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The Voltage-Gated Calcium Channel γ Subunits: A Review of the Literature

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Members of the voltage-gated calcium channel γ subunit gene family (Cacng), have been rapidly discovered since the discovery of the identification of the mouse $\gamma 2$ gene (Cacng2) and its association with the *stargazer* mutant mouse line. The fact that this mutant mouse line exists has allowed researchers to gain insights into the function of the $\gamma 2$ subunit. For example, *stargazer* mice have elevated levels of neuropeptide Y production, very low cerebellar brain derived neurotrophic factor production, and diminished cerebellar $GABA_A$ $\alpha 6$ and $\beta 3$ production. Study of this mutant mouse line has also revealed that the $\gamma 2$ subunit is involved in AMPA receptor trafficking and targeting to the synaptic membrane. For the most part, the effect of the γ subunits on the electrophysiology of voltage-gated calcium channels is to downregulate calcium channel activity by causing a hyperpolarizing shift in the inactivation curve. This finding and the association of these subunits with AMPA receptor trafficking has led some researchers to question the actual role of the γ subunits. This article reviews the discovery, cellular localization, tissue distribution, and function of the eight members of the Cacng family.

KEY WORDS: Voltage-gated calcium channel; γ subunit; *stargazer*; stargazin; epilepsy; absence seizure; PDZ domain; AMPA receptor.

INTRODUCTION

The purpose of this review is to describe the discovery, cellular localization, tissue distribution, and function of the eight putative members of the voltage-gated calcium channel (VGCC) γ subunit gene family (Cacng). To a significant extent, findings from the mutant mouse line, *stargazer*, have informed the characteristics and functions of this gene family, so a review of the mouse phenotype will be included. The study of this gene family has yielded important insights into how the vertebrate genome evolved so this will also be reviewed. Finally, despite the fact the a mutation in the mouse (Cacng2) causes an absence seizure phenotype, no diseases have been linked to any member of the γ subunit Cacng family. Testing of clinical hypotheses will also be reviewed.

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DISCOVERY OF THE VGCC γ SUBUNIT

The voltage-gated calcium channel (VGCC) γ subunit protein was identified in muscle by Glossmann *et al.* (1987) upon the purification of the 1,4-dihydropyridine (DHP) receptor from guinea pig skeletal muscle T-tubule membranes. Four polypeptides were copurified with this receptor which ultimately became known as the L-type skeletal muscle VGCC. The subunits were named $\alpha 1$, $\alpha 2$, β, and γ subunits, on the basis of size because they migrated at 180–190 kDa, 150–155 kDa, 55–60 kDa, and 28–35 kDa on SDS-PAGE, respectively. Sharp and Campbell (1989) and Campbell *et al.* (1989) obtained similar results. The γ subunit has since become known as the $\gamma 1$ subunit. Jay *et al.* (1990) described the nucleotide and amino acid sequence of the $\gamma 1$ subunit derived rabbit skeletal muscle cDNA. It was isolated from a cDNA library utilizing purified guinea pig polyclonal antiserum specific for purified $\gamma 1$ subunit protein. Overlapping cDNA clones yielded a 666 nucleotide opened reading frame encoding 222 amino acids with a calculated

0145-479X/03/1200-0649/0

molecular weight slightly over 25 kDa. Hydrophobicity plots predicted a 4 transmembrane subunit with intracellularly located amino- and carboxyl-termini. Electrophoretic analysis suggested a role for disulfide bonds in the three-dimensional confirmation of the protein. Ten cysteine residues were identified in the deduced protein. Bosse *et al.* (1990) similarly identified the cDNA for the rabbit $\gamma 1$ subunit and noted that the $\gamma 1$ transcript did not encode a signal peptide sequence.

Powers *et al.* (1993) described the cDNA for the human $\gamma 1$ subunit, which was isolated from a fetal skeletal muscle cDNA library using rabbit $\gamma 1$ cDNA as a probe. The isolated cDNA also predicted a 222-amino acid peptide (Fig. 1). Cosmids containing the human Cacng1 were also isolated revealing a gene with four exons spanning 12.5 kb of genomic DNA. Chromosomal localization of the human Cacng1 was to 17q23 (Powers *et al.*, 1993) and 17q24 (Iles *et al.*, 1993). Eberst *et al.* (1997) described the isolation of the rat skeletal muscle $\gamma 1$ subunit cDNA which encoded a 223-amino acid-protein that shared 84 and 79% identity, respectively, with the human and rabbit skeletal muscle $\gamma 1$ subunits. Wissenbach *et al.* (1998) isolated and characterized the mouse Cacng1 and found it had characteristics similar to the other species' $\gamma 1$ subunits. Amplification of $\gamma 1$ subunit cDNA yielded a low abundance of a second smaller fragment of about 500 bp in length in addition to full-length γ cDNA. The splice variant was named γ-b. It uses a second splice site within exon 1 leading to omission of part of exon 1 by causing a frameshift leading to a stop codon 180 nucleotides thereafter. The γ-b translated polypeptide was 68 amino acid residues long, hydrophilic, and lacked the typical membrane-spanning segments and glycosylation sites.

In 1998 Letts *et al.* (1998) discovered a mouse gene which encoded a neuronal VGCC γ subunit protein in the *stargazer* mutant mouse line which will be described below. The encoded protein was at first named "stargazin" and its discovery proved to be a watershed event leading to the discovery of six additional subunits in this family. For purposes of clarity, stargazin will be referred to $\gamma 2$ throughout this paper. The authors found the Cacng2 by building upon previous research that mapped the *stargazer* mutant to a 1.3 Mb contig of chromosome 15 (Letts *et al.*, 1997). The *stargazer* mutant was shown to have an early transposon insertion in the Cacng2 intron 2. Analysis of the unmutated Cacng2 predicted a 969 bp open reading frame encoding a protein of 323 amino acids with a molecular weight of 36 kDa. The Cacng2 and encoded protein had striking similarities to Cacng1 in terms of gene structure, intron sizes, and hydrophobicity plot although there was only modest amino acid identity (25% over 200 amino acids). The authors noted that human PAC dJ293L6 and PAC dJ1119A7 contained a possible homolog for mouse Cacng2 exons 1, 3, and 4. Black and Lennon (1999) built upon these results by cloning human $\gamma 2$ and $\gamma 3$ subunits from cerebellar cDNA. These authors reported the full-length amino acid sequence and predicted secondary structure of the human $\gamma 2$ and $\gamma 3$ subunits located on chromosomes 22q13.1 and 16p12-p13.1, respectively. They conducted BLASTN searches using the mouse $\gamma 2$ sequence found in the Letts *et al.* (1998) article. Burgess *et al.* (1999) and Green *et al.* (2001) subsequently confirmed the identification of the human Cacng3, Cacng4, and Cacng5 located on chromosome 17q24. Burgess *et al.* (1999) noted that the amino acid identity between the $\gamma 1$ subunit and $\gamma 3$, $\gamma 4$, and $\gamma 5$ subunits was between 22 and 26%. However, amino acid identity between the $\gamma 2$ subunit and $\gamma 3$, $\gamma 4$, and $\gamma 5$ was 84, 64, and 32%, respectively. The γ subunits were shown to exhibit low percentage amino acid identity but similar hydrophobicity profiles to claudin proteins, lens intrinsic membrane protein MP20, and peripheral myelin protein PMP22 (Sanders, *et al.*, 2001).

