\beta$ increase $Ca_V\alpha_1$ current amplitude remains controversial, either attributed to increased trafficking, increased maximum open probability, or both[53,54]. On the one side, some research groups have showed that $Ca_V\beta$ subunits increased localisation of functional channels at the plasma membrane and suggested a "chaperone-like" effect of $Ca_V\beta$ subunits[55,56], probably by releasing $Ca_V\alpha_1$ subunits from the endoplasmic reticulum[56]. On the other side, another group has argued that the effect of $Ca_V\beta$ subunits on $Ca_V\alpha_1$ subunits is to improve the coupling between voltage sensor movement and pore opening manifested as an increase in the ionic-current to charge-movement ratio (I/Q) rather than to increase the gating charge movement which would imply an increased number of functional channels inserted in the plasma membrane[57-59]. One of the reasons for these discrepancy may be attributed to the heterologous expression system used in some of the studies[58,59]. *Xenopus* oocytes have showed to express endogenous $Ca_V\beta$ subunits[52] that could be present in sufficient concentration to saturate the effect of $Ca_V\beta$s on trafficking while not saturating the effect on

voltage-dependence processes (as both effects could be equally mediated by $Ca_V\beta$), since there is a difference in the concentration-dependence of these two processes[60].

All $Ca_V\beta$ subunits, except the splice variant β_{2a}, accelerate channel inactivation rate and left-shift voltage-dependence of steady-sate inactivation curves[61,62]. $Ca_V\beta_{2a}$, which is indistinguishable from its homologues in terms of $Ca_V\alpha_1$ modulation of activation, slows Ca^{2+} channel inactivation rate and right-shifts voltage-dependence of steady-state inactivation[61-64]. These differential regulatory properties have been classically associated with the palmitoylation of two cysteins at its N-terminus that renders $Ca_V\beta_{2a}$ membrane tethered[65,66].

A molecular modelling study based on protein sequence homology showed that $Ca_V\beta$ subunits consist of two conserved protein-protein interaction domains; a Src homology 3 (SH3) domain and a guanylate kinase (GK) domain, corresponding to D2 and D4 conserved regions respectively[67], suggesting that $Ca_V\beta$ belong to the family of Membrane-Associated Guanylate Kinases (MAGUKs)[53] (see Figure 5A, adapted from[68]). MAGUKs are a protein scaffold family that organizes signalling components near membranes, usually contain N-terminal PDZ domain(s), followed by a SH3 domain, a flexible linker termed the HOOK, and a nucleotide kinase domain[69]. The presence of MAGUK-like domains in $Ca_V\beta$ subunits suggest that they might have a role in scaffolding multiple signalling pathways around the channel[70] (see below).

All $Ca_V\beta$s have a primary high affinity binding site on $Ca_V\alpha_1$ subunits of 18 amino acids located in the cytoplasmic loop between domains I and II (I-II loop) of $Ca_V\alpha_1$ subunits, referred to as the α_1-interaction domain (AID), which has a characteristic consensus motif[71] (see Figure 5B, adapted from[68]). Mutagenesis and biochemical studies determined that a minimal

sequence of 41 conserved amino acids (located in the N-terminus of the second conserved domain of $Ca_V\beta$s and termed β-interaction domain (BID)) was required both to bind AID and to influence $Ca_V\alpha_1$ subunit expression[72,73].

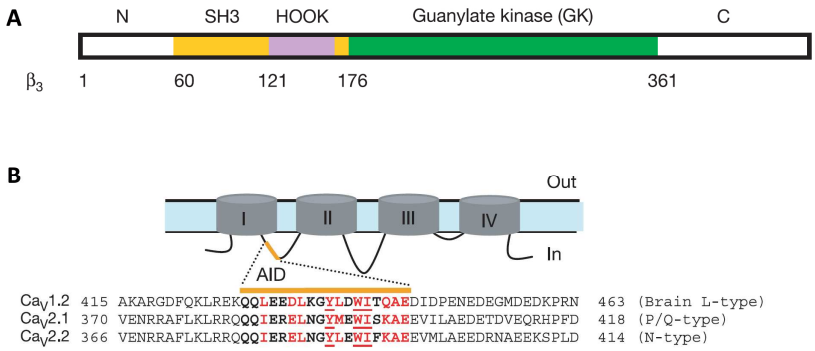

Figure 5. $Ca_V\beta$ subunit domains and AID sequence alignment. A. Schematic diagram of $Ca_V\beta_3$ subunit domain organization based on the crystal structure. **B.** Schematic domain organization of α_1 and alignment of the 49-amino-acid sequence of I-II loop from $Ca_V1.2c$ (M67515), $Ca_V2.1$ (X57477) and $Ca_V2.2$ (D14157). Residues forming the AID are in bold and those involved in interactions with the $Ca_V\beta$ subunit are in red, with the three most critical residues underlined.

Three recent X-ray crystallographic studies have now solved the core (SH3-HOOK-GK) domains of three $Ca_V\beta$ subunits, alone and/or in complex with AID, providing new interesting structural and functional insights about AID-$Ca_V\beta$ interaction[68,74,75]. Crystal structures have showed that AID in complex with $Ca_V\beta$ folds into an amphiphatic α-helix[68,74,75] and that AID transition from coil to helix is forced by the association to $Ca_V\beta$[75]. Conserved aromatic residues face to one side of the helix and strongly interact with a deep hydrophobic groove located in the GK domain of $Ca_V\beta$s now dubbed the AID-binding pocket (ABP)[68,74,75] rather than with $Ca_V\beta$-BID domain as previously reported[72] (see Figure 6A and B, redrawn

from[54,74]). From these results one could speculate that GK module itself may preserve some if not all the modulatory capabilities of the full $Ca_V\beta$ subunit. Alternatively, it is worth to note that as seen from the crystals AID occupies only a very small area of $Ca_V\beta$ core and therefore, much of its surface is free and available for interactions with other regions of α_1 or other proteins. In partial favour to the first view, whole-cell recordings in *Xenopus* oocytes confirmed that $Ca_V\beta_3$-GK module alone was sufficient at least to potentiate $Ca_V2.1$ channel currents[68]. Similarly, $Ca_V\beta$-GK constructs had previously shown to significantly stimulate Ca_V current although not to the same extent as wild-type (WT) $Ca_V\beta$, while long deletions on GK domain caused significant loss of Ca_V currents[72]. However, it is a still a matter of great debate whether GK module suffices to recapitulate all the aspects of $Ca_V\beta$-dependent modulation of $Ca_V\alpha_1$ subunits and the putative participation of the other domains of $Ca_V\beta$s on this modulation via alternative contacts (of lower affinity than those with AID) with $Ca_V\alpha_1$ subunits.

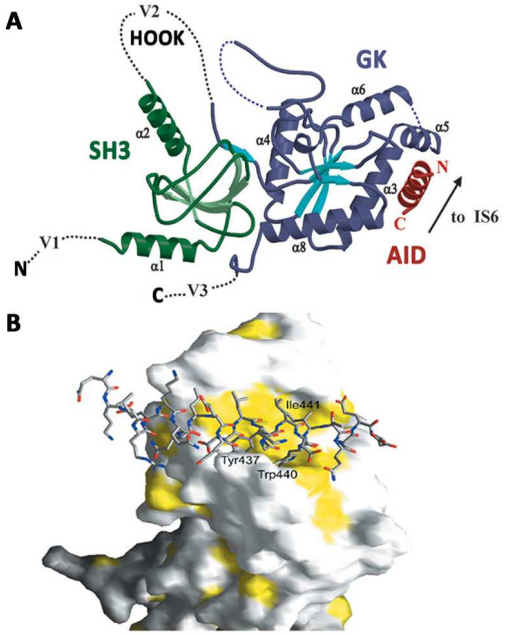

Figure 6. Structure of the $Ca_V\beta_{2a}$–$Ca_V1.2$ AID complex. **A.** Ribbon diagram of the complex. Dashed lines indicate regions absent from the structures. **B.** Surface representation of the $Ca_V\beta3$ subunit (hydrophobic regions of ABP in yellow) with bound AID peptide (stick representation). The three most crucial AID residues for binding are labelled.

A host of biochemical and functional data previous to crystallization of $Ca_V\beta$-AID complex has linked $Ca_V\beta$ short variable regions as well as $Ca_V\alpha_1$ cytoplasmic regions (in addition to I-II loop) to the $Ca_V\beta$-mediated inactivation process. The first evidences that the $Ca_V\beta$ N-terminal region was an important determinant for inactivation were supported by the fact that $Ca_V\beta_{2a}$, the solely non-inactivating $Ca_V\beta$, has N-terminal modification whose mutation reverses its phenotype[66]. Additional data has favoured the idea that not only $Ca_V\beta$ N-terminal[61,76,77] but also HOOK regions[77,78] are linked to $Ca_V\beta$ modulation of inactivation. Concerning $Ca_V\alpha_1$, contacts between N- and C-terminus of α_{1A} subunit with the low conserved C-terminus of $Ca_V\beta_4$ have shown to be important for the regulation of inactivation[79-81]. Similarly, N-terminus of α_{1B} was shown to contribute to $Ca_V\beta_{2a}$-mediated retardation of voltage-dependent inactivation[82]. All these data suggest the existence of isoform-specific interactions other than $Ca_V\beta$-GK with AID that provide the unique regulatory characteristics for each α_1- $Ca_V\beta$ pair.

Soon after the crystal structures of $Ca_V\beta$ core interacting with AID motif came up, important works combining fine molecular, biochemical, and electrophysiological approaches have prompted to more accurately define the exact molecular determinants of $Ca_V\beta$s (and in one case also of $Ca_V\alpha_{1S}$) that account for $Ca_V\beta$-α_1 functional interaction leading to contradictory but quite intriguing conclusions.

Some authors have seen that GK module binding to AID is necessary and sufficient to support $Ca_V\beta$ chaperone role in the increase of channel surface expression[83,84]. However, the specific $Ca_V\beta$ roles on gating are supported by secondary contacts of $Ca_V\beta$ N-term[83] and/or HOOK[83,84] modules to a yet undetermined region of $Ca_V\alpha_1$.

Others have demonstrated that $Ca_V\beta$s require an interaction between SH3 and GK modules to recapitulate regulatory functions on functional cell surface channel expression[85] or inactivation properties[86].

Additional striking experiments have shown that $Ca_V\beta$s regulation of surface expression and channel gating properties are AID-independent and require SH3 module binding to a different from AID site within the distal part of I-II loop of $Ca_V\alpha_1$. This new scenario might place $Ca_V\beta$-GK-AID binding as a mere pre-requisite that orientates and concentrates $Ca_V\beta$s near α_1 to permit an additional interaction between $Ca_V\beta$-SH3 and α_1-I-II loop that underlies the particular regulation seen with each $Ca_V\beta$-α_1 pair[87].

Recently, a nice work has shed new light on the matter demonstrating that in fact just $Ca_V\beta$-GK module suffices to modulate channel gating[88]. In this report, refolded GK domains from two functionally distinct Cavβs, β_{1b} and β_{2a} (which are know to facilitate or inhibit channel inactivation respectively), were found to equally inhibit inactivation[88]. Moreover, they showed that slow GK inactivation pattern switched to fast during channel biogenesis suggesting that GK module acts as a brake to impair voltage-dependent inactivation and that facilitation of inactivation requires additional structural determinants acquired by post-translational modifications that in the case of β_{2a} may not occur because palmitoylation sequesters it to other membranous compartments early in the course of biogenesis[88]. Finally, they suggest that whereas GK regulates Ca^{2+} entrance, the SH3 domain may link channel activity to other cellular processes by binding to additional proteins[88].

In line with this hypothesis there has recently been established that $Ca_V\beta$ subunits can exert α_1-independent functions that may or may not affect voltage-gated Ca^{2+} channel regulation indirectly. Previous work by the

same group has demonstrated that $Ca_V\beta$-SH3 can interact with dynamin (a GTPase involved in receptor-mediated endocytosis) and reduce the number of Ca_V channels in the plasma membrane but only when its binding to AID is prevented[89]. Associaton of $Ca_V\beta$ subunits to other small GTPases had been previously shown to decrease Ca_V channels at the plasma membrane by inhibiting $Ca_V\beta$ association to α_1[90]. All these $Ca_V\beta$ new functions are further supported by the already demonstrated physiological unbinding of $Ca_V\beta$ subunits from α_1[60,91,92].

1.4.3 Molecular Determinants of Voltage-dependent Inactivation in HVA Ca^{2+} Channels

Voltage-dependent inactivation is a key regulatory mechanism for the control of intracellular Ca^{2+} increases in response to AP and is, therefore, believed to play an important role in Ca^{2+} signalling and neuronal excitability[93,94].

In voltage-gated K^+ and Na^+ channels the molecular determinants that govern fast inactivation involve pore block by a cytoplasmic gating particle: the N-terminus of the four α subunits in the case of tetrameric K^+ channels (i.e. “ball and chain” or “N” inactivation) or the III-IV loop of the α subunit in the case of Na^+ channels (i.e. “hinged lid” inactivation)[94].

In the case of voltage-gated Ca^{2+} channels the general inactivation mechanism may be reminiscent of that of K^+ or Na^+ channels, but its molecular determinants appear to be more widely spread over the α_1 subunit and show a much more complex regulation[93,94].

Work from a number of laboratories has implicated both cytoplasmic regions and the pore-lining S6 transmembrane helices in the inactivation

process. The importance of pore-lining S6 transmembrane helices in Ca^{2+} channel inactivation process has been first deduced from chimeric studies where substitution of different S6 segments from different α_1 domains between Ca_V channels showing different inactivation kinetics was sufficient to induce a change in the inactivating phenotype[95,96]. Further evidence has arisen from the observed alteration of inactivation kinetics due to mutations located in the IIS6, IIIS6 and IVS6 regions of $Ca_V2.1$ channels[97,98]. Taken together, these findings indicate that all four S6 segments of α_1 subunit contribute to the inactivation process. Furthermore, all four transmembrane domains have shown to contribute in determining the overall voltage-dependence inactivation[99]. In sum, these evidences support a "pore collapse" model of inactivation that may originate from a global structural change of the four domains of α_1 subunit where the S6 segments, arranged as an inverted "tepee" analogous to the crystal structure of KcsA channel[100], would have a key role in inactivation gating by determining the pore constriction. In this model, cytoplasmic regions of α_1 such as the I-II loop (which, as previously mentioned, has been intensively linked to the regulation of inactivation by $Ca_V\beta$ subunits) may further contribute to the inactivation process by affecting the orientation of pore-forming segments and/or restrict their mobility[93].

In contrast, a number of additional observations provide support for a classic "hinged-lid-type" mechanism of Ca^{2+} channel inactivation that would involve the participation of α_1 cytoplasmic regions[94]. In this respect, the fact that $Ca_V\beta$ subunits (the major determinants governing Ca^{2+} channel gating properties) bind to the α_1 I-II loop[71] suggest a crucial role of this cytoplasmic region as a crossroad for gating channel regulation[94,101]. Additional evidences support a role of the I-II loop in Ca^{2+}

channel inactivation. For instance, insertion of a single valine residue in the $Ca_V2.1$ I-II loop dramatically slows the rate of inactivation of this channel, shifting it from a Q-type to a P-type phenotype[27]. Chimeric substitutions between different Ca_V channels and site-directed mutagenesis of the I-II loop result in significant changes in the rates of inactivation[94]. Furthermore, overexpression of I-II loop peptides accelerates the inactivation kinetics of expressed $Ca_V2.1$ channels[102]. Taken together, these findings suggest a role of the I-II loop as a putative hinged-lid gating particle. In this model, S6 segments (which probably line the inner mouth of the pore) could form part of the docking site for the inactivation gate[94]. Mobility restriction of the I-II loop by interactions with $Ca_V\beta$ subunits or other cytoplasmic domains would differentially regulate inactivation[94]. Accordingly, it has been suggested that the exclusive ability of $Ca_V\beta_{2a}$ to prevent inactivation may be attributed to anchoring of this subunit to the plasma membrane, that would be expected to restrict I-II loop mobility toward the pore[76,94]. However, this hypothesis might need to be contrasted with current molecular and functional data indicating that inhibition of inactivation is an intrinsic property of all $Ca_V\beta$-GK domains[88].

As previously mentioned, in addition to the I-II loop, other α_1 cytoplasmic regions such as the N-terminus[82], the II-III[103], the III-IV loop[104], and the C-terminus[81] have been also linked to inactivation. Interestingly, it has been proposed that regulation of channel inactivation is mediated by an interaction between I-II and III-IV loops of the $Ca_V2.1$ channel and that $Ca_V\beta$ subunits modulate inactivation by modifying this interaction via binding to AID[105]. In contrast, the contribution of N- and C-terminal regions to inactivation may arise indirectly from their interaction with $Ca_V\beta$s[81,82,105].

The extent to which these cytoplasmic regions either restrict S6 mobility (i.e. in the "pore collapse" model) or I-II loop mobility (i.e. in the "hinged-lid" model) to control channel gating is further increased by the reported interactions between the various cytoplasmic regions and between those cytoplasmic regions and other regulatory proteins (e.g. $Ca_V\beta$s, SNARE proteins, etc.) adding additional steps of intricacy to the two proposed models of Ca^{2+} channel inactivation[93,94].

2. NEURONAL VOLTAGE-GATED Ca^{2+} CHANNEL ROLE IN SYNAPTIC TRANSMISSION

2.1. The Presynaptic Ca_V2 Channel Signalling Complexes

$Ca_V2.1$ (P/Q) channels are located in presynaptic terminals and somatodendritic membranes throughout the mammalian brain[106] and although at many central synapses N- and R-type Ca^{2+} channels also cooperate in controlling neurotransmitter release, they display a preferential role in initiating AP-evoked neurotransmitter release because of a more efficient coupling to the exocytotic machinery[107-109]. The somatodendritic $Ca_V2.1$ channel localization points to additional postsynaptic roles (e.g. control of Ca^{2+}-dependent gene expression[110]).

Ca^{2+} entering neurons by the opening of voltage-gated Ca^{2+} channels in response to APs forms a transient Ca^{2+} microdomain in the presynaptic nerve terminal[111,112]. Vesicle fusion and neurotransmitter release is initiated within 200 μs after the arrival of the AP and requires a brief (<1 ms) but high increase in intracellular Ca^{2+} concentration with a threshold of 10 μM and near maximal activation at 50 μM[113].

Presynaptic proteins of the vesicle-docking/fusion machinery, including plasma membrane SNARE proteins (i.e. syntaxin 1A and SNAP-25) and synaptotagmin must be located near Ca^{2+} channels in order to receive the Ca^{2+} signal. In many cases, this close localization is achieved by direct interaction with the intracellular domains of Ca^{2+} channels, which serve as signal transduction platforms for cytosolic Ca^{2+} signalling[4]. The signalling complexes of presynaptic Ca^{2+} channels contain SNARE proteins involved in exocytosis, G proteins involved in feedback regulation of Ca^{2+} channels, and many Ca^{2+}-binding proteins involved in regulation of channel activity and initiation of Ca^{2+}-dependent responses.

2.2. Regulation of Ca_V2 Channels by G Proteins

For the neuronal Ca_V channels, particulary N- and P/Q-types, a major mechanism of inhibitory modulation occurs via activation of heterotrimeric G proteins by seven transmembrane G protein-coupled receptors (GPCRs). GPCRs activation was first found to reduce AP duration, and subsequently this effect was found to result from inhibition of presynaptic voltage-gated Ca^{2+} channels in many types of neurons[34]. GPCRs in presynaptic nerve terminals bind released neurotransmitters and provide a negative feed-back to inhibit N- and P/Q-type and thereby reduce synaptic transmission[114]. G protein negative regulation of neurotransmitter release is very potent because of the power law relationship between Ca^{2+} influx and synaptic transmission[115]. Most neurotransmitters inhibit Ca^{2+} currents in this manner, including acetylcholine, glutamate, GABA, biogenic amines, and many neuropeptides. Autoreceptors in one nerve terminal bind neurotransmitter(s) released from that terminal, whereas other G

protein-coupled receptors in the same nerve terminal may respond to neurotransmitters released by nearby nerve terminals from other neurons[29].

The key features that typify this inhibition are a positive shift in the voltage dependence and a slowing of channel activation[116]. These effects are relieved by strong depolarization resulting in facilitation of Ca^{2+} currents because of a shift between two channel states with different gating properties: “reluctant” and “willing” to open states[116]. Faster removal of inhibition can also be induced by the application of a strong depolarizing prepulse immediately before the test pulse[117]. Looking from a more physiological point of view, given that G protein inhibition is voltage-dependent, synaptic release may be strongly inhibited for a single AP, but inhibition relieved by a train of APs[118]. This prediction has been positively confirmed in microisland cultures of hippocampal neurons in which autapses are formed by single hippocampal pyramidal neurons[119]. In this preparation, trains of AP-like stimuli relieved the inhibition of synaptic transmission caused by activation of GABA-B receptors resulting in facilitation of synaptic transmission by 1.5 fold, which was subsequently blocked by selective blockade of P/Q-type Ca^{2+} channels with peptide neurotoxins[119].

The above described modulation of Ca_V2 channels in most native tissues is usually mediated by heterotrimeric G protein subunits released from the pertussis toxin-sensitive G_i/G_o class[114]. At the molecular level, although G protein α subunits confer specificity in receptor coupling, voltage-dependent inhibition of Ca^{2+} channels is a membrane-delimited event mediated by G protein $\beta\gamma$ dimers ($G\beta\gamma$) who, by itself, are able to mimic GPCR agonists-mediated inhibition when transfected either in primary neurons or cell lines[120,121].

Relief from G-protein inhibition by strong depolarization causes a dissociation of Gβγ from the channel α_1 subunit, a highly voltage- and time-dependent kinetic process commonly termed as facilitation development. Furthermore, re-establishment of inhibition after a "facilitating" prepulse, during a period at the holding potential, is likely to result from rebinding of Gβγ to the α_1 subunit, and can be measured as facilitation decay rate. Whether these two kinetic processes actually involve physical dissociation and reassociation to the α_1 subunit remains to be established[34].

Direct measurements of ionic and gating currents in HEK293 cells transfected with $Ca_V2.2$ channel after G protein activation have shown slowness of the latter and a induction of ~20 mV separation between the voltage-dependent activation of gating charge movement and ionic current suggesting that the underlying biophysical mechanism of Gβγ is to restrain the movement of the S4 voltage sensors of Ca_V2 channels and the transduction of voltage-sensor activation into channel opening[122]. Prolonged depolarization to more positive membrane potentials can overcome this effect by forcing voltage sensor movement and thus favour Ca^{2+} channel activation[4].

The effects of Gβγ subunits might be mediated by binding to one or more sites on the Ca^{2+} channel α_1 subunit (see Figure 7, redrawn from[29]). Possible sites of Gβγ subunit interaction with Ca_V channels have been extensively studied by construction and analysis of channel chimeras, by G protein-binding experiments, and by site-directed mutagenesis and expression. Initially, most data pointed to the α_1 I-II loop as an essential determinant for Gβγ-mediated modulation[101,123,124]. Overlay and mutagenesis assays showed the existence of two Gβγ putative binding sites in this α_1 intracellular domain[101,123]: one inside AID sequence with a characteristic QQIER amino acid motif and a second more complex

domain comprised of several microsites in the distal part of the I-II loop[123]. Peptide competition as well as mutagenesis of these sites prevented channel inhibition by Gβγ[101,123,124].

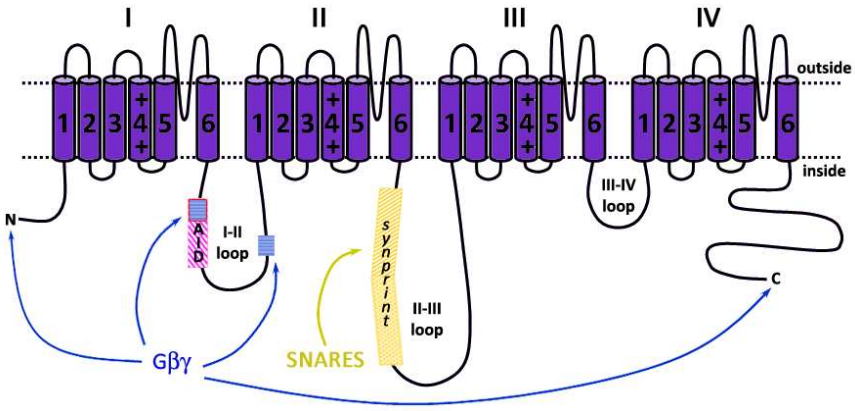

Figure 7. Interaction sites of Gβγ and SNARE proteins on α_1 subunit. Gβγ subunits interact with a QQIER amino acid motif inside the AID sequence and a more distal second domain (blue wefted squares) in the I-II loop, the N- and the C-terminus. The SNARE proteins interact with the *synprint* site (amino acids 718-963 of the α_{1B} subunit and amino acids 722-1036 of the BI isoform of α_{1A} bind both syntaxin 1A and SNAP-25, while amino acids 724-981 of the rbA isoform of α_{1A} only interact with SNAP-25) located inside the II-III loop.

However, further studies using chimeras containing different segments of channels with different levels of G protein-mediated inhibition (i.e. $Ca_V2.2$ are strongly inhibited while $Ca_V2.1$ present less strong inhibition) or chimeras between those and $Ca_V1.2$ channels which are not inhibited by G protein at all found that I-II loop was either not essential for G protein modulation or not the most critical region[125-127]. Instead, N-terminus of Ca_V2 α_1 subunits was found to be more important in mediating G protein modulation[126,128]. Specifically, an 11-amino acid motif YKQSIAQRART in $Ca_V2.2$ N-terminus (that is also highly conserved in $Ca_V2.1$ and $Ca_V2.3$) was shown to be essential for G protein modulation, and mutation of either YKQ or RAR to AAA abolished G protein modulation[126].

Additional studies using chimeric channels have either implicated[125,129] or ruled out[130] α_1 C-terminus region as a putative structural determinant and/or binding site to promote G protein mediated inhibition.

The fact that Gβγ share common molecular determinants with $Ca_V\beta$ subunits for its functional interaction with α_1 subunits as well as the notion that their effects upon Ca_V2 channels could be considered as opposite has led to many investigators to explore the possibility that $Ca_V\beta$ subunits might be involved in G protein modulation and compete with Gβγ for the binding to α_1[34]. In initial studies in native neurons, antisense oligonucleotides against $Ca_V\beta$s resulted in enhanced G protein-mediated inhibition of Ca^{2+} currents leading to the conclusion that $Ca_V\beta$ counteracted Gβγ action[131].

Additional experiments in *Xenopus* oocytes showed less or even complete loss of G protein inhibition following coexpression of a $Ca_V\beta$ subunit further reinforcing that hypothesis[129], although these studies only examined inhibition at a single potential and need to be interpreted with caution because of the presence of endogenous $Ca_V\beta$ subunits[34]. Further studies studying the voltage-dependence of G protein inhibition in the presence or absence of coexpressed $Ca_V\beta$s in Xenopus oocytes showed that the voltage at which G proteins produced maximum amount of inhibition was displaced by $Ca_V\beta$ subunits suggesting that the functional antagonism between Gβγ and $Ca_V\beta$ rather than competitive is dynamic and depends on the membrane voltage[132].

