MODULATION OF AMPA RECEPTOR DESENSITIZATION BY NOOTROPIC DRUGS AND ENDOGENOUS PROTEINS

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MODULATION OF AMPA RECEPTOR

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ENDOGENOUS PROTEINS

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TABLE OF CONTENTS

Chapter **Page**

LIST OF TABLES

Table **Page**

LIST OF DIAGRAMS

Diagram **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

aa	amino acid
ABP	AMPA receptor binding protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	adenosine triphosphate
CaMKII	calcium-calmodulin dependent kinase II
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
CNS	central nervous system
CTZ	cyclothiazide
D1	domain 1 of the extracellular portion of the AMPA receptor, forms a dimer interface with D1 of adjacent AMPA receptor subunit
D2	domain 2 of the extracellular portion of the AMPA receptor
DIV	days *in vitro*
EPSC	excitatory postsynaptic current
GRASP-1	GRIP associated protein - 1
GRIP1/2	glutamate receptor interacting protein 1/2
HF	hydroflumethiazide
Hz	hertz (per second)
LTP	long term potentiation
LTD	long term depression
mEPSC	miniature excitatory postsynaptic current
ms	millisecond
nA	nanoAmperes
NBQX	2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)qunoxaline
NMDA	N-methyl-D-aspartate
pA	picoAmperes

pF	picoFarads
PICK1	protein interacting with C Kinase 1
PKA	protein kinase A
PKCα	protein kinase Cα
PSD-95	postsynaptic density protein, 95kD
PDZ domain	PSD-95, Discs-large/DLG, ZO-1 domain, common protein-protein binding motif
SAP90	synapse associated protein, 90kD; also termed PSD-95
stg	stargazin
TARP	transmembrane AMPA receptor regulatory protein
TCM	trichlormethiazide
TM	transmembrane domain
μs	microsecond
VGCC	voltage-gated calcium channel

Chapter I

INTRODUCTION AND LITERATURE REVIEW

1.1 The AMPA Subtype of Ionotropic Glutamate Receptors

The amino acid neurotransmitter glutamate mediates most fast excitatory synaptic transmission in the mammalian central nervous system (CNS). Glutamate gates three subtypes of ionotropic receptors named for their selective agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate. Kainate receptors are primarily presynaptic and modulate neurotransmitter release in the CNS (Huettner, 2003) while NMDA and AMPA receptors co-localize postsynaptically. The AMPA receptor is the primary mediator of fast synaptic transmission in the mammalian CNS while NMDA receptors play a predominantly modulatory role (McBain and Mayer, 1994; Dingledine et al., 1999).

AMPA receptors are tetrameric assemblies (Rosenmund et al., 1998) composed of various homomeric and heteromeric combinations of the subunits GluR1, 2, 3, and 4 (Boulter et al., 1990), also termed GluR-A, B, C, and D (Keinanen et al., 1990). Mature subunit proteins are approximately 900 amino acids in length, with a molecular weight of 98-100 kD (Hollmann and Heinemann, 1994). All four subunits share the same membrane topology (Diagram 1), namely a large amino-terminal extracellular domain, an intracellular carboxyl-terminus, three full membrane spanning domains (TM1, 3 and 4) with the second hydrophobic sequence forming a re-entrant loop lining the channel pore

(TM2) and an extracellular loop between TM3 and TM4 (Hollmann et al., 1994). Stern-Bach et al. (1994) demonstrated that the membrane proximal region of the first extracellular domain (termed S1) and the initial portion of the extracellular loop (S2) form the agonist binding domain, also referred to as the ligand binding core.

All four subunits naturally occur as one of two alternative splice variants, *flip* or *flop* (Sommer et al., 1990), that exhibit characteristic responses to agonist binding. The *flip*/*flop* splice cassette is located in the latter portion of the extracellular loop between TM3 and TM4 (Diagram 1). The subunits also vary in length of the C-terminal tail, with GluR2 and 3 occurring primarily in short form, and GluR1 and 4 in long forms (Diagram 7, Dingledine et al., 1999). The short form of GluR4 is expressed primarily in cerebellum and referred to as GluR4c (Gallo et al., 1992).

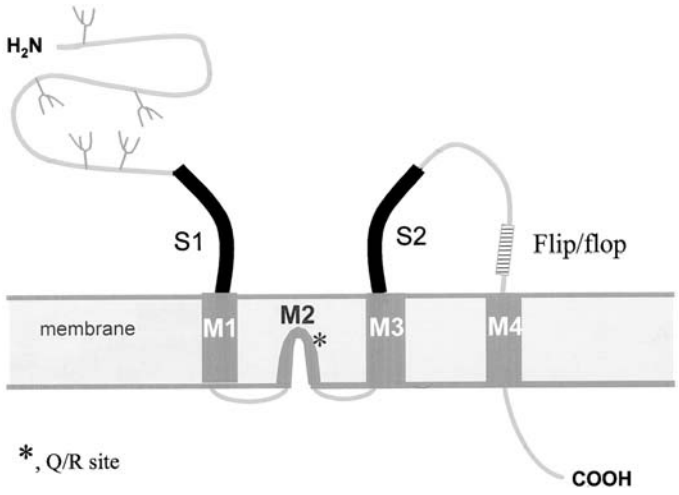


Diagram 1: Membrane topology of AMPA receptor subunits (from Dingledine et al., 1999)

1.1.1 AMPA Receptor Functional Properties Depend Upon Receptor Composition

While most neuronal AMPA receptors are Ca^{2+} impermeable, homomeric channels composed of GluR1, 3 and 4 are highly Ca^{2+} permeable. Homomeric GluR2 channels,

however, are impermeable to Ca^{2+} ions (Hollmann et al., 1991; Sommer et al., 1991). Interestingly, while the genetic sequence of the channel-lining TM2 region is identical between the four subunits, nearly 100% of GluR2 mRNA undergoes editing by dsRNA adenosine deaminase, converting a glutamine (CAG → Q) to an arginine (CIG → R; Sommer et al., 1991). Inclusion of a single edited GluR2/GluR-B subunit in a tetramer dramatically decreases calcium permeability of the channel (Hume et al., 1991; Burnashev et al., 1992). This was one of the first demonstrations that mRNA editing, in addition to alternative splicing, is a mechanism by which the cell can generate a diverse protein population from a single gene. The presence of an arginine residue in the pore also prevents channel block by endogenous polyamines and polyamine spider toxins, creating a non-rectifying channel that equally passes inward and outward current composed primarily of Na^{+} ions. Homomeric receptors composed of GluR1, 3 or 4, on the other hand, are Ca^{2+} permeable, strongly inwardly rectifying and blocked by polyamines (Verdoorn et al., 1991). mRNA editing of an R/G site found in the second extracellular domain before the *flip/flop* cassette in GluR2, 3, and 4 speeds recovery from and reduces the amount of steady-state desensitization (Lomeli et al., 1994; Mosbacher et al., 1994).

An important characteristic of AMPA receptors is the profound desensitization that occurs in the continued presence of agonist. Desensitization of G-protein coupled receptors has a time course of seconds to minutes and involves phosphorylation and subsequent uncoupling of the activated receptor from second messengers; however, desensitization of AMPA receptors occurs in milliseconds and involves a conformational change in tertiary and quaternary structure that restricts current flow through the channel.

The rate of onset of desensitization and degree of steady-state desensitization vary with subunit and *flip/flop* composition (Lomeli et al., 1994; Mosbacher et al., 1994). For example, although *flip* subunits in general desensitize to a lesser degree than do *flop* subunits (Diagram 2A), the *flip* splice variants of GluR1 and GluR3 desensitize more strongly than do *flip* splice variants of GluR2 and GluR4 (Diagram 2B). At certain synapses, the rapidity of AMPA receptor desensitization may affect the time course and amplitude of synaptic responses, especially under conditions of high frequency synaptic activity (Trussell et al., 1993; Otis et al., 1996; Arai and Lynch, 1998). In addition, desensitization may serve to limit nerve cell death induced by pathologically elevated levels of glutamate release during ischemic events (Brorson et al., 1995).

1.1.2 AMPA Receptor Pharmacology

Various agonists elicit different degrees of desensitization at the AMPA receptor (Diagram 3). Binding of the endogenous agonist glutamate or the selective agonist AMPA initiates a brief high conductance opening of the channel followed by rapid desensitization (τ 1-10 ms), resulting in a steady-state current that is typically < 2% of the peak response (Tang et al., 1989; Patneau and Mayer, 1991). Quisqualate is a non-selective full agonist that activates both ionotropic and metabotropic glutamate receptors, and produces strongly desensitizing responses at AMPA receptors. The willardiines and the partial agonist kainate evoke more weakly desensitizing responses at the AMPA receptor, paradoxically producing greater steady-state currents than those seen in the presence of the full agonists glutamate, AMPA or quisqualate (Boulter et al., 1990; Patneau and Mayer, 1990; Patneau et al., 1992).

