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Nonchannel Functions of the Calcium Channel γ Subunit: Insight From Research on the *Stargazer* Mutant

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Voltage-dependent calcium channels (VDCC) are essential regulators of intracellular calcium concentration, which in turn influences a broad spectrum of cellular functions especially in neurons. Identification of several calcium channel mutations as the cause of neurological disorders in human and mouse indicates the importance of the integrity of these channels to neuronal function. Studies of mutant mice, each carrying a disrupted gene of a different VDCC subunit, have revealed many unexpected roles of these molecules and have significantly advanced our knowledge of subunit function in the last few years. This review addresses recent discoveries of the function of the $\gamma 2$ subunit, also named stargazin, with special emphasis on roles other than calcium conductance.

KEY WORDS: Calcium channels; stargazin; γ subunit; cerebellar granule cells; AMPA receptor; synaptic targeting; postsynaptic density; PDZ-binding protein; phosphorylation.

INTRODUCTION

Calcium channels are unique among the superfamily of voltage-dependent ion channels because intracellular Ca^{2+} serves as a ubiquitous second messenger. Calcium ions play a key role in regulating a broad spectrum of cellular functions in both developing and mature central nervous systems. Such functions include cell proliferation and differentiation, cell–cell interactions, coupling electrical excitation to gene transcription, modulation of neurite outgrowth, synaptogenesis, neurotransmitter release, and synaptic plasticity. The complex distribution and diverse functionality of calcium ions are associated with a variety of voltage-dependent calcium channels (VDCC) and multiple genes encoding calcium channel subunits. VDCC comprise a diverse family of hetero-multimeric complexes, each composed of a large pore-forming $\alpha 1$ subunit with a voltage sensor and three auxiliary subunits, $\alpha 2/\delta$, β, and γ, which target and/or anchor the channel to the membrane, and modulate its voltage-dependent kinetics (Catterall, 2000; Jones, 1998).

Mutations in the genes that encode various VDCC subunits lead to defective channel function and result in neurological disorders in humans as well as in animals. To date at least seven spontaneous mutant mouse strains (*tottering*, *leaner*, *rolling*, *rocker*, *lethargic*, *ducky*, and *stargazer*) with similar inherited neurological phenotype of generalized spike-wave epilepsy and cerebellar ataxia have been linked to mutations of genes encoding all four VDCC subunits in mice. Mutational analyses of these mutants have led to the identification of several novel VDCC subunits (see review by Burgess and Noebels, 1999; Fletcher *et al*., 1998). Investigation of such mutants has furthered our understanding of the role of VDCC genes in neuronal function and provided insights into the mechanisms underlying inherited neurological diseases. One such example is the γ subunit family. The discovery of the $\gamma 2$ subunit mutation in *stargazer* mouse has transformed the traditional view of γ subunit as just a VDCC auxiliary subunit. Accumulating evidence has shown that $\gamma 2$ also functions as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor chaperone for synaptic targeting and that it shares homology with proteins regulating protein folding and trafficking, and cell–cell contact such as PMP-22 and claudins. The

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current review will focus on recent research of *stargazer* mutants with special emphasis on the noncalcium channel related features of the stargazin gene.

SPONTANEOUS MOUSE MUTANT *STARGAZER*

The *stargazer* mouse (gene symbol, *stg*) is among the best characterized animal models of inherited neurological disease. The *stg* mutation arose spontaneously in the A/J inbred strain in the Animal Resources colony of the Jackson Laboratory in the 1980s (Noebels *et al*., 1990). The mutant was identified because of its unusual upward head-tossing movements and ataxic gait. Initial genetic evaluation of the mutant located the mutation on chromosome 15 with an autosomal recessive pattern of inheritance (Noebels *et al*., 1990). Two new alleles, stg^{wag} (*waggler*) and stg^{3J}, recently identified at the Jackson Laboratory arose in different mouse strains (Sweer *et al*., 1991). The ataxia in *waggler* mice is very similar to but a little milder than that in *stargazer* without periodic head-tossing movements (Qiao *et al*., 1998a).

Severe Behavioral Defects

Abnormal behavioral phenotype in homozygous *stg* mutants represents a peculiar motor syndrome. Ataxic gait is one of the most noticeable neurological deficits, which can first be recognized at postnatal day 14 (Noebels *et al*., 1990). Periodic head-tossing movements occur both at rest and during locomotion. Hyperactivity and agitated circling motion are often associated with any environmental stimulation. Quantitative tests of motor performance showed severely disturbed righting responses while swimming, and impairment of motor coordination and balance on RotarRod (Noebels *et al*., 1990; Qiao *et al*., 1996). Sensory–motor tests of classical eyeblink conditioning is also severely impaired in two of the *stg* alleles tested implicating defects in cerebellar learning (Bao *et al*., 1998; Qiao *et al*., 1998b). Reports of sensory deficits in *stargazer* from different research groups are mixed. On one hand, normal pain perception and tone-induced neuronal responses in auditory cortex were found in *stg* and stg^{wag} mice (Bao *et al*., 1998). On the other hand, the acoustic startle test showed that *stargazer* might be deaf (Khan *et al*., 2002).

