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In vivo regulation of AMPA receptors by their TARP auxiliary subunits

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In vivo regulation of AMPA receptors by their TARP auxiliary subunits

by

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Dedicated to my parents and my sister

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Contributions

The work in Chapters 3-5 was done in collaboration with other authors. The information in Chapter 2 describes the methods used by Karen as well as the other authors in Chapters 2-5 and some of this text is included in manuscripts submitted or accepted for publication.

All electrophysiological data in Chapter 3 was collected by Karen. She also generated the RNAi constructs and γ-2 constructs. Jessica O'Brien, a technician in the laboratory of Roger Nicoll, performed immunoblot experiments and prepared the majority of dissociated neuron cultures. Siavash Karimzadegan, a technician in David Bredt's laboratory, generated the γ-3 and γ-4 knockout mice.

For Chapter 4, Karen conceived of the experiments and carried out the majority of the data collection. The biochemistry and immunohistochemistry data presented in Figure 2 was collected by Jessica O'Brien. Generation of the γ-3 knockout mice was done by Siavash Karimzadegan. This work has been submitted for publication

Experiments in Chapter 5 were carried out by Karen and Franklin Hays. Karen collected the electrophysiological data, and Franklin carried out the crystal structure analysis. Franklin is a member of the Robert Stroud laboratory. This work has been accepted for publication in Science Magazine.

In vivo regulation of AMPA receptors by their TARP auxiliary subunits

Karen Menuz

Abstract

Ion channels are often modulated by auxiliary subunits. Transmembrane AMPA receptor regulatory proteins (TARPs) are auxiliary subunits for AMPA-type glutamate receptors. These receptors are responsible for much of the fast excitatory synaptic transmission in the brain, and their mobility is thought to contribute to memory storage mechanisms. Although TARPs are known to modify AMPA receptor trafficking and gating *in vitro*, their contribution to *in vivo* AMPA receptor function is less clear.

By generating mice lacking multiple TARP family members, we found that TARPs are functionally redundant. Single TARP knockout mice are viable, while those lacking multiple isoforms are often lethal. Consistent with molecular redundancy, AMPA receptor transmission in cerebellar Golgi cells is unaffected in single TARP knockout mice, but nearly eliminated in double knockouts. Unexpectedly, the remaining AMPA receptors have a different subunit composition, suggesting that TARPs may preferentially traffic GluR2 containing receptors.

Our studies also highlight a role for TARPs in inhibitory neurons because AMPA receptor function in cerebellar Purkinje cells and Golgi cells is reduced in TARP knockout mice. Additionally, the loss of TARPs reduces the decay time of interneuron EPSCs. This indicates that AMPA receptor biophysical properties make a significant contribution to the time course of synaptic events, even at fast-decaying interneuron synapses.

Our studies investigating TARP function *in vivo* also led to the discovery that TARPs profoundly change AMPA receptor pharmacology. Whereas CNQX is a competitive antagonist on AMPA receptors alone, it is a partial agonist on receptors containing any member of the TARP family and elicits depolarizing currents in neurons throughout the brain. By obtaining the crystal structure of CNQX bound to the AMPA receptor ligand-binding domain, we determined that CNQX induces a small amount of domain closure. Based on our findings, we propose an expanded model of AMPA receptor gating such that TARPs increase the likelihood that domain closure leads to channel opening, i.e. they increase the coupling efficiency. Together our studies demonstrate that TARPs are an integral component of AMPA receptors in the central nervous system.

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Chapter 1:

General Introduction

Ion channels are membrane-bound pore-forming proteins that permit the regulated movement of ions across cellular membranes. They are essential for the function of the nervous system as well as skeletal and cardiac muscle contraction, amongst other biological functions. From voltage-gated calcium channels that allow for neurotransmitter release to ligand-gated acetylcholine receptors that trigger muscle contraction, there is a large diversity of ion channels that arose through gene duplication and modification of ancestral channels. The primary ion conducting subunits of related ion channels often form heteromeric channels, which are further modulated by the presence of auxiliary subunits. Thus, the modular nature of channels allows the combinatorial generation of channels with distinct and specialized functions.

Auxiliary subunits influence primary subunit function

The primary pore-forming α subunits of voltage-gated channels are often associated with auxiliary subunits that modulate their activity. There are criteria that a protein must meet to be considered an auxiliary subunit, rather than a protein that simply interacts with the primary α subunit (Arikkath and Campbell, 2003). An auxiliary subunit should form a stable association with the α subunit as shown by its presence in purified channel complexes, bind directly to the α subunit, and alter the trafficking and/or gating properties of the α subunit. Until recently, auxiliary subunits had not been described for ligand-gated ion channels, and therefore the bulk of the work describing their function had been done on voltage-gated ion channels. The importance of auxiliary subunits *in vivo* is highlighted by their association with some inherited human disorders including

familial epilepsy, ataxia, and long QT syndrome (Arikkath and Campbell, 2003; Isom, 2001; Yu et al., 2005).

While some auxiliary subunits are transmembrane proteins, such as the β subunits of voltage-gated sodium channels, others are cytosolic proteins, such as the β subunits of voltage-gated potassium channels (Isom et al., 1994). There are often many different isoforms of a given auxiliary subunit that can interact either promiscuously or with limited members of a given voltage-gated ion channel family, in part determined by their developmental and cell-type expression pattern (Dolphin, 2003; Isom, 2001; Li et al., 2006). Additionally, each α subunit may interact with multiple different auxiliary subunits such as voltage-gated calcium channel α subunits that bind $\alpha_2\delta$, γ, and β subunits (Isom et al., 1994). Together the diversity of the α subunits, auxiliary subunits, and their interactions generates a large number of possible channel complexes. This allows channels to fit precisely the signaling role required, and reflects the modular nature of proteins and complexes often seen in nature.

Auxiliary subunits that modulate the trafficking of α subunits often do so by increasing the surface expression of receptors, and sometimes by affecting their subcellular localization. For example, some β auxiliary subunit isoforms increase the surface expression of associated calcium, sodium, and potassium channel α subunits, perhaps by masking endoplasmic reticulum (ER) retention motifs on the α subunit (Arikkath and Campbell, 2003; Dolphin, 2003; Hanlon and Wallace, 2002). Sodium channel β subunits can interact with specific extracellular matrix proteins thereby localizing channels to specific subcellular regions such as nodes of Ranvier and axon initial segment (Hanlon and Wallace, 2002; Lai and Jan, 2006). The creation of chimeric

and point mutants has allowed the identification of the specific sites of interaction between many auxiliary and α subunits, such as the identification of the precise interaction between calcium channel α and β subunits (Arikkath and Campbell, 2003; Hanlon and Wallace, 2002; Isom, 2001; Walker and De Waard, 1998).

Many gating properties of voltage-gated ion channels can be affected by the presence of auxiliary subunits, including their activation and inactivation kinetics and their voltage-dependence. For example, both the β1 sodium channel auxiliary subunit and the β1 and β3 potassium channel subunits speed inactivation of their associated α subunits (Hanlon and Wallace, 2002; Isom et al., 1994). Different auxiliary subunit isoforms can have modulatory effects differing in magnitude or even direction, such as calcium channel subunits β1b, β2 and β4 which slow inactivation to varying degrees while β3 speeds inactivation (Dolphin, 2003). Each individual auxiliary subunit often affects multiple gating properties.

Auxiliary subunits with similar regulatory properties have recently been identified for the ligand-gated AMPA receptors. I will first describe what is known about the properties and function of AMPA receptors in the absence of transmembrane AMPA receptor regulatory proteins (TARPs), and then describe the ways in which TARPs modulate their function.

AMPA receptors in the CNS

General Background

AMPA and NMDA receptors are the two families of ionotropic glutamate receptors responsible for the majority of fast synaptic transmission in the CNS. Both

receptor-types are key players in synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), processes that may underlie learning and memory (Whitlock et al., 2006). In LTP, specific patterns of synaptic activity lead to an NMDA receptor dependent increase in synaptic strength due to the movement of AMPA receptors into the synapse, while in LTD, different activity patterns lead to an NMDA receptor dependent removal of AMPA receptors to lower synaptic strength (Bredt and Nicoll, 2003). Thus due to their role in both synaptic plasticity and moment-to-moment neuronal signaling, the regulation of AMPA receptors by interacting proteins and cell signaling pathways has been an active field of study.

AMPA receptors are tetrameric monovalent ion channels formed by either homomeric or heteromeric combinations of the subunits GluR1-4 (Figure 1A) (Boulter et al., 1990; Keinanen et al., 1990; Rosenmund et al., 1998; Sommer et al., 1990). Each GluR subunit is approximately 900 amino acids in length and can be functionally separated into an extracellular N terminal domain, a ligand-binding domain composed of S1 and S2, three transmembrane domains and a reentrant pore loop, and an intracellular C terminal tail (Figure 1B). The ligand-binding domain is found in two regions of the receptor: S1 is found just before the first transmembrane domain, and S2 is located between the second and third transmembrane domains. Each subunit binds one glutamate molecule, allowing the tetrameric receptor to bind up to four glutamate molecules simultaneously.

Differences in the expression patterns, trafficking and biophysical properties of individual GluR subunits contribute to the diversity of AMPA receptors observed in neurons (Geiger et al., 1995; Lambolez et al., 1996). The functional variety of AMPA

receptors is increased through splice variants of GluR1-4 and RNA editing of GluR2. The flip and flop splice variants are alternatively spliced in a short region in the S2 domain and affect both receptor kinetics and channel pharmacology (Mosbacher et al., 1994; Partin et al., 1994; Sommer et al., 1990). Most AMPA receptor subunits have a conserved glutamine (Q) residue in their pore, but nearly all native GluR2 subunits have an arginine (R) instead due to RNA editing (Sommer et al., 1991). The presence of GluR2(R) subunits generates receptors that are calcium-impermeable and have a linear current-voltage relationship, while receptors with only GluR(Q) subunits are calcium-permeable and inwardly rectifying (Hollmann et al., 1991; Mansour et al., 2001; Verdoorn et al., 1991).

AMPA receptor pharmacology

Glutamate is the endogenous agonist for many ionotropic and metabotropic receptors in the CNS. Therefore, for several decades before their cloning, the function of glutamate receptors in the CNS was studied based on their pharmacology. Receptors were distinguished by selective antagonists and rank-order comparisons of agonist potency (Collingridge and Lester, 1989). For many years, it was unclear whether AMPA receptors were different than kainate receptors, and they were grouped together as the non-NMDA receptors (Watkins et al., 1990). As with kainate and NMDA receptors, AMPA receptors were named for exogenous agonists that preferentially activate them (Watkins et al., 1990).

Until the identification of the high affinity quinoxalinedione family of non-NMDA receptor competitive antagonists, including CNQX and DNQX, good selective

antagonists were only known for NMDA receptors, and this confounded the study of AMPA receptors (Honore et al., 1988; Watkins et al., 1990). Further pharmacological distinction between AMPA and kainate receptors depended upon the development of the 2,3-benzodiazepine family of non-competitive antagonists, including GYKI 53655 and GYKI 52466, which selectively block AMPA receptors (Donevan and Rogawski, 1993; Tarnawa et al., 1993).

Functional studies on the cloned AMPA receptor subunits have refined and increased our knowledge about their pharmacology. Full-agonists such as glutamate, quisqualate and AMPA maximally activate receptors, and the responses to these agonists rapidly and strongly desensitize in the prolonged presence of these agonists (Patneau and Mayer, 1990; Sommer et al., 1990; Trussell et al., 1988). In contrast, partial-agonists such as kainate and domoate evoke weakly desensitizing, submaximal responses even at saturating concentrations due to their lower efficacy (Patneau and Mayer, 1990; Patneau et al., 1993; Sommer et al., 1990; Trussell et al., 1988).

Some allosteric compounds, including cyclothiazide, aniracetam, and trichloromethiazide, partially or fully slow and prevent AMPA receptor desensitization depending in part on the GluR subunit isoform. Cyclothiazide modulates flip splice variants with little effect on flop variants, and this modulation is determined by a single amino acid in the S2 region (Partin et al., 1995; Partin et al., 1994). Some naturally occurring toxins, such as philanthotoxin and Joro spider toxin, selectively block GluR2(R) lacking channels (Blaschke et al., 1993; Brackley et al., 1993). Unfortunately, few compounds that can easily distinguish between the other subunits have been developed (Nilsen and England, 2007). New drugs will continue to become available as

many compounds targeting AMPA receptors are currently under development for use as cognitive enhancers and the treatment of disorders such as Parkinson's, epilepsy, and stroke (Lynch, 2006; Rogawski, 2006).

Biophysical properties and structure-function relationship of AMPA receptors

Synaptic AMPA receptor mediated currents decay within a few milliseconds, due to the fast removal of glutamate from the synaptic cleft and the intrinsic biophysical properties of AMPA receptors. AMPA receptors have a relatively low affinity for glutamate compared to NMDA receptors. In response to short pulses of glutamate, AMPA receptors deactivate quickly, ($\tau < 1$ ms), governed by the time course of agonist dissociation, which is inversely related to agonist affinity (Erreger et al., 2004; Zhang et al., 2006). During prolonged glutamate applications, the peak current rapidly decays to a steady state current (desensitization, $\tau \sim 1$-6 ms), in which the agonist- bound receptor is thought to be in equilibrium between open and desensitized receptor conformations (Erreger et al., 2004). As the desensitized state is thought to be non-conducting, the steady-state currents are only a small fraction of the peak currents.

Subunit composition also affects AMPA receptor kinetics. The presence of flop subunits increases the rate of desensitization compared to flip receptors (Mosbacher et al., 1994; Sommer et al., 1990). GluR4 receptors desensitize and deactivate faster than other receptors, while GluR2(R) containing receptors generally have slower kinetics (Geiger et al., 1995; Mosbacher et al., 1994). As a result, the time course of synaptic EPSCs is sometimes partly dependent upon the subunit composition (Jonas, 2000).

High-resolution crystal structures of the isolated AMPA receptor ligand-binding domain have provided insight into their structure-function relationship (Figure 1C). The ligand-binding domain contains two lobes that both contain a section of the S1 and S2 domains, which are artificially connected by a linker to replace the pore-forming regions. The two lobes are arranged in a clamshell structure, with the ligand-binding site in the inner hinge region. The ligand-binding domain is usually crystallized as a back-to-back dimer of two S1S2 protomers, with the upper lobes of each protomer forming a stable interface. Agonist binding induces movement of the lower and upper lobes towards each other (domain closure), and the degree of domain closure correlates with the efficacy of the agonist (Armstrong and Gouaux, 2000; Jin et al., 2003) (Figure 1C). Full agonists induce ~20º of domain closure relative to the *apo* state, while partial agonists induce intermediate states (Armstrong and Gouaux, 2000; Armstrong et al., 1998). Competitive antagonists stabilize the structure in a relatively open conformation (Armstrong and Gouaux, 2000; Hogner et al., 2003).

The initial step of domain closure moves the lower lobe up and away from the lower lobe of the other protomer, and this separation also correlates with agonist efficacy (Jin et al., 2003). This movement of the lower lobes is thought to induce strain on the pore-forming domains in the full-length subunits and thereby pull the channel open (Jin et al., 2003; Sun et al., 2002). This tension can alternatively lead to a separation of the dimer interface between the two upper lobes, thus relieving the strain in an agonist-bound non-conducting state, the desensitized receptor (Armstrong et al., 2006; Sun et al., 2002). Supporting this model, cyclothiazide binds in the interface between the two upper lobes and stabilizes their association, and a leucine to tyrosine point mutation (amino acid 507

in GluR3) that blocks receptor desensitization is found in a similar location (Stern-Bach et al., 1998; Sun et al., 2002). Furthermore, desensitization increases the distance between the two upper lobes (Armstrong et al., 2006).

Each GluR subunit within the tetrameric channel is thought to gate relatively independently of the other subunits. There is thought to be a direct correlation between the number of agonists bound and the conductance state of the receptor (Rosenmund et al., 1998). As a result, lower concentrations of agonist lead to a greater proportion of time spent in lower subconductance states (Rosenmund et al., 1998; Smith et al., 2000). Similar conductance levels are found for full and partial agonists, but the fraction of time spent in high conductance states is greater for full agonists (Jin et al., 2003). This suggests that each subunit only exists in either an open or a closed state, with larger degrees of domain closure translating into a greater probability of individual subunit opening (increased coupling efficiency) (Jin et al., 2003).

Biogenesis and trafficking of AMPA receptors

AMPA receptors are assembled from heteromeric and homomeric combinations of GluR1-4 (Figure 1A). In the early steps of biogenesis, AMPA receptor subunit mRNA can both be alternatively spliced to form flip and flop isoforms and undergo RNA editing, in particular at the Q/R site in GluR2 (Greger et al., 2003). After mRNA translation in the ER, AMPA receptors first assemble as dimers due to interactions of their of N terminal domains which allows them to only form receptors with members of the same receptor family, but not kainate or NMDA receptors (Ayalon and Stern-Bach, 2001). Heteromeric receptors with different GluR subunits and/or splice variants are preferentially formed

compared to heteromers (Brorson et al., 2004; Mansour et al., 2001). These dimers then dimerize to form the functional tetrameric receptor, a process enhanced by RNA editing at the R/G site in the S2 domain (Ayalon and Stern-Bach, 2001; Greger et al., 2006). Due to electro-repulsive charge interactions, RNA editing at the Q/R site in GluR2 prevents GluR2(R) tetramers from forming, although it is unknown whether this occurs at the dimerization or tetramerization step (Greger et al., 2003). Only properly assembled tetrameric channels are then exported from the ER to the Golgi, with flip splice variants trafficking better than flop variants (Coleman et al., 2006).

Receptor subunit composition is thought to regulate the post-ER trafficking of AMPA receptors. In particular, AMPA receptors with short C terminal tails (GluR2 and GluR3) are thought to be constitutively trafficked into synapses, while those with long C terminal tails (GluR1 and GluR4) require activity dependent synaptic plasticity such as LTP (Passafaro et al., 2001; Shi et al., 2001). AMPA receptors move laterally between the extrasynaptic membrane and the synapse, where they are stabilized by interactions with post-synaptic density (PSD) associated proteins such as PSD-95 (Bats et al., 2007; Elias et al., 2006; Groc et al., 2004).

Regulation of AMPA receptors by interacting proteins and phosphorylation

Given the importance of AMPA receptor signaling for synaptic transmission and plasticity, it is not surprising that they are tightly regulated by interacting proteins and phosphorylation. The C terminal tails of AMPA receptors are the least conserved part of the receptors, and it is these regions that have been studied for their involvement in protein interactions. Specific phosphorylation sites on the tails have been identified, such

as Ser831 and Ser845 on GluR1, and Ser880 on GluR2 (Matsuda et al., 1999; Roche et al., 1996). Phosphorylation of GluR1 augments receptor activity, and LTP may involve phosphorylation of Ser831 (Barria et al., 1997a; Barria et al., 1997b; Lee et al., 2003; Roche et al., 1996). The phosphorylation of GluR2, which is located within a PDZ binding motif, regulates the binding of PDZ proteins (Chung et al., 2000; Matsuda et al., 1999).

Cytosolic proteins containing multiple PDZ binding proteins such as GRIP and PICK1 can bind the C terminal tails of GluR2 and GluR3, while SAP-97 binds GluR1 (Dong et al., 1997; Leonard et al., 1998; Xia et al., 1999). Non-PDZ containing proteins that bind AMPA receptor C terminal tails include 4.1N that binds GluR1 and NSF that binds GluR2 (Song and Huganir, 2002). These protein interactions are proposed to regulate the synaptic localization of AMPA receptors, in particular during LTP and LTD (Bredt and Nicoll, 2003).

TARPs are AMPA receptor auxiliary subunits

Stargazer mice led to discovery of TARPs

Although many cytosolic proteins modulate AMPA receptor function, they are not considered to be auxiliary subunits. Indeed, until recently auxiliary subunits were primarily known for non-ligand-gated ion channels. This changed with the identification of TARPs as AMPA receptor auxiliary subunits (Figure 1D and 1E). Unlike the study of cytosolic interacting proteins, the discovery of TARPs was not through the systematic search for AMPA receptor interacting proteins, but began instead with a spontaneous mutation on the inbred A/J mouse strain at Jackson Laboratories (Noebels et al., 1990).

Mice homozygous for a recessive mutation were noticed due to their head-tossing and ataxia and were named stargazer. Further studies found that the mice had epileptic non-convulsive seizures more frequent and prolonged than any other mouse strain, and led to their use as a rodent model for absence epilepsy (Letts, 2005; Noebels et al., 1990). These generalized seizures, which occurred together with behavioral arrest, consisted of short bursts of 6/sec spike wave discharges most likely due to synchronized bursting in thalamocortical networks and were attenuated by ethosuximide, an anti-absence drug (Aizawa et al., 1997; Noebels et al., 1990; Qiao and Noebels, 1993). Two other spontaneous mutant mice strains were discovered, waggler and stargazer3J, that were allelic to the Stargzer mutation but produced less severe ataxia and seizures (Letts et al., 2003). As is common in seizure-prone animals, sprouting was seen in mossy fibers from hippocampal dentate granule cells in the adult stargazer mice (Qiao and Noebels, 1993). Cerebellar and vestibular disorders were also reported (Khan et al., 2004; Meng et al., 2006; Qiao et al., 1998; Qiao et al., 1996).

Genetic analysis indicated that the stargazer mutation was due to a transposon insertion in a novel gene, known as stargazin, which prevented normal mRNA splicing and production of the stargazin protein (Letts et al., 1998). Stargazin is 323 amino acid, ~36-28 kD four transmembrane protein with both its N and C terminus located in the cytoplasm (Letts et al., 1998). It contains a large extracellular loop between the first and second transmembrane domains, known as Ex1, and a ~110 amino acid long cytoplasmic C terminal tail (Figure 1E). The stargazin protein sequence shared ~20% identity with the skeletal calcium channel auxiliary subunit γ-1, and was therefore named γ-2 (Letts et al., 1998). A mutation in a calcium channel gene was not unexpected, as other rodent models

of epilepsy have mutations in calcium channel auxiliary subunits (Burgess et al., 1997; Fletcher et al., 1996). Later studies identified further related subunits γ-3 through γ-8 (Burgess et al., 1999; Burgess et al., 2001; Chu et al., 2001; Klugbauer et al., 2000).

The effects of γ-2 on calcium channel activity have been controversial, with disputed minimal effects on the gating of $Ca_V2.1$ (α1a, P/Q type) and T type channels in heterologous systems (Green et al., 2001; Kang et al., 2001; Klugbauer et al., 2000; Letts et al., 1998; Moss et al., 2003; Rousset et al., 2001). Calcium channels have been co-purified with γ-2 from brain tissue, but these complexes may also contain AMPA receptors (Kang et al., 2001; Kang et al., 2006; Sharp et al., 2001). The *in vivo* calcium channel modulation by γ-2 is equally unclear and may only occur in some neuron populations (Chen et al., 2000; Zhang et al., 2002).

The nearly complete loss of AMPA receptor mediated synaptic transmission in cerebellar granule cells from stargazer and waggler mice led to the finding that γ-2 acts as an AMPA receptor auxiliary subunit (Figure 1D) (Chen et al., 1999; Chen et al., 2000; Hashimoto et al., 1999). The interaction of γ-2 is highly specific as it modulates AMPA receptors, but not the closely related kainate receptors (Chen et al., 2003). AMPA receptor expression in stargazer granule cells was rescued by the expression of γ-2, or select members of the γ family (γ-3, γ-4, or γ-8) (Chen et al., 2000; Tomita et al., 2003). This group of proteins was then labeled the transmembrane AMPA receptor regulatory proteins (TARPs). TARPs are much more related to each other (48-75% amino acid identity) than to other γ subunits (~20% identity) and all TARPs contain a PDZ binding motif on their C terminal tails, while γ-1 and γ-6 do not (Chu et al., 2001). The roles of

TARPs in AMPA receptor regulation is now widely accepted, in contrast to their possible involvement in calcium channel modulation.

Distribution of TARPs

In adult animals, the four TARP family members are found primarily in the brain, although there are some reports of γ-4 expression outside the CNS in the prostate, lung, and small intestine (Burgess et al., 2001; Chu et al., 2001; Green et al., 2001; Klugbauer et al., 2000; Moss et al., 2003). TARP expression has also been detected in the spinal cord (Burgess et al., 2001; Green et al., 2001; Moss et al., 2003). Within the CNS, most TARPs are only expressed in neurons, with the exception of γ-4 that is also found in glia (Fukaya et al., 2005; Tomita et al., 2003).

Within the brain, TARP expression is generally widespread and associated with the expression of AMPA receptors. The highest expression of γ-2 is found in the cerebellum, and moderate levels have been reported in the cerebral cortex, hippocampus, olfactory bulb, striatum, thalamus, and hypothalamus (Fukaya et al., 2005; Green et al., 2001; Klugbauer et al., 2000; Moss et al., 2003; Tomita et al., 2003). High levels of γ-3 are found in the cerebral cortex and hippocampus, and moderate expression in the olfactory bulb, striatum, and hypothalamus (Fukaya et al., 2005; Green et al., 2001; Klugbauer et al., 2000; Moss et al., 2003; Tomita et al., 2003). Expression of γ-4 is highest in the striatum, and it is also present in the olfactory bulb, cerebellum, thalamus, hypothalamus, as well as various glia (Fukaya et al., 2005; Green et al., 2001; Klugbauer et al., 2000; Letts et al., 2005; Tomita et al., 2003). The presence of γ-4 has also been reported at lower levels in other regions, but this is likely due to its expression in glia

(Fukaya et al., 2005). γ-8 levels are clearly highest in the hippocampus, although it also present in cerebral cortex (Fukaya et al., 2005; Tomita et al., 2003).

The developmental expression of TARPs is unclear. Although some studies have detected the presence of multiple TARPs in fetal brain extracts (Burgess et al., 2001; Fukaya et al., 2005; Green et al., 2001), others have only detected the presence of γ-4 (Klugbauer et al., 2000; Letts et al., 2005; Tomita et al., 2003). This may be due to the use of rodent versus human tissue or different detection methods. The embryonic regional expression patterns of TARPs are similar to that in adults, with the exception of γ-4 that is more widely expressed in younger animals (Fukaya et al., 2005).

TARPs promote the surface expression of AMPA receptors

Like other pore-forming ion channel subunits, AMPA receptors can traffic to the plasma membrane in the absence of auxiliary subunits, but the presence of auxiliary subunits can promote trafficking. Native neuronal AMPA receptors can be co-purified with TARPs and all native γ-2 protein was shown to be associated with AMPA receptors (Fukata et al., 2005; Tomita et al., 2003; Vandenberghe et al., 2005b). The complex is likely to be tightly associated, as an unbiased mass-spectrometry analysis of proteins bound to native AMPA receptors identified all four TARPs (Nakagawa 2005). In contrast, quantitative analysis of native receptor complexes did not find significant binding to other AMPA receptor C terminus interacting proteins such as GRIP, PICK1, NSF, and SAP-97 although binding had been reported in other studies (Barry and Ziff, 2002; Fukata et al., 2005; Nakagawa et al., 2005; Vandenberghe et al., 2005b). Thus,

TARPs meet the auxiliary subunit criteria for formation of direct tightly associated complexes.

The first indication that TARPs are involved in AMPA receptor trafficking was the finding that stargazer cerebellar granule cells lack AMPA receptor EPSCs, despite normal synapse formation indicated by the unaffected NMDA receptor EPSCs (Chen et al., 1999; Chen et al., 2000; Hashimoto et al., 1999). Biotinylation assays of cultured stargazer cerebellar granule cells revealed a large reduction of surface AMPA receptor protein, which correlated with their decreased response to exogenously applied glutamate (Chen et al., 2000; Tomita et al., 2003). An approximately 90% reduction of total AMPA receptor protein levels also occurs in the hippocampus of γ-8$^{-/-}$ mice (Rouach et al., 2005). All members of the TARP family are able to rescue AMPA receptor surface expression in cultured stargazer granule cells (Chen et al., 2000; Tomita et al., 2003). Furthermore, overexpression of either γ-2 or γ-8 increases extrasynaptic AMPA receptor currents in hippocampal pyramidal cells, and co-expression of γ-2 increases AMPA receptor surface levels in heterologous cells such as *Xenopus* oocytes, HEK293 cells, and COS cells (Chen et al., 2003; Rouach et al., 2005; Schnell et al., 2002; Tomita et al., 2005a; Tomita et al., 2005b; Turetsky et al., 2005; Vandenberghe et al., 2005a).