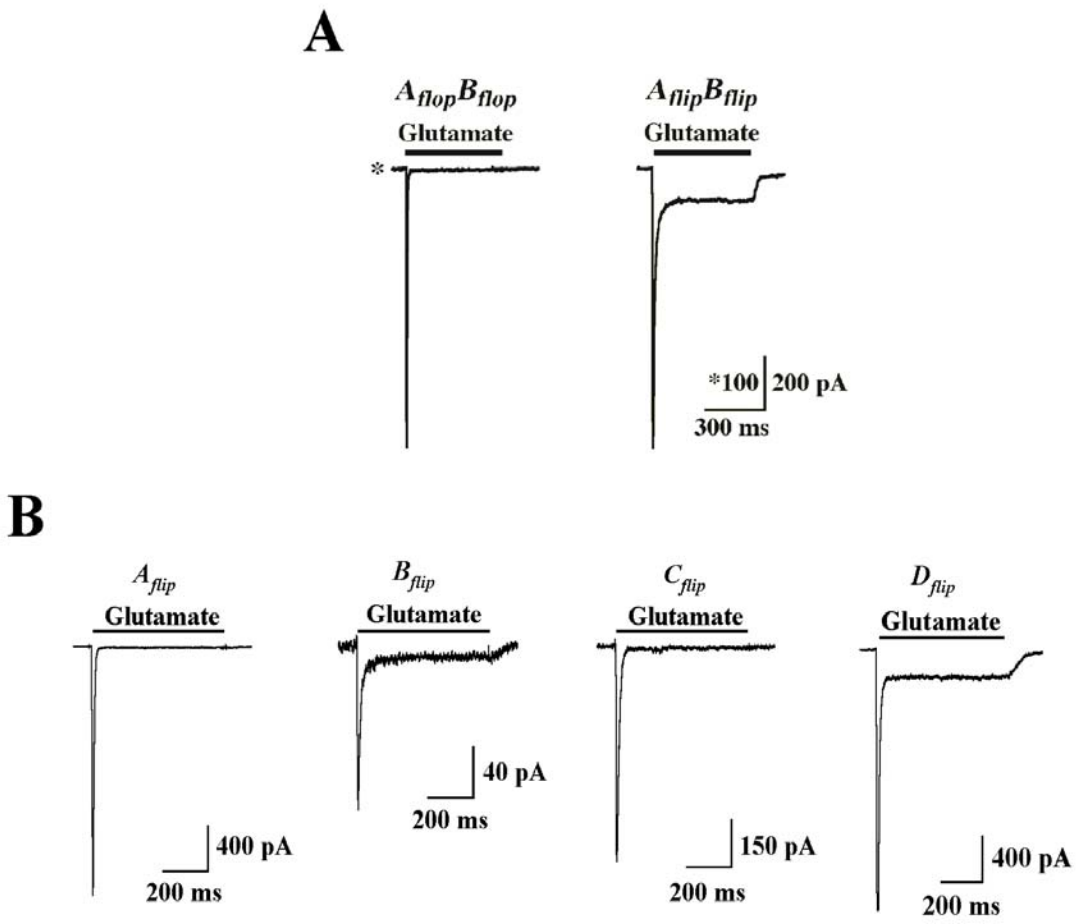


Diagram 2: Unique desensitization profiles of various heteromeric and homomeric AMPA receptor subunit combinations. A. Heteromers composed of pure *flop* isoforms exhibit more strongly desensitizing responses resulting in smaller steady-state currents than receptors composed purely of *flip* isoforms. B. Subunit specific desensitization profiles. Note that the *flip* isoforms of GluR-A and C are more strongly desensitizing than the *flip* isoforms of GluR-B and D. Traces were obtained by rapid application of 3 mM glutamate for 500-600 ms to HEK293 cells transiently transfected with GFP and the indicated AMPA receptor subunit combination. Note that GluR-A is also referred to as GluR1, GluR-B as GluR2, GluR-C as GluR3, and GluR-D as GluR4.

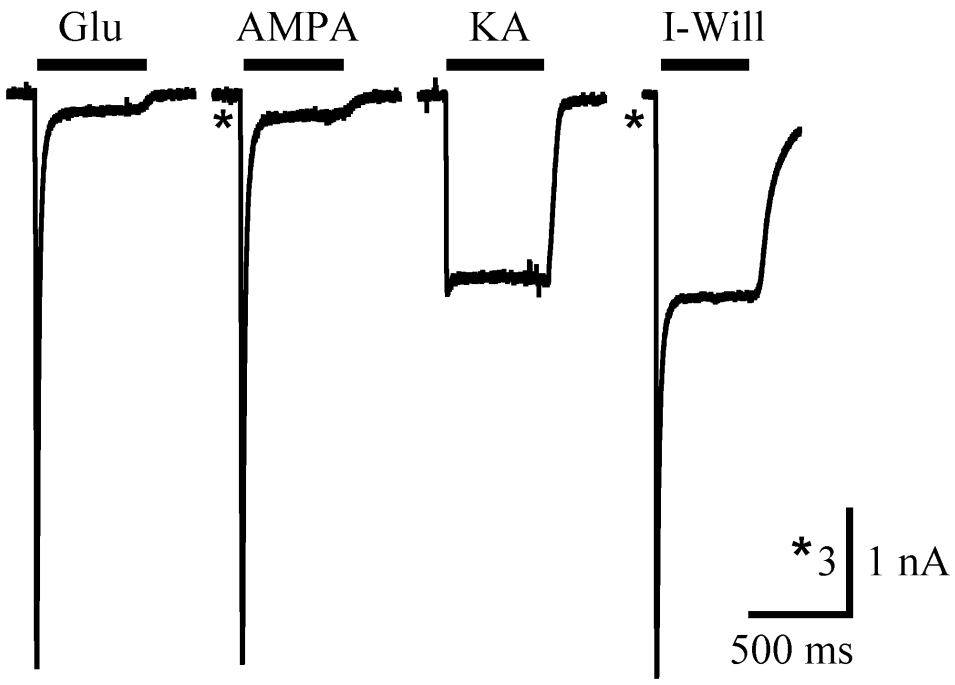
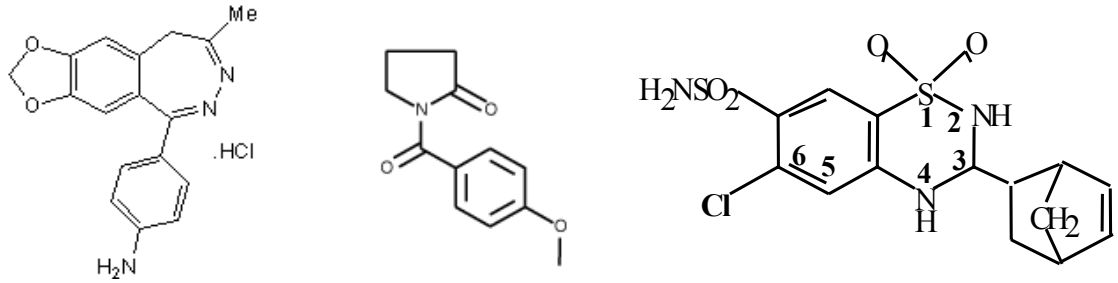


Diagram 3: Representative traces of full and partial agonists at the AMPA receptor. Responses obtained by 500 ms applications of agonists producing varying degrees of desensitization to hippocampal neurons. Glutamate and AMPA are strongly desensitizing full agonists of the AMPA receptor while kainate is a weakly desensitizing partial agonist. At neuronal AMPA receptors, iodowillardiine produces peak responses comparable to glutamate, but with much less steady-state desensitization (from Patneau et al., 1992).

There are a variety of antagonists of the AMPA receptor. CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione) and NBQX (2, 3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)qunoxaline) are potent competitive antagonists (Honore et al., 1988; Watkins et al., 1990; Gill et al., 1992; Zeman and Lodge, 1992), while argiotoxin and Joro spider toxin are channel blockers (Kawai et al., 1982; Budd et al., 1988). Interestingly, block by

the spider toxins is dependent upon Q/R editing: channels lacking edited GluR2 are susceptible to block, while the presence of an edited GluR2 confers insensitivity to toxin binding, just as it eliminates block by internal polyamines (Blaschke et al., 1993; Herlitze et al., 1993). Unlike the 1,4-benzodiazepines, which potentiate responses of $GABA_A$ receptors, the 2,3-benzodiazepines are noncompetitive antagonists of the AMPA receptor (Donevan and Rogawski, 1993) that appear to selectively bind and hold the receptor in the desensitized state (Turetsky et al., 2004). The derivative GYKI 52466 has both anticonvulsant properties in animal models of epilepsy and neuroprotective properties in focal and global ischemia (Chapman et al., 1991; Smith et al., 1991; Smith and Meldrum, 1992; May and Robison, 1993; Yamaguchi et al., 1993; Gill, 1994).



GYKI 52466 **Aniracetam** **Cyclothiazide**

Diagram 4: Chemical structures of various allosteric modulators of the AMPA receptor. GYKI 52466 inhibits agonist-evoked responses at AMPA receptors while aniracetam and cyclothiazide are positive allosteric modulators.

Positive allosteric modulators of the AMPA receptor bind at a site distinct from the agonist binding site and enhance current flow through the ion channel. Positive allosteric modulators of the AMPA receptor exert their effects through a variety of mechanisms.

The classic benzoylpiperidine aniracetam increases single channel open time (Tang et al., 1991; Vyklicky et al., 1991; Partin et al., 1996; Lawrence et al., 2003) and possibly single channel conductance (Staubli et al., 1992) by slowing the rate of receptor deactivation. Aniracetam is more efficacious at *flop* splice isoforms (Johansen et al., 1995). CX516 (BDP-12) and CX546, also benzoylpiperidines, were developed as memory-enhancing drugs and are known as Ampakines (Staubli et al., 1994b; Staubli et al., 1994a; Lynch et al., 1996; Ingvar et al., 1997; Hampson et al., 1998). CX516 and CX546 modulate AMPA receptor mediated currents by accelerating channel opening and slowing channel closing, respectively (Arai et al., 2002).

