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In Vivo Analysis of Voltage-Dependent Calcium Channels

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The molecular cloning of calcium channel subunits has identified an unexpectedly large number of genes and splicing variants, many of which have complex expression patterns: a central problem of calcium channel biology is to understand the functional significance of this genetic complexity. The genetic analysis of voltage-dependent calcium channels (VDCCs) provides an approach to defining channel function that is complimentary to pharmacological, electrophysiological, and other molecular methods. By discovering or creating alleles of VDCC genes, one can gain an understanding of the VDCC function at the whole animal level. Of particular interest are mutations in the α_1 genes that encode the pore forming subunits, as they define the specific channel subtypes. In fact, a variety of calcium channelopathies and targeted mutations have been described for these genes in the last 6 years. The mutant alleles described below illustrate how phenotype analysis of these alleles has uncovered very specific functional roles that can be localized to specific synapses or cells.

KEY WORDS: Voltage-dependent calcium channels; migraine; ataxia; epilepsy; night blindness; mouse; knockouts; mutations.

INTRODUCTION

The molecular cloning of calcium channel subunits has identified an unexpectedly large number of genes and splicing variants, many of which have complex expression patterns: a central problem of calcium channel biology is to understand the functional significance of this genetic complexity. The biophysical characterization of different genes and isoforms has largely been accomplished using heterologous expression systems, e.g., HEK cells or *Xenopus* oocytes. While these systems provide certain technical advantages, they do not fully replicate the in vivo milieu. This is evident from the fact that biophysical properties of channels may differ from those seen in neurons. There may be many reasons; certainly these preparations don't express the full repertoire of auxiliary subunits found in tissues. In addition, analysis of synaptic and higher network function can only be performed in intact systems. The focus of this review will be to summarize and discuss the α_1 alleles, spontaneous and targeted, and their functional characterization in vivo.

Understanding the in vivo function of VDCCs will require manipulation of channel function in intact animals. This can be accomplished in two ways, through the discovery of spontaneously occurring variants or through the construction of engineered alleles. Spontaneous variants come in two flavors: alleles that contribute to polygenic disorders and mutations that underlie monogenic traits. Identification of loci that contribute to polygenic disorders requires sophisticated analyses, such as association or sib pair studies. Monogenic loci can be identified through linkage analysis. Even if monogenic alleles contribute only to rare diseases, this approach can identify candidate genes that can be screened for "milder" variants that may contribute to polygenic disease. Engineered alleles comprise transgenes, knockouts, and knockins. These alleles can be designed to test the role of a gene in a specific tissue or to alter the repertoire of isoforms that are expressed. In the case where a spontaneous null allele doesn't exist, one can be generated to define the loss of function phenotype and identify a requirement for gene

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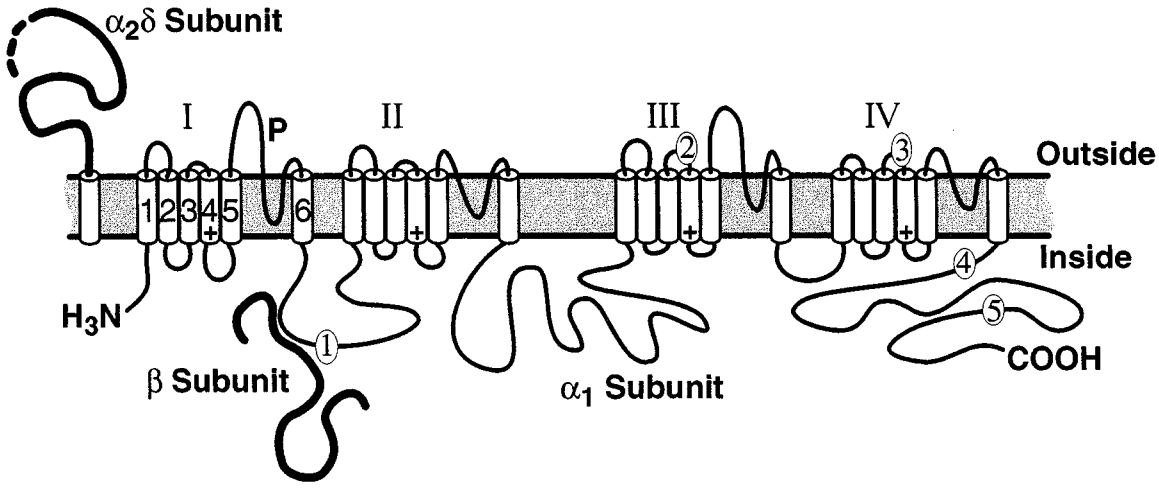

Fig. 1. *Structure of the voltage-dependent calcium channel.* The prototypical HVA voltage-dependent calcium channel consists of at least three subunits. The α_1 poreforming subunit is a transmembrane protein organized into four domains (I–IV), each containing six transmembrane segments (1–6). The P loops line the channel. The cytoplasmic β subunits bind to the I–II loop. The transmembrane $\alpha_2\delta$ (proteolytically cleaved into α_2 and δ) associates with the channel as well, although the mechanism is not defined. In muscle a transmembrane γ subunit is also present, but data supporting neuronal γ subunits is less clear. Positions of alternate splice forms are indicated by circled numbers:(1) alternate splice acceptors; (2) alternate exon; (3) alternate exon; (4) alternate EF hand encoding exons; (5) alternate splice acceptors.

function in normal development. In some cases specific human disease alleles can be expressed in the mouse to provide an experimental resource to define the pathophysiology of a human disease. Examples will be discussed in terms of the insight they provide into the function of particular calcium channel subtypes.

As defined by their electrophysiological properties, these channels are identified as high voltage activated (HVA) or low voltage activated (LVA). The prototypical HVA VDCC channel comprises multiple subunits: the α_1 poreforming unit, and several cytoplasmic or transmembrane modulatory subunits, β, α_2–δ, and γ (Fig. 1). Molecular cloning of the HVA VDCC subunit genes has now identified seven α_1 (α_{1A}–α_{1F}, α_{1S}) genes, and, in addition, 4β (β_1–β_4), 3 $\alpha_2\delta$ ($\alpha_2\delta1$–$\alpha_2\delta3$), and 8γ ($\gamma1$–$\gamma8$) modulatory subunit genes (Catterall, 1998; Nargeot *et al.*, 1997; Perez-Reyes and Schneider, 1995; Tsien *et al.*, 1991; Zhang and O'Neil, 1999). Among the HVA channels there are two groups (L- and non-L-type) based on the sensitivity to dihydropyridines (DHP), a class of organic compounds that bind to and modulate the activity of L-type channels. The non-L-type channels, as defined by pharmacological and electrophysiological criteria, include the P/Q, N, and R types. Thus, the α_1 subunits of the L-type channels are encoded by four genes (α_{1C}, α_{1D}, α_{1F}, α_{1S}), the P/Q-type by one (α_{1A}), the N-type by one (α_{1B}), and the R-type by one (α_{1E}). The LVA channel is referred to as the T-type, and is encoded by three genes (α_{1G}, α_{1H}, α_{1I}). The LVA channel is apparently composed of only the α_1 subunit.