Mild Morphological Defects

Interestingly, the severe behavioral abnormalities are not reflected in morphology of the central nervous system in general. With slight reduction of gross wet weight (Qiao *et al*., 1996), initial neuropathological screening revealed no obvious cytological or architectural lesions in the *stg* brain (Noebels *et al*., 1990). Subtle morphological changes were noticed later with specific histochemical staining methods, at different developmental time points, and in electron micrographs in some brain regions. A striking pattern of aberrant mossy fiber sprouting and increased neuropeptide-Y expression in the hippocampus were linked to the prolonged seizure activity in the aged mutants (Chafetz *et al*., 1995; Qiao and Noebels, 1993). While there was no major primary neuronal cell loss, a mild selective loss of hilar interneurons was found in the dentate gyrus of hippocampus (Qiao and Noebels, 1993). In cerebellum, delayed disappearance of external granule cells during development and persistent presence of immature granule cells in adults were evident with normal granule cell density in adult mutants (Qiao *et al*., 1998b). Abnormal synaptic ultrastructure has also been reported (Qiao, 2001). In stg^{wag} mice, reduced cerebellar granule cell size was observed in Golgi-stained sections (Chen *et al*., 1999).

Multifunctional Defects

Functional defects in the *stg* brain are complex and show regional differences. In the frontal cortex, abnormally increased neuronal activity and excitability are the major themes at systemic as well as at cellular levels. Spontaneous spike-wave epileptic discharges can be recorded by electrocorticograph (ECoG) in several cortical and subcortical regions including neocortex, olfactory bulb, basal ganglia, thalamus, and hippocampus (Noebels *et al*., 1990; Qiao and Noebels, 1991, unpublished results). The generalized seizure activity is accompanied by absence like behavioral immobility without head-tossing movement. Similar to petit mal seizure in humans, the seizures are sensitive to classical antiabsence drugs, such as ethosuximide but not to anticonvulsants for grant mal seizures such as Dilantin (Qiao and Noebels, unpublished results). It has also been reported that P-[3-aminopropyl]-P-cyclohexylmethylphosphinic acid (CGP 46381), a γ-aminobutyric acid $GABA_B$ receptor antagonist, and 5-methyl-10,11-dihydro-5*H*-dibenzo[*a*, *b*]cyclohepten-5,10-imine hydrogen maleate (MK-801), a noncompetitive *N*-methyl-D-aspartate (NMDA) antagonist, suppresses spike-wave discharges significantly in *stg* mice (Aizawa *et al*., 1997). Electrophysiological studies revealed that the epileptic discharge is associated with hyperexcitability, spontaneous neuronal firing, and decreased after hyperpolerization in thalamocortical networks (Di Pasquale *et al*., 1997).

Table I. Comparison of Distinctive Defects in the Different Brain Regions of *Stargazer*

	Frontal brain	Cerebellum
Behavior		
Seizure activity	Frequent spike-wave epilepsy (Noebels *et al*., 1990)	None (Qiao and Noebels, unpublished data)
Motor performance	No obvious defect	Ataxic gait (Noebels *et al*., 1990)
Learning and memory	Normal fear conditioning (Bao *et al*., 1998)	Severe impaired eyeblink conditioning (Qiao *et al*., 1998a,b).
Morphology		
Aberrant fiber out growth	Hippocampal mossy fiber sprouting (Qiao and Noebels, 1993)	None
Development	N/D[a]	Delayed GC migration, incomplete maturation (Qiao *et al*., 1998a,b)
Ultrastructure	N/D[a]	Abnormal granule cell synapses (Qiao, 2001)
Function		
Cellular activity	Increased excitability (Di Pasquale *et al*., 1997)	Decreased cell activity
AMPA receptor	Normal (Hashimoto *et al*., 1999)	Absent (Hashimoto *et al*., 1999)
Inhibitory activity	Decreased AHP (Di Pasquale *et al*., 1997)	Slow $GABA_A$ response (Chen *et al*., 1999)

[a] N/D, not determined.

In contrast, the nature of functional defects in *stg* cerebellum is the opposite. Cerebellum is the only brain region that shows no seizure activity in ECoG recordings, and no spontaneous synchronized activity can be recorded in cerebellar slices in vitro (unpublished results). In fact, decreased cellular activity or actually silent synapses due of lack of AMPA receptor-mediated responses in cerebellar granule cells is the main feature in electrophysiological recordings of cerebellar cortex in both *stg* and stg^{wag} mice (Chen *et al*., 1999; Hashimoto *et al*., 1999). A defect in GABAergic synaptic transmission between Golgi and granule cells was also found in adult stg^{wag} mutants (Chen *et al*., 1999).