The classic benzothiadiazine cyclothiazide acts at a site distinct from that of the 2,3-benzodiazepines (Kessler et al., 1996; Donevan and Rogawski, 1998) and significantly inhibits AMPA receptor desensitization (Patneau et al., 1993; Yamada and Tang, 1993). Unlike aniracetam, the benzothiadiazines act preferentially at receptors composed of *flip* subunits (Partin et al., 1994), with the selectivity determined by a single amino acid residue in the *flip/flop* domain (Table 2; Partin et al., 1995; Partin et al., 1996). Residues outside of the *flip/flop* region have also been shown to influence cyclothiazide modulation (Partin, 2001). The mechanism of action of cyclothiazide is frequently described as reduction or attenuation of desensitization (Nagarajan et al., 2001; Horning and Mayer, 2004).

1.2 Synaptic Physiology

1.2.1 Varying AMPA Receptor Subunit Composition Produces Functional Heterogeneity

AMPA receptors mediate the majority of fast synaptic transmission in the mammalian nervous system; however, the specific subunit composition of the receptor varies between neurons, and even between different synapses on a single neuron (Zhao et al., 1997), resulting in unique postsynaptic responses. For example, cerebellar Purkinje and granule cells express GluR2 and GluR4, while GluR1 is relatively absent in cerebellar neurons (Hack et al., 1995; Zhao et al., 1997; Brorson et al., 1999; Huh and Wenthold, 1999). Inclusion of GluR2 in a tetramer determines the physiological properties of the receptor channel: lower channel conductance, minimal Ca^{2+} permeability, and slower deactivation and desensitization kinetics. Certain mature auditory, visual and cerebellar neurons primarily express the *flop* isoform of GluR4, resulting in Ca^{2+} permeable channels with extremely fast deactivation and desensitization kinetics (Geiger et al., 1995; Rubio and Wenthold, 1997; Gardner et al., 1999; Lawrence and Trussell, 2000; Gardner et al., 2001; Wall et al., 2002; Veruki et al., 2003; Joshi et al., 2004).

Excitatory pyramidal neurons in the CA1, CA2 and dentate gyrus regions of the hippocampus primarily express heteromeric combinations of GluR1/GluR2 and GluR2/GluR3 as well as a small population of homomeric GluR1 receptors (Geiger et al., 1995; Wenthold et al., 1996). Assembly of GluR1/2 heteromers preferentially includes two GluR1 and two GluR2 subunits assembled so each GluR1 subunit is flanked by GluR2 and vice versa (Mansour et al., 2001). GluR1 and GluR2 in hippocampus and

neocortex are found as both *flip* and *flop* splice variants (Hestrin, 1993; Geiger et al., 1995; Fleck et al., 1996). Heteromeric receptors preferentially include both *flip* and *flop* isoforms in a single tetramer (Brorson et al., 2004), suggesting native hippocampal AMPA receptors are composed of GluR1*flop*/GluR2*flip* and/or GluR1*flip*/GluR2*flop*. More mature synapses in cultured hippocampal neurons have a relatively greater proportion of GluR2 than do immature synapses (Pickard et al., 2000), suggesting AMPA receptors with higher Ca^{2+} permeability may be important during neuronal and synaptic development in the hippocampus. Additionally, the AMPA receptor subunit content of inhibitory GABAergic interneurons is unique from that in excitatory neurons. Hippocampal and neocortical interneurons desensitize faster and have greater Ca^{2+} permeability than pyramidal neurons in the corresponding area due to elevated expression of GluR4*flop* and reduced expression of GluR2 (Hestrin, 1993).

Regional differences and developmentally regulated changes in AMPA receptor subunit composition have been studied extensively in brainstem nuclei involved in auditory processing. Before synapse formation occurs, AMPA receptors in neurons from avian nucleus magnocellularis (nMAG) desensitize relatively slowly, are Ca^{2+} impermeable and are not sensitive to polyamine channel block, indicating incorporation of GluR2. After synapse formation, AMPA receptors in these cells desensitize rapidly, have increased Ca^{2+} permeability and sensitivity to polyamine block as a consequence of GluR2 downregulation and GluR4 upregulation (Lawrence and Trussell, 2000). Neurons in the cochlear nuclei, the mammalian homologue of nMAG, receive input from both auditory nerves and axons of local granule cells. Neurons directly involved in auditory processing exhibit fast, Ca^{2+} permeable synaptic responses mediated primarily by

GluR4*flop*, while those synapsing with local fibers exhibit slower, Ca^{2+} impermeable responses due to the presence of GluR2 (Gardner et al., 1999, 2001; Joshi et al., 2004).

1.2.2 Desensitization During Normal Synaptic Transmission

The duration of excitatory postsynaptic currents (EPSCs) varies between 100 μs and 2 ms (Raman and Trussell, 1995; Kinney et al., 1997), while the time constants for AMPA receptor desensitization range from 2-12 ms in various brain regions (summary in Raman et al., 1994). Desensitization plays a role in auditory and cerebellar synaptic transmission (Trussell and Fischbach, 1989; Trussell et al., 1993; Kinney et al., 1997; Wall et al., 2002). For example, in the cerebellum the rapidity of desensitization of GluR4*flop* receptors may shape EPSCs at mature mossy fiber/granule cell synapses and mossy fiber/unipolar brush cell synapses in rat cerebellum (Kinney et al., 1997; Wall et al., 2002). However, the time course of normal synaptic transmission in most brain regions is believed to be determined primarily by deactivation rather than desensitization kinetics of the AMPA receptor (Jonas and Spruston, 1994; Conti and Weinberg, 1999).

AMPA receptor desensitization affects postsynaptic responses during the prolonged presence of glutamate in the cleft and/or high frequency stimulation. A common feature of high frequency stimulation is a decrement in the "second response", which can be abolished by cyclothiazide (Otis et al., 1996), a positive modulator that inhibits AMPA receptor desensitization (Patneau et al., 1993; Yamada and Tang, 1993). Thirty-five to forty percent of AMPA receptors in avian neurons are desensitized after a single synaptic event (Otis et al., 1996). The relatively high affinity of glutamate for the desensitized state slows dissociation of neurotransmitter, suggesting the extent of desensitization of a

synaptic population of AMPA receptors can define synaptic responses during high frequency stimulation. Desensitization therefore limits the frequency at which full amplitude responses can be elicited at a particular synapse (Trussell and Fischbach, 1989; Jones and Westbrook, 1996).

1.3 Conformational Changes Involved in Agonist Binding, Channel Gating and Desensitization

Crystallization of the extracellular domains of the GluR2 subunit of the AMPA receptor in complex with various ligands (Armstrong et al., 1998; Armstrong and Gouaux, 2000; Hogner et al., 2002; Sun et al., 2002) has significantly enhanced our understanding of the mechanism of AMPA receptor gating and desensitization. The propensity of the extracellular domains to form dimers in solution suggests native tetramers are assembled as a pair of dimmers (Diagram 5). Interestingly, dimerization is enhanced by mutations that disrupt desensitization (Stern-Bach et al., 1998), as well as by binding of the positive allosteric modulator cyclothiazide (Sun et al., 2002). The extracellular portion of each AMPA receptor subunit has two distinct regions, termed domain 1 (D1) and domain 2 (D2) that are assembled into a "clamshell" conformation (Stern-Bach et al., 1994; Armstrong et al., 1998; Armstrong and Gouaux, 2000; Mayer et al., 2001; Hogner et al., 2002; Sun et al., 2002). As pictured in Diagram 5, D2 consists primarily of the extracellular domain between TM3 and TM4 while D1 is made up of both the first ~150 amino acids of the extracellular domain preceding TM1 and of the alternatively spliced *flip/flop* domain found in S2 (Armstrong et al., 1998). The agonist binding site is formed at the junction of D1 and D2.

Each tetrameric AMPA receptor has two dimer interfaces, resulting in two-fold rotational symmetry of the extracellular domains. This symmetry has been shown to extend to the outermost portion of the channel (Sobolevsky et al., 2004), while the interior and cytosolic portion of the channel is still assumed to have four-fold symmetry (Stern-Bach, 2004). Helix D of S1, helix J of S2 and an interdomain β strand of S1 from one subunit interact in a complementary fashion with the same structures in an adjacent subunit to form the dimer interface (Diagram 5, Table 1). A considerable hydrogen bond network, two salt bridges and hydrophobic van der Waals interactions maintain the interface (Armstrong et al., 1998; Armstrong and Gouaux, 2000; Partin, 2001; Hogner et al., 2002). Table 1 identifies interactions between specific residues in these domains. Of note, helix J consists of amino acids 741-756 of the ~38 amino acid *flip/flop* region in GluR2 (Armstrong et al., 1998; Partin, 2001), a pivotal region in defining the desensitization profile of the receptor (Sommer et al., 1990).

Crystallization of the soluble extracellular domains of GluR2 shows that two molecules of the positive allosteric modulator cyclothiazide bind at the dimer interface through interactions with residues near the agonist binding site and in the *flip/flop* region (Partin et al., 1996; Partin, 2001; Sun et al., 2002; Leever et al., 2003). This significantly increases the stability of the dimer interface, suggesting that stabilization of inter-subunit interactions determines the degree of receptor desensitization. Stern-Bach and colleagues (1998) have identified a point mutation in GluR3 (L507Y) that abolishes desensitization. Examination of the crystallized extracellular domains reveals that the homologous amino acid (L483) in GluR2 is a primary participant in dimer interface stabilization. Substitution of a tyrosine for a leucine at position 483 in GluR2 facilitates hydrophobic

interactions between this residue and L748 and K752 on the adjacent helix J (See Table 2). The ring structures of the tyrosine side chain also allows favorable cation-π interactions (Sun et al., 2002), further strengthening the dimer interface and significantly shifting the K_D of dimerization to the left.