Klugbauer *et al.* (2000) identified mouse Cacng3, Cacng4, and Cacng5 which had characteristics similar to previously described subunits, with the exception of Cacng5 (AJ272046), which had a sequence quite unlike the human Cacng5 sequence and its gene had only two exons. Chu *et al.* (2001) described three new human and rat subunits Cacng, including human $\gamma 6$, $\gamma 7$, and $\gamma 8$ on chromosome 19q13.4 and conducted a phylogenetic analysis of all the known members of the human, mouse, and rat γ subunit Cacng family. They identified an extra intron in rat Cacng5 sequence which led them to suggested an additional coding region for the human ortholog whereby the extra sequence that exists in an intron in the rat sequence is also an intron in human. Thus the Cacng5 subunit is the only member of the gene family with five exons. The authors proposed that the mouse subunit, identified as Cacng5 (AJ272046) by Klugbauer *et al.* (2000), and related rat (AF361355) and human (AF361356) sequences be referred to as a mouse *p*roteins distantly *r*elated (pr) to the γ subunit Cacng family because these genes share only one common sequence motif (GLWXXC) with the other γ subunits and that motif is not unique to γ subunits since it is found in other four transmembrane domain proteins (e.g. lens intrinsic membrane proteins MP20). In addition, the exon/intron configuration of these genes contains only two exons. Nonetheless, the mouse pr protein was shown to modulate calcium current when expressed with $\alpha 1$G subunits by Klugbauer *et al.* (2000). Cacng8 was found to have an atypical initiation codon, -3AXXCTGG $+4$ and encodes an elongated intracellular C-terminal region terminated by a PDZ-(*P*SD-95/**S**AP-90, *D*iscs-large,

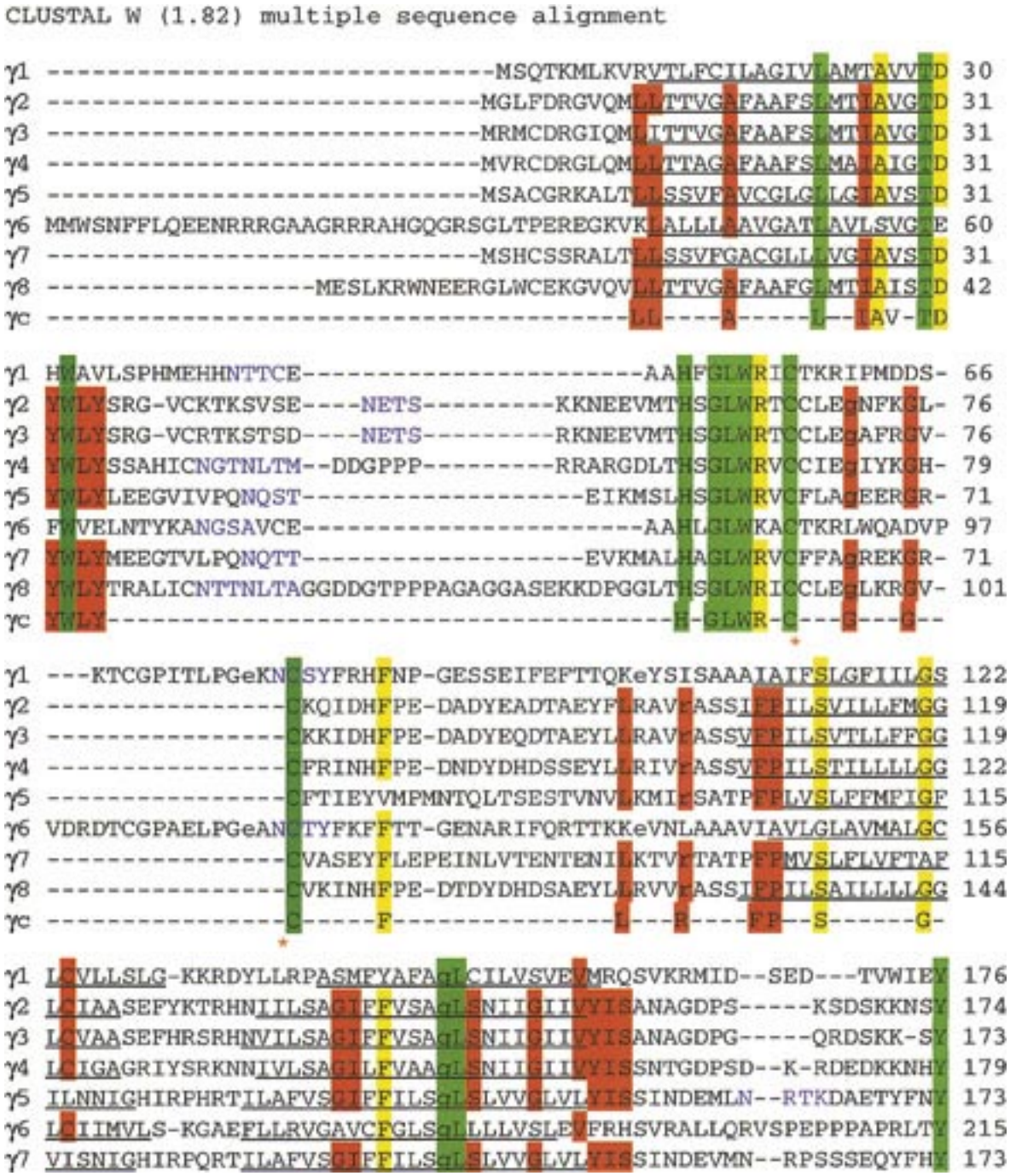

Fig. 1. γ1-8 amino acid sequences compiled by CLUSTAL W (1.82) program (http://www.ebi.ac.uk/clustalw/). Consensus sequence shows amino acids present in 6 (red), 7 (yellow), or all 8 (green) of the γ subunits. *Consensus cysteine residues might form disulfide bonds. Intron-exon spice sites determined by comparing sequenced cDNA data (γ1–5, 8) to GenBank data using BLAST resources. Lower case letter identifies splice codon sites. Blue: N-glycosylation site patterns predicted use ProSite resources (http://www.expasy.ch/prosite/). Red: PDZ consensus sequence. Underlined amino acids are predicted transmembrane regions of the proteins. Double underlined is the predicted Protein Phosphate A phosphorylation site. GenBank sequences used in this analysis: γ1-NM000727, γ2-NM006078, γ3-NM006539, γ4-NM014405, γ5-AF361351, γ6-AF361352, γ7-AF361353, and γ8-AF351354.