The new nomenclature groups the channels into L-type (Ca_v 1.1–1.4), non-L-type (Ca_v 2.1–2.3), and T-type (Ca_v 3.1–3.3) as indicated in Table I (Ertel *et al.*, 2000). The corresponding poreforming units use the α_1 prefix. As shown in Fig. 1, the calcium channel α_1 subunit is a single polypeptide containing four repeats (domains I–IV) of six transmembrane segments and a pore lining loop.

In the 6 years since the identification of mutations in the α_1 2.1 gene there has been increasing interest in using genetic methods to define the in vivo roles of each channel subtype. As described below, these efforts have already yielded some intriguing insights. Additional phenotyping, as well as the generation of tissue-specific and inducible knockouts/knockins, will further refine our understanding of VDCC function.

L-TYPE VDCC, α_1 1.2

The L-type channel Ca_v 1.2 plays a key role in calcium regulation in the heart. Its role in diseases, such as hypertrophy and heart failure, is controversial even though calcium misregulation is clearly part of the pathological mechanism. To clarify the role of the Ca_v 1.2 channel in cardiac and smooth muscle, where it is the predominant

Table I. Targeted and Spontaneous Alleles of α_1 Genes

Gene	Alias	Channel	Allele type	Disease	Lesion type	Phenotype
α_1 1.1	α1S	L-type	Spontaneous	HOKPP	Mis	Periodic paralysis
			Spontaneous	*mdg*	Del	Muscular dysgenesis
α_1 1.2	α1C	L-type	Targeted		Del	Embryonic lethality
			Transgenic		OE	Cardiac hypertrophy
α_1 1.3	α1D	L-type	Targeted		Del	Deafness, sinoatrial node dysfunction, β cell developmental defect
α_1 1.4	α1F	L-type	Spontaneous	CSNB	Non, Del, SS	Vision defects
α_1 2.1	α1A	P/Q-type	Spontaneous	FHM	Mis,	Migraine, ataxia
			Spontaneous	EA2	Non, Del, Mis, SS, CAG	Episodic ataxia
			Spontaneous	SCA6	CAG expansion	Cerebellar degeneration
			Spontaneous	Epilepsy	Non	Epilepsy
			Spontaneous	*tg*	Mis, SS	Ataxia, epilepsy, cerebellar degeneration, dyskinesia, altered nociception
			Targeted		Del	Ataxia, epilepsy, cerebellar degeneration
α_1 2.2	α1B	N-type	Targeted		Del	Altered nociception, sympathetic NS dysfunction
α_1 2.3	α1E	R-type (?)	Targeted		Del	Hyperglycemia, memory defect, anxiety, sperm defect, altered nociception
α_1 3.1	α1G	T-type	Targeted		Del	Thalamic burst firing defect

Note. Mis–missense; Del–deletion; OE–over expression; Non–nonsense; SS–splice site; CAG–CAG expansion.

channel, a null mutation was created in the α_1 1.2 gene (Seisenberger *et al.*, 2000). Homozygous null mice die in utero between days 12.5 and 14.5 postcoitum (p.c.). An unexpected finding was that homozygous null cardiomyocytes beat. Further investigation revealed a novel, L-type like VDCC with a low affinity for the DHP nisoldipine. The authors present compelling arguments that none of the known VDCCs can account for this current, and suggest that an as of yet unidentified α_1 gene is responsible. Further experimentation will be required to define the requirement for Ca_v 1.2 in survival and development past 14.5 days p.c. A conditional null allele will need to be generated in order to investigate the role of Ca_v 1.2 in adult tissues.

Another approach to defining the role of the VDCC in disease has been to overexpress α_1 1.2 using a transgene driven by a myosin promoter (Muth *et al.*, 1999, 2001). Transgenic mice showed a progressive cardiac hypertrophy and death before 1 year of age. Western blot, current density, and contractility measurements showed increases that confirmed increased activity of Ca_v 1.2. Histological analysis revealed hypertrophic myopathy, disorganized myocytes, and calcium deposits. Isolated cells from transgenic animals did not respond to isoproterenol or forskolin. Investigation of the mechanism of decreased symbol β adrenergic receptor (βAR) response revealed significant activation of PKCα, which phosphorylates, and thus inhibits, βAR and βAR kinase. Given that this mouse model has the hallmarks of human cardiomegaly it will be a useful resource for understanding the role of calcium in this pathological mechanism.

L-TYPE VDCC, α_1 1.3

Because of the difficulties in selectively isolating L-type currents encoded by α_1 1.2 and 1.3 using pharmacological tools, Platzer *et al.* (2000) generated a null allele in α_1 1.3. Unlike α_1 1.2, viable and fertile null mice were recovered. The relative contribution of α_1 1.3 in neurons, muscle, and neuroendocrine cells was examined. The mice were found to be deaf and histological analysis revealed degeneration of inner hair cells (IHC) and outer hair cells (OHC) between postnatal days 14 and 35, after the onset

of deafness. Patch clamp analysis of IHCs revealed that α_1 1.3 contributes >90% of the VDCC current. Deafness is explained by the lack of coupling of mechanically induced depolarization to neurotransmitter release in IHCs. The known expression of α_1 1.3 in atrial cardiomyocytes prompted an investigation of cardiac function. Abnormalities included brachycardia, arrhythmia, and AV block. No morphological alterations were detected. Denervation and pharmacological treatments showed the phenotype was due to an intrinsic defect in sinoatrial node pacemaker activity. The authors reported normal fasting serum glucose and insulin levels in 5–8-week-old mice with normal glucose and insulin tolerance. A later report showed deficiencies in postnatal expansion of pancreatic β cells in null mice (Namkung *et al.*, 2001) in spite of compensatory upregulation of α_1 1.2. This group reported that null mice were hypoinsulinemic and glucose intolerant at 12–14 weeks of age. The discrepancy might be due to age or the different genetic backgrounds of the mutant mice.

L-TYPE VDCC, α_1 1.4

Disease Alleles in Humans

Positional cloning efforts by two groups identified incomplete X-linked congenital stationary night blindness (CSNB) as a novel α_1 gene encoding an L-type VDCC (Bech-Hansen *et al.*, 1998; Strom *et al.*, 1998). This disease is identified as a nonprogressive retinopathy that results in night blindness, reduced visual acuity, myopia, nystagmus, and strabismus. Although electroretinograms are abnormal, residual rod and cone function is evident. The disease is thought to reflect a defect in retinal neurotransmission (Hood and Greenstein, 1990). The tissue specificity of the phenotype is readily explained by the restricted expression of α_1 1.4 which is detected only in the outer and inner nuclear layers of the retina and the ganglion cell layer but no other tissues.

A very large collection of disease alleles of the α_1 1.4 gene has been identified in patients with CSNB (Boycott *et al.*, 2001; Jacobi *et al.*, 2003; Strom *et al.*, 1998; Wutz *et al.*, 2002; Zito *et al.*, 2003) (Table II).

The majority of these alleles are truncating mutations and they are distributed throughout the coding sequence. A minority (19 of 47 or 40%) are missense or inframe deletion mutations and they are also distributed throughout the coding sequence. Since α_1 1.4 is X-linked, these mutations disrupt the only copy of the gene in males, suggesting the disease is caused by simple loss of function. A phenotype in carrier females, who might be mosaic for the disruption, has not been reported. Substantial clinical variability is seen and further genetic analysis might identify interacting genes and environmental factors (Boycott *et al.*, 2000).