TARPs are not involved in AMPA receptor oligomerization and bind tetrameric receptors rather than monomers or dimers (Vandenberghe et al., 2005b). TARPs associate with GluR tetramers in the ER and promote export from the ER the Golgi, a trafficking step that is often tightly regulated (Tomita et al., 2003). Thus, many of the remaining AMPA receptors in stargazer cerebellar granule cells are immaturely glycoslyated, consistent with an inability to exit the ER, whereas most receptors are mature in wild-

type mice (Tomita et al., 2003). TARPs appear to act as chaperones for AMPA receptors, and perhaps promote proper folding, as the unfolded protein response is upregulated in cells expressing AMPA receptors without TARPs (Vandenberghe et al., 2005a). The expression of γ-2 seems to be limiting for the formation of γ-2 / AMPA receptor complexes in wild-type mice as the ~50% of AMPA receptors not bound to TARPs are immature (Fukata et al., 2005; Vandenberghe et al., 2005b).

A region within the C terminal tail of TARPs is critical for its ability to promote AMPA receptor expression. By making chimeric domain swap constructs between γ-2 and γ-5, a related protein that does not affect AMPA receptor trafficking or function, it was shown that a γ-5 construct with the γ-2 C terminal tail (2Cyto) was able to increase AMPA receptor surface expression in oocytes to the same extent as full length γ-2, while a mirror chimeric construct (γ-2 with the γ-5 tail (Cyto)) was not (Tomita et al., 2005a). Furthermore, truncation of the γ-2 C terminus just after the fourth transmembrane domain also prevented enhancement of AMPA receptor expression in heterologous cells (Tomita et al., 2004; Turetsky et al., 2005). This suggests that the TARP C terminal tail is both necessary and sufficient for promoting surface trafficking.

Further mapping of the region within the C terminal that promotes receptor trafficking demonstrated the TARP PDZ binding motif is not required as γ-2 constructs missing the PDZ motif were able to rescue AMPA receptor surface expression in stargazer granule cells and increase extrasynaptic expression in hippocampal neurons (Chen et al., 2000; Schnell et al., 2002). Analysis of membrane trafficking in HEK cells indicates that the region between amino acids 212-288 is critical (Turetsky et al., 2005).

Less is known about the regions of AMPA receptors necessary for TARP binding and promotion of membrane trafficking. TARP binding and trafficking does not require the presence of the AMPA receptor N terminal domain or RNA editing of the Q/R site within the pore (Bedoukian et al., 2006; Korber et al., 2007a; Tomita et al., 2007). Although AMPA receptors with a truncation of their C terminal tail are nearly unable to traffic to the cell surface, γ-2 can still promote expression of these constructs (Bedoukian et al., 2006). TARPs are able to increase expression of both flip and flop AMPA receptor subunits, though they may particularly enhance the trafficking of flop subunits that can have difficulty exiting the ER in heterologous cells (Bedoukian et al., 2006; Coleman et al., 2006; Turetsky et al., 2005).

TARPs localize AMPA receptors to synapses

Another way in which TARPs act as auxiliary subunits is that they affect AMPA receptor localization within the neuron. Like AMPA receptors, TARPs are found on extrasynaptic membranes, but preferentially clustered at excitatory synapses (Chen et al., 2000; Cuadra et al., 2004; Fukaya et al., 2006; Inamura et al., 2006; Schnell et al., 2002; Tomita et al., 2003). TARPs target AMPA receptors to synapses via an interaction with synapse associated PDZ containing proteins, in particular membrane-associated guanylate kinase (MAGUK) proteins (Chen et al., 2000; Dakoji et al., 2003; Schnell et al., 2002). MAGUKs are cytosolic proteins that contain three PDZ binding domains, an SH3 domain and a guanylate kinase domain and include PSD-95, PSD-93, SAP-102, and SAP-97 (Tomita et al., 2001). *In vivo* TARPs may primarily interact with PSD-95 and PSD-93, but they are capable of binding SAP-102 and SAP-97 in heterologous systems

(Chen et al., 2000; Dakoji et al., 2003; Schnell et al., 2002). Consistent with this targeting mechanism, diffusion of γ-2 away from synapses to extrasynaptic sites is prevented by association with PSD-95 (Bats et al., 2007).

TARPs contain a type I PDZ binding motif (TTPV) on their C terminal tail which can interact with either of the first two PDZ domains in PSD-95 (Chen et al., 2000; Schnell et al., 2002). γ-2 constructs lacking the TTPV sequence (γ-2ΔC) are unable to rescue synaptic AMPA receptor currents in stargazer cerebellar granule cells, unlike full-length γ-2 (Chen et al., 2000). Furthermore, transfection of γ-2ΔC into wild-type cerebellar granule cells or hippocampal pyramidal neurons reduces synaptic AMPA receptor levels, presumably by competing with native γ-2 for the binding of AMPA receptors (Bats et al., 2007; Chen et al., 2000; Schnell et al., 2002). Compensatory mutations in both γ-2 and PSD-95 added further evidence that this direct interaction is sufficient to mediate AMPA receptor synaptic targeting and stabilize AMPA receptors at synapses (Bats et al., 2007; Schnell et al., 2002).

The amount of MAGUKs may be limiting for synaptic AMPA receptor expression as overexpression of PSD-95 increases synaptic AMPA receptor currents, while overexpression of either γ-2 or γ-8 does not (Rouach et al., 2005; Schnell et al., 2002). However, the localization of AMPA receptors to synapses by TARPs is a dynamic process and is regulated by phosphorylation of nine serine residues in the TARP C terminal tail by PKC and CaMKII (Tomita et al., 2005b; Tsui and Malenka, 2006). The phosphorylation of γ-2 is required for synaptic trafficking, but not membrane trafficking. Thus, overexpression of γ-2 with the nine serines replaced by phosphorylation mimic aspartate residues (S9D) increased synaptic AMPA receptor currents, an effect dependent

upon the PDZ binding motif. TARP phosphorylation stabilizes receptors in the synapse, as removal of AMPA receptors during LTD is blocked by the overexpression of the S9D construct (Tomita et al., 2005b). Phosphorylation of TARPs may also be necessary for LTP (Tomita et al., 2005b). PKA can phosphorylate the second threonine within the γ-2 TTPV sequence in heterologous cells, and this phosphorylation blocks interactions with PSD-95 leading to the removal of receptors from synapses (Chetkovich et al., 2002; Choi et al., 2002). However, it is unclear whether this site is phosphorylated *in vivo*.

TARPs modulate AMPA receptor gating

Like many auxiliary subunits, TARPs also modulate the gating of AMPA receptors, generally in ways that augment their activity. All TARPs increase glutamate evoked currents from both flip and flop GluR1 and GluR2 homomers far more than predicted by the concomitant increase in surface expression (Priel et al., 2005; Tomita et al., 2005a; Turetsky et al., 2005; Yamazaki et al., 2004). Additionally, TARPs increase the efficacy of the partial agonist kainate such that the ratio of kainate and glutamate evoked currents resembles that seen in neurons (Tomita et al., 2005a; Turetsky et al., 2005). It is unknown how this occurs, but may result from greater kainate-induced AMPA receptor domain closure or increased coupling of the domain closure to channel opening. TARPs also lower the EC_{50} for both glutamate and kainate (Priel et al., 2005; Tomita et al., 2005a; Turetsky et al., 2005; Yamazaki et al., 2004).

The effects of TARPs on glutamate induced gating may be due to their slowing of desensitization and deactivation, and reduction of the extent of desensitization such that larger steady state currents are observed (Priel et al., 2005; Tomita et al., 2005a; Turetsky

et al., 2005). It has also been reported that γ-2 speeds recovery from desensitization (Priel et al., 2005; Turetsky et al., 2005). On the single channel level, γ-2 increases the frequency of GluR4 openings to larger conductance levels and increases burst lengths, without changing the conductance levels themselves or affecting open times (Tomita et al., 2005a). Given that desensitization of AMPA receptors is thought to involve a rearrangement of the dimer interface, TARPs may stabilize the dimer interface and thus slow entry into desensitization (Armstrong et al., 2006; Sun et al., 2002). Another possibility is that TARPs destabilize the desensitized state, thus promoting faster recovery from desensitization (Priel et al., 2005; Turetsky et al., 2005). At synapses, these effects are likely to shape AMPA receptor mediated EPSCs and to enhance summation of closely spaced events.

This modulation of biophysical properties by γ-2 was reported for GluR1, GluR2 and GluR4 homomers, with lesser effects on GluR3 (Kott et al., 2007; Priel et al., 2005; Tomita et al., 2005a; Turetsky et al., 2005). Although the qualitative changes were generally similar, the degree of modulation depends on the exact combination of the AMPA receptor homomers (flip and flop versions of GluR1-4) or heteromers with the 4 different TARPs (Kott et al., 2007; Turetsky et al., 2005). Most noticeably, γ-4 slows the rates of desensitization and deactivation more than other TARPs, and this effect can be detected in neuronal miniature EPSCs (Korber et al., 2007b; Milstein et al., 2007).

Although the precise site where TARPs interact with AMPA receptors is unknown, modulation of gating properties does not require the AMPA receptor N terminal domain nor most of their C terminal tail, suggesting that TARP modulation depends on interactions with the AMPA receptor pore, linker regions or extracellular

ligand binding domain (Bedoukian et al., 2006; Tomita et al., 2007). This is consistent with the finding that both modulation of desensitization and kainate efficacy depend on the γ-2 Ex1 region that is located extracellularly (Tomita et al., 2005a; Turetsky et al., 2005). It is unclear whether the γ-2 tail is involved in modulating desensitization, but it is not required for increasing kainate efficacy (Priel et al., 2005; Tomita et al., 2005a; Turetsky et al., 2005). Further studies will be needed to identify the exact amino acid residues involved in TARP-AMPA receptor interactions.

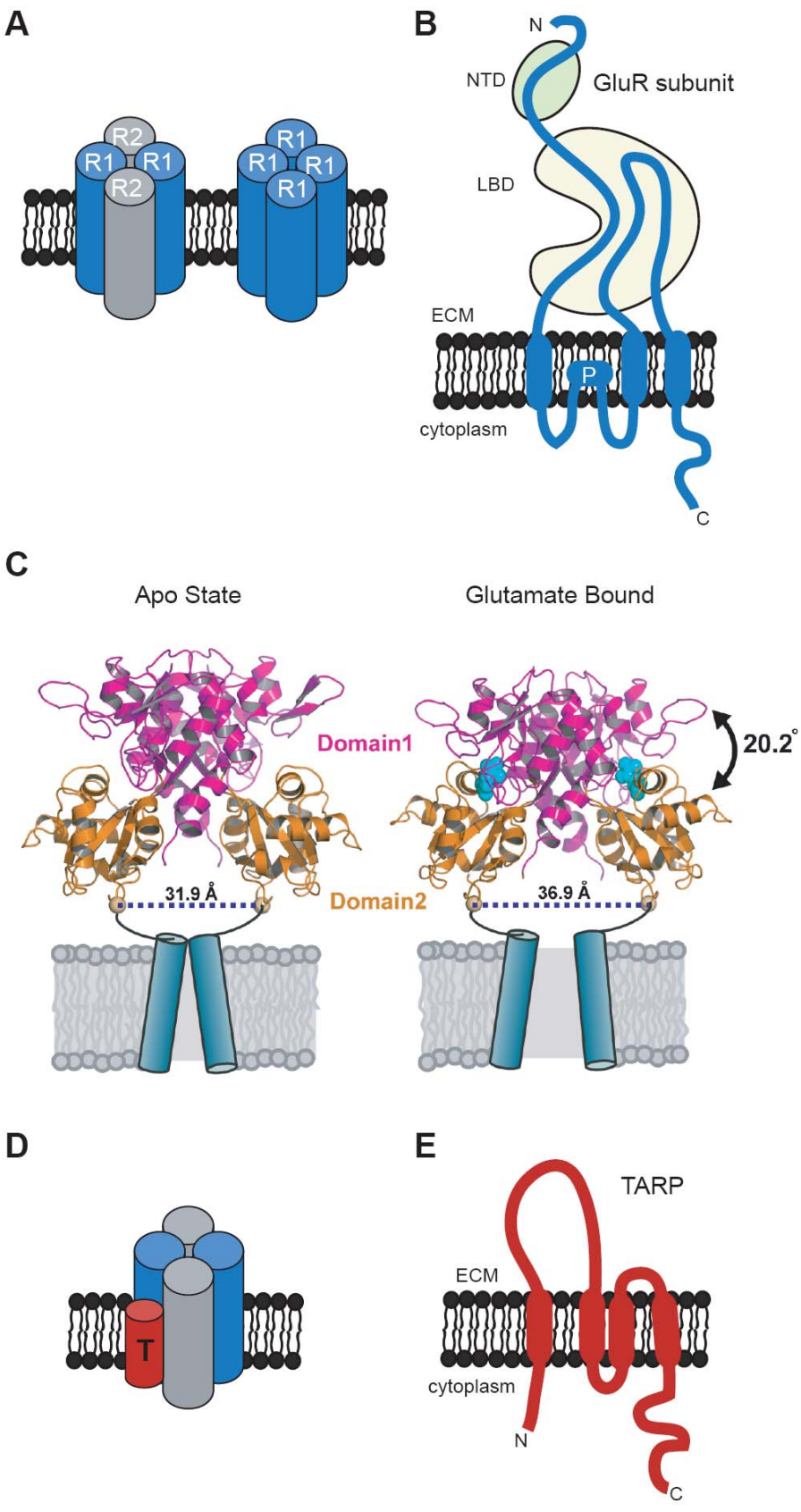
Phylogenic and sequence homology of TARPs

Comparison of the mouse, rat and human TARP ortholog sequences demonstrated that these proteins are highly conserved with ~98% amino acid identity (Chu et al., 2001). Furthermore, phylogenic analysis suggests that all γ genes arose from a common ancestor, before the split of rodent and primate lineages (Chu et al., 2001). More recently a set of proteins which share 21-25% homology with γ-2 were identified as orthologs in *Caenorhabditis elegans, Drosophilia melanogaster*, and *Apis mellifera* (honey bee) (Walker et al., 2006a). Despite their relatively low similarity to mammalian TARPs, they were each able to enhance rat GluR1 activity (Walker et al., 2006a). However, the function of STG-1, the *C. elegans* ortholog, may differ from mammalian TARPs given that it does not greatly increase receptor trafficking (Walker et al., 2006a). STG-1 does promote some aspect of the *C. elegans* GLR-1 gating, either by slowing desensitization or allowing the channel to enter the open state (Walker et al., 2006b). *C. elegans* GLR receptors also differ from those in mammals in that their activity depends upon an unrelated auxiliary subunit SOL1 (Walker et al., 2006b; Zheng et al., 2004).

The finding of TARPs in animals from *C. elegans* to humans suggests that TARP association is a critical component of normal AMPA receptor function. Unlike other AMPA receptor interacting proteins, TARPs meet the criteria of being auxiliary subunits by being tightly associated and modulating trafficking, localization, and gating. Although it is unknown whether native synaptic AMPA receptors are universally found with TARPs, the properties of neuronal AMPA receptors are most consistent with TARP modulation. It is currently unknown how the presence of TARPs fits with previous work on TARP-less receptors, such as interactions with cytoplasmic proteins, trafficking rules for long versus short tailed receptors, and AMPA receptor phosphorylation in synaptic plasticity. Thus far, the properties of AMPA receptors with TARPs have been primarily studied in heterologous systems, but they are likely to have critical roles *in vivo*.

Figure 1. AMPA receptor and TARP structures.

(A) AMPA receptors are formed by either homomeric or heteromeric assemblies of GluR subunits, such as GluR1/2 or GluR1 containing receptors. Each subunit can bind glutamate and is thought to contribute to the pore. **(B)** An individual GluR subunit contains three transmembrane helices and a re-entrant pore loop (P), a cytosolic C terminal tail, an extraceullar N terminal domain (NTD), and an extracellular ligand-binding domain (LBD), shaded in yellow. The LBD has an approximately clamshell-like shape. **(C)** The isolated LBD (S1S2) has been used for crystal structure studies of AMPA receptors. S1S2 is created by removing the transmembrane domains, NTD and C terminal tail and adding an artificial linker where the first and second transmembrane domains would be. S1S2 crystalizes as a pair of back-to-back dimers. Depicted are the two S1S2 protomers with the upper half of each clamshell (Domain 1) in magenta and the lower half (Domain 2) in yellow. For orientation, two transmembrane helices and their connection to S1S2 were added by hand. When comparing the *apo* and glutamate-bound states, we see that Domain 2 approaches Domain 1 in the glutamate-bound state (domain closure) and the separation between the linkers increases, likely corresponding to channel opening (PDB codes 1FT0 and 1FTJ, respectively (Armstrong and Gouaux, 2000)). **(D)** TARPs (red) are transmembrane proteins tightly associated with AMPA receptors, but are not required to form the pore or to bind glutamate. (E) TARP topology includes four transmembrane helices, with intracellular N and C termini, and two extracellular loops. The longer loop, known as Ex1, is thought to be particularly important for regulation of AMPA receptor gating, while the C-terminal tail interacts with PDZ binding proteins.


A
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R1
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NTD
GluR subunit
LBD
ECM
P
cytoplasm
C
C
Apo State
Glutamate Bound
Domain1
20.2°
31.9 Å
Domain2
36.9 Å
D
T
E
TARP
ECM
cytoplasm
N
C

Chapter 2:

Methods

Knockout mice

All experiments followed animal welfare guidelines established by the University of California, San Francisco I.A.C.U.C. Stargazer mice (γ-$2^{-/-}$ mice), γ-$4^{-/-}$ mice, and γ-$8^{-/-}$ mice have been described previously (Letts et al., 1998; Milstein et al., 2007; Rouach et al., 2005). TARP γ-$3^{-/-}$ mice were generated by standard knockout technology. Southern blot analysis was used to verify proper targeting. Genomic mouse tail DNA was digested with XbaI and blotted with a P^{32} labeled γ-3 probe that was located 5' to exon 2. PCR primers for the γ-3 Southern probe were: forward: TTCATAGATGGCCTTTCC; reverse: CCAACATTCCACTCTGGG. Multiple knockout mice were generated by crossing single knockout mice. PCR genotyping of mouse tail DNA was performed with the following primers:

for γ-$2^{-/-}$:

F-WT: CATTTGTTATACATGCTCTAG R-WT: ACTGTCACTCTATCTGGAATC

F-KO: GAGCAAGCAGGTTTCAGGC R-KO: ACTGTCACTCTATCTGGAATC

for γ-3:

F-WT: AACTAGGTTCCCAGATAGCC R-WT: GCTTCTAATGGGTTGCGCCC

F-KO: GGCTGCTCTTTGGTTAATCGG R-KO: TACCCGGTAGAATTGACCTGC

for γ-$4^{-/-}$:

F-WT: GGACTCCTGGGAGAGATGCC R-WT: CGGCTGTAGATCCTCCCAGC

F-KO: GGTGATGGCGTTCAGTGCACGG R-KO: TACCCGGTAGAATTGACCTGC

and for γ-$8^{-/-}$:

F-WT: TCGCGCTTTCCTCTCGTCCC R-WT: GCTGCCACGAACAGGATCCC

F-KO: CGTTTAGGATCTACCCAGATC R-KO: TACCCGGTAGAATTGACCTGC

Antibodies

The rabbit polyclonal antibodies to γ-3, γ-8 and the pan-TARP antibody used for detecting γ-2 have been characterized previously (Tomita et al., 2003). The following commercial antibodies were used: rabbit polyclonal antibodies to GluR1, GluR2/3, and GluR4 (Chemicon) and monoclonal antibodies to NR1 (Pharmingen) and tubulin (Sigma).

Constructs

The previously described γ-2/γ-5 chimera construct was subcloned in tandem with IRES-EGFP into the pSCA1 Semliki forest virus vector (Tomita et al., 2005a; Tomita et al., 2005b). The γ-2 tail construct was generated by using PCR to amplify the region from amino acid 607 through amino acid 957 and then subcloning into the pLLox3.7 lentiviral vector together with IRES-EGFP. The short hairpin RNAi oligonucleotides were inserted into pLLox3.7 and this vector was used to transfect HEK293 cells. Full-length γ-2, γ-3, γ-4, and γ-8 constructs were subcloned into pIRES2-EGFP (pIR2) (Clonetech), which uses a cytomegalovirus promoter to drive expression of the inserted construct and contains an IRES sequence before an EGFP coding region to label transfected cells. GluR1(Q) flip (gift from R. Huganir) and GluR1(Q) flop constructs were PCR subcloned into the pIRC vector. The pIRC vector was created by exchanging the EGFP in pIR2 with mCherry (gift of R. Tsien, Stanford University).

Dissociated neuron cultures

Dissociated cerebellar granule neurons were prepared from postnatal days 5-7

γ-2$^{-/+}$ mouse cerebella and plated on coverslips precoated with poly-D-lysine in 24-well dishes. Cultures were maintained in MEM (5.3 K^+) (Invitrogen) supplemented with glucose (0.5%), transferrin (0.1 mg/ml), insulin (0.025 mg/ml), glutamine (2 mM), and 10% FBS (Invitrogen). Cytosine arabinoside was added with fresh media after two days in culture.

Other dissociated neuronal cultures were prepared from the hippocampi of E16-E19 wild-type and γ-8$^{-/-}$ mice, spinal cord of E13-E16 wild-type and γ-2$^{-/-}$;γ-3$^{-/-}$;γ-4$^{-/-}$ mice, and cerebral cortices of E14-E18 γ-2$^{-/-}$;γ-3$^{-/-}$;γ-4$^{-/-}$ mice and their littermates. The tissues were dissociated by papain digestion and brief mechanical trituration. Cells were plated on poly-D-lysine–coated glass coverslips (12 mm). Hippocampal and cortical cultures were plated in Neurobasal media (Gibco) with B-27 and 5% FBS (Invitrogen) (Craven et al., 1999). After three days, the media was replaced by serum free media. Spinal cultures were plated and maintained in L15 media with FBS. After 3 days in culture, cytosine arabinoside was added.

Viral particles

Viral particles for γ-2/γ-5-pSCA1 were produced by transfecting pSCA1 and pHelper into HEK293 cells. Supernatants were harvested 48 hours after transfection and stored at −80°C. Chymotrypsin treatment was used to active viruses prior to infection. 10-50 μL of virus were added per well of cultured granule cells and cells were recorded one day post infection. Lentiviral particles were produced by transfecting pLLox3.7-γ-2 tail and helper vectors pDelta8.9 and pVSVg into HEK293 cells. Supernatants were collected after 48 hours, concentrated by ultrafiltration in Centricon Plus 100 (Millipore)

and stored at −80°C. For infection of cultured granule cells, 10-50 μL was added per well and cells were recorded 2-3 weeks post infection.

Immunoblotting

Brain regions from mice (P25-P45) were homogenized in 4.5 volumes of 320 mM sucrose buffer and then sonicated in 2% final SDS. Similar treatments were done for acute spinal cord and hippocampus tissue from P2 mice. Equal amounts (20 μg) of protein were separated on 8% polyacrylamide gels followed by transfer to PVDF membranes. For HEK293 cell immunoblots, 35 mm confluent dishes were suspended in PBS and resuspended in SDS buffer. 12% polyacrylamide gels were loaded with ~5% of the total protein. Similar methods were used for immunoblotting spinal cord cultures on 10 mm glass coverslips. Proteins were detected by immunoblotting using the HRP-ECL kit from Amersham. The densitometry function of ImageJ software, available from the NIH, was used to determine the relative amounts of AMPA and NMDA receptor protein. Statistical significance was determined by either a Student's paired *t*–test or a one-way repeated measures ANOVA, followed by the Tukey's post-hoc test.

Immunohistochemistry

Anesthetized mice ages P18-22 were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 minutes. Brain tissues were post-fixed for 4 hours followed by a 2 hour incubation in 10% sucrose/PBS and two 8 hour incubations in 20% sucrose/PBS solutions. Sagittal sections (35 μm) were cut with a freezing microtome and were then

blocked for one hour in 3% normal goat serum and incubated overnight in GluR1, GluR2/3, and GluR4 antibodies at 4°C. The sections were processed with Vectastain ABC kit using DAB (3,3'-diaminobenzidine) as substrate.

Electrophysiology in acute slices from juvenile animals

For cerebellar Golgi cell and granule cell recordings, parasagittal cerebellar slices (200 μm) from 2 to 3 week old mice were cut in cold (4-6° C) ACSF containing (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 25 glucose, 4 $MgCl_2$, and 1 $CaCl_2$ saturated with 95% O_2-5% CO_2. Slices were incubated at 30-34° C for 1 hour, then moved to room temperature for 30 minutes, and finally stored in the recording solution at room temperature. The recording solution was identical to the cutting solution, except that the concentration of $MgCl_2$ and $CaCl_2$ were 1 mM and 2 mM respectively. Parasaggital cerebellar slices (300 μm) from 2 to 4 week old mice for Purkinje cell recordings and transverse hippocampal slices (300 μm) from P11-P21 mice were prepared similarly with the following differences. Slices for Purkinje cell recordings were cut in ice cold (0-4° C) ACSF, incubated at 30-34° C for 15 minutes, and then kept at room temperature prior to use. Hippocampal slices were incubated at 30-34°C for 30 minutes, and then maintained at room temperature. The cutting and recording ACSF for Purkinje cells contained the following (in mM): 119 NaCl, 2.5 KCl, 26.3 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 1.3 $MgCl_2$, and 2.5 $CaCl_2$. The solutions for hippocampal slices in Chapter 4 were the same except that the recording solution contained 4 mM $MgCl_2$ and 4 mM $CaCl_2$.

All synaptic recording solutions contained 100 μM picrotoxin (Sigma), except for recording sIPSCs in cerebellar granule cells. For Golgi cell recordings, 3 μM strychnine (Sigma) was added. For recording holding current changes in Chapter 5, 100 μM picrotoxin (Sigma), 500 nM TTX (Tocris), 50 D-μM APV (Tocris) were added to the recording solution. In some experiments (see text), 500 μM trichloromethiazide (TCM) (Sigma) or 10 μM GYKI 53655 (custom synthesis by Tocris) was added to the recording solution. DNQX (Tocris), CNQX (Tocris), and NBQX (Tocris) were bath applied at a concentration of 10 μM.

Whole cell recordings were obtained using glass electrodes (1.5-6 MΩ). Purkinje cells, hippocampal pyramidal cells, and cerebellar granule cells were visually identified. Cerebellar Golgi cells and mature (<600 MΩ input resistance) dentate granule cells were identified according to previously established criteria (Dieudonne, 1998; Liu et al., 2000; Overstreet-Wadiche and Westbrook, 2006). The internal pipette solution for recording synaptic responses in Purkinje cells contained (in mM): 115 Cs methanesulfonate, 20 CsCl, 10 HEPES, 2.5 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine, 0.6 EGTA, 0.1 spermine, and 5 QX-314, pH 7.2-3, adjusted to 305-315 mOsm. For outside-out patches, the pipette solution contained (in mM): 135 Cs methanesulfonate, 10 CsCl, 10 Hepes, 2 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP, 1 EGTA, pH 7.2-3, adjusted to 305-315 mOsm. Purkinje cells in holding current change experiments and all hippocampal pyramidal cells were recorded with a pipette solution consisting of (in mM): 110 Cs methanesulfonate, 10 CsCl, 10 Hepes, 2 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP, 10 Cs_4-BAPTA, pH 7.2-3, 295-315 mOsm. The solution usually contained 5 mM QX-314 and 0.1 mM spermine. Golgi cell recordings used the same internal solution as hippocampal

cells, except that 0.1% Lucifer yellow was added, the osmolarity was adjusted to 305-315 mOsm, and for recordings of mEPSCs QX-314 and spermine were omitted. The liquid junction potential was not corrected for.