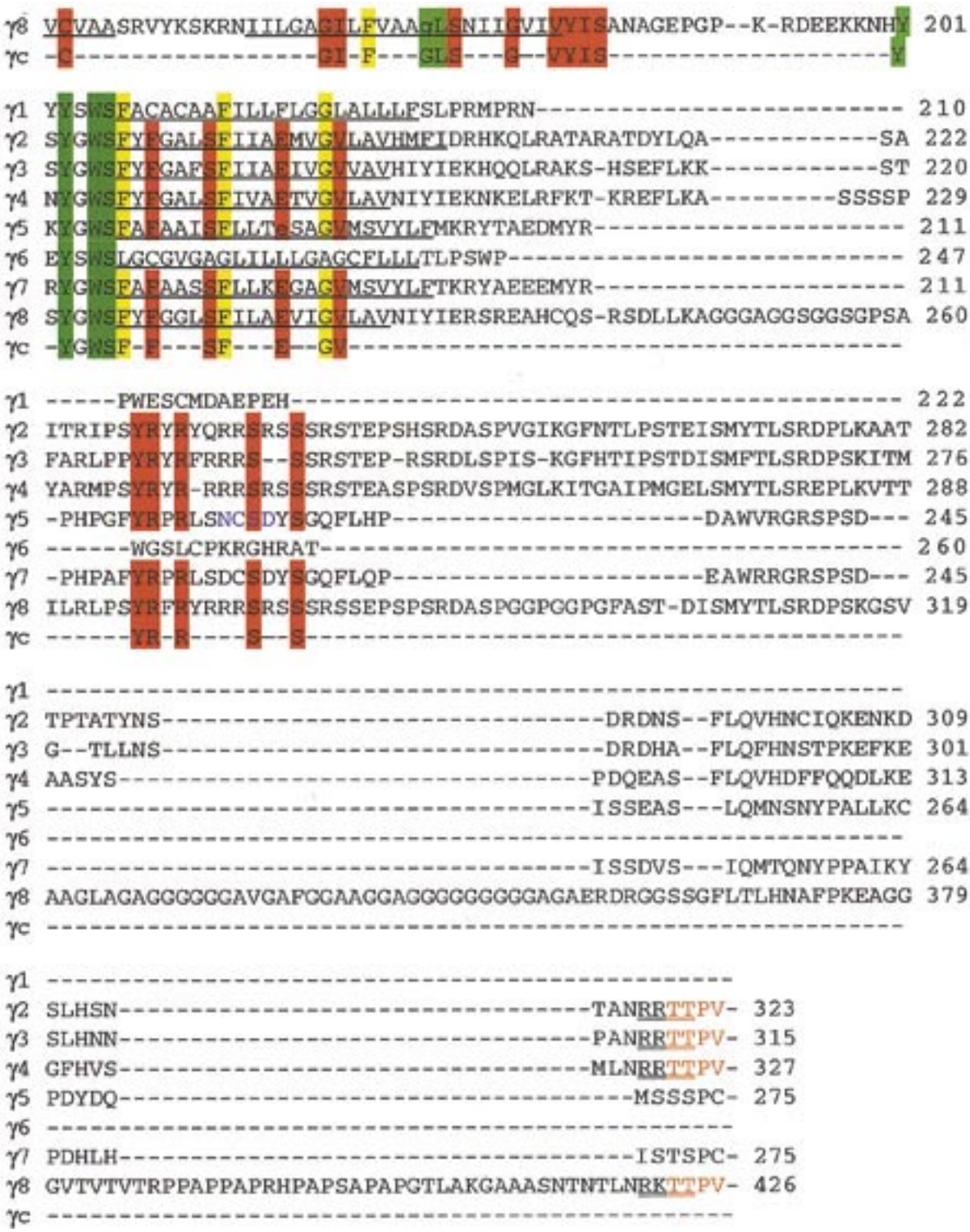

Fig. 1. *(Continued)*

ZO-1) binding motif similar to that seen in $\gamma 2$, $\gamma 3$, and $\gamma 4$ subunits. Cacng6 was found to have two isoforms because of alternative splicing detected on RT-PCR. The short form was comprised of only-three exons and analysis suggests that the amino acid sequence may contain only two membrane-spanning regions.

Burgess *et al.* (2001) also described the cluster Cacng6, Cacng7, and Cacng8 subunit gene on human chromosome 19q13.4. Cacng8 was noted to have an extensive C/G-nucleotide-rich region in the 5′ and 3′ portions of the gene. The predicted coding region of exon 4 was 228 bp longer than its most closely related γ subunit gene, Cacng4. The authors were unable of produce PCR products spanning exon 4 that would confirm the sequence of the entire cDNA likely due to the G/C-nucleotide content. Thus, they were unable to confirm splice sites and the entire sequence by analysis of cDNA. Phylogenetic analysis revealed γ subunits 2, 3, 4, and 8 to be closely related. $\gamma 5$ and $\gamma 7$ were also closely related as were $\gamma 1$ and $\gamma 6$ (Fig. 2).

Several regions in the proteins with high levels of sequence homology have been observed (Fig. 1). These are thought to represent sequences essential for basic function such as transmembrane segments, N-glycosylation motifs, conserved extracellular cystine residues, and interactional domains like the PDZ-binding domain identified in the C-terminus of $\gamma 2$, $\gamma 3$, $\gamma 4$, and $\gamma 8$ (Burgess *et al.*, 2001).

Consistent across several studies were the observations by Sharp *et al.* (2001) that these subunits are highly glycosylated. They uniformly have electrophoretic mobility patterns of heavier proteins than predicted by amino acid sequence. Sharp *et al.* (2001) proved glycosylation of the $\gamma 2$, $\gamma 3$, and $\gamma 4$ isoforms expressed in HEK 293 cells by comparing weights of these subunits with or without the N-glycosylation inhibitor, tunicamycin. Western blot analysis of $\gamma 2$ with tunicamycin showed a band at 26 kDa and in the absence of tunicamycin showed a band of 33–38 kDa. Similarly $\gamma 3$ was 24 kDa versus 32–35 kDa, respectively, and γ was 36.5 kDa versus 39–51 kDa, respectively.

In summary, the subunit Cacng family contains identified eight members (Fig. 1). These members are technically considered to be part of the claudin family of proteins. There was a considerable lag in time between the characterization of Cacng1 and subsequent genes due to great difference in the amino acid composition of the members of this family. The predicted proteins span the membrane four times and have their amino- and carboxyl-termini located intracellularly. The genes have four exons with the exception of Cacng5, which has five. The first exon tends to be large. CLUSTAL W analysis reveals many regions of amino acid identity. The majority of high homology regions are located around membrane spanning domains, however, there is a HXGLWRXC motif that is located in region between transmembrane sequences 1 and 2 and is predicted to lie extracellularly. There are also two cysteine residues located in this region that might form disulfide bonds. The carboxyl-termini of these proteins is of particular interest given that $\gamma 2$, $\gamma 3$, $\gamma 4$, and $\gamma 8$ have a type 1 PDZ-binding motif and $\gamma 5$ and $\gamma 7$ have a SS/TSPC motif which may also be involved in protein interactions (Chu *et al.*, 2001). All of the subunits have N-glycosylation sites, again, located in the first extracellular spanning region of the encoded proteins.