Photoreceptor cells are hyperpolarized by light and consequently reduce neurotransmitter release. By analogy, it has been suggested that decreased calcium mediated neurotransmitter release from photoreceptors, due to the loss of α_1 1.4, would keep ON-bipolar cells depolarized and limit dark adaptation. An explanation for nystagmus, myopia, and strabismus in these patients remains to be elucidated.

P/Q-TYPE VDCC, α_1 2.1

Disease Alleles in Humans

The discovery of disease-associated mutations in the α_1 2.1 gene has led to unexpected and compelling observations about the in vivo function of the P/Q VDCC. As reviewed previously (Felix, 2000; Fletcher *et al.*, 1998; Jen, 1999; Pietrobon, 2002), in 1996 mutations causing familial hemiplegic migraine (FHM, OMIM #141500) and episodic ataxia type 2 (EA2, OMIM # 108500), located on chromosome 19p13 (Joutel *et al.*, 1993; Kramer *et al.*, 1995; Teh *et al.* 1995; von Brederlow *et al.*, 1995), were identified in the α_1 2.1 gene (Ophoff *et al.*, 1996). Soon after, another genetic screening strategy identified expanded triplet repeats in the α_1 2.1 gene in eight unrelated patients suffering from progressive ataxia, termed spinocerebellar ataxia type 6 (SCA6, OMIM # 183086) (Zhuchenko *et al.*, 1997).

In the few years since the original observations, significant mutation hunting efforts have identified additional FHM, EA2, and SCA6 alleles (Table III) (Battistini *et al.*, 1999; Carrera *et al.*, 1999; Denier *et al.*, 1999, 2001; Ducros *et al.*, 1999, 2001; Friend *et al.*, 1999; Gardner, 1999; Guida *et al.*, 2001; Jen *et al.*, 1998, 1999, 2000; Jodice *et al.*, 1997; Jouvenceau *et al.*, 2001; Kors *et al.*, 2001; Matsuyama *et al.*, 2003; Scoggan *et al.*, 2001; Vahedi *et al.*, 2000; van den Maagdenberg *et al.*, 2002; Yue *et al.*, 1997, 1998). Recently, epilepsy has also been associated with the CACNAIA gene, with a mutation being detected in one case (Chioza *et al.*, 2001, 2002; Jouvenceau *et al.*, 2001).

Clinical and Genetic Heterogeneity

This large collection of mutant alleles allows one to examine genotype/phenotype reationships and determine

Table II. CSNB Disease Alleles in $\alpha_1$1.4

	Nucleotide mutation[a]	Change	Position[b]	Reference
1	C148T	R50stop	NH2	Boycott *et al.*, 2001
2	AGAAA151-155del	FS		Wutz *et al.*, 2002
3	T220C	C74R	NH	Wutz *et al.*, 2002
4	C244T	R82stop	NH	Boycott *et al.*, 2001
5	AGCG466-del, ins34	Ins	I S2-3	Boycott *et al.*, 2001
6	Intron4, 2A > G	SS	I S3-4	Boycott *et al.*, 2001
7	T685C	S229P		Wutz *et al.*, 2002
8	G832T	E257stop		Zito *et al.*, 2003
9	G781A	G261R		Wutz *et al.*, 2002
10	CTT951-953del	F318del	PI	Boycott *et al.*, 2001
11	G1106A	G369D	I S6	Boycott *et al.*, 2001
12	C1218del	FS	I-II	Boycott *et al.*, 2001
13	C1282T	Q428stop		Wutz *et al.*, 2002
14	G1523A	R508Q	I-II	Strom *et al.*, 1998
15	C1840T	R614stop	II S4	Boycott *et al.*, 2001
16	G1988A	G663D	II S5	Boycott *et al.*, 2001
17	T225G	F742C		Wutz *et al.*, 2002
18	Intron19, G > C	SS		Wutz *et al.*, 2002
19	T2546C	L849P		Wutz *et al.*, 2002
20	C2650T	R884stop	III S1-2	Boycott *et al.*, 2001
21	Intron21, G > A	SS		Wutz *et al.*, 2002
22	Intron21, CA > del	SS		Wutz *et al.*, 2002
23	C2750A	A917D	III S2	Boycott *et al.*, 2001
24	C2172C	R958stop	III S4	Strom *et al.*, 1998
25	Intron24, G > A	SS	III S4-5	Boycott *et al.*, 2001
26	CAT2973-2975del	1992del	III S5	Boycott *et al.*, 2001
27	G3019A	G1007R		Wutz *et al.*, 2002
28	G3092del	FS	PIII	Boycott *et al.*, 2001
29	3133insC	FS	PIII	Strom *et al.*, 1998
30	C3145T	R1049W	PIII	Strom *et al.*, 1998
31	T3203C	L1068P		Wutz *et al.*, 2002
32	C3640del	FS	IV S2	Boycott *et al.*, 2001
33	Del3658-3669	12 bp del	IV S2	Strom *et al.*, 1998
34	C3862T	R1288stop	IV S4	Boycott *et al.*, 2001
35	Intron 33, T > A,C	SS		Wutz *et al.*, 2002
36	C4042T	Q1348stop	PIV	Strom *et al.*, 1998
37	T4091A	L1364H	PIV	Strom *et al.*, 1998
38	Intron35, G > C	SS		Wutz *et al.*, 2002
39	G4320A	W1440stop	IV S6	Boycott *et al.*, 2001
40	T4462C	C1488R		Wutz *et al.*, 2002
41	C4466G	P1489R		Wutz *et al.*, 2002
42	T4490C	L1497P		Wutz *et al.*, 2002
43	C4548de1	FS		Jacobi *et al.*, 2003
44	Intron40, 2A > G	SS	COOH	Boycott *et al.*, 2001
45	A4771T	K1591stop	COOH	Strom *et al.*, 1998
46	C5446T	R1816stop		Wutz *et al.*, 2002
47	G5632del	FS	COOH	Boycott *et al.*, 2001

[a] All mutations were remapped onto NM_005183, using the beginning of the open reading frame as position 1.
[b] Position indicated by domain and segment.

the penetrance of the phenotype. Thus, it is important to determine if clinically distinguishable diseases are caused by different types of alleles, and also if a single lesion, or type of lesion, can give rise to a variable phenotype. A related question is penetrance, i.e., how often do carriers not express disease symptoms? These investigations can provide hints about the existence of interacting alleles or environmental factors that modify the course of the disease. A complex picture has emerged from these studies.