As summarized in Table 1, previous studies on the *stg* mutant have revealed several unique features of this animal model. First, the characteristic dual phenotype of epilepsy and ataxia is associated with distinctive regionally restricted defects affecting different parts of the brain. Second, the behavioral phenotype and severe functional defects do not parallel with the mild morphological abnormalities. These results indicate that a single gene mutation may elicit different pathological changes in different brain regions and that dual cellular and molecular mechanisms may underlie the dual phenotype seen in the *stg* mouse.

STARGAZIN GENE AND γ SUBUNIT FAMILY

Genetics and Structure

Much effort has been devoted to the final identification of the *stargazer* gene. Serious genotyping of a large intersubspecific cross first mapped the *stargazer* locus between D15Mit30 and the parvalbumin gene after excluding several candidate genes (Letts *et al*., 1997). Subsequent Southern blot analyses led to the identification of an approximately 6-kb early transposon (ETn) insertion which was only associated with the *stargazer* mutation (Letts *et al*., 1998). A novel gene of four exons with a large first intron of 50–90 kb spanning and an ETn insertion in intron 2 was found to reside at the *stargazer* locus. Secondary-structure analysis suggested that the *stargazer* gene encodes a membrane-spanning protein with four transmembrane domains and both amino and carboxy cytosolic termini (Letts *et al*., 1998). Subsequent analysis of the gene product, stargazin, revealed considerable homology with the skeletal muscle VDCC $\gamma 1$ subunit in its first 200 amino acids (25% identity, 38% similarity), exon–intron organization, predicted protein secondary structure including four transmembrane segments, as well as its modulatory effect on the activity of VDCC in vitro. It resulted in classification of stargazin as a VDCC $\gamma 2$ subunit (Cacng2; Letts *et al*., 1998).

VDCC γ Subunits and Stargazin Isoforms

The original knowledge of γ subunits was derived from purification and sequencing of the $\gamma 1$ subunit from rabbit skeletal muscle in 1990 (Bosse *et al*., 1990; Jay *et al*., 1990). The muscle-specific $\gamma 1$ subunit remained the sole member of the γ family for several years until the isolation of the defective gene in *stargazer* (Letts *et al*., 1998). The addition of the neuron-specific $\gamma 2$ subunit to the family opened the door for subsequent cloning of several γ subunits. Homologue search quickly led to the identification of a corresponding $\gamma 2$ subunit in human as well as three new isoforms, $\gamma 3$, $\gamma 4$, and $\gamma 5$

(Black & Lennon, 1999; Burgess *et al*., 1999; Klugbauger *et al*., 2000). Three additional isoforms, $\gamma 6$–$\gamma 8$ were soon discovered (Burgess *et al*., 2001; Chu *et al*., 2001; Moss *et al*., 2002).

The γ subunits are glycoproteins containing four transmembrane domains with intracellular N- and C-termini, most encoded by a gene assembled from four exons (Burgess *et al*., 1999). $\gamma 7$ is the only γ subunit reported to be the product of a five exon gene with a very different and much longer C-terminus (Moss *et al*., 2002). Phylogenetic analysis of the 24 γ subunits in mouse, rat, and human suggests that they evolved from a common ancestral γ subunit via gene duplication (Chu *et al*., 2001). Tissue distribution of eight γ family members in rat revealed widspread expression including brain, heart, lung, testi, and skeletal muscle (Chu *et al*., 2001). Except for $\gamma 1$, the other seven recently identified γ subunits are all expressed in the brain, some exclusively ($\gamma 2$, $\gamma 3$, and $\gamma 5$) and some more extensively in other tissues and organs ($\gamma 4$, $\gamma 6$, and $\gamma 7$). While all γ subunits share several conserved regions and a very similar transmembrane topology, some specific pairs of γ subunits show closer relationships to each other than to others in the family (Fig. 1). Stargazin ($\gamma 2$) and its related isoforms ($\gamma 3$, $\gamma 4$, and $\gamma 8$) form a specific subfamily because of their close amino acid identity and presence of a PDZ-binding motif (Chu *et al*., 2001; Klugbauger *et al*., 2000).

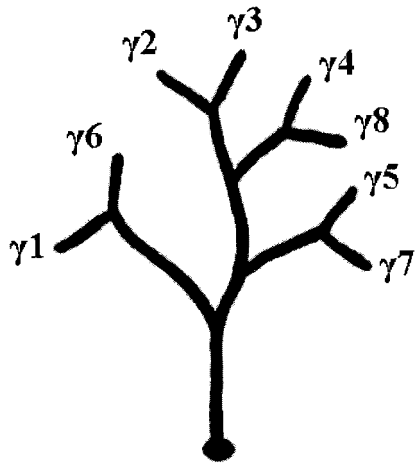

Fig. 1. Phylogenetic tree of members of calcium channel γ subunit family, constructed from sequence comparisons by Chu *et al*. (2001). Common branching points reflect shared ancestor sequences. Basically, there are two subgroups of γ subunits, the $\gamma 1$ and $\gamma 6$ subfamily, and the other γ subunits. The specific pairs indicate closer relationships to each other than to others in the family.