The experiments in Chapter 4 were carried out as follows: Purkinje cells were voltage-clamped at –70 mV using an Axopatch-1D amplifier (Axon Instruments). Climbing fiber EPSCs were evoked by electrical stimulation in the granule cell layer and were identified by their large amplitude all-or-none response. Dual-component EPSCs composed of AMPA and kainate receptor currents were recorded in 10 μM GYKI 53655 (Tocris, custom synthesis) to prevent voltage escape. Pure kainate receptor EPSCs were then isolated by increasing GYKI 53655 to 100 μM and the difference was used to obtain a measurement of the pure AMPA receptor current. Parallel fiber AMPA receptor EPSCs were evoked by stimulation in the molecular layer, then 10 μM CNQX (Tocris) was added and mGluR dependent responses were recorded by stimulating 10 times at 100 Hz at the same stimulation intensity (Tempia et al., 1998). Outside-out patches were pulled from Purkinje cells and held at –60 mV. A Valve-Link 8 controller and pinch-valves connected to a 360 μm flowpipe (AutoMate Scientific) were used for rapid solution exchange. The control line contained (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 $CaCl_2$, 4 $MgCl_2$, 250 μM cyclothiazide (Tocris), and 25 μM (RS)-CPP (Tocris). The test solution additionally included 10 mM glutamate.

Hippocampal CA1 pyramidal cells were voltage-clamped at +40 mV, and dual component EPSCs were evoked by stimulating the stratum radiatum. NMDA receptors were then blocked by adding (RS)-CPP to obtain a pure AMPA receptor EPSC, and the NMDA receptor EPSC was obtained by subtraction. Paired-pulse facilitation was

measured by stimulating twice at 25 Hz at–60 mV. Rectification was measured by obtaining the AMPA receptor EPSCs at +40 mV and –60 mV. The rectification index (RI) was defined as (I_{+40}/I_{-60}) x $[(-60 - E_{rev})/(40-E_{rev})]$ such that a linear response would have a (RI) of 1 and a fully rectifying response would have a value of 0.

Evoked currents were obtained in Golgi cells similarly. EPSCs were evoked by stimulating the molecular layer while holding the cell at –70 mV. Dual-component responses were evoked while holding the cell at +40 mV, and then the AMPA receptor current was isolated by adding 50 μM D-APV (Tocris). The paired-pulse ratio and rectification index was measured in the same way as for pyramidal cells, with the exception that the holding current was –70 mV. For recording mEPSCs, 500 nM TTX (Tocris) and 50 μM D-APV (Tocris) were additionally added to the recording solution. Customized IgorPro (Wavemetrics Inc.) software was used to analyze mEPSCs off line with a threshold of 10 pA.

For the holding current change experiments in Chapter 5, cells were voltage-clamped at –70 mV except for: 1) cerebellar granule cells, which were held at 0 mV to isolate $GABA_A$ mediated sIPSCs by collapsing the driving force for sEPSCs, and 2) Purkinje cells, which were held at –10 mV. Cells were sampled every 10 seconds for holding current experiments, while continuous recordings were used for sIPSC experiments. Traces were filtered at 1-2 kHz, digitized at 5-10 kHz, and analyzed using IgorPro (Wavemetrics Inc.). sIPSCs were analyzed using customized IgorPro software using a threshold amplitude of 10 pA. sIPSC frequency was compared between a 3 minute baseline epoch just before CNQX application, and a 3 minute epoch 2 minutes after CNQX entered the recording chamber. Cells not having at least 3 sIPSCs in the

baseline epoch were discarded. The holding current change was measured as the average holding current over 5 minutes after CNQX had been in the recording chamber for 5 minutes (time 10-15 minutes on the graphs).

Statistical significance was determined by either a Student's t-test for comparisons between two groups or a one-way ANOVA for comparisons between multiple groups. If significant, the ANOVA was followed by either the Tukey's or Games-Howell post hoc tests depending on whether the data met the assumption of equal variance according to the Levene statistic. All data shown are the mean ± SEM.

HEK293 cell transfection and recordings

HEK293T cells were grown in DMEM supplemented with 10% FBS and penicillin-streptomycin and were maintained according to standard protocols. Lipofectamine (Invitrogen) was used for transfection of HEK293 cells at 60-80% confluency. For each 35 mm well, a total of 2 μg DNA and 5 μl Lipofectamine was gently mixed in 300 μl OPTI-MEM and incubated for 15 to 20 minutes. The DNA for transfection consisted of 1 μg $GluR1_i$-pIRC or $GluR1_o$-pIRC together with 1 μg of one of the following constructs: pIR2, γ-2-pIR2, γ-3-pIR2, γ-4-pIR2, or γ-8-pIR2. The culture media was removed from the wells and the DNA/OPTI-MEM mix together with an additional 500 μl OPTI-MEM was added for 2-4 hours. Then the transfection media was replaced with a modified culture media containing MEM Eagle's with Earle's BSS, without L-Glutamine in place of DMEM to prevent tonic activation of AMPA receptors by trace amounts of glutamate in the media. Cells were split onto glass coverslips after 16-40 hours and recorded 20-48 hours post transfection. For transfection of RNAi

constructs, 0.25 μg γ-2 and 0.75 μg lentiviral vector were mixed with 4 μl Lipofectamine in a total of 200 μl OPTI-MEM. This mixture was added to 1 mL of maintanence HEK media for 3 hours, and replaced with fresh HEK media. Cells were used for immunoblotting 48 hours post-transfection.

For electrophysiological recordings, cells were placed in a recording chamber containing room temperature ACSF (in mM): 119 NaCl, 2.5 KCl, 26.3 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 1.3 $MgCl_2$, and 2.5 $CaCl_2$. Whole-cell voltage-clamp recordings used glass pipettes (3-6 MΩ) filled with the same internal solution used for recording hippocampal neurons. HEK293 cells were voltage clamped to –70mV and data acquired using IgorPro as described above for acute slices.

A brief two or five second application of ligand containing solutions was achieved using a perfusion system consisting of a Valve-Link 8 controller and pinch-valves connected to a 360 μm flowpipe (AutoMate Scientific). The control line contained (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 4 $CaCl_2$, and 4 $MgCl_2$ and usually included 500 μM TCM and sometimes 100 μM GYKI 53655 (see text). The test solution was made by adding 10 μM CNQX, DNQX, or NBQX to an aliquot of the control solution. A second test solution contained 1 mM glutamate in the control solution. Opening and closing of lines was controlled via the Valvelink 8. Transfected cells were identified as GFP positive. However, this only ensured that the pIR2 vector was expressed in these cells. To verify that the GluR1 construct was also expressed, we recorded glutamate evoked currents and discarded cells that had a response < 500 pA to ensure that a sufficient number of AMPA receptors were expressed on the cell surface. In the case of GluR1 flop receptors, cells were discarded if there was less than 20 pA of

steady state current. For the comparison of current in the presence and absence of TCM, cells were only used if they had a sufficient amount of CNQX-induced current in the presence of TCM to be able to detect a current in the absence of TCM. Averages of 3-4 applications were used to determine the peak response to a given test solution. Note, the same data for CNQX application on GluR1 with γ–2 is shown in Chapter5, Fig. 4A and B to allow better comparison of the different conditions. Similarly, the data for DNQX on GluR1 with γ–2 is the same as in Fig. 2. Statistical significance was determined using a Student's t-test. All data shown are the mean ± SEM.

Cultured neuron electrophysiology

Whole-cell patch-clamp recordings were obtained from 10-19 DIV neurons using 3 to 6 MΩ glass electrodes filled with an internal solution containing (in mM): 115 Cs methanesulfonate, 20 CsCl, 10 HEPES, 2.5 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine, 0.6 EGTA, 0.1 spermine, and 5 QX-314, pH 7.2-3, adjusted to 295- 305 mOSM. In the recording chamber, the cells were bathed in a solution containing (in mM): 119 NaCl, 2.5 KCl, 26.3 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 1.3 $MgCl_2$, and 2.5 $CaCl_2$. For experiments except those involving outside-out patches, 100 μM picrotoxin (Sigma) and 500 nM TTX (Tocris) were added. Additionally, 3 μM strychnine (Sigma) was added for recording mEPSCs in cultured spinal neurons. Neurons were voltage-clamped from –60 to –70 mV. For mEPSC recordings, a 5 pA threshold was used and otherwise analyzed as described above.

Fast agonist application was achieved using a gravity perfusion system as described above. The control line always contained (in mM): 140 NaCl, 2.4 KCl, 10

HEPES, 10 glucose, 2.5 $CaCl_2$, and 4 $MgCl_2$. For granule cell recordings, 100 μM picrotoxin (Sigma), 500 nM TTX (Tocris), and 50 μM RS-CPP (Tocris) were added. For cortical neuron and hippocampal neuron outside-out patch recordings, 250 μM cyclothiazide (Tocris) was added to the control solution. The agonist solution was made by adding 500 μM AMPA (Tocris) to an aliquot of the control solution. Transfected or infected cells were identified by GFP expression.

Electrophysiology in acute slices from E16.5-P1 mice

Slices were prepared similarly to those from juvenile animals as described above. Hippocampal slices were cut in the same manner as described above for hippocampal slices, except that P0-P1 animals were used. For cortical and spinal cord slice preparation, timed-pregnant mother mice were sacrificed by cervical dislocation, and the E16.5-E18.5 embryos were removed. Those that did not move in response to touch were assumed to be triple knockout mice. Slices were prepared from one triple knockout mouse and one littermate and the genotypes were determined at a later time point. For recording cortical neurons, coronal brain slices (300 μm) were prepared in the same manner as for hippocampal slices. For spinal cord slices, the meninges surround the spinal cord were removed and the spinal cord was set into an agarose block for cutting slices (300-400 μm). The cutting solution was the same as described above, at a temperature of 0-4 ºC. As before, slices were incubated at 37 ºC and then left at room temperature prior to recordings.

In the recording chamber, slices were bathed with ACSF containing (in mM): 119 NaCl, 2.5 KCl, 26.3 $NaHCO_3$, 1 NaH_2PO_4, 11 glucose, 1.3 $MgCl_2$, and 2.5 $CaCl_2$. For

whole cell application of agonists to spinal cord and cortical neurons, 100 μM picrotoxin (Sigma) and 500 nM TTX (Tocris) were added to the ACSF. Strychnine (3 μM) (Sigma) was added to spinal cord ACSF. Visually identified neurons were patched with an electrode containing (in mM): 115 Cs methanesulfonate, 20 CsCl, 10 Hepes, 2.5 $MgCl_2$, 4 Na_2-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine, 0.6 EGTA, 0.1 spermine, and 5 QX-314, pH 7.2-3, adjusted to 295-305 mOsm.

Fast agonist application was achieved using a gravity perfusion system as described above. The control line always contained (in mM): 140 NaCl, 2.4 KCl, 10 HEPES, 10 glucose, 2.5 $CaCl_2$, and 4 $MgCl_2$. For spinal cord and cortical neurons, 250 μM cyclothiazide was added, while 100 μM cyclothizide was added for hippocampal pyramidal cell outside-out patches. The agonist solution was made by adding 500 μM AMPA (Tocris) to an aliquot of the control solution and was applied for 2-5 seconds.

S1S2J expression, purification and crystallization

The S1S2J construct, generously provided by E. Gouaux and described previously (Chen et al., 1998) contains residues N392-K506 of S1 and P632-S775 of S2 with numbering according to the full length receptor excluding the signal peptide sequence. The DNA sequence encoding S1S2J was cloned into a modified pET32 T7 expression plasmid with a trypsin cleavable eight histidine tag. This construct was expressed and purified as previously described (Furukawa and Gouaux, 2003) with some modifications. Briefly, the plasmid containing S1S2J was transformed into Origami B (DE3) cells (Novagen) with transformants selected using LB-agar plates containing 50 μg/ml carbenicillin and 25 μg/ml kanamycin. Cells were grown in 2x LB media in a Sartorius

15L fermentor with a 10L working volume at 8 liters per minute of house air and 250 rpm stir and 37 °C to an OD600≈0.6 at which point the temperature was reduced to 20 °C and, were induced with 0.8 mM isopropyl-β-D-thiogalactoside. The cells were incubated overnight following induction. Buffer A contains 50 mM TRIS-HCl, pH 7.4RT, 150 mM NaCl and 1mM fresh phenylmethanesulphonylfluoride. Cells were harvested by centrifugation at 5000 X g for 15 minutes and resuspended in 5 ml lysis buffer (Buffer A plus 10 mM imidazole) per gram of wet cell weight. Cells were fully resuspended using a homogenizer and then lysed in a C3 Emulsiflex microfluidizer via four complete passes at 15,000 psi. Lysate was immediately spun down at 40000xg for 60 minutes and supernatant recovered for incubation with Ni-NTA resin (Qiagen). Following a two hour incubation with IMAC resin on a nutator at 4 °C the protein was purified with 20 column volumes of Buffer A with 10 mM imidazole, 20 column volumes of Buffer A with 40 mM imidazole and then eluted with Buffer A and 250 mM imidazole. Eluted fraction was immediately exchanged into 50 mM Tris-HCl pH 7.8RT using a 10DG desalting column (Bio-Rad). Tag cleavage was accomplished by incubation overnight at 4 °C with 50 μg trypsin and subsequently removed with benzamidine resin followed by a second Ni-NTA purification. Following elution the cleaved S1S2 was exchanged into 20 mM MES pH 5.5 and 10 mM NaCl for the final SP ion exchange purification step. No exogenous glutamate was introduced to the sample during purification.

Crystallization, data collection and refinement

Purified S1S2 was dialyzed for 48 hours against five fold molar excess of CNQX (Ascent Scientific Asc-044) dissolved in 10 mM HEPES pH 7.0 and 20 mM NaCl.

Retentate was concentrated to 10 mg/ml in a 10 kDa Amicon Ultra (Millipore) concentrator. Crystals were grown at 4 °C by the hanging drop vapor diffusion method in a 1:1 ratio of protein and reservoir solution which contained 40 mM sodium citrate, 10 mM ammonium sulfate and 1% PEG 400. Prior to data collection crystals were flash frozen in liquid nitrogen and cryo-protected with 30% ethylene glycol. Diffraction intensities were collected on Advanced Light Source Beamline 8.3.1 using an ADSC Quantum-Q210 CCD detector and processed using HKL2000 (Otwinowski and Minor, 1997). Beamline 8.3.1 was funded by the National Science Foundation, the University of California and Henry Wheeler. The ALS is supported by the Director, Office of Science, Office of Basic Energy Sciences, Materials Sciences Division, of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098 at Lawrence Berkeley National Laboratory. Molecular replacement was used to determine initial phases using a DNQX dimer (Protein Data Bank code 1FTJ) as an initial search model in Phaser (Read, 2001). Initial molecular replacement solution was subjected to simulated-annealing in CNS to minimize model bias and subsequently moved to CCP4 (Collaborative Computational Project, 1994) and Refmac5 (Murshudov et al., 1997). Iterative cycles of manual model building in Coot (Emsley and Cowtan, 2004) and positional/temperature factor refinement in Refmac5 were performed until R values stabilized. Protomers were compared and tight NCS restraints implemented along with TLS and manual solvent building with CNQX ligands added towards the end of refinement.

Chapter 3:

Testing AMPA receptor function in the absence of TARPs

Introduction

Fast excitatory synaptic transmission in the central nervous system (CNS) is primarily mediated by glutamate acting on AMPA and NMDA receptors. The activity-dependent trafficking of AMPA receptors in and out of synapses is thought to underlie learning and memory. AMPA receptors are tetrameric channels formed by homo- and heteromeric combinations of the pore-forming subunits GluRs1-4. Although the GluR subunits were first cloned nearly two decades ago, only relatively recently has γ-2 (stargazin) been identified as auxiliary subunit that can modulate GluR subunit function (Chen et al., 2000; Tomita et al., 2005a). After the initial cloning of γ-2, a family of structurally similar proteins were cloned and named based on their similarity to the calcium channel subunit γ-1 (γ-3 through γ-8) (Burgess et al., 1999; Burgess et al., 2001; Klugbauer et al., 2000). However, only a subset (γ-3, γ-4, and γ-8) had the ability to increase AMPA receptor expression in cerebellar granule cells and and together with γ-2 were named the transmembrane AMPA receptor regulatory proteins (TARPs) (Tomita et al., 2003).

The easily apparent ataxia and distinctive head movements led to the serendipitous discovery of the spontaneous mutant mouse Stargazer, which lacks γ-2, at Jackson Laboratories (Noebels et al., 1990). Due to their frequent seizures, they have been used as a rodent model for epilepsy (Frankel, 1999; Noebels et al., 1990). These profound behavioral abnormalities suggest that TARPs are critical for maintaining AMPA receptor function. In contrast, when researchers generated knockout mice for both γ-4 and γ-8 (Letts et al., 1998; Milstein et al., 2007; Rouach et al., 2005), each was behaviorally indistinguishable from littermates. Given that γ-2, γ-4, and γ-8 are found in

different as well as overlapping neuron populations, which could have AMPA receptors of varying subunit composition, TARPs may only be required for the function of specific groups of AMPA receptors or in certain types of neurons.

However, it is also possible that TARPs are molecularly redundant and their overlapping expression profile allows other TARPs to compensate for the loss of any single TARP, thus preventing behavioral abnormalities. Unfortunately, the localization, timecourse and relative amount of TARP expression in individual neurons and brain regions is not fully known, in part due to the variation in reports from different research groups (Burgess et al., 2001; Fukaya et al., 2005; Klugbauer et al., 2000; Lein et al., 2007; Moss et al., 2003; Tomita et al., 2003). Most studies have relied upon *in-situ* hybridization, as it has been difficult to develop antibodies that do not cross-react among family members. However, it is clear that TARPs are mainly expressed in the CNSf, except for the expression of γ-4 in many tissues early in development. Most studies have shown that γ-2 is found in virtually all brain regions, while expression of γ-3 is highest in the cortex, γ-4 in the olfactory bulb, striatum, and glia, and γ-8 in the hippocampus (Fukaya et al., 2005; Klugbauer et al., 2000; Lein et al., 2007; Tomita et al., 2003). However, γ-3, γ-4, and γ-8 are also found in other regions at lower expression levels (Fukaya et al., 2005; Lein et al., 2007). It is unknown whether expression levels of other TARPs are increased when one TARP is knocked out.

Many key synaptic proteins exist as members of families, such that the loss of one protein can be compensated for by the presence of another. As a result, single knockout mice often do not have the phenotype predicted by previous *in vitro* studies. For example, PSD-95 is a founding member of the family of MAGUK proteins that anchor

neurotransmitter receptors in synapses, yet knockout mice lacking PSD-95 do not have synaptic transmission defects (Elias et al., 2006). To observe a loss of synaptic AMPA receptors in PSD-95 knockout mice, it was necessary to combine this strain with mice lacking a related family member PSD-93 or to acutely knockdown PSD-95 expression using RNAi (Elias et al., 2006). Likewise, single neuroligin knockout mice appear behaviorally normal, while triple neuroligin knockout mice die shortly after birth (Varoqueaux et al., 2006) and mice lacking either MALS1 or MALS2 are indistinguishable from littermates (Misawa et al., 2001) while triple MALS knockout mice die perinatally and exhibit synaptic transmission abnormalities (Olsen et al., 2005). Thus to determine the *in vivo* importance of TARPs, it would be helpful to generate mice and/or neurons entirely lacking TARP expression.

Given the relative normality of single TARP knockout mice, our goal was to determine whether TARPs are required for proper AMPA receptor function. To do this, we examined three potential methods of reducing TARP expression. In the first technique, we tested putative dominant negative constructs that might outcompete native TARPs, thus reducing the pool of functional TARPs. Our second method was to examine AMPA receptor mediated synaptic transmission in multiple TARP knockout mice. Finally, we used RNAi to reduce TARP expression in individual neurons.

Results

Putative dominant negative TARP constructs

Given that there are multiple TARP family members, we first attempted to generate dominant negative constructs that could potentially reduce functionality of all

TARPs. Stargazin is a four pass transmembrane protein that contains a PDZ-binding motif on its intracellular C-terminal tail (Figure 1A), similar to other members of the TARP family. γ-5 is structurally related to TARPs, but is unable to modulate AMPA receptor function (Tomita et al., 2005a; Tomita et al., 2003; Tomita et al., 2004). Previously a chimeric construct containing γ-2 with the γ-5 C-terminus (also known as "cyto", here referred to as γ-2/γ-5) (Figure 1A) was shown to modulate AMPA receptor gating, but not promote their surface expression (Tomita et al., 2005a). Therefore, this construct may compete with native TARPs for AMPA receptor binding, and cause a reduction in AMPA receptor expression.

To determine whether this construct acts in a dominant negative manner, we expressed the γ-2/γ-5 construct in cultured cerebellar granule cells. Cerebellar granule cells from γ-$2^{-/-}$ mice lack synaptic and extrasynaptic AMPA receptors in culture (Chen et al., 2000). Thus if the construct competes with γ-2 for AMPA receptor binding, it should reduce AMPA receptor expression in these cells. We expressed the γ-2/γ-5 construct together with Green Fluorescent Protein (GFP) using a Semiliki forest virus. We found that the extrasynaptic AMPA receptor content measured by recording the whole-cell response to brief applications of 500 μM AMPA was not different between GFP positive and GFP negative neurons (GFP negative 69.9 ± 6.8 pA, GFP positive 91.6 ± 8.8 pA, n = 7 each, P = 0.074) (Figure 1B), suggesting that this construct does not act as a dominant negative.

As the γ-2 C-terminal tail is necessary for promoting AMPA receptor surface trafficking, it may interact with cytosolic proteins involved in membrane trafficking (Tomita et al., 2005a). Overexpression of the γ-2 tail alone may sequester necessary

TARP binding partners, thereby interfering with normal TARP function. Therefore we generated a γ-2 tail construct that contained the γ-2 C-terminal tail after the fourth transmembrane domain (Figure 1A). We also deleted the last 4 amino acids (TTPV) which constitute the PDZ binding motif as the full-length tail non-specifically reduces both AMPA and NMDA receptor function, most likely due to interactions with multiple PDZ binding proteins in the absence of the full-length protein (Schnell et al., 2002; Songyang et al., 1997). We expressed this construct together with GFP in cultured cerebellar granule cells using a lentiviral system. Extrasynaptic AMPA receptor function was not changed in cells expressing the γ-2 tail construct, as measured by recording the whole-cell response to brief applications of 500 μM AMPA (GFP negative 75.8 ± 8.0 pA, GFP positive 67.6 ± 6.8 pA, n = 6 and 7 respectively, P = 0.44) (Figure 1C). Therefore, this construct was also unable to act in a dominant negative fashion.

Generation of multiple TARP knockout mice

We next attempted to remove TARP expression by using multiple TARP knockout mice. We crossed previously generated knockout mice lacking γ-3, γ-4, and γ-8 (Milstein et al., 2007; Rouach et al., 2005) to stargazer mice, which lack γ-2, in order to create multiple knockout mice. We found that γ-$2^{-/-}$;γ-$3^{-/-}$ mice were sick and generally died in the first post-natal week (Chapter 4), while γ-$2^{-/-}$;γ-$4^{-/-}$ mice, γ-$2^{-/-}$;γ-$8^{-/-}$ mice, and γ-$3^{-/-}$;γ-$4^{-/-}$ mice did not show obvious behavioral abnormalities. In contrast, we did not find γ-$2^{-/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ or γ-$2^{-/-}$;γ-$3^{-/-}$;γ-$8^{-/-}$ pups that survived past birth, suggesting that TARPs are molecularly redundant.

AMPA receptors in spinal cord neurons from γ-2$^{-/-}$;γ-3$^{-/-}$;γ-4$^{-/-}$ mice

We decided to focus on γ-2$^{-/-}$;γ-3$^{-/-}$;γ-4$^{-/-}$ mice (also referred to as γ-2,3,4 knockout mice and triple knockouts). We found that there were significantly fewer triple knockout mice than predicted by Mendelian inheritance just before birth (E17-18.5) (Figure 2A), indicating that this knockout combination is often embryonic lethal. Using timed-pregnant mothers, we obtained E18.5 triple knockout pups for experiments. We were able to identify knockout pups by their lack of movement in response to touch, unlike most littermates. This apparent paralysis led us to study the effects of the triple knockout on spinal cord neurons.

We voltage-clamped spinal neurons (generally interneurons) from E18.5 spinal cord slices and measured the response to brief whole-cell application of 500 μM AMPA in the presence of cyclothiazide. We did not find any significant difference between triple knockout mice and their littermates (γ-2$^{-/+}$ or γ-2$^{+/+}$ and γ-3$^{-/-}$;γ-4$^{-/-}$) or wild-type mice (WT 822 ± 279 pA, γ-2,3,4 littermates 1525 ± 293 pA, γ-2,3,4 knockouts 965 ± 240 pA, n = 5, 4, and 8 respectively, ANOVA P = 0.26) (Figure 2B). However, the health of the slices prevented us from readily obtaining synaptic AMPA receptor mediated currents. We therefore used cultured dissociated spinal cord neurons to investigate synaptic AMPA receptor currents in triple knockout mice. No difference was found in the amplitude, frequency, or decay kinetics of AMPA receptor mediated miniature EPSCs (mEPSCs) in triple knockouts compared to wild-types (amplitude: WT 17.7 ± 1.2 pA, γ-2,3,4 knockouts 19.8 ± 1.8 pA; frequency: WT 1.94 ± 0.43 Hz, γ-2,3,4 knockouts 1.91 ± 0.69 Hz; decay: WT 3.00 ± 0.31 ms, γ-2,3,4 knockouts 2.59 ± 0.29 Hz, n= 12 WT and 9 γ-2,3,4 knockouts, P > 0.3 for each measurement) (Figure 2C). Therefore, both

extrasynaptic and synaptic AMPA receptors appear normal in spinal neurons from γ-2,3,4 knockout mice, despite their inability to move.

AMPA receptors in cortical neurons from γ-2,3,4 knockout mice

We next measured AMPA receptor function in acute and cultured dissociated cortical neurons. We first recorded whole-cell responses to brief applications of AMPA with cyclothiazide in cortical plate neurons in slices prepared from E18.5 triple knockout mice, their littermates, and wild-type mice. No difference was found in the average peak amplitude (WT 238 ± 70 pA, γ-2,3,4 littermates 214 ± 35 pA, γ-2,3,4 knockouts 219 ± 74 pA, n = 14, 20, and 9 respectively, ANOVA P = 0.94) (Figure 3A), suggesting that there was no reduction in extrasynaptic AMPA receptor content. We also applied AMPA in the presence of cyclothiazide to outside-out patches pulled from cultured cortical neurons. Surprisingly, we found that there was increase in peak amplitude in the triple knockouts compared to littermates (γ-2,3,4 littermates 178 ± 44 pA, γ-2,3,4 knockouts 389 ± 85 pA, n = 11 and 6 respectively, $P < 0.03$) (Figure 3B). This increase could be an artifact due to the combination of a low sample size and/or the use of cultured cortical neurons, which are not a uniform population. We therefore analyzed the subpopulation of neurons that gave non-desensitizing responses to AMPA which formed approximately 2/3 of the neurons, and again found a significant increase in the AMPA induced current in outside-out patches (γ-2,3,4 littermates 216 ± 52 pA, γ-2,3,4 knockouts 458 ± 110 pA, n = 7 and 4 respectively, $P < 0.05$). This surprising result suggests that over time cultured neurons compensate for the loss of TARPs, either by upregulating expression of the remaining TARP γ-8 or by using a TARP-independent mechanism.

To determine whether the increase in extrasynaptic AMPA receptor function led to a corresponding increase in synaptic AMPA receptors, we assayed synaptic transmission in cultured cortical neurons by measuring mEPSCs. Both the amplitude and frequency of mEPSCs were similar in triple knockout mice and their littermates (amplitude: γ-2,3,4 littermates 19.3 ± 1.5 pA, γ-2,3,4 knockouts 19.2 ± 1.1 pA; frequency: γ-2,3,4 littermates 5.46 ± 0.97 Hz, γ-2,3,4 knockouts 5.63 ± 1.21 Hz, n= 10 and 9 respectively, $P > 0.9$ for each measurement) (Figure 3C). Thus, loss of TARPs γ-2, 3, and 4 does not affect expression of synaptic AMPA receptors in cortical neurons.