Table III. $\alpha_1$2.1 Human and Mouse Disease Alleles

	Mutation[a]	Change	Phenotype	Position[b]	Reference
1	G850A	R192Q	FHM	I S4	Ophoff *et al.*, 1996
2	G859A	R195K	FHM	I S4	Ducros *et al.*, 2001
3	C928T	S218L	FHM/Edema	I S4/5	Kors *et al.*, 2001
4	C1032T	*H253Y*	EA2	PI	van den Maagdenberg *et al.*, 2002
5	G1152A	G293R	PCA	PI	Yue *et al.*, 1997
6	Intron11,G > A	SS	EA2	II S2	Denier *et al.*, 1999
7	G2023A	R583Q	FHM/AX	II S4	Battistini *et al.*, 1999
8	TTCA2145-8*del*	FS	EA2	II S5	van den Maagdenberg *et al.*, 2002
9	2214-6	P647L[c]	AX/AE	PII	Fletcher *et al.*, 1996
10	C2272T	T666M	FHM/AX	PII	Ophoff *et al.*, 1996
11	AG2317-8*del*	FS	EA2	PII	Denier *et al.*, 1999; van den Maagdenberg *et al.*, 2002
12	T2416C	V714A	FHM	II S6	Ophoff *et al.*, 1996
13	C2420G	D715E	FHM/AX	II S6	Ducros *et al.*, 1999
14	*Ins*3092G	FS/1067stop	EA2	II-III	Scoggan *et al.*, 2001
15	*Ins*3689C	FS/1144stop	EA2	II-III	Matsuyama *et al.*, 2003
16	C4073*del*	FS/1294stop	EA2	III S1	Denier *et al.*, 1999; Ophoff *et al.*, 1996
17	C4110T	R1279stop	EA2	III S2	Yue *et al.*, 1998
18	Intron24,G > A	SS	EA2	III S3	Ophoff *et al.*, 1996
19	A4281G	K1336E	FHM	III S3/4	Ducros *et al.*, 2001
20	4353-5	R1360G[c]	AX	III S4	Mori *et al.*, 2000
21	A4429G	Y1385C	FHM/AX	III S5	Vahedi *et al.*, 2000
22	C4451*del*	FS	EA2	III S5	van den Maagdenberg *et al.*, 2002
23	T4486G	*F1404C*	EA2	III S5/PIII	Jen *et al.*, 2001
24	Intron26, G > A	SS	EA2	III S5/PIII	Denier *et al.*, 1999
25	4497-9	T1407K[c]	AX/AE	PIII	Zwingman *et al.*, 2001
26	C4607G	Y1444stop	EA2	PIII	Denier *et al.*, 1999
27	G4644T	V1457L	FHM	PIII	Carrera *et al.*, 1999
28	T4747C	*F1491S*	EA2	III S6	Guida *et al.*, 2001
29	C4914T	R1547stop	EA2	III/IV	Denier *et al.* 1999; Jen *et al.*, 1999
30	CTT5056-8*del*	*AY1593/4D*	EA2	IV S1/2	Denier *et al.*, 1999
31	G5123*del*	FS/1624stop	EA2	IV S2	Scoggan *et al.*, 2001
32	G5260A	*R1662H*	EA2	IV S4	Friend *et al.*, 1999
33	C5277T	R1668W	FHM/AX	IV S4	Ducros *et al.*, 2001
34	T5320C	L1682P	FHM/AX	IV S4	Gardner, 1999
35	T5325C	W1684R	FHM/AX	IV S4	Ducros *et al.*, 2001
36	G5361A	V1696I	FHM	IV S5	Ducros *et al.*, 2001
37	G5544A	*E1757K*	EA2	PIV	Denier *et al.*, 2001
38	A5706C	I1811L	FHM/AX	IV S6	Ophoff *et al.*, 1996
39	C5733T	R1820stop	AE	IV S6	Jouvenceau *et al.*, 2001
40	Intron42	FS[c]	AX/AE	COOH	Fletcher *et al.*, 1996
41	7213-7245	N-CAG	SCA/EA2	COOH	Zhuchenko *et al.*, 1997

Note. FHM –Familial hemiplegic migraine; PCA –progressive cerebellar ataxia; AX –ataxia; AE –absence epilepsy; EA2 –episodic ataxia 2; SCA –spinocerebellar ataxia 6.

[a] Mutations were mapped to X99897 as a common reference, numbered from nucleotide 1, ORF begins at 276.

[b] Position indicated by domain and segment.

[c] Mouse allele.

FHM is recognized by dominantly inherited migraine with aura and ictal hemiparesis. Severe atypical attacks can include fever, coma, or seizures (Ducros *et al.*, 2001). In many cases there are also cerebellar deficits, including cerebellar atrophy. EA2 is recognized by stress- or exerise-induced ataxic attacks associated with interictal nystagmus. Often this disease is responsive to acetazolamide. In some cases progressive vermal cerebellar atrophy is also detected. SCA6 is recognized by permanent ataxia that progresses over 20–30 years, nystagmus, and cerebellar atrophy. On further investigation it is clear that there is significant overlap in these diseases. As more patients harboring α_1 2.1 mutations were identified and examined, it became clear that FHM patients could be grouped into those with pure migraine and migraine with cerebellar signs (Ducros *et al.*, 2001) (Table III). Interictal

signs include nystagmus, ataxia, and dysarthria. As larger collections of genetically diagnosed patients have been identified, it is also clear that there is significant clinical variability even among patients harboring the same disease allele (Alonso *et al.*, 2003; Kors *et al.*, 2003). EA2 is similarly heterogeneous, encompassing paroxysmal attacks as well as permanent and progressive symptoms (Baloh *et al.*, 1997; Denier *et al.*, 1999). Similarly, it is recognized that frank SCA6 symptoms can be preceded by and coexist with paroxysmal episodes. The most significant differences between EA2 and SCA6 are the extracerebellar signs unique to SCA6 (Frontali, 2001) but these are present only in a small subset of patients. Since the onset of SCA6 is later on average than EA2, it has been suggested that these differences could be related simply to the progression of the disease.

In general, FHM alleles are missense mutations, and thus are not obviously nulls. They are not randomly distributed as they tend to cluster in S4, P, and S6 segments. There is no obvious difference that readily explains pure FHM versus FHM with ataxia. There is however a correlation between specific alleles and severity. For example, patients with T666M have a higher frequency of attacks compared to patients with the D715E allele (Ducros *et al.*, 2001). The preponderance of EA2 alleles are truncating mutations (12/18 are nonsense, frameshift, or splice site) but several EA2 missense mutations have also been identified. Interestingly, all EA2 alleles tested so far, including three missense and two truncating mutations, show severe loss of function (see below). The exceptional mutation is the G293R missense allele that gives rise to either a milder episodic/progressive ataxia or severe progressive ataxia in different individuals. Although the episodes were stress induced they are refractory to acetazolmide. Importantly, these patients did not have extracerebellar signs that are seen in SCA6 (Yue *et al.*, 1997).

The CAG repeats range from 4 to 18 in controls and 19 to 30 in affected patients. The expansion is present near the 3′ end of the α_1 2.1 gene and affects coding sequence in only one isoform of the mRNA which, by virtue of a five nucleotide insertion just before the stop codon, extends the open reading frame into what had previously been considered 3′ untranslated sequence. This observation has been confirmed in European and Japanese pedigrees with consistent molecular features: this expansion may account for as much as 30% of ataxia cases in Japan (Matsumura *et al.*, 2003; Matsuyama *et al.*, 1997). The CAG tracts in SCA6 patients are also not unstable, unlike other triplet expansions, which tend to increase in size between generations; anticipation has not been noted in SCA6. The large collection of patients harboring CAG expansions has confirmed an inverse correlation of onset with repeat size (Frontali, 2001; Geschwind *et al.*, 1997). In addition, gene dosage has an effect, with homozygous patients showing earlier onset and more severe symptoms (Kato *et al.*, 2000; Matsumura *et al.*, 2003). Interestingly, the smaller CAG expansions can present as EA2 (Jen *et al.*, 1998; Jodice *et al.*, 1997; Koh *et al.*, 2001). It has been proposed that the function of the channel per se is affected. This alternate splice form is present in the mouse, although no glutamates are present. In fact, the COOH terminus is the region of lowest sequence similarity between mouse and human (Fig. 2).