Homology With Other Molecules

In addition to having high homology with other VDCC γ subunits, stargazin is also distantly related to several tetraspan membrane proteins such as peripheral myelin protein 22 (PMP22) and claudin proteins. PMP22 is a critical component of myelin in the peripheral nervous system (Jetten & Suter, 2000). Charcot Marie Tooth Disease type 1A (CMTD) is associated with defects in PMP22, which disrupt protein folding and trafficking (Warner *et al*., 1999). A modest sequence homology was observed between PMP22 and stargazin as well as other members of the VDCC γ subunits (Sanders *et al*., 2001). Claudin is a protein family with more than 20 members, and is one of two components of the tight junction proteins in cpithelial cells (Gonzalez-Mariscal *et al*., 2003). Similar to C-terminal PSD-95/DLG/ZO-1 (PDZ)-binding sites on stargazin, many claudin isoforms terminate with the residues Tyr-Val, which bind to the first PDZ domain of the other tight junction protein, occludin (Itoh *et al*., 1999). Occludin belongs to the family of membrane-associated guanylate kinase (MAGUK), which also includes PSD-95. It has been hypothesized that there is a general role for claudin/stargazin–MAGUK protein complexes in regulating or mediating cellular communication (Tomita *et al*., 2001). Additional study of the homology between stargazin and other tetraspan membrane proteins may provide more insight concerning the functions of stargazin and other VDCC γ subunits.

FUNCTION OF $\gamma 2$ SUBUNIT

The function of the neuronal $\gamma 2$ subunit appears to be much more complex than its muscle counterpart. Similar to $\gamma 1$, neuronal $\gamma 2$ has been found to modulate calcium kinetics in nonneuronal cells in vitro. Its functional effect is relatively minor but significant in most cells tested. Electrophysiological analysis showed that addition of the $\gamma 2$ subunit increased steady-state inactivation of P/Q-type $\alpha 1$A calcium channels (Klugbauer *et al*., 2000; Letts *et al*., 1998; Rousset *et al*., 2001), caused a significant decrease in the current amplitude of both $\alpha 1$A and $\alpha 1$B calcium channels (Kang *et al*., 2001), and substantially slowed the rate of deactivation of T-type $\alpha 1$I channels (Green *et al*., 2001). Although it is still not clear which type of calcium channel it is associated with in the brain, the $\gamma 2$ subunit has been shown to form complexes in vivo with VDCC (Kang *et al*., 2001; Sharp *et al*., 2001). In addition to these modulatory effects on VDCC, several recent studies also show that $\gamma 2$ is involved in other biological functions,

which are not directly related to calcium or calcium channels.

STARGAZIN IN REGULATING AMPA RECEPTOR FUNCTION

The initial linkage between $\gamma 2$ and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors was from the observation of a selective absence of postsynaptic AMPA currents in cerebellar granule cells of *stargazer* mutants (Hashimoto *et al*., 1999). Specifically, the excitatory postsynaptic currents (EPSCs) at mossy fiber to granule cell synapses are devoid of the fast component mediated by AMPA receptors while the slow component mediated by NMDA receptors is normal. The defect in AMPA receptor function is specific to cerebellar granule neurons, as the AMPA-mediated component is normal in CA1 pyramidal cells of the *stg* mutant. Measurement of glutamate release from presynaptic terminals during synaptic transmission to granule cells has ruled out a possible defect in neurotransmitter release. In fact, these neurons are insensitive to exogenously applied AMPA (Hashimoto *et al*., 1999). Interestingly, the same functional defects were also found in cerebellar granule cells of another *stargazer* allele, *waggler* mouse, which arose independently in a different genetic background (Bao *et al*., 1998; Chen *et al*., 1999; Qiao *et al*., 1998a). These results indicate that the stargazin gene mutation somehow leads to absence of functional AMPA receptors at the postsynaptic membrane in cerebellar granule cells.

AMPA receptors are hetero-tetramers composed of variable combinations of four subunits, glutamate receptor 1 (GluRl)–GluR4 (Hollmann & Heinemann, 1994; Seebburg, 1993). Cerebellar granule cells express predominantly GluR2 and GluR4 in adults with transient expression of GluRl and GluR3 during development (Hollmann & Heinemann, 1994). It would be hard to imagine that a single gene is capable of silencing four molecules located on different chromosomes. Indeed, analysis of two $\gamma 2$ mutant alleles revealed that selective AMPA receptor dysfunction is not associated with altered expression of any AMPA receptor subunit at either mRNA or protein levels (Chen *et al*., 1999; Hashimoto *et al*., 1999). As each AMPA receptor subunit has at least one different alternative splicing variant and the flip form is about five times more potent than the flop in terms of current flow (Hollmann & Heinemann, 1994), failure of a developmental switch from the flop to flip form is another possibility, which could account for the AMPA receptor defects. This hypothesis was ruled out when in situ hybridization using splice-form-specific oligo probes revealed no significant difference in abundance of either the flip or flop form of any AMPA receptor subunit (Qiao *et al*., 1998a). The lack of AMPA receptor synaptic currents in the *stargazer* mouse has been proven to be an autonomous granule cell defect, independent of cerebellar circuitry, caused by selective absence of functional AMPA receptors on both synaptic and extrasynaptic membrane (Chen *et al*., 2000).