Expression of γ-8 in young mice

Together our data from γ-2,3,4 knockout mice do not support the idea that TARPs are critical for AMPA receptor trafficking or synaptic localization. However, the lethality associated with multiple TARP knockout mice suggests that the family members are functionally redundant. It is known that cortical neurons express γ-8 after the first post-natal week, and it is possible that neurons also express this TARP earlier in development (Fukaya et al., 2005; Tomita et al., 2003). This TARP is expressed in many brain regions (Fukaya et al., 2005; Lein et al., 2007), but it is unclear whether it is found in the spinal cord. By immunoblotting with an antibody against γ-8, we detected γ-8 expression in cultured spinal cord neurons and in acute hippocampal and spinal cord extracts from P2 wild-type mice (Figure 4A). We were not able to detect a band in γ-$8^{-/-}$ mice, demonstrating that the antibody is specific.

It has been shown that loss of γ-8 greatly decreases AMPA receptor expression in neurons from hippocampal slices. To verify that γ-8 can also regulate AMPA receptor

function in dissociated neurons, we applied AMPA in the presence of cyclothizide to outside-out patches from cultured dissociated hippocampal neurons. We found that γ-8$^{-/-}$ mice have an approximately 50% reduction of AMPA receptor expression (WT 638 ± 103 pA, γ-8$^{-/-}$ 310 ± 60 pA, n = 20 and 14 respectively, $P < 0.02$) (Figure 4B). This is somewhat less than seen in acute slices (~90% reduction) (Rouach et al., 2005), although expression of other TARPs may be upregulated in dissociated neurons given their long time *in vitro*. To determine whether γ-8 regulates AMPA receptor expression at an age similar to when we examined neurons from acute slices from γ-2,3,4 knockout mice, we pulled outside-out patches from P0-P1 CA1 pyramidal cells in acute hippocampal slices. There was a 65% reduction of AMPA receptor currents in γ-8$^{-/-}$ mice compared to wild-type controls (WT 377 ± 108 pA, γ-8$^{-/-}$ 134 ± 48 pA, n = 6 and 8 respectively, $P < 0.05$) (Figure 4C). Our data thus suggest that γ-8 is expressed already at birth in sufficient levels to regulate AMPA receptor function. Furthermore its expression in the spinal cord and spinal cultures, and possible expression in the cortex, may allow it to compensate for the loss of other TARPs in γ-2,3,4 knockout mice.

Use of RNAi to reduce TARP expression

To circumvent difficulties associated with TARP knockout lethality, we decided to combine TARP knockout mice with RNAi to reduce expression of all TARPs. We generated γ-3$^{-/-}$;γ-4$^{-/-}$;γ-8$^{-/-}$ mice, which were both viable and fertile. We designed RNAi constructs against γ-2 with the goal of eventually eliminating γ-2 expression in γ-3$^{-/-}$;γ-4$^{-/-}$;γ-8$^{-/-}$ mice to generate neurons lacking all TARPs (Figure 5A). To test the efficacy of these constructs, we co-expressed individual RNAi constructs in a lentiviral vector

together with γ-2 in cultured human embryonic kidney cells (HEK293 cells). By immunoblotting with an antibody against γ-2, we determined that only construct number 2 significantly reduced γ-2 expression compared to the lentiviral vector alone (Figure 5A). However, this construct did not reduce γ-2 entirely, as some γ-2 was detected unlike in HEK293 cells not transfected with γ-2.

To determine whether the γ-2 RNAi #2 construct could reduce γ-2 levels in neurons, we transfected cultured cerebellar granule cells with the RNAi #2 containing vector, which also expresses GFP. Mice lacking γ-2 have a nearly complete elimination of extrasynaptic AMPA receptors in cerebellar granule cells, allowing them to serve as a indicator of the efficacy of γ-2 knockdown. We measured extrasynaptic AMPA receptor currents induced by brief whole-cell application of AMPA to cerebellar granule cells. The response to AMPA was reduced ~30% in cells expressing RNAi #2 (GFP negative 75.2 ± 6.9 pA, GFP positive 52.1 ± 6.1 pA, n = 12 each, $P < 0.03$). However, the remaining AMPA receptor function was much larger than seen in γ-2$^{-/-}$ mice suggesting that the remaining γ-2 expression in RNAi expressing cells was sufficient to traffic AMPA receptors.

Discussion

Our data are inconclusive in regards to the necessity of TARPs for AMPA receptor function. Although TARPs are critical for postnatal life, we did not find a reduction in AMPA receptor expression in either cortical or spinal neurons. However, the expression of γ-8 in these cells may maintain AMPA receptor function in the absence of other TARPs.

We were unable to generate constructs that act as dominant negatives to reduce the function of all TARP isoforms. In particular, we were surprised that the γ-2/γ-5 construct did not outcompete native TARPs. This construct binds AMPA receptors and modulates their gating, but does not increase their membrane trafficking. However, it is possible that this construct binds to AMPA receptors with a lower affinity than native TARPs, and thus cannot outcompete native TARPs even when expressed at high levels. Another possibility is that the γ-2/γ-5 construct is competent to promote membrane trafficking in neurons, but not in oocytes.

We found one RNAi construct that was successful in reducing γ-2 protein levels in HEK293 cells, but it did not reduce expression in cerebellar granule cells to a sufficient level to reproduce the phenotype of γ-$2^{-/-}$ mice. This is perhaps not entirely unexpected as small quantities of TARPs appear to be sufficient for increasing AMPA receptor surface expression in oocytes and RNAi does not completely eliminate gene expression. Therefore we turned to knockout mice.

By generating various knockout mice, it became clear that TARPs are functionally redundant. Whereas most single knockout mice are indistinguishable from littermates, multiple knockout mice are sick and die postnatally. However, expression of γ-2 appears to be particularly critical as γ-$3^{-/-}$;γ-$4^{-/-}$;γ-$8^{-/-}$ mice are viable, in contrast to γ-$2^{-/-}$;γ-$3^{-/-}$;γ-$8^{-/-}$ and γ-$2^{-/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ mice, which die before or at birth. This is perhaps not surprising, given that γ-2 is widely expressed in the brain (Fukaya et al., 2005; Lein et al., 2007). Although TARPs can compensate for the loss of other family members, the critical function of TARPs is unclear given that AMPA receptor synaptic and extrasynaptic function was not reduced in γ-$2^{-/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ mice. There are at least two

possibilities for this finding. On one hand, TARPs may play a yet undiscovered role in neurons, in addition to or instead of their role in AMPA receptor regulation. The lack of TARPs may then lead to abnormalities in this critical function. On the other hand, γ-8 may be either present or upregulated in the triple knockout neurons that we examined: cortical and spinal neurons. It is possible that only select subsets of neurons are unable to express γ-8, leading to abnormal AMPA receptor expression in those cells.

Unfortunately the expression pattern of TARPs at birth is unclear. Although it was originally shown that only γ-4 is present at birth (Tomita et al., 2003), the reduction of hippocampal AMPA receptor expression at in neonatal γ-$8^{-/-}$ mice suggests that γ-8 is expressed at birth, at least in the hippocampus. We were also able to detect γ-8 protein in P2 spinal cord neurons. In the mature brain, γ-8 is expressed most highly in the hippocampus, but is also found in the cortex and olfactory bulb, but only at low levels in the thalamus and brain stem (Fukaya et al., 2005; Lein et al., 2007). Thus the lethality associated with the γ-$2^{/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ genotype may be due to a reduction of AMPA receptors in the thalamus or brain stem, while γ-8 maintains AMPA receptor function in the spinal cord and cortex. This would be analogous to the situation in γ-$2^{/-}$ mice in which cerebellar granule cells have a profound reduction of AMPA receptors, as they only express γ-2, while hippocampal neurons are unaffected. Thus it is difficult to determine whether the γ-$2^{/-}$;γ-$3^{-/-}$;γ-$4^{-/-}$ lethality is due to the effects of TARPs on AMPA receptors or some unknown function. In order to circumvent the difficulty of breeding quadruple knockout mice, we decided to take a different approach and examine the effects of multiple knockout mice on specific populations of neurons in which the expression of various TARPs is known.

Figure 1. Expression of putative TARP dominant negative constructs in cerebellar granule cells.

(A) On the left is a diagram of γ-2 with four transmembrane domains and a TTPV PDZ-binding motif on its C terminal tail. On the right are schematics of the topology of γ-2 and the structurally similar γ-5, drawn approximately to scale. The γ-2/γ-5 and γ-2 tail constructs are also depicted. **(B)** The γ-2/γ-5 construct together with GFP was expressed in cultured cerebellar granule cells from γ-$2^{-/+}$ mice using a Semliki forest virus. The response to fast local application of AMPA was measured in infected cells and their neighbors, and the peak amplitudes were quantified. No significant difference was found. **(C)** AMPA was briefly applied to cultured γ-$2^{-/+}$ cerebellar granule cells infected with a lentivirus expressing the γ-2 tail construct and GFP. No difference was observed between infected cells and their neighbors.

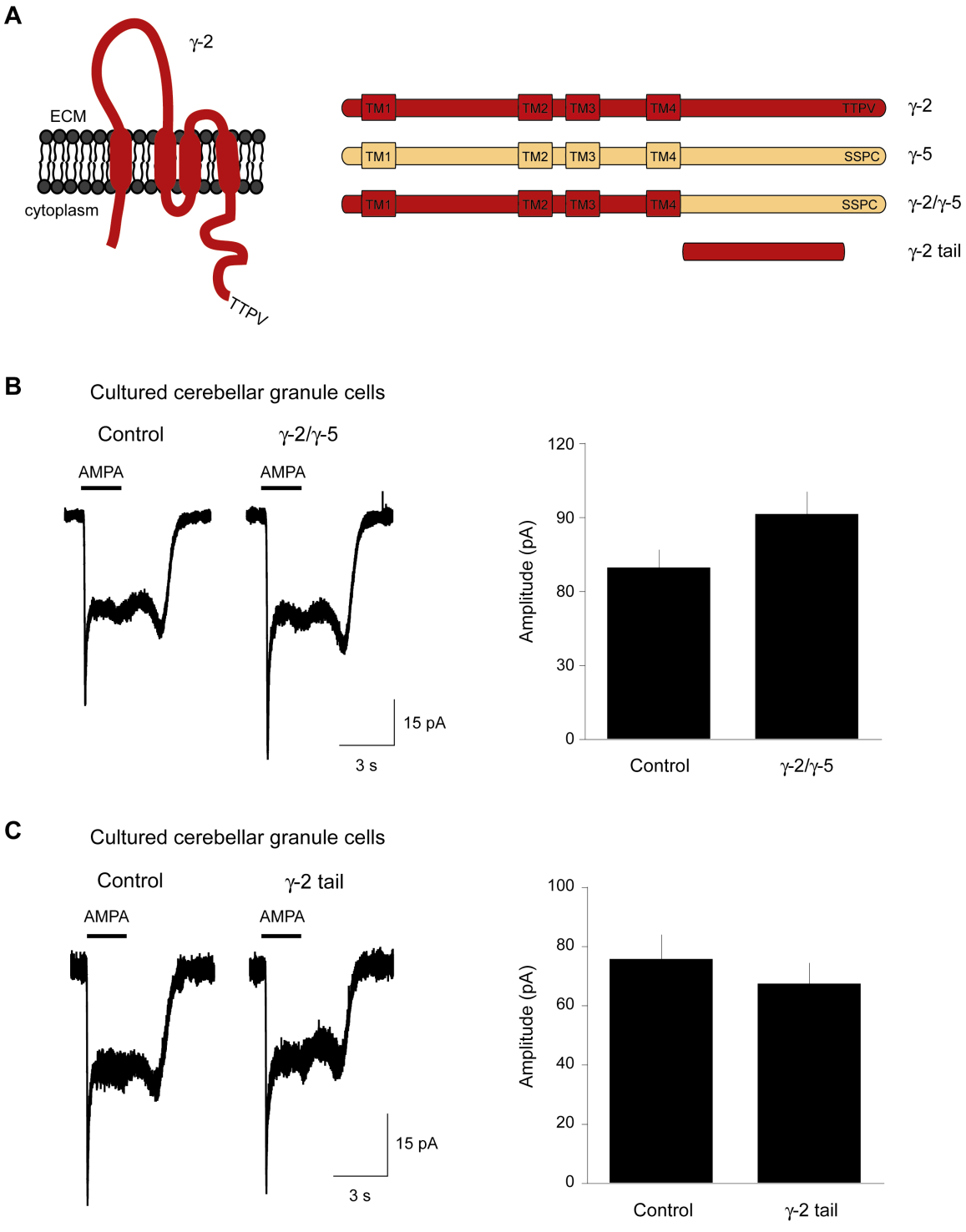

A
γ-2
ECM
cytoplasm
TTPV
TM1
TM2
TM3
TM4
TTPV
γ-2
SSPC
γ-5
SSPC
γ-2/γ-5
γ-2 tail
B
Cultured cerebellar granule cells
Control
γ-2/γ-5
AMPA
AMPA
15 pA
3 s
Amplitude (pA)
120
90
80
30
0
Control
γ-2/γ-5
C
Cultured cerebellar granule cells
Control
γ-2 tail
AMPA
AMPA
15 pA
3 s
Amplitude (pA)
100
80
60
40
20
0
Control
γ-2 tail

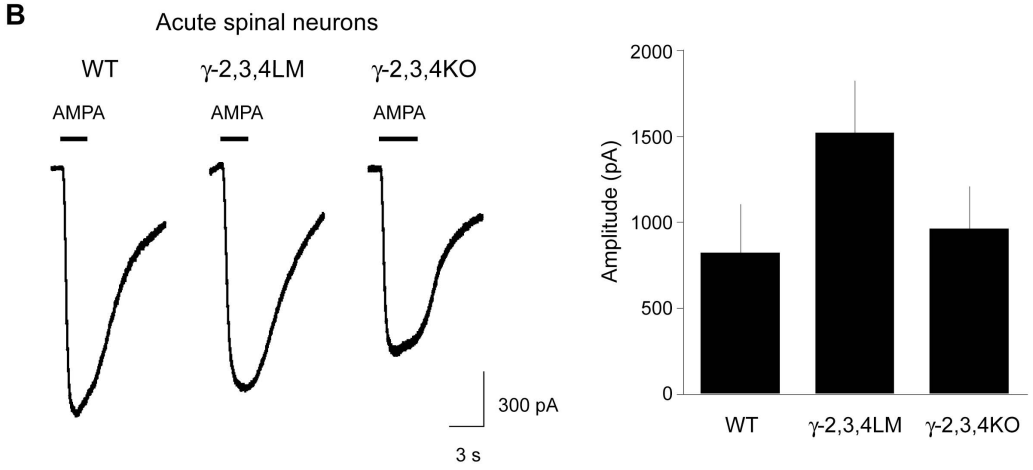
Figure 2. AMPA receptors in spinal neurons from γ-2,3,4 triple knockout mice.

(A) Table showing the genotypes of litters produced by crossing γ-$2^{-/+}$;γ-$3^{-/-}$;γ-$4^{-/-}$ mice. Pups were removed at ~E18.5 for experiments and all pups in the litter were genotyped. Fewer triple knockout mice were found than predicted by Mendelian genetics. **(B)** Short pulses of AMPA in the presence of cyclothiazide were applied to neurons from E18.5 spinal cord slices. No significant difference in the average holding current change was observed between wild-type, γ-2,3,4 knockouts, and γ-2,3,4 littermates. **(C)** Neurons were cultured from spinal cords extracted from either wild-type or γ-2,3,4 knockout mice. Typical mEPSC activity is shown by three consecutive two second sweeps. On the right the average mEPSC for a neuron from each genotype is shown. Neurons were only included for analysis if at least 50 mEPSCs were detected. The average amplitude and frequency of mEPSCs was similar in wild-types and triple knockouts.

A

	γ-2$^{+/+}$; γ-3$^{-/-}$; γ-4$^{-/-}$	γ-2$^{+/-}$; γ-3$^{-/-}$; γ-4$^{-/-}$	γ-2$^{-/-}$; γ-3$^{-/-}$; γ-4$^{-/-}$	litters	Chi square
At E18.5	15 (27%)	36 (64%)	5 (9%)	10	P < 0.02

B



C

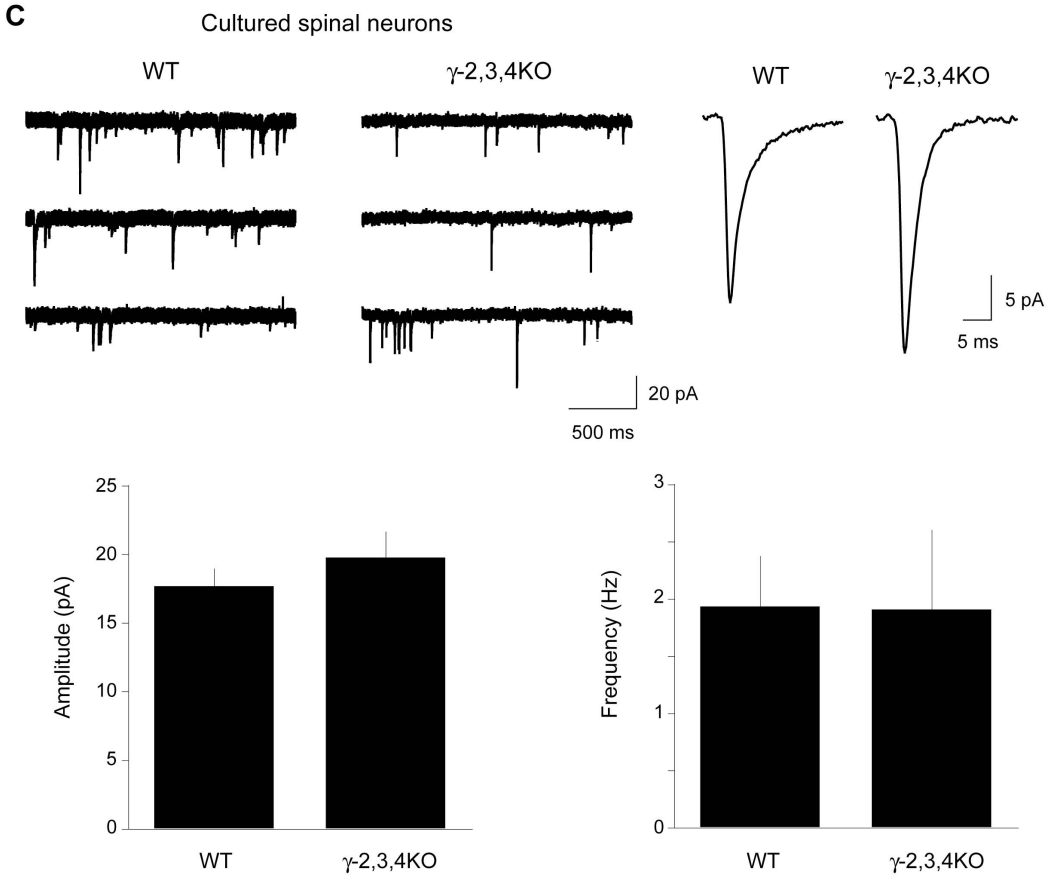


Figure 3. AMPA receptor function in cortical neurons from triple knockout mice.

(A) Acute coronal slices were prepared from E18.5 wild-types, γ-2,3,4 triple knockouts, and γ-2,3,4 littermates. Whole-cell recordings were obtained from cortical neurons and AMPA was briefly applied to the whole cell in the presence of cyclothiazide. The average holding current change was similar in the three genotypes. **(B)** In cyclothiazide, AMPA was briefly applied to outside-out patches from dissociated cultured cortical neurons from γ-2,3,4 knockouts, and γ-2,3,4 littermates. The average amplitude in triple knockout mice was significantly increased to approximately twice the size of littermates. **(C)** On the upper left are three consecutive traces of mEPSCs from γ-2,3,4 littermates and γ-2,3,4 knockouts. On the upper right is the average mEPSC from representative cells of each genotype. Only cells with at least 100 mEPSCs were used for analysis. On the bottom are bar graphs showing that no change in amplitude or frequency was detected in the triple knockouts compared to their littermates.

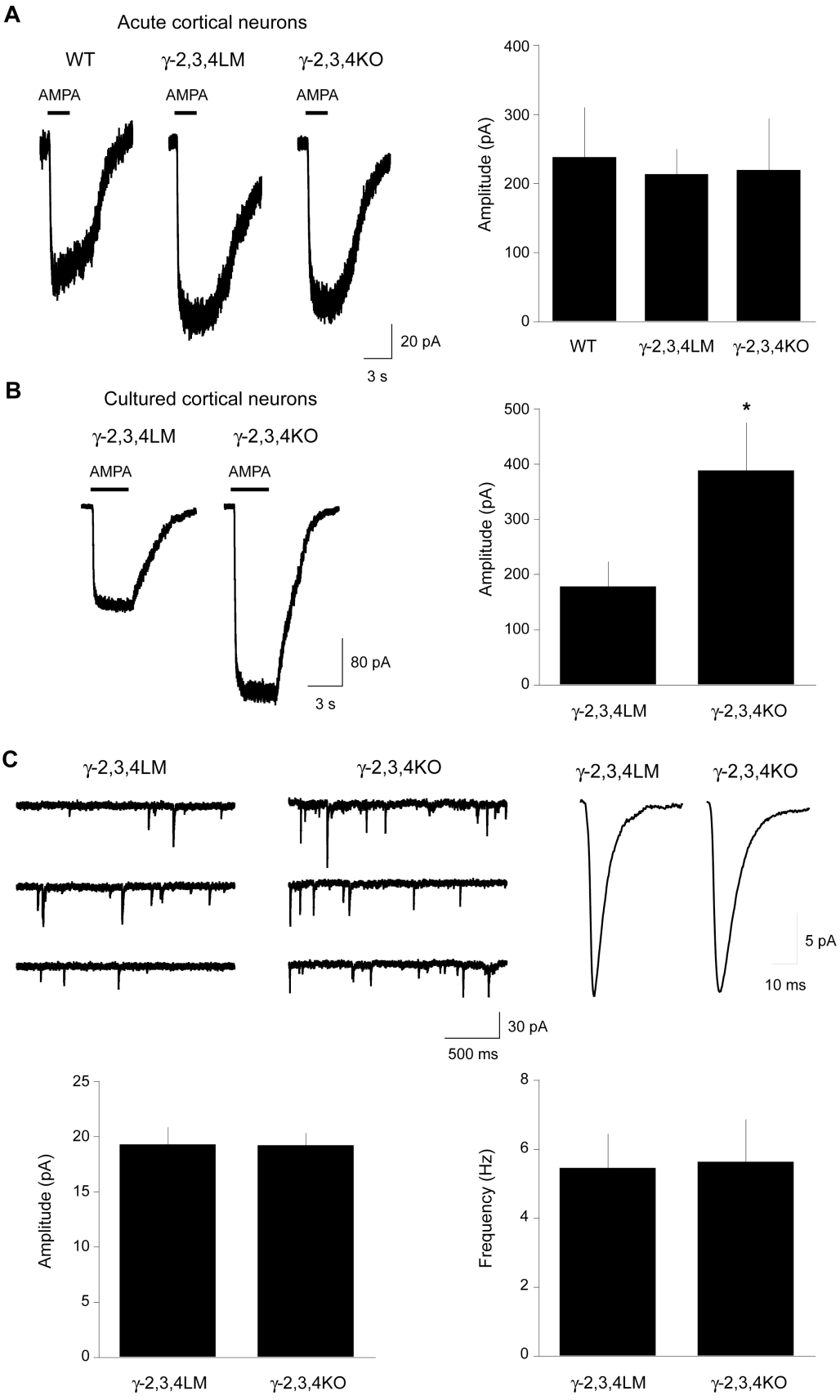

A
Acute cortical neurons
WT
γ-2,3,4LM
γ-2,3,4KO
AMPA
AMPA
AMPA
20 pA
3 s
Amplitude (pA)
400
300
200
100
0
WT
γ-2,3,4LM
γ-2,3,4KO
B
Cultured cortical neurons
γ-2,3,4LM
γ-2,3,4KO
AMPA
AMPA
80 pA
3 s
Amplitude (pA)
500
400
300
200
100
0
*
γ-2,3,4LM
γ-2,3,4KO
C
γ-2,3,4LM
γ-2,3,4KO
γ-2,3,4LM
γ-2,3,4KO
5 pA
10 ms
30 pA
500 ms
Amplitude (pA)
25
20
15
10
5
0
γ-2,3,4LM
γ-2,3,4KO
Frequency (Hz)
8
6
4
2
0
γ-2,3,4LM
γ-2,3,4KO

Figure 4. γ-8 is present in young animals and dissociated neurons.

(A) Immunoblots from either spinal cord cultures (left) or P2 hippocampi or spinal cords (right) were blotted with an antibody that detects γ-8. γ-8 is detectable in all conditions, except in γ-8 knockout mice. **(B)** Outside-out patches were pulled from cultured dissociated hippocampal neurons prepared from wild-type and γ-8 knockout mice. The response to a brief application of AMPA in the presence of cyclothiazide was significantly reduced ~50% in γ-8 knockout mice. **(C)** Acute hippocampal slices were prepared from P0-1 wild-type and γ-8 knockout mice and CA1 pyramidal neurons were recorded. The response to local application of AMPA in the presence of cyclothiazide was significantly reduced in γ-8 knockout mice.