Recent studies have demonstrated that abrogation of $Ca_V\beta$ interaction with Ca_V2 α_1 by AID mutagenesis do not affect the availability of Gβγ inhibition but prevent current facilitation by a depolarizing prepulse[133], and in a similar way, in the absence of $Ca_V\beta$s, coexpression of $Ca_V\beta$-GK domain constructs is sufficient to restore voltage-dependence of G protein

inhibition[134]. Additionally, FRET studies have demonstrated that $Ca_V\beta$ and $G\beta\gamma$ are able to bind α_1 at the same time[135]. Taken together, these results suggest that $Ca_V\beta$ subunits do not displace $G\beta\gamma$ but instead modulate the voltage dependence of G protein modulation process via their GK domain[134].

2.3 Regulation of Ca_V2 Channels by SNARE Proteins

Neurotransmitter exocytosis is a highly regulated, multi-step process that may occur through a host of molecular mechanisms that culminate with the fusion of transmitter-containing vesicles at the presynaptic active zone of nerve terminal membranes[136]. Active zones are small (i.e. 200-300 nm) specialized presynaptic membrane compartments that contain two essential "gates" for neurotransmission: one for Ca^{2+} entry, which is the voltage-gated Ca^{2+} channel, and the other for neurotransmitter exit, which is the synaptic vesicle fusion site. The performance of a synapse depends on the number of vesicles docked and primed at those sites to be immediately available for fusion (i.e. the size of the ready releasable pool) following the arrival of an AP[137].

One crucial step of neurotransmitter release depends on the formation of the SNARE (soluble N-sensitive factor attachment receptor) complex (spatially and temporally organized by SM (Sec1/Munc 18-like) proteins) that tethers neurotransmitter-laden vesicles close to the site of Ca^{2+} entry and promotes its priming[138,139]. In response to AP-induced Ca^{2+} influx the SNARE complex suffers a conformational change that facilitates the fusion between vesicle and nerve terminal membranes to release neurotransmitter into the synapse. The minimal determinants of this protein complex include vesicle-bound SNARE protein (v-SNARE)

synaptobrevin and target plasma membrane SNARE proteins (t-SNAREs) syntaxin 1A and synaptosomal-associated protein 25 (SNAP-25). Each contributes one (synaptobrevin and syntaxin) or two (SNAP-25) α-helices to a four-helix bundle of such high affinity that the resulting complex is resistant to denaturation by sodium dodecyl sulfate (SDS) (see Figure 8A, redrawn from *web.mit.edu*).

Formation of SNARE complex is absolutely required for vesicle fusion competence, but increasing evidence suggest that SNARE proteins may also be involved in vesicle recruitment[140]. In fact, recruitment of synaptic vesicles to sites where Ca^{2+} channel cluster, rather than fusion competence, is a limiting step for rapid neurotransmitter release in response to presynaptic APs[140].

Biochemical studies demonstrate that both $Ca_V2.1$ and $Ca_V2.2$ channels colocalize densely with syntaxin 1 at the presynaptic nerve terminals[106] and can be isolated as a complex with SNARE proteins[141,142]. Pull-down and binding assays demonstrated that plasma membrane SNARE proteins syntaxin 1A and SNAP-25, but not the synaptic vesicle SNARE synaptobrevin, specifically interact with the $Ca_V2.2$ channel by binding to the II-III loop of the α_{1B} subunit via an 87 amino acid sequence termed *syn*aptic *pr*otein *int*eraction (*synprint*) site (amino acids 718-963 of α_{1B})[143,144](see Figure 7, redrawn from[29]).

This interaction is Ca^{2+} dependent, with a maximal binding at 20 μM Ca^{2+} and reduced binding at lower or higher concentrations[144], suggesting sequential steps of association and dissociation of SNARE proteins with $Ca_V2.2$ channels as a function of Ca^{2+} concentration[29]. $Ca_V2.1$ channels also have an analogous *synprint* site, and different alternative splicing channel isoforms have distinct interactions with syntaxin 1A and/or SNAP-25 that do not show Ca^{2+} dependence[145,146].

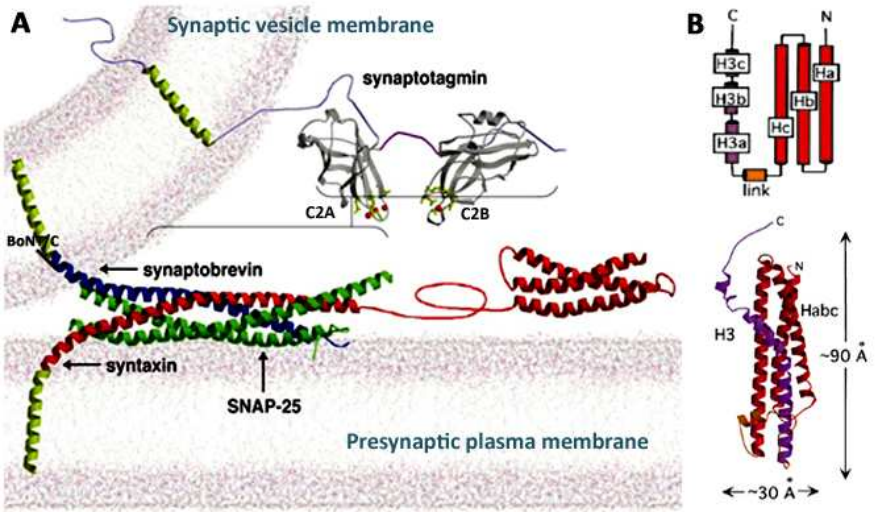

Figure 8. A. Cartoon depicting the SNARE core complex and Synaptotagmin. Note the four helix boundle formed by syntaxin (red), SNAP-25 (green) and synaptobrevin (blue). Transmembrane domains of syntaxin and synaptobrevin are indicated in yellow. Ca^{2+} ions bound to synaptotagmin C2 domains are shown in red. **B. Topology and ribbon diagrams of syntaxin 1A in "closed" conformation.** The Habc domain is shown in red, the Habc/H3 linker in orange and the H3 region in purple.

The BI isoform (previously cloned from rabbit brain but also present in rat brain) of α_{1A} binds both syntaxin 1A and SNAP-25 via two adjacent segments of the II-III loop between amino acids 722 and 1036 while only interaction with SNAP-25 can be detected for the rbA isoform of α_{1A} (previously cloned from rat brain but also present in rabbit brain) in its correspondent amino acid region (amino acids 724-981)[145](see Figure 7, redrawn from[29]).

Other synaptic proteins with a relevant role in the exocytotic process are also able to interact with Ca_V2 channels. The synaptotagmins are a vesicle-associated family of proteins that contain two Ca^{2+}-binding domains (C2A and C2B) which serve as the putative Ca^{2+} sensors necessary to permit the

final step of fast, synchronous vesicle release[136,139] (see Figure 8A, redrawn from *web.mit.edu*). The C2B of synaptotagmin 1 binds to the *synprint* sites of both $Ca_V2.1$ and $Ca_V2.2$ channels[146,147]. Moreover, syntaxin 1A interacts competitively with either *synprint* or synaptotagmin 1 in a Ca^{2+}-dependent way, such that at low Ca^{2+} concentrations syntaxin 1A bounds synprint with higher affinity, whereas at high Ca^{2+} concentration (>30 µM) its association to synaptotagmin increases, suggesting that this sequential binding accounts for stepwise protein interactions that occur during exocytosis[146,147].

Second-messenger regulation of neurotransmitter release via modulation of the interactions of proteins with the exocytotic apparatus has a potential role in synaptic plasticity[29]. P/Q-type and N-type Ca^{2+} channels as well as SNARE proteins are subjected to phosphorylation by several presynaptic protein kinases. It has been shown that phosphorylation of a recombinant N-type *synprint* peptide by protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase type II (CaMKII) inhibit its interaction with native rat brain SNARE complexes containing syntaxin 1A and SNAP-25 suggesting that phosphorylation of the synprint site by these protein kinases may serve as a biochemical switch controlling SNARE-*synprint* interaction[148]. Furthermore, this mechanism provides a potential functional link between neurotransmitter-activated protein phosphorylation and tethering docked synaptic vesicles in an optimal position to respond to the Ca^{2+} signal from presynaptic Ca_V2 channels[29].

In addition to linking presynaptic Ca^{2+} channels to the vesicle release machinery, syntaxin 1A and SNAP-25 regulate channel function. As observed in electrophysiological recordings, coexpression of syntaxin 1A or SNAP-25 with $Ca_V2.2$ or rbA $Ca_V2.1$ in heterologous expression systems sharply decreases the availability of these channels due to a SNARE-induced

shift of voltage dependence of inactivation towards more negative potentials without affecting voltage-dependent activation[149,150].

The inhibitory effects of syntaxin 1A on $Ca_V2.2$ channels can be reversed by coexpression of SNAP-25[151,152] or synaptotagmin 1[153], and the inhibitory effects of SNAP-25 on rbA $Ca_V2.1$ channels can be completely relieved by coexpressing syntaxin and synaptotagmin 1[150]. In sum, these results show that the formation of a mature SNARE complex containing SNAP-25, syntaxin 1A and synaptotagmin relieves the channel from single SNARE protein-induced inhibition[150,151,153]. In a nerve terminal, this reversible negative shift in the voltage dependence of inactivation would provide a molecular switch to inhibit N- or P/Q-type Ca^{2+} channels when associated with an immature single SNARE protein-containing complex, but reactivate them as the synaptic core complex matures in preparation for vesicle release, thus providing a potential mechanism to increase release probability of synaptic vesicles that are docked close to Ca_V2 channels[150,154] (see Figure 9).

Syntaxin 1A (35 kDa) is comprised of four α-helical domains in its cytosolic N-terminus (Ha, Hb, Hc, and H3a,b,c), and a short C-terminal transmembrane domain[155] (see Figure 8B, adapted from[155]).

With respect to the molecular determinants of syntaxin 1A governing Ca_V2 channels modulation, the first biochemical studies showed that the interaction takes place with the C-terminal one third of syntaxin 1A (residues 181-288, comprising SH3 and the transmembrane domain)[143]. Further studies combining mutagenesis of either syntaxin 1A or the pore-forming α_{1B} subunit of N-type Ca^{2+} channels with functional assays in a Xenopus oocyte expression system and biochemical binding experiments, showed that transmembrane region and a short region within the H3 helical cytoplasmic domain of syntaxin (containing residues Ala-240 and

Val-244) were critical for channel modulation but not for binding to the *synprint* site[156]. Surprisingly, deletions within the channel II-III loop that completely eliminated the *synprint* site weakened syntaxin 1A modulation, but by no means abolished it[156].

Taken together, these results suggest that syntaxin's modulatory action on channel gating is facilitated by the presence of the II-III loop but this channel motif cannot be solely responsible for the interaction of syntaxin 1A with Ca_V2 α_1 subunits[149]. The authors wisely propose I-II loop and/or C-terminus as putative candidates for this additional SNARE binding site because of their implication in both channel inactivation and regulation by several effector proteins (e.g. $Ca_V\beta$s or $G\beta\gamma$ subunits)[149].

In addition to modulate $Ca_V2.2$ channel gating, syntaxin 1A also promotes tonic G protein inhibition of those channels[157]. This phenomenon was indirectly observed for the first time in chick giant calyces that became insensitive to G protein inhibition after treatment with botulinum toxin C1 (which cleaves syntaxin 1A)[158]. Subsequent studies in heterologous expression systems showed that syntaxin 1A was able to interact with $G\beta\gamma$ subunits and colocalize them near N-type Ca^{2+} channels thus facilitating effective G protein inhibition of the channels[157] and this cooperative functional interaction was further demonstrated in chick dorsal root ganglion neurons[159]. The same group prompted next to evaluate the molecular determinants of syntaxin 1A with relevance in the modulation of N-type channels concerning its functional interactions both with the *synprint* site and the $G\beta\gamma$ subunits[160].

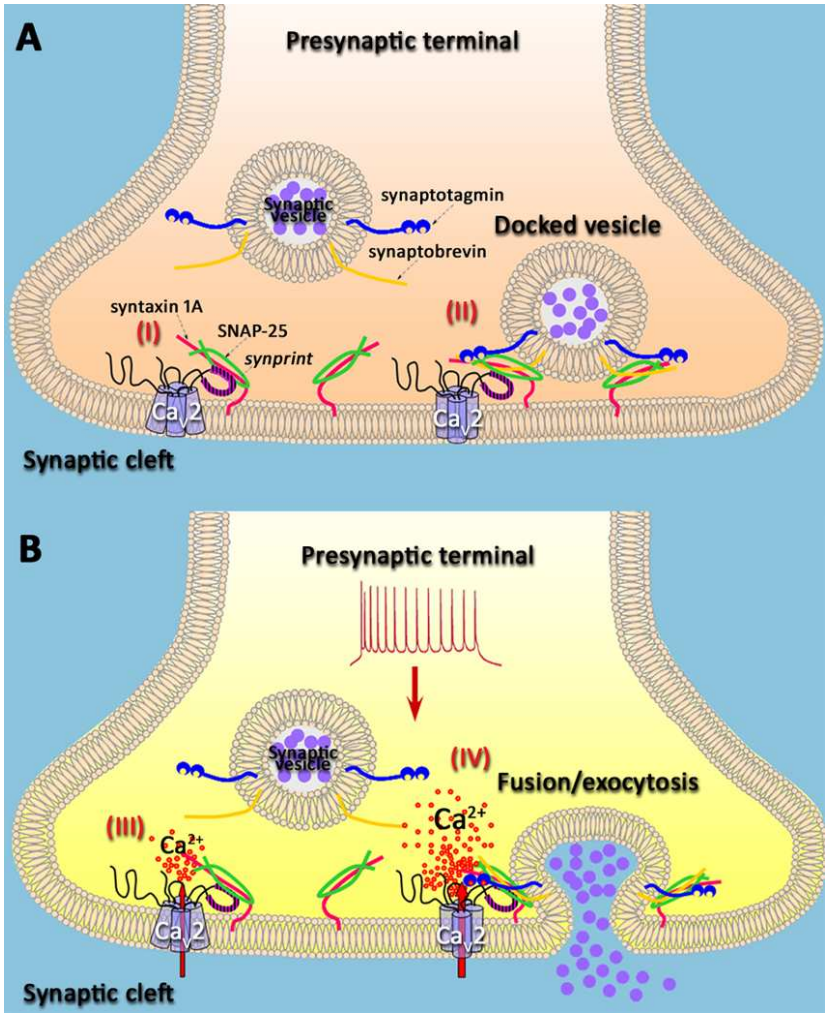

Figure 9. Proposal of SNARE regulatory mechanism of Ca_V2 channels in the control of neurotransmission efficiency. A. In the absence of a docked vesicle, SNARE proteins syntaxin or SNAP-25 alone exert inhibitory effects on Ca_V2 channels by hyperpolarizing its voltage dependent inactivation (I). This inhibition is relieved when the channel is associated with the entire SNARE complex (II). **B.** In response to a train of action potentials, Ca^{2+} influx will be most favoured through Ca_V2 channels coupled to docked synaptic vesicles that are ready for release (compare III and IV), providing a potential mechanism to increase the release probability of synaptic vesicles that are docked close to Ca_V2 channels.

Using an *in vitro* binding strategy, they found that distinct domains of syntaxin 1A participate in each interaction, with the C-terminus (residues 183-230) binding to Gβ but not Gγ subunit and the N-terminus (residues 1-69) binding to the *synprint* site[160]. Surprisingly, these results disagree with previous data showing that syntaxin 1A functionally interacts with the *synprint* site via its C-terminal domain (SH3 + TMD)[143].

Inconsistencies aside, the above mentioned studies suggest that susceptibility to modulation by G proteins may require the association of Ca_V2 channels with the transmitter release machinery, which might ensure that channels tethered at release sites are preferentially regulated by G protein second messenger pathways, providing an additional mechanism by which SNARE and/or G proteins may in its turn fine tune synaptic efficiency[161].

2.3.1. Impact of SNARE-*synprint* Interaction on Synaptic Transmission

Analysis of the functional consequences of Ca_V2 channel-regulation by SNARE proteins in intact neurons is an important step toward understanding these regulatory processes in their physiological context that may provide relevant information about the real impact of SNARE modulation on synaptic transmission. However, it demands the design of quite challenging experiments due to the difficulty of specifically manipulating Ca^{2+} channel-SNARE interaction in the presynaptic terminal. Different approaches have been successfully used: injection of synprint peptides to compete for such interaction into the presynaptic cell, expression of syntaxin or mutant Ca_V2 channels by injecting the cDNA

into the presynaptic cell, and the use of several peptide toxins in order to cleave specific SNARE proteins or to block specific Ca^{2+} channel types.

As demonstrated in the initial studies identifying syntaxin1A-binding site, peptides derived from the *synprint* are able to competitively inhibit Ca_V2-SNARE interaction *in vitro*[143]. Injection of *synprint* peptides from $Ca_V2.2$ channels in presynaptic superior cervical ganglion neurons (SCGNs) in culture significantly reduced the cholinergic excitatory postsynaptic response of neighbouring neurons by competitive uncoupling of the endogenous Ca^{2+} channel-SNARE interaction at nerve terminals[162]. Fast excitatory postsynaptic potentials (EPSPs) due to synchronous transmitter release were inhibited between 23% and 42%, while late EPSPs arising from asynchronous release[3] following a train of APs and paired-pulse facilitation (PPF)[4] were increased (reflecting reduced synaptic strength)[162]. Similarly, injection of $Ca_V2.2$ *synprint* peptides into embryonic Xenopus spinal neurons stimulated under physiological Ca^{2+} concentrations (~1.8 mM) caused a 25% reduction of relative transmitter release corresponding to an uncoupling of ~70% of the formerly linked vesicles by the *synprint* peptide as correlated by the authors with a model of Ca^{2+}-dependent synaptic transmission[165]. Increases in the extracellular Ca^{2+} concentration effectively rescued the reduction in synaptic transmission, implying that docked vesicles competitively displaced from Ca^{2+} channels by *synprint* peptides can be released thanks to flooding the presynaptic terminal with Ca^{2+} in a way that exceeds the threshold of release[165].

[3]It has been postulated that a delayed asynchronous release is triggered by the buildup of intracellular Ca^{2+} during repetitive stimulation and, as such, must rely on a slow Ca^{2+} sensor with high-affinity Ca^{2+}-binding sites[163].

[4]PPF is the increase in EPSP evoked by a second impulse. Increases in PPF following repetitive stimulation reflect short-term synaptic plasticity impairment[164].

If syntaxin 1A inhibits Ca^{2+} channel by stabilization of the inactivated state, disruption of native syntaxin-channel interaction by the *synprint* peptide would therefore lead to an increase in Ca^{2+} influx in injected terminals compared to control thus underestimating the physiological effect of this disruption on neurotransmitter release. In the study with SCGNs, the authors suggest that a potential increase in Ca^{2+} influx in terminals of *synprint*-injected neurons may counteract the decrease in synchronous release (thus explaining the incomplete inhibition of neurotransmission, between 23% and 42%), but may contribute to the observed increases in both asynchronous transmitter release and paired-pulse facilitation in injected terminals compared to control[162]. However, neither increase in peak Ca^{2+} currents nor shifts in voltage dependent inactivation were observed due to blocking the interaction of syntaxin with N-type Ca^{2+} channels by *synprint* injection in cell body recordings[162]. Analogously, fura-2 measurements indicated that the ratio of relative Ca^{2+} influx into injected and control synaptic terminals of *Xenopus* was virtually identical suggesting that *synprint* peptide exerts its effects by reducing the efficiency of neurotransmitter release without affecting the Ca^{2+} influx through the channels[165]. Put together, these experiments underscore the physiological importance of the interaction between presynaptic Ca^{2+} channels and members of the docking and fusion machinery and that interruption of this physical link does not completely abolish synaptic transmission[162,165], but makes it less probable by shifting its Ca^{2+}-dependence to higher values[165]. Nevertheless, evidence supporting syntaxin 1A modulation of Ca_V2 channels *in vivo* is missing in these two reports.

In a subsequent study, syntaxin 1A modulation of Ca^{2+} channels was evaluated in rat neocortical synaptosomes by applying Botulinum

neurotoxin type C1 (BoNtC1)[5] in a sufficient concentration to block neurotransmission and monitor Ca^{2+} entry following neuronal stimulation. Whereas the initial Ca^{2+} rise induced by repetitive firing or steady depolarizations was unchanged, late increase in Ca^{2+} entry (through members of Ca_V2 family) was significantly augmented by syntaxin cleavage[166]. These experiments provided the first evidence that a SNARE protein can influence Ca^{2+} influx through Ca_V2 channels in vertebrate nerve terminals[166].

At a first sight, one could perceive that interactions of Ca_V2 channels with SNARE proteins have two opposing effects: tethering synaptic vesicles near the source of Ca^{2+} entry would increase synaptic transmission, whereas enhancing voltage dependent Ca^{2+} channel inactivation would reduce synaptic transmission[29,164]. These effects were dissected by use of competing $Ca_V2.2$ *synprint* peptides and mutant syntaxin in *Xenopus* oocyte neuromuscular junction[164]. Injection of competing *synprint* peptides into developing neuromuscular junctions reduced the basal efficiency of synaptic transmission, as reflected in increased paired-pulse facilitation and reduced quantal content of synaptic transmission[164]. The effect of the *synprint* peptide to reduce the number of docked vesicles linked to Ca^{2+} channels is predominant because its potentially opposing effect to relieve channel inhibition would be occluded in the subset of channels that could participate in vesicle release as it must be assumed that they would interact with a complete SNARE complex, where SNAP-25 and synaptotagmin are present. In contrast, overexpression of a syntaxin mutant that is unable to regulate Ca^{2+} channels (i.e. A240V, V244A), but still binds to them[156], increased the efficiency of synaptic transmission, as reflected in reduced paired-pulse facilitation and

[5]BoNtC1 cleaves syntaxin 1A via its C-terminal region (see Figure 8A).

increased quantal content compared both to control and *synprint* conditions[164]. In this case, the mutant syntaxin most likely relieves $Ca_V2.2$ channels from endogenous syntaxin-mediated negative regulation of voltage dependent inactivation, thereby increasing Ca^{2+} entry and synaptic transmission, but does not alter linkage of docked vesicles to $Ca_V2.2$ channels. These results demonstrate a bidirectional regulation of synaptic transmission in vivo by interactions of SNARE proteins with $Ca_V2.2$ channels.

The effects of disrupting syntaxin interaction with Ca_V2 channels on their coupling to vesicle exocytosis have been further evaluated by transient transfection of WT or *synprint*-deleted $Ca_V2.2$ channels into mouse pheochromocytoma (MPC) cell line 9/3L, a cell line that has the machinery required for rapid stimulation but lacks endogenous voltage-dependent Ca^{2+} channels. Total secretion and the rate of secretion, determined by capacitance measurements, were measured from MPC transfected cells showing that both components were significantly reduced in the synprint deletion-expressing cells[167]. These results confirm that *synprint* site is necessary for the efficient coupling of Ca^{2+} influx to synaptic transmission/vesicle exocytosis. Nonetheless, there are some exceptions to this “nearly universal” rule.

First evidence against the requirement of *synprint* site to allow exocytosis comes strikingly from a previous paper by the same group showing that expressed $Ca_V1.2$ channels and $Ca_V3.1$, which do not express *synprint* sites, expressed appropriate currents and supported rapid exocytosis with a stimated efficiency similar to *synprint*-containing $Ca_V2.2$ channels[168]. In fact, as authors shrewdly mention in the discussion, L-type channels can initiate release in some specific cell types (e.g. pancreatic beta cells). In a similar way, Ca^{2+} channels that mediate neurotransmitter

release in some invertebrate animals like *Drosophila*, *Lymnaea* or *C. Elegans* have no recognizable *synprint* site. Yet, the injection of rat *synprint* peptides can still disrupt synaptic transmission in *Lymnaea*[169].

These observations raise the possibility that physical coupling between presynaptic Ca^{2+} channels and release machinery may not be always obligatory to support neurotransmitter release. If this was true, *synprint* peptides may disrupt neurotransmitter release in *Lymnaea* by a non-specific action (e.g. preventing vesicle recruitment or vesicle docking by blocking the binding between different members of the release machinery). A more likely explanation suggests that syntaxin and/or SNAP-25 may bind voltage-dependent Ca^{2+} channels via alternative sites rather than or in addition to *synprint* site in the II-III loop[156]. In this case, *synprint* peptides would still be able to disrupt transmitter release mediated by Ca^{2+} channels without *synprint* sites (i.e. the fact that SNARE proteins bind to *synprint* peptide[143,145,147] not only abolishes the interaction with the *synprint* site but likely hinders additional SNARE interactions in $Ca_V\alpha_1$ subunit). Clearly, our current understanding of the intricate interactions between Ca^{2+} channels and the synaptic release machinery is in its infancy. A great deal of work, especially in native neuronal settings, remains to be done in order to decipher the complexities of synaptic protein regulation on Ca^{2+} channel physiology.

3. NEUROBIOLOGY OF MIGRAINE

3.1. Genetic Component of Migraine

Migraine is a common and disabling brain disorder characterized by episodic attacks of headache often accompanied by hypersensitivity to

light and sound, nausea and/or vomiting (migraine without aura; MO), and, in at least one third of the cases, preceded by reversible neurological aura symptoms (migraine with aura; MA). Auras are most frequently visual, but can involve other senses, or occasionally cause motor or speech deficits (see Table 2).

Migraine affects about a 15% of people in developed countries and has a profound effect on wellbeing, not only during the attack, but also in terms of work performance, family and social relationships, and school achievement. It has been estimated to be the most costly neurological disorder in Europe[170]. The remarkably prevailing occurrence of migraine (cumulative lifetime incidence of 43% in women and 18% in men in a recent population study)[171] suggests that it may involve relatively minor perturbations of normal brain function and may, therefore, have much to teach us about the basic physiology of the nervous system[172].

Familial clustering and twin studies show that migraine has a strong genetic component, with a likely multifactorial polygenic pattern of inheritance[173]. Although several susceptibility genes and loci both for MA and MO have been reported in case-control studies and genome-wide scan analysis respectively, causative genes have not yet been identified, except for familial hemiplegic migraine (FHM), a rare, autosomal dominant subtype of MA that includes some degree of hemiparesis (see Table 3, redrawn from[170,174]).

Mutations in genes *CACNA1A* and *SCNA1A*, encoding the pore-forming α_1 subunits of the neuronal voltage-gated $Ca_V2.1$ (P/Q-type) channels and $Na_V1.1$ channels, are responsible for FHM1 and FHM3, respectively, whereas mutations in *ATP1A2*, encoding the α_2 subunit of the Na^+, K^+ ATPase, are responsible for FHM2.

Table 2: International Headache Society diagnostic criteria for MO and MA[175].

1.1	**Migraine without aura**
A	At least five attacks fulfilling B–D
B	Headache attacks lasting 4–72 h (untreated or unsuccessfully treated)
C	Headache has at least two of the following characteristics:
	Unilateral location
	Pulsating quality
	Moderate or severe intensity (inhibits or prohibits daily activities)
	Aggravation by walking stairs or similar routine physical activity
D	During headache at least one of the following:
	Nausea and/or vomiting
	Photophobia and phonophobia
E	Not attributed to another disorder
1.2.1	**Typical aura with migraine headache**
A	At least two attacks fulfilling B–D
B	Fully reversible visual, sensory, or aphasic aura symptoms (but no motor weakness)
C	At least two of the following:
	Homonymous positive features and/or unilateral sensory symptoms
	At least one symptom develops gradually over more than 5 min or two or more occur in succession
	Each symptom lasts 5–60 min
D	Headache follows aura with a free interval of less than 60 min (it may also begin before or simultaneously with the aura)
E	Not attributed to another disorder

Note that most patients with migraine have migraines either with or without aura throughout their life, but some have attacks of both types. Not all patients who have migraine with aura have attacks that fulfil criteria B–D, and some with the diagnosis of typical aura with migraine headache also have attacks that fulfil the criteria for migraine without aura.