Interaction of Stargazin With AMPA Receptor

The initial clue regarding the potential receptor targeting defect was provided by an immunolocalization study of AMPA receptor subunits in cerebellar granule cells (Chen *et al*., 2000). Both immunocytochemical staining and immunogold electron microscopic analysis revealed lack of synaptic labeling of AMPA receptor subunits despite the presence of cytoplasmic staining. The physical association of stargazin with AMPA receptor subunits was first established by co-immunoprecipitation. Chen *et al*. (2000) have shown that in co-transfected COS cells, stargazin co-immunoprecipitates with GluRl, 2, and 4. Such direct interaction appears to be specific to AMPA receptors, as stargazin does not bind to NMDA receptor subunit 1 or Kvl.4, a voltage-dependent potassium channel. Later studies by Sharp *et al*. (2001) also confirmed the co-immunoprecipitation of stargazin with GluRl from solubilized mouse brain homogenates. The most direct evidence came from reversal of mutant cerebellar granule cell dysfunction by adding the $\gamma 2$ molecule. Transfecting stargazin rescues AMPAR-mediated synaptic currents in *stg* cerebellar granule cells (Chen *et al*., 2000), thus indicating that stargazin regulates AMPA receptor function.

C-Terminal PDZ-Binding Domain Mediates the Interaction

Proteins that contain PSD-95/DLG/ZO-1 (PDZ) homologous domain motifs play key roles in scaffolding receptors and signaling elements at synapses. Detailed analysis of stargazin structure revealed the basis of such interactions in the regulation of AMPA receptors. The cytosolic carboxy terminal tail of stargazin contains a PDZ-binding site, which could bind to type 1 PDZ domains of several PDZ proteins. Indeed, a series of studies showed that the PDZ binding site is critical for synaptic targeting of AMPA receptors (Chen *et al*., 2000).

Stargazin is able to associate through the PDZ-binding site with several synaptic PDZ proteins including PSD-95, SAP-97, PSD-93, and SAP-102 in vitro. Co-transfecting experiments have further confirmed that stargazin regulates AMPA receptor function by targeting and clustering AMPA receptors through its C-terminal interaction with PDZ proteins (Chen *et al*., 2000). Co-transfecting stargazin and PSD-95 induces clusters of GluR4 at the cell surface, while deleting the final four amino acids of stargazin (stargazinΔC) disrupts interaction with PSD-95 and prevents clustering of GluR4. In electrophysiological studies, it is possible to distinguish the function of synaptic versus extrasynaptic AMPA receptors by measuring AMPA receptor-mediated synaptic currents and glutamate-evoked responses. The stargazin mutation not only disrupts synaptic AMPA receptor function but also abolishes AMPA receptor responses at extrasynaptic sites (Chen *et al*., 2000). That the C-terminal of stargazin is required for synaptic clustering of AMPA receptors and AMPA-mediated synaptic currents was demonstrated by Chen *et al* (2000) who showed that a truncated-form of stargazin failed to rescue AMPA receptor-mediated synaptic currents in *stg* cerebellar granule cells. Interestingly, the glutamate-evoked response in *stargazer* granule neurons can be restored by both full length as well as stargazinΔC transfection. These results clearly indicate that the stargazin molecule regulates AMPA receptor function through two separate processes: membrane targeting and synaptic clustering. In the first step, stargazin interacts with AMPA receptors in a C-terminal-independent manner and regulates the delivery of AMPA receptors to the cell surface. Second, stargazin interacts through its C-terminal PDZ-binding site with PSD-95 or other PDZ proteins and causes AMPA receptors to cluster at synapses. These findings were further confirmed in a gene transfection study on hippocampal slice culture by Schnell *et al*. (2002). They showed that increasing synaptic PSD-95 levels selectively increases the number of synaptic AMPA receptors, while overexpression of stargazin massively increases the number of extrasynaptic AMPA receptors without affecting the synaptic response. Through analyzing a series of mutant and deletion constructs, they also showed that direct interactions between the stargazin C-terminal PDZ-binding site and the first two PDZ domains of PSD-95 mediate the synaptic delivery of AMPA receptors.