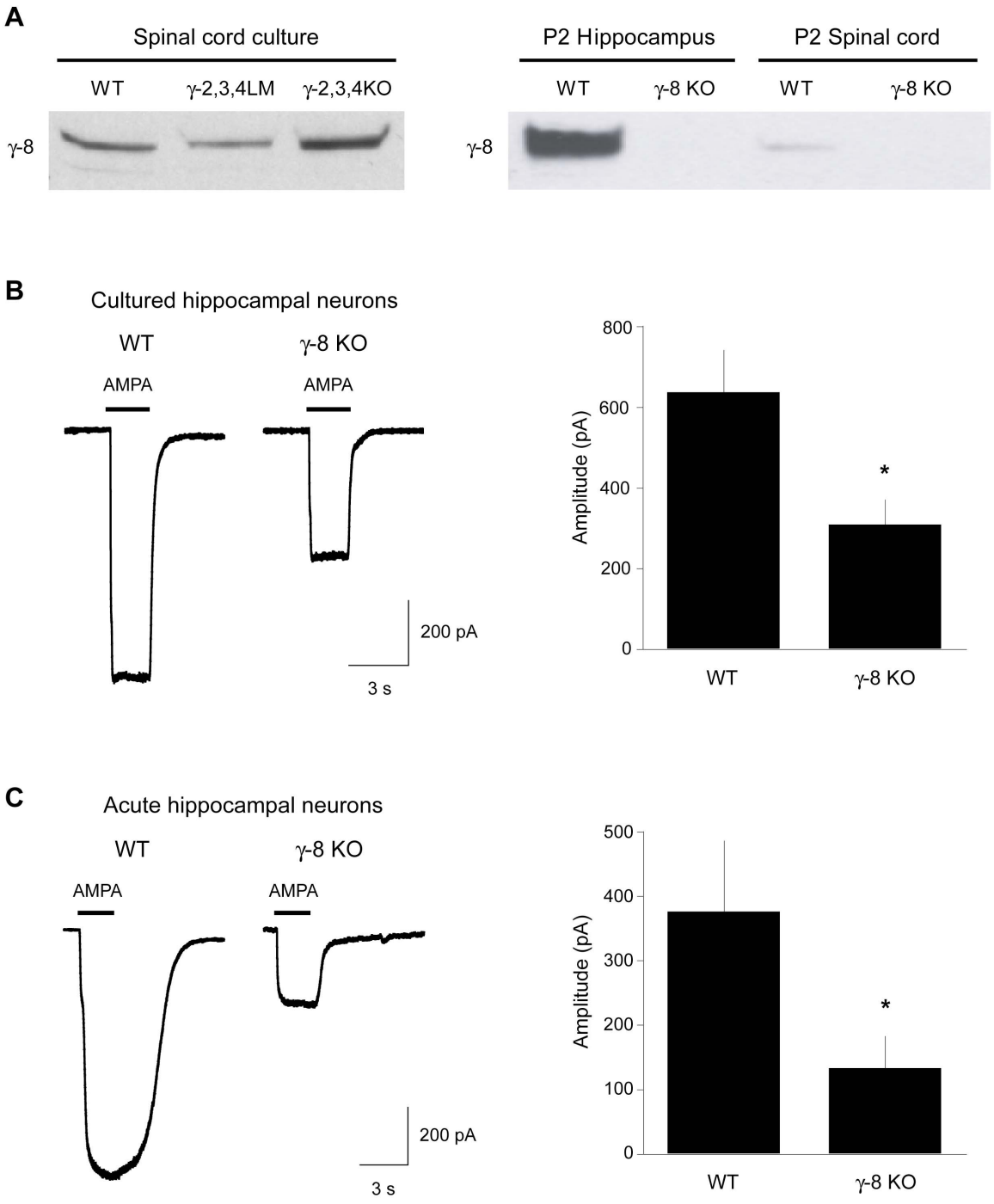
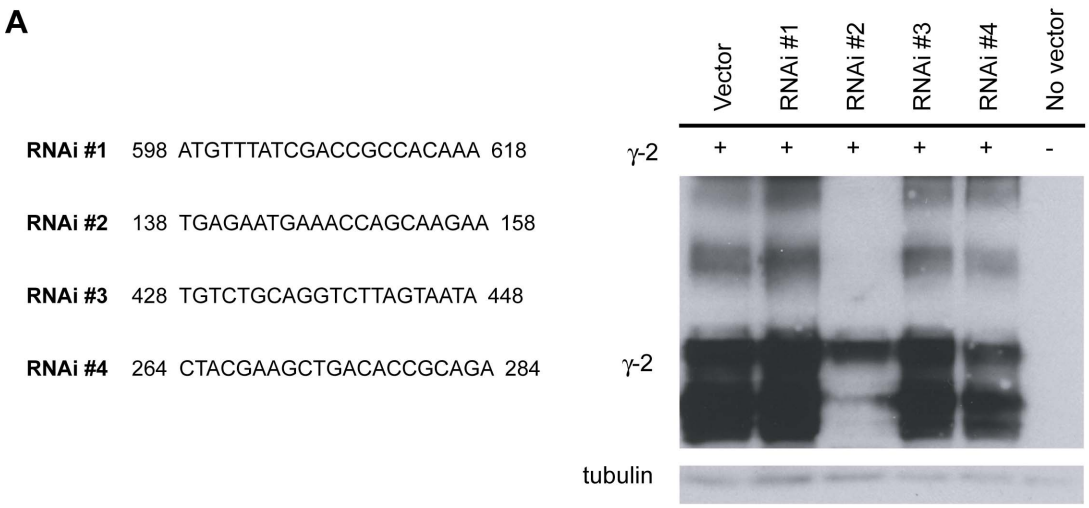
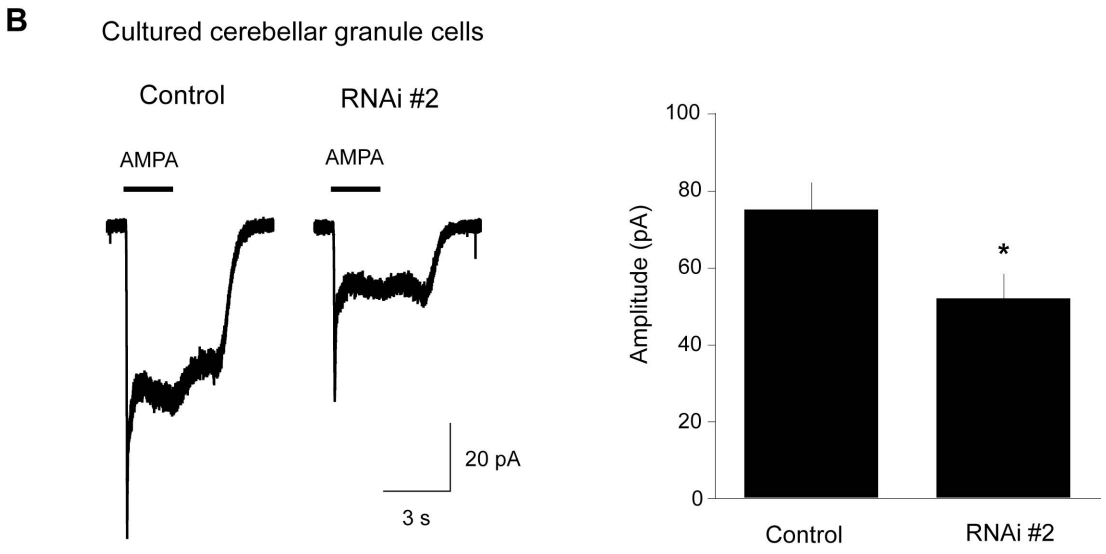

A
Spinal cord culture
WT
γ-2,3,4LM
γ-2,3,4KO
γ-8
P2 Hippocampus
P2 Spinal cord
WT
γ-8 KO
WT
γ-8 KO
γ-8
B
Cultured hippocampal neurons
WT
γ-8 KO
AMPA
AMPA
200 pA
3 s
Amplitude (pA)
800
600
400
200
0
*
WT
γ-8 KO
C
Acute hippocampal neurons
WT
γ-8 KO
AMPA
AMPA
200 pA
3 s
Amplitude (pA)
500
400
300
200
100
0
*
WT
γ-8 KO

Figure 5. RNAi in HEK293 cells and cerebellar granule cells.

(A) Four RNAi constructs were designed and tested for their ability to knockdown γ-2 expression in HEK293 cells. The sequences that the RNAi constructs target within γ-2 are shown on the left. On the right are immunoblots of extracts from HEK293 cells transfected with γ-2 and either an empty vector or the vector expressing one of the RNAi constructs. An antibody that detected γ-2 was used. On the far right are extracts from untransfected HEK293 cells. Tubulin expression was used as a loading control. **(B)** Cultured cerebellar granule cells from γ-$2^{-/+}$ mice were transfected with RNAi #2. The peak response of GFP positive transfected cells to a two second local perfusion of AMPA in cyclothiazide was compared to neighboring untransfected cells. A significant ~30% reduction was observed in cells expressing RNAi #2.


A
RNAi #1 598 ATGTTTATCGACCGCCACAAA 618
RNAi #2 138 TGAGAATGAAACCAGCAAGAA 158
RNAi #3 428 TGTCTGCAGGTCTTAGTAATA 448
RNAi #4 264 CTACGAAGCTGACACCGCAGA 284
Vector
RNAi #1
RNAi #2
RNAi #3
RNAi #4
No vector
γ-2
+ + + + + -
γ-2
tubulin


B
Cultured cerebellar granule cells
Control
RNAi #2
AMPA
AMPA
20 pA
3 s
Amplitude (pA)
100
80
60
40
20
0
*
Control
RNAi #2

Chapter 4:

TARPs govern AMPA receptor synaptic targeting and subunit composition in inhibitory neurons

Introduction

Voltage- and ligand-gated ion channels form the foundation of neuronal signaling in the brain. Research has primarily focused on pore-forming principal subunits of ion channels; however, auxiliary subunits can dramatically modify trafficking, localization, and gating of voltage-gated channels (Isom et al., 1994; Levitan, 2006; Yu et al., 2005). Until recently, few ligand-gated ion channels were known to possess auxiliary subunits, but this changed with the identification of transmembrane AMPA receptor regulatory proteins (TARPs) as auxiliary subunits for the ligand-gated AMPA receptors (Chen et al., 2000; Nicoll et al., 2006; Ziff, 2007). As AMPA receptors underlie the majority of fast excitatory synaptic transmission in the brain and their mobility contributes to learning and memory, it is critical to understand how TARPs regulate AMPA receptor activity.

TARPs are a family of related four-pass transmembrane proteins (Letts et al., 1998), including γ-2, γ-3, γ-4, γ-7, and γ-8, which promote AMPA receptor channel function (Tomita et al., 2003, Kato 2007). Like classical auxiliary subunits of voltage-gated channels, TARPs regulate many functional aspects of AMPA receptors. TARPs augment AMPA receptor surface trafficking, enhance synaptic clustering, increase glutamate affinity, increase kainate efficacy, determine CNQX pharmacology and slow channel deactivation and desensitization (Kott et al., 2007; Menuz et al., 2007; Priel et al., 2005; Tomita et al., 2005a; Turetsky et al., 2005; Yamazaki et al., 2004; Zhang et al., 2006). Research on TARP knockout mice has shown that TARPs are important for both extrasynaptic and synaptic AMPA receptor expression in certain excitatory neurons. Cerebellar granule cells from γ-2$^{-/-}$ (stargazer) mice have a nearly complete loss of both synaptic and extrasynaptic AMPA receptors (Chen et al., 1999; Chen et al., 2000;

Hashimoto et al., 1999), and hippocampal pyramidal cells from γ-8$^{-/-}$ mice have a profound loss of extrasynaptic receptors, but only a small impairment of AMPA receptor mediated synaptic transmission (Fukaya et al., 2006; Rouach et al., 2005). It remains unclear whether TARPs directly regulate AMPA receptor synaptic targeting or whether the loss of synaptic receptors results secondarily from the severe depletion of cell surface receptors. Indeed, the distinct AMPA receptor trafficking defects in γ-2$^{-/-}$ and γ-8$^{-/-}$ mice together with the differential subcellular localization of γ-2 and γ-8, suggest that TARPs may have specialized subcellular functions (Inamura et al., 2006).

Stargazer mice, which lack γ-2, are ataxic and display absence epilepsy characterized by spike-wave discharges coupled to behavioral arrest (Frankel, 1999; Noebels et al., 1990). Loss of AMPA receptor function in cerebellar granule cells likely explains stargazer ataxia. However, defects in excitatory transmission do not readily fit with the stargazer seizure disorder (Osten and Stern-Bach, 2006). One possibility is that inhibitory neurons may require TARPs to maintain normal synaptic AMPA receptor function, and the resulting disinhibition in γ-2$^{-/-}$ mice may underlie seizure generation. However, TARP knockout mice have not been reported to lack synaptic AMPA receptors in inhibitory neurons, and knockout mice lacking γ-4 or γ-8 do not have visible behavioral abnormalities (Letts et al., 2005; Rouach et al., 2005).

This study employs multiple TARP knockout mice to address key questions regarding the *in vivo* regulation of AMPA receptors by TARPs. We demonstrate functional redundancy of TARPs, which explains the maintenance of AMPA receptor function in single TARP knockout mice. Importantly, we find that AMPA receptor activity in inhibitory neurons requires TARPs and show that TARPs slow interneuron

AMPA receptor kinetics. Surprisingly, we find a role for TARPs in regulating AMPA receptor subunit composition. We further show that TARPs can specifically regulate synaptic localization of AMPA receptors independent of their effects on receptor surface trafficking.

Results

γ-2,3$^{-/-}$ mice fail to thrive

To investigate the essential *in vivo* function of TARPs, we first generated mice deficient in γ-3 (Figures 1A–1C), which is widely expressed in brain (Lein et al., 2007). As these knockout mice were indistinguishable from wild-type (wt) littermates (Figures 1D and 1E), γ-3 is either not required for normal brain function or another TARP compensates for its loss.

Given that γ-2 and γ-3 are closely related, we tested for molecular redundancy by breeding together γ-2$^{-/-}$ (stargazer) and γ-3$^{-/-}$ mice to generate γ-2$^{-/-}$;γ-3$^{-/-}$ mice (abbreviated γ-2,3$^{-/-}$). We found that γ-2,3$^{-/-}$ mice are born at predicted Mendelian ratios, but that the majority of γ-2,3$^{-/-}$ die within a few days after birth (Table 1). By culling littermates, some γ-2,3$^{-/-}$ mice lived for a few weeks, but rarely survived beyond the 4th post-natal week. At P14, surviving γ-2,3$^{-/-}$ mice were ~55% the weight of γ-3$^{-/-}$ littermates, while γ-2$^{-/-}$ mice were ~75% the weight of their wild-type littermates (Figures 1D and 1E). No significant difference in weight was observed between γ-3$^{-/-}$ and wild-type mice. In addition to their smaller size, γ-2,3$^{-/-}$ mice displayed profound ataxia, which was considerably more severe than in γ-2$^{-/-}$ mice (Suppl. movies 1-4). At the whole

animal level, the loss of γ-3 exaggerates the γ-2 phenotype suggesting that these two TARPs may serve redundant functions.

Loss of cerebellar AMPA receptor expression in γ-2,3$^{-/-}$ mice

TARPs chaperone AMPA receptors for proper protein folding and membrane trafficking (Vandenberghe et al., 2005a). We therefore asked whether mutation of γ-2 and γ-3 yields a secondary loss of AMPA receptor protein levels, as occurs in the hippocampus of γ-8$^{-/-}$ mice (Rouach et al., 2005). Immunohistochemical staining of sagittal brain slices using antibodies raised against the AMPA receptor subunits GluR1, GluR2/3 and GluR4 did not reveal any gross localization differences between wild-type and γ-2,3$^{-/-}$ mice (Figure 2A). We then used immunoblot analysis to quantify changes in AMPA receptor protein levels. Although γ-2 and γ-3 both occur in forebrain cortical regions, no changes in AMPA receptor subunit levels were found in either the cortex or the hippocampus (Figure 2B). In contrast, loss of γ-2 and γ-3 did reduce AMPA receptor levels in the cerebellum (Figure 3C). Cerebellar protein levels of both GluR1 and GluR4 were reduced by ~25% in γ-2$^{-/-}$ mice, although no further reduction occurred with the additional loss of γ-3. In contrast, GluR2/3 levels were unaltered in γ-3$^{-/-}$ mice, were reduced by ~30% in γ-2$^{-/-}$ mice and were reduced by ~70% in γ-2,3$^{-/-}$ mice. This synergistic reduction in GluR2/3 levels in the γ-2,3$^{-/-}$ mice implies a functional redundancy for these two closely-related TARPs.

TARPs can regulate synaptic AMPA receptors in interneurons

The loss of cerebellar AMPA receptors and severe ataxia in γ-2,3$^{-/-}$ mice suggested impaired synaptic transmission in at least one population of cerebellar neurons. We investigated AMPA receptor mediated synaptic transmission in cerebellar Golgi cells (Palay and Chan-Palay, 1974), local interneurons that express high levels of γ-2 and γ-3 (Fukaya et al., 2005; Lein et al., 2007) and whose ablation causes ataxia (Watanabe et al., 1998). We quantified Golgi cell AMPA receptor mediated transmission by stimulating parallel fibers to evoke a compound EPSC composed of NMDA and AMPA receptor mediated currents at a holding potential of +40 mV. We then pharmacologically separated the currents by applying 50 μM D-APV and measured the ratio of I_{AMPA} to I_{NMDA}. Double knockout mice had a nearly 90% reduction of I_{AMPA}/I_{NMDA} whereas no change was seen in either single knockout (Figure 3A). No difference was observed in the paired-pulse ratio, a measure of pre-synaptic function, consistent with the predicted postsynaptic loss of receptors (Figure 3B).

The reduction in I_{AMPA} in γ-2,3$^{-/-}$ mice may reflect decreased AMPA receptors in all Golgi synapses or selective loss from a subset of synapses. If the former is true, then both the amplitude and the frequency of miniature EPSCs (mEPSCs) should be reduced, as many events would fall below the threshold for detection. If the later were true, only the mEPSC frequency would diminish. To distinguish between these possibilities, we measured Golgi cell mEPSCs. In contrast to wild-type or single knockout mice, mEPSCs rarely appeared in γ-2,3$^{-/-}$ mice, and the few remaining had amplitudes just above the 10 pA detection threshold (Figures 3C and 3D). This ~90% decrease in frequency and large change in amplitude suggest a generalized loss of synaptic AMPA receptors in the double

knockout mice; we cannot rule out an additional loss of total synapses. Together, our evoked transmission and mEPSC data indicate that either γ-2 or γ-3 can support normal AMPA receptor-mediated synaptic transmission in Golgi cells.

Loss of TARPs speeds AMPA receptor kinetics and changes subunit composition

Previous work showed that TARPs slow the rates of AMPA receptor deactivation and desensitization both in heterologous systems and in some excitatory neurons (Cho et al., 2007; Milstein et al., 2007; Priel et al., 2005; Tomita et al., 2005a). However, it is unclear if TARPs can also slow AMPA receptor kinetics in interneurons given that the kinetics in interneurons are often markedly faster than those of excitatory neurons. Although some factors underlying the faster kinetics in interneurons have been identified including expression of specific AMPA receptor subunits and highly synchronized glutamate release (Jonas, 2000; Jonas et al., 2004), a role for TARPs has not been examined. Like many interneurons, Golgi cell mEPSCs have extremely rapid decay kinetics, average decay time constant ~1.7 ms. We find that the decay of mEPSCs in γ-2,3$^{-/-}$ mice was nearly twice as fast as those in single KO and wild-type mice (Figure 4A). Thus, TARPs can contribute significantly to AMPA receptor kinetics at fast interneuron synapses.

We were surprised to find that the remaining synaptic AMPA receptors in γ-2,3$^{-/-}$ mice have a different subunit composition than in wild-type mice. Whereas the I-V relationships of synaptic AMPA receptor-mediated currents in wild-type and single-knockout mice were linear (rectification index of 1), the I-V curves in γ-2,3$^{-/-}$ mice were rectifying (Figure 4B). Given that native AMPA receptors require GluR2 subunits to

generate a linear I-V relationship, our data suggest that AMPA receptors in control animals contain GluR2, whereas synapses in γ-2,3$^{-/-}$ mice contain a mixed population of GluR2-containing and GluR2-lacking AMPA receptors. Thus specifying the composition of synaptic AMPA receptors is yet another mechanism by which TARPs modulate AMPA receptor function.

TARPs control synaptic localization of AMPA receptors

Due to the profound loss of cell surface AMPA receptors in TARP knockout neurons, it has remained uncertain whether TARPs specifically mediate synaptic targeting of AMPA receptors or whether the loss of synaptic AMPA receptors in TARP knockouts occurs secondary to the diminished levels of surface receptors. Given that our immunoblot experiments indicated that γ-2,3$^{-/-}$ mice had a large loss of GluR2/3 protein, we sought to dissociate these TARP roles in studies of cerebellar Purkinje cells because they primarily express γ-2 and our immunoblot experiments indicated that γ-2$^{-/-}$ mice did not exhibit a large change in cerebellar AMPA receptor protein levels (Figure 2C). Consistent with this finding, extrasynaptic AMPA receptor levels in Purkinje cells from γ-2$^{-/-}$ mice, measured by applying 500 μM AMPA to outside-out patches, were not significantly different from wild-type mice (Figure 5A). Despite normal levels of surface AMPA receptors, we found a substantial loss of synaptic AMPA receptors at both climbing fiber and parallel fiber synapses in γ-2$^{-/-}$ mice (see methods for details) (Figures 5B and 5C). At climbing fiber synapses, the ratio of I_{AMPA} to $I_{kainate}$ was decreased ~70%, and at parallel fiber synapses the ratio of I_{AMPA} to I_{mGluR} was reduced by ~50%. The climbing fiber evoked $I_{kainate}$ was comparable between wild-type and γ-2$^{-/-}$ mice

suggesting that the number of synapses was not decreased, but rather the number of AMPA receptors at each synapse was reduced. No further decrease in synaptic AMPA receptors was found with the additional removal of γ-3, as predicted by its lack of expression in Purkinje cells. Together our data demonstrate that loss of TARPs can reduce synaptic AMPA receptor targeting independent of changes in receptor surface expression.

Evidence against differential TARP subtype function in receptor trafficking

It is unclear why many neurons express multiple TARP family members. A recent electron microscopy study of the hippocampus found that γ-2 is only located at synapses while γ-8 is found both synaptically and extrasynaptically (Inamura et al., 2006). One possibility is that the highly homologous TARPs γ-2 and γ-3 may maintain synaptic AMPA receptor localization, while γ-8 may primarily mediate extrasynaptic expression. This model fits with the relatively minor reduction of hippocampal synaptic AMPA receptors in γ-8$^{-/-}$ mice, the nearly complete loss of extrasynaptic receptors in γ-8$^{-/-}$ mice (Rouach et al., 2005), and the synaptic loss of receptors in the cerebellum of γ-2$^{-/-}$ and γ-2,3$^{-/-}$ mice (Figures 3 and 5).

To test whether γ-2 and γ-3 are specialized to regulate synaptic AMPA receptor expression, we examined AMPA receptor mediated transmission in hippocampal CA1 pyramidal cells that express γ-2, γ-3 and γ-8. We found that the ratio of I_{AMPA} to I_{NMDA} was unchanged in γ-2,3$^{-/-}$ mice compared to controls (Figure 6A). There was also no change in the paired pulse ratio (Figure 6B) or the rectification index (Figure 6C). These data argue against a model of synaptic versus extrasynaptic TARP specializations and

suggests that, similar to Golgi cells, either γ-2 or γ-8 can maintain AMPA receptor synaptic function.

Discussion

Our results demonstrate that TARPs are essential genes that control AMPA receptors in diverse neuronal types throughout the brain. We show that TARPs γ-2 and γ-3 are redundant, which allows neurons to maintain AMPA receptor function in the absence of a single TARP family member. Furthermore, we show that TARPs can specifically control synaptic targeting and EPSC decay kinetics of AMPA receptors in inhibitory neurons. Finally, our data reveal an unexpected role for TARPs in regulating AMPA receptor subunit composition.

Molecular redundancy of TARP family members

Although γ-$2^{-/-}$ mice (stargazer) have a clear behavioral phenotype, other TARP knockout mice, including the γ-$3^{-/-}$ mice reported here, do not show obvious behavioral abnormalities. Given the demonstrated importance of TARPs in regulating AMPA receptor maturation, trafficking, and gating, one might have expected greater behavioral abnormalities. Alternatively, because many neurons express multiple TARP family members, the loss of an individual TARP might be functionally compensated by other TARPs. Our study of γ-$2,3^{-/-}$ mice supports this latter possibility, as loss of both TARPs results in early postnatal lethality and severe ataxia. Furthermore, our experiments in cerebellar Golgi cells directly demonstrate that these two TARPs are molecularly redundant, as these neurons maintain wild-type levels of synaptic AMPA receptors in the

absence of either γ-2 or γ-3, but lose ~90% of receptors if both TARPs are removed. The changes in rectification and mEPSC decay in double knockout mice support the idea that these TARPs fulfill similar functions.

Recently, γ-7 was identified as a new member of the TARP family, and it is expressed in both cerebellar Golgi cells and Purkinje cells (Kato et al., 2007). It is noteworthy that γ-7 cannot maintain full synaptic AMPA receptor expression in the absence of γ-2 and γ-3 in either Golgi or Purkinje cells, consistent with the finding that γ-7 does not increase membrane trafficking of AMPA receptors to the same extent as γ-2 in dissociated cerebellar granule cells (Kato et al., 2007). It is possible that while TARPs γ-2 and γ-3 have similar functions, TARP γ-7 is important for other aspects of AMPA receptor regulation.

Analysis of the remaining synaptic receptors in Golgi cells found that the decay kinetics in γ-2,3$^{-/-}$ mice ($\tau \sim 0.85$ ms) were approximately twice as fast as in wild type ($\tau \sim 1.6$-1.7 ms). These decay kinetics resemble the values previously published for deactivation of receptors in the absence and presence of TARPs. That is, in heterologous expression systems, AMPA receptor subunits and splice variants have deactivation time constants of ~0.6-1.2 ms (Dingledine et al., 1999; Sekiguchi et al., 2002), and receptors with TARPs decay in the range of ~1.4-2.4 ms (Kato et al., 2007; Tomita et al., 2005a; Zhang et al., 2006). Thus, the 10% remaining AMPA receptors in Golgi cells of γ-2,3$^{-/-}$ mice may not be associated with TARPs. Other mechanisms have been proposed to influence the membrane trafficking and synaptic targeting of AMPA receptors including their C-terminal tail interactions with various cytoplasmic proteins (Barry and Ziff, 2002; Ziff, 2007). Future genetic studies will be needed to address these possibilities.

Regulation of synaptic and extrasynaptic AMPA receptors by TARPs

TARPs are thought to target AMPA receptors to synapses by associating with the synapse-localized MAGUK family of proteins via the PDZ-binding motif on the TARP C-terminal tail (Bats et al., 2007; Elias and Nicoll, 2007; Schnell et al., 2002). However, in the hippocampus of γ-8$^{-/-}$ mice, the ~90% loss of extrasynaptic AMPA receptors compared to the ~35% loss of synaptic receptors suggested that TARPs may primarily function to increase surface expression of AMPA receptors *in vivo* (Rouach et al., 2005). Our findings in the Purkinje cells of γ-2$^{-/-}$ mice suggest the opposite: no significant decrease in either extrasynaptic receptor expression or in total AMPA receptor protein levels, but a ~60-70% decrease in synaptic AMPA receptor currents. As post-synaptic kainate receptor mediated responses remained intact, simple synaptic loss seems unlikely. These results demonstrate that γ-2 can specifically regulate AMPA receptor expression at synapses.

Together, our data from γ-2$^{-/-}$ Purkinje cells and γ-2,3$^{-/-}$ Golgi cells, combined with previous findings in γ-8$^{-/-}$ hippocampal neurons raise the possibility that γ-2 and γ-3 mediate synaptic AMPA receptor trafficking whereas γ-8 controls cell surface trafficking. An electron microscopy study demonstrating the synaptic localization of γ-2, but extrasynaptic and synaptic localization of γ-8, provides support for this idea (Inamura et al., 2006). However, we found that loss of γ-2 and γ-3 did not reduce synaptic AMPA receptor expression in hippocampal pyramidal cells, suggesting that γ-8 can target AMPA receptors to synapses. It remains uncertain whether extrasynaptic AMPA receptor expression depends primarily upon TARP γ-8. If true, the lack of γ-8 expression in the

cerebellum may explain the relatively low density of extrasynaptic AMPA receptors in outside-out patches from cerebellar Purkinje, stellate, and granule cells (Hausser and Roth, 1997; Liu and Cull-Candy, 2000; Silver et al., 1996) compared to hippocampal neurons (Jonas and Sakmann, 1992; Rouach et al., 2005).

TARPs may govern AMPA receptor subunit composition

The change from linear to rectifying synaptic responses in Golgi cells from γ-2,3$^{-/-}$ mice suggests that TARPs may regulate AMPA receptor subunit composition, as TARPs do not appreciably change the I-V relationship of AMPA receptors composed of single subunits (Tomita et al., 2005a). The increased rectification indicates a partial loss of GluR2-containing receptors, which fits with the loss of GluR2/3 protein (Figure 2C). This change in subunit composition was surprising, as TARPs can functionally interact with all four AMPA receptor subunits (Turetsky et al., 2005) and no change in rectification was found at hippocampal synapses in γ-8$^{-/-}$ mice (Rouach et al., 2005).

How might TARPs specify subunit composition? TARPs may preferentially associate with GluR2-containing receptors early in the biosynthetic pathway, enhance the trafficking of GluR2-containing receptors to the cell surface, or increase the insertion of GluR2-containing receptors into synapses. Previous surface biotinylation assays have indicated that γ-2 enhances the plasma membrane expression of both GluR1 and GluR2 homomeric receptors in heterologous systems (Turetsky et al., 2005; Yamazaki et al., 2004). This may support a role for TARPs in specifically targeting GluR2-containing receptors to synapses, but further studies will be needed to definitively distinguish between these and other mechanisms.

At least two possibilities may account for the appearance of synaptic GluR2-lacking receptors in γ-2,3$^{-/-}$ mice. These receptors may have been present in control synapses and remained after the selective loss of GluR2-containing receptors, or homeostatic mechanisms may traffic GluR2-lacking receptors to compensate for the massive loss of synaptic AMPA receptors. Given that approximately half of synaptic AMPA receptors in double knockout mice are GluR2-lacking and only ~10% of total receptors remain, our measurements of the rectification index are not sensitive enough to rule out the presence of a small minority of GluR2-lacking receptors in wild-type mice.