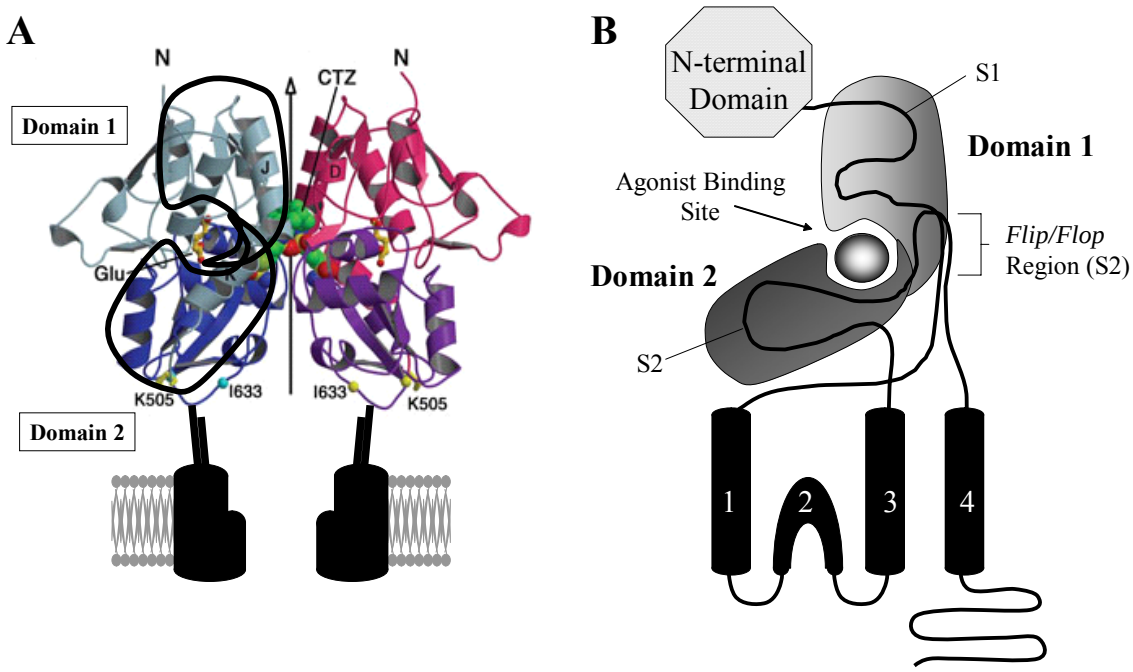


Diagram 5: Models of the extracellular domains of the AMPA receptor subunit. A. Crystallized structure of the extracellular domains of two AMPA receptor subunits bound by the benzothiadiazine cyclothiazide (CTZ) and the agonist glutamate (Glu). Domain 1 (D1) and domain 2 (D2) from a single subunit are outlined, with D1 in light blue and D2 in dark blue (from Sun et al., 2002). D1 and D2 of the adjacent subunit are shown in pink and purple, respectively. Note the location of helix J and helix D at the dimer interface. For reference, the channel-forming transmembrane domains, which have not been crystallized, are also depicted. B. Schematic of the location of the S1 and S2 regions relative to domain 1 and domain 2. Also shown are the channel-forming transmembrane portions of the receptor and the N-terminal domain (~400 amino acids). The N-terminal domain, which is not necessary for agonist binding or desensitization, plays a role in tetramer assembly (Leuschner and Hoch, 1999; Pasternack et al., 2002).

TABLE I

Amino Acid Interactions at the GluR2 (GluR-B) Dimer Interface

Domain 1		Adjacent Domain 1
L483 (helix D) ♦	⟷	L748, L751, K752 (helix J)
E486 (helix D)	⟷	K493 * (interdomain β strand)
N754 (helix J) ♦	⟷	S729 (near interdomain hinge)

All amino acid numbers refer to the mature GluR2/GluR-B protein

* Conserved in all AMPA and kainate receptor subunits (Armstrong and Gouaux, 2000)

♦ Residues found at the dimer interface that strongly influence desensitization

TABLE II

Summary of Residues Affecting Agonist Binding and Desensitization

Agonist Binding Site (GluR2)		*Flip/Flop* residue determining cyclothiazide sensitivity	Non-Desensitizing Mutants
S1	Y405 Y450 P478 • T480 • R485 • *	GluR1 S/N750 GluR2 S/N754 ♦	GluR1 L497Y GluR2 L483Y ♦ GluR3 L507Y GluR4 L484Y
S2	L650 ° S654 • T655 L703 E705 •	NOTE: Kainate receptors have a Q at this site and are insensitive to modulation by cyclothiazide	

• Glutamate, AMPA and kainate interact similarly with the α-carboxyl and -amino groups of these residues (Armstrong et al., 1998; Armstrong and Gouaux, 2000)

* Conserved in all ionotropic glutamate receptors (Armstrong and Gouaux, 2000)

° Mutation to threonine left-shifts the EC_{50} of kainate and increases domain closure (Armstrong et al., 2003)

♦ Residues found at the dimer interface that strongly influence desensitization. The comparable amino acids in GluR1, 3, and 4 are predicted to have similar importance in desensitization of these subunits.

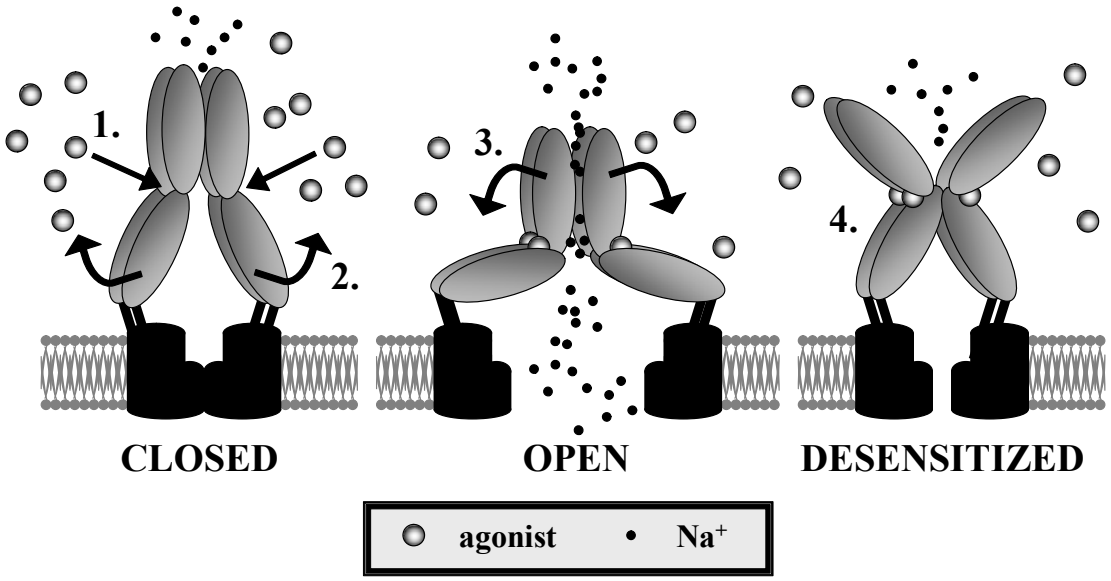


Diagram 6: Model of agonist binding and desensitization. First, agonist binds in the ligand binding cleft (1), inducing movement in the D2 domain that decreases the angle between D1 and D2 (2), which is coupled to channel opening. Further domain closure places strain on the dimer interface (3), initiating a conformational change into the lower energy desensitized state and closing the channel (4).

The current working model of AMPA receptor desensitization centers around the stability of the dimer interface. In the resting state, two adjacent subunits are associated via interactions between the D1 domains, forming a dimer (Diagram 6). Agonist binding at the ligand binding core near the hinge region of the clamshell produces movement of the D2 domain, which is communicated to the transmembrane domains to open the channel. Further closure of the "clamshell" destabilizes the dimer interface, facilitating transition into the lower energy desensitized state and closing the channel (Armstrong et al., 1998; Sun et al., 2002; Horning and Mayer, 2004). In verification of this model, studies have demonstrated that mutations affecting interactions at the dimer interface

predictably alter the kinetics and magnitude of receptor desensitization during agonist-evoked responses (Sun et al., 2002; Horning and Mayer, 2004).

A major mechanistic question is how agonist-induced conformational changes in the extracellular domains are coupled to various open states of the AMPA receptor. Interestingly, binding of the full agonists glutamate and AMPA appear to induce 20° closure between D1 and D2, while the partial agonist kainate causes only a 12° domain closure (Armstrong and Gouaux, 2000). Binding of the willardiines, a family of compounds that acts as partial agonists at GluR2 homomers, also induce lesser domain closure than the full agonists glutamate and AMPA (Jin et al., 2003). The extent of domain closure between D1 and D2 directly correlates with the degree of channel opening (Armstrong et al., 2003; Jin et al., 2003). Glutamate and AMPA elicit large peak but strongly desensitized steady-state responses, while kainate responses are only weakly desensitizing with a minimal peak (see Diagram 3). It is likely that a greater degree of domain closure is initially translated into higher conductance open states but causes greater strain on the dimer interface. This accelerates the transition into the desensitized state, resulting in smaller steady-state currents than those produced by agonists that induce a lesser degree of domain closure. This model suggests that agonist-specific conformations of the extracellular domains translate into a characteristic profile of subconductance states.

1.4 Glutamate Receptors and Models of Learning and Memory

The synapse is the functional unit of the nervous system. The basic process of neurotransmitter release, binding and activation of postsynaptic receptor with subsequent

synaptic clearance is repeated innumerable times in the mammalian brain; however, the identity of the neurotransmitter, the nature, number and coupling of the postsynaptic receptor and the rate of neurotransmitter clearance makes each synapse a unique and dynamic entity. One of the fundamental goals of neuroscience is to determine how tuning of these parameters allows individual synaptic events to encode the alterations in cognition defined as learning and memory.