CELLULAR LOCALIZATION AND VGCC CHANNEL ASSOCIATIONAL DATA

$\gamma 1$ was identified because of its tight association with the heteromultimeric skeletal muscle VGCC (Glossmann *et al.*, 1987; Sharp and Campbell 1989). Letts *et al.* (1998) confirmed that the mouse $\gamma 2$ protein localized to synaptic membranes utilizing a polyclonal antibody to the last 15 amino acids of the protein that included the consensus site. Klugbauer *et al.* (2000) determined the cellular localization of the myc-fusion $\gamma 2$ subunit in HEK293 cells utilizing anti-myc antibodies, which also indicated synaptic localization. Green *et al.* (2001) confirmed the validity of the hydrophobicity plots for $\gamma 2$ by using a myc epitope labeled C-terminus of $\gamma 2$ which was found to be

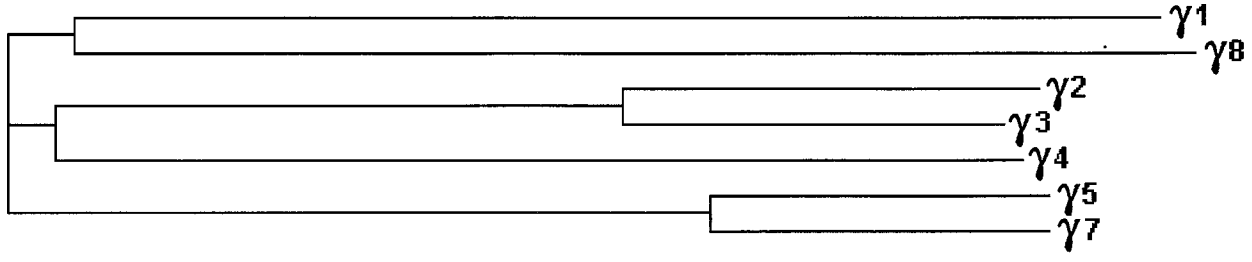

Fig. 2. Phylogram of human $\gamma 1$ through $\gamma 8$ using CLUSTAL W (1.82) available at http:/www.ebi.ac.uk/clustalw/.

intracellular and a myc-labeled region between the first and second transmembrane domain which was found to be located extracellularly. Kang *et al.* (2001) did immunoprecipitation studies to show that in rabbit brain $\gamma 2$ and $\gamma 3$ subunits bind to neuronal calcium channel complexes composed of $\alpha 1$A/B, $\alpha 2\delta$, and β subunits. Sharp *et al.* (2001) used electron microscopy to localized γ subunit immunostaining to dendritic structures of hippocampal mossy fiber synapses, especially within the postsynaptic densities. They also assessed the association of native γ subunits with VGCC by detergent-solubilizing mouse forebrain and using antibodies against highly conserved C-terminal epitopes for $\gamma 2$, $\gamma 3$, and $\gamma 4$ subunits to immunoprecipitate $\alpha 1$B subunits. Furthermore, anti-$\gamma 2$ antibodies and anti-$\alpha 1$B antibodies independently immunoprecipitated AMPA receptor subunit GluRl from these mouse forebrain homogenates.

In summary, there is evidence that $\gamma 2$, $\gamma 3$, and $\gamma 4$ subunits locate in the membrane, that their proteins are oriented as predicted by hydrophobicity plots, and that they are associated with $\alpha 1$A, $\alpha 1$B, and AMPA receptors. It has not been shown that the remaining members of this protein family have the same properties. Finally, interaction domains for these subunits has not been elucidated.

TISSUE EXPRESSION OF γ SUBUNITS

Powers *et al.* (1993) found mouse $\gamma 1$ cDNA was expressed in muscle by RT-PCR while no expression was observed in heart, liver, kidney, spleen, stomach, or brain. Letts *et al.* (1998) found mouse $\gamma 2$ mRNA to be expressed in adult mouse brain abundantly with highest expression in cerebellum, olfactory bulb, cerebral cortex, thalamus, and CA3 and dentate gyrus regions of the hippocampus by in situ hybridization. Northern hybridization failed to show this transcript in heart, spleen, lung, liver, muscle, kidney, or testes.

Klugbauer *et al.* (2000) used Northern blots to show that $\gamma 3$ and $\gamma 4$ were expressed exclusively in brain whereas $\gamma 5$ was expressed in liver, kidney, heart, lung, skeletal muscle, and testes. In situ hybridization revealed $\gamma 2$ mRNA to be strongly detected in cerebellum and moderately expressed in hippocampus, cerebral cortex, thalamus, and olfactory bulb. $\gamma 3$ transcript was strongly expressed in hippocampus, cerebral cortex and moderately expressed in olfactory bulb and caudate putamen. $\gamma 4$ mRNA was highly expressed in caudate putamen, olfactory bulb, habenulae and less so in cerebellum and thalamus. $\gamma 2$ and $\gamma 4$ expression in cerebellum came predominantly from Purkinje cells.

Green *et al.* (2001) used TaqMan assays to identify $\gamma 2$, $\gamma 3$, and $\gamma 4$ subunit mRNA expression patterns in 28 brain regions and peripheral tissues. The three γ subunits were expressed as follows: $\gamma 2$ was found to be expressed in all brain regions with higher expression in the cerebellum, fetal brain, and nucleus accumbens. There was also expression in testes. $\gamma 3$ was expressed predominately in the nucleus accumbens, putamen, amygdala, caudate nucleus, temporal cortex, hippocampus, and to a lesser extent in fetal brain. There was a small amount of expression in testes. $\gamma 4$ was expressed broadly in brain with very strong expression in fetal brain and some increased expression in caudate nucleus, putamen, and thalamus. There was substantial expression in prostate and lung and less expression in testes, stomach, pancreas, small intestine, placenta, and uterus. Because $\gamma 4$ was highly expressed in fetal brain, it is hypothesized to have a potential role during development. This hypothesis is supported by Kious *et al.* (2002) who found $\gamma 4$ expression in the chick cranial neural plate and in the cranial and dorsal root ganglia. Timing of expression correlates precisely with the onset of neuronal differentiation. $\gamma 4$ expression is also observed in the myotome and a subpopulation of differentiating myoblasts in the limb bud. In the distal cranial ganglia, $\gamma 4$ expression was detected in cells destined to become neurons and neural crest cells. The authors hypothesize that $\gamma 4$'s subtle effects on VGCC calcium transients effects neuronal differentiation.

Chu *et al.* (2001) did expression analysis of rat γ transcripts in a panel of 10 mRNA libraries (atrium, ventricle, aorta, skeletal muscle, brain, kidney, lung, liver, spleen, and testis). $\gamma 1$ was shown to expresses in skeletal muscle and weakly in aorta. $\gamma 2$, $\gamma 3$, and $\gamma 5$ were found only in brain. $\gamma 4$ was found in atrium, aorta, brain, and lung. The long splice variant of $\gamma 6$ was found in atrium, ventricle, skeletal muscle and the short splice variant was found in atrium, ventricle, aorta, brain, and lung. $\gamma 7$ was expressed in all tissues except aorta, kidney, liver, and spleen. $\gamma 8$ was found only in brain and testis. The authors noted that one or another γ subunit mRNA was expressed in all of the tissues studied except spleen and kidney.