TARP regulation of AMPA receptors in inhibitory neurons and epilepsy

Our data from Golgi and Purkinje cells unequivocally demonstrate that TARPs regulate AMPA receptor expression in inhibitory neurons. Together with previous data in excitatory neurons, such as cerebellar granule cells and hippocampal pyramidal cells, our results suggest that the role for TARPs in AMPA receptor function is widespread throughout the brain. In particular, the loss of synaptic AMPA receptors in Golgi cells indicates a role for TARPs in local interneurons. Many studies have sought to explain the remarkably fast decay kinetics seen in interneurons, and a variety of factors have been found that account for their fast time course. Our data show that mEPSC decay kinetics become ~2 fold faster in the absence of TARPs, suggesting that TARPs actually slow mEPSC kinetics in interneurons.

Stargazer (γ-2$^{-/-}$) mice serve as a rodent model for absence epilepsy due to their characteristic seizures coupled with behavioral arrest (Frankel, 1999). These seizures are

due to abnormal thalamocortical network activity, in which thalamocortical oscillations convert into spike wave discharge seizures. Many single-gene mutations that cause epilepsy involve mutations in ion channels, such as $GABA_A$ receptors, the loss of which results in increased neuronal excitability. It has been more difficult to explain how loss of AMPA receptors in stargazer mice leads to seizure generation. Our finding that interneurons may also require TARPs for functional AMPA receptors suggests a model in which disinhibition may underlie seizure activity. In this model, a loss of AMPA receptors from interneurons within the thalamocortical network, perhaps thalamic reticular neurons or cortical interneurons, may occur if a specific subpopulation of interneurons only express TARP γ-2. This loss may lead to disinhibition, a decrease in inhibitory activity, and thereby create an overly active thalamic/cortical network. Recently a similar mechanism of disinhibition was proposed to underlie the seizures caused by mutations in the sodium channel gene *Scn1a*, which has been implicated in diseases such as generalized epilepsy with febrile seizures and severe myoclonic epilepsy (Ogiwara et al., 2007; Yu et al., 2006). Further studies will be needed to expand our findings to thalamocortical interneurons in stargazer mice. Our data together with the *Scn1a* findings suggest that an understanding of epilepsy will require knowledge of both the identity of mutated genes and the expression pattern of these gene products.

Table 1. Survival of γ-2,3$^{-/-}$ mice in the absence of culling littermates.

	γ-2$^{+/+}$; γ-3$^{-/-}$	γ-2$^{+/-}$; γ-3$^{-/-}$	γ-2$^{-/-}$; γ-3$^{-/-}$	litters	Chi square
At birth	59 (28%)	111 (53%)	41 (19%)	37	p = 0.162
At P14 (pups)	62 (35%)	106 (61%)	7 (4%)	46	p < 0.0001
Died < P14	5 (9%)	23 (42%)	27 (49%)	30	p < 0.0001

The number of progeny of each genotype resulting from crossing γ-2$^{+/-}$;γ-3$^{-/-}$ parents is shown. The percentage of animals of each genotype is given in parentheses.

Figure 1. Generation of TARP γ-3$^{-/-}$ and γ-2,3$^{-/-}$ mice.

(A) Schematic diagram of the γ-3 protein, wild-type genomic locus, targeting vector, and targeted allele. Transmembrane domains 2 and 3 were disrupted by homologous recombination. A small box marks the location of the 5' probe used for Southern blot analysis. Exons are marked as black boxes and abbreviated Ex. **(B)** Southern blot analysis of mouse tail DNA digested with XbaI using the 5' probe shown in (A) detects both a 6.5 kb wild-type fragment and the 4.2 kb knockout fragment in γ-3$^{+/-}$ mice. **(C)** Immunoblot analysis of brain lysates from γ-3$^{+/+}$, γ-3$^{+/-}$, and γ-3$^{-/-}$ mice demonstrates the absence of γ-3 protein in γ-3$^{-/-}$ mice. Tubulin was used as a loading control. **(D, E)** The average weight of P14-15 γ-2$^{-/-}$ mice is only slightly less than that of wild-type littermates. In contrast, the few γ-2,3$^{-/-}$ mice that survive weigh around half of their γ-3$^{-/-}$ littermates, which were indistinguishable from wild-type mice (wt 8.49 ± 0.17 g, γ-3$^{-/-}$ 7.97 ± 0.15 g, γ-2$^{-/-}$ 6.20 ± 0.27 g , γ-2,3$^{-/-}$ 4.59 ± 0.40 g; n = 86, 94, 14, and 15 respectively; $P < 0.05$ for γ-2,3$^{-/-}$ compared to γ-3$^{-/-}$, γ-2$^{-/-}$ and wt, $P < 0.0001$ for γ-2$^{-/-}$ versus wt littermates, and $P > 0.05$ for γ-3$^{-/-}$ versus wt mice). For weight measurements, γ-2$^{-/-}$ littermates (γ-2$^{+/+}$ and γ-2$^{+/-}$) were grouped as wild-type (wt) while γ-2,3$^{-/-}$ littermates (γ-2$^{+/+}$,3$^{-/-}$ and γ-2$^{+/-}$,3$^{-/-}$) were grouped as γ-3$^{-/-}$ mice because no differences between littermates were observed.

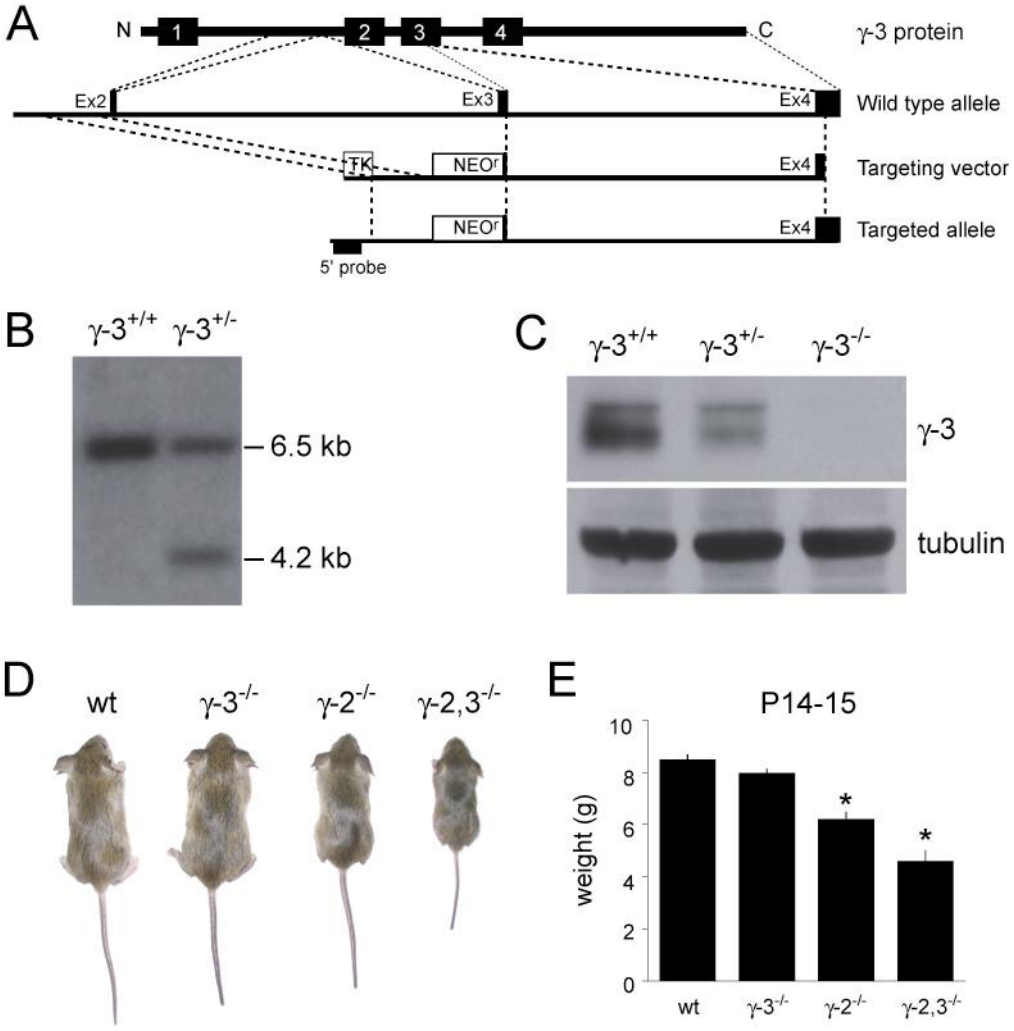

A
N
1
2
3
4
C
γ-3 protein
Ex2
Ex3
Ex4
Wild type allele
TK
NEOr
Ex4
Targeting vector
NEOr
Ex4
Targeted allele
5' probe
B
γ-3+/+
γ-3+/-
6.5 kb
4.2 kb
C
γ-3+/+
γ-3+/-
γ-3-/-
γ-3
tubulin
D
wt
γ-3-/-
γ-2-/-
γ-2,3-/-
E
P14-15
weight (g)
10
8
6
4
2
0
*
*
wt
γ-3-/-
γ-2-/-
γ-2,3-/-

Figure 2. AMPA receptor expression in γ-2,3$^{-/-}$ mice.

(A) The expression levels and localization of AMPA receptor subunits GluR1, GluR2/3 and GluR4 appear similar in immunohistochemical stains of sagittal brain sections from wild-type and γ-2,3$^{-/-}$ mice. Shown are representative sections from one of three experiments. **(B)** Immunoblot analysis of total AMPA receptor subunit protein from both cortical and hippocampal extracts reveals no difference in expression in γ-2,3$^{-/-}$ mice compared to age-matched wild-type mice (hippocampus: n = 3 and 4 blots for GluR1 and GluR2/3 respectively, $P > 0.5$ for each; cortex: n = 4 and 5 blots for GluR1 and GluR2/3 respectively, $P > 0.3$ for each). As expected, no changes are seen in the expression of the NMDA receptor subunit NR1. **(C)** The protein levels of AMPA receptor subunits were also unchanged in the cerebellum of γ-3$^{-/-}$ mice (n= 3, 5, and 5 for GluR1, GluR2/3 and GluR4 respectively; $P > 0.2$ for each compared to wild-type). However, cerebellar expression levels of both GluR1 and GluR4 are significantly reduced in γ-2$^{-/-}$ mice without further loss in double knockout mice (n = 5 and 4 blots for GluR1 and GluR4 respectively; $P < 0.002$ for either γ-2$^{-/-}$ or γ-2,3$^{-/-}$ versus wild-type and $P > 0.6$ for γ-2$^{-/-}$ versus γ-2,3$^{-/-}$ for both GluR1 and GluR4). The protein level of GluR2/3 is decreased by ~30% in γ-2$^{-/-}$ mice and ~70% in γ-2,3$^{-/-}$ mice compared to wild-type (n = 7 blots; $P < 0.002$ for either γ-2$^{-/-}$ or γ-2,3$^{-/-}$ versus wild-type, $P < 0.001$ for γ-2$^{-/-}$ versus γ-2,3$^{-/-}$). Hash marks on the bar graphs indicate that separate blots were used for γ-3$^{-/-}$ and wild-type comparisons.

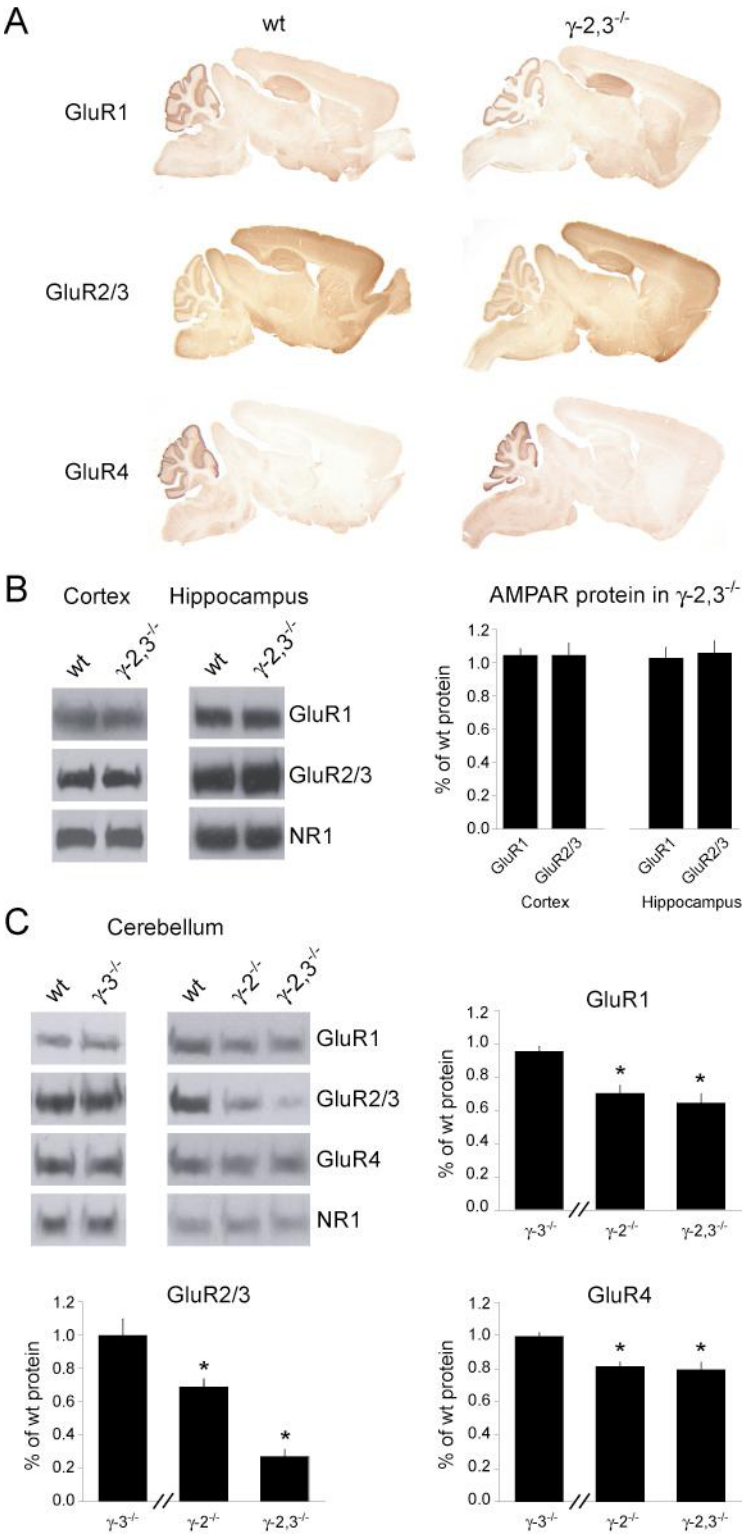

A
wt
γ-2,3-/-
GluR1
GluR2/3
GluR4
B
Cortex
Hippocampus
wt
γ-2,3-/-
GluR1
GluR2/3
NR1
AMPAR protein in γ-2,3-/-
% of wt protein
GluR1
GluR2/3
Cortex
Hippocampus
C
Cerebellum
wt
γ-3-/-
γ-2-/-
γ-2,3-/-
GluR1
GluR2/3
GluR4
NR1
GluR1
GluR2/3
GluR4
% of wt protein

Figure 3. Severe reduction in AMPA receptor mediated synaptic transmission in cerebellar Golgi cells from γ-2,3$^{-/-}$ mice.

(A) Dual component EPSCs (gray) evoked in wild-type, γ-2$^{-/-}$, γ-3$^{-/-}$ and γ-2,3$^{-/-}$ mice are shown on the left. I_{AMPA} (red) and I_{NMDA} (black) were pharmacologically separated and the ratio of I_{AMPA} / I_{NMDA} was measured for each cell. Whereas the I_{AMPA} / I_{NMDA} ratio was similar in wild-type, γ-2$^{-/-}$ and γ-3$^{-/-}$ mice, it was severely compromised in γ-2,3$^{-/-}$ mice (wt 2.56 ± 0.39, γ-2$^{-/-}$ 2.09 ± 0.60, γ-3$^{-/-}$ 2.97 ± 0.73, γ-2,3$^{-/-}$ 0.27 ± 0.06; n = 9, 6, 7, and 7 respectively; $P < 0.002$ for wild-type compared to γ-2,3$^{-/-}$, $P > 0.9$ for each other genotype compared to each wild-type). **(B)** Representative traces show that the paired-pulse facilitation measured at a 40 ms interval was unchanged in Golgi cells from γ-2,3$^{-/-}$ mice (wt 1.91 ± 0.15, γ-2$^{-/-}$ 1.62 ± 0.11, γ-3$^{-/-}$ 1.80 ± 0.10, γ-2,3$^{-/-}$ 1.75 ± 0.14; n = 11, 8, 10, and 8 respectively; $P > 0.4$). **(C)** The average amplitude of AMPA receptor mEPSCs in Golgi cells in γ-2,3$^{-/-}$ mice is reduced to nearly the threshold of detection (10 pA, dotted line on bar graph), while amplitudes in wild-type, γ-2$^{-/-}$ and γ-3$^{-/-}$ mice are similar (wt 20.1 ± 1.0 pA, γ-2$^{-/-}$ 22.3 ± 1.6 pA, γ-3$^{-/-}$ 21.3 ± 1.0 pA, γ-2,3$^{-/-}$ 13.8 ± 0.6 pA; n = 14, 9, 10, and 8 respectively; $P < 0.03$ for each genotype compared to γ-2,3$^{-/-}$, $P > 0.4$ for each other genotype compared to each other). Sample traces are the average mEPSC from representative cells for each genotype. **(D)** The mEPSC frequency is severely reduced in γ-2,3$^{-/-}$ mice compared to controls (wt 1.30 ± 0.15 Hz, γ-2$^{-/-}$ 1.85 ± 0.40 Hz, γ-3$^{-/-}$ 1.31 ± 0.23 Hz, γ-2,3$^{-/-}$ 0.16 ± 0.04 Hz; n = 13, 9, 10, and 11 respectively; $P < 0.02$ for each genotype compared to γ-2,3$^{-/-}$, $P > 0.5$ for each other genotype compared to each other). Sample traces consisting of three overlaid consecutive sweeps are shown for each genotype.

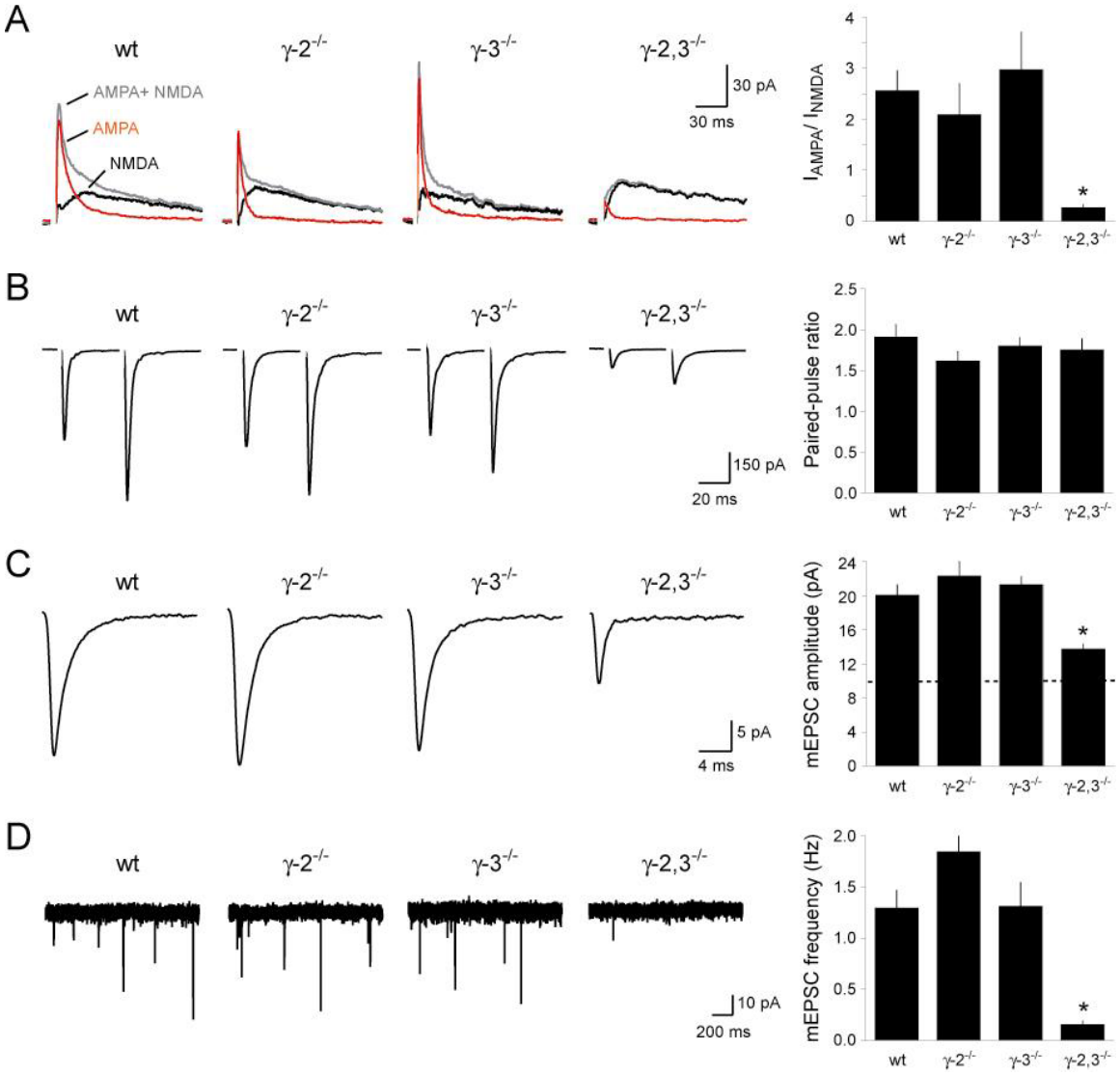

A
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
AMPA+ NMDA
AMPA
NMDA
30 pA
30 ms
I AMPA/ I NMDA
B
150 pA
20 ms
Paired-pulse ratio
C
5 pA
4 ms
mEPSC amplitude (pA)
D
10 pA
200 ms
mEPSC frequency (Hz)

Figure 4. TARPs affect synaptic AMPA receptor kinetics and subunit composition. **(A)** The decay kinetics of the average mEPSC in Golgi cells from γ-2,3$^{-/-}$ mice are nearly twice as fast as in single knockout or wild-type mice (wt 1.73 ± 0.14 ms, γ-2$^{-/-}$ 1.72 ± 0.23 ms, γ-3$^{-/-}$ 1.59 ± 0.16 ms, γ-2,3$^{-/-}$ 0.85 ± 0.04 ms; n = 14, 9, 10, and 8 respectively; $P < 0.03$ for each genotype compared to γ-2,3$^{-/-}$, $P > 0.9$ for each other genotype compared to each other). Sample traces show the peak-normalized average mEPSC from a representative cell from each genotype. A wild-type trace (dotted line) is superimposed over the trace from a γ-2,3$^{-/-}$ Golgi cell to better illustrate the change in kinetics. **(B)** The rectification index (RI) of evoked AMPA receptor mediated currents is linear in wild-type and single knockout mice, but partially rectifying in double knockout mice (wt 1.05 ± 0.05, γ-2$^{-/-}$ 1.05 ± 0.08, γ-3$^{-/-}$ 1.03 ± 0.05, γ-2,3$^{-/-}$ 0.54 ± 0.05; n = 9, 7, 8, and 7 respectively; $P < 0.0001$ for each genotype compared to γ-2,3$^{-/-}$, $P > 0.9$ for each other genotype compared to each other). Traces from a sample cell held at –70 mV, E_{rev}, and +40 mV are shown for each genotype. The corresponding I-V relationship is plotted below each cell, and the rectification index for that cell is also given.

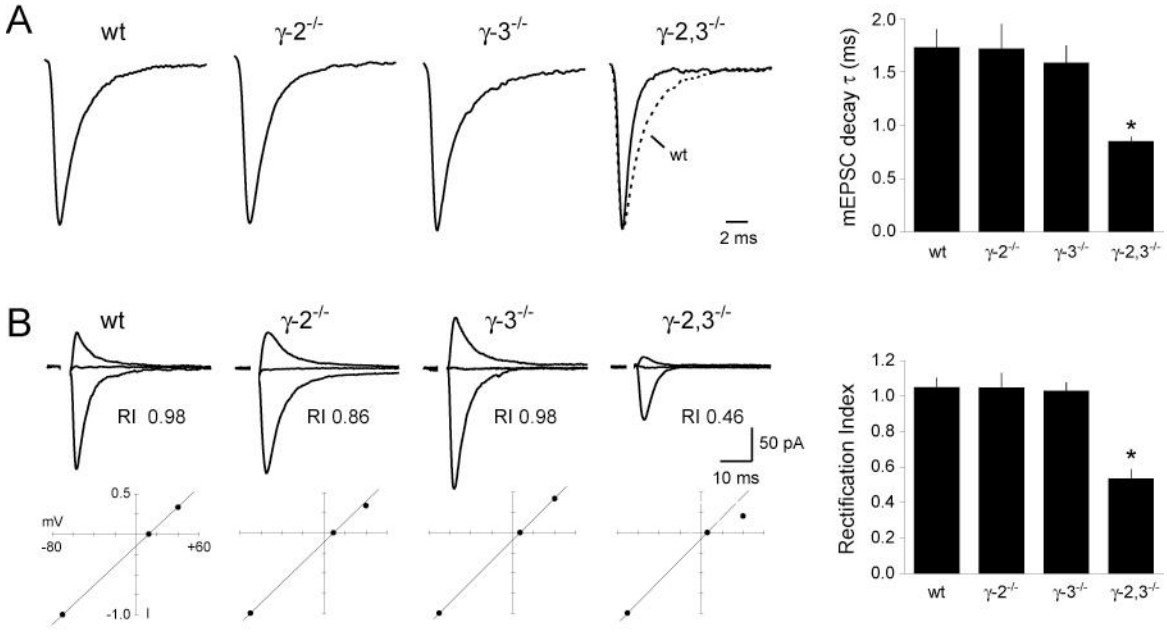

A
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
wt
2 ms
mEPSC decay τ (ms)
2.0
1.5
1.0
0.5
0.0
*
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
B
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
RI 0.98
RI 0.86
RI 0.98
RI 0.46
50 pA
10 ms
0.5
mV
-80
+60
-1.0
I
Rectification Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
wt
γ-2-/-
γ-3-/-
γ-2,3-/-

Figure 5. TARPs regulate synaptic localization of AMPA receptors independent of surface trafficking in Purkinje cells.

(A) Sample traces of the response to a 2 second 500 μM application of AMPA in the presence of cylcothiazide to outside-out patches pulled from Purkinje cells and voltage-clamped at -60 mV. The average amplitude is not significantly different in γ-$2^{-/-}$ mice compared to wild-types, suggesting that AMPA receptors traffic normally to the cell surface. As expected given the lack of γ-3 expression in these cells, no change is seen with the additional loss of γ-3 (wt 38.4 ± 9.2, γ-$2^{-/-}$ 32.6 ± 5.7, γ-$3^{-/-}$ 25.4 ± 4.7, γ-$2,3^{-/-}$ 23.9 ± 10.4; n = 18, 17, 31, and 8 respectively, P = 0.44). **(B)** Synaptic AMPA receptor content at climbing fiber synapses in γ-$2^{-/-}$ mice. Dual component AMPA and kainate receptor mediated EPSCs (gray) were recorded in 10 μM GYKI; kainate EPSCs (black) were then pharmacologically isolated with 100 μM GYKI, and the difference reflected the AMPA EPSC (red). The AMPA/kainate ratio was decreased in γ-$2^{-/-}$ mice, but unaffected by the loss of γ-3 (wt 4.03 ± 0.29, γ-$2^{-/-}$ 1.28 ± 0.24, γ-$3^{-/-}$ 3.72 ± 0.65, γ-$2,3^{-/-}$ 1.43 ± 0.13; n = 7, 5, 5, and 6 respectively, P < 0.001 for either γ-$2^{-/-}$ or γ-$2,3^{-/-}$ compared to either wt or γ-$3^{-/-}$, P > 0.7 for wt versus γ-$3^{-/-}$, P > 0.9 for γ-$2^{-/-}$ versus γ-$2,3^{-/-}$). **(C)** Similarly, the ratio of AMPA EPSCs to mGluR induced EPSCs at parallel fiber synapses onto Purkinje cells was reduced by >50% in γ-$2^{-/-}$, and unaffected by the loss of γ-3 (wt 5.83 ± 0.82, γ-$2^{-/-}$ 2.88 ± 0.59, γ-$3^{-/-}$ 6.38 ± 1.20, γ-$2,3^{-/-}$ 2.25 ± 0.42; n = 8, 8, 6, and 9 respectively, P < 0.05 for either γ-$2^{-/-}$ or γ-$2,3^{-/-}$ compared to either wt or γ-$3^{-/-}$, P > 0.9 for wt versus γ-$3^{-/-}$, P > 0.9 for γ-$2^{-/-}$ versus γ-$2,3^{-/-}$). For synaptic experiments, γ-$2^{+/-}$ and γ-$2^{+/+}$ were combined, as they were not distinguishable.