1.4.1 A Historical Perspective of Learning and Memory

In the late nineteenth and early twentieth century Ivan Pavlov defined criteria for a type of vertebrate learning known as the conditioned response. In this paradigm a conditioned stimulus (such as a bell tone) is temporally linked to an unconditioned stimulus (food presentation) that produces a conditioned physiological response (salivation) in such a way that the animal "learns" to respond physiologically to the conditioned stimulus alone. Pavlov firmly established that successsful associative learning is critically dependent upon the order of stimulus presentation (conditioned stimulus preceding unconditioned stimulus) and the latency between stimuli (Pavlov, 1940); thus, learning is an orderly, time-dependent process. Pavlov's behavioral studies begged the question: What physiological changes in the CNS accompany and maintain associative learning?

Early in the 20th century, the famous neurohistologist Santiago Ramon y Cahal proposed the Neuron Doctrine, which identifies the neuron as the fundamental physiological and anatomic unit of the nervous system. He hypothesized that neurons store information through changes in synaptic strength (Ramon y Cajal, 1913). Less than

a decade after the publication of Pavlov's codification of associative learning, the psychologist Donald Hebb elaborated upon Cajal's theory and Pavlov's results, postulating that:

> When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased (Hebb, 1949).

Hebb proposed that elevated activity at a particular synapse increases the strength of the synapse. In other words, learning and memory cause or are a consequence of a persistent, activity-dependent molecular change in the synapse.

1.4.2 NMDA Receptors as Coincidence Detectors

Glutamate receptors play a central role in the activity-dependent molecular events believed to underlie learning and memory. As previously mentioned, AMPA receptors synaptically colocalize with NMDA receptors, which, unlike most AMPA receptors, are permeable to Ca^{2+} ions. NMDA receptors are unique in that they are both ligand- and voltage-gated. Glutamate released into the synapse binds and activates NMDA receptors; however, the channel is blocked by a Mg^{2+} ion until the membrane is sufficiently depolarized (-45 to -35 mV). This singular arrangement ensures that only strong postsynaptic depolarization activates the NMDA receptor, allowing it to detect the coincidence of pre- and postsynaptic activity that occurs during high frequency neurotransmission. Influx of Ca^{2+} ions through the receptor activates a variety of enzymes, including calcium-calmodulin dependent kinase II (CaMKII) and protein kinase

A (PKA), that activate downstream cascades that alter synaptic strength in an activity-dependent manner (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Lynch, 2004).

1.4.3 Long Term Potentiation (LTP) and Long Term Depression (LTD)

An initial question that sparked great debate in the neuroscience community was whether the modifications effecting synaptic plasticity were found pre- or postsynaptically. At the center of the dispute were "silent synapses" (Nicoll and Malenka, 1999), which are synapses that appear functionally mature upon visual examination but do not participate in normal synaptic transmission. During neuronal development, AMPA receptor expression lags behind that of NMDA receptors, resulting in a significant number of "silent" synapses containing only NMDA receptors, which are unable to mediate basal neurotransmission. Conversion of silent synapses to active synapses after appropriate stimulation was initially interpreted as evidence of alterations in neurotransmitter release; however, during development these synapses become functional due to AMPA receptor incorporation (Rao et al., 1998; Petralia et al., 1999). Trafficking of AMPA receptors into the synapse allows the released neurotransmitter to effect postsynaptic depolarization, creating an active contact from a "silent synapse". The overwhelming majority of research in the past ten years indicates that postsynaptic events, specifically increased trafficking of AMPA receptors into the synapse, are the primary mediators of synaptic plasticity (reviewed in Malinow and Malenka, 2002).

Research focusing on the molecular events underlying synaptic plasticity has primarily drawn inferences about intact brain function from studies examining long term potentiation (LTP) in hippocampal slice preparations, which preserve hippocampal

circuitry. Induction of LTP in hippocampal slices involves brief (1 s to 1.5 min), high frequency (2-100 Hz) stimulation of a presynaptic neuron often paired with depolarization of the postsynaptic neuron (-5 to 5 mV). This protocol produces prolonged increases in postsynaptic responses that can persist hours beyond the initial stimulus. LTP-like alterations *in vivo* have been shown to persist for days after induction (Bliss and Lomo, 1970).

The primary mechanism of elevated postsynaptic responses is rapid incorporation of AMPA receptors into the postsynaptic density (Shi et al., 1999). LTP induction in the hippocampus, for example, initiates an interaction between PDZ domain-containing proteins and the long C-terminal tail of GluR1 that drives synaptic insertion of GluR1/GluR2 heteromers (Shi et al., 1999; Hayashi et al., 2000). The associativity, input specificity, and persistence over time of LTP (Bliss and Collingridge, 1993) make this process an excellent model for the processes underlying learning and memory. Several studies have found evidence of LTP-like synaptic plasticity resulting from experience-related changes in vertebrate cortex (reviewed by Lynch, 2004). Also, certain drugs that facilitate LTP also enhance learning (Staubli et al., 1994b; Staubli et al., 1994a; Lynch et al., 1996; Hampson et al., 1998).

Long term depression (LTD) involves regulated removal of synaptic AMPA receptors to reduce synaptic strength (reviewed in Song and Huganir, 2002). The classic form of LTD is observed at parallel fiber/Purkinje cell synapses in the cerebellum and is NMDA receptor-independent, relying instead upon activation of metabotropic glutamate receptors and Ca^{2+} influx through voltage-gated calcium channels (reviewed in Daniel et al., 1998; Ito, 2001). Hippocampal neurons exhibit NMDA receptor-dependent LTD

after low (1 Hz), rather than high, frequency stimulation that involves activation of protein phosphatases such as calcineurin (reviewed in Malenka and Bear, 2004).

It is clear that the mechanisms implementing long-term alterations of synaptic strength differ between brain regions and can be mediated by a myriad of overlapping mechanisms; however, rapid insertion and removal of AMPA receptors appears to be a fundamental mechanism of synaptic modification (Martin et al., 2000; Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Lynch, 2004).

1.5 Glutamate Receptors and Pathophysiology

According to the most recent statistics from the American Heart Association, 70.1 million Americans have cardiovascular disease (2005). Of these, 7.1 million individuals have experienced a heart attack and 5.4 million have suffered from stroke. Excitotoxicity, a process in which over-activation of glutamate receptors leads to neuronal death, is a major cause of brain damage resulting from these hypoxic-ischemic insults. Focal or global ischemia in the CNS inhibits ATP production, causing a breakdown in ionic gradients. This leads to elevated vesicular release of glutamate and impaired uptake of the neurotransmitter. Synaptic and extra-synaptic AMPA receptors mediate an initial Na^+ influx that causes osmotic swelling, cytoplasmic dilution, organelle disruption, and, if perpetuated, cell lysis. Prolonged depolarization of the postsynaptic membrane activates NMDA receptors, allowing abnormally elevated Ca^{2+} entry into the cell. Unregulated levels of Ca^{2+} activate a variety of enzymes, including various proteases, kinases, phosphatases, endonucleases and, in particular, nitric oxide synthase, resulting in disturbance of the electron transport chain, production of toxic amounts of

free radicals, and peroxidation of cell membranes, ultimately causing neuronal death (Choi, 1988).

While Ca^{2+} flux through NMDA receptors is thought to initiate neuronal death during excitotoxic events, NMDA receptor antagonists are less neuroprotective than AMPA receptor antagonists in models of ischemia (Buchan et al., 1991). Additionally, the selective vulnerability of cerebellar Purkinje cells, which express AMPA but not NMDA receptors (Crepel et al., 1982; Cull-Candy et al., 1998), to ischemia suggests a key role for AMPA receptors in excitotoxic events. The neuroprotective effect if AMPA receptor antagonists in *in vivo* models of ischemia (Sheardown et al., 1990; Gill, 1994) suggests that AMPA receptor inhibition is a clinically relevant treatment option for ischemic events such as heart attack and stroke.

Excitotoxic events are also implicated in neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS, also known as Lou Gehrig's disease). ALS is an adult onset disease characterized by progressive degeneration of upper (corticospinal) and lower (spinomuscular) motor neurons leading to death within two to five years after diagnosis, normally from respiratory failure (Doble, 1999). In the United States, ALS incidence is between 1 and 2 per 100,000 individuals and is most commonly diagnosed in middle age, affecting men more frequently than women (Walling, 1999). The glutamate release inhibitor riluzole modestly increases ALS patient survival time and slows the rate of motor function deterioration (Doble, 1999). Excitotoxic events, and thus glutamate receptor involvement, are also implicated in other neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases as well as such disorders as epilepsy

and schizophrenia (Hollmann and Heinemann, 1994; Yamada, 1998; Johnson et al., 1999; Lees, 2000; Goff et al., 2001; Danysz, 2002b).

1.6 Modulation of AMPA Receptor Desensitization by Exogenous Drugs

The initial portion of this thesis project focuses on defining the interaction of the benzothiadiazine modulators with AMPA receptor desensitization. The nootropic effects of positive allosteric modulators of the AMPA receptor have been demonstrated in both animal and human models of learning and memory (Johnson et al., 1999; Goff et al., 2001; Danysz, 2002b) and are being explored as novel therapies for cognitive disorders such as schizophrenia (Danysz, 2002a) and Alzheimer's disease (Derkach et al., 1999). Rational drug design to enhance learning or reduce deleterious effects of stroke or other excitotoxic events relies upon a clear understanding of the modulator mechanism of action. For example, a modulator with high affinity in the absence but rapid dissociation in the continued presence of agonist may act as an ideal nootropic by increasing the efficacy of synaptic responses without danger of excitotoxic cell death.