Burgess *et al.* (2001) conducted RT-PCR assays to examine the expression profile of each member of the human γ subunit family using mRNA master panels of 24 tissues including brain, heart, kidney, liver, lung, colon, bone marrow, small intestine, spleen, stomach, thymus, prostate, skeletal muscle, testis, uterus, fetal brain, fetal liver, spinal cord, placenta, adrenal gland, pancreas, salivary gland, trachea, and mammary gland. $\gamma 1$ was detected in multiple tissues including brain, kidney, liver, and stomach. This was surprising because previously published reports had revealed $\gamma 1$ transcripts almost exclusively in skeletal muscle. Subsequent template dilution removed all signals for $\gamma 1$ from nonskeletal muscle tissues suggesting

a low abundance of this transcript in nonskeletal muscle tissues. $\gamma 2$ expression was detected in brain, liver, colon, small intestine, testis, fetal brain, fetal liver, spinal cord, and adrenal gland. $\gamma 3$ expression was detected in adult and fetal brain. $\gamma 4$ detection occurred in brain, kidney, lung, colon, small intestine, stomach, prostate, testis, uterus, fetal brain, spinal cord, fetal liver, placenta, adrenal gland, pancreas, salivary gland, trachea, and mammary gland. $\gamma 5$ detection occurred in brain, kidney, thymus, prostate, testis, fetal brain, and spinal cord. $\gamma 6$ analysis revealed two splice variants. The amount of expression of each of the splice variants varied according to the tissue being tested. For example small intestine and spleen revealed the larger splice variant almost exclusively whereas bone marrow, salivary gland, skeletal muscle, and prostate revealed the smaller splice variant. Overall some expression of $\gamma 6$ gene activity was present in brain, kidney, lung, bone marrow, small intestine, spleen, stomach, thymus, prostate, skeletal muscle, testis, uterus, fetal brain, fetal liver, spinal cord, placenta, adrenal gland, salivary gland, trachea, and mammary gland. $\gamma 7$ expression was present in all tissues except heart, bone marrow, uterus, placenta, pancreas, or salivary gland. $\gamma 8$ expression was seen only in brain, fetal brain. Finally, a lack of identification of the γ subunit expressed in heart was quite unexpected and suggests that an additional γ subunit gene may be yet to be identified.

Sharp *et al.* (2001) explored the distribution of $\gamma 2$, $\gamma 3$, and $\gamma 4$ subunits in mouse brain at the protein level. Antipeptide antibodies to the three γ isoforms were produced. Regional expression of γ isoforms was determined by Western blotting of brain protein extracts. $\gamma 2$ was detected in mouse forebrain and cerebellum but not in thymus, heart, lung, skeletal muscle, liver, or kidney by Western blot. $\gamma 2$ amounts were highest in cerebellum and cortex, moderate in midbrain, thalamus, hippocampus, and striatum, and lowest in pons and brain stem. $\gamma 3$ was detected in highest levels in the cortex, moderate in the midbrain and striatum, and lowest in the thalamus and hippocampus and not at all in the cerebellum, pons, or brainstem. $\gamma 4$ was highest in the cortex, midbrain, hippocampus, and striatum and lowest in thalamus, pons, brainstem, and cerebellum. Immunohistochemistry confirmed Western blot findings. The authors note that subunit signals were detected in regions containing highly enriched excitatory glutaminergic synapses and was only faintly detectable in cell bodies suggesting a role for γ in synaptic functioning.

In summary, the various members of the Cacng family are expressed in most tissues, however, the results obtained from the authors cited above are sometimes in conflict and most involved detection of mRNA which does not always reflect the degree of tissue expression of a given gene. The use of γ subunit-specific antibodies correlation with RT-PCR may provide the most accurate information on tissue expression. The role of $\gamma 4$ in development is strongly suggested by its temporal expression profile.

EFFECTS ON VGCC ELECTROPHYSIOLOGY

Wei *et al.* (1991) studied the electrophysiologic interactions between rabbit skeletal muscle β and $\gamma 1$ subunits with rabbit cardiac $\alpha 1$C subunits in a Xenopus oocyte expression system using Ba^{2+} as a charge carrier. Coexpression of skeletal muscle $\gamma 1$ subunit with $\alpha 1$C subunits did not significantly alter currents. However, coexpression of $\alpha 1$C and β subunits with $\gamma 1$ increased the peak currents and rates of activation at more negative potentials. Thus, the β and $\gamma 1$ subunits can modulate the biophysical properties of $\alpha 1$C. Eberst *et al.* (1997) demonstrated that HEK293 cells' transiently cotransfection with $\alpha 1$C-a/$\beta 2$a/$\alpha 2\delta/\gamma 1$ subunits resulted in a shift of the activation curve to negative potentials and accelerated current inactivation without changing other voltage-dependent properties of the channel.

Letts *et al.* (1998) used a BHK cell line stably transfected with neuronal $\alpha 1$A/$\beta 1$a/$\alpha 2\delta$ to show that the current–voltage relationship of calcium currents were very similar between groups but coexpression of $\gamma 2$ did alter the balance between channel availability and inactivation. By studying cells at a variety of potentials exposed to 5 s inactivation prepulses, they demonstrated current amplitudes that were decreased as the channels became increasingly inactivated. $\gamma 2$ accentuated inhibition by shifting the voltage-dependence of channel availability toward negative potentials. Midpoint voltage was more negative in cells transfected with $\gamma 2$ resulting in a hyperpolarizing shift of 7 mV.

Klugbauer *et al.* (2000) studied the effects of $\gamma 2$, $\gamma 4$, and $\gamma 5$ (later designated as a pr protein as described above) on $\alpha 1$A, $\alpha 1$C, and $\alpha 1$G function using transfected HEK293 cells with Ba^{2+} or Ca^{2+} as charge carriers. With the subunit combination $\alpha 1$A/$\beta 1$a/$\alpha 2\delta$-1, only $\gamma 2$ showed a small but significant shift of the voltage-dependent activation to more positive potentials. It also shifted the steady-state inactivation curve toward a more hyperpolarized potential. The steady-state inactivation curve was also observed with $\gamma 4$ but not with $\gamma 5$. The same experiment using $\alpha 1$A/$\beta 2$a/$\alpha 2\delta$-1 with $\gamma 2$ had the same effect on steady-state inactivation. However, the negative shift in the voltage-dependence of inactivation only occurred when Ca^{2+}, but not Ba^{2+}, was used as a charge carrier. The use of Ba^{2+} caused a shift to more positive

potentials. α1C/β2a/α2δ-1/γ1 caused the steady-state inactivation curve to shift 30 mV to the negative while current inactivation was accelerated. Using γ2 in place of γ1 and using Ba^{2+} as the charge carrier led to small shifts of the voltage-dependence of activation and inactivation but γ5 did not have this effect. The results suggested an interaction between γ2 and α1C. α1G coexpression with γ4 lead to significant shifting of steady-state inactivation curves to more positive voltages. The speed of recovery from voltage-dependent inactivation was slowed by γ2 and γ4. γ5 lead to the time course of both current activation and inactivation during a depolarizing pulse to be significantly accelerated over a range of voltages suggesting that γ5 may be an auxiliary subunit of the low voltage activation channel α1G.