The stargazin-mediated AMPA receptor targeting mechanism appears to be generally utilized in excitatory synapses in the central nervous system. First, a similar clustering effect is also seen in hippocampal neurons (Chen *et al*., 2000). The stargazinΔC transfection disrupts AMPA receptor responses in wild-type cerebellar granule cells as well as hippocampal neurons by a significant reduction of both the amplitude and frequency of AMPA synaptic events (Chen *et al*., 2000).

Second, most of the γ subunits, except γ1 and γ6, have a PDZ binding motif (Chu *et al*., 2001). Among these subunits, γ3, γ4, and γ8 share high sequence homology with stargazin, and γ3 and γ4 have been found to rescue AMPA receptor function in *stargazer* cerebellar granule cells (Chen *et al*., 2001). Although no compensatory increase of another γ subunit was evident in the *stargazer* mouse (Sharp *et al*., 2001), reliance predominantly on the γ2 isoform may explain the restriction of AMPA defects to cerebellar granule cells. It would be interesting to determine whether all PDZ containing γ subunits can regulate AMPA receptor targeting and clustering.

Although in vitro COS cell studies demonstrate that the stargazin C-terminal PDZ-binding sequence is necessary for proper AMPA receptor targeting to synapses, a subsequent question is which molecules bind stargazin and mediate AMPA receptor targeting and clustering under physiological conditions. Several candidate genes have been pulled out using yeast two-hybrid screening of a rat brain library (Chetkovich *et al*., 2001). These include the Protein Interacting Specifically with TC10 (PIST) molecule as well as several well-characterized PDZ proteins including PSD-93, PSD-95, SAP-97, and SAP-102. While other PDZ proteins interact with stargazin via the PDZ domain, PIST binds to stargazin independently of its PDZ-binding domain. This raises the possibility that PIST may be responsible for C-terminal-independent AMPA receptor membrane targeting. Further experiments are necessary to sort out which part of the stargazin molecule interacts with PIST, and the functional consequences of such an interaction.

REGULATION OF STARGAZIN INTERACTION WITH PDZ BINDING PROTEIN

As the stargazin C-terminus proved to be crucial to synaptic clustering, much attention has been focused on the C-terminal region of stargazin. Detailed analysis of the stargazin C-terminal sequence revealed that the threonine residue (T321) is critical for PDZ binding, and is a potential phosphorylation site for multiple protein kinases including cAMP-dependent protein kinase A (PKA). Indeed, T321 of stargazin has been shown to be phosphorylated by PKA in vitro (Chetkovich *et al*., 2002; Choi *et al*., 2002). Using phosphorylation-site-specific stargazin antibodies, Choi *et al*. (2002) also showed that the stargazin C-terminus is phosphorylated at the T321 residue in vivo. From earlier work on other molecules with

similar structure it was predicted that phosphorylation of T321 might disrupt interactions between stargazin and the PDZ-binding proteins. To test this hypothesis, Choi *et al*. (2002) created mutations (T321D, T321E) mimicking stargazin phosphorylation. The mutated proteins not only failed to interact with PSD-95 and SAP-97 in the yeast two-hybrid assay, but also abolished biochemical association and co-clustering between stargazin and PSD-95 in co-immunoprecipitation experiments. In triply transfected COS cells with stargazin, PSD-95, and the PKA catalytic subunit, PSD-95 antibodies brought down a significant amount of total stargazin protein without a detectable amount of phosphorylated stargazin. The importance of the T321 is also confirmed by a transfection study on hippocampal slice culture using the construct T321F stargazin point mutation (Schnell *et al*., 2002). Stargazin T321F strongly depressed AMPA receptor-mediated synaptic currents, which mimics the stargazinΔC result, and prevented the enhancement of the AMPA currents induced by PSD-95 overexpression. Interestingly, T321 phosphorylation does not affect PIST binding with stargazin, further confirming that PIST does not interact with stargazin at its C-terminal PDZ-binding domain (Chetkovich *et al*., 2001). These findings demonstrate that phosphorylation of stargazin at the C-terminal T321 residue specifically inhibits its interaction with PDZ binding proteins.

Stargazin phosphorylation also affects synaptic localization of stargazin. Chen *et al*. (2000) have shown that stargazin is enriched in detergent-insoluble postsynaptic density (PSD) fractions along with AMPA receptor subunits and PSD-95. Immunoblot analysis of PSD fractions of rat brain revealed a significant enrichment of total stargazin in all PSD fractions with phosphorylated stargazin decreasing to nondetectable levels in the core PSD III fraction (Choi *et al*., 2002). This suggests that phosphorylated stargazin is less tightly associated with PSD. Chetkovich *et al*. (2002) also showed recently that stargazin T321 phosphorylation disrupts synaptic AMPA receptor clustering and downregulates AMPA synaptic currents in cultured hippocampal neurons. This further establishes that the phosphorylation site is an important regulator of synaptic density and function of AMPA receptors. As the C-terminal phosphorylation sequence of stargazin is a consensus for several different protein kinases, additional experiments are needed to determine if other protein kinases are involved in regulating stargazin and PDZ-binding protein interactions. Also, the exact signal transduction pathways connecting stargazin phosphorylation with dynamic regulation of synaptic AMPA receptors remain to be worked out.