1.7 Intracellular AMPA Receptor Associated Proteins

In recent years a number of proteins have been identified that associate with AMPA receptors (Bredt and Nicoll, 2003). These proteins are primarily cytosolic and appear to be involved either in anchoring the receptor at the synapse and maintaining basal synaptic strength, or in trafficking events associated with long-term modifications of synaptic strength. Regulated insertion and removal of AMPA receptors from the synapse is a

major mechanism of synaptic plasticity and is likely to be involved in the molecular changes intrinsic to learning and memory formation (see Section 1.4.3).

Glutamate receptor subunits with short C-terminal tails (GluR2, 3 and 4c) have a type II PDZ binding domain at the extreme C-terminus that mediates binding to the cytosolic proteins GRIP1 (glutamate receptor interacting protein 1), GRIP2/ABP (AMPA receptor binding protein), and PICK1 (protein interacting with C kinase) (Dong et al., 1997; Srivastava et al., 1998; Dev et al., 1999; Dong et al., 1999). PDZ domains, named after three proteins containing the motif (PSD-95, Discs-large/DLG, ZO-1), are ~90 amino acids long and mediate a vast array of protein-protein interactions, typically by recognition of short sequences in the C-terminus of their binding partner. GluR2 also contains a short non-PDZ sequence that binds NSF (N-ethylmaleimide-sensitive factor), a membrane fusion ATPase associated with SNARE complex disassembly (Nishimune et al., 1998; Osten et al., 1998). GluR1 and 4 interact via a non-PDZ domain interaction with the cytosolic actin-binding band 4.1 proteins (Shen et al., 2000; Coleman et al., 2003; Diagrams 7 and 8). AMPA receptor subunits also associate with the transmembrane AMPA receptor regulatory proteins, or TARPs, which include stargazin/γ2, γ3, γ4, and γ8 (Tomita et al., 2003). The following sections focus on the role of PICK1, GRIP1 and stargazin in AMPA receptor trafficking and long term modifications of synaptic strength.

Diagram 7: Short and long C-terminal domains of selected AMPA receptor subunits. Single residues highlighted in green are sites of phosphorylation. Amino acids highlighted in blue are sites of protein-protein interactions. The black line delimits the C-terminal PDZ binding domain. Enzymes are shown in bold italics, binding proteins in bold. CaMKII, calcium calmodulin dependent kinase II; PKC, protein kinase C; PKA, protein kinase A; SAP97, synapse associated protein 97; NSF, N-ethylmaleimide-sensitive factor; GRIP1, glutamate receptor interacting protein 1; ABP, AMPA receptor binding protein; PICK1, protein interacting with C kinase. (Modified from Malinow and Malenka, 2002.)

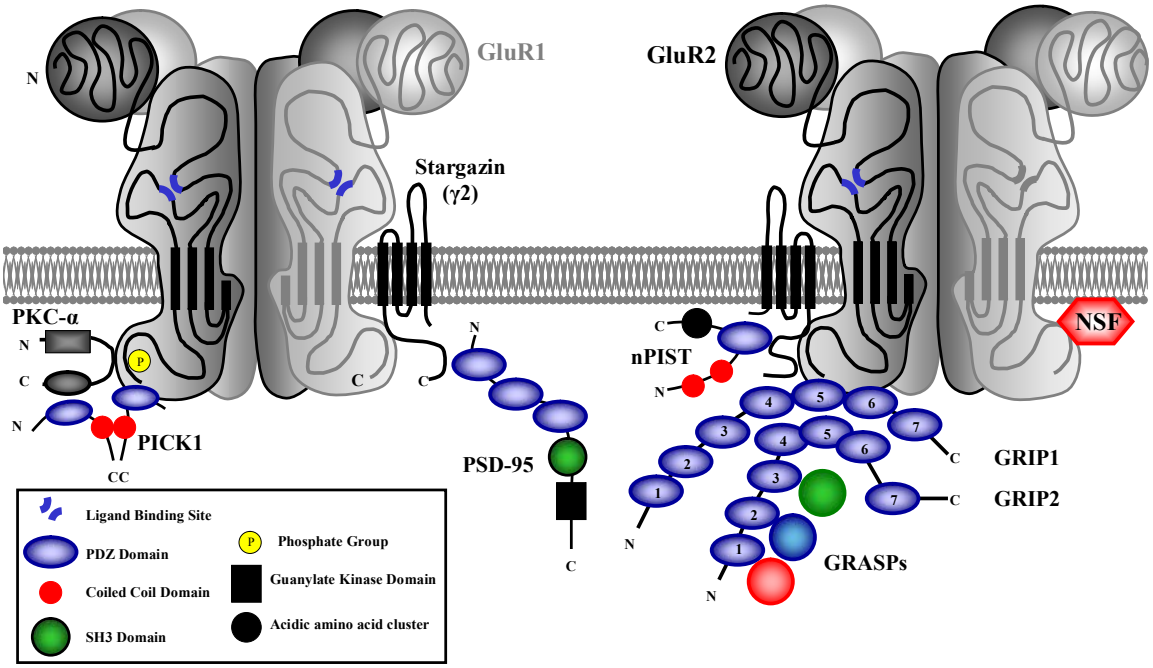


Diagram 8: Association of synaptic AMPA receptors with various proteins. PICK1, GRIP1 and GRIP2 associate with GluR2 via PDZ binding domains, while NSF binds to GluR1 via a non-PDZ domain mediated interaction. Stargazin/γ2 also associates with the receptor via a non-PDZ domain interaction. Also shown is association of PSD-95 and nPIST with stargazin (γ2). Proteins depicted: PKC-α, protein kinase C-α; PICK1, protein interacting with C kinase 1; PSD-95, postsynaptic density protein, 95kD; nPIST, neuronal isoform of protein-interacting specifically with TC10; GRIP1 and 2, glutamate receptor interacting proteins 1 and 2; GRASP, GRIP associated protein; NSF, N-ethylmaleimide-sensitive factor. Other abbreviations: PDZ, PSD-95, Discs large/DLG, ZO-1 domain (~90 amino acids); SH3, Src homology 3 domain (~60 amino acids); acidic amino acid cluster sequence is EESEE. (Modified from Song and Huganir, 2002).

1.7.1 The Glutamate Receptor Interacting Protein (GRIP) Family

While GRIP1 has no catalytic activity, it contains 7 PDZ domains, allowing it to interact with numerous proteins (Table 3). GRIP1 interacts with the extreme C-terminus of GluR2 and GluR3 via PDZ domains 4 and 5 (Dong et al., 1997) and colocalizes with synaptic AMPA receptors (Dong et al., 1997; Wyszynski et al., 1999). GRIP1 is enriched in the postsynaptic density, with a small but significant cytosolic presence (Wyszynski et al., 1998; Dong et al., 1999). Its numerous PDZ domains allow multiple and diverse binding partners, suggesting possible functions as an anchoring protein, or as a bridge juxtaposing receptors with regulatory enzymes or trafficking complexes.

A highly homologous protein called ABP or GRIP2 is also synaptically localized and binds GluR2 and GluR3 via PDZ domains 3, 5 and 6. ABP/GRIP2 forms homomultimers as well as heteromultimers with GRIP1 (Srivastava et al., 1998; Dong et al., 1999; Im et al., 2003), compounding the number and extent of possible protein interactions. In rat cerebrum ABP and GRIP1 infrequently colocalize, with complementary expression patterns both neuronally and synaptically (Burette et al., 2001; Takamiya et al., 2004). GRIP1 expression early in embryonic development precedes that of AMPA receptors, while GRIP2 is expressed in parallel with the receptor [(Dong et al., 1999), but see also (Bruckner et al., 1999)]. A shorter splice variant called GRIP1c 4-7 contains 4 PDZ domains identical to PDZ domains 4-7 of GRIP1 and also colocalizes with AMPA receptors at excitatory synapses in the hippocampus (Charych et al., 2004).

ABP is found in rat as three splice variants: a shorter version with 6 PDZ domains, a longer version with 7 PDZ domains called ABP-L or GRIP2 (Srivastava et al., 1998; Dong et al., 1999) and an N-terminal palmitoylated variant of ABP-L (deSouza et al.,

2002). Similar variants have been identified in mouse brain (Yamazaki et al., 2001). A region between PDZ3 and PDZ7 in non-palmitoylated ABP-L/GRIP2 targets and clusters GluR2-containing receptors at intracellular membranes, possibly creating and maintaining a subsynaptic pool of AMPA receptors available for activity-dependent insertion (Fu et al., 2003). Palmitoylated ABP-L is localized on the surface of dendritic spines, implying a synaptic presence (deSouza et al., 2002). Palmitoylation is a reversible modification (Magee et al., 1987; Milligan et al., 1995), similar to phosphorylation, and is associated with appropriate membrane localization and stabilization of molecules ranging from small G proteins to morphogens involved in embryonic development (Dudler and Gelb, 1996; Go and Mitchell, 2003; Chen et al., 2004); thus, palmitoylation of ABP may constitute a mechanism by which neurons can regulate synaptic AMPA receptor content.

1.7.2 GRIP Binding Partners

GRIP1 is specifically bound by several GRIP-associated proteins (GRASPs). GRASP-1 is a neuron-specific guanine nucleotide exchange factor regulated by NMDA receptor activation (Ye et al., 2000). In hippocampal cultures, over-expression of GRASP-1 reduces synaptic AMPA receptor clustering. Signaling through this pathway may comprise an unexamined component of synaptic plasticity or a possible protective mechanism during excitotoxic events (Ye et al., 2000).