Rousset *et al.* (2001) studied the electrophysiology of *Xenopus* oocytes injected with various combinations of subunits. All oocytes were injected with α1A/α2δ. The oocytes were then transfected with β1, β2, β3, β4, or no β subunit and each of these lines was subsequently transfected with either γ2, γ3, γ4, or no γ subunit. Electrophysiologic recordings were done Ba^{2+} currents. They found that when no β subunit was expressed, γ2 and γ3 induced a small negative shift of the inactivation curve and an acceleration of inactivation kinetics. When β subunits were expressed, these same effects remained but in rare oocytes calcium channels displaying unusual slow inactivation that was dependent upon the presence of β subunits. The γ subunits increased the number of oocytes expressing the slow inactivation calcium currents. When the slow inactivation currents were removed from analysis, the basic effect of γ subunits appeared to be an acceleration of the inactivation of calcium channels and a small hyperpolarizing shift in the inactivation curve. The only exception to a negative depolarizing shift was the combination of β2/γ2 which caused a positive shift. The unusual slow-inactivating currents recorded were relatively rare (3/72 oocytes) and suggested that γ subunits may act as a catalysts for phosphorylation of α1A subunits by optimizing channels' secondary/tertiary structure for modification.

Green *et al.* (2001) studied the functional effects of the γ2, γ3, and γ4 subunit on T-type channels encoded by α1I by cotransfecting HEK293. Electrophysiologic studies revealed that none of the γ subunits produce large changes in current amplitude although γ2 elicited a small but nonsignificant increase in current amplitude. Furthermore there was no detectable change in current activation kinetics for all tested potentials between -50 and $+20$ mV. Steady-state inactivation and rate of inactivation of α1I channels was also not markedly affected by γ subunits. Deactivation of α1I calcium channels was significantly slowed in the presence of γ2. This effect was absent with γ3 and γ4.

Kang *et al.* (2001) found that γ2 decreased current amplitude of α1B and α1A calcium channels when coexpressed with β3/α2δ subunit. The inhibitory effects were dependent upon the presence of the α2δ subunit. Both γ1 and γ2 decelerated activation kinetics of α1B-class calcium channels. The results suggest that the γ2 subunit is a part of the neuronal calcium channel complex and that it plays a role in downregulating calcium channel activity.

In summary, the effects of the various γ subunits on VGCC activity is small and some of the results cited are in conflict. The consensus seems to be that most of the γ subunits studied downregulate calcium channel activity by causing a hyperpolarizing shift in the inactivation curve. Furthermore, some of the research involved the use of the a putative mouse γ5 subunit which has been renamed to a pr protein and is no longer considered part of the γ subunit family. The conflicting results reported may be due to differences in recording techniques, charge carriers, cells transfected, or possibly the vectors used in transfection experiments.

FUNCTIONS FOR THE γ2 SUBUNIT DEDUCED FROM *STARGAZER* STUDIES

The mouse γ2 subunit was discovered in the *stargazer* mouse mutant (Letts *et al.*, 1998). Even before this mutation was shown to be etiologic to the phenotype, a great deal of work was done to describe this mutant mouse line. This information will be reviewed next because it has lead to important discoveries about the γ2 subunit's role in CNS function. However, the research raises additional questions about the γ2 subunit's role in neurogenesis, neuroplasticity, and epilepsy.

THE *STARGAZER* PHENOTYPE

Noebels *et al.* (1990) described the inheritance pattern, initial linkage mapping, and phenotype of the *stargazer* mutant. This mutation arose in the A/J inbred strains of the Jackson Laboratory and has an autosomal recessive pattern of inheritance. Initial linkage analysis showed the *stargazer* mutation to map near the middle of chromosome 15 and this was later refined to a 150 kb interval by Letts *et al.* (1997). Homozygous mutants have reduced body size and by a mildly ataxic gait by postnatal day 14. By 1 month of age, spontaneous abnormal head movements can be seen at rest and during locomotion,

which resembled choreiform movements. The mutant's name is derived from the fact that it periodically elevates its head producing an upward gaze that is sustained for several seconds. Righting responses while swimming are severely disturbed. The neurological symptoms show a steady progression in the frequency of head tossing movements with increased age. While females are fertile, males are not. The mutant also has absence seizures. EEG recordings show prolonged high-amplitude, bilaterally symmetrical discharges in the neocortex that are accompanied by sudden behavioral arrests lasting the duration of the discharge. Paroxysmal spike bursts at a frequency of 6–7 spikes/s lasting a mean duration of 6 s are recorded. An average of 125 discharges/h were recorded leading to abnormal cortical synchronization being present 21% of any sampling period. A second type of seizure pattern was recorded less than one time per day, on average, with EEG characteristics of brief acceleration of cortical spite frequency from the usual fixed interval of 6–7 spikes/second to a faster, irregular rate of 9–14 spikes/second. Motor movements associated with this pattern included slow locomotor activity or repetitive forepaw grooming. The head elevations have no temporal relationship to the abnormal cortical discharges. Letts *et al.* (1998) demonstrated a mutation in the Cacng2 caused by an early transposon that prevented expression downstream of the first exon. Another Cacng2 mutation was strongly suggested in the *waggler* mouse line which has a very similar phenotype (Chen *et al.*, 1999 Letts *et al.*, 1998). Sharp *et al.* (2001) showed that mutation in the mouse Cacng2 in the *stargazer* mouse resulted in loss of $\gamma 2$ protein as predicted. No compensatory changes in the expression of $\gamma 3$ or $\gamma 4$ protein were evident. A loss of $\gamma 2$ from cerebellar mossy fiber synapse in the *stargazer* mouse line makes this region vulnerable to dysfunction. However the mechanism of this vulnerability is unknown given that immunostaining of the cerebellar granule cell layer of *stargazer* mice continues to reveal some immunoreactivity suggesting that a nonmutated γ isoform, perhaps $\gamma 4$, is expressed in the granule cell layer Sharp *et al.* (2001).

ABNORMALITIES IN NEUROTROPHIC FACTORS

Chafetz *et al.* (1995) examined the expression of neuropeptide Y in the brain of *stargazer* mice to determine if activity-induced gene expression might alter neurotransmitter phenotypes in abnormally bursting networks of the *stargazer* line. The authors determined if endogenous synaptic activity patterns was sufficient to change granule cell neuropeptide gene expression without cellular injury over the long term. Immunocytochemistry of NPY, glial fibrillary acidic protein, and 72 kDa heat shock protein was done utilizing the avidin–biotin method. The principal finding was that elevated levels of NPY appeared in the hippocampus following the development of spontaneous seizure discharges in *stargazer* mice. The authors hypothesized that the *stargazer* gene locus itself had no primary effect on granule cell NPY phenotype but felt that the ectopic NPY expression was due to abnormal synaptic activity patterns during spike-wave synchronization.