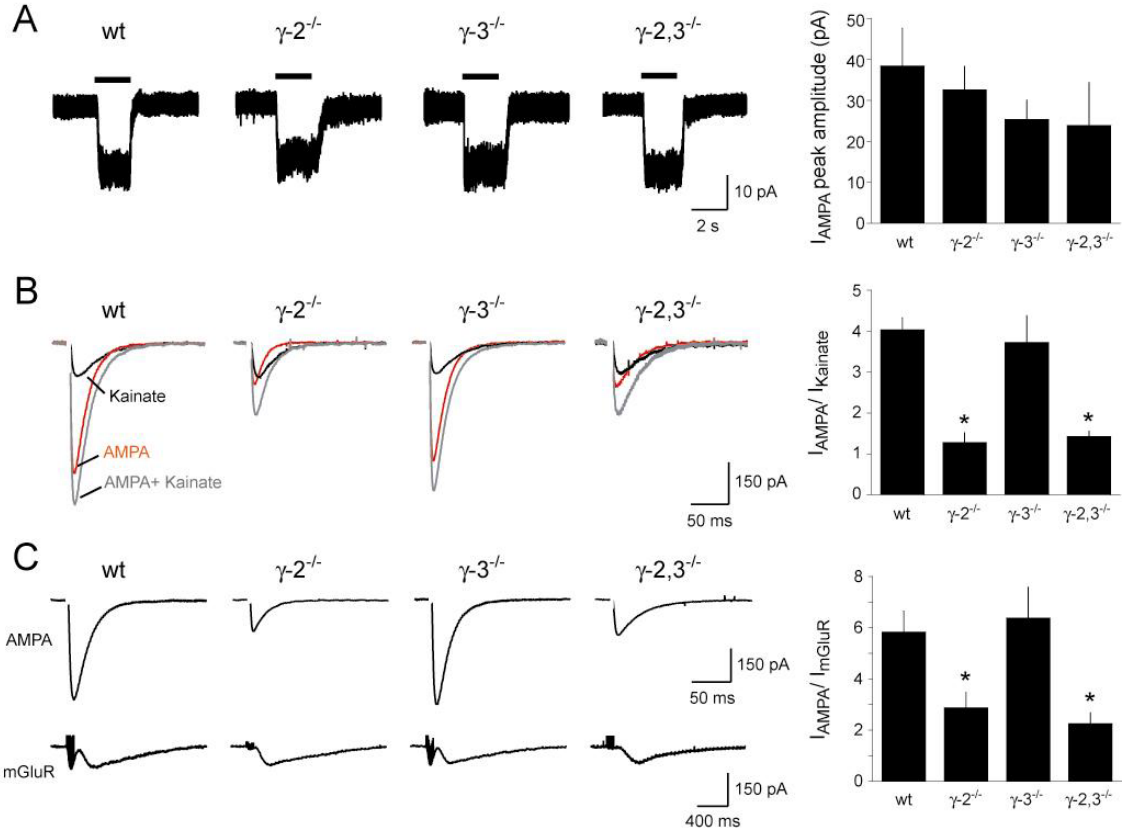
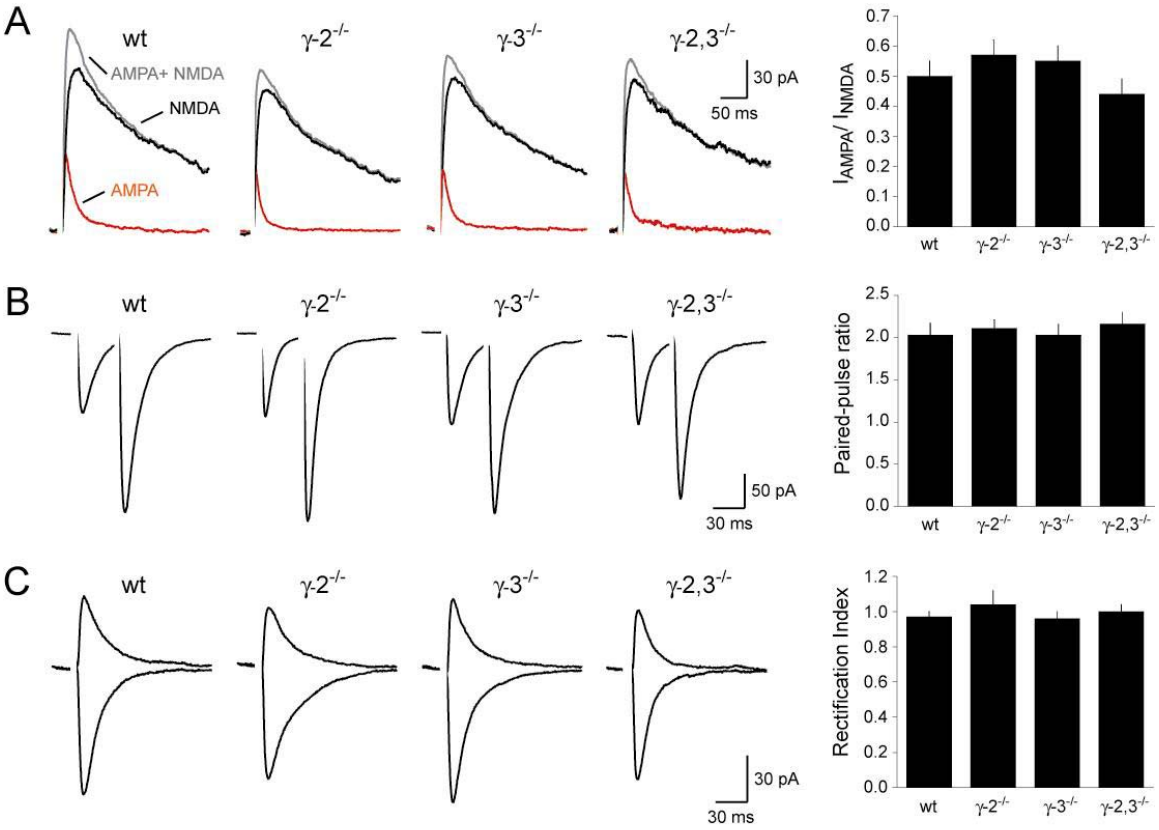

A
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
10 pA
2 s
I AMPA peak amplitude (pA)
50
40
30
20
10
0
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
B
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
Kainate
AMPA
AMPA+ Kainate
150 pA
50 ms
I AMPA/ I Kainate
5
4
3
2
1
0
*
*
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
C
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
AMPA
150 pA
50 ms
mGluR
150 pA
400 ms
I AMPA/ I mGluR
8
6
4
2
0
*
*
wt
γ-2-/-
γ-3-/-
γ-2,3-/-

Figure 6. TARPs γ-2 and γ-3 are not specifically required for synaptic trafficking of AMPA receptors in hippocampal neurons.

(A) Sample traces of dual-component EPSCs (gray) in hippocampal CA1 pyramidal cells held at +40 mV were pharmacologically separated into their AMPA (red) and NMDA (black) mediated components. No changes are seen in the AMPA/NMDA ratio in any knockout strain (wt 0.50 ± 0.05, γ-2$^{-/-}$ 0.57 ± 0.05, γ-3$^{-/-}$ 0.55 ± 0.05, γ-2,3$^{-/-}$ 0.44 ± 0.05; n = 12, 10, 9, and 9 respectively; P = 0.32). **(B)** Paired-pulse facilitation measured at a 40 ms interval in pyramidal cells held at –60 mV is unchanged in all knockout strains (wt 2.03 ± 0.14, γ-2$^{-/-}$ 2.11 ± 0.10, γ-3$^{-/-}$ 2.03 ± 0.13, γ-2,3$^{-/-}$ 2.16 ± 0.14; n = 12, 10, 9, and 9 respectively; P = 0.86). **(C)** Synaptic AMPA receptors in γ-2,3$^{-/-}$ mice have a linear IV curve like wild-type mice, such that the rectification index is ~1 (wt 0.97 ± 0.03, γ-2$^{-/-}$ 1.04 ± 0.08, γ-3$^{-/-}$ 0.96 ± 0.04, γ-2,3$^{-/-}$ 1.00 ± 0.04; n = 12, 9, 9, and 8 respectively; P = 0.68). Shown are EPSCs from cells held at –60 mV and +40 mV.


A
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
AMPA+ NMDA
NMDA
AMPA
30 pA
50 ms
I AMPA/ I NMDA
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
B
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
50 pA
30 ms
Paired-pulse ratio
2.5
2.0
1.5
1.0
0.5
0.0
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
C
wt
γ-2-/-
γ-3-/-
γ-2,3-/-
30 pA
30 ms
Rectification Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
wt
γ-2-/-
γ-3-/-
γ-2,3-/-

Chapter 5:

TARP auxiliary subunits switch AMPA receptor antagonists into partial agonists

Introduction

Excitatory synaptic transmission in the brain is mediated by glutamate acting on two classes of ionotropic receptors: AMPA and *N*-methyl-D-aspartate (NMDA) receptors. A major breakthrough in the field of excitatory synaptic transmission came with the discovery of the quinoxalinedione series of competitive AMPA receptor antagonists in 1988 (Honore et al., 1988; Sheardown et al., 1990; Watkins et al., 1990). These drugs, CNQX, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), potently and selectively block AMPA receptors and have been indispensable in characterizing excitatory synaptic transmission in the central nervous system (CNS).

As competitive antagonists, quinoxalinediones interact with AMPA receptors in the same binding pocket as agonists, thereby occluding agonist binding. Unlike agonists, competitive antagonists have no efficacy, meaning that binding does not lead to opening of the ion channel (gating). Despite the current widespread use of quinoxalinediones as AMPA receptor antagonists to study synaptic transmission, a few reports indicate that these compounds can have excitatory actions on a subset of interneuron populations, though not on excitatory neurons (Brickley et al., 2001; Hashimoto et al., 2004; Maccaferri and Dingledine, 2002; McBain et al., 1992). The mechanism for this effect remains unexplained.

Results

While recording cerebellar granule cells, we also observed that application of CNQX (10 μM) increased sIPSC frequency (0.22 ± 0.07 Hz to 1.58 ± 0.56 Hz, n = 5

cells, $P < 0.05$) without a change in amplitude (21.1 ± 6.1 pA to 19.3 ± 3.1 pA, n = 5 cells) (Fig. 1, A and B) (Brickley et al., 2001), implying increased excitability of the pre-synaptic interneurons, cerebellar Golgi cells (Palay and Chan-Palay). Application of CNQX to Golgi cells reliably evoked an inward current in voltage-clamp recordings (-37.9 ± 4.4 pA, n = 11), indicating a non-canonical depolarizing effect of CNQX on these cells (Fig. 1C). However, we found that antagonizing AMPA receptors does not simply lead to depolarization as application of GYKI 53655 (10 μM), a non-competitive AMPA receptor antagonist (Tarnawa et al., 1993), did not induce an inward current in Golgi cells (Fig. 2). Given that the CNQX-induced depolarization was recorded in $GABA_A$, NMDA, and glycine receptor antagonists as well as TTX to block network activity, our data suggested that CNQX was not simply acting through another neuronal receptor, but instead that CNQX may act as an AMPA receptor agonist on these cells.

We therefore tested whether a non-competitive AMPA receptor antagonist could block the CNQX-induced depolarization and whether a positive allosteric modulator could potentiate the response. Pre-incubation of cerebellar slices with GYKI 53655 blocked the CNQX-induced current (CNQX induced current in GYKI = 0.4 ± 2.5 pA, n = 5, $P < 0.001$ compared to CNQX alone) (Fig. 1C). Furthermore, trichloromethiazide (TCM) (500 μM), a positive modulator structurally similar to cyclothiazide (Mitchell and Fleck, 2007; Yamada and Tang, 1993), increased the response to CNQX (-88.7 ± 21.6 pA, n = 5, $P < 0.01$ compared to CNQX alone) (Fig. 1D). Thus CNQX appears to act as an agonist on these AMPA receptors, despite its previous characterization as a competitive antagonist.

Although AMPA receptors are expressed on most, if not all neurons in the brain, a depolarizing action of CNQX was not previously reported for excitatory neurons (Brickley et al., 2001; Maccaferri and Dingledine, 2002). This raised the possibility that AMPA receptors on interneurons somehow differ from those on other types of neurons. We therefore tested whether CNQX can act as an agonist on AMPA receptors expressed by hippocampal CA1 pyramidal cells. In the absence of TCM, CNQX did not evoke a significant inward current (-1.9 ± 2.6 pA, n = 5) (Fig. 1E) as was reported previously (Lester et al., 1989; Maccaferri and Dingledine, 2002). However a CNQX-induced current was observed in the presence of TCM (-103.5 ± 43.3 pA, n = 5). A CNQX-induced current was also detected in the presence of TCM in dentate granule cells (-12.2 ± 5.1 pA, n = 5) (Fig. 1F) and cerebellar Purkinje cells (–131.7 ± 27.2 pA, n = 5) (Fig. 1G). Given that CNQX could induce depolarizing currents in all neuron types tested, the agonist activity of CNQX is most likely a general property of CNS neurons and AMPA receptors. The lack of detection of CNQX-induced currents previously was most likely because CNQX was not tested in the presence of TCM.

To conclusively attribute the depolarizing current to AMPA receptor activation, we next attempted to reconstitute the agonist-activity of CNQX on AMPA receptors expressed in cultured human embryonic kidney (HEK293) cells. A brief application of CNQX evoked an inward current on HEK293 cells transfected with the AMPA receptor pore-forming subunit GluR1(Q) flop and γ-2, a member of the TARP family of AMPA receptor auxiliary subunits (Nicoll et al., 2006; Osten and Stern-Bach, 2006; Ziff, 2007) (-12.7 ± 5.7 pA, n = 6) (Fig. 3A). Furthermore, the CNQX-induced current in HEK293 cells had many properties consistent with AMPA receptor activation. The response to

CNQX was significantly enhanced in the presence of TCM (-287.0 ± 104.6 pA, n = 6, P < 0.04) (Fig. 3A). For ease of measurement, all further experiments in HEK293 cells were carried out in the presence of TCM. Comparison of the currents evoked by CNQX and glutamate (1 mM) indicated that CNQX is acting as a partial agonist (Fig. 3A). Partial agonists such as kainate bind AMPA receptors, but only induce a fraction of the activation induced by full agonists such as glutamate. As in Golgi cells, GYKI 53655 blocked the CNQX-induced inward current in HEK293 cells (inhibition 99.3 ± 0.7%, n = 4) (Fig. 3B), and the effect of GYKI was reversible. Furthermore, the current elicited by CNQX had the current-voltage (I-V) relationship expected for GluR1(Q) AMPA receptors (n = 4) (Fig. 3C) (Verdoorn et al., 1991). Together our data indicated that CNQX acts as a partial agonist in a heterologous expression system.

We sought to determine whether other members of the quinoxalinedione family also have agonist activity. A brief application of DNQX elicited an inward current in transfected HEK293 cells (-284.0 ± 99.4 pA, n = 13) not different from that seen with CNQX (-211.1 ± 69.9 pA, n = 11, P = 0.57) (Fig. 3D and 6A). In contrast, NBQX did not elicit an inward current in HEK293 cells (23.2 ± 7.0 pA, n = 9) (Fig. 3D). We observed similar effects in cerebellar Golgi cells in the absence of TCM (DNQX -10.0 ± 3.0 pA, n = 6; NBQX 2.1 ± 2.1 pA, n = 5) (Fig. 3E). Therefore, NBQX acts purely as a competitive AMPA receptor antagonist, whereas both CNQX and DNQX act as partial agonists.

Models of channel activation and desensitization have been developed through structural studies of isolated iGluR ligand binding domains (LBD). These LBDs consist of two domains arranged in a clamshell-like manner that undergo a conformational rearrangement, typically movement of domain 1 towards domain 2 (domain closure),

upon ligand binding (Fig. 4). Binding of full agonists such as glutamate induce maximal domain closure (~21°) whereas partial agonists such as kainate induce partial closure (12°) relative to the unbound *apo* state (Jin et al., 2003). Therefore, channel activation is correlated with the degree of domain closure upon ligand binding (Hansen et al., 2007; Jin et al., 2003). Agonist efficacy also correlates with the length of separation between the linker regions of each subunit, which connect the ligand-binding domains to the pore-forming transmembrane segments in the full-length receptor (Fig. 4) (Armstrong et al., 2003; Hansen et al., 2007; Jin et al., 2003; Mayer and Armstrong, 2004).

To test whether CNQX induces domain closure and linker separation consistent with agonist activity, we obtained the crystal structure of CNQX bound to the GluR2 LBD, also referred to as 'S1S2' (Fig. 4C and 5, Table 1). The CNQX-bound structure was ~6.4° closed relative to the *apo* state (~7.7° and ~5.1° for the two protomers) which is consistent with partial agonist activity. However, the linker separation in the CNQX-bound structure (29.7 Å) was similar to the *apo* state (29.4 Å), suggesting CNQX may not transduce its domain closure into channel opening, and is therefore inconsistent with the partial agonist activity we observed. Our CNQX-bound structure was similar to the structure of DNQX bound to the GluR2 LBD (Cα RMSD = 0.71 Å) (Hansen et al., 2007).

A key difference between the CNQX-bound LBD structure and native receptors is that the latter are co-expressed with TARPs, which include γ-2, γ-3, γ-4, and γ-8. To determine whether co-expression of TARPs is required for CNQX-induced receptor activation, we compared the CNQX-induced currents in HEK293 cells transfected with GluR1 and γ-2 to those transfected with GluR1 alone. Neither CNQX nor DNQX activated AMPA receptors in the absence of γ-2, even in the presence of TCM (CNQX –

1.63 ± 1.10 pA, n = 8; DNQX –1.08 ± 0.56 pA, n = 12 (Fig. 6A). This was not due to a lack of AMPA receptor expression as the average glutamate-evoked currents in cells transfected with GluR1 alone were similar in the two conditions (GluR1 alone 2941 ± 485 pA, n = 20; γ-2 + GluR1 3386 ± 409 pA, n = 24; P = 0.48). Furthermore, the use of TCM to both slow and block AMPA receptor desensitization (Mitchell and Fleck, 2007; Yamada and Tang, 1993), suggests that the lack of CNQX-induced currents in the absence of γ-2 is not simply due to a detection difficulty due to rapid desensitization. This indicates that CNQX and DNQX act as pure competitive antagonists on AMPA receptors in the absence of γ-2, but as agonists in the presence of γ-2. Other members of the TARP family produced similar changes in CNQX activity (Fig. 6B). Therefore, inclusion of any TARP family member in an AMPA receptor complex switches the nature of CNQX and DNQX from competitive antagonists to partial agonists.

Discussion

In light of our results, the structurally similar quinoxalinediones, CNQX and DNQX, are perhaps best considered to be partial agonists rather than competitive antagonists, given that most neurons in the CNS express TARPs (Lein et al., 2007; Tomita et al., 2003). The lack of CNQX agonist activity in the absence of TCM in hippocampal pyramidal cells (Fig. 1E) (Maccaferri and Dingledine, 2002) and other excitatory neurons may be due to rapid desensitization of CNQX-induced currents in these cells due to the expression of the flip splice variant of AMPA receptor subunits (Bertolino et al., 1993; Geiger et al., 1995). The current induced by CNQX on GluR1 flip receptors was only ~3% of the current induced by CNQX in the presence of TCM (Fig.

3A and 7B). If hippocampal pyramidal cells primarily express flip receptors, we would expect only 3 pA of CNQX-induced current in the absence of TCM, which is similar to the trend we observed (-1. 9 ± 2.6 pA). In contrast, the weaker TCM potentiation in Golgi cells may be due to expression of the flop variants of AMPA receptors as the CNQX-induced currents from GluR1 flop receptors were only moderately potentiated by TCM (Fig. 7). This moderate potentiation agrees with the known weaker potency of cyclothiazide, a congener of TCM, on flop receptors (Partin et al., 1994).

Although CNQX acts as a weak partial agonist, its occupancy of the AMPA receptor ligand-binding site would prevent binding of other agonists and further activation. Thus, interpretations of previous experiments using CNQX to antagonize AMPA receptors are valid, except perhaps in the case when neurons were incubated for extended periods with CNQX, which may lead to cell death and/or receptor internalization due to chronic AMPA receptor activity. Given that quinoxalinedione analogs, as well as other competitive antagonists of AMPA receptors, have undergone clinical testing for the treatment of numerous diseases, including epilepsy and stroke (Bialer et al., 2007; Catarzi et al., 2007; Rogawski, 2006), better predictive activity of *in vivo* function may benefit from the inclusion of TARPs in *in vitro* drug screening assays.

Although previous work had shown that TARPs increase the efficacy of AMPA receptor agonists (Priel et al., 2005; Tomita et al., 2005a; Turetsky et al., 2005), their relationship to our structure-function based understanding of AMPA receptor gating had remained entirely unknown. Based on our current findings, we put forward a model in which TARPs either strengthen the coupling between agonist-induced domain closure and channel opening, perhaps by promoting linker separation or directly enhance the

degree of domain closure induced by CNQX. Further structural studies are required to distinguish between these two alternatives.

Table 1. Crystallographic data collection and refinement statistics.

Data Collection	
Space group	*P1*
Unit Cell	
a, *b*, *c* (Å)	66.6, 97.6, 101.0
α, β, γ angles (°)	81.9, 89.1, 77.9
Total reflections	78300
Resolution (Å)	40-2.5
Completeness(%)[a]	96.7 (95.8)
I/sigma(I)[a]	6.9 (2.4)
R_{merge}(%)[a,b]	9.3 (33.3)
Refinement Statistics	
Resolution (Å)	40-2.5
R_{cryst} (R_{free}) (%)[c]	25.1 (28.2)
Protein (Solvent) atoms	16153 (651)
Average B-Factors (Å^2) Protein, Solvent, Ligand atoms	20.5, 29.1, 40.8
RMSD bond lengths (Å)[d], angles (°)[d]	0.006, 0.94

[a]Values in parentheses refer to the highest resolution shell

[b]$R_{merge} = \Sigma_{hkl} \Sigma_i | I_{hkl,\,i} - \langle I \rangle_{hkl} | / \Sigma_{hkl} \Sigma_i | I_{hkl,\,i} |$ where I_{hkl} is the intensity of a reflection and $\langle I \rangle_{hkl}$ is the average of all observations of this reflection and its symmetry equivalents.

[c]$R_{cryst} = \Sigma_{hkl} | F_{obs} - kF_{calc} | / \Sigma_{hkl} |F_{obs}|$. $R_{free} = R_{cryst}$ for 5% of reflections that were not used in refinement.

[d] Root mean square deviation of bond lengths and angles from ideal values.

Figure 1. Depolarizing current elicited by CNQX in neurons.

(A, B) The frequency of sIPSCs in cerebellar granule cells voltage-clamped at 0 mV (EPSC reversal potential) was measured before and after bath application of CNQX ($n = 5$ cells, $P < 0.05$). **(C, D)** The holding current needed to voltage-clamp cerebellar Golgi cells to –70 mV was measured as CNQX was applied in the absence or presence of either 10 μM GYKI 53655 ($n = 11, 5$ respectively, $P < 0.001$) or 500 μM TCM ($n = 11, 5$, respectively, $P < 0.01$). **(E)** Similarly, CNQX was applied to hippocampal CA1 pyramidal cells in the presence and absence of TCM ($n = 5$ each). **(F, G)** CNQX was also applied to hippocampal dentate granule cells ($n = 5$) and Purkinje neurons in the presence of TCM ($n = 5$). Hippocampal neurons were voltage-clamped at –70 mV; Purkinje neurons were held at –10 mV to prevent voltage escape.

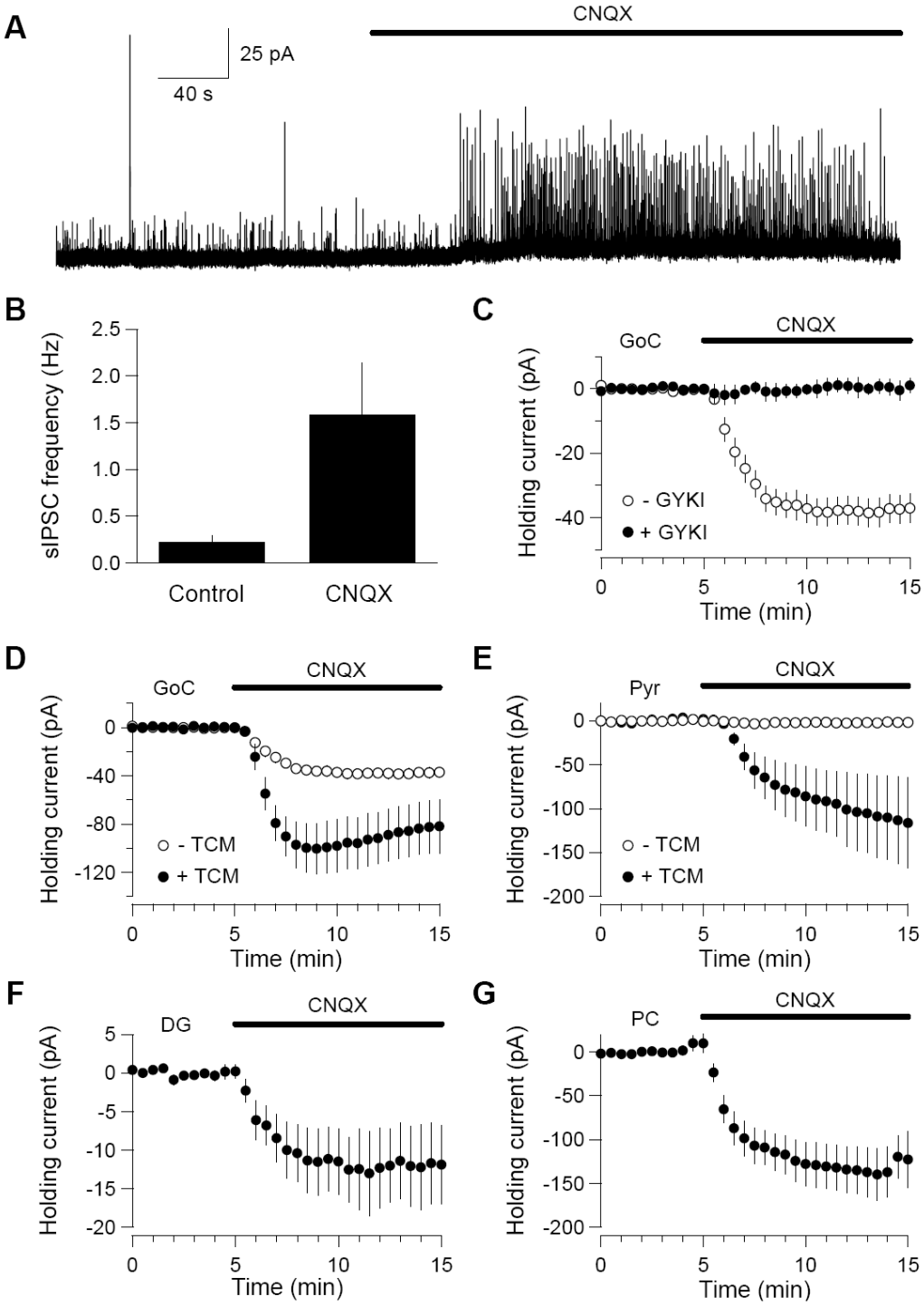

A
CNQX
25 pA
40 s
B
sIPSC frequency (Hz)
2.5
2.0
1.5
1.0
0.5
0.0
Control
CNQX
C
GoC
CNQX
Holding current (pA)
0
-20
-40
- GYKI
+ GYKI
0
5
10
15
Time (min)
D
GoC
CNQX
Holding current (pA)
0
-40
-80
-120
- TCM
+ TCM
0
5
10
15
Time (min)
E
Pyr
CNQX
Holding current (pA)
0
-50
-100
-150
-200
- TCM
+ TCM
0
5
10
15
Time (min)
F
DG
CNQX
Holding current (pA)
5
0
-5
-10
-15
-20
0
5
10
15
Time (min)
G
PC
CNQX
Holding current (pA)
0
-50
-100
-150
-200
0
5
10
15
Time (min)

Figure 2. **Change in holding current elicited by GYKI and CNQX.**

(A) The holding current needed to voltage-clamp cerebellar Golgi cells to –70 mV was measured as either 10 μM GYKI 53655 (left) or 10 μM CNQX (right) was bath applied (n = 11 for GYKI). The panel on the right is the same data as in figure 1C, and shown again for comparison.