The genetics of FHM have lead to interesting hypothesis concerning the molecular mechanisms of migraine attacks in more common types of migraine yet they might share the same pathophysiological pathways. So far, there has not been definitive evidence for the involvement of the three genes responsible for FHM in common forms of migraine although it has been speculated that, like FHM, common migraine may also involve differences in genes involved in ion transport[170,176].

However, a recent extensive screen of a European population concluded that common variants in ion transport genes do not play a major role in susceptibility to common migraine[177].

Most of the migraine association studies are single reports and await confirmatory replication which would be essential to show that the already reported associations are indeed reflecting a biological relation with migraine[170]. The C677T polymorphism of MTHFR is the only genetic variant to date associated with MA in several populations, including

patients identified from a random population sample, although negative findings have been also reported in two studies[170].

Table 3: Genetic Component of Migraine: genes causing FHM, candidate genes and polymorphisms associated to common forms of migraine, and susceptibility loci identified in genome-wide scans in common types of migraine.

LOCI	GENE	PRODUCT	MUTATION/ POLYMORPHISM	DISASE	REF.
19p13.2-p13.1	*CACNA1A*	P/Q Ca^{2+} channel α_{1A} subunit	25 mutations	FHM 1, SHM	(Ophoff et al., 1996)
1q23	*ATP1A2*	Na^+, K^+-ATPase pump α_2 subunit	47 mutations	FHM 2, SHM and BM	(De et al., 2003)
2q24.3	*SCN1A*	Na1.1 neuronal Na^+ channel α_1 subunit	5 mutation	FHM3	(Dichgans et al., 2005)
6q25.1	*ESR1, ESR2*	Estrogen receptor 1 and 2	ESR1 (G594A, G325C, and G2014A) and ESR2 (A2100G) polymorphisms	MO, MA	(Colson et al., 2004; Oterino et al., 2008; Kaunisto et al. 2006)
11q22-q23	*PR*	Progesterone receptor	*Alu* insertion in exon 7	MO, MA	(Colson et al., 2005)
19p13.3-p13.2	*INSR*	Insulin receptor	4 SNPs within the gene	MO, MA	(McCarthy et al., 2001; Netzer et al. 2008)
1p36.3	*MTHFR*	Methylenetetra-hydrofolate reductase	C677T polymorphism, protective effect of 677T	MA	(Lea et al., 2004; Scher et al. 2006; Kaunisto et al. 2006; Rubino et al. 2007; Schürks et al. 2008).
6p21.3	*TNFA, TNFB*	Tumor Necrosis Factor α and β	-308 G/A polymorphism (α), G252A (β)	MO	(Rainero et al., 2004; Asuni et al. 2009)
10q32.1	*ACE*	Angiotensin-converting enzyme	1 insertion/deletion polymorphism	MO	(Paterna et al., 2000)
19p13.2	*LDLR*	Low-density lipoprotein receptor	Triallelic TA repeat in the 3'UTR	MO	(Mochi et al., 2003)
6p21.3	*HLA-DRB1*	Human Leucocyte Antigen- beta chain 1	HLA-DRB1*16 allele	MO	(Rainero et al., 2005)
4q21, 4q24	?	---	---	MO, MA	(Bjornsson et al., 2003; Wessman et al., 2002)
6p12.2-p21.1	?	---	---	MO, MA	(Carlsson et al., 2002)
11q24	?	---	---	MA	(Cader et al., 2003)
14q21.2-q22.3	?	---	---	MO	(Soragna et al., 2003)
FHM genes					
Candidate genes that present polymorphic variants associated with migraine in studies published since 2000 and including at least 200 patients and 200 controls					
Susceptibility loci identified in genome-wide scans					

FHM=familial hemiplegic migraine, SHM=sporadic hemiplegic migraine, BM= basilar-type migraine, MA=migraine with aura, MO=migraine without aura

The many linkage peaks detected in genome-wide scans support the hypothesis that migraine traits are heterogeneous; while some relatively strong penetrant variants appear on a family level, a relatively modest effect is shown on a population level and, therefore, the endpoint phenotypes commonly used in those studies (MA and MO) may not be optimum.

Latent class[6] and trait component[7] analyses might help to reclassify the phenotypes to facilitate the identification of common genetic variants predisposing to headache disorders[170].

3.1.1. Comorbidity

Some rare inherited neurological diseases are comorbid to migraine and might serve as models to study migraine-related mechanisms and new therapeutic aspects[170]. The term comorbidity does not necessarily imply direction or causation. Mechanisms of comorbidity include unidirectional or bidirectional predisposition as well as shared pathophysiological, genetic, and/or environmental risk factors.

In particular, the association of epilepsy and migraine has been long recognized; both are among the most prevalent neurological conditions sharing pathophysiological mechanisms and common clinical features[178]. Several studies have identified common genetic and molecular substrates for migraine and epilepsy, including phenotypic-genotypic

[6] Latent class analysis groupings consist of individuals with different numbers and combinations of symptoms and thus reflect a measure of severity that differs from the traditional endpoint diagnoses based on the strict IHS diagnostic criteria.

[7] Trait component analysis is another approach to use individual traits and trait groups of the IHS criteria instead of the end diagnosis used in linkage or association studies.

correlations with mutations in *CACNA1A*, *ATP1A2* and *SCN1A* genes[178]. Interestingly, antiepileptic drugs are increasingly recommended for migraine profilaxis: valproate or divalproex, topiramate, and gabapentin have demonstrated efficacy in double-blind controlled trials[179].

Of worth mentioning is also the association of migraine and depression that co-occur far more commonly within patients than would be expected by chance. The comorbidity is bidirectional (i.e. people with migraine have an increased risk of depression, and vice versa) which suggest that shared molecular or other mechanisms are involved[180]. As in the case of anticonvulsants, antidepressants are effective drugs in migraine preventive therapy[179].

3.2. Introduction to Migraine Pathophysiology

A migraine attack is a spectacularly complex brain event that can produce an array of neurological and systemic symptoms. Although headache is typically the most prominent feature, a migraine may include multiple other symptoms that occur before, during, or after the pain. The characterization of a migraine attack is further complicated by the considerable variability in the clinical symptoms from one individual to the next, and from attack to attack within a given individual. As in any complex disease, the genetic load can be seen as determining an inherent migraine threshold that itself is modulated by internal and external factors (migraine triggers). Exercise, stress, diet and sleeping habits have been pointed as plausible elements that might generate a migraine attack.

What is clear, however, is that migraine pathophysiology involves multifaceted molecular, cellular, neuroanatomic, and neurochemical

mechanisms[172,181]. The site where the initial activation of these mechanisms occurs has not been determined with certainty. The idea that dilation of cerebral vessels is a primary cause of migraine pain has been challenged by a variety of evidence whereas "trigeminovascular system" (TGVS) activation continues to be widely accepted as an important component of the headache (see below).

3.2.1. Primary Cause of the Migraine Headache: The Trigeminal Pathway

The neuronal structures and pathways involved in the transmission and modulation of cephalic pain are schematically illustrated in Figure 10 (redrawn from[182]). Within the skull, pain sensitivity is principally restricted to the meningeal blood vessels, which are densely innervated by the ophthalmic division of the trigeminal nerve. It is generally accepted that migraine pain is initiated by the activation of these neurovascular afferents (i.e. the TGVS) that synapse in the trigeminal nucleus pars caudalis (TNC) and in upper cervical dorsal horns. Impulses are then carried rostrally to the brain structures involved in the perception of pain, including thalamic nuclei and the periaqueductal grey region (PAG). The PAG controls craniovascular pain in two ways, by ascending projections to the thalamus (the last relay site on the way to the cortex for almost all sensory information) and descending inhibitory modulation of nociceptive afferent activation[182].

Simultaneously, the release of vasoactive neuropeptides such as calcitonin gene-related peptide (CGRP) or substance P (SP) from the trigeminal peripheral nerve endings initiates a large inflammatory process (i.e. neurogenic inflammation) characterized by meningeal

vasodilatation, plasma extravasation, and mast cell degranulation (with further secretion of other proinflammatory substances in the dura). Trigeminal nerve activation also leads to vasodilatation of meningeal blood vessels through activation of a parasympathetic reflex at the level of the superior salivatory nucleus (SSN)[182].

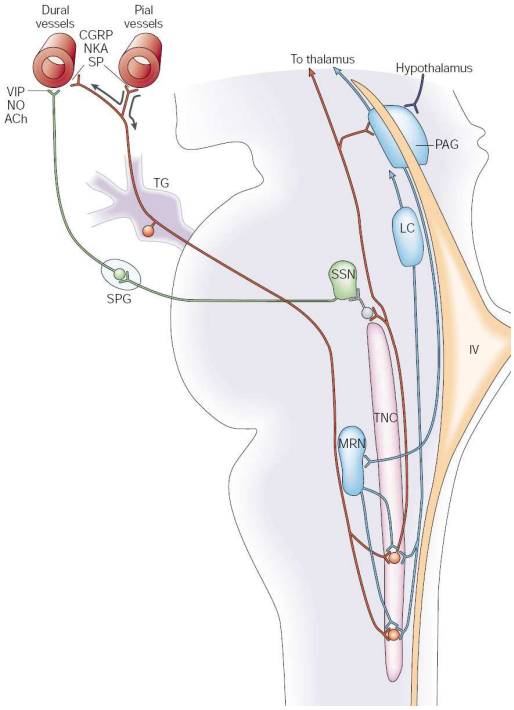

Figure 10. Neuronal pathways involved in trigeminovascular activation and pain processing. IV, fourth ventricle; ACh, acetylcholine; CGRP, calcitonin gene-related peptide; LC, locus coeruleus; PAG, periaqueductal grey region; MRN, magnus raphe nucleus; NKA, neurokinin A; NO, nitric oxide; SP, substance P; SPG, superior sphenopalatine ganglion; SSN, superior salivatory nucleus; TG, trigeminal ganglion; TNC, trigeminal nucleus pars caudalis; VIP, vasoactive intestinal peptide.

Evidence that activation of the TGVS occurs in humans is provided by the increased level of CGRP in jugular venous blood during a migraine attack, and its subsequent return to normal levels after treatment with the antimigraine drug sumatriptan[8] and subsequent headache relief[183].

[8] Sumatriptan is a $5HT_{1B/1D}$ serotonergic receptor agonist belonging to the triptan class of antimigraine drugs. The mechanism of action of these drugs is to inhibit both CGRP release from the peripheral TGVS nerve endings and neurotransmitter release from the central nerve endings of TGV afferents thus blocking neurogenic inflammation and central sensitization (see below).

The crucial question that has been bothering researchers until present is the primary mechanism that leads to the activation of TGVS and consequently to migraine headache. Until the 90's, migraine and other primary headache syndromes were described as vascular pathologies. According to the once widely accepted "vascular theory of migraine" (proposed by HG Wolff in the 1930s), aura symptoms arose from a transient ischemia induced by vasoconstriction, and consequently, the headache resulted from rebound abnormal vasodilatation of intracranial arteries that mechanically activated perivascular sensory fibers[182].

Alhough intracranial vasodilatation is an appealing simple explanation for migraine pain, this hypothesis has never been capable of explaining the wide range of symptoms that may precede, accompany, or follow the pain. Multiple imaging studies have now confirmed that vasodilatation is not required for migraine headache. Xenon blood flow studies, single-photon emission computed tomography (SPECT), and functional magnetic resonance imaging (fMRI) studies show significant hypoperfusion, in some cases followed by sustained hypoperfusion during a migraine attack[184-186]. In addition, blood flow velocities (an indirect marker of vessel diameter) measured to migraneurs with Doppler sonography do not show a significant increase in the diameter of the middle cerebral artery during migraine attacks[187], and what is more, migraine attacks can be induced without dilatation of this artery[188]. These findings are not consistent with vasodilatation as a primary trigger for pain, and, to the contrary, suggest that headache may be triggered by hypoperfusion.

At present, most authors support the "neurogenic theory", which states that migraine arises from a primary brain dysfunction responsible for the activation and sensitization (i.e., progressive amplification of a response

following repetition of a stimulus) of the TGVS[172,182,189,190]. Although the cellular and molecular mechanisms remain largely unknown, two main migraine generators have been proposed: the cerebral cortex and the brainstem.

- The Role of the Cerebral Cortex

There is substantial clinical and imaging evidence for changes in cortical activity associated with migraine. Migraine is associated with a variety of symptoms that can be attributed to changes in cortical function. The most prominent among these are the visual changes associated with migraine aura that arise from altered function in the occipital lobe. The neuropsychologist Karl Lashley made in 1941 detailed suggestions about how brain functions may cause characteristic visual aura symptoms (see Figure 11A, from[182]). He postulated that the scotoma (i.e., an area of lost vision surrounded by an area of less depressed or normal vision) resulted from a region of depressed neural activity in the visual cerebral cortex, and scintillations (i.e., bright spot lights), resulted from a region of intense cortical excitation that propagated slowly across the cortex[182]. A few years later, the electrophysiological correlate was reported by Aristides Leaõ in the rabbit cerebral cortex and was termed cortical spreading depression (CSD)[191].

In animals, CSD can be triggered by focal -electrical, mechanical, or high K^+- stimulation of the cerebral cortex and it involves a short-lasting depolarization wave that propagates slowly across the cortex (i.e., 3-5 mm/min) followed by prolonged nerve cell depression synchronously with a dramatic failure of brain ion homeostasis, efflux of excitatory amino acids from nerve cells and enhanced energy metabolism[192]. The depolarization phase is associated with an increase in blood flow (BF),

whereas the neural depression phase is associated with a decrease[192] likewise the changes in regional cerebral BF observed during a migraine aura propagating at a rate of 2-3 mm/min in humans[193]. These similarities lead to the hypothesis that CSD was responsible of migraine aura[192].

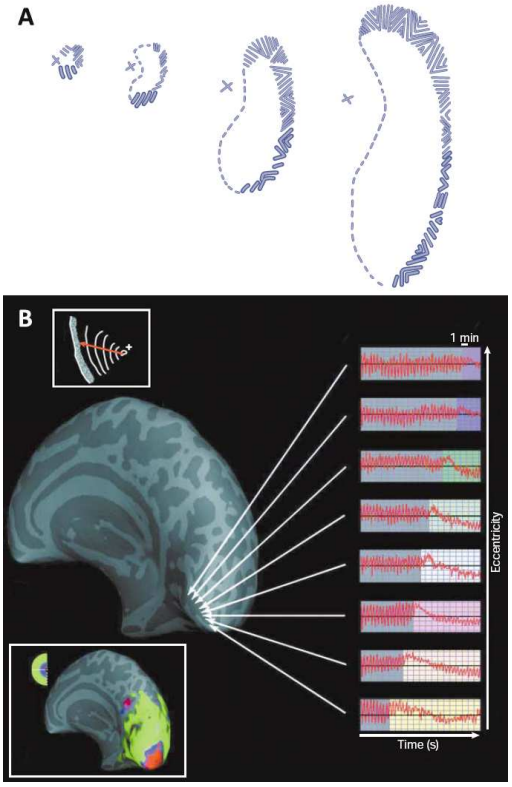

Figure 11. Spreading suppression of cortical activity during migraine aura. A. Original drawing by Lashley illustrating the progression of his own visual aura over time, consisting of a scotoma (within dashed line) and a scintillating border with typical fortifications. **B.** BOLD fMRI showing CSD-typical cerebrovascular changes in a reconstruction of patient's brain while experiencing a visual aura. A similar BOLD response was found within all of the extrastriate areas, differing only in the time of onset of the MR perturbation. Upper box illustrates the visual defect of the patient studied with brain imaging. Lower box shows a colour representation of the retinotopic topography of the perturbations' progression that start in the fovea (in red). Parafoveal eccentricities are shown in blue, and more peripheral eccentricities are shown in green.

Intriguing blood oxygenation level-dependent functional magnetic resonance imaging (BOLD fMRI) studies showed cerebrovascular changes that propagated with temporal and spatial characteristics remarkably similar to those of CSD in the cortex of migraineurs while experiencing a visual aura[194,195] (see Figure 11B, redrawn from[194]). Further evidence that CSD underlies visual aura has been obtained with magnetoencephalography studies: first in animals[196], and then in humans[197]. Likely, auras with sensory symptoms might result from CSD-like events within sensory cortices[182].

Interestingly, recent animal studies support the idea that CSD may also initiate migraine[198,199]. Despite the lack of electrophysiological evidences of enhanced firing of TNC after CSD, the available animal studies strongly support the conclusion that CSD, implicated in migraine visual aura, activates TGV afferents and evokes a series of cortical meningeal and brainstem events consistent with the development of headache[182,200,201].

However, it continues to be a matter of debate whether similar cortical phenomena could also occur in MO[15, 32-34]. Even some authors have suggested the possibility that CSD in MO could originate in a clinically silent area of the cerebral cortex[182].

The precise mechanism by which a CSD propagating across the cortical gray matter might activate the meningeal nociceptors is not entirely clear. It has been shown that CSD produces substantial changes (lasting minutes) in the composition of the extracellular fluid in the rat cortex (large increases in the composition of glutamate, K^+ ions, H^+ ions, NO, arachidonic acid and prostanglandins) that might be able to activate and/or sensitize the TGVS, either directly or by causing perivascular inflammation[202]. Moreover, recent findings show that CSD also up-

regulates certain proteins and genes, suggesting long-term changes in TGVS sensitivity[200,203,204].

Nevertheless, crucial questions still remain unanswered: what triggers CSD events in human brain? And, which mechanisms make the brain of migraineurs more susceptible to episodic CSD in response to these specific triggers?

A number of diverse stimuli have been proposed (e.g. direct cortical trauma, exposure to high concentrations of excitatory amino acids, increases in extracellular K^+ concentration, etc.) as potential triggers of CSD[202]. Migraineurs are hypersensitive to any kind of sensory overload and there is strong evidence for altered cortical excitability and abnormal processing of sensory information in their brain in the period between migraine attacks[205-207]. Genetic and environmental factors may modulate individual susceptibility by lowering CSD threshold, and cortical excitation may cause sufficient elevation in extracelular K^+ and glutamate to initiate CSD[208]. However, the mechanisms underlying the interictal abnormalities in cortical activity are controversial and their relationship to susceptibility and/or occurrence of CSD is unclear[209].

Important insights into the molecular and cellular mechanisms that may lead to altered cortical excitability and increased susceptibility to CSD in migraineurs have come from the discovery of the genes causing FHM and from the functional analysis of the disease causing-mutations in heterologous systems as well as in animal models of the disease. The main findings in the genetics and neurobiology of FHM and their implications for migraine mechanisms will be presented in detail at the third part of this section.

Nonetheless, CSD is a profound neural event, involving massive neuronal and glial depolarization as well as dramatic and sustained changes in

vascular function and some authors have questioned how such a profound event could occur in the absence of more significant neurological impairment[210]. The often subtle neurological symptoms of migraine aura, or the absence of symptoms altogether, are hard to reconcile with the extreme perturbations in cellular function that occur in large areas of the cortex with CSD[210]. If the changes in cortical function are not caused by CSD, are there any alternative explanations?

It has been shown that astrocytes are capable of producing intracellular Ca^{2+} waves in response to mechanical stimulation and glutamate that propagate with temporal and spatial characteristics that are remarkably similar to those of CSD, and, more interestingly, these waves consistently occur in association with spreading depression, both *in vitro* and *in vivo*[211,212]. Astrocytes are abundant cells in the central nervous system that have been traditionally viewed as playing only a passive and supportive role in nervous system function. But recent studies demonstrate that astrocytes are capable of extensive intercellular signalling that can modulate both neuronal and vascular activity suggesting that astrocytes are ideally positioned to modulate widely propagated changes in both vascular and neuronal activity with the pattern that is observed in migraine patients[212]. Thus, a speculative alternative to classical CSD as a mechanism of the propagated cortical changes in migraine is a phenomenon that involves astrocyte waves that could explain both the propagated changes in blood flow and metabolism that are observed with functional imaging studies, as well as the cortical symptoms that in most patients are not as profound as might be expected to result from classical CSD[210].

- The Involvement of the Brainstem

There is also strong evidence supporting that brainstem nuclei play a significant role in the pathophysiology of migraine. Nausea, vertigo, and autonomic symptoms are among the clinical features of migraine that may arise from an alteration of signalling in the brainstem[212].

As mentioned before, it is clear from the clinical symptoms of patients and from imaging studies that propagated waves of cortical activity can occur as part of migraine, especially while experiencing aura symptoms. However, some observations argue against CSD as the primary generator of a migraine attack. First, the majority of migraine attacks are not preceded by aura or other clearly propagated cortical symptoms; instead aura can occur after the onset of the pain, and even MA patients commonly experience attacks without aura[213]. Second, therapeutic intervention may abolish migraine aura in some patients yet not prevent their headaches, and the other way around[214]. These observations confirm the lack of sequential relationship between aura and headache. Additionally, premonitory symptoms (i.e. cognitive or physical symptoms by which migraineurs can reliably predict an impeding attack) such as fatigue, difficulty concentrating, and neck pain may occur up to several hours before the onset of headache (and aura)[215]. Taken together, these evidences indicate that at least in some patients, different brain regions are activated well before the onset of the cortical waves that occur in MA (and maybe in MO). Therefore, an alternative view proposes that the primary cause of migraine is an episodic alteration of brainstem, hypothalamus or other subcortical areas that may precede changes in brain activity and clinical symptoms indicative of CSD[216]. Two main findings have been considered to provide indirect support of this

hypothesis. First, placement of electrodes in PAG for the treatment of chronic pain produced migraine-like headaches in non-migraineurs[217]. Second, positron emission tomography (PET) and fMRI studies show consistent activation of brainstem regions during migraine attacks, particularly the region of the dorsolateral pons[218-220]. These evidences suggest a role for brainstem areas in migraine pain process but do not provide clear evidence of how brainstem dysfunction can activate TGVS, or whether there is a relationship with the observed abnormal cortical activity. The most widely accepted hypothesis is that the brainstem nuclei might act in a permissive way by favouring central hiperexcitability of trigeminal pathways[182,189].

3.2.2. Pain Mechanisms

Two pain mechanisms have been considered in migraine: neurogenic inflammation and peripheral and central trigeminal pathway sensitization. In regard to the latter, the brainstem may have an important role in the maintenance of migraine pain after TGVS activation (i.e. central sensitization).

Animal studies have demonstrated that inflammatory mediators produce activation of TGV afferents, a long-lasting increase in their activity, and an increase in their responsiveness to mechanical stimuli (peripheral sensitization)[221,222] whereas chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in second-order brainstem trigeminal neurons (central sensitization)[223]. On the basis of these findings, it has been postulated that peripheral and central sensitization are manifested, respectively, as pulsatile or throbbing headache (and its worsening after coughing or other normally innocuous

activities that increase intracranial pressure) and cutaneous allodynia (i.e., cutaneous pain resulting from a nonnoxious stimulus to the skin), the last reported in more than 70% of migraineurs[224,225].

Pharmacological research on more efficient antimigraine drugs has given much information on this subject. It was not until the late 80's, when researchers started to test the efficiency of triptans (serotonin 5-$HT_{1B/1D/1F}$ receptor agonists) against migraine headache[226]. These drugs inhibit neurogenic inflammation and meningeal vasodilatation by preventing the release of vasoactive neuropeptides, mainly CGRP, from the peripheral nerve endings of TGV afferents[227,228]. Triptans also inhibit neurotransmitter release from the central nerve endings of TGV afferents and transmission of nociceptive impulses to second-order neurons of the trigeminocervical complex (central sensitization)[227-229].

Evidence has been accumulating that a vascular effect of acute antimigraine compounds might be unnecessary for the termination of migraine pain suggesting that neurogenic inflammation may not be sufficient to maintain migraine pain[181]. In agreement with this idea, the serotonin 5-HT_{1F} selective agonist that lacks vasoconstrictive effects has demonstrated a high clinical efficacy in phase II trials[230] suggesting that the key mechanism in maintaining the severe prolonged pain of migraine may underlie the phenomenon of central sensitization.

3.2.3. An Integrated Model for Migraine Pathophysiology

The following is a hypothesized model for the sequence of events leading to the generation of a migraine attack:

In the cortex of migraineurs, a variety of different factors (genetic, neurochemical, ionic, and/or hormonal) lead to a dysregulation of

interictal excitability. Under certain triggers, this altered excitability exceeds the threshold for CSD initiation. Subsequent propagation of CSD (and possibly astrocyte Ca^{2+} waves too) in somatosensory cortical areas produces aura symptoms and promotes propagated changes in meningeal vascular caliber, and also breakdown of the BBB[203] (that may eventually contribute to worsen the pain[212]).

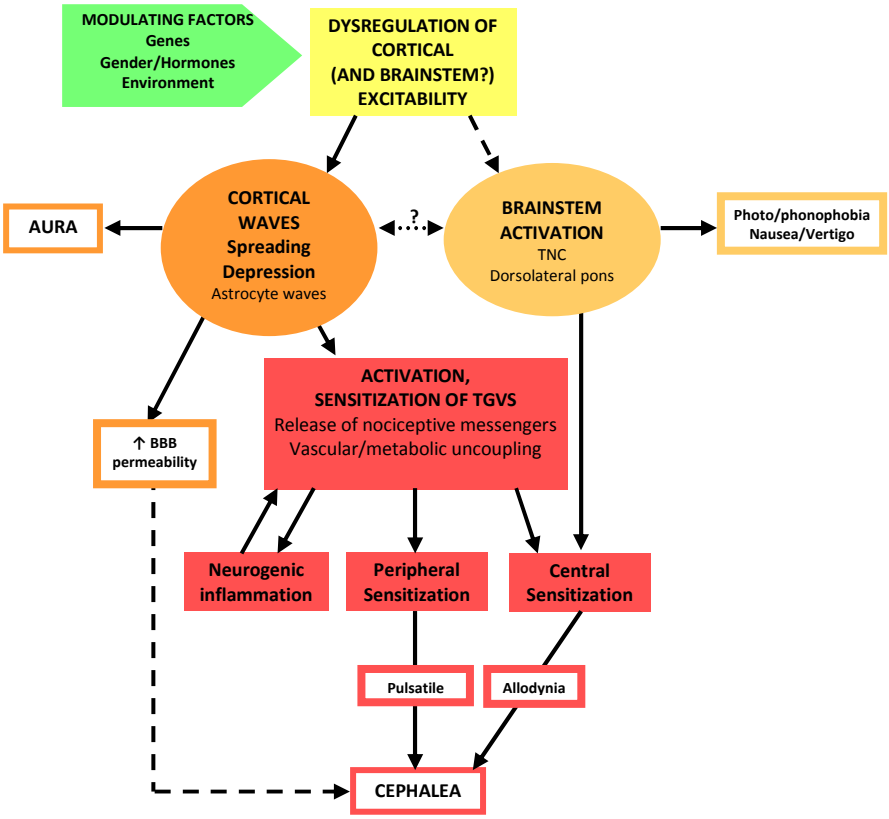

Figure 12. Proposed pathophysiological mechanisms involved in the generation of migraine headache. Current evidence indicates that CSD is the most probable primary event in TGVS activation in MA and, perhaps, also in MO. Brainstem nuclei dysfunction involved in the control of central pain might exert a permissive role by favouring central trigeminal hyperexcitability. The relationship between abnormal brainstem function and CSD remains hypothetical and unclear, hence the dashed line and question mark.