Qiao *et al.* (1996) characterized *stargazer* cerebellar histolopathology and brain derived neurotrophic factor (BDNF) expression patterns at various stages of development. The cerebellum had a 14% reduction in gross cerebellar net weight in mutants because of generalized reduction in all anatomical elements but a normal cytoarchitectural pattern. Northern hybridization revealed early failure of BDNF mRNA expression in the *stargazer* throughout the entire cerebellar cortex but no significant decrease in frontal cortex or olfactory bulb expression. No changes were found in in situ hybridization experiments measuring full-length and truncated BDNF TrkB receptors. Nerve growth factor and neurotrophin 3 levels were not changed in *stargazer* mice. The lack of BDNF was hypothesized to lead to ataxia in the *stargazer* mutant because (1) ataxia occurs at a point in cerebellar development at which the BDNF gene is normally activated (2) BDNF knockout mice have essentially the same ataxic phenotype and cerebellar findings, (3) BDNF has been shown to play a role in synaptogenesis and adaptive changes in connectivity. Qiao *et al.* (1998) studied impaired eyeblink conditioning in *stargazer* mutant mice. BDNF mRNA expression in normal cerebellum is not apparent until postnatal day 15 and peaks at approximately postnatal day 20 in the rat. This peak coincides with granule cell migration and maturation. Lack of BDNF appeared to cause impairment in cerebellar granule cell maturation and cerebellar learning. Thompson *et al.* (1998) found decreased expression of $GABA_A$ receptor $\alpha 6$ and $\beta 3$ subunits in *stargazer* mutant mouse cerebellum suggesting a possible role for BDNF in the regulation of cerebellar $GABA_A$ receptor expression.

In summary, elevated levels of neuropeptide Y were observed after seizure onset and there is a striking reduction in the production of BDNF production in cerebellar granule cells but normal expression in frontal cortex and olfactory bulb in the *stargazer* mutant line. The cause of these findings is not known. While they may be related to seizure activity, the probability that these findings are due to mutated Cacng2 cannot be excluded. The lack of BDNF probably interferes with granule cell maturation and may

be related to the reduction in cerebellar $GABA_A$ receptor $\alpha 6$ and $\beta 3$ subunits.

CHARACTERIZATION OF AMPA RECEPTOR TRAFFICKING

Study of the *stargazer* line has helped to elucidate the mechanism by which AMPA receptors are trafficked to the synaptic membrane. Hashimoto *et al.* (1999) showed that excitatory postsynaptic currents (EPSCs) at mossy fiber cerebellar granule cell synapses in the *stargazer* mutant lacked AMPA receptor-mediated fast component but had a normal NMDA receptor-mediated slow component. This abnormality was attributed to a defect in postsynaptic AMPA receptor function because presynaptic glutamate release was normal. BDNF-deficit mice had normal AMPA receptor-mediated EPSC component so the defect in AMPA receptor function in the *stargazer* mutant was not due to a lack of BDNF production. The non-NMDA receptor-mediated EPSC component in the CAl pyramidal cells of the *stargazer* mutant mouse was normal. The four AMPA receptor subunit mRNAs, GluRα1-4 were expressed normally regionally in the *stargazer* mouse brain as measured by in situ hybridization. Furthermore, the mutant cerebellum expressed normal levels of GluRα4 protein as measured by immunoblot. Hippocampal synapses of the *stargazer* mutant mouse line had normal AMPA receptor-mediated responses. Thus, the failure of AMPA receptor function is specific to the cerebellar mossy fiber granule cell synapse. The AMPA receptor abnormalities seen in the cerebellum of the *stargazer* mice creates a functional deafferentation of the cerebellum, which could explain the motor dysfunction observed in *stargazer* mice including impaired acquisition of eyeblink conditioning.

Chen *et al.* (2000) reported that mouse $\gamma 2$ regulates synaptic targeting of AMPA receptors by two distinct mechanisms. $\gamma 2$ was found to interact with AMPA receptors and PDZ proteins. $\gamma 2$ and GluR4 co-immunoprecipitate when cotransfected into COS cells. $\gamma 2$ also interacts with transfected GluRl and GluR2 subunits but not NMDA receptor subunits. However, the authors were unable to co-immunoprecipitate $\gamma 2$ and with GluR from brain extracts. $\gamma 2$ contains a type 1 PDZ-binding site (Fig. 1). The authors found that $\gamma 2$ interacts with PSD-95 and SAP-102. Deletion of the PDZ-binding site from $\gamma 2$ (called stargazinΔC) disrupted the interaction with PSD-95 but did not interfere with binding to GluR4. Transfection of $\gamma 2$, GluR4, and PSD-95 caused redistribution of proteins into patch-like clusters at the cell surface that were dependent upon the PDZ domain being present in the $\gamma 2$ protein. When $\gamma 2$ was transfected into *stargazer* mouse neurons the intact $\gamma 2$ rescued AMPA receptor function in these neurons. Utilization of stargazinΔC failed to restore synaptic responses. Interestingly, in wild-type cells, stargazinΔC had little effect on glutamate stimulated AMPA receptor responses or NMDA receptor responses. However, there was a marked reduction in the amplitude and frequency of spontaneous AMPA receptor synaptic events indicating that the stargazinΔC disrupted synaptic localization of AMPA receptors. StargazinΔC down-regulated hippocampal AMPA receptor EPSPs. Cerebellar granule cells appear to be particularly sensitive to the mutated $\gamma 2$ subunit found in *stargazer* because the cells appear to only express $\gamma 2$ whereas Purkinje cells and hippocampal neurons expressed another member of the γ subunit family that being $\gamma 3$ which may compensate for the hypomorphic $\gamma 2$ subunit.

Choi *et al.*. (2002) note that the RRTT sequence in the type 1 PDZ-binding motif (RRTTPV) is also a consensus sequence for phosphorylation by protein kinase A (PKA) (R/KR/XXS/T) such that T321 (RRTTPV) can be phosphorylated by PKA. This phosphorylation was hypothesized to weaken the interaction between $\gamma 2$ and PSD-95. Antibodies specific for phosphorylated and nonphosphorylated T321 of the $\gamma 2$ protein were generated. A yeast two-hybrid analysis system was used with constructs containing the last 121 amino acid residues of $\gamma 2$. The C-terminus was mutated strategically in order to test the ability of $\gamma 2$ to interact with PDZ domains (RRTDPV, RRTEPV, and RRTTPA). COS cells were transfected and coimmunoprecipitation and clustering assays were done. The findings were that phosphorylation of $\gamma 2$ at T321 by PKA inhibits interactions with PSD-95 suggesting that $\gamma 3$, $\gamma 4$, and $\gamma 8$ may similarly interact with PSD-95 and be regulated by phosphorylation. Downstream effects of this regulation are that $\gamma 2$ phosphorylation may (1) regulate synaptic targeting of AMPA receptors; (2) regulate the stability of $\gamma 2$ on synaptic surface membranes by limiting the ability of $\gamma 2$ to interact with PDZ anchors; (3) regulate translocation of AMPA receptors to the cell surface.