The point mutation described in the epileptic patient introduces a premature stop codon just after domain IV (Jouvenceau *et al.*, 2001). The patient presented with epilepsy, episodic ataxia, and chronic progressive ataxia. The epilepsy included generalized tonic-clonic seizures at an early age, but only absence episodes continued in adulthood.

In summary, it has become very clear that a genotype–phenotype correlation cannot be established for these disease alleles. However, the identification of these mutations raises several questions: (1) what is the mechanism that triggers these episodes?;(2) is there a mechanistic difference between the permanent and progressive symptoms?; and (3) what underlies the differences in the tissue specificity?

Mouse Models

In 1996, a positional cloning effort identified the mouse *Cacnala* gene as the tottering (*tg*) locus. Currently four alleles of tottering have been described. The tottering strain exhibits mild ataxia and paroxysmal dyskinesia. Leaner (tg^{la}), has severe ataxia and progressive degeneration of the anterior vermis of the cerebellum. Rolling mouse Nagoya, identified in 1973 (Oda, 1973), presents with a wobbling gait. Recently, a new allele, rocker, was described (Zwingman *et al.*, 2001). The phenotype of the rocker mouse (tg^{rkr}) includes, in addition to ataxia and absence seizures, aberrant "weeping" Purkinje cell dendrites. In addition, unlike the other alleles, rocker does not show ectopic spines, axonal torpedoes, or persistent tyrosine hydroxylase expression. The other known allele, tg^{3j}, is not widely available and little is known about it. Electroencephalograph recordings show frequent (l/min) spontaneous generalized spike and wave discharges associated with behavioral arrest in these mice (Noebels and Sidman, 1979). Tottering alleles are considered a model of absence epilepsy.

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ASMPRLPAENQRRRGRPRGNNLS|TISDTSPMKRSASVLGPKARRLDDYSL
ASMPRLPAENQRRRGRPRGNDLS|TISDTSPMKRSASVLGPKARRLDDYSL