RELATIONSHIP BETWEEN STARGAZIN CALCIUM CHANNEL FUNCTION AND AMPA TARGETING

It is still controversial whether the stargazin molecule can serve as a calcium channel subunit modulating calcium kinetics and at the same time as a membrane protein regulating AMPA receptor targeting and synaptic strength. Anatomically, VDCC complexes are localized to both cell bodies and neuronal processes including postsynaptic regions (Hillman *et al*., 1991; Ludwig *et al*., 1997), while the $\gamma 2$ subunit (Sharp *et al*., 2001) is localized to dendrites and postsynaptic densities. Evidence from co-immunoprecipitation studies also reveals a biological association of stargazin with both the VDCC complex (Kang *et al*., 2001; Sharp *et al*., 2001) as well as AMPA receptors (Chen *et al*., 2000; Sharp *et al*., 2001). The establishment of a complex between VDCC $\alpha 1$B subunit and GluRl protein in solubilized mouse brain homogenates provided the first physical link between VDCC subunits and AMPA receptors (Sharp *et al*., 2001). These results suggest close association of the VDCC channel-forming subunit with stargazin and AMPA receptors. On one hand, stargazin effects on AMPA receptor function appear to be independent of calcium channel function, as blockade of VDCC does not affect stargazin AMPA receptor clustering (Chen *et al*., 2000). On the other hand, there is indirect evidence that VDCC activation induces clustering of PSD-Zip45, a member of the Homer/Vesl protein family (Okabe *et al*., 2001), indicating that increased intracellular calcium regulates PDZ binding proteins. Borgdorff and Choquet (2002) have reported recently that raising intracellular calcium triggers rapid AMPA receptor immobilization and local accumulation on the neuronal surface. This suggests that AMPA receptor lateral movements are also regulated by calcium influx. Although it is still not clear at this point if stargazin and/or associated γ subunits play any role in the process, it presents the intriguing possibility that the γ subunit may be a structural and functional link between AMPA receptors and VDCC, integrating calcium-channel-induced intracellular signaling and synaptic localization of AMPA receptors.

OTHER BIOLOGICAL EFFECTS OF STARGAZIN

The stargazin mutation also affects GABAergic neurotransmission in *stg* cerebellar granule cells. It was found that expression of the $\alpha 6$ and $\beta 3$ subunits of the $GABA_A$ receptor is significantly decreased (Thompson *et al*., 1998), and [^{3}H] musimol binding assay showed 39% of control level in *stg* cerebellar granule neurons (Thompson *et al*., 2002). Functional studies revealed slower $GABA_A$

synaptic currents in *stg* cerebellar granule cells (Chen *et al*., 1999). It is also suggested that $GABA_A$ receptor trafficking may be compromised in *stg* cerebellar granule cells, as cell surface expression of $\alpha 6$ and δ subunits was downregulated in *stargazer*, and stargazin can be co-immunoprecipitated with $GABA_A$ δ (Thompson *et al*., 2002). While there is evidence that the stargazin molecule is not colocalized with inhibitory boutons in the hippocampus (Chen *et al*., 2000), further study is necessary to determine if similar receptor-targeting mechanisms to those in glutamate transmission are involved in GABA synaptic transmission.

The specific role of stargazin in the selective failure of BDNF expression in cerebellar granule cells of *stargazer* is also not clear (Qiao *et al*., 1998b). It is obvious that the transmembrane protein stargazin does not regulate BDNF gene expression directly. However, it is well known that regulation of BDNF expression is highly dependent upon neuronal activity. Both VDCC and AMPA receptors are heavily involved in neuronal activity and can induce BDNF expression in several ways. VDCC-evoked calcium influx activates multiple calcium sensitive transcription factors and regulates BDNF gene transcription (Shieh *et al*., 1998; Tabuchi *et al*., 2000; Tao *et al*., 1998, 2002). An AMPA receptor-mediated increase in BDNF mRNA has been reported to involve a mitogen-activated protein (MAP) kinase signaling pathway that is independent of ionotropic activity and Ca^{2+} influx in cerebellar cultures (Hayashi *et al*., 1999). In some neurons, AMPA receptors and VDCC may work consecutively. Legutko *et al* (2001) showed that AMPA receptor-mediated increases in BDNF mRNA involve activation of both L-VDCCs and MAP kinases in cerebellar granule cells. Further experiments are needed to explore these possibilities and to sort out whether the selective lack of BDNF seen in the *stargazer* cerebellum is caused by developmental silence of AMPA receptors or if it is linked to alterations of calcium channel kinetics, or both.