Cortical waves are also associated with release of a wide variety of neurotransmitters and neuromodulators, as well as changes in the ionic composition of the extracellular space that can activate perivascular trigeminal nociceptive afferents (i.e. the TGVS) responsible for the development and maintenance of migraine headache.

Pain is transmitted via peripheral these trigeminal pathways to the brainstem where second-order neurons are activated and eventually become sensitized. Activation of TGVS also leads to neurogenic inflammation further sensitize trigeminal pathways by a rebound mechanism. Peripheral and central sensitization of trigeminal pathways is manifested, respectively, as pulsatile/throbbing headache and allodynia.

Alternatively, second-order neurons in the brainstem are activated in parallel to, or even in the absence of, cortical phenomena by changes in cellular excitability that may be similar to those described in the cortex. Third-order neurons in the thalamus and cortex are then activated and sensitized (see Figure 12, redrawn from[181,212]).

3.3. Familial Hemiplegic Migraine

The main symptoms of headache and aura (as well as the accompanying symptoms of nausea, photophobia and phonophobia) of FHM attacks are very similar to those of MA, and both types of attack might alternate in patients and co-occur within families. FHM is characterized by obligatory motor aura symptoms that consist of motor weakness or paralysis, which is often, but not always, unilateral. Three or four aura symptoms are nearly always present in FHM attacks, and usually in the temporal order: visual, sensory, motor and aphasic symptoms, and they last longer than in MA[231]. Also the duration of the headache is usually longer. In contrast

to other types of migraine, some FHM patients have atypical severe attacks with signs of diffuse encephalopathy, impairment of consciousness (coma) or confusion, prolonged hemiplegia lasting several days, or in a few cases seizures[232]. Moreover, about 20% of FHM families show permanent cerebellar symptoms of progressive ataxia and/or nistagmus[231]. Emotional stress and minor head trauma are among the most common triggers of FHM attacks[232].

A new era started fifteen years ago when Ophoff and co-workers were able to show that FHM, linked to chromosome 19p13, is caused by missense mutations in the *CACNA1A* gene[233]. So far, three genes –the ion-channel genes *CACNA1A* and *SCNA1*, and one encoding the Na^+, K^+-ATP exchanger- have been found to underlie FHM1, 3, and 2, respectively (see Table 3). These mutations probably contribute to neuronal hyperexcitability by leading either to an increased release (i.e. gain-of-function mutations in *CACNA1A* and *SCNA1*) or to an inefficient clearing of synaptic glutamate (i.e. loss-of-function mutations in *ATP1A2*)[181,182] (see Figure 13 and main text below).

The first mutations in the gene encoding the α_2 subunit isoform of the Na^+, K^+-ATPase responsible for FHM2 were identified in 2003[234], and since then have been described over thirty mutations that mostly involve amino acid changes, although there are also small deletions and a mutation that causes an extension fo the protein by affecting the stop codon[174]. Mutations in *ATP1A2* gene can also cause permanent mental retardation in children with a family history of HM, and epilepsy[170].

The Na^+, K^+-ATPase is a P-type pump that utilizes the energy of ATP to actively transport Na^+ ions out and K^+ ions into the cell thus allowing to maintain resting membrane potential and cell volume and providing the driving force for nutrients and neurotransmitter uptake. It is usually

composed of two subunits, a catalytic α subunit consisting of 10 transmembrane segments (that contains the ATP and ions binding sites), and a regulatory β subunit. Most FHM2 mutations in the transporter are localized within the large intracellular loop between segments 4-5 (which undergoes major conformational changes during enzymatic cycle and contains the hydrolase and ATP-binding domains), critical for its correct function.

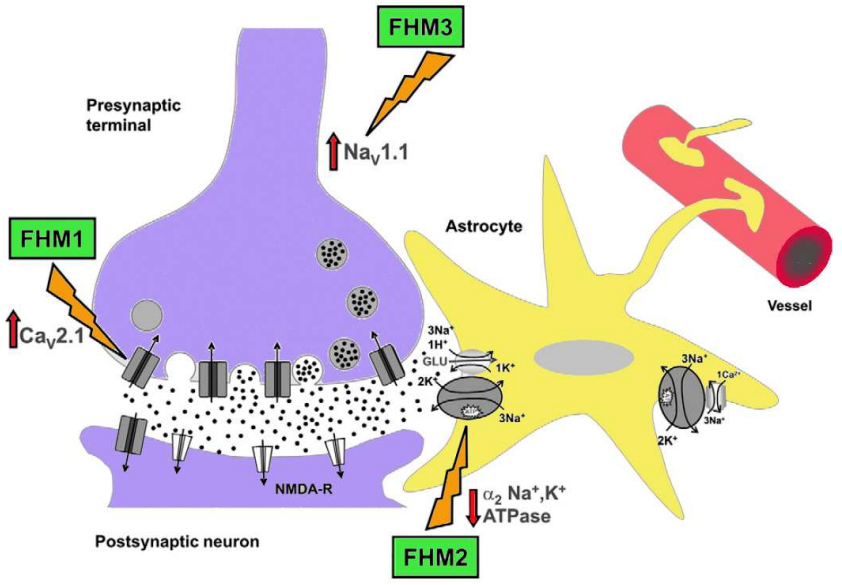

Figure 13. Gain-of-function mutations in $Ca_V2.1$ channels (FHM1) or $Na_V1.1$ channels (FHM3) and loss-of-function mutations in α_2 Na^+, K^+-ATPase (FHM2) may all render the brain more susceptible to CSD (adapted from[236]).

Active transport of glutamate into astrocytes and neurons is driven by both Na^+ and K^+ gradients. Particularly, colocalization of α_2 Na^+, K^+-ATPase subunit isoform with glutamate transporters exclusively in glial cells has been shown in adult somatosensory cortex[235], suggesting a specific functional coupling of the α_2 Na^+, K^+-ATPase with astrocytic

glutamate transporters and a precise role of this pump in glutamate clearance by glial cells in the adult cortex.

The functional consequences of a large number of FHM2 mutations have been investigated and have been shown to either produce: severe or complete loss-of-function, reduced affinity for extracellular K^+ or decreased catalytic turnover with increased affinity for extracellular K^+[174]. Impaired clearance of K^+ and glutamate by astrocytes during cortical neuronal activity, consequent to a decreased number of functional Na^+, K^+-ATPases, or a decreased affinity or decreased catalytic turnover of Na^+, K^+-ATPase pumps, would depolarize neurons and enhance glutamate concentration in the synaptic cleft, thus impairing recovery from excitatory neuronal impulses. Therefore, one would predict that the pathophysiological mechanism of FHM2 mutations is to enhance neuronal susceptibility to undergo CSD and/or stimulate astroglia to produce propagating Ca^{2+} waves (see Figure 13).

Recently, the discovery of *SCN1A*, the third gene linked to FHM3, encoding the α_1 subunit of the neuronal voltage-gated Na^+ channel $Na_V1.1$, further reinforced the view of migraine as a channelopathy that has to do with neuronal excitability[237]. The pore-forming α_1 subunit of Na_V channels shares the fundamental design of six transmembrane segments repeated in four homologous domains (similar to Ca_V channels).

So far, five FHM3 mutations have been identified in SCN1A and for three of them the functional consequences have been analyzed[174]. Initially, two FHM3 mutations (Q1489K and L1649) introduced in the cardiac $Na_V1.5$ revealed various gain-of-function effects[73,74]. However, when included in the more appropriate $Na_V1.1$ protein they resulted in loss-of-channel activity due to either enhanced inactivation or reduced cell surface

expression[238]. On the contrary, the third FHM3 mutation analysed (L263V) produces gain-of-function features on $Na_V1.1$ channels[238]. Furthermore, when Q1489K mutation was expressed in cultured neurons its effects on channel gating could be consistent with either enhanced or reduced neuronal excitability, depending on which stimulation paradigm was used[239]. Therefore, defining the role of mutant $Na_V1.1$ in FHM3 is not an easy task due to the lack of knowledge regarding the channel expression pattern and its contribution to cortical excitability. However, it has been hypothesized that loss-of-function effects will primarily disturb the functioning of inhibitory GABAergic interneurons (where $Na_V1.1$ is normally expressed[75]), whereas gain-of-channel activity will have a predominant effect on excitatory neurons[238] (see Figure 13).

3.4. Familial Hemiplegic Migraine Type 1

Roughly half of the cases of FHM are due to mutations in the *CACNA1A* gene[174,233]. All the 21 FHM1 mutations reported so far (including the Y1245C *de novo* mutation identified in a sporadic patient without other affected cases in the family[240]) are missense mutations that produce substitutions of conserved amino acids in important functional regions of $Ca_V2.1$ channel including the pore-lining segments and the voltage sensor regions (see Figure 14). Pure FHM1 and FHM1 with cerebellar symptoms are associated with distinct mutations[232]. The fact that single disease-causing mutations give rise to different clinical phenotypes suggests the existence of genetic or environmental modifiers.

Presynaptic $Ca_V2.1$ channels are efficiently coupled to the exocytotic machinery and play a prominent role in initiating action potential-evoked

neurotransmitter release with an increased relative contribution with postnatal age at many CNS synapses[106-109,241].

$Ca_V2.1$ channels are expressed in all the brain structures that have been implicated in migraine pathogenesis and/or migraine pain, including the cerebral cortex, the trigeminal ganglia, and brainstem nuclei involved in the control of nociception[236].

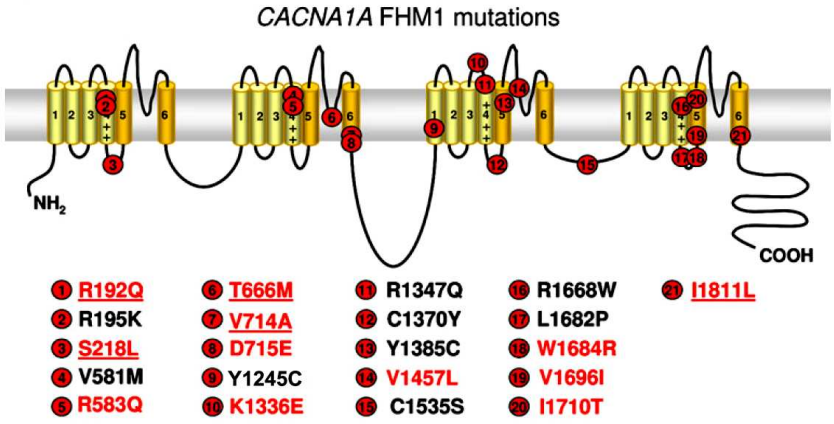

Figure 14. Locations of human FHM1 mutations in the secondary structure of the $Ca_V2.1$ α_1 subunit (Genbank Ac. nr. X99897). In red, FHM1 missense mutations whose functional consequences have been studied on recombinant $Ca_V2.1$ channels in heterologous expression systems previous to this thesis; the functional consequences of the underlined FHM1 mutations have also been studied in neurons from $Ca_V2.1^{-/-}$ mice expressing human $Ca_V2.1\alpha_1$ subunits.

In cerebral cortex, excitatory synaptic transmission at pyramidal cell synapses in different cortical areas depends predominantly on P/Q-type Ca^{2+} channels and, although P/Q-type Ca^{2+} channels are less significant regulators of GABA release, a role of P/Q-type Ca^{2+} channels in mediating GABA release from fast-spiking (FS) interneurons to pyramidal cells has been demonstrated recently in rat cortex[209,242-244].

In the trigeminovascular system, P/Q-type Ca^{2+} channels are involved in the control of release of vasoactive neuropeptides from perivascular

terminals of meningeal nociceptors[245,246], and both of glutamate and CGRP release from trigeminal ganglion neurons in culture[247]. P/Q-type Ca^{2+} channels are also involved in controlling tonic inhibition of TNC neurons with input from the dura[248].

A possible important whilst complex role of P/Q channels in central sensitization of the trigeminovascular system is suggested by pharmacological evidence in the spinal cord where spinal specific inhibition of P/Q Ca^{2+} channel can prevent and/or attenuate allodynia[249], and by the analysis of pain responses in *Cacna1A*$^{-/-}$ mice that show on one hand a pronociceptive role of P/Q Ca^{2+} channels in inflammatory and neuropathic pain, and, on the other hand, antinociceptive role of P/Q Ca^{2+} channels in response to acute noninjurious noxious thermal stimuli[250]. This complex picture likely reflects the preeminent role of P/Q-type Ca^{2+} channels in controlling release of both excitatory and inhibitory neurotransmitters and their wide distribution in different regions involved in pain[251].

About half of FHM1 mutations also cause progressive cerebellar symptoms. The expression $Ca_V2.1$ channels is particularly high in both Purkinje and granule cells of the mammalian cerebellum, where they exert key pre- and postsynaptic functions in neuronal signal transmission and processing, respectively[182]. Mice with null mutation in *Cacna1a* gene show severe cerebellar ataxia and dystonia, together with selective progressive cerebellar degeneration. Different mouse strains with spontaneous *Cacna1a* mutations all suffer from ataxia and exhibit reduced P/Q-type currents in Purkinje cells[251]. In humans, other neurological disorders with cerebellar disfunction, as episodic ataxia type 2 and spinocerebellar ataxia type 6, are caused by mutations in *CACNA1A*[233,252].

3.4.1. Functional Consequences of FHM1 Mutations

The functional consequences of 12 FHM1 mutations (in red in Figure 14) have been investigated in heterologous expression systems expressing recombinant $Ca_V2.1$ channels[97,98,253-261]; five of these mutations (underlined in Figure 14) have been also investigated in neurons from *Cacna1A*$^{-/-}$ mice expressing human $Ca_V2.1\alpha_1$ subunits[259,260,262,263]. Recently, the generation of knockin (KI) mice carrying two different FHM1 mutations (R192Q and S218L) allowed the first analysis of mutant channels expressed at their endogenous level in neurons[242,264-266,266].

The studies in heterologous expression systems show that the FHM1 mutations alter many biophysical properties of human $Ca_V2.1$ channels in a complex way, but collectively, a consistent gain-of-function effect in the activation properties of the channel can be concluded from these studies.

Single-channel analysis of eight mutant $Ca_V2.1$ channels revealed a homogeneous effect of enhanced open probability in a wide-range of depolarizations, mainly due to a shift to lower voltages of channel activation[255,259,260] (see Figure 15A, taken from[251]). Consistent (and significant for the majority of the 12 FHM1 mutations analyzed until the development of the present doctoral thesis) shifts to lower voltages of activation of the mutant channels were also revealed by measurements of whole-cell currents in heterologous systems and KI mouse neurons[97,98,253,255-261] (see Figure 15B, taken from[251]). For certain mutations, the magnitude (or even the presence) of the shift in activation depended on the α_{1A} splice variant and type of coexpressed $Ca_V\beta$ subunit[253,257].

Interestingly, a reduction in the direct inhibition of $Ca_V2.1$ channels by Gβγ following G-protein-coupled receptor stimulation has been reported for two different FHM1 mutations, an effect that may lead to further gain-of-function of Ca^{2+} currents through mutant channels during synaptic transmission[256,258,261].

The FHM1 mutations also affect the kinetics of $Ca_V2.1$ channel inactivation when expressed in heterologous systems or in neurons but the effects are highly heterogeneous and difficult to interpret. FHM1 mutations increase, decrease or unaffect kinetics of inactivation[97,98,253,255,258,260] and produce negative shifts of voltage dependent steady-state inactivation[257,258], and in some cases, either the presence or magnitude of these effects is dependent on the α_{1A} splice variant expressing the FHM1 mutation and $Ca_V\beta$ subunit coexpressed[253,257]. The pathophysiological impact of the changed inactivation properties in FHM1 remains to be elucidated.

Evidence that some FHM1 mutations decrease the density of functional channels in the membrane has been subject to much debate because this effect may be in apparent contradiction to the mechanisms of migraine pathogenesis. When the mutant channels were expressed in HEK293 cells, a decreased maximal whole-cell Ca^{2+} current density were found for some FHM1 mutations compared to WT with the exception of R192Q mutation, and when the same mutations were expressed in $Ca_V2.1^{-/-}$ neurons, a consistent decrease in maximal whole-cell P/Q-type Ca^{2+} channel density was found for all (including R192Q) mutants investigated suggesting a loss-of-function effect of FHM1 mutations in the expression of functional channels in the membrane[254,255,259,260,262,263].

This controversy has been recently solved by the generation and functional characterization of the two different FHM1 KI mouse models

(R192Q and S218L) because it has allowed studying the functional consequences of these FHM1 mutations on $Ca_V2.1$ channels and synaptic transmission in neurons expressing the channels at their endogenous levels[242,264-266].

In humans, whereas mutation R192Q causes pure FHM, mutation S218L causes a particularly dramatic clinical syndrome, that may consist of, in addition to attacks of hemiplegic migraine, slowly progressive cerebellar ataxia and atrophy, epileptic seizures, coma or profound stupor and severe, sometimes fatal, cerebral oedema which can be triggered by only a trivial head trauma[267]. In mice, while homozygous R192Q (RQ/RQ) and heterozygous S218L (SL/WT) KI models did not exhibit an overt phenotype, homozygous S218L (SL/SL) KI model exhibited mild permanent cerebellar ataxia, spontaneous attacks of hemiparesis and/or (sometimes fatal) generalized seizures, and brain oedema after only a mild head impact, thus modelling the main features of the severe S218L clinical syndrome[264,265].

Electrophysiological studies in cerebellar granule cells and cortical pyramidal cells have revealed larger P/Q-type Ca^{2+} current densities in R192Q and S218L KI neurons compared to WT neurons in a wide range of relatively mild depolarizations, reflecting an 8-9 mV shift in the voltage-dependence of activation to more negative voltages, and similar P/Q-type Ca^{2+} current densities in KI and WT neurons at higher voltages, indicating equivalent densities of functional $Ca_V2.1$ channels[242,264,265] (see Figure 15C, taken from[251]).

In agreement with the lower threshold of activation of human S218L $Ca_V2.1$ channels compared to R192Q $Ca_V2.1$ channels found in heterologous systems[260], the gain of function of P/Q current at low voltages was larger in S218L than in R192Q KI mice[264,265], and this gain-

of-function was about twice as large in homozygous compared to heterozygous KI mice, revealing and allele-dosage effect consistent with the dominance of the mutation in FHM1 patients[264]. The densities of the other HVA Ca^{2+} currents were similar in WT and KI mice neurons, indicating the absence of compensatory mechanisms[242,264,265].

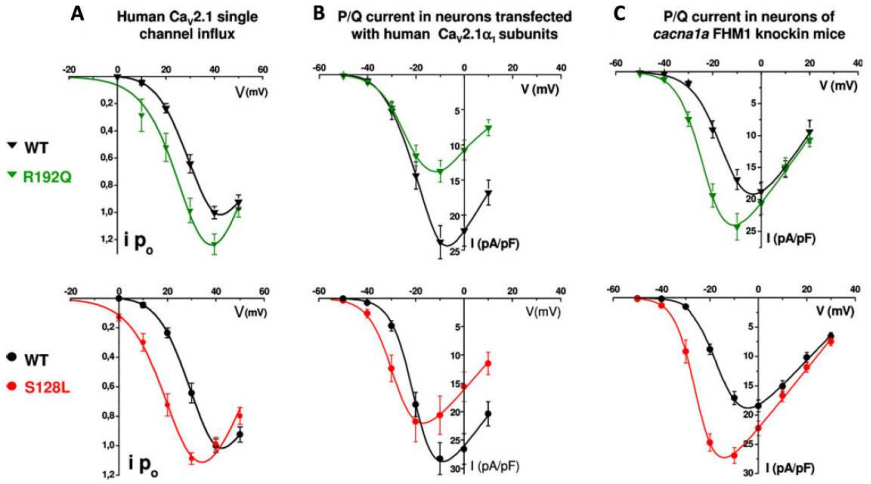

Figure 15. FHM1 mutations produce gain-of-function effect on $Ca_V2.1$ channel activation. A. Single channel influx through human $Ca_V2.1$ channels as measured by the product of single channel current, **i**, and the open probability, $\mathbf{p_o}$, from cell-attached recordings (90 mM $BaCl_2$) on HEK293 cells expressing WT or mutant $Ca_V2.1\alpha_1$ subunits. **B.** P/Q current density (5 mM $BaCl_2$) in cerebellar granule cells from $Ca_V2.1^{-/-}$ mice transfected with WT or FHM1 mutant $Ca_V2.1\alpha_1$ subunits. **C.** P/Q current density (5 mM $BaCl_2$) in cerebellar granule cells from WT and homozygous FHM1 KI mice.

The gain of function of FHM1 mutations on $Ca_V2.1$ channel activation correlates with enhanced evoked neurotransmitter release at the neuromuscular junction (NMJ)[264,265]. In some types of neurons, the enhancement in neurotransmission depends on the shape of the evoked action potential, as suggested by neurotransmission studies in brain slices of the Calyx of Held and pyramidal cell layer 2/3 of R192Q KI

mice[266]. More recently, cortical excitatory neurotransmission was found to be increased both in neuronal microcultures and brain slices of R192Q KI mice due to enhanced probability of glutamate release at cortical pyramidal cell synapses[242].

The investigation of experimental CSD, elicited either by electrical stimulation of the cortex in vivo or high $[K^+]_o$ in cortical slices, has revealed a lower threshold for CSD induction and increased velocity of propagation in both KI mouse models[242,264,265] (see Figure 16), but the facilitation of both induction and propagation of CSD is larger in S218L[264] and the post-CSD motor deficits are more severe (including generalized seizures and a unique increased susceptibility to repetitive successive CSD events following a single CSD-inducing stimulus)[264,268].

Interestingly and in agreement with the higher incidence of migraine in females, the velocity of propagation and the frequency of CSDs were larger in females than in males of both mutant strains; the sex difference was abrogated by ovariectomy and enhanced by orchiectomy, suggesting that female and male gonadal hormones differentially modulate CSD susceptibility[268,269].

Conjointly, the studies of experimental CSD in FHM1 KI mice reinforce the view of CSD as a key player in the pathogenesis of migraine and support a key role of $Ca_V2.1$ channels in the initiation and propagation of CSD. Accordingly, previous studies had showed an increased threshold for initiation and a decreased propagation velocity of experimental CSD in *leaner* and *tottering* mice both carrying spontaneous loss-of-function mutations in *Cacna1a*[243].

The finding of increased glutamate release at pyramidal synapses of KI mice has provided a plausible mechanism to explain the increased facilitation of CSD in FHM1 mutant mice[242]. Moreover, it has been

demonstrated that facilitated CSD KI phenotype can be reverted by bringing glutamate release back to WT values using a subsaturating concentration of the P/Q-type channel blocker ω-AgaIVA[242].

In migraineurs, CSD arises "spontaneously" in response to specific triggers that eventually overwhelm regulatory mechanisms of neuronal excitability and ignites CSD[209]. Insights of how this might occur have been provided by the interesting finding that, in contrast to excitatory neurotransmission, inhibitory neurotransmission at synapses between FS interneurons and pyramidal cells is not altered in R192Q KI mice, despite being initiated by P/Q-type Ca^{2+} channels[242].

It appears reasonable to hypothesize that, as a consequence, in certain conditions (i.e. under certain migraine triggers) the cortical excitation-inhibition balance may be disrupted due to excessive recurrent excitation, resulting in overexcitation and neuronal hyperactivity that may increase $[K^+]_o$ above the critical value for CSD ignition[209].

The studies of FHM1 mice support the view of migraine as brain disorder of excitability with episodic disruptions of the excitation-inhibition balance and hyperactivity of cortical circuits in response to migraine triggers at the basis for vulnerability to CSD ignition[209,242](see Figure 16, redrawn from[209]). Given the remarkable clinical and genetic heterogeneity of migraine, it is possible that episodic disruptions of the excitation–inhibition balance in response to migraine triggers may occur independently or in parallel in multiple brain regions and different cellular types that altogether converge in activating the TGVS[212].

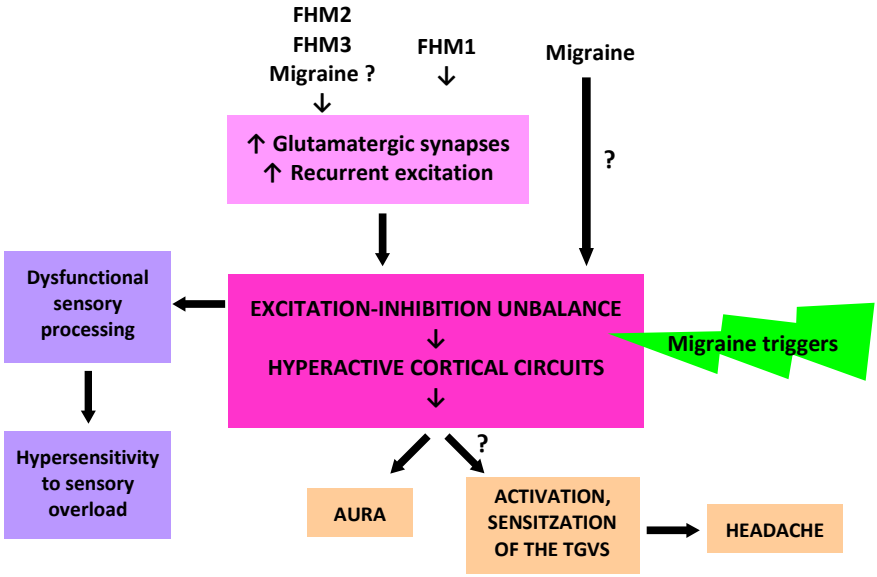

Figure 16. Proposed pathophysiological mechanism for FHM1 and migraine. In FHM1 (and probably, FHM2, FHM3 and a fraction of migraine cases) excitation–inhibition imbalance results from enhanced glutamatergic neurotransmission and enhanced recurrent cortical excitation. In migraine, different mechanisms, that remain to be elucidated, may result in excitation–inhibition breakdown of cortical (and/or subcortical areas) that have been hypothesized to activate the trigeminovascular system.

II.OBJECTIVES

Mutations in the gene encoding the pore-forming α_{1A} subunit of $Ca_V2.1$ (P/Q-type) Ca^{2+} channels (*CACNA1A*) cause familial hemiplegic migraine (FHM1) most probably by promoting channel activation that in turn would favour the initiation and propagation of cortical spreading depression, an abnormal increase of cortical activity –followed by a long-lasting neuronal suppression wave- that propagates across the cortex. Decreased channel inhibition by direct G-protein modulation as exhibited by two FHM1 mutations may contribute to this persistent state of neuronal hyperactivity.

In this doctoral thesis two novel migraine-related mutations in CACNA1A have been characterized: Y1245C and A454T. Mutation Y1245C is the first migraine-associated genetic alteration described in any S1 segment of α_{1A} reported in a sporadic hemiplegic migraine (SHM) patient that debuted with childhood periodic syndromes (CPS). Mutation A454T is located in the I-II intracellular loop of α_{1A} and, although not causative of the disease, is associated with the absence of sensorimotor symptoms in a migraine with aura pedigree.