ERVPPEENQRHHQRRRDRSHRASERSLGRYTDVDT|GLGTDLSMTTQSGDL
ERVPPEENQRYHQRRRDRGHRTSERSLGRYTDVDT|GLGTDLSMTTQSGDL

PSKERDQERGRPKDRKHRQHHHHHHHHHHPPPPDKDRYAQERPDHGRARAR
PSKDRDQDRGRPKDRKHRPHHHHHHHHHHPPAPDRDRYAQERPDTGRARAR

DQRWSRSPSEGREHMAHRQGS|SSVSGSPAPSTSGTSTPRRGRRQLPQTPS
EQRWSRSPSEGREHTTHRQGS|SSVSGSPAPSTSGTSTPRRGRRQLPQTPC

TPRPHVSYSPVIRKAGGSGPPQQQQQQQQQQQAVARPGRAATSGPRRYPGP
TPRPLVSYSPAPR----------RP------AARKMAGP

TAEPLAGDRPPTGGHSSGRSPRMERRVPGPARSESPRACRHGGARWPASGP
AAPPGG----------SPRGCRR-APRWPAHAP

HVSEGPPGPRHHGYYRGSDYDEADGPGSGGGEEAMAGAYDAPPPVRHASSG
---EGPR-P------RGADYTEPDSP-----REPPGGAHDPAP--------

ATGRSPRTPRASGPACASPSRHGRRLPNGYYPAHGLARPRGPGSRKGLHEP
---RSPRTPRAAG--CASP-RHGRRLPNGYYAGHGAPRPR--TARRGAHDA

YSESDDDWCN
YSESEDDWC
```

Fig. 2. *Aligned human and mouse amino acid sequence at the carboxy terminus.* The human (top) and mouse (bottom) protein sequences are shown. Vertical bars indicate the boundaries of exons. The last exon encodes a polyglutamate repeat in the human protein (polyQ) which is not present in the mouse. The protein sequences encoded by the human and mouse *Cacna1a* genes generally show > 95% similarity, with two exceptions, part of the II–III linker and the carboxy terminus.

Tottering is a missense mutation (P649L) in the second pore lining region and tg^{la}, the most severe allele, is a truncation of the protein near the carboxy terminus (Fletcher *et al.*, 1996). Rolling Nagoya (tg^{rmn}) was found to be a missense mutation in the S4 segment of domain III (R1310G) (Mori *et al.*, 2000). The molecular lesion in rocker is a missense mutation (T1358K) in the third pore lining domain. Thus, similar to the human mutations, missense alleles are less severe than truncations. Unlike the human, the mouse mutations are recessive (Fig. 3).

Because none of the spontaneous alleles seemed likely to be null mutations, two null mutations were engineered and characterized (Fletcher *et al.*, 2001; Jun *et al.*, 1999). The null allele shows degeneration, indicating that cell death is likely not caused by gain of function mutation in leaner mice.

Functional Characterization of Human Disease Mutations

Functional characterization of human disease alleles has relied on heterologous expression systems (HEK cells or *Xenopus* oocytes) in which engineered α_{1A} constructs and selected modulatory subunits are coexpressed. To date seven FHM missense mutations have been analyzed (Hans *et al.*, 1999; Kraus *et al.*, 1998, 2000) Melliti *et al.*, 2003. In all cases a hyperpolarizing shift in activation was found, but effects on current density, conductance, and inactivation properties were different for each mutation. Somewhat more consistent effects were observed when mutant channels were expressed by transfection of neurons from α_{1A} null mice, as channel density was consistently reduced (Tottene *et al.*, 2002). Tottene *et al.* speculate that

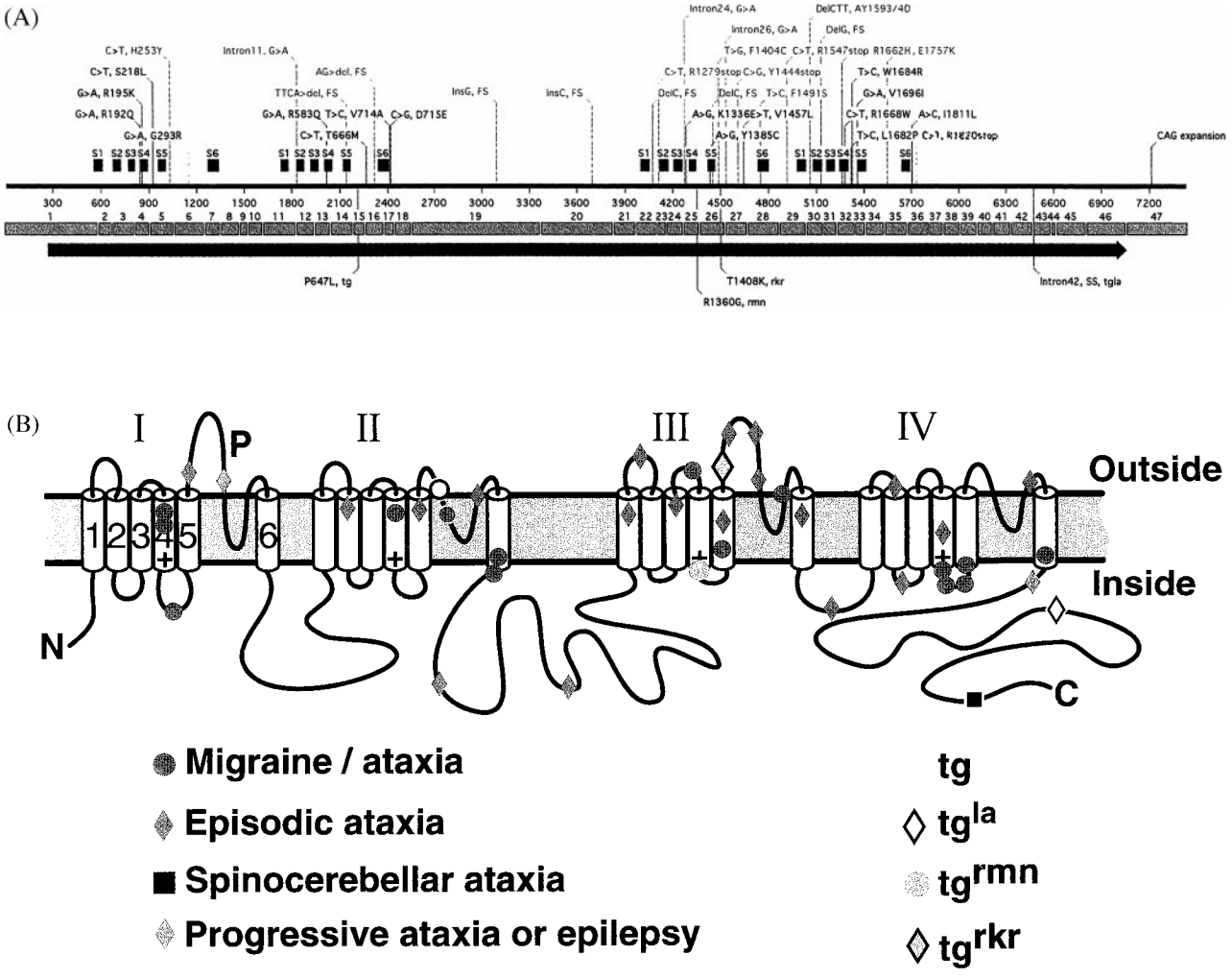

Fig. 3. *Summary of CACNAIA mutations in man and mouse.* (A) The top lines show the positions of human mutations in the CACNAIA cDNA. Blue lines are FHM, red lines are EA2 associated mutations. The progressive ataxia and epilepsy mutations are shown in green. The transmembrane domains are labeled and shown as blue boxes. The exon structure is indicated below the ruler with exons shown as red boxes. The open reading frame is indicated as a solid green arrow, and does not include the alternate splice that extends the ORF into the 3′ UTR. Mouse mutations are shown below. Migraine/ataxia mutations appear to cluster in the S4, P, and S6 segments. (B) The mutations are indicated on a topological representation of the *Cacna1a* protein.

equivalent trafficking of mutant and wild-type channels to the synapse results in increased calcium flux and network hyperexcitability. This is consistent with what is known about cortical spreading depression, in relation to aura, as reviewed in (Pietrobon and Striessing (2003). Another mechanism of hyperexcitability has been described for the R192Q mutation, which is resistant to G-protein-mediated inhibition (Melliti *et al.*, 2003).

Several missense and nonsense mutations (R1279stop, F1404C, R1547stop, AY1593/1594D, F1491S) associated with EA-2 have also been characterized and they consistently show pronounced loss of function (Guida *et al.*, 2001; Jen *et al.*, 2001; Wappl *et al.*, 2002). Specifically, the R1279 construct, when transfected into tsA-201 or COS7 cells showed no, or vastly reduced, current (R1279stop = 0.4 vs. wt = 56 pA/pF) (Jen *et al.*, 2001; Wappl *et al.*, 2002). The R1404C and R1547stop construct gave similar results in COS7 cells (0.88 and 0.45 pA/pF, respectively) (Jen *et al.*, 2001). The AY1593/1594D construct yielded no current in tsA-201 cells, however, injection of AY1593/1594D encoding RNA into *Xenopus* oocytes yielded channels with altered biophysical properties (depolarizing shift in voltage dependence of activation, increased voltage-dependent

inactivation) (Wappl *et al.*, 2002). Likewise, the F1491S construct yielded no current above background in HEK 293 cells (Guida *et al.*, 2001). The R1279stop, F1491S, and AY1593/1594D constructs expressed protein, as detected by Western blot or immunocytochemistry (Guida *et al.*, 2001; Wappl *et al.*, 2002). To test the hypothesis that truncated protein could interfere with the function of the wild-type channel, RNA encoding the R1279stop allele was coinjected with wild-type RNA, but a dominant negative effect was not observed (see R1820stop below) (Wappl *et al.*, 2002). Thus, it appears that EA2 mutations show a more severe loss of function than FHM associated mutations.

Wappl *et al.* also characterized the G293R mutation that has the unusual feature of being associated with either episodic, acetazolamide-insensitive or progressive ataxia in different individuals. In *Xenopus* oocytes these channels showed decreased current, a depolarizing shift in voltage dependence of activation and increased inactivation. In HEK cells decreased current density and mean open time was observed. Thus, similar to EA2 mutations, this allele shows loss of function.

There is significant discordance among characterizations of CAG expansions. When expressed in HEK cells, these mutant alleles showed an increased current density but otherwise normal properties (Piedras-Renteria *et al.*, 2001) or a decreased current density and a hyperpolarizing shift in the voltage dependence of inactivation (Matsuyama *et al.*, 1999). In one report, mutant alleles showed a negative or positive shift in inactivation dependence, depending on which splice isoform (+ or −NP) was expressed (Toru *et al.*, 2000). Finally, a fourth report described a β subunit dependent effect of the mutation (Restituito *et al.*, 2000). The differences between these results highlight the need for careful analysis of channel mutants in the proper neuronal environment.

Since the R1820stop mutation is located distal to domain IV it was hypothesized that this truncated protein could still form a channel. However, when the R1820stop construct was injected into Xenopus oocytes no voltage-dependent calcium current was detected. When coinjected with wild-type RNA the mutant construct suppressed the wild-type channel (Jouvenceau *et al.*, 2001). The R1820stop allele thus appears to act as a dominant negative, but the molecular mechanism is unknown.

In summary, it is tempting to speculate that combined gain and loss of function effects (taken as calcium flux) at the single channel and whole cell levels, which depend on depolarization, could account for the complexity of the FHM/AX phenotype (Pietrobon and Striessinig, 2003; Tottene *et al.*, 2002). Further, one would hypothesize that ataxia of FHM/AX and EA2 would be principally due to loss of function. Further work will be required to evaluate this suggestion.

Functional Characterization of Mutant Channels in Mouse