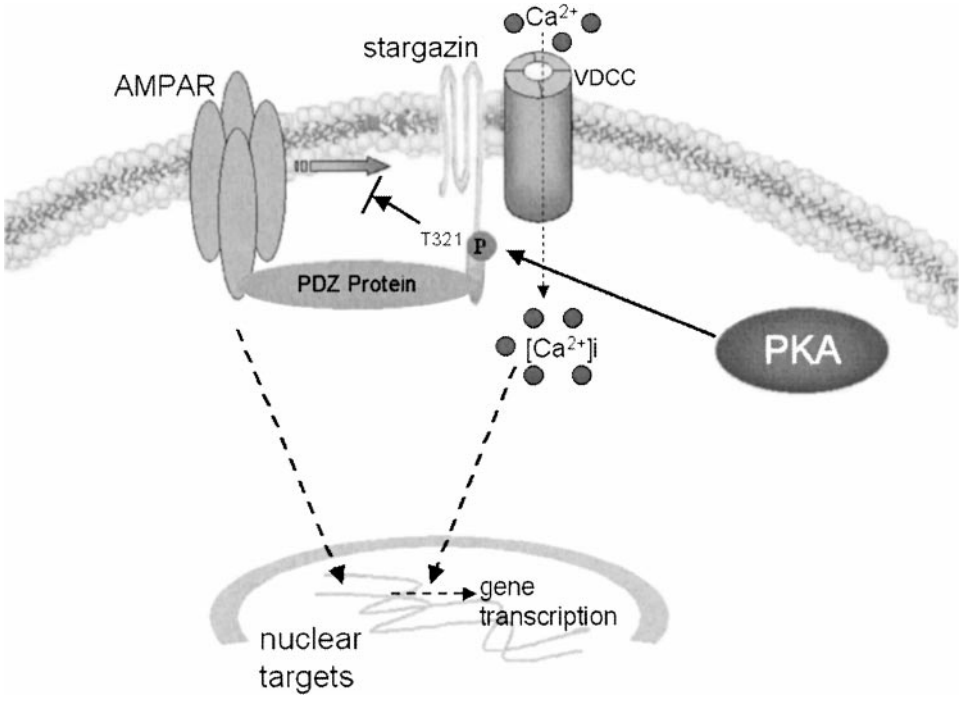

Fig. 2. Potential role of stargazin in modulation of calcium channel function and AMPA receptor clustering. Stargazin interacts through its C-terminal PDZ domain with PDZ proteins and brings AMPA receptors to postsynaptic sites. The interaction of stargazin with PDZ proteins can be modulated by PKA phosphorylation on the C-terminal T321 residue, which in turn will regulate AMPA receptor clustering. The association of stargazin with both VDCC and the AMPA receptor provides a basis to integrate the AMPA receptor-mediated synaptic response with calcium channel activation-induced increases in intracellular calcium levels. These postsynaptic changes will regulate the efficacy of synaptic transmission and affect gene transcription through multiple signal transduction pathways. VDCC: voltage-dependent calcium channels; AMPAR: AMPA receptor; PKA: cAMP-dependent protein kinase A; P: phosphorylation.

BDNF plays an important role in postnatal maturation of cerebellar granule cells. Both $GABA_A$ receptor subunit composition and electrophysiological behavior of *stargazer* granule cells are characteristic of immature status during early development (Chen *et al*., 1999; Thompson *et al*., 1998). These immature features along with the delayed cerebellar granule cell migration and immature granule cell ultrastructure (Qiao, 2001; Qiao *et al*., 1998b) can be partly explained as consequences of the BDNF defect. This possibility is supported by several similar abnormalities being found in the cerebellum of BDNF knockout mice (Conover *et al*., 1995; Schwartz *et al*., 1997). Additional studies are necessary to address the molecular mechanisms underlying these abnormalities.

In summary, recent studies clearly indicate that in addition to its classical function in modulation of calcium kinetics, stargazin as well as several of its associated γ isoforms are involved in AMPA receptor trafficking. This stargazin-mediated AMPA receptor synaptic targeting includes at least two steps, C-terminal-independent membrane insertion and C-terminal/PDZ-binding-dependent synaptic clustering. The interaction of stargazin with PDZ-binding proteins can be modulated by PKA phosphorylation on the C-terminal T321 residue. These findings, plus the evidence of the biological association of stargazin with both VDCC $\alpha 1$ subunits and AMPA receptors suggest that stargazin may be a critical link providing intriguing connections between calcium channels and AMPA receptors (Fig. 2). Such integration of two essential elements in the postsynaptic membrane provides a powerful potential mechanism to regulate synaptic transmission and synaptic plasticity. The crucial role of stargazin in synaptic transmission and subsequent effects on intracellular signal transduction and gene expression may explain the severe biological defects seen in the *stargazer* mutant. Studies of the stargazin molecule have provided valuable insights into the normal structure and function of γ subunits and will help us to identify its exact role in cellular disease processes. Better understanding of the physiological and pathophysiological function of each individual calcium channel subunit will promote the design of new treatment strategies, which would be beneficial for patients with similar types of disorders in the future.

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