The association of GRIP1 with the heavy chain of the motor protein kinesin via a non-PDZ domain interaction may contribute to trafficking GluR2-containing AMPA receptors from the soma to dendritic locations (Setou et al., 2002). Association with

MAP-1B (microtubule associated protein-1B) may allow GRIP1 to anchor synaptic AMPA receptors to the cytoskeleton (Seog, 2004). GRIP1 and ABP/GRIP2 also mediate synaptic anchoring of AMPA receptors via a PDZ domain 6 association with liprin-α, a protein that interacts with a family of receptor protein tyrosine phosphatases. Co-immunoprecipitation of liprin-α, GRIP1 and GluR2/3 from rat cortex shows a strong, physiologically relevant association between the three proteins. Disrupting the GRIP-liprin-α interaction in cultured hippocampal neurons severely diminishes GluR2 synaptic localization, suggesting that association with liprin-α is necessary for GRIP1 to act as a membrane anchor (Wyszynski et al., 2002).

GRIP family members associate with a broad array of neuronal proteins (Table 3). While a large percentage of GluR2/3 in adult rat brain is believed to associate with GRIP family members (Wyszynski et al., 1999; Burette et al., 2001), only 30-50% of GRIPs are associated with AMPA receptor subunits (Wyszynski et al., 1998; Wyszynski et al., 1999). It is clear from the multitude of GRIP1 binding partners that this protein family has diverse roles in neuronal and non-neuronal cells. For example, GRIPs clearly associate with GABAergic as well as excitatory synapses (Burette et al., 1999; Dong et al., 1999; Wyszynski et al., 1999; Charych et al., 2004). GRIP1 also interacts with the intracellular C-terminus of the extracellular matrix protein Fras1 to play a crucial role in embryonic epithelial development, digit morphogenesis and kidney formation.

TABLE III

Summary of GRIP1 and PICK1 Binding Partners

	Binding Partner	via PDZ Domain:	Comments:	Reference/s
GRIP1	GluR2, 3, 4c	4, 5	Targeting and anchoring AMPA receptors in synaptic and intracellular membranes	(Dong et al., 1997; Dong et al., 1999; Osten et al., 2000; Braithwaite et al., 2002)
	GRIP1/2	4, 5, 6	Create scaffolding for protein anchoring	(Dong et al., 1999; Ye et al., 2000)
	GRASP-1	7	A neuronal ras-GEF regulated by NMDA receptor activity	(Ye et al., 2000)
	Liprin-α	6	AMPA receptor synaptic targeting	(Wyszynski et al., 2002)
	EphrinB1 ligand Eph Receptors	6, 7	Scaffolding for multiprotein signaling complexes	(Torres et al., 1998; Bruckner et al., 1999)
	KIF5	Linker between 6 and 7	Somatodendritic receptor trafficking	(Setou et al., 2002)
	MAP-1B LC	Region containing PDZ2	Synaptic anchoring of AMPA receptors	(Seog, 2004)
	NG2	7	Glial-neuronal recognition and signaling in glial progenitor cells	(Stegmuller et al., 2003)
	Fras1	1, 2, 3	Limb morphogenesis, kidney genesis, epithelial adhesion (animal model of Fraser Syndrome)	(Takamiya et al., 2004)
PICK1	GluR2	1	Clustering and activity-dependent removal of GluR2 subunits	(Dev et al., 1999; Xia et al., 1999; Terashima et al., 2004)
	PKCα	1	Localization of enzyme to synaptic sites	(Staudinger et al., 1997; Perez et al., 2001)
	mGluR7a	1	Clustering of metabotropic GluRs at presynaptic sites	(Boudin et al., 2000)
	EphB2, ephrin-B1, EphA7	1	Clustering Eph receptors and ligands, possibly at synapses	(Torres et al., 1998)
	DAT, NET	1	Localization, expression, and function of monoamine transporters	(Torres et al., 2001)

GEF, guanine nucleotide exchange factor; KIF5, heavy chains of conventional kinesin; MAP-1B LC, light chain of microtubule association protein-1B (Setou et al., 2002); NG2, single pass transmembrane proteoglycan expressed in oligodendrocyte progenitor cells (Stegmuller et al., 2003); Fras1, extracellular matrix protein mutated in animal model of Fraser syndrome, which is characterized by blistering of embryonic but not adult skin, renal agenesis and cryptophthalamos (Takamiya et al., 2004); mGluR7a, metabotropic glutamate receptor 7a; DAT, dopamine transporter; NET, norepinephrine transporter

1.7.3 Protein Interacting with C Kinase (PICK1)

PICK1 was originally isolated by its association with the extreme C-terminus of PKCα (Staudinger et al., 1995). Via its single PDZ domain, PICK1 also binds the extreme C-terminus of GluR2 and GluR3 (Dev et al., 1999; Xia et al., 1999). While GRIP1 associates with GluR2 through a type II PDZ domain interaction (Dong et al., 1997), the PICK1 PDZ domain accommodates both a type II interaction with GluR2 and a non-conventional type I interaction with PCKα (Staudinger et al., 1997; Perez et al., 2001). Mutation of one amino acid (K27E) in its single PDZ domain disrupts GluR2 but not PKCα binding (Dev et al., 2004). PICK1 is capable of homo-oligomerization via interactions distinct from those mediating PCKα association, and can simultaneously interact with AMPA receptor subunits and PKCα (Staudinger et al., 1997; Xia et al., 1999; Perez et al., 2001). PICK1 is capable of clustering AMPA receptors in heterologous cells (Dev et al., 1999; Xia et al., 1999). Unlike the GRIPs, PICK1 appears to colocalize with AMPA receptors only at excitatory synapses on dendritic spines and does not colocalize with markers for inhibitory synapses (Xia et al., 1999).

1.7.4 Differential Interactions of GRIP and PICK1 with GluR2

Interactions between GRIP1, PICK1 and GluR2 are believed to be important in regulating the removal, insertion and stabilization of AMPA receptors at synaptic and internal membrane sites during induction of long term depression. Over-expression of the C-terminal GluR2 PDZ domain in cultured rat neurons interferes with binding of endogenous GRIP1 and/or PICK1 with AMPA receptors and disrupts AMPA receptor clustering (Dong et al., 1997) without affecting extrasynaptic receptor distribution or

synaptic density (Dong et al., 1997; Osten et al., 2000; Shi et al., 2001). While PICK1 and GRIP1 bind the same C-terminal PDZ domain in GluR2, phosphorylation of serine 880 (GluR2-S^{880}) in the GluR2 C-terminus dramatically interferes with GRIP1 but not PICK1 binding (Dong et al., 1997; Chung et al., 2000; Matsuda et al., 2000). A construct distinguishing between these proteins demonstrates that GRIP1 association is responsible for stabilizing GluR2-containing AMPA receptors at the synapse (Osten et al., 2000). Mutant GluR2 subunits that interfere with GRIP1 but not PICK1 binding are poorly synaptically localized in hippocampal slice cultures (Seidenman et al., 2003), further supporting a role for GRIP1 as a synaptic membrane anchor. Interestingly, association of ABP-L/GRIP2, which appears to stabilize AMPA receptors in subsynaptic membranes, interferes with phosphorylation of serine 880 (Fu et al., 2003), suggesting a feedback mechanism by which of GRIP2 association with GluR2 maintains intracellular localization of AMPA receptors and thus down-regulation of synaptic receptors.

Ligand binding studies of AMPA receptor surface expression after NMDA-induced LTD in cultured hippocampal neurons support a role for PICK1 in activity-dependent AMPA receptor internalization (Iwakura et al., 2001). Disruption of GRIP1 and/or PICK1 binding in hippocampal slices and cerebellar Purkinje cells using a peptide corresponding to the GluR2 PDZ binding domain (pep2-SVKI) blocks induction of LTD (Daw et al., 2000; Xia et al., 2000; Kim et al., 2001). While these studies generated conflicting evidence about the exact involvement of GRIP1 and PICK1 in hippocampal and cerebellar LTD, both groups propose models in which the phosphorylation-mediated differential association of PICK1 and GRIP1 with GluR2 is involved in destabilizing (PICK1) and stabilizing (GRIP1) AMPA receptors in preparation for or in maintenance

of LTD, respectively. The GRIPs appear to act as membrane anchors at both surface and internal membranes. Phosphorylation of GluR2-S^{880} may disrupt GRIP1 association with synaptic receptors and facilitate PICK1 binding, leading to internalization of GluR2-containing receptors. Subsequent dephosphorylation would promote association of GRIP1 with AMPA receptors at subsynaptic membranes to maintain synaptic depression. Distinct signaling cascades mediating hippocampal and cerebellar LTD raise the possibility that these proteins play slightly different roles in the two brain regions.

Several studies have shown active involvement of PICK1 in regulation of GluR2 surface expression. PICK1 preferentially associates with active PKCα, an enzyme associated with cerebellar LTD that can phosphorylate GluR2-S^{880} *in vitro*. Pharmacological activation of endogenous neuronal PKCα redistributes the diffusely localized enzyme so it colocalizes with PICK1 in dendritic spines (Perez et al., 2001). In both hippocampal and cortical neurons, PKCα activation results in AMPA receptor internalization, decreased GluR2 surface expression, and serine 880 phosphorylation of most internalized GluR2 (Chung et al., 2000; Perez et al., 2001). PKCα activation does not affect GRIP distribution in hippocampal neurons (Chung et al., 2000) or heterologous cells (Matsuda et al., 2000). These studies suggest a model in which PKCα activation initiates its association with PICK1. Close proximity to the C-terminal tail then facilitates phosphorylation of serine 880, dissociating GRIP from the receptor, and allowing receptor internalization (Perez et al., 2001). While evidence exists that PKCα is not the kinase mediating GluR2-S^{880} phosphorylation (Daw et al., 2000; Kim et al., 2001), phosphorylation of this residue is necessary for induction of hippocampal LTD (Kim et al., 2001; Chung et al., 2003; Seidenman et al., 2003), suggesting association of

PICK1 with phosphorylated GluR2 mediates activity-dependent down-regulation of synaptic AMPA receptors.