The main objectives of this doctoral thesis have been:

1. To study of the functional consequences of both mutations on $Ca_V2.1$ channel activation, inactivation, and deactivation using two different functional $Ca_V\beta$ subunits (i.e. β_3 and β_{2a}).
2. To analyze the effect of Y1245C mutation on $Ca_V2.1$ channel modulation by $G\beta\gamma$ subunits and the relevance of such effect under physiological stimuli.
3. To examine the effect of A454T mutation on the functional interaction among $Ca_V2.1$ channels and SNARE proteins and its physiological impact on exocytosis.

III.RESULTS

1. ARTICLE A:

The hemiplegic migraine-associated Y1245C mutation in CACNA1A results in a gain of channel function due to its effect on the voltage sensor and G-protein-mediated inhibition

Selma A. Serra, Noèlia Fernàndez-Castillo, Alfons Macaya, Bru Cormand, Miguel A. Valverde, and José M. Fernández-Fernández

Pflugers Arch. 2009 Jul;458(3):489-502.

PubMed ID:19189122

Serra SA, Fernàndez-Castillo N, Macaya A, Cormand B, Valverde MA, Fernández-Fernández JM. The hemiplegic migraine-associated Y1245C mutation in CACNA1A results in a gain of channel function due to its effect on the voltage sensor amd G-protein-mediated inhibition. Pflugers Arch. 2009; 458(3): 489-502.

2. ARTICLE B:

A mutation in the first intracellular loop of CACNA1A prevents P/Q channel modulation by SNARE proteins and lowers exocytosis

Selma A. Serra, Ester Cuenca-León, Artur Llobet, Francisca Rubio-Moscardo, Cristina Plata, Oriel Carreño, Noèlia Fernàndez-Castillo, Roser Corominas, Miguel A. Valverde, Alfons Macaya, Bru Cormand,and José M. Fernández-Fernández

Proc. Natl. Acad. Sci. U S A. 2010 Jan 26;107(4):1672-7

PubMed ID: 20080591

Serra SA, Cuenca-León E, Llobet A, Rubio-Moscardo F, Plata C, Carreño O, et al. A mutation in the first intracellular loop of CACNA1A prevents P/Q channel modulation by SNARE proteins and lowers exocytosis. Proc Natl Acad Sci U S A. 2010; 107(4): 1672-7.

IV. DISCUSSION

1. GENOTYPE-PHENOTYPE CORRELATIONS IN FHM1

FHM1 is characterized by striking clinical variability. Age at onset, frequency, duration, and features of attacks may vary from one patient to another, even among affected members of a given family who carry the same mutation in the same gene[1]. This variability suggests that complex interactions between the functional consequences of the mutation and environmental factors or modifying genetic components are involved in shaping FHM1 clinical phenotype. To date, no genes have been proposed as putative candidates in modifying migraine phenotype.

Now, we have reported the CACNA1A A454T mutation in two out of seven family members affected by dominant migraine with aura (MA) whose genetic cause could not be associated to any of the three FHM genes (i.e. *CACNA1A*, *ATP1A2* or *SCN1A*). Interestingly, while A454T carriers only displayed visual symptoms in their aura, the other five MA-affected individuals presented, in addition to visual symptoms, either sensory (i.e. facial and tongue paresthesia) or motor symptoms (i.e. hemiplegia) during their aura phase suggesting that A454T mutation could cause a modification of the aura phenotype.

Some periodic syndromes, including cyclical vomiting, abdominal migraine, and benign paroxysmal vertigo in childhood (BPV) have been recently considered by the International Headache Society (IHS) as childhood periodic syndromes (CPS) that are precursor to migraine in young patients[2]. A fourth CPS, benign paroxysmal torticollis of infancy (BPT), is not included in this classification but is presented in its Appendix[2].

Although the existence of these disorders has been known for decades, their suggested relationship to migraine as well as their aetiology

remains to be elucidated[3]. Recently, two reports have suggested that BPT might be the earliest manifestation of migraine in life, with a possible genetic linkage to *CACNA1A* mutations in a hemiplegic migraine background although functional evidence in order to consider *CACNA1A* alteration as the cause of BPT is lacking in these reports[4,5].

We have now have performed the first functional characterization of a CACNA1A mutation (Y1245C) found in a patient suffering childhood periodic syndromes (BPT and BPV) that evolved into HM which had been previously reported in one of these articles[5]. Our data reveals an overall gain-of-$Ca_V2.1$ channel function due to mutation Y1245C that may explain the observed clinical phenotypes. Our results give support to the validation of BPT as another childhood migraine equivalent that may be caused by *CACNA1A* genetic variation as well.

2. FHM MUTATIONS AFFECT DIFFERENT BIOPHYSICAL ASPECTS OF $Ca_V2.1$ CHANNEL ACTIVITY AND REGULATION

2.1. Localization of FHM1 Mutations on α_{1A} Subunit

FHM1 mutations have been shown to affect conserved amino acids in important functional regions of the voltage-gated calcium channel α_{1A} subunit, mainly around the line pore (S5-P-S6 segments), in the loops connecting S3–S4 or S4–S5 segments, the S5 and S6 segments, and in the S4 segment of the voltage sensors of its four transmembrane domains[6] (see Figure 17, modified from[6]).

Interestingly, we have described the first mutation associated to HM reported in any S1 segment of α_{1A} subunit: Y1245C. Besides, Y1245

residue is highly conserved in evolution at the interspecies level as well as among the Ca_V2 family of voltage-gated human Ca^{2+} channels, indicating that amino acid Y1245 has an important functional and/or structural role in the gating properties of Ca_V2 channels.

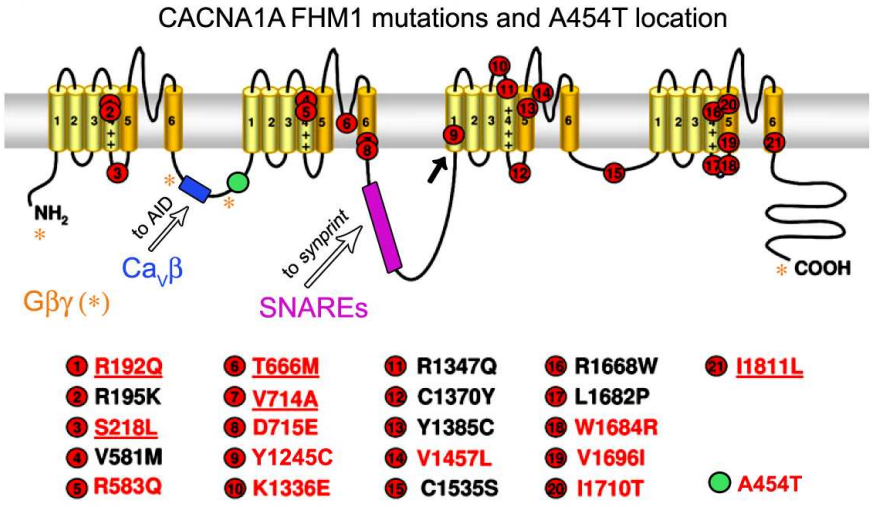

Figure 17. CACNA1A FHM1 mutations and A454T location in the secondary structure of CACNA1A subunit (Genbank Ac. nr. X99897). In red, mutations of which the functional consequences have been analysed so far. A green round shows the location of A454T mutation within α_{1A} I-II loop. Depicted in blue and lilac, AID and *synprint* motifs, respectively. Gβγ binding sites are indicated with orange asterisks.

Similar to Y1245C, the interesting location of A454T in the I-II loop of α_{1A} as well as the high evolutionary conservation of amino acid A454 points to the importance of this residue in the biophysical properties of Ca_V2 channels, notably in those controlled by regulatory proteins binding to the intracellular segments of the channel. Significantly, its location fits well with a non-causative role for A454T mutation in migraine pathology.

2.2. Biophysical Consequences of FHM1 Mutations on $Ca_V2.1$ Channel Activation and Channel Expression

An important consistent effect of all the FHM1 mutations investigated so far and confirmed by the two available KI mouse models is to produce a hyperpolarizing shift in the potential for half-maximal activation ($V_{1/2\ act}$) of $Ca_V2.1$ channels, resulting in an increased channel activity[6]. The functional effects of FHM1 mutations on $Ca_V2.1$ channel activation are consistent with the implication of S4 segments in voltage-sensing and the driving of conformational alterations that open the channel's gate, and that the S4–S5 linker, the S5–S6 segments, and the P-loop play an important role in the gate conformation and/or the coupling between voltage-sensing and channel-gating[7].

We found that mutation Y1245C also causes a negative shift of $V_{1/2\ act}$ by ~9 mV. Additionally, we observed accelerated activation kinetics and slowed deactivation kinetics of Y1245C mutant channels within a wide range of depolarizations, altogether rendering an overall gain-of-function due to left-shifted faster opening and higher accumulation in the open state of mutant compared to WT channels.

Previous functional analysis in heterologous expression systems has shown that some FHM1 mutations also alter the density of functional channels at the membrane. However, the recent analysis of $Ca_V2.1$ channels in neurons of the KI mouse expressing R192Q and S518L FHM1 mutations suggests that such alterations may be an artefact caused by overexpression[8-10]. Under our experimental conditions current densities resulting from heterologous expression of mutant Y1245C $Ca_V2.1$ channels were comparable to those of WT channels. Nevertheless, we can not rule out the possibility that Y1245C mutation

increases functional channel expression at the plasma membrane in endogenous systems as this has been the case for R192Q and S518L mutant channel current densities when analyzed at their endogenous levels in KI mouse neurons[8,10] compared to transient expression in $Ca_V2.1^{-/-}$ mice neurons[11,12].

In agreement with a non-causative role for in migraine pathology, A454T mutation has no effects on $Ca_V2.1$ current density, voltage dependence of activation or activation and deactivation kinetics.

2.2.1. Functional Effects of FHM1 Mutations on the Modulation of $Ca_V2.1$ Channel Gating by G Proteins

One of the major inhibitory pathway controlling voltage-gated Ca^{2+} channels at the presynaptic level is mediated by G protein-coupled receptor activation[13]. As previously reported for R192Q and S218L FHM1 mutations, Y1245C also reduces G protein-mediated inhibition of $Ca_V2.1$ channels. The conclusion that another common effect of FHM1 mutations, in addition to the shift to lower voltages of channel activation, is a reduction of inhibitory modulation by G proteins, may have important implications for migraine pathogenesis.

However, an apparent controversy concerning the mechanism by which R192Q and S218L FHM1 mutations reduce G protein inhibition of P/Q channels arises from these two previous reports. Initially, it was proposed that R192Q reduced the magnitude of G protein inhibition by altering the molecular mechanisms by which $G\beta\gamma$ dimers interaction with the channel affect voltage-sensor movement in response to changes in membrane potential but without affecting the rates of binding (facilitation decay) and unbinding (facilitation development) of $G\beta\gamma$

dimmers to α_{1A} subunit[14]. Conversely, a more detailed study has shown that in fact both R192Q and S218L FHM1 mutations promote accelerated Gβγ dissociation from the activated channel in a range of low depolarizing voltages (−10 to +30 mV for S218L, and −10 to 0 mV for R192Q) without affecting the maximal G protein inhibition of P/Q channels measured at the start of the depolarization[15]. Such discrepancies seem to be due to the time point where the extent of G protein inhibition was measured.

This inhibition is recognized by strong ionic current inhibition derived from the binding of Gβγ to the channel in its closed conformation (ON effect). The process of channel activation produces deinhibition, which is characterized by slowed current kinetics and a more or less pronounced extent of current recovery (OFF effects) depending on the electric gradient promoted by the applied potential. Hence, two important factors have to be taken into account: 1) the time constant of G protein dissociation follows the voltage dependence of channel opening, and 2) the level of inhibition measured at the peak of current amplitude after depolarization include some fraction of current that has already been recovered.

Therefore, to avoid the second factor, the extent of G-protein inhibition has to be measured at the start of the depolarization. The reduction in Gβγ-mediated inhibition and facilitation of R192Q channel mutants in the first report was measured at 25 ms from the beginning of the test depolarizing pulses, time point where significant relief from inhibition may already have occurred giving the impression of reduced G protein inhibition in the R192Q mutant channel if faster Gγβ dissociation occurs[14,15]. Now, we have shown functional consequences on both ON and OFF effects for Y1245C mutation: 1) a decrease in the maximal

voltage-dependent G protein-mediated current inhibition, and 2) accelerated Gβγ dissociation rate at intermediate physiological voltages (+10 mV to +30 mV) by ~1.7 fold. However, as we measured facilitation ratios at 20 ms from the beginning of the test depolarizing pulses, we believe that the accelerated Gβγ dissociation accounts for the decreased levels of G protein inhibition observed in mutant channels, as at that time point some recovery from Gβγ-mediated inhibition has already happened. Besides, we cannot exclude that the lower activation threshold of Y1245C channels could also contribute to accelerate the recovery from G protein inhibition since it has been previously shown that the time constant of G protein dissociation follows the voltage of channel opening[16].

To provide novel insights in disease mechanisms, it appears essential to show that FHM1 mutations reduce G protein modulation of $Ca_V2.1$ channels under physiological stimuli (e.g. during single action potential (AP) and/or during trains of APs). Moreover, the impact on AP-evoked P/Q-type Ca^{2+} current might be different depending on the mechanism by which the mutation reduces the G protein inhibition.

In order to address this issue, we have evaluated G protein modulation in WT and Y1245C mutant channels using two different protocols that mimic physiological stimuli: a) trains of short depolarizing pulses to + 30 mV and + 100 mV, delivered at 71.4 Hz, and b) AP-like waveforms (APW) of different durations representing a wide range of action-potential durations present in neurones[17]. Mutant channels showed nearly absent current facilitation measured at the end of the trains compared to WT, and a significant lower degree of P/Q current inhibition during all APW tested (WT current reduction was ~90% in all APWs tested compared to a 50-74% reduction of Y1245C currents). The observation of lower degree

of current reduction by Gβγ in mutant channels at medium and slow APWs (50%) compared with fast APW (74%) confirms that the lower G protein-mediated inhibition in Y1245C mutant channels may be due to faster dissociation of Gβγ (because with longer stimulus there is more time to produce Gβγ dissociation in Y1245C channels) and suggests that this might be the general pathophysiological mechanism by which FHM1 mutations modify $Ca_V2.1$ channel modulation by G protein signalling pathway.

At the molecular level, it is complicated to speculate about the mechanism by which the introduction of a cystein residue near the inner mouth of IIIS1 may affect the channel's gate conformation and/or the coupling between voltage-sensing and channel-gating along with the regulation of both processes by Gβγ subunits.

It is well-known that voltage dependence is conferred by the voltage sensor region that has been identified in the four transmembrane segments (S1–S4). Extensive structure-function studies, structure determination, and molecular modelling are now converging on a "sliding-helix mechanism" for electromechanical coupling in which outward movement of positive gating charges in the S4 transmembrane segments catalyzed by sequential formation of ion pairs with negatively charged amino acid residues in the neighbouring S1, S2, and/or S3 segments pulls the S4-S5 linker, bends the S6 segment, and opens the pore[18]. It has been demonstrated that two isoleucine residues in S1 and S2 segments positioned close to the external channel mouth of Shaker K channel constitute a hydrophobic plug encountered by the first most extracellular gating charge in S4 in the channel's closed conformation[19]. These results suggest that other hydrophobic residues in S1 segments may constrain gating charges of the S4 segment during its inward and

outward movement in response to changes in membrane potential. We hypothesize that the introduction of a cystein at position 1245 alters one structural constraint resulting in a more energetically favourable movement of the gating charges across the transmembrane environment. Therefore, less depolarization would be needed to open the mutant channel. As mentioned before, we cannot discard that the two major effects of Y1245C (lower voltage threshold of channel activation and increased Gβγ dissociation rate) may be explained by the better coupling of voltage-sensing to mutant channel opening itself since it has been previously shown that the time constant of G protein dissociation follows the voltage of channel opening[16].

Interestingly, we have been able to revert both the negative shift on the voltage dependence of activation and the reduction in G protein inhibition of mutant channels by adding the sulphydryl-reducing agent dithiothreitol (DTT) to the intracellular solution. Our results strongly suggest the formation of spontaneous disulfide bridge between cystein 1245 and another cystein located near the cytosolic region of the voltage-sensor. However, we have neither been able to establish which of the 27 native cysteins in α_{1A} subunit is participating in the formation of the disulfide bridge nor the mechanism by which such new bridge may facilitate S4 movement.

2.3. Biophysical Consequences of FHM1 Mutations on Voltage-dependent $Ca_V2.1$ Channel Inactivation

Voltage-dependent inactivation of Ca^{2+} channels is a key physiological mechanism for regulating intracellular Ca^{2+} levels and neuronal excitability by contributing to short-term depression of neurosecretion[20-

[22]. As outlined in the introduction of this thesis, our current understanding of fast inactivation of Ca^{2+} channels may resemble that of the families of sodium (Na^{+}) and potassium (K^{+}) channels; however, the molecular details governing this biophysical parameter in voltage-dependent Ca^{2+} channels appear to be much more complex and remains less understood.

The fact that most of the FHM1 mutations analysed so far (as well as some CACNA1A mutations associated to ataxia) affect the properties of $Ca_V2.1$ channel inactivation underscore the importance of voltage-dependent inactivation in the aetiology of neurological disorders. However, the reported effects of FHM1 on $Ca_V2.1$ channel inactivation are highly heterogeneous involving both gain- and loss-of-function[6] making difficult to establish the consequences of these effects in a generalized pathophysiological context. In some cases, some hallmarks of the clinical phenotype conferred by a particular FHM1 mutation have been interpreted in terms of the observed effects in voltage-dependent inactivation. Two good paradigms subjected to such interpretation have been T666M and S218L mutations. On the one hand, T666M mutation, reported in FHM1 families with cerebellar ataxia, has little effects on activation but accelerates $Ca_V2.1$ kinetics of fast inactivation, slows kinetics of recovery from inactivation and increases channel accumulation on the inactivated state predicting a decrease of Ca^{2+} channel influx into cerebellar neurons that might explain the T666M-associated ataxic phenotype[12,23,24]. On the other hand, S218L mutation, that produces a severe clinical phenotype with delayed cerebral oedema and coma after minor head trauma, causes the largest shift to lower voltages of $Ca_V2.1$ channel activation showing significant current densities at voltages close to the resting potential of many neurons[11]. In

addition, S218L has unique effects on the kinetics of channel inactivation because it introduces a large component of current that inactivates very slowly, and increases the rate of recovery from inactivation[11]. The particular combination of low threshold of activation and slow inactivation may lie beneath the singularly large facilitation of both induction and propagation of cortical spreading depression (CSD) reported in S218L KI mice[8].

Regarding the molecular determinants, the localization of several FHM1 affecting inactivation has put some light on the subject implicating pore-forming S5, the connecting pore loops and especially S6 segments in determining the overall voltage-dependence of Ca^{2+} channel fast inactivation. The implication of S6 segments is further supported by chimeric and mutant channel studies[25,26].

In contrast to the majority of FHM1 causing mutations, Y1245C has neither effect on the kinetics of fast $Ca_V2.1$ channel inactivation nor on the recovery from fast inactivation suggesting that S1 segments may not be implicated in the control of this biophysical process. Likewise Y1245C, we have shown that A454T mutation has no effects on fast inactivation kinetics. This is a particularly striking finding since A454T mutation is located in the intracellular I-II loop of α_{1A} which has been extensively reported to have a fundamental role in $Ca_V2.1$ fast inactivation process[20]. In addition, it has been shown that small deletion of a potential calmodulin binding site in $Ca_V1.2$ channels (Δ520-532) which includes the equivalent conserved alanine residue of $Ca_V2.1$ A454T mutation (i.e. A521), alters fast inactivation kinetics when $Ca_V1.2$ channels co-expressed with $Ca_V\beta_{1a}$ and $Ca_V\beta_3$ but not β_{2a}[27]. Although the contribution of the deleted region to Ca^{2+}-dependent inactivation mediated by calmodulin via binding to this region subunit was ruled out[28], this region

was shown to be a potential binding site for SH3 module of $Ca_V\beta$ subunits in the control of fast inactivation kinetics[27]. These results further underscore the already known role of the I-II loop as an important molecular determinant of Ca^{2+} channel inactivation and suggest the existence of an AID-independent $Ca_V\beta$ binding site in the I-II loop that may account for some $Ca_V\beta$-mediated modulation of Ca^{2+} channel gating[27]. However, further confirmation of the functional role of $Ca_V\beta$-SH3 binding to the corresponding region in $Ca_V2.1$ channels is needed.

As mentioned above, our results demonstrate that structural alteration mediated by A454T mutation in this region do not affect the modulation of $Ca_V2.1$ fast inactivation kinetics mediated by two different $Ca_V\beta$ subunits (i.e. β_3 and β_{2a}).

Conversely, both Y1245C and A454T mutations affect steady-state voltage dependence of slow $Ca_V2.1$ channel inactivation. Evidence for slow inactivation in Ca^{2+} channels comes from the slow phase in channel recovery kinetics after sustained membrane depolarization. Therefore, the functional relevance of changes in steady-sate slow inactivation may build-up during and after high-frequency neuronal firing, such as those occurring during CSD in migraine patients[29].

It has been shown that the conformationally distinct Ca^{2+} channel populations (closed/resting, open and inactivated) are closely interrelated[26]. In particular, there is evidence that voltage-dependent fast inactivation of Ca^{2+} channels also occurs from the resting state[30] and that slow inactivation in Ca^{2+} channels occurs not only after entry into the fast inactivated state but also to a significant extent directly from the open state[31]. Thus, although fast and slow inactivated states appear to be connected, the link needs not to be a direct one. In fact, these two processes can be distinguished by large differences in speed of onset and

recovery (hundreds of milliseconds vs. tens of seconds or minutes), the rate of development with regard to membrane depolarization, temperature dependence and the finding that SNARE proteins can selectively act on a specific aspect of inactivation (i.e. slow inactivation)[26,32]. Moreover, previous reports analyzing the functional consequences of FHM1 mutations (and our two reports) show that these two parameters can change independently[33].

Table 4 summarizes the main points of contrast between fast and slow inactivation on $Ca_V2.2$ channels which might be quite similar in $Ca_V2.1$ channels.

Although slow inactivation is a nearly universal feature of voltage-gated Ca^{2+} channels and presented also in voltage-gated Na^+ and K^+ channels, not much is known for sure about its molecular nature or the relationship between fast and slow inactivated states.

Table 4. Contrasting properties of fast and slow inactivation of Ca_V2 channels

Fast inactivation	Slow inactivation
Rapidly developing, rapidly recovering	Slowly developing, slowly recovering
Less negative voltage midpoint	More negative voltage midpoint
Rate voltage-dependent over wide range of membrane potentials	Rate voltage-independent beyond levels where fast inactivation is saturated
Temperature-dependent	Onset not intrinsically temperature-dependent; recovery strongly temperature-dependent
Onset accelerated by repetitive pulsing	Onset accelerated by repetitive pulsing
Unaffected by syntaxin	Strongly promoted by syntaxin

Slow inactivation of N-type Ca^{2+} channels is accompanied by a striking increase in affinity for externally acting ω-conotoxins[34] and in P/Q-type channels it is also affected by some FHM1 mutations[33,35] suggesting that it might be influenced by changes in the external pore and voltage sensor residues.

Regarding Y1245C mutation effects on voltage-dependent steady-sate inactivation we observed a left shift to more hyperpolarized voltages (~15 mV) accompanied by a significant increase in steepness suggesting a direct effect of the mutation on the voltage sensor in the channel transition to slow inactivated state. To our knowledge, this is the first evidence suggesting the participation of S1 segments in Ca^{2+} channel slow inactivation.

There is also evidence of $Ca_V\beta$-specific modulation of slow inactivation in $Ca_V2.1$ channels. All $Ca_V\beta$ subunits, except the splice variant $Ca_V\beta_{2a}$ shift voltage dependence of steady-state inactivation towards more negative potentials[36]. Tissue-specific expression patterns of different $Ca_V\beta$ subunits or changes in the subunit assembly during development or disease states appear, therefore, as significant determinants of slow inactivation in $Ca_V2.1$ channels and could account for the complex genotype-phenotype relationship observed in neuronal Ca^{2+} channelopathies. For instance, one report analyzing the functional consequences of 4 FHM1 mutations showed that upon mutant channel-$Ca_V\beta_4$ co-expression (predominantly expressed in cerebellar neurons[37]), just the mutation associated to permanent cerebellar shifted voltage-dependent steady-state inactivation towards more negative potentials suggesting a decreased channel availability in cerebellar neurons which may result in the observed clinical phenotype[33].

Our functional characterization of A454T mutation has also shown $Ca_V\beta$-dependent effects on voltage-dependent steady-state inactivation which may result on either increased or decreased channel availability upon mutant channel co-expression with β_3 or β_{2a}, respectively; however, at present it is difficult to establish a correlation between these changes and the observed clinical phenotype in A454T carriers. On the other

hand, our results suggest that the modulation of slow inactivation by $Ca_V\beta$ subunits might be independent from their binding to the AID motif. Considering that the region around A454 may be a putative binding site for $Ca_V\beta$-SH3 domain[27] we can hypothesize that $Ca_V\beta$ subunits modulate slow inactivation by establishing AID-independent contacts between their SH3 domains and the distal part of the I-II loop. A454T mutation may modify these putative functional interactions by altering the distal structure of the I-II loop, therefore uncoupling the effect of $Ca_V\beta$ subunits on the voltage-dependence of steady-state inactivation and placing the slow inactivation curves closer to the expected value for $Ca_V2.1$ channels lacking regulatory $Ca_V\beta$s[36]. An alternative explanation for the effect of A454T mutation on the regulation of slow channel inactivation by $Ca_V\beta$s may rely in the modification of the intramolecular transduction mechanisms by which $Ca_V\beta$-α_{1A} interaction modulates inactivation due to a structural change of the I-II loop.

2.3.1. Modulation of Slow Inactivation by SNARE Proteins

The role of SNARE proteins as specific physiological modulators of slow inactivation in Ca^{2+} channels has been extensively demonstrated[38-40] and suggest that the II-III loop of $Ca_V\alpha_1$ subunits may be an essential molecular determinant of slow inactivation. Accordingly, natural occurring spliced variants of $Ca_V2.2$ and $Ca_V2.1$ channels lacking the *synprint* site have been found to produce shifts toward more depolarized potentials on the steady-state voltage dependence of slow inactivation *per se*[41,42]. On the other hand, properties of N- or P/Q-type Ca^{2+} channels were not significantly affected by attempts at disrupting their interaction

with syntaxin in rat superior cervical ganglion cells subjected to botulinum toxin type C (BTX C) treatment or injection of *synprint* peptide[43,44], and in calyx terminals at chick ciliary ganglion synapses treated with BoNtC1[45], among others. Possible explanations for these discrepancies can be suggested on the basis of studies in Xenopus oocytes and synaptosomes[32,46]. First, evidence that syntaxin modulation is specific for slow inactivation draws attention to the experimental procedure, which must allow enough time for the slow inactivated state to develop[32]. Second, in the case of recordings of N-type currents in sympathetic neuron cell bodies, it is not clear whether syntaxin was present in sufficient quantity to generate a significant inhibition[44]. Third, in nerve terminal preparations where syntaxin is likely to be abundant, the inhibitory effect might be tonically prevented by interactions with other synaptic proteins[47]. Thus, observing a syntaxin effect might require a significant degree of vesicle turnover[46]. Fourth, some discrepancies might arise from differences in subunit isotypes or splice variants that do not bind syntaxin[48] as opposed to other splice variants[40]. If syntaxin modulation of Ca_V2 channels was re-evaluated in these systems it would be interesting to apply trains of pulses to promote hidden inactivation because this seems to be particularly effective in supporting the SNARE negative effect on Ca_V2 channel slow inactivation[32,49].