Several papers have reported characterizations of calcium current in tottering, leaner, and rolling Nagoya mice. In two reports, leaner Purkinje cells show a significant (60%) decrease in current density and no other changes (Dove *et al.*, 1998; Lorenzon *et al.*, 1998). In primary cultures, Wakamori *et al.* report a less severe reduction in tottering Purkinje cell current density (40%) compared to leaner (60%), consistent with the milder tottering phenotype. Leaner also showed depolarizing shifts in voltage dependence of activation and inactivation; this was specific to the longer leaner isoform in transfected cells (Wakamori *et al.*, 1998). Similarly, Purkinje cells from rolling mice showed reduced current density and a depolarizing shift in voltage dependence of activation (Mori *et al.*, 2000). Thus, biophysical alterations, causing loss of function, correlate with phenotype severity. Two α_1 2.1 targeted null alleles have been generated, deleting either exons 3–4 (NH terminus) or exons 14–17 (domain II) (Fletcher *et al.*, 2001; Jun *et al.*, 1999). In both cases, the mice were severely affected, usually not surviving past weaning. In mice heterozygous for the 5' deletion there was upregulation of the wild-type allele, in that normal levels of P/Q current were detected. In contrast, mice heterozygous for the domain II deletion did not show compensation, rather, a 50% reduction in P/Q current was observed. Careful examination of these mice detected no deficits. This result suggests that the reduction in current density seen in tottering mice cannot alone account for the phenotype, and the pathological mechanism is yet to be described. Cerebellar neurodegeneration can be caused by loss of function, as this was observed in aged homozygous null mice. Interestingly, both groups reported an increase of N- and L-type currents, showing that compensatory changes in VDCCs can occur.

Characterization of Network Function

The phenotypes of epilepsy and migraine aura indicate that these mutations have an effect at the network level as well. Emerging evidence has strengthened the suggestion that cortical spreading depression (CSD) underlies aura (Hadjikhani *et al.*, 2001; James *et al.*, 2001). While it has not been possible to study the electrophysiology of CSD directly in human, this problem could be approached by engineering the human migraine alleles in the mouse.

Interestingly, it has recently been shown that tottering mice are impaired in the initiation and propagation of evoked CSD (Ayata *et al.*, 2000). A key difference between the human and mouse mutant alleles is the opposite shift in voltage dependence of activation and knockin mice harboring human mutations will be invaluable for examining the relationship between the migraine alleles and CSD.

Absence epilepsy is a common feature of α_1 2.1 mutations in mice. Synaptic function has also been examined and in several studies a reduction in excitatory but not inhibitory synaptic transmission was uncovered (Caddick *et al.*, 1999; Helekar and Noebels, 1994; Leenders *et al.*, 2002). These observations suggested a mechanism by which these mutations could contribute to network hyperexcitability. In this model, a relative increase in inhibitory input is critical, since inhibitory interneurons play a key role in synchronizing discharges in the thalamocortical circuit that underlies absence seizures.

N-TYPE VDCC, α_1 2.2

Characterization of Targeted Null Allele

Historically the Ca_v 2.2 channel has been shown to play a key role in neurotransmitter release in central and peripheral synapses. In addition, a large number of studies have implicated the Ca_v 2.2 channel in nociception, not only because of its expression pattern but also because of the pain blocking effects of N-type channel antagonists. Thus a nociception phenotype was of significant interest to the groups that have constructed and characterized α_1 2.2 null mice (Hatakeyama *et al.*, 2001; Ino *et al.*, 2001; Kim *et al.*, 2001; Saegusa *et al.*, 2001). Somewhat unexpectedly, α_1 2.2 homozygous null mice proved to be viable and lack any overt phenotype. Several assays for pain perception have identified deficits in the null mice (Table IV). Although there are some discrepancies between the reports, the null mice consistently showed diminished pain responses in various assays. First, spinal reflexes to mechanical (von Frey filaments) and thermal (tail and paw flick) stimuli were examined. Two of three reports showed no changes in response to acute mechanical stimuli. While two groups reported decreased sensitivity to thermal stimulus of tail only one reported a change in paw flick. Supraspinal involvement was assessed by hotplate and no change was observed by two of the three groups. All groups reported a deficit in response to inflammatory pain in the formalin assay as reflected in a diminished response in the late phase of the assay. The groups who investigated the response to visceral inflammatory pain (writhing assay) differed in their observations. Activation of the descending antinociceptive pathway by pre-

Table IV. Phenotypes Reported for $\alpha_1$2.2 Homozygous Null Mice

Assay	Saegusa *et al.*, 2001	Kim *et al.*, 2001	Hatakeyama *et al.*, 2001
von Frey	No change	−Response	No change
Tail flick	+Latency	+Latency	
Paw flick	No change	+Latency	
Hot plate	No change	No change	+Latency
Formalin Ph I	No change	No change	No change
Formalin Ph II	−Reaction	−Reaction	−Reaction
Writhing	No change	Reduced	
Antinociception	Impaired		
Neuropathic	Impaired		

conditioning noxious visceral stimulation showed a deficit in null mice. Importantly, a deficit in neuropathic pain (spinal nerve ligation paradigm) was also reported in null mice.

In a general search for altered behavior in null mice several assays for anxiety and startle were performed. A significant decrease in anxiety related behaviors was reported (Saegusa *et al.*, 2001). A high degree of exploratory behavior shown by mice placed in a brightly lit arena (open field assay) or on an elevated platform with open and enclosed arms (elevated plus maze) is considered to be inversely related to anxiety. By these measures, homozygous null α_1 2.2 mice showed decreased anxiety.

Finally, sympathetic nerve activity was shown to be markedly diminished in null mice (Ino *et al.*, 2001). First, the carotid baroreflex was impaired in null mice. Second, direct stimulation of sympathetic nerves elicited no response in isolated atria, in contrast, function of the parasympathetic system was normal. Unexpectedly, the mice had chronic elevation of heart rate and blood pressure. This occurred in the absence of increased serum norepinephrine or upregulation of β-adrenergic receptors. Further characterization of the consequences of sympathetic nervous system failure in these mice has been proposed.

R-TYPE VDCC, α_1 2.3

Characterization of Targeted Null Allele

The molecular basis of the R-type VDCC has been difficult to elucidate, in part because of the lack of pharmacological agents that specifically block its function. A recently identified inhibitor (SNX-482) blocks α_1 2.3 encoded R-type current in heterologous systems, but not all R-type current in neuronal preparations (Newcomb *et al.*,

1998). Because heterologously expressed α_1 2.3 gives rise to channels that do not always have a neuronal conterpart another strategy was adopted. Using antisense oligonucleotides targeting α_1 2.3, two groups were able to inhibit R-type current in transfected cerebellar neurons, although to different degrees (Piedras-Renteria and Tsien, 1998; Tottene *et al.*, 2000). Interestingly, Tottene *et al.* showed antisense inhibition of SNX-482 sensitive and insensitive R-type currents. The genetic approach of ablating the α_1 2.3 gene should provide insight into what component of the R-type current is encoded by α_1 2.3, and reveal the functional role of α_1 2.3 encoded channels. Several groups have generated α_1 2.3 knockouts and pursued different analyses of each (Lee *et al.*, 2002; Saegusa *et al.*, 2000; Wilson *et al.*, 2000).

Interestingly, the α_1 2.3 gene is not required for development as viable and overtly normal homozygous null mice were recovered. Because of the expression of α_1 2.3 in primary afferent neurons in the dorsal root ganglia (DRG) and dorsal horn of the spinal cord Saegusa *et al.* investigated a possible effect on nociception. While normal responses were observed to acute mechanical, thermal, and chemical stimuli, null mice were deficient in response to inflammatory pain. Presensitization studies further indicated a deficit in antinociceptive pathways. Both α_1 2.2 and α_1 2.3 null mice show this deficit. The authors speculate that these channels are involved in controlling the excitability of neurons in the descending antinociceptive pathways (periaqueductal grey matter and/or raphe magnus neurons).