In summary, it is through the study of *stargazer* that a major component of AMPA receptor trafficking was defined. $\gamma 2$ is the first transmembrane protein found to interact with glutamate receptors. Wild-type $\gamma 2$ protein rescues mutant granule cells. Removal of the PDZ-binding motif does not rescue synaptic responses. $\gamma 2$ without the PDZ-binding domain does allow extra synaptic AMPA receptor responses, however. Thus, the transmembrane domains of $\gamma 2$ interact with AMPA receptors and regulate

the delivery of AMPA receptors to the postsynaptic cell membrane. Also, through interaction with the $\gamma 2$ PDZ-binding site and PSD-95 or related target proteins delivery of AMPA receptors to the synapses occurs (Tomita *et al.*, 2001). Presumably the other $\gamma 3$, $\gamma 4$, and $\gamma 8$ are involved in targeting other proteins to the postsynaptic density but the exact nature of this interaction has yet to be described.

EVOLUTION OF THE Cacng SUBUNIT FAMILY AND MAMMALIAN GENOME

Burgess *et al.* (1999) conducted phylogenetic and chromosomal analysis for Cacng1 though Cacng5, which supported evolution for the Cacng family by two episodes of the ancient tandem duplications and at least two chromosomal duplication events. Burgess *et al.* (2001) later expanded this study to describe the Cacng6, Cacng7, and Cacng8 cluster located in tandem array on chromosome 19q 13.4. This cluster is paralogous to the cluster containing Cacng1, Cacng4, and Cacng5 on chromosome 17q24. Through analysis of the Cacng3, Cacng4, Cacng5 genes on chromosome 16p12-13 and 17q24 the authors found that flanking genes served as a marker for the adjacent Cacng subunit genes. For example the protein kinase C α gene is located at 17q24 whereas the protein kinase C β gene is located on 16p11.2 and the protein kinase C γ gene is located on 19q13.4. This led the authors to successfully search that region for additional Cacng members. The evolutionary relationship of the Cacng clusters were analyzed to show a consistent relationship between the protein kinase C genes in tandem with Cacng across chromosomes. These consistent results suggested gene cluster evolution characterized by both tandem and chromosome segment duplications. These results were used to support the hypothesis that there were two rounds of whole-genome duplication that occurred early in vertebrate history to generate the modern vertebrate genome. Chu *et al.* (2001) also researched the phylogenetic characteristics of the eight members of the human, mouse, and rat Cacng family and similarly concluded that the mammalian γ subunits are derived from a single ancestral gene with at least two episodes of tandem duplication and several episodes of chromosomal duplication resulting in Cacng members being present on four human chromosomes. The ancestral form of the Cacng appeared before the division of rodents and primates. No γ subunit-like sequences have been found in *Caenorhabditis elegans*. The authors conclude that local tandem gene duplication occurred before duplication of the gene clusters that resulted from either chromosomal duplication or large-scale genome duplication. In summary, the fact that no γ subunit genes have been identified in *C. elegans* indicates that the ancestral gene evolved more recently. The fact that there are eight γ subunits organized throughout the genome with the consistent finding of flanking genes from another protein family is very strong evidence that tandem duplication followed by either chromosomal or whole-genome duplication.

HUMAN CLINICAL RESEARCH

The mouse Cacng2 gene is associated with a absence seizure phenotype so one would expect that there the human Cacng2 might be associated with a seizure disorder. So far this is not the case. Cacng2 has been studied as a candidate gene for three genetic disorders to date: Malignant hyperthermia, spinocerebellar ataxia 10 (SCA10), and velocardiofacial syndrome (VCFS).

Iles *et al.* (1993) tested the hypothesis that the human $\beta 1$ and γ subunit VGCC genes were responsible for malignant hyperthermia susceptibility by doing pedigrees analysis using markers for the $\beta 1$ Cacng1 and (Cacng1). They excluded these genes as candidates genes for malignant hyperthermia susceptibility. Sudbrak *et al.* (1993) similarly failed to confirm linkage with this disease. Finally, Lynch *et al.* (1995) screened the Cacng1 for the causative mutation for MHS patients using the single-strand confirmation polymorphism method and found three variants, one that changed the amino acid sequence, G196S (G657A). However this polymorphism was detected in normal samples as well, thus, excluding it as a disease-causing allele.

Burgess *et al.* (2000) looked for an association between the Cacng2 gene and SCA10 which had been mapped to chromosome 22q13. Cacng2 was an excellent candidate given that a small CAG expansion in the VGCC $\alpha 1$A subunit gene is associated with spinocerebellar ataxia (SCA6). Utilizing mapping procedures involving polymorphic microsatellite markers, the gene was excluded as a viable candidate for SCA10.

Finally, Black *et al.* (2001) found that Cacng2 was not deleted in VCFS with or without schizophrenia using fluorescent in situ hybridization probes. The research was done because the gene is located near a marker linked to schizophrenia and because it is in the vicinity of the VCFS microdeletion region. No deletion was found using FISH probes in VCFS cases with or without schizophrenia.

In summary, no human diseases have been associated with mutations or polymorphisms of the genes in the γ subunit family Cacng. However, a number of interesting

diseases are found in the vicinity of these genes including familial infantile convulsions and paroxysmal choreoathetosis which is found in the vicinity of Cacng3.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

The Cacng subunit family contains eight members. Although a good deal is known about Cacng1 and Cacng2, and the proteins they encode, the role for the other γ subunits is less well defined. Area for future research should include (1) Analysis of tissue expression at the protein level given that mRNA expression studies are yielding confusing results. (2) Definition of the peptide domains involved in interactions with the AMPA receptor subunits and VGCC subunits is needed. (3) A comprehensive list of proteins that interact with the γ subunits is needed. It is likely that other proteins are targeted to the postsynaptic membrane by these proteins. (4) A standardized assessment of the electrochemical effects of the γ subunits on all members of the high- and low-voltage-activated calcium channels is needed. Standardization for charge carrier and technique might eliminate some of the variable results that are being observed. (5) The neuropeptide Y, BDNF, and $GABA_A$ receptor findings in *stargazer* need further research in light of the known association with the mutation of the mouse Cacng2. The question that has not been fully explored is whether these findings are secondary to the gene mutation or due to the seizure phenotype. (6) Finally, calcium channels are strongly represented in mouse seizure phenotypes (Burgess and Noebels, 1999) and there is a growing list of channelopathies that are associated with human neurological disorders, especially seizure disorders. It is recommended that Cacng family members be considered candidate genes for any neurological disorders showing linking to 16p12-p13.1, 17q24, 19q13.4, or 22q13.1.

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