We have demonstrated that A454T mutation prevents $Ca_V2.1$ modulation by two SNARE proteins (syntaxin 1A and SNAP-25) using three different approaches. First, we have measured I_{Ca} availability in HEK293 cells (evoked by 20 ms test pulses to +20 mV) derived with series of 30 s prepulses applied from V_h = -80 mV in ascending order (from -100 mV to +10 mV) or just two 30 s prepulses to -80 mV or -20 mV in WT or A454T $Ca_V2.1$ channels-expressing cells in the presence or absence of co-

expressed syntaxin 1A or SNAP-25. Second, we have measured I_{Ca} availability in HEK293 cells transfected with WT $Ca_V2.1$ channels co-expressing either WT or A454T I-II loop in the presence or absence of syntaxin 1A using the two prepulse protocol. Third, we have measured I_{Ca} decay during a 200-Hz train of 2-ms depolarizations from −80 mV to +20 mV in a mouse pheochromocytoma (MPC) cell line expressing endogenous SNARE proteins transfected with WT or A454T channels in the presence or absence of co-expressed BTX C. As previously reported, by using this train of pulses protocol, current remaining at the end of the train shows steady-state inactivation[49].

In HEK cells, the SNARE-induced hyperpolarizing shift on voltage-dependent steady-sate inactivation was prevented in cells expressing either A454T channels or the combination of WT channels and A454T I-II loop. In MPC cells, expression of the neurotoxin BTX C prevented decay of WT but not A454T P/Q currents suggesting that the negative modulation of endogenous SNARE proteins was present in WT channels but prevented in A454T mutant channels in agreement with the data obtained in HEK293 cells.

However, how can a single point mutation in the I-II loop abolish the modulation of a protein binding to the II-III loop?

At first glance, there are two possibilities: 1) A454T mutation, by altering the I-II loop structure, disrupts the three-dimensional positioning of the intracellular domains of α_{1A} resulting in impaired SNARE binding to the *synprint* site, or 2) A454T mutation leads to the loss of a yet unknown SNARE-stabilizing/interacting motif which might be additional to the already known *synprint* site.

A previous report has shown that the transmembrane region of syntaxin 1A and a short region of within H3 helical cytoplasmic domain of

syntaxin, containing residues A240 and V244, appear critical for $Ca_V2.2$ channel modulation but not for biochemical association to the *synprint* site suggesting that syntaxin and the α_{1B} subunit engage in two kind of interactions: an anchoring interaction via *synprint* site and a modulatory interaction via another site located elsewhere in the channel sequence[50]. The authors propose the I-II loop and the C-terminal regions of α_{1B} subunit as good candidates implicated in the transduction of SNARE functional effect on inactivation[50]. In line with this view, our results clearly show the importance of I-II loop structural integrity to support $Ca_V2.1$ modulation of slow inactivation by SNARE proteins. However, our experiments co-expressing *in trans* WT or A454T I-II loop together with WT channels suggest that A454T effect is not due to a loss of a SNARE-interacting/stabilizing motif in the I-II loop. If that was the case, A454T I-II loop could have not eliminated the modulation of channels containing the WT I-II loop to syntaxin 1A whereas WT I-II loop should have partially prevented SNARE modulation by competing for their binding. Instead, we agree with the possibility suggesting that the modification of I-II loop structure by A454T mutation may spoil the three-dimensional arrangement of CACNA1A intracellular domains, which, in turn, alters the interaction pattern between α_{1A} cytoplasmic domains.

Our hypothesis is in concert with recent reports that view cross-talks between cytosolic regions of α_{1A} as important molecular determinants of voltage-dependent Ca^{2+} channel inactivation[20,51], and supports the idea that regulation of voltage-gated Ca^{2+} channels by SNARE proteins require the integrity of channel domains additional to the *synprint* site[50].

To date, this is the first evidence of a natural occurring mutation affecting one of the most important specialized regulations of presynaptic $Ca_V2.1$ channels.

▪ Consequences of an Impaired Coupling between $Ca_V2.1$ Channels and the Exocytotic Machinery

Ca_V2 channel/SNARE interaction not only modulates channel activity itself but it is also thought to drive plasma membrane vesicle docking near the Ca^{2+} entry pathway, optimizing neurotransmitter release[38,49,52].

It has been broadly demonstrated that the disruption of SNARE/$Ca_V2\alpha_1$ interactions using *synprint* peptide competition or *synprint* deletion strategies inhibits release both in neurons and in phechromocytoma cells suggesting that this site is required for coupling Ca^{2+} ion influx to rapid exocytosis[44,53-55]. On the other hand, it has been shown that expression of a mutant syntaxin 1A (i.e. A240V, A244V), which reduces Ca^{2+} channel modulation but not binding *in vitro*, increases synaptic strength at *Xenopus* tadpole tail neuromuscular synapses *in vivo* while injection of synprint peptides in the same synapses has the opposite effect[54].

However, as deduced from all these studies, the effect of severing the physical interaction between *synprint* site and SNARE proteins do not totally prevent synaptic transmission. For instance, competing Ca^{2+} channel/SNARE interaction by injection of s*ynprint* peptides (from $Ca_V2.2$) in two independent studies carried out in superior cervical ganglion neurons and frog neuromuscular junction neurons produced a maximal (whilst reversible) transmitter release inhibition of only 42% and 25%, respectively[44,53] although the authors from the latter study by modelling their results proposed that ~70% of the previously linked synaptic vesicles became uncoupled from Ca^{2+} channels due to the action of *synprint* peptides[53]. In a similar way, expression of $Ca_V2.2$ channels with a partial deletion in their *synprint* site (that presumably eliminates SNAP-25 interaction with α_{1B} subunit) in MPC cells produced and average of

~50% reduction in exocytosis when the cells were stimulated with trains of five depolarizing voltage steps to +20 mV (duration 200 ms, interpulse 50 ms)[55]. In this report, a 45% reduction in capacitance produced by the first depolarization was measured in cells expressing the α_{1B} deletion mutant compared to α_{1B} WT supporting the idea that Ca^{2+} channel/SNARE interaction via *synprint* may be required for optimally locate vesicles near Ca^{2+} entry sites[55].

By using the same cellular model, we have shown that MPC cells expressing A454T mutant $Ca_V2.1$ channels display a 49% reduction in secretory efficiency following an equivalent train of 5 depolarizing pulses to +20 mV, with a significant ~44% decrease already detected from the first pulse. This first pulse mainly mobilizes those vesicles docked closely to release sites (i.e. vesicles defining the readily releasable pool or RRP) and the subsequent pulses would allow the recruitment of more distant vesicles thanks to Ca^{2+} diffusion. Our results strongly suggest that A454T mutation prevents the interaction between α_{1A} and SNARE proteins thus resulting in synaptic vesicle mislocalization in $Ca_V2.1$ mutant channel-expressing cells. If A454T $Ca_V2.1$ mutant channels were still able to bind syntaxin 1A or SNAP-25 proteins although having lost their negative modulation we should expect enhanced rather than reduced exocytosis due to higher Ca^{2+} entry in response to repetitive stimulation as deduced from the expression of A240V, A244V mutant synataxin at the neuromuscular synapses of *Xenopus* tadpole[54].

However, it should be noted that the putative positive effects on Ca^{2+} influx consequent to the loss of SNARE modulation in *synprint* injected neurons have never underestimated the negative effects on transmitter release[44,48,54]. On the other hand, one should be extremely cautious about the conclusions drawn from the studies using *synprint* deleted

channels because it has been shown that this site might be necessary for both the localization and functional coupling of exogenous Ca^{2+} channels at sites of neurotransmitter release[56]. Therefore, the *synprint* deleted channel-dependent reduction in transmitter release could be in part attributed to a reduction in Ca^{2+} influx due to the loss of functional channel expression. In fact, the above mentioned study in MPC cells showed an apparent 50% reduction in *synprint* deleted channel current density (pA/pF) although these differences were strikingly lost after their Ca^{2+} influx normalization[55].

In this regard, due to the non-linear relationship between a Ca^{2+} concentration and release[57], small differences in Ca^{2+} charge could translate into large reductions in exocytosis. In our case, despite the absence of differences in both P/Q currents and Ca^{2+} charge density (calculated by integrating the area under the current traces and normalizing by cell size) between $Ca_V2.1$ WT and A454T channels, MPC cells transfected with mutant channels showed significant lower secretory efficiency at intermediate Ca^{2+} buffering conditions.

The incomplete action of the *synprint* to achieve complete block of synaptic transmission has been attributed the limited peptide concentration that can be introduced in the cell body or the barriers to diffusion of the peptides (i.e. the extensive network of varicosities) to all presynaptic active zones[44]. Alternatively, the effect of moving vesicles away from local channels upon *synprint* injection would reach a maximum after which high density of surrounding Ca^{2+} channels in the active zones may be high enough to exceed the threshold of release [53].

In this respect, increasing the $[Ca^{2+}]_e$ up to 10 mM rescues the decrease in transmitter release observed in *synprint* injected frog neuromuscular neurons at physiological Ca^{2+} concentration (1.8 mM)[53]. Analogously, by

varying the intracellular Ca^{2+} buffering by adding different EGTA concentrations in the pipette solution, we have shown that A454T mutation shifts the Ca^{2+} dependence of the exocytotic process to higher values (see Figure 18).

Also relevant to the argument about the importance of the SNARE-channel interaction on secretion is the fact that vesicle recruitment near voltage-gated Ca^{2+} channels during repetitive or long-lasting depolarizations also involves SNARE proteins[58], although the underlying molecular mechanism is currently unknown. According to our data we propose that SNARE-channel interaction may participate in the recruitment of these vesicles, thereby explaining why A454T mutation, through an alteration of Ca^{2+} channel-SNARE interaction, affects not only the coupling between $Ca_V2.1$ channels and fusion-competent vesicles from the RRP but also the recruitment of new vesicles in response to consecutive pulses.

We cannot discard the possibility, however, that A454T mutation somehow alters the sensitivity of the Ca^{2+} sensor of release without affecting vesicle localization as we have not unequivocally demonstrated the existence of vesicle uncoupling from mutant channels in MPC cells. This alternative hypothesis could explain the apparent lower sensitivity to the secretory response of A454T compared to WT channel-expressing cells when increasing EGTA from 1 mM to 5 mM. Such an excess in Ca^{2+} buffering conditions would rather impair the fusion of those vesicles not linked to Ca^{2+} channels and, hence, we should expect a larger relative reduction of secretion in A454T channel-expressing cells in response to this manoeuvre.

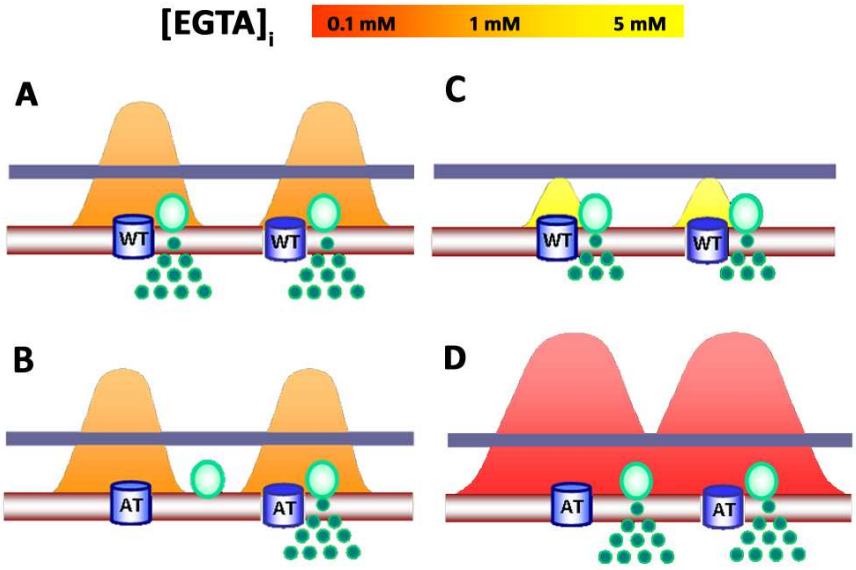

Figure 18. Hypothetical schematic comparision of different buffering conditions in WT vs. A454T channel-expressing cells during exocytosis. Under near physiological concentrations of intracellular Ca^{2+} buffering (1 mM EGTA), if indeed almost all vesicles are linked to WT channels, only minimal Ca^{2+} domain is needed to reach a minimum threshold for release (A). The short distance between the Ca^{2+} source and the Ca^{2+} sensor of release (supported by biochemical data that identified the Ca^{2+} binding protein synaptotagmin as an integral member of the SNARE complex[59]) would give the Ca^{2+} buffer little time to remove the high Ca^{2+} at the inner mouth of the channel and consequently increase the probability of transmitter release. Conversely, as not all mutant channels would be coupled to docked vesicles secretory efficiency would be significantly reduced under the same conditions of Ca^{2+} buffering (B). Addition of an excess of exogenous Ca^{2+} buffer to the cytoplasm rapidly restricts Ca^{2+} signals and impairs vesicle recruitment to the release-ready state[60]. In consequence, dialysis with 5 mM EGTA strongly reduces the secretion evoked by a train of stimuli in both α_{1A} A454T and WT-expressing cells. In this case, the drastic restriction of Ca^{2+} domains in WT channel-expressing cells likely mimics the effect induced by A454T mutation in the 1 mM EGTA condition (C). In contrast, the secretion of A454T channels-expressing cells can be rescued to WT values by decreasing intracellular EGTA concentration down to 0.1 mM (D).

3. RELEVANCE OF Y1245C AND A454T MUTATIONS IN MIGRAINE PATHOPHYSIOLOGY

As discussed in the introduction of this PhD thesis, cortical spreading depression (CSD), the physiological phenomenon underlying the aura symptoms, might activate the trigeminovascular system (TGVS) involved in the initiation and maintenance of cephalic pain giving rise to a migraine attack[29]. Besides, brainstem dysfunction might also participate in maintaining the "hyperexcitable brain status" of migraineurs during the interictal periods and in the mechanism of central sensitization occurring in the attacks[61].

Analogously to the other FHM1 mutations analysed so far and in concert with FHM1 animal models, Y1245C functional consequences fit well with the causative role of the mutation. The two main findings with Y1245C $Ca_V2.1$ channels expressed in HEK293 cells (i.e. left shift in the current–voltage activation curve and reduced G protein inhibition) suggest that presynaptic Y1245C $Ca_V2.1$ channels would have a lower threshold for initiating excitatory transmitter release which, in turn, may exhibit a diminished feedback regulation by G protein signalling pathway. Our data gain relevance by the fact that Y1245C $Ca_V2.1$ channel gain-of-function due to G protein misregulation is also evident in response to physiological input. Therefore, it seems accurate to propose that CSD would be more likely to occur in the cortex of Y1245C carriers under certain triggers that increase neuronal firing.

It is a shade more difficult to figure out how Y1245C mutation can cause the two childhood periodic syndromes associated to this particular mutation: benign paroxysmal torticollis (BPT) and benign paroxysmal vertigo (BPV) in childhood.

The paroxystic nature of these syndromes, their frequent association to migraine, and the effectiveness of nonanalgesic migraine therapy in CPS, share common physiopathological mechanisms[3]. In this regard, a PET study has shown decrease of glucose metabolism in both cerebellar cortex and basal ganglia, and a decrease of perfusion in the basal and temporal cortex during attacks of idiopathic neck dystonia (i.e. torticollis) in a child, consistent with brainstem metabolic dysfuntion and cortical CSD-like changes, respectively[62]. It has also been demonstrated that children with childhood periodic syndromes exhibited visual-evoked responses similar to those of children with migraine during interictal periods[3].

Although the etiopathogenesis of both paroxysmal syndromes is not well known, some authors have suggested the involvement of the central vestibular regions and vestibule-cerebellar connections[3]. Moreover, although the vestibular cortical system is one of the most complex systems of the human brain, several evidences point to some areas in the parietal cortex in the processing of vestibular information[63].

We hypothesize that during infancy, gain-of-function of $Ca_V2.1$ Y1245C channels would favour CSD triggering more readily in parietal cortical areas leading to CPS syndromes, whereas somatosensory and motor cortices may become more susceptible to CSD leading to HM symptoms in the adulthood; yet, further mechanistic insights into the pathophysiology of CPS are needed in order to support such hypothesis. It is unknown how a given mutation may be responsible of a variety of clinical manifestations at different ages although one possibility points to different spliced isoforms, functions and/or subcellular targeting of P/Q Ca^{2+} channels during brain development[64]. This is particularly important,

as alternative splicing of α_{1A} and its coupling to different $Ca_V\beta$ isoforms affects the functional impact of FHM1 mutations[33,65].

Further environmental and/or genetic factors may shape the phenotypic expression of migraine symptoms by worsening or improving the functional consequences of migraine causative mutations. Interestingly, the clinical spectrum of the family reported in our functional study of A454T suggested a modulatory role of this mutation in the phenotypic expression of aura symptoms. Unfortunately, the genetic cause of migraine in this family could not be ascertained. Even so, whichever mutation is causing migraine in this family it would presumably lead to a lower threshold for CSD initiation and propagation.

The main finding of a robust reduction of secretory efficiency in pheochromocytoma cells expressing either rabbit or human A454T $Ca_V2.1$ channels suggest that neurotransmitter release coupled to presynaptic A454T mutant channels may also be reduced in A454T carriers. Therefore, the presence of these mutant channels in a migraine genetic background (likely to reduce the threshold for CSD initiation) would restrict CSD propagation. It has been shown that the occipital cortex (involved in visual stimuli integration) has the lowest threshold for CSD initiation whereas CSD it is more reluctant to occur in sensorimotor cortices[66]. Accordingly, it seems appealing to speculate that A454T would not prevent the initiation of CSD in the visual cortex but would impede its propagation to sensory and motor cortices.

Nevertheless, in spite of the attenuated aura symptoms in A454T carriers they still suffer from migraine headache. For various reasons, however—including the existence of aura without pain, an inconsistent relationship between the aura and pain lateralization, the occurrence of aura with other primary headaches, and the observation that some treatments

improve aura but do not affect pain[67]—it seems that the aura–pain relationship cannot be explained by a simple cause–effect association. In fact, the precise mechanism that links CSD in the cortical gray matter (causing the aura) to activation of the meningeal nociceptors (causing the headache) is not entirely clear[68]. Recently, it has been shown that in the rat and mouse cortex dilatation of cortical surface arterioles propagated with a significantly greater intrinsic velocity and into areas beyond the spread of parenchymal CSD[69]. Besides, vasomotor activity could be experimentally dissociated from the parenchymal CSD wave[69]. These results suggest a mechanism, intrinsic to the vasculature, for propagation of vasodilatation associated with CSD. Hence, it seems plausible that the putative decreased propagation of CSD in A454T carriers may not be able to avoid trigeminovascular nociceptor activation due to the independent nature of neurovascular conduction of CSD.

The negative effect of A454T mutation on exocytosis depends on the cytosolic Ca^{2+}-buffering conditions. Because presynaptic Ca^{2+} microdomains can be modified by the particular endogenous Ca^{2+} buffers[70] the impact of A454T on neurotransmitter release might vary at different synapses expressing distinct endogenous Ca^{2+} buffers. At present, it is difficult to predict the impact of A454T mutation in the physiology of native P/Q channels expressed in the different brain areas that have been implicated in migraine pathophysiology.

CSD undoubtedly provides a possible target mechanism for the laboratory-based development of new migraine medicines. Currently, the mechanism of action of some preventive and acute migraine therapies is thought to be related, directly or indirectly, to blocking of CSD. For instance, the gap-junction blocker tonabersat, an inducible nitric oxide synthase inhibitor and botulinum toxin A are all being investigated in

clinical trials as preventive therapies[71]. In this respect, our results highlight a novel target for both therapeutic design and the identification of pathological mechanisms and risk factors: the presynaptic complex $Ca_V2.1$channel-SNAREs. Accordingly, we and others have assessed the contribution of the *STX1A* gene (encoding the syntaxin 1A SNARE protein) to common forms of migraine through case-control association studies in two different populations and identified risk variants[72,73] (see Annex).

There is still a long way to go in this area of neurology but important steps have been already been taken. In the future, it is essential to persevere in the development of new animal models for migraine since the first two FHM1 KI mouse models have provided extremely valuable information in the understanding of migraine pathophysiology. Sooner or later, the improvement of brain imaging techniques resolution would surely help to comprehend the intricate nature of the phenomena occurring in the brains of migraineurs between and during migraine attacks. In the meantime, we expect that functional analysis of new mutations associated to migraine may continue to provide interesting novel insights into the research field of voltage-gated Ca^{2+} channels, a good example of which are the two papers presented in this PhD thesis.

V. CONCLUSIONS

1) The CACNA1A mutation Y1245C is the first to be found in any S1 segment of α_{1A} subunit and is associated to childhood periodic syndromes and hemiplegic migraine. It produces an overall gain of $Ca_V2.1$ channel function mainly due to a reduction in the threshold for channel activation and the inhibition mediated by $G\beta\gamma$ subunits. Both effects manifest under action potential-like stimuli.

2) The changes in the voltage-dependent steady-state inactivation induced by Y1245C mutation suggest an alteration on the voltage sensor region although the pathophysiological relevance of this effect in respect to other alterations induced by this mutation remains to be elucidated.

3) The formation of a new disulfide bridge between cysteines may contribute to the effects of Y1245C on activation and $G\beta\gamma$-mediated inhibition of $Ca_V2.1$ channel, as they were significantly reverted by the sulphydryl-reducing agent dithiothreitol.

4) The reported functional impact of Y1245C mutation on $Ca_V2.1$ channel function is consistent with a pathogenic role of the mutation and gives further support to the validation of benign paroxysmal torticollis of infancy as a migraine precursor caused by genetic alteration in *CACNA1A*.

5) CACNA1A mutation A454T is localized in the I-II loop of α_{1A} and has no effects on the activation, deactivation and fast inactivation properties of $Ca_V2.1$ channels in agreement with the non-causative role of this mutation in migraine, although is associated to the attenuation of aura symptoms.

6) The functional consequences of A454T mutation in the voltage dependence of $Ca_V2.1$ steady-state inactivation are $Ca_V\beta$-dependent and their physiological relevance would mainly build up during and after the high-frequency neuronal firing that may occur in migraine attacks triggered by cortical spreading depression.

7) The loss of functional interaction between t-SNARE proteins and A454T $Ca_V2.1$ channels impairs channel coupling to vesicle release due to a shift of secretion efficiency to higher Ca^{2+} levels. This result highlights the relevance of the I-II loop as an integrator of channel regulatory mechanisms that also include SNARE protein binding and modulation of $Ca_V2.1$ channels during transmitter release.

8) The negative impact of A454T mutation on $Ca_V2.1$ channel function may result in decreased propagation of cortical spreading depression thus explaining the absence of sensorimotor auras in A454T carriers.

9) Based on the clinical and functional data regarding A454T mutation, we propose *CACNA1A* not only as a migraine disease-causing gene but also as a migraine phenotype modifier gene as well.

VI.ANNEX

1. ARTICLE C:

Contribution of syntaxin 1A to the genetic susceptibility to migraine: a case-control association study in the Spanish population

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VII. REFERENCES

1. Berridge, M. J., Bootman, M. D. & Roderick, H. L. Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* **4**, 517-529 (2003).

2. Berridge, M. J., Lipp, P. & Bootman, M. D. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **1**, 11-21 (2000).

3. Hille, B. *Ion Channels of Excitable Membranes*. (Sinauer Associates Inc., Sunderland, Massachusetts,2001).

4. Catterall, W. A. Structure and regulation of voltage-gated Ca2+ channels. *Annu. Rev. Cell Dev. Biol.* **16**, 521-555 (2000).

5. Tsien, R. W. & Barrett, C. F. *Voltage-gated Calcium Channels*. Zamponi, G. W. (ed.), pp. 27-47 (Kluwer Academic/Plenum,2011).

6. Yu, F. H. & Catterall, W. A. The VGL-chanome: a protein superfamily specialized for electrical signaling and ionic homeostasis. *Sci. STKE.* **2004**, re15 (2004).

7. Hille, B. *Ion Channels of Excitable Membranes*. Sinauer Associates Inc., Sunderland, Massachusetts (2001).

8. Dolphin, A. C. A short history of voltage-gated calcium channels. *Br. J. Pharmacol.* **147 Suppl 1**, S56-S62 (2006).

9. FATT, P. & KATZ, B. The electrical properties of crustacean muscle fibres. *J. Physiol* **120**, 171-204 (1953).

10. FATT, P. & GINSBORG, B. L. The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol* **142**, 516-543 (1958).

11. Hagiwara, S. & Nakajima, S. Differences in Na and Ca spikes as examined by application of tetrodotoxin, procaine, and manganese ions. *J. Gen. Physiol* **49**, 793-806 (1966).

12. Hagiwara, S., Ozawa, S. & Sand, O. Voltage clamp analysis of two inward current mechanisms in the egg cell membrane of a starfish. *J. Gen. Physiol* **65**, 617-644 (1975).

13. Reuter, H. The dependence of slow inward current in Purkinje fibres on the extracellular calcium-concentration. *J. Physiol* **192**, 479-492 (1967).

14. Fleckenstein, A. History of calcium antagonists. *Circ. Res.* **52**, I3-16 (1983).

15. Tsien, R. W., Lipscombe, D., Madison, D. V., Bley, K. R. & Fox, A. P. Multiple types of neuronal calcium channels and their selective modulation. *Trends Neurosci.* **11**, 431-438 (1988).

16. Reuter, H. Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* **301**, 569-574 (1983).

17. Nowycky, M. C., Fox, A. P. & Tsien, R. W. Long-opening mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. *Proc. Natl. Acad. Sci. U. S. A* **82**, 2178-2182 (1985).

18. Carbone, E. & Lux, H. D. A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. *Nature* **310**, 501-502 (1984).

19. Nowycky, M. C., Fox, A. P. & Tsien, R. W. Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* **316**, 440-443 (1985).

20. Nilius, B., Hess, P., Lansman, J. B. & Tsien, R. W. A novel type of cardiac calcium channel in ventricular cells. *Nature* **316**, 443-446 (1985).

21. McCleskey, E. W. *et al.* Omega-conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proc. Natl. Acad. Sci. U. S. A* **84**, 4327-4331 (1987).

22. Llinas, R. R., Sugimori, M. & Cherksey, B. Voltage-dependent calcium conductances in mammalian neurons. The P channel. *Ann. N. Y. Acad. Sci.* **560**, 103-111 (1989).

23. Mintz, I. M. *et al.* P-type calcium channels blocked by the spider toxin omega-Aga-IVA. *Nature* **355**, 827-829 (1992).

24. Randall, A. & Tsien, R. W. Pharmacological dissection of multiple types of Ca2+ channel currents in rat cerebellar granule neurons. *J. Neurosci.* **15**, 2995-3012 (1995).

25. Mori, Y. *et al.* Primary structure and functional expression from complementary DNA of a brain calcium channel. *Nature* **350**, 398-402 (1991).