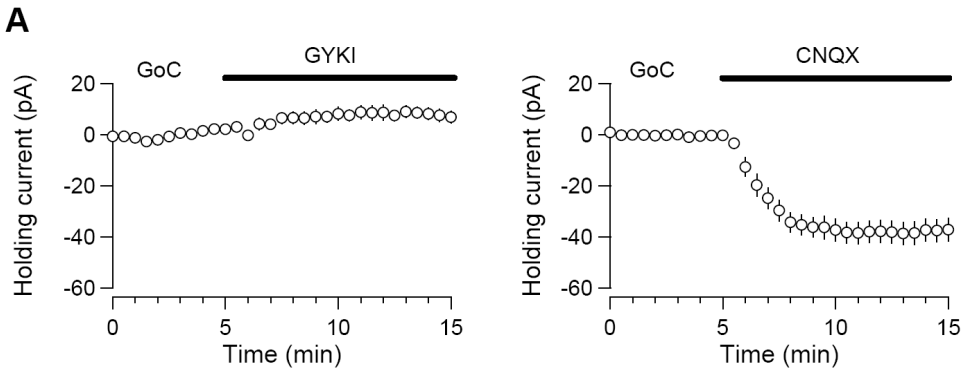

A
GoC
GYKI
Holding current (pA)
20
0
-20
-40
-60
0
5
10
15
Time (min)
GoC
CNQX
Holding current (pA)
20
0
-20
-40
-60
0
5
10
15
Time (min)

Figure 3. AMPA receptor activation by CNQX and DNQX, but not NBQX, in a heterologous system.

(A) The change in holding current elicited by a brief application of 10 μM CNQX to voltage-clamped (-70 mV) HEK293 cells expressing GluR1 flop and γ-2 was measured in the absence and presence of 500 μM TCM. A brief application of 1 mM glutamate in TCM was later applied (n = 6). **(B)** The CNQX-induced change in holding current was measured before, during, and after the application of 100 μM GYKI 53655 (n = 4). **(C)** The current-voltage (I-V) relationship of the CNQX-induced current in TCM is shown (n = 4). **(D)** The effect of 10 μM DNQX and 10 μM NBQX were also measured in HEK 293 cells co-transfected with GluR1 and γ-2 (n = 11 DNQX, 9 NBQX) and **(E)** in cerebellar Golgi cells in the absence of TCM (n = 6 DNQX, 5 NBQX).

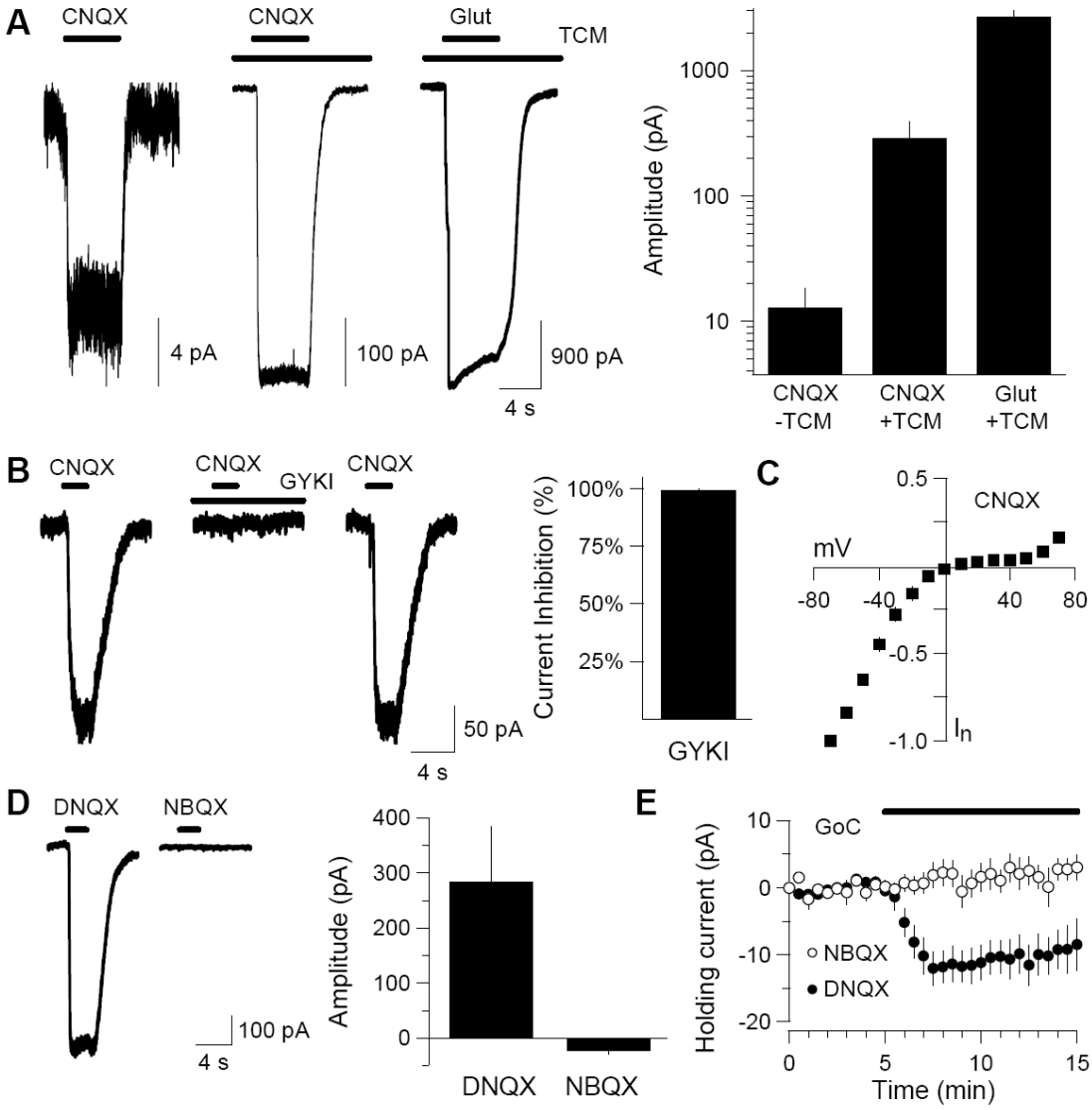

A
CNQX
CNQX
Glut
TCM
4 pA
100 pA
900 pA
4 s
Amplitude (pA)
1000
100
10
CNQX -TCM
CNQX +TCM
Glut +TCM
B
CNQX
CNQX
GYKI
CNQX
50 pA
4 s
Current Inhibition (%)
100%
75%
50%
25%
GYKI
C
0.5
CNQX
mV
-80
-40
40
80
-0.5
-1.0
In
D
DNQX
NBQX
100 pA
4 s
Amplitude (pA)
400
300
200
100
0
DNQX
NBQX
E
GoC
10
0
-10
-20
NBQX
DNQX
Holding current (pA)
0
5
10
15
Time (min)

Figure 4. Crystal structure of CNQX bound to the AMPA receptor LBD.

iGluR monomers are composed of three transmembrane helices and one re-entrant loop, an N-terminal domain and a ligand binding domain. Shown are two pore-forming helices (rendered as green cylinders) within a membrane bilayer and the LBD rendered as a ribbon diagram and colored in magenta (Domain 1) and orange (Domain 2). The LBD is a back-to-back dimer of two protomers each of which contains a Domain 1 and Domain 2. **(A,B)** GluR2 LBD crystal structure in the *apo* and glutamate-bound states (PDB codes 1FT0 and 1FTJ, respectively (Armstrong and Gouaux, 2000)). Glutamate is rendered as a Corey, Pauling, Koltun (CPK) model and colored cyan in panel B. Domain closure denotes the degree of Domain 2 movement towards Domain 1 relative to the *apo* state (20.2°). Linker distance is measured between the Cα atoms of Pro632 in each protomer. In the full-length receptor the artificial linker (orange ball) is connected to the pore-forming helices. Glutamate binding induces domain closure, increases the linker distance, and opens the channel pore. **(C)** GluR2 LBD crystal structure in the CNQX-bound state (rendered as a CPK model and colored cyan).

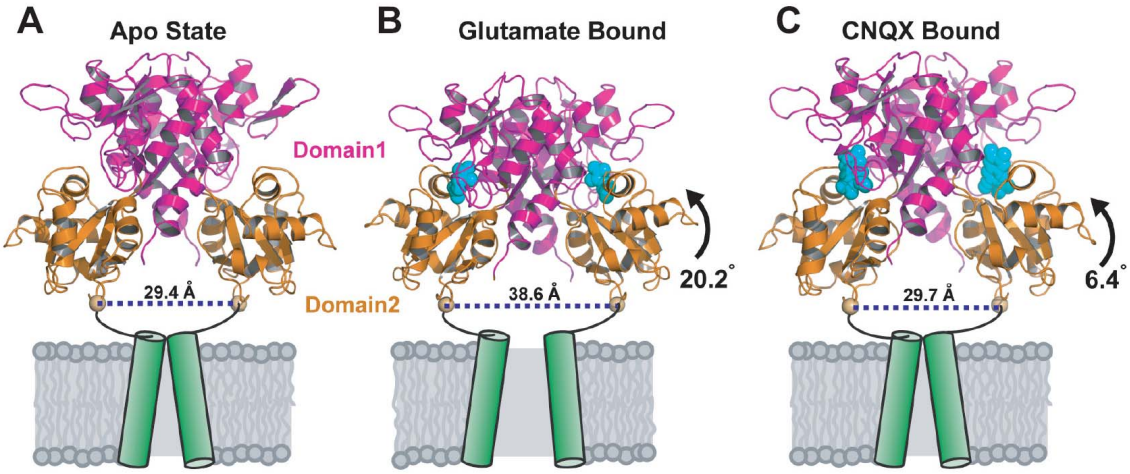
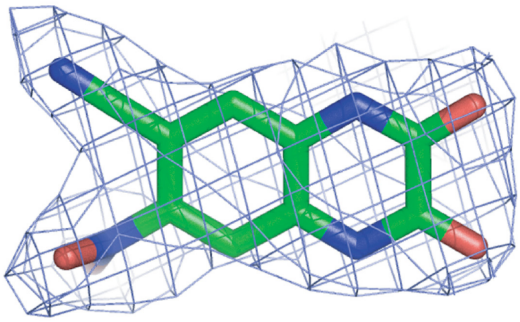

A
Apo State
B
Glutamate Bound
C
CNQX Bound
Domain1
Domain2
29.4 Å
38.6 Å
29.7 Å
20.2°
6.4°

Figure 5. **Final and omit electron density maps for CNQX bound to LBD.** CNQX is rendered as sticks with nitrogen colored in blue, oxygen in red and carbon in green. **(A)** 2Fo-Fc electron density map (blue) calculated with the final refined LBD-CNQX model contoured at 1.0 σ. **(B)** Simulated annealing omit map (red) for CNQX shown in panel A contoured at 1.0 σ. Both panels created with Bobscript (Esnouf, 1999).

A



B

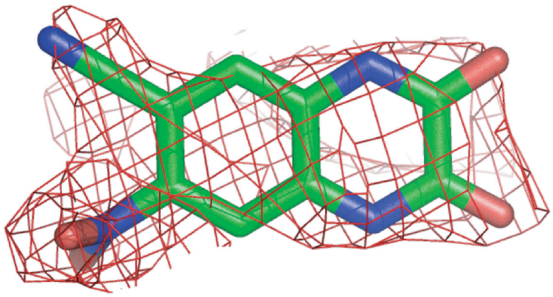


Figure 6. Effects of CNQX and DNQX in the absence and presence of TARPs.

(A) Either CNQX or DNQX was applied in the presence of TCM to HEK293 cells co-transfected with GluR1 and γ-2 or with GluR1 alone (CNQX GluR1+ γ-2 (n = 11), GluR1 alone (n = 8); DNQX GluR1+ γ-2 (n = 13), GluR1 alone (n = 12)). **(B)** CNQX-induced holding current changes in cells transfected GluR1 and either TARP γ-2, γ-3, γ-4, or γ-8 (n = 11, 4, 5, 4).

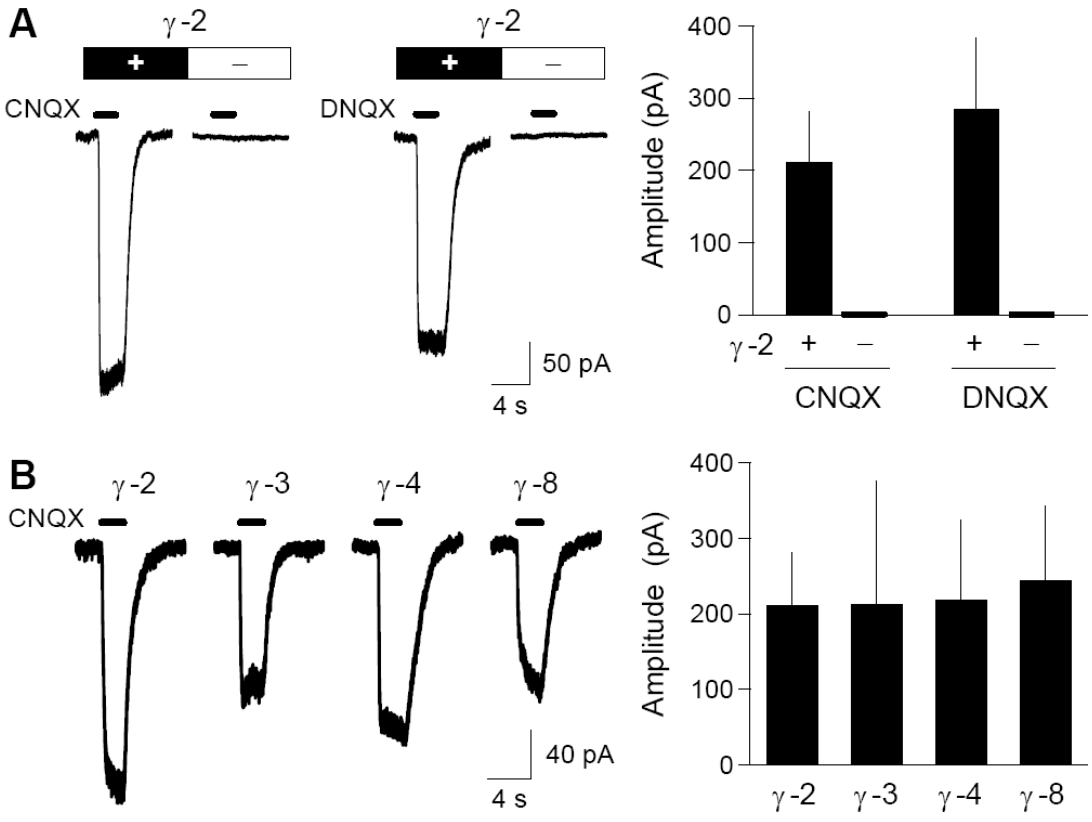
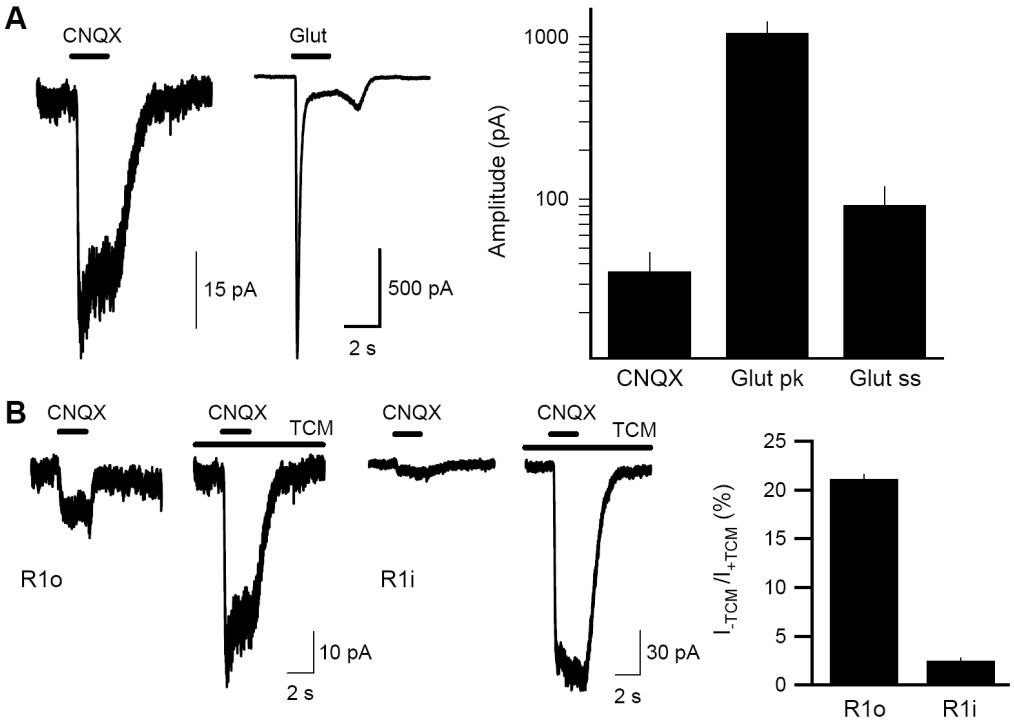

A
γ-2
+
–
CNQX
γ-2
+
–
DNQX
50 pA
4 s
Amplitude (pA)
400
300
200
100
0
γ-2
+
–
+
–
CNQX
DNQX
B
CNQX
γ-2
γ-3
γ-4
γ-8
40 pA
4 s
Amplitude (pA)
400
300
200
100
0
γ-2
γ-3
γ-4
γ-8

Figure 7. CNQX-induced current through GluR1 flip and GluR1 flop AMPA receptors.

(A) The change in holding current elicited by a brief application of either 10 μM CNQX or 1 mM glutamate to voltage-clamped (-70 mV) HEK293 cells expressing GluR1 flop receptors and γ-2 was measured in the presence of 500 μM TCM. The graph on the right depicts the average peak CNQX current and both the average peak and steady state glutamate current (n = 6). **(B)** CNQX was applied to HEK293 cells expressing γ-2 and either GluR1 flop (R1o) or GluR1 flip (R1i) in the absence and presence of TCM. The ratio of the current in the absence of TCM to the current in the presence of TCM is shown in the bar graph on the right (n = 5 for R1i and 4 for R1o, $P < 0.0001$).


A
CNQX
Glut
15 pA
500 pA
2 s
Amplitude (pA)
1000
100
CNQX
Glut pk
Glut ss
B
CNQX
CNQX
TCM
CNQX
CNQX
TCM
R1o
R1i
10 pA
2 s
30 pA
2 s
I-TCM /I+TCM (%)
25
20
15
10
5
0
R1o
R1i

Chapter 6:

General Discussion

Protein function is diversified by the combination of modular components. These modular components can be within the protein itself, such as PDZ binding domains, or due to the interactions of separate proteins, such as the modulation of primary ion channel subunits by auxiliary subunits. Ionotropic glutamate receptors, including AMPA receptors, carry out much of the excitatory moment-to-moment signaling within the brain. Specialization is achieved by the generation of tetrameric receptors with different combinations of various GluR splice variants, RNA editing, and differential interactions with cytoplasmic proteins. Here we demonstrate that TARPs, AMPA receptor auxiliary subunits, add another layer to specialization of AMPA receptor function.

Our data suggest that the native AMPA receptors in the brain are generally associated with TARPs. We have shown that TARPs are functionally redundant *in vivo* as cerebellar Golgi cells maintain AMPA receptors in single but not double TARP knockout mice. Accordingly, AMPA receptor protein levels detected by immunoblotting are greatly decreased in double knockout mice. Our data in Purkinje cells suggest that TARPs can regulate synaptic localization of AMPA receptors independent of their role in surface trafficking. The defect in γ-$2^{-/-}$ Purkinje cells appears to be the opposite of that in γ-$8^{-/-}$ hippocampal neurons where surface expression is more greatly affected than synaptic expression.

Our data further show that TARPs modulate the biophysical properties of AMPA receptors, not only *in vitro* but also *in vivo*. The decay kinetics of EPSCs at interneuron synapses were thought to primarily reflect the time course of glutamate in the synapse and the AMPA receptor GluR subunit composition (Jonas, 2000). Our results clearly demonstrate that TARPs can make a considerable contribution to EPSC decay times.

TARP influence on AMPA receptor gating

We also show that TARPs greatly affect the pharmacology of AMPA receptors. Two quinoxalinedione compounds, CNQX and DNQX, that were characterized as competitive antagonists on GluR tetrameric receptors act as partial agonists with the addition of TARPs. Although this was quite surprising, del Castillo and Katz already put forth in 1957 the idea that competitive antagonists and agonists only vary in their efficacy. They proposed a two-step reaction in which the receptor R first binds an agonist S to form SR, which is then converted into an active complex SR'.

> We may represent this, for instance, by
>
> $$S + R \Leftrightarrow SR \Leftrightarrow SR'$$
>
> where SR' is the depolarizing compound whose nature and transformation are, at present, unknown.
> According to this concept, whether a substrate acts as a depolarizer or a competitive inhibitor would depend solely on the rate constants of the two steps . . . Moreover a substance which has a relatively weak or slow depolarizing action . . . may, at the same time, antagonize the depolarization produced by fast and powerful agents (Del Castillo and Katz, 1957).

In this sense, CNQX can be considered to be an infinitely weak partial agonist whose efficacy is increased in the presence of TARPs. Similarly, kainate, a partial agonist on AMPA receptors in the absence of TARPs, has increased efficacy in the presence of TARPs (Tomita et al., 2005a).

The quote above also foretells the ability of weak partial agonists, such as CNQX acting on AMPA receptors with TARPs, to antagonize the response to agonists with greater efficacy, such as glutamate. Thus, although we find that CNQX is a weak partial agonist on neurons throughout the nervous system, this does not invalidate the majority of studies in which CNQX was used to selectively antagonize AMPA receptor function.

Until recently, the structure-function relationship of AMPA receptors had been considered only in the context of the GluR subunits. Through crystal structure studies of the isolate AMPA receptor ligand-binding domain, it has been proposed that agonist binding leads to domain closure, which increases intramolecular strain, and thus leads to channel opening (Mayer and Armstrong, 2004). Partial agonists induce less domain closure than full agonists, while competitive antagonists were thought to induce little to no domain closure. Our structure of CNQX bound to the TARP-less AMPA receptor ligand-binding domain suggests that CNQX only induces a small amount of domain closure, similar to DNQX (Armstrong and Gouaux, 2000).

The presence of TARPs may serve to either increase the amount of domain closure induced by CNQX or to increase the coupling efficiency between domain closure and channel opening. A similar mechanism may occur when kainate binds AMPA receptors in the presence of TARPs. Previous work has suggested that the domain closure induced by kainate is limited by its isopropenyl group, which would sterically clash with Leu-650 if further domain closure occurred (Armstrong and Gouaux, 2000). Substitution of threonine for Leu-650 increases kainate efficacy and domain closure (Armstrong et al., 2003). Therefore, I think it is unlikely that TARPs increase the amount of domain closure, and instead favor the model of increased coupling between a fixed amount of domain closure and channel opening. As transmembrane proteins, TARPs are positioned to interact with the GluR ligand-binding domain, the pore-region, as well as the short linkers that couple these two domains. The extracellular Ex1 domain of TARPs is involved in its modulation of kainate efficacy and other gating properties (Tomita et al., 2005a; Turetsky et al., 2005), and may also be involved in the conversion of CNQX into

a partial agonist. A more detailed picture of these mechanisms may come from crystal structure studies, but unfortunately, it has not yet been possible to crystallize AMPA receptors in the presence of TARPs, as this may require the difficult crystallization of full-length transmembrane complexes.

Functional redundancy or specialized functions?

Like many other synaptic proteins, TARPs consist of a family of related molecularly redundant proteins. This redundancy serves in part to maintain normal AMPA receptor function in most neurons in the absence of any one family member. As a result, most single TARP knockout mice appear indistinguishable from littermates while multiple knockout mice are often lethal. Cerebellar granule cells only express γ-2, and as a result lose AMPA receptor expression in γ-$2^{-/-}$ mice (Chen et al., 2000). In contrast, we and others have shown that hippocampal pyramidal cells, which express γ-2, γ-3, and γ-8, retain the majority of synaptic AMPA receptor function in single knockout mice (Hashimoto et al., 1999; Rouach et al., 2005). Similar compensation occurs with many synaptic proteins (Elias et al., 2006; Olsen et al., 2005; Varoqueaux et al., 2006).

Although it may be expedient to express a second functionally similar "backup" molecule, it is more difficult to envision the need for further members of the same family if they are functionally identical. Hippocampal pyramidal neurons express three TARPs, and it is possible that they are only molecularly redundant in some aspects. In this manner, the modulation of AMPA receptors would depend not only on the presence or absence of TARPs but also on the specific TARP associated with the channel. Recently it has been shown that γ-4 has a greater effect on AMPA receptor kinetics than other family

members, and that this effect is detectable in synaptic EPSCs (Cho et al., 2007; Korber et al., 2007b; Milstein et al., 2007). It is likely that all TARPs are found with synaptic receptors, but γ-8 may be preferentially localized with extrasynaptic receptors (Fukaya et al., 2006; Inamura et al., 2006; Rouach et al., 2005). Finally, a recently identified member of the TARP family, γ-7, enhances the trafficking and gating properties of AMPA receptors to a smaller extent than other TARP family members (Kato et al., 2007).

Another possibility is that specific TARP isoforms traffic and modulate the gating of some AMPA receptor GluR subunits preferentially. Although each TARP is able to bind each GluR isoform (Kott et al., 2007; Turetsky et al., 2005), the ability of individual TARPs to promote the surface trafficking of different GluR isoforms and modulate their biophysical properties may differ. For instance, there is some evidence that TARPs are less effective at regulating the GluR3 flop isoform (Kott et al., 2007) and γ-7 may preferentially regulate GluR1 containing AMPA receptors (Kato et al., 2007).

Based on the current-voltage relationship, we found that the remaining AMPA receptors in Golgi cell from γ-2,3$^{-/-}$ mice contain a mixture of GluR2-lacking and GluR2-containing AMPA receptors, in contrast to wild-type mice in which all receptors contain GluR2. As TARPs are thought to interact with AMPA receptors in the ER after they have formed tetramers, it is unlikely that TARPS directly influence the GluR subunit composition of receptors during biogenesis. Instead it is more likely that TARPs either selectively interact with or more strongly promote surface trafficking of GluR2-containing AMPA receptors. Studies in heterologous systems have not suggested these selective interactions as TARPs readily promote the trafficking of homomeric GluR1 tetramers and other non-GluR2-containing receptors (Chen et al., 2003; Tomita et al.,

2005a; Turetsky et al., 2005). However, TARP interactions with and trafficking of AMPA receptors may differ in neurons, in particular due to a possible competition between GluR2-containing and GluR2-lacking receptors for TARP binding. Future studies will need to combine *in vitro* and *in vivo* studies on TARPs to fully elucidate their common and unique roles in AMPA receptor regulation.

Chapter 7:

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