Because the mice appeared to be "timid" or "anxious" various tests for anxiety were performed (described above). Increased anxious behavior was detected in the open field test although no difference was observed in elevated plus maze or startle tests. The α_1 2.3 gene is also highly expressed in the hippocampus and therefore learning and memory was evaluated in these mice (Kubota *et al.*, 2001). Cue and contextual memory in a fear conditioning paradigm was normal in null mice. A deficit in spatial memory was revealed by Morris water maze, although this may be influenced by the anxious nature of the mutants. No deficit in long-term potentiation was observed in hippocampal slice preparations. Two other biological systems were investigated based on the expression pattern of α_1 2.3 First, expression in the pancreas suggested a possible effect on insulin and glucose homeostasis (Matsuda *et al.*, 2001). In fact null mice exhibit hyperglycemia. Second, the role of α_1 2.3 in sperm function was examined (Sakata *et al.*, 2001, 2002). Because of previous questions about the role of α_1 2.3 in LVA currents detected in immature sperm, electrophysiological characterization of the null mice was performed. In pachytene stage spermocytes the Ca_v 2.3 channel makes no contribution to LVA currents. A requirement for α_1 2.3 function in mature sperm was identified given that sperm from null mice show aberrant calcium transients and unusual motility. Thus, these studies have revealed a range of subtle but intriguing deficits in α_1 2.3 null mice.

Two groups have pursued the relationship between α_1 2.3 encoded channels and R-type current. Wilson *et al.* reported loss of only the SNX-482 sensitive component of the R-type current in α_1 2.3 homozygous null mice. This was a minor fraction of the R-type current in the cells (cerebellar granule and DRG neurons) that were examined. Lee *et al.* reported a decrease of 84% of R-type current in central amygdala neurons. Since they didn't report on SNX-482 sensitivity, there may not be a conflict in the data. Further examination of R-type currents in other neurons should resolve this question.

T-TYPE VDCC, $\alpha_1$3.1

Characterization of Targeted Null Allele

The LVA calcium channels have several unique properties, including activation at very negative potentials, and the ability to promote burst firing in neurons. This characteristic, which is due to the inactivation properties of these channels, has been implicated in synchronized network discharges in the thalamus that underline sleep spindles and absence epilepsy seizures (spike and wave discharge, SWD) (Coulter *et al.*, 1989). Recently, there has been eroding support for the role of LVA currents (Leresche *et al.*, 1998; Pinault *et al.*, 1998; Steriade and Contreras 1995). In addition, controversy over cortical versus thalamic origins of SWDs has not been resolved (Steriade and Contreras, 1998). To identify a requirement for $\alpha_1$3.1 in thalamocortical (TC) relay neurons and define its function role in seizures, Kim *et al.* (2001) generated homozygous null mutant mice. The histology of the mutant brain is reported to be normal. Physiological analysis shows that LVA calcium currents are completely absent in the TC neurons of $\alpha_1$3.1 null mice, indicating that $\alpha_1$3.1 encodes the principal component of the T-type Ca^{2+} channels in TC neurons. No compensatory changes were observed in the activities of HVA VDCCs. Interestingly, the $\alpha_1$3.1 mutation only affects the rebound burst-mode potentials, which are absent in the TC neurons, as no difference was observed in tonic-mode firings. $\alpha_1$3.1 null mice are resistant to the genesis of spike and wave discharges induced by baclofen or γ-butyrolactone, however, the mutant mice exhibited SWD-like activities in response to systemic administration of bicuculline with

complex types of behavioral seizures. Administration of 4-aminopyridine at 10 mg/kg demonstrated that the mice developed tonic-clonic seizures Thus, the $\alpha_1$3.1 calcium channel plays a critical role in the generation of absence seizures in the thalamocortical network, but is not required for other types of seizures. The resistance of $\alpha_1$3.1 null mice to the generation of SWDs induced by baclofen is likely due to the absence of burst-mode firing generated by $GABA_B$ receptor-mediated membrane hyperpolarization.

SUMMARY

The genetic analysis of VDCCs provides an approach to defining channel function that is complimentary to pharmacological, electrophysiological, and other molecular methods. By discovering or creating alleles of VDCC genes, one can gain an understanding of the VDCC function at the whole animal level. The mutations described above illustrate how one can define the function of different channels in a complex biological system. For example, Ca_v 1.2, 1.3, and 2.1 play different roles in cardiac function, as reflected in their respective phenotypes of hypertrophy, node dysfunction, and sympathetic nervous system defects. An effect on the neuroendocrine system is seen in Ca_v 1.3 and 2.3 null mice; their phenotypes are lack of β cell proliferation and reduced insulin sensitivity with hyperglycemia, respectively. Likewise, Ca_v 2.2 and 2.3 are involved in the nociceptive system. However, there are some significant differences in the presensitization assays that may be related to different expression patterns in the nucleus raphe magnus. Interestingly, Ca_v 2.2, and 2.3 mutations appear to have opposite effects on the emotional state of the mice. Finally, loss of Ca_v 2.1 is associated with absence seizures, while Ca_v 3.1 is required for seizure events. The emerging picture is that the knockouts identify very specific deficits associated with particular channels. Thus it is now possible to define the functional role of the channel, localized to a particular cell or synapse, in the context of a biological process or system.

Interestingly, these genetic studies provide some support for biochemical and molecular studies on the association of specific β subunits and α1 poreforming proteins. Thus, certain mutant mice show significant phenotypic overlap. These include the Ca_v 2.1 and β4 strains, which both have ataxia and absence epilepsy. Only the β2 null mice show retinal degeneration (Ball *et al.*, 2002). The β3 null mice show deficits in nociception, similar to the Ca_v 2.2 and 2.3 null strains. As further detailed phenotyping is accomplished, using combination of different mutations, it is likely that more insight will be gained into the identity of particular channel complexes in vivo.

Only in a few cases is there an understanding of the pathological mechanism. For example, the loss of Ca_v 1.4 in the retina, Ca_v 1.3 in the ear, and Ca_v 2.2 from sympathetic neurons results in defects due to loss of neurotransmission at identified synapses. In other cases, such as Ca_v 2.1 associated diseases, tremendous progress has been made in the characterization of mutant alleles, but further work must be accomplished.

In the case of Ca_v 1.2 and 2.3, results from the null alleles suggest there are novel currents that have been uncovered. Thus, in the $\alpha_1$1.2 null a novel L-type like current has been identified. The $\alpha_1$2.3 null seems to indicate that not all R-type current is carried by this gene product. Breeding of double or triple knockouts may provide some answers.

Finally, perhaps the most surprising finding was that so many of the null alleles result in viable, fertile, and essentially normal mice. It does not appear that this can be explained by compensatory changes in other channels as this was not seen in many cases.

Ultimately, these data build a very promising foundation for drug development aimed at alleviating widespread and debilitating diseases, including autonomic system failure, metabolic syndrome, heart disease, ataxia, migraine, pain, and epilepsy.

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