26. Starr, T. V., Prystay, W. & Snutch, T. P. Primary structure of a calcium channel that is highly expressed in the rat cerebellum. *Proc. Natl. Acad. Sci. U. S. A* **88**, 5621-5625 (1991).

27. Bourinet, E. *et al.* Splicing of alpha 1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. *Nat. Neurosci.* **2**, 407-415 (1999).

28. Takahashi, M., Seagar, M. J., Jones, J. F., Reber, B. F. & Catterall, W. A. Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A* **84**, 5478-5482 (1987).

29. Catterall, W. A. & Few, A. P. Calcium channel regulation and presynaptic plasticity. *Neuron* **59**, 882-901 (2008).

30. Perez-Reyes, E. Molecular characterization of T-type calcium channels. *Cell Calcium* **40**, 89-96 (2006).

31. Arikkath, J. & Campbell, K. P. Auxiliary subunits: essential components of the voltage-gated calcium channel complex. *Curr. Opin. Neurobiol.* **13**, 298-307 (2003).

32. Catterall, W. A., Perez-Reyes, E., Snutch, T. P. & Striessnig, J. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol. Rev.* **57**, 411-425 (2005).

33. Ertel, E. A. *et al.* Nomenclature of voltage-gated calcium channels. *Neuron* **25**, 533-535 (2000).

34. Dolphin, A. C. G protein modulation of voltage-gated calcium channels. *Pharmacol. Rev.* **55**, 607-627 (2003).

35. Davies, A. *et al.* Functional biology of the alpha(2)delta subunits of voltage-gated calcium channels. *Trends Pharmacol. Sci.* **28**, 220-228 (2007).

36. Felix, R., Gurnett, C. A., De Waard M. & Campbell, K. P. Dissection of functional domains of the voltage-dependent Ca2+ channel alpha2delta subunit. *J. Neurosci.* **17**, 6884-6891 (1997).

37. Davies, A. *et al.* The alpha2delta subunits of voltage-gated calcium channels form GPI-anchored proteins, a posttranslational modification essential for function. *Proc. Natl. Acad. Sci. U. S. A* **107**, 1654-1659 (2010).

38. Bauer, C. S., Tran-Van-Minh, A., Kadurin, I. & Dolphin, A. C. A new look at calcium channel alpha2delta subunits. *Curr. Opin. Neurobiol.* **20**, 563-571 (2010).

39. Gurnett, C. A., Felix, R. & Campbell, K. P. Extracellular interaction of the voltage-dependent Ca2+ channel alpha2delta and alpha1 subunits. *J. Biol. Chem.* **272**, 18508-18512 (1997).

40. Canti, C. *et al.* The metal-ion-dependent adhesion site in the Von Willebrand factor-A domain of alpha2delta subunits is key to trafficking voltage-gated Ca2+ channels. *Proc. Natl. Acad. Sci. U. S. A* **102**, 11230-11235 (2005).

41. Barclay, J. *et al.* Ducky mouse phenotype of epilepsy and ataxia is associated with mutations in the Cacna2d2 gene and decreased calcium channel current in cerebellar Purkinje cells. *J. Neurosci.* **21**, 6095-6104 (2001).

42. Donato, R. *et al.* The ducky(2J) mutation in Cacna2d2 results in reduced spontaneous Purkinje cell activity and altered gene expression. *J. Neurosci.* **26**, 12576-12586 (2006).

43. Hendrich, J. *et al.* Pharmacological disruption of calcium channel trafficking by the alpha2delta ligand gabapentin. *Proc. Natl. Acad. Sci. U. S. A* **105**, 3628-3633 (2008).

44. Witcher, D. R. *et al.* Subunit identification and reconstitution of the N-type Ca2+ channel complex purified from brain. *Science* **261**, 486-489 (1993).

45. Letts, V. A. *et al.* The mouse stargazer gene encodes a neuronal Ca2+-channel gamma subunit. *Nat. Genet.* **19**, 340-347 (1998).

46. Kang, M. G. & Campbell, K. P. Gamma subunit of voltage-activated calcium channels. *J. Biol. Chem.* **278**, 21315-21318 (2003).

47. Chu, P. J., Robertson, H. M. & Best, P. M. Calcium channel gamma subunits provide insights into the evolution of this gene family. *Gene* **280**, 37-48 (2001).

48. Chen, L. *et al.* Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* **408**, 936-943 (2000).

49. Kang, M. G. *et al.* Biochemical and biophysical evidence for gamma 2 subunit association with neuronal voltage-activated Ca2+ channels. *J. Biol. Chem.* **276**, 32917-32924 (2001).

50. Zhang, Y., Mori, M., Burgess, D. L. & Noebels, J. L. Mutations in high-voltage-activated calcium channel genes stimulate low-voltage-activated currents in mouse thalamic relay neurons. *J. Neurosci.* **22**, 6362-6371 (2002).

51. Ferron, L. *et al.* The stargazin-related protein gamma 7 interacts with the mRNA-binding protein heterogeneous nuclear ribonucleoprotein A2 and regulates the stability of specific mRNAs, including CaV2.2. *J. Neurosci.* **28**, 10604-10617 (2008).

52. Birnbaumer, L. *et al.* Structures and functions of calcium channel beta subunits. *J. Bioenerg. Biomembr.* **30**, 357-375 (1998).

53. Dolphin, A. C. Beta subunits of voltage-gated calcium channels. *J. Bioenerg. Biomembr.* **35**, 599-620 (2003).

54. Richards, M. W., Butcher, A. J. & Dolphin, A. C. Ca2+ channel beta-subunits: structural insights AID our understanding. *Trends Pharmacol. Sci.* **25**, 626-632 (2004).

55. Brice, N. L. *et al.* Importance of the different beta subunits in the membrane expression of the alpha1A and alpha2 calcium channel subunits: studies using a depolarization-sensitive alpha1A antibody. *Eur. J. Neurosci.* **9**, 749-759 (1997).

56. Bichet, D. *et al.* The I-II loop of the Ca2+ channel alpha1 subunit contains an endoplasmic reticulum retention signal antagonized by the beta subunit. *Neuron* **25**, 177-190 (2000).

57. Olcese, R. *et al.* Coupling between charge movement and pore opening in vertebrate neuronal alpha 1E calcium channels. *J. Physiol* **497 (Pt 3)**, 675-686 (1996).

58. Neely, A., Wei, X., Olcese, R., Birnbaumer, L. & Stefani, E. Potentiation by the beta subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science* **262**, 575-578 (1993).

59. Neely, A., Garcia-Olivares, J., Voswinkel, S., Horstkott, H. & Hidalgo, P. Folding of active calcium channel beta(1b) -subunit by size-exclusion chromatography and its role on channel function. *J. Biol. Chem.* **279**, 21689-21694 (2004).

60. Canti, C. *et al.* Evidence for two concentration-dependent processes for beta-subunit effects on alpha1B calcium channels. *Biophys. J.* **81**, 1439-1451 (2001).

61. Olcese, R. *et al.* The amino terminus of a calcium channel beta subunit sets rates of channel inactivation independently of the subunit's effect on activation. *Neuron* **13**, 1433-1438 (1994).

62. Stea, A. *et al.* Localization and functional properties of a rat brain alpha 1A calcium channel reflect similarities to neuronal Q- and P-type channels. *Proc. Natl. Acad. Sci. U. S. A* **91**, 10576-10580 (1994).

63. Sokolov, S., Weiss, R. G., Timin, E. N. & Hering, S. Modulation of slow inactivation in class A Ca2+ channels by beta-subunits. *J. Physiol* **527 Pt 3**, 445-454 (2000).

64. De Waard M. & Campbell, K. P. Subunit regulation of the neuronal alpha 1A Ca2+ channel expressed in Xenopus oocytes. *J. Physiol* **485 (Pt 3)**, 619-634 (1995).

65. Chien, A. J., Carr, K. M., Shirokov, R. E., Rios, E. & Hosey, M. M. Identification of palmitoylation sites within the L-type calcium channel beta2a subunit and effects on channel function. *J. Biol. Chem.* **271**, 26465-26468 (1996).

66. Qin, N. *et al.* Unique regulatory properties of the type 2a Ca2+ channel beta subunit caused by palmitoylation. *Proc. Natl. Acad. Sci. U. S. A* **95**, 4690-4695 (1998).

67. Hanlon, M. R., Berrow, N. S., Dolphin, A. C. & Wallace, B. A. Modelling of a voltage-dependent Ca2+ channel beta subunit as a basis for understanding its functional properties. *FEBS Lett.* **445**, 366-370 (1999).

68. Chen, Y. H. *et al.* Structural basis of the alpha1-beta subunit interaction of voltage-gated Ca2+ channels. *Nature* **429**, 675-680 (2004).

69. Dimitratos, S. D., Woods, D. F., Stathakis, D. G. & Bryant, P. J. Signaling pathways are focused at specialized regions of the plasma membrane by scaffolding proteins of the MAGUK family. *Bioessays* **21**, 912-921 (1999).

70. Rousset, M., Cens, T. & Charnet, P. Alone at last! New functions for Ca2+ channel beta subunits? *Sci. STKE.* **2005**, e11 (2005).

71. Pragnell, M. *et al.* Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha 1-subunit. *Nature* **368**, 67-70 (1994).

72. De Waard M., Pragnell, M. & Campbell, K. P. Ca2+ channel regulation by a conserved beta subunit domain. *Neuron* **13**, 495-503 (1994).

73. De Waard M., Scott, V. E., Pragnell, M. & Campbell, K. P. Identification of critical amino acids involved in alpha1-beta interaction in voltage-dependent Ca2+ channels. *FEBS Lett.* **380**, 272-276 (1996).

74. Van, P. F., Clark, K. A., Chatelain, F. C. & Minor, D. L., Jr. Structure of a complex between a voltage-gated calcium channel beta-subunit and an alpha-subunit domain. *Nature* **429**, 671-675 (2004).

75. Opatowsky, Y., Chen, C. C., Campbell, K. P. & Hirsch, J. A. Structural analysis of the voltage-dependent calcium channel beta subunit functional core and its complex with the alpha 1 interaction domain. *Neuron* **42**, 387-399 (2004).

76. Restituito, S. *et al.* The [beta]2a subunit is a molecular groom for the Ca2+ channel inactivation gate. *J. Neurosci.* **20**, 9046-9052 (2000).

77. Stotz, S. C. *et al.* Several structural domains contribute to the regulation of N-type calcium channel inactivation by the beta 3 subunit. *J. Biol. Chem.* **279**, 3793-3800 (2004).

78. Qin, N. *et al.* Identification of a second region of the beta-subunit involved in regulation of calcium channel inactivation. *Am. J. Physiol* **271**, C1539-C1545 (1996).

79. Walker, D., Bichet, D., Campbell, K. P. & De Waard M. A beta 4 isoform-specific interaction site in the carboxyl-terminal region of the voltage-dependent Ca2+ channel alpha 1A subunit. *J. Biol. Chem.* **273**, 2361-2367 (1998).

80. Walker, D. *et al.* A new beta subtype-specific interaction in alpha1A subunit controls P/Q-type Ca2+ channel activation. *J. Biol. Chem.* **274**, 12383-12390 (1999).

81. Sandoz, G. *et al.* Distinct properties and differential beta subunit regulation of two C-terminal isoforms of the P/Q-type Ca(2+)-channel alpha(1A) subunit. *Eur. J. Neurosci.* **14**, 987-997 (2001).

82. Stephens, G. J., Page, K. M., Bogdanov, Y. & Dolphin, A. C. The alpha1B Ca2+ channel amino terminus contributes determinants for beta subunit-mediated voltage-dependent inactivation properties. *J. Physiol* **525 Pt 2**, 377-390 (2000).

83. He, L. L., Zhang, Y., Chen, Y. H., Yamada, Y. & Yang, J. Functional modularity of the beta-subunit of voltage-gated Ca2+ channels. *Biophys. J.* **93**, 834-845 (2007).

84. Richards, M. W., Leroy, J., Pratt, W. S. & Dolphin, A. C. The HOOK-domain between the SH3 and the GK domains of Cavbeta subunits contains key determinants controlling calcium channel inactivation. *Channels (Austin.)* **1**, 92-101 (2007).

85. Takahashi, S. X., Miriyala, J. & Colecraft, H. M. Membrane-associated guanylate kinase-like properties of beta-subunits required for modulation of voltage-dependent Ca2+ channels. *Proc. Natl. Acad. Sci. U. S. A* **101**, 7193-7198 (2004).

86. McGee, A. W. *et al.* Calcium channel function regulated by the SH3-GK module in beta subunits. *Neuron* **42**, 89-99 (2004).

87. Maltez, J. M., Nunziato, D. A., Kim, J. & Pitt, G. S. Essential Ca(V)beta modulatory properties are AID-independent. *Nat. Struct. Mol. Biol.* **12**, 372-377 (2005).

88. Gonzalez-Gutierrez, G. *et al.* The guanylate kinase domain of the beta-subunit of voltage-gated calcium channels suffices to modulate gating. *Proc. Natl. Acad. Sci. U. S. A* **105**, 14198-14203 (2008).

89. Gonzalez-Gutierrez, G., Miranda-Laferte, E., Neely, A. & Hidalgo, P. The Src homology 3 domain of the beta-subunit of voltage-gated calcium channels promotes endocytosis via dynamin interaction. *J. Biol. Chem.* **282**, 2156-2162 (2007).

90. Beguin, P. *et al.* Regulation of Ca2+ channel expression at the cell surface by the small G-protein kir/Gem. *Nature* **411**, 701-706 (2001).

91. Restituito, S., Cens, T., Rousset, M. & Charnet, P. Ca(2+) channel inactivation heterogeneity reveals physiological unbinding of auxiliary beta subunits. *Biophys. J.* **81**, 89-96 (2001).

92. Bichet, D., Lecomte, C., Sabatier, J. M., Felix, R. & De Waard M. Reversibility of the Ca(2+) channel alpha(1)-beta subunit interaction. *Biochem. Biophys. Res. Commun.* **277**, 729-735 (2000).

93. Hering, S. *et al.* Molecular determinants of inactivation in voltage-gated Ca2+ channels. *J. Physiol* **528 Pt 2**, 237-249 (2000).

94. Stotz, S. C., Jarvis, S. E. & Zamponi, G. W. Functional roles of cytoplasmic loops and pore lining transmembrane helices in the voltage-dependent inactivation of HVA calcium channels. *J. Physiol* **554**, 263-273 (2004).

95. Zhang, J. F., Ellinor, P. T., Aldrich, R. W. & Tsien, R. W. Molecular determinants of voltage-dependent inactivation in calcium channels. *Nature* **372**, 97-100 (1994).

96. Stotz, S. C., Hamid, J., Spaetgens, R. L., Jarvis, S. E. & Zamponi, G. W. Fast inactivation of voltage-dependent calcium channels. A hinged-lid mechanism? *J. Biol. Chem.* **275**, 24575-24582 (2000).

97. Kraus, R. L., Sinnegger, M. J., Glossmann, H., Hering, S. & Striessnig, J. Familial hemiplegic migraine mutations change alpha1A Ca2+ channel kinetics. *J. Biol. Chem.* **273**, 5586-5590 (1998).

98. Kraus, R. L. *et al.* Three new familial hemiplegic migraine mutants affect P/Q-type Ca(2+) channel kinetics. *J. Biol. Chem.* **275**, 9239-9243 (2000).

99. Spaetgens, R. L. & Zamponi, G. W. Multiple structural domains contribute to voltage-dependent inactivation of rat brain alpha(1E) calcium channels. *J. Biol. Chem.* **274**, 22428-22436 (1999).

100. Doyle, D. A. *et al.* The structure of the potassium channel: molecular basis of K+ conduction and selectivity. *Science* **280**, 69-77 (1998).

101. Zamponi, G. W., Bourinet, E., Nelson, D., Nargeot, J. & Snutch, T. P. Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. *Nature* **385**, 442-446 (1997).

102. Cens, T., Restituito, S., Galas, S. & Charnet, P. Voltage and calcium use the same molecular determinants to inactivate calcium channels. *J. Biol. Chem.* **274**, 5483-5490 (1999).

103. Kaneko, S. *et al.* Identification and characterization of novel human Ca(v)2.2 (alpha 1B) calcium channel variants lacking the synaptic protein interaction site. *J. Neurosci.* **22**, 82-92 (2002).

104. Fathallah, M. *et al.* Modelling of the III-IV loop, a domain involved in calcium channel Ca(v)2.1 inactivation, highlights a structural homology with the gamma subunit of G proteins. *Eur. J. Neurosci.* **16**, 219-228 (2002).

105. Geib, S. *et al.* The interaction between the I-II loop and the III-IV loop of Cav2.1 contributes to voltage-dependent inactivation in a

beta -dependent manner. *J. Biol. Chem.* **277**, 10003-10013 (2002).

106. Westenbroek, R. E. *et al.* Immunochemical identification and subcellular distribution of the alpha 1A subunits of brain calcium channels. *J. Neurosci.* **15**, 6403-6418 (1995).

107. Mintz, I. M., Sabatini, B. L. & Regehr, W. G. Calcium control of transmitter release at a cerebellar synapse. *Neuron* **15**, 675-688 (1995).

108. Wu, L. G., Westenbroek, R. E., Borst, J. G., Catterall, W. A. & Sakmann, B. Calcium channel types with distinct presynaptic localization couple differentially to transmitter release in single calyx-type synapses. *J. Neurosci.* **19**, 726-736 (1999).

109. Qian, J. & Noebels, J. L. Presynaptic Ca2+ channels and neurotransmitter release at the terminal of a mouse cortical neuron. *J. Neurosci.* **21**, 3721-3728 (2001).

110. Sutton, K. G., McRory, J. E., Guthrie, H., Murphy, T. H. & Snutch, T. P. P/Q-type calcium channels mediate the activity-dependent feedback of syntaxin-1A. *Nature* **401**, 800-804 (1999).

111. Llinas, R., Sugimori, M. & Silver, R. B. Microdomains of high calcium concentration in a presynaptic terminal. *Science* **256**, 677-679 (1992).

112. Stanley, E. F. The calcium channel and the organization of the presynaptic transmitter release face. *Trends Neurosci.* **20**, 404-409 (1997).

113. Schneggenburger, R. & Neher, E. Presynaptic calcium and control of vesicle fusion. *Curr. Opin. Neurobiol.* **15**, 266-274 (2005).

114. Hille, B. Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci.* **17**, 531-536 (1994).

115. Dodge, F. A., Jr. & Rahamimoff, R. Co-operative action a calcium ions in transmitter release at the neuromuscular junction. *J. Physiol* **193**, 419-432 (1967).

116. Bean, B. P. Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* **340**, 153-156 (1989).

117. Ikeda, S. R. Double-pulse calcium channel current facilitation in adult rat sympathetic neurones. *J. Physiol* **439**, 181-214 (1991).

118. Elmslie, K. S. Neurotransmitter modulation of neuronal calcium channels. *J. Bioenerg. Biomembr.* **35**, 477-489 (2003).

119. Brody, D. L. & Yue, D. T. Relief of G-protein inhibition of calcium channels and short-term synaptic facilitation in cultured hippocampal neurons. *J. Neurosci.* **20**, 889-898 (2000).

120. Herlitze, S. *et al.* Modulation of Ca2+ channels by G-protein beta gamma subunits. *Nature* **380**, 258-262 (1996).

121. Ikeda, S. R. Voltage-dependent modulation of N-type calcium channels by G-protein beta gamma subunits. *Nature* **380**, 255-258 (1996).

122. Jones, L. P., Patil, P. G., Snutch, T. P. & Yue, D. T. G-protein modulation of N-type calcium channel gating current in human embryonic kidney cells (HEK 293). *J. Physiol* **498 (Pt 3)**, 601-610 (1997).

123. De Waard M. *et al.* Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. *Nature* **385**, 446-450 (1997).

124. Herlitze, S., Hockerman, G. H., Scheuer, T. & Catterall, W. A. Molecular determinants of inactivation and G protein modulation in the intracellular loop connecting domains I and II of the calcium channel alpha1A subunit. *Proc. Natl. Acad. Sci. U. S. A* **94**, 1512-1516 (1997).

125. Zhang, J. F., Ellinor, P. T., Aldrich, R. W. & Tsien, R. W. Multiple structural elements in voltage-dependent Ca2+ channels support their inhibition by G proteins. *Neuron* **17**, 991-1003 (1996).

126. Canti, C., Page, K. M., Stephens, G. J. & Dolphin, A. C. Identification of residues in the N terminus of alpha1B critical for

inhibition of the voltage-dependent calcium channel by Gbeta gamma. *J. Neurosci.* **19**, 6855-6864 (1999).

127. Page, K. M., Stephens, G. J., Berrow, N. S. & Dolphin, A. C. The intracellular loop between domains I and II of the B-type calcium channel confers aspects of G-protein sensitivity to the E-type calcium channel. *J. Neurosci.* **17**, 1330-1338 (1997).

128. Page, K. M., Canti, C., Stephens, G. J., Berrow, N. S. & Dolphin, A. C. Identification of the amino terminus of neuronal Ca2+ channel alpha1 subunits alpha1B and alpha1E as an essential determinant of G-protein modulation. *J. Neurosci.* **18**, 4815-4824 (1998).

129. Qin, N., Platano, D., Olcese, R., Stefani, E. & Birnbaumer, L. Direct interaction of gbetagamma with a C-terminal gbetagamma-binding domain of the Ca2+ channel alpha1 subunit is responsible for channel inhibition by G protein-coupled receptors. *Proc. Natl. Acad. Sci. U. S. A* **94**, 8866-8871 (1997).

130. Stephens, G. J., Canti, C., Page, K. M. & Dolphin, A. C. Role of domain I of neuronal Ca2+ channel alpha1 subunits in G protein modulation. *J. Physiol* **509 (Pt 1)**, 163-169 (1998).

131. Campbell, V., Berrow, N. S., Fitzgerald, E. M., Brickley, K. & Dolphin, A. C. Inhibition of the interaction of G protein G(o) with calcium channels by the calcium channel beta-subunit in rat neurones. *J. Physiol* **485 (Pt 2)**, 365-372 (1995).

132. Canti, C., Bogdanov, Y. & Dolphin, A. C. Interaction between G proteins and accessory subunits in the regulation of 1B calcium channels in Xenopus oocytes. *J. Physiol* **527 Pt 3**, 419-432 (2000).

133. Leroy, J. *et al.* Interaction via a key tryptophan in the I-II linker of N-type calcium channels is required for beta1 but not for palmitoylated beta2, implicating an additional binding site in the regulation of channel voltage-dependent properties. *J. Neurosci.* **25**, 6984-6996 (2005).

134. Dresviannikov, A. V., Page, K. M., Leroy, J., Pratt, W. S. & Dolphin, A. C. Determinants of the voltage dependence of G protein modulation within calcium channel beta subunits. *Pflugers Arch.* **457**, 743-756 (2009).

135. Sandoz, G. *et al.* Cavbeta-subunit displacement is a key step to induce the reluctant state of P/Q calcium channels by direct G protein regulation. *Proc. Natl. Acad. Sci. U. S. A* **101**, 6267-6272 (2004).

136. Sudhof, T. C. The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27**, 509-547 (2004).

137. Rosenmund, C., Rettig, J. & Brose, N. Molecular mechanisms of active zone function. *Curr. Opin. Neurobiol.* **13**, 509-519 (2003).

138. Sudhof, T. C. The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**, 645-653 (1995).

139. Sudhof, T. C. & Rothman, J. E. Membrane fusion: grappling with SNARE and SM proteins. *Science* **323**, 474-477 (2009).

140. Wadel, K., Neher, E. & Sakaba, T. The coupling between synaptic vesicles and Ca2+ channels determines fast neurotransmitter release. *Neuron* **53**, 563-575 (2007).

141. Martin-Moutot, N. *et al.* Interaction of SNARE complexes with P/Q-type calcium channels in rat cerebellar synaptosomes. *J. Biol. Chem.* **271**, 6567-6570 (1996).

142. Leveque, C. *et al.* Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis. *J. Biol. Chem.* **269**, 6306-6312 (1994).

143. Sheng, Z. H., Rettig, J., Takahashi, M. & Catterall, W. A. Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**, 1303-1313 (1994).

144. Sheng, Z. H., Rettig, J., Cook, T. & Catterall, W. A. Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. *Nature* **379**, 451-454 (1996).

145. Rettig, J. *et al.* Isoform-specific interaction of the alpha1A subunits of brain Ca2+ channels with the presynaptic proteins syntaxin and SNAP-25. *Proc. Natl. Acad. Sci. U. S. A* **93**, 7363-7368 (1996).

146. Kim, D. K. & Catterall, W. A. Ca2+-dependent and -independent interactions of the isoforms of the alpha1A subunit of brain Ca2+ channels with presynaptic SNARE proteins. *Proc. Natl. Acad. Sci. U. S. A* **94**, 14782-14786 (1997).

147. Sheng, Z. H., Yokoyama, C. T. & Catterall, W. A. Interaction of the synprint site of N-type Ca2+ channels with the C2B domain of synaptotagmin I. *Proc. Natl. Acad. Sci. U. S. A* **94**, 5405-5410 (1997).

148. Yokoyama, C. T., Sheng, Z. H. & Catterall, W. A. Phosphorylation of the synaptic protein interaction site on N-type calcium channels inhibits interactions with SNARE proteins. *J. Neurosci.* **17**, 6929-6938 (1997).

149. Bezprozvanny, I., Scheller, R. H. & Tsien, R. W. Functional impact of syntaxin on gating of N-type and Q-type calcium channels. *Nature* **378**, 623-626 (1995).

150. Zhong, H., Yokoyama, C. T., Scheuer, T. & Catterall, W. A. Reciprocal regulation of P/Q-type Ca2+ channels by SNAP-25, syntaxin and synaptotagmin. *Nat. Neurosci.* **2**, 939-941 (1999).

151. Wiser, O., Bennett, M. K. & Atlas, D. Functional interaction of syntaxin and SNAP-25 with voltage-sensitive L- and N-type Ca2+ channels. *EMBO J.* **15**, 4100-4110 (1996).

152. Jarvis, S. E. & Zamponi, G. W. Distinct molecular determinants govern syntaxin 1A-mediated inactivation and G-protein inhibition of N-type calcium channels. *J. Neurosci.* **21**, 2939-2948 (2001).

153. Wiser, O., Tobi, D., Trus, M. & Atlas, D. Synaptotagmin restores kinetic properties of a syntaxin-associated N-type voltage sensitive calcium channel. *FEBS Lett.* **404**, 203-207 (1997).

154. Catterall, W. A. Interactions of presynaptic Ca2+ channels and snare proteins in neurotransmitter release. *Ann. N. Y. Acad. Sci.* **868**, 144-159 (1999).

155. Misura, K. M., Scheller, R. H. & Weis, W. I. Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* **404**, 355-362 (2000).

156. Bezprozvanny, I., Zhong, P., Scheller, R. H. & Tsien, R. W. Molecular determinants of the functional interaction between syntaxin and N-type Ca2+ channel gating. *Proc. Natl. Acad. Sci. U. S. A* **97**, 13943-13948 (2000).

157. Jarvis, S. E., Magga, J. M., Beedle, A. M., Braun, J. E. & Zamponi, G. W. G protein modulation of N-type calcium channels is facilitated by physical interactions between syntaxin 1A and Gbetagamma. *J. Biol. Chem.* **275**, 6388-6394 (2000).

158. Stanley, E. F. & Mirotznik, R. R. Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels. *Nature* **385**, 340-343 (1997).

159. Lu, Q. *et al.* Syntaxin 1A supports voltage-dependent inhibition of alpha1B Ca2+ channels by Gbetagamma in chick sensory neurons. *J. Neurosci.* **21**, 2949-2957 (2001).

160. Jarvis, S. E., Barr, W., Feng, Z. P., Hamid, J. & Zamponi, G. W. Molecular determinants of syntaxin 1 modulation of N-type calcium channels. *J. Biol. Chem.* **277**, 44399-44407 (2002).

161. Spafford, J. D. & Zamponi, G. W. Functional interactions between presynaptic calcium channels and the neurotransmitter release machinery. *Curr. Opin. Neurobiol.* **13**, 308-314 (2003).

162. Mochida, S., Sheng, Z. H., Baker, C., Kobayashi, H. & Catterall, W. A. Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca2+ channels. *Neuron* **17**, 781-788 (1996).

163. Sun, J. *et al.* A dual-Ca2+-sensor model for neurotransmitter release in a central synapse. *Nature* **450**, 676-682 (2007).

164. Keith, R. K., Poage, R. E., Yokoyama, C. T., Catterall, W. A. & Meriney, S. D. Bidirectional modulation of transmitter release by calcium channel/syntaxin interactions in vivo. *J. Neurosci.* **27**, 265-269 (2007).

165. Rettig, J. *et al.* Alteration of Ca2+ dependence of neurotransmitter release by disruption of Ca2+ channel/syntaxin interaction. *J. Neurosci.* **17**, 6647-6656 (1997).

166. Bergsman, J. B. & Tsien, R. W. Syntaxin modulation of calcium channels in cortical synaptosomes as revealed by botulinum toxin C1. *J. Neurosci.* **20**, 4368-4378 (2000).

167. Harkins, A. B., Cahill, A. L., Powers, J. F., Tischler, A. S. & Fox, A. P. Deletion of the synaptic protein interaction site of the N-type (CaV2.2) calcium channel inhibits secretion in mouse pheochromocytoma cells. *Proc. Natl. Acad. Sci. U. S. A* **101**, 15219-15224 (2004).

168. Harkins, A. B., Cahill, A. L., Powers, J. F., Tischler, A. S. & Fox, A. P. Expression of recombinant calcium channels support secretion in a mouse pheochromocytoma cell line. *J. Neurophysiol.* **90**, 2325-2333 (2003).

169. Zamponi, G. W. Regulation of presynaptic calcium channels by synaptic proteins. *J. Pharmacol. Sci.* **92**, 79-83 (2003).

170. Wessman, M., Terwindt, G. M., Kaunisto, M. A., Palotie, A. & Ophoff, R. A. Migraine: a complex genetic disorder. *Lancet Neurol.* **6**, 521-532 (2007).

171. Stewart, W. F., Wood, C., Reed, M. L., Roy, J. & Lipton, R. B. Cumulative lifetime migraine incidence in women and men. *Cephalalgia* **28**, 1170-1178 (2008).

172. Charles, A. Advances in the basic and clinical science of migraine. *Ann. Neurol.* **65**, 491-498 (2009).

173. Wessman, M., Kaunisto, M. A., Kallela, M. & Palotie, A. The molecular genetics of migraine. *Ann. Med.* **36**, 462-473 (2004).

174. De Vries B., Frants, R. R., Ferrari, M. D. & van den Maagdenberg, A. M. Molecular genetics of migraine. *Hum. Genet.* **126**, 115-132 (2009).

175. The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* **24 Suppl 1**, 9-160 (2004).

176. Montagna, P. The physiopathology of migraine: the contribution of genetics. *Neurol. Sci.* **25 Suppl 3**, S93-S96 (2004).

177. Nyholt, D. R. *et al.* A high-density association screen of 155 ion transport genes for involvement with common migraine. *Hum. Mol. Genet.* **17**, 3318-3331 (2008).

178. Bianchin, M. M., Londero, R. G., Lima, J. E. & Bigal, M. E. Migraine and epilepsy: a focus on overlapping clinical, pathophysiological, molecular, and therapeutic aspects. *Curr. Pain Headache Rep.* **14**, 276-283 (2010).

179. Silberstein, S. D. & Goadsby, P. J. Migraine: preventive treatment. *Cephalalgia* **22**, 491-512 (2002).

180. Breslau, N., Lipton, R. B., Stewart, W. F., Schultz, L. R. & Welch, K. M. Comorbidity of migraine and depression: investigating potential etiology and prognosis. *Neurology* **60**, 1308-1312 (2003).

181. Pietrobon, D. Migraine: new molecular mechanisms. *Neuroscientist.* **11**, 373-386 (2005).

182. Pietrobon, D. & Striessnig, J. Neurobiology of migraine. *Nat. Rev. Neurosci.* **4**, 386-398 (2003).

183. Goadsby, P. J. & Edvinsson, L. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. *Ann. Neurol.* **33**, 48-56 (1993).

184. Olesen, J. *et al.* Timing and topography of cerebral blood flow, aura, and headache during migraine attacks. *Ann. Neurol.* **28**, 791-798 (1990).

185. Woods, R. P., Iacoboni, M. & Mazziotta, J. C. Brief report: bilateral spreading cerebral hypoperfusion during spontaneous migraine headache. *N. Engl. J. Med.* **331**, 1689-1692 (1994).

186. Denuelle, M., Fabre, N., Payoux, P., Chollet, F. & Geraud, G. Posterior cerebral hypoperfusion in migraine without aura. *Cephalalgia* **28**, 856-862 (2008).

187. Limmroth, V. *et al.* Changes in cerebral blood flow velocity after treatment with sumatriptan or placebo and implications for the pathophysiology of migraine. *J. Neurol. Sci.* **138**, 60-65 (1996).

188. Kruuse, C., Thomsen, L. L., Birk, S. & Olesen, J. Migraine can be induced by sildenafil without changes in middle cerebral artery diameter. *Brain* **126**, 241-247 (2003).

189. Goadsby, P. J., Lipton, R. B. & Ferrari, M. D. Migraine--current understanding and treatment. *N. Engl. J. Med.* **346**, 257-270 (2002).

190. Silberstein, S. D. Migraine. *Lancet* **363**, 381-391 (2004).

191. Leao, A. A. P. Spreading depression of activity in the cerebral cortex. *J. Neurophysiol.* **7**, 359-390 (1944).

192. Lauritzen, M. Pathophysiology of the migraine aura. The spreading depression theory. *Brain* **117 (Pt 1)**, 199-210 (1994).

193. Olesen, J., Larsen, B. & Lauritzen, M. Focal hyperemia followed by spreading oligemia and impaired activation of rCBF in classic migraine. *Ann. Neurol.* **9**, 344-352 (1981).

194. Hadjikhani, N. *et al.* Mechanisms of migraine aura revealed by functional MRI in human visual cortex. *Proc. Natl. Acad. Sci. U. S. A* **98**, 4687-4692 (2001).

195. Welch, K. M., Cao, Y., Aurora, S., Wiggins, G. & Vikingstad, E. M. MRI of the occipital cortex, red nucleus, and substantia nigra during visual aura of migraine. *Neurology* **51**, 1465-1469 (1998).

196. Bowyer, S. M. *et al.* Analysis of MEG signals of spreading cortical depression with propagation constrained to a rectangular cortical strip. I. Lissencephalic rabbit model. *Brain Res.* **843**, 71-78 (1999).

197. Bowyer, S. M., Aurora, K. S., Moran, J. E., Tepley, N. & Welch, K. M. Magnetoencephalographic fields from patients with spontaneous and induced migraine aura. *Ann. Neurol.* **50**, 582-587 (2001).

198. Bolay, H. *et al.* Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. *Nat. Med.* **8**, 136-142 (2002).

199. Ayata, C., Jin, H., Kudo, C., Dalkara, T. & Moskowitz, M. A. Suppression of cortical spreading depression in migraine prophylaxis. *Ann. Neurol.* **59**, 652-661 (2006).

200. Parsons, A. A. Cortical spreading depression: its role in migraine pathogenesis and possible therapeutic intervention strategies. *Curr. Pain Headache Rep.* **8**, 410-416 (2004).

201. Dalkara, T., Zervas, N. T. & Moskowitz, M. A. From spreading depression to the trigeminovascular system. *Neurol. Sci.* **27 Suppl 2**, S86-S90 (2006).

202. Somjen, G. G. Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. *Physiol Rev.* **81**, 1065-1096 (2001).

203. Gursoy-Ozdemir, Y. *et al.* Cortical spreading depression activates and upregulates MMP-9. *J. Clin. Invest* **113**, 1447-1455 (2004).

204. Sanchez-Del-Rio, M. & Reuter, U. Migraine aura: new information on underlying mechanisms. *Curr. Opin. Neurol.* **17**, 289-293 (2004).

205. Welch, K. M. Brain hyperexcitability: the basis for antiepileptic drugs in migraine prevention. *Headache* **45 Suppl 1**, S25-S32 (2005).

206. Aurora, S. K. & Wilkinson, F. The brain is hyperexcitable in migraine. *Cephalalgia* **27**, 1442-1453 (2007).

207. Coppola, G., Pierelli, F. & Schoenen, J. Is the cerebral cortex hyperexcitable or hyperresponsive in migraine? *Cephalalgia* **27**, 1427-1439 (2007).

208. Moskowitz, M. A., Bolay, H. & Dalkara, T. Deciphering migraine mechanisms: clues from familial hemiplegic migraine genotypes. *Ann. Neurol.* **55**, 276-280 (2004).

209. Pietrobon, D. Insights into migraine mechanisms and CaV2.1 calcium channel function from mouse models of familial hemiplegic migraine. *J. Physiol* **588**, 1871-1878 (2010).

210. Charles, A. Does cortical spreading depression initiate a migraine attack? Maybe not . . *Headache* **50**, 731-733 (2010).

211. Charles, A. Intercellular calcium waves in glia. *Glia* **24**, 39-49 (1998).

212. Charles, A. & Brennan, K. C. The neurobiology of migraine. *Handb. Clin. Neurol.* **97**, 99-108 (2010).

213. Stewart, W. F., Linet, M. S., Celentano, D. D., Van, N. M. & Ziegler, D. Age- and sex-specific incidence rates of migraine with and without visual aura. *Am. J. Epidemiol.* **134**, 1111-1120 (1991).

214. Wolthausen, J., Sternberg, S., Gerloff, C. & May, A. Are cortical spreading depression and headache in migraine causally linked? *Cephalalgia* **29**, 244-249 (2009).

215. Kelman, L. The premonitory symptoms (prodrome): a tertiary care study of 893 migraineurs. *Headache* **44**, 865-872 (2004).

216. Goadsby, P. J. Migraine, aura, and cortical spreading depression: why are we still talking about it? *Ann. Neurol.* **49**, 4-6 (2001).

217. Raskin, N. H., Hosobuchi, Y. & Lamb, S. Headache may arise from perturbation of brain. *Headache* **27**, 416-420 (1987).

218. Weiller, C. *et al.* Brain stem activation in spontaneous human migraine attacks. *Nat. Med.* **1**, 658-660 (1995).

219. Bahra, A., Matharu, M. S., Buchel, C., Frackowiak, R. S. & Goadsby, P. J. Brainstem activation specific to migraine headache. *Lancet* **357**, 1016-1017 (2001).

220. Denuelle, M., Fabre, N., Payoux, P., Chollet, F. & Geraud, G. Hypothalamic activation in spontaneous migraine attacks. *Headache* **47**, 1418-1426 (2007).

221. Levy, D., Jakubowski, M. & Burstein, R. Disruption of communication between peripheral and central trigeminovascular neurons mediates the antimigraine action of 5HT 1B/1D receptor agonists. *Proc. Natl. Acad. Sci. U. S. A* **101**, 4274-4279 (2004).

222. Strassman, A. M., Raymond, S. A. & Burstein, R. Sensitization of meningeal sensory neurons and the origin of headaches. *Nature* **384**, 560-564 (1996).

223. Burstein, R., Yamamura, H., Malick, A. & Strassman, A. M. Chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in brain stem trigeminal neurons. *J. Neurophysiol.* **79**, 964-982 (1998).

224. Burstein, R. Deconstructing migraine headache into peripheral and central sensitization. *Pain* **89**, 107-110 (2001).

225. Kaube, H. *et al.* Acute migraine headache: possible sensitization of neurons in the spinal trigeminal nucleus? *Neurology* **58**, 1234-1238 (2002).

226. Doenicke, A., Brand, J. & Perrin, V. L. Possible benefit of GR43175, a novel 5-HT1-like receptor agonist, for the acute treatment of severe migraine. *Lancet* **1**, 1309-1311 (1988).

227. Goadsby, P. J. The pharmacology of headache. *Prog. Neurobiol.* **62**, 509-525 (2000).

228. Waeber, C. & Moskowitz, M. A. Therapeutic implications of central and peripheral neurologic mechanisms in migraine. *Neurology* **61**, S9-20 (2003).

229. Jennings, E. A., Ryan, R. M. & Christie, M. J. Effects of sumatriptan on rat medullary dorsal horn neurons. *Pain* **111**, 30-37 (2004).

230. Goldstein, D. J. *et al.* Selective seratonin 1F (5-HT(1F)) receptor agonist LY334370 for acute migraine: a randomised controlled trial. *Lancet* **358**, 1230-1234 (2001).

231. Thomsen, L. L. *et al.* A population-based study of familial hemiplegic migraine suggests revised diagnostic criteria. *Brain* **125**, 1379-1391 (2002).

232. Ducros, A. *et al.* The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel. *N. Engl. J. Med.* **345**, 17-24 (2001).

233. Ophoff, R. A. *et al.* Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. *Cell* **87**, 543-552 (1996).

234. De Fusco M. *et al.* Haploinsufficiency of ATP1A2 encoding the Na+/K+ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat. Genet.* **33**, 192-196 (2003).

235. Cholet, N., Pellerin, L., Magistretti, P. J. & Hamel, E. Similar perisynaptic glial localization for the Na+,K+-ATPase alpha 2 subunit and the glutamate transporters GLAST and GLT-1 in the rat somatosensory cortex. *Cereb. Cortex* **12**, 515-525 (2002).

236. Pietrobon, D. Familial hemiplegic migraine. *Neurotherapeutics.* **4**, 274-284 (2007).

237. Dichgans, M. *et al.* Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine. *Lancet* **366**, 371-377 (2005).

238. Kahlig, K. M. *et al.* Divergent sodium channel defects in familial hemiplegic migraine. *Proc. Natl. Acad. Sci. U. S. A* **105**, 9799-9804 (2008).

239. Cestele, S. *et al.* Self-limited hyperexcitability: functional effect of a familial hemiplegic migraine mutation of the Nav1.1 (SCN1A) Na+ channel. *J. Neurosci.* **28**, 7273-7283 (2008).

240. Cuenca-Leon, E. *et al.* Genetic analysis of 27 Spanish patients with hemiplegic migraine, basilar-type migraine and childhood periodic syndromes. *Cephalalgia* **28**, 1039-1047 (2008).

241. Iwasaki, S., Momiyama, A., Uchitel, O. D. & Takahashi, T. Developmental changes in calcium channel types mediating central synaptic transmission. *J. Neurosci.* **20**, 59-65 (2000).

242. Tottene, A. *et al.* Enhanced excitatory transmission at cortical synapses as the basis for facilitated spreading depression in Ca(v)2.1 knockin migraine mice. *Neuron* **61**, 762-773 (2009).

243. Ayata, C., Shimizu-Sasamata, M., Lo, E. H., Noebels, J. L. & Moskowitz, M. A. Impaired neurotransmitter release and elevated threshold for cortical spreading depression in mice with

mutations in the alpha1A subunit of P/Q type calcium channels. *Neuroscience* **95**, 639-645 (2000).

244. Zaitsev, A. V., Povysheva, N. V., Lewis, D. A. & Krimer, L. S. P/Q-type, but not N-type, calcium channels mediate GABA release from fast-spiking interneurons to pyramidal cells in rat prefrontal cortex. *J. Neurophysiol.* **97**, 3567-3573 (2007).

245. Asakura, K. *et al.* alpha-eudesmol, a P/Q-type Ca(2+) channel blocker, inhibits neurogenic vasodilation and extravasation following electrical stimulation of trigeminal ganglion. *Brain Res.* **873**, 94-101 (2000).

246. Akerman, S., Williamson, D. J. & Goadsby, P. J. Voltage-dependent calcium channels are involved in neurogenic dural vasodilatation via a presynaptic transmitter release mechanism. *Br. J. Pharmacol.* **140**, 558-566 (2003).

247. Xiao, Y., Richter, J. A. & Hurley, J. H. Release of glutamate and CGRP from trigeminal ganglion neurons: Role of calcium channels and 5-HT1 receptor signaling. *Mol. Pain* **4**, 12 (2008).

248. Ebersberger, A., Portz, S., Meissner, W., Schaible, H. G. & Richter, F. Effects of N-, P/Q- and L-type calcium channel blockers on nociceptive neurones of the trigeminal nucleus with input from the dura. *Cephalalgia* **24**, 250-261 (2004).

249. Vanegas, H. & Schaible, H. Effects of antagonists to high-threshold calcium channels upon spinal mechanisms of pain, hyperalgesia and allodynia. *Pain* **85**, 9-18 (2000).

250. Luvisetto, S. *et al.* Pain sensitivity in mice lacking the Ca(v)2.1alpha(1) subunit of P/Q-type Ca(2+) channels. *Neuroscience* (2006).

251. Pietrobon, D. CaV2.1 channelopathies. *Pflugers Arch.* **460**, 375-393 (2010).

252. Zhuchenko, O. *et al.* Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nat. Genet.* **15**, 62-69 (1997).

253. Adams, P. J. *et al.* Ca(V)2.1 P/Q-type calcium channel alternative splicing affects the functional impact of familial hemiplegic migraine mutations: implications for calcium channelopathies. *Channels (Austin.)* **3**, 110-121 (2009).

254. Barrett, C. F., Cao, Y. Q. & Tsien, R. W. Gating deficiency in a familial hemiplegic migraine type 1 mutant P/Q-type calcium channel. *J. Biol. Chem.* **280**, 24064-24071 (2005).

255. Hans, M. *et al.* Functional consequences of mutations in the human alpha1A calcium channel subunit linked to familial hemiplegic migraine. *J. Neurosci.* **19**, 1610-1619 (1999).

256. Melliti, K., Grabner, M. & Seabrook, G. R. The familial hemiplegic migraine mutation R192Q reduces G-protein-mediated inhibition of P/Q-type (Ca(V)2.1) calcium channels expressed in human embryonic kidney cells. *J. Physiol* **546**, 337-347 (2003).

257. Mullner, C., Broos, L. A., van den Maagdenberg, A. M. & Striessnig, J. Familial hemiplegic migraine type 1 mutations K1336E, W1684R, and V1696I alter Cav2.1 Ca2+ channel gating: evidence for beta-subunit isoform-specific effects. *J. Biol. Chem.* **279**, 51844-51850 (2004).

258. Serra, S. A. *et al.* The hemiplegic migraine-associated Y1245C mutation in CACNA1A results in a gain of channel function due to its effect on the voltage sensor and G-protein-mediated inhibition. *Pflugers Arch.* **458**, 489-502 (2009).

259. Tottene, A. *et al.* Familial hemiplegic migraine mutations increase Ca(2+) influx through single human CaV2.1 channels and decrease maximal CaV2.1 current density in neurons. *Proc. Natl. Acad. Sci. U. S. A* **99**, 13284-13289 (2002).

260. Tottene, A. *et al.* Specific kinetic alterations of human CaV2.1 calcium channels produced by mutation S218L causing familial hemiplegic migraine and delayed cerebral edema and coma after minor head trauma. *J. Biol. Chem.* **280**, 17678-17686 (2005).

261. Weiss, N., Sandoval, A., Felix, R., Van den, M. A. & De Waard M. The S218L familial hemiplegic migraine mutation promotes deinhibition of Ca(v)2.1 calcium channels during direct G-protein regulation. *Pflugers Arch.* **457**, 315-326 (2008).

262. Cao, Y. Q. *et al.* Presynaptic Ca2+ channels compete for channel type-preferring slots in altered neurotransmission arising from Ca2+ channelopathy. *Neuron* **43**, 387-400 (2004).

263. Cao, Y. Q. & Tsien, R. W. Effects of familial hemiplegic migraine type 1 mutations on neuronal P/Q-type Ca2+ channel activity and inhibitory synaptic transmission. *Proc. Natl. Acad. Sci. U. S. A* **102**, 2590-2595 (2005).

264. van den Maagdenberg, A. M. *et al.* High cortical spreading depression susceptibility and migraine-associated symptoms in Ca(v)2.1 S218L mice. *Ann. Neurol.* **67**, 85-98 (2010).

265. van den Maagdenberg, A. M. *et al.* A Cacna1a knockin migraine mouse model with increased susceptibility to cortical spreading depression. *Neuron* **41**, 701-710 (2004).

266. Inchauspe, C. G. *et al.* Gain of function in FHM-1 Ca(V)2.1 knock-in mice is related to the shape of the action potential. *J. Neurophysiol.* **104**, 291-299 (2010).

267. Kors, E. E. *et al.* Delayed cerebral edema and fatal coma after minor head trauma: role of the CACNA1A calcium channel subunit gene and relationship with familial hemiplegic migraine. *Ann. Neurol.* **49**, 753-760 (2001).

268. Eikermann-Haerter, K. *et al.* Genetic and hormonal factors modulate spreading depression and transient hemiparesis in mouse models of familial hemiplegic migraine type 1. *J. Clin. Invest* **119**, 99-109 (2009).

269. Eikermann-Haerter, K. *et al.* Androgenic suppression of spreading depression in familial hemiplegic migraine type 1 mutant mice. *Ann. Neurol.* **66**, 564-568 (2009).

270. Cuvellier, J. C. & Lepine, A. Childhood periodic syndromes. *Pediatr. Neurol.* **42**, 1-11 (2010).

271. Giffin, N. J., Benton, S. & Goadsby, P. J. Benign paroxysmal torticollis of infancy: four new cases and linkage to CACNA1A mutation. *Dev. Med. Child Neurol.* **44**, 490-493 (2002).

272. Tombola, F., Pathak, M. M. & Isacoff, E. Y. How does voltage open an ion channel? *Annu. Rev. Cell Dev. Biol.* **22**, 23-52 (2006).

273. Weiss, N., Arnoult, C., Feltz, A. & De Waard M. Contribution of the kinetics of G protein dissociation to the characteristic modifications of N-type calcium channel activity. *Neurosci. Res.* **56**, 332-343 (2006).

274. Pattillo, J. M., Artim, D. E., Simples, J. E., Jr. & Meriney, S. D. Variations in onset of action potential broadening: effects on calcium current studied in chick ciliary ganglion neurones. *J. Physiol* **514 (Pt 3)**, 719-728 (1999).

275. Catterall, W. A. Ion channel voltage sensors: structure, function, and pathophysiology. *Neuron* **67**, 915-928 (2010).

276. Campos, F. V., Chanda, B., Roux, B. & Bezanilla, F. Two atomic constraints unambiguously position the S4 segment relative to S1 and S2 segments in the closed state of Shaker K channel. *Proc. Natl. Acad. Sci. U. S. A* **104**, 7904-7909 (2007).

277. Branchaw, J. L., Banks, M. I. & Jackson, M. B. Ca2+- and voltage-dependent inactivation of Ca2+ channels in nerve terminals of the neurohypophysis. *J. Neurosci.* **17**, 5772-5781 (1997).

278. Forsythe, I. D., Tsujimoto, T., Barnes-Davies, M., Cuttle, M. F. & Takahashi, T. Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. *Neuron* **20**, 797-807 (1998).

279. Stotz, S. C. & Zamponi, G. W. Identification of inactivation determinants in the domain IIS6 region of high voltage-activated calcium channels. *J. Biol. Chem.* **276**, 33001-33010 (2001).

280. Pitt, G. S. *et al.* Molecular basis of calmodulin tethering and Ca2+-dependent inactivation of L-type Ca2+ channels. *J. Biol. Chem.* **276**, 30794-30802 (2001).

281. Patil, P. G., Brody, D. L. & Yue, D. T. Preferential closed-state inactivation of neuronal calcium channels. *Neuron* **20**, 1027-1038 (1998).

282. Degtiar, V. E., Scheller, R. H. & Tsien, R. W. Syntaxin modulation of slow inactivation of N-type calcium channels. *J. Neurosci.* **20**, 4355-4367 (2000).

283. Stocker, J. W., Nadasdi, L., Aldrich, R. W. & Tsien, R. W. Preferential interaction of omega-conotoxins with inactivated N-type Ca2+ channels. *J. Neurosci.* **17**, 3002-3013 (1997).

284. Ludwig, A., Flockerzi, V. & Hofmann, F. Regional expression and cellular localization of the alpha1 and beta subunit of high voltage-activated calcium channels in rat brain. *J. Neurosci.* **17**, 1339-1349 (1997).

285. Rajapaksha, W. R. *et al.* Novel splice variants of rat CaV2.1 that lack much of the synaptic protein interaction site are expressed in neuroendocrine cells. *J. Biol. Chem.* **283**, 15997-16003 (2008).

286. Mochida, S., Saisu, H., Kobayashi, H. & Abe, T. Impairment of syntaxin by botulinum neurotoxin C1 or antibodies inhibits acetylcholine release but not Ca2+ channel activity. *Neuroscience* **65**, 905-915 (1995).

287. Davies, J. N. & Zamponi, G. W. Old proteins, developing roles: The regulation of calcium channels by synaptic proteins. *Channels (Austin.)* **2**, 130-138 (2008).

288. Mochida, S. *et al.* Requirement for the synaptic protein interaction site for reconstitution of synaptic transmission by P/Q-type calcium channels. *Proc. Natl. Acad. Sci. U. S. A* **100**, 2819-2824 (2003).

289. Moser, T. & Neher, E. Rapid exocytosis in single chromaffin cells recorded from mouse adrenal slices. *J. Neurosci.* **17**, 2314-2323 (1997).

290. John, B., Klemm, E. & Haverkamp, F. Evidence for altered basal ganglia and cortical functions in transient idiopathic dystonia. *J. Child Neurol.* **15**, 820-822 (2000).

291. Tascioglu, A. B. Brief review of vestibular system anatomy and its hig.her order projections. *Neuroanatomy* **4**, 24-27 (2005).

292. Chang, S. Y. *et al.* Age and gender-dependent alternative splicing of P/Q-type calcium channel EF-hand. *Neuroscience* **145**, 1026-1036 (2007).

293. Brennan, K. C. *et al.* Distinct vascular conduction with cortical spreading depression. *J. Neurophysiol.* **97**, 4143-4151 (2007).

294. Neher, E. & Sakaba, T. Multiple roles of calcium ions in the regulation of neurotransmitter release. *Neuron* **59**, 861-872 (2008).

295. Goadsby, P. J. Emerging therapies for migraine. *Nat. Clin. Pract. Neurol.* **3**, 610-619 (2007).

296. Corominas, R. *et al.* Contribution of syntaxin 1A to the genetic susceptibility to migraine: a case-control association study in the Spanish population. *Neurosci. Lett.* **455**, 105-109 (2009).

297. Lemos, C. *et al.* Evidence of syntaxin 1A involvement in migraine susceptibility: a Portuguese study. *Arch. Neurol.* **67**, 422-427 (2010).