PICK1 may also be involved in regulating basal GluR2 surface expression. In an attempt to clearly define the interaction of PICK1 with GluR2, Collingridge and colleagues (2004) found that, in agreement with Perez et al. (2001) and Chung et al. (2000), expression of a PICK1-Sindbis virus construct in rat hippocampal slices selectively down-regulates synaptic GluR2. This occurs without changing GluR2 internal distribution, total GluR1 and GluR2 levels or, interestingly, GluR1 surface expression. Paradoxically, the reduced presence of GluR2 in the synapse increases the magnitude of EPSCs, possibly due to the greater channel conductance of GluR2-lacking receptors (Terashima et al., 2004). It is clear that the availability of PICK1 can affect basal synaptic transmission; however, both the physiological significance of this finding and confirmation of the more dynamic role of this protein in LTD remains open to further experimentation.

GRIP1 and PICK1 have been implicated in processes beyond cerebellar and hippocampal LTD. There is some evidence that GRIP1 plays a slightly more active role in LTP-like events in the spinal cord. Li and colleagues (1999) found that peptide-mediated disruption of GRIP1 but not PICK1 binding blocked the serotonin-mediated conversion of silent synapses to functional connections between sensory afferents and dorsal horn neurons, an effect that appeared to be mediated by PKC. Elsewhere in the nervous system, GRIP1 and PICK1 may be involved in development of schizophrenia. A recent study reported elevated levels of GRIP1 mRNA in the dorsolateral prefrontal cortex and occipital lobe of elderly schizophrenics (Dracheva et al., 2005). The PICK1

gene has been mapped to an area associated with increased susceptibility to schizophrenia and incidence of a single polymorphism of this gene is elevated in schizophrenic individuals (Hong et al., 2004).

1.8 Modulation of AMPA Receptor Desensensitization by GRIP1/PICK1

Reversible modifications such as phosphorylation have been shown to change channel properties: phosphorylation of GluR1 at serine 831 by CaMKII increases single channel conductance (Derkach et al., 1999) while phosphorylation of the same subunit at serine 845 by PKA increases channel open probability (Banke et al., 2000). Reversible protein associations may also alter functional properties. The burgeoning number of proteins involved in AMPA receptor trafficking yield a field of promising candidates. It has been previously shown that the PDZ-domain mediated association of PSD-95/SAP90 with kainate receptors significantly slows the rate of glutamate-evoked desensitization (Garcia et al., 1998; Bowie et al., 2003), raising the possibility that interactions of the AMPA receptor with PDZ domain-containing proteins could alter channel properties. The differential association of a large protein such as GRIP1, GRIP2 or PICK1 may influence parameters such as desensitization or channel conductance. If cytoplasmic proteins can, by physical association, alter AMPA receptor channel function, a new parameter must be included in models of receptor kinetics, basal synaptic transmission and synaptic plasticity.

1.9 The Transmembrane AMPA Receptor Regulatory Protein (TARP) Family

The *stargazer* mutant mouse exhibits symptoms of severe cerebellar dysfunction, including ataxia as well as impaired eyeblink conditioning, a motor learning paradigm dependent on intact cerebellar function (Noebels et al., 1990; Qiao et al., 1998). The spontaneous viral insertion of a 6-kb early transposon into intron 2 of the *stargazer* gene prematurely arrests transcription, causing complete ablation of protein expression (Letts et al., 1998). Stargazin/γ2 was initially grouped by homology in the voltage-gated calcium channel γ family (Letts et al., 1998); subsequent experiments demonstrated that the mutant phenotype is due primarily to a severe deficit of AMPA receptor surface trafficking in cerebellar granule cells (Chen et al., 2000).

Based on homology and the ability to rescue AMPA receptor-mediated currents in *stargazer* cerebellar granule cells, a family of tetraspanning transmembrane AMPA receptor regulatory proteins (TARPs) has been identified that includes stargazin/γ2, γ3, γ4, and γ8 (Tomita et al., 2003). Western blotting and *in situ* hybridization show high levels of expression of stargazin/γ2 in the cerebellum, γ3 in cerebral cortex, γ4 in caudate putamen, and γ8 in hippocampus. γ4 is expressed in both neurons and glia while expression of the remaining family members is exclusively neuronal. While γ2 is the only TARP expressed in cerebellar granule cells, there is overlap in TARP distribution in other brain regions. The TARPs appear to be developmentally regulated; γ4 expression peaks early in post-natal maturation while γ2, γ3, and γ8 mRNA appears later and increases into adulthood until maximum levels are obtained (Tomita et al., 2003).

Evidence exists that the members of the TARP family function as auxiliary subunits of the AMPA receptor. TARPs co-purify with synaptic AMPA receptors in rat brain

(Nakagawa et al., 2005; Vandenberghe et al., 2005b), and single particle electron microscopy indicates the transmembrane regions of TARPs are closely associated with the transmembrane regions of the native AMPA receptors (Nakagawa et al., 2005). TARP family members do not coimmunoprecipitate, which suggests they do not intermix at a single AMPA receptor (Tomita et al., 2003); however, the number of TARPs associated with an individual AMPA receptor is unknown.

1.9.1 Augmentation of AMPA Receptor Surface Expression by Stargazin/γ2

Electrophysiological recordings from *stargazer* (*stg, -/-*) cerebellar granule cells revealed a critical reduction in both synaptic and extrasynaptic AMPA receptor currents (Chen et al., 2000). Transcription and translation of AMPA receptor subunit genes is comparable between mutant and wild-type cells; therefore, the profound deficit in AMPA receptor mediated currents in *stargazin* mutant mice is due to defective surface trafficking of the ion channel (Hashimoto et al., 1999). Expression of stargazin enhances glutamate-evoked responses between 5- and 10-fold in both neurons and heterologous systems (Schnell et al., 2002; Chen et al., 2003) and performs a vital role in AMPA receptor surface trafficking. γ3, γ4 and γ8 are believed to function similarly in other brain regions such as cortex and hippocampus.

AMPA receptors from *stargazer* cerebellar granule cells exhibit immature glycosylation patterns, suggesting stargazin may in part function as an ER chaperone (Tomita et al., 2003). The unfolded protein response (UPR) is a process by which accumulation of unfolded proteins in the endoplasmic reticulum (ER) stimulates transcription of chaperone proteins like BiP (Ig binding potein). Stimulation of the UPR

increases surface expression of GluR1 in heterologous cells. In cells cotransfected with GluR1 and stargazin there is no further increase in GluR1 surface expression upon stimulation of the UPR. This suggests stargazin and the UPR have similar mechanisms of action, namely facilitating proper protein folding and exit from the ER (Vandenberghe et al., 2005a).

1.9.2 Stargazin/γ2 and Synaptic Trafficking of AMPA Receptors

Stargazin mutant mice display severely diminished transmission at cerebellar mossy fiber/granule cell synapses (Hashimoto et al., 1999) which can be restored by exogenous expression of γ2 (Chen et al., 2000). Transfection of a truncated stargazin construct restores extrasynaptic responses in *stg* cerebellar granule cells to the same degree as full-length stargazin; however, this construct slightly decreases synaptic AMPA receptor responses in both wild-type cerebellar granule cells and hippocampal neurons (Chen et al., 2000). The truncated four C-terminal amino acids comprise a PDZ domain that binds PSD-95 (postsynaptic density protein, 95kD; see Diagram 6), a protein that does not directly interact with AMPA receptors but enhances AMPA receptor synaptic transmission (Schnell et al., 2002) and limits the incorporation of AMPA recepters into the synapse during activity-dependent synaptic plasticity (Ehrlich and Malinow, 2004). Over-expression of stargazin in hippocampal neurons increases current density mediated by extrasynaptic receptors but does not alter EPSCs (Schnell et al., 2002), suggesting that synaptic AMPA receptor trafficking involves both stargazin and PSD-95. Association of stargazin with the receptor is sufficient for surface trafficking, while a subsequent

interaction between stargazin and PSD-95 is necessary to move the receptor into the synapse (Chen et al., 2000; Schnell et al., 2002).

Stargazin can associate with various other proteins via regions of its C-terminus distinct from the PDZ domain. Stargazin interacts with nPIST, the neuronal isoform of protein-interacting specifically with T10 (Cuadra et al., 2004; Ives et al., 2004). nPIST binds the stargazin C-terminus between amino acids 243-283 and, like PSD-95, appears to be necessary for AMPA receptor synaptic targeting (Cuadra et al., 2004; Diagram 8). Stargazin also binds LC2 (light chain 2 of microtubule associated protein) via a C-terminal non-PDZ domain mediated interaction. The association of LC2 with non-synaptic stargazin/AMPA receptor complexes in cerebellar neurons suggests LC2 participates in early stages of AMPA receptor surface trafficking (Ives et al., 2004).

The C-terminal tail of stargazin has multiple phosphorylation sites. Phosphorylation of threonine 321 in the PDZ binding domain disrupts PSD-95 mediated receptor clustering (Chetkovich et al., 2002). Basal levels of stargazin T321 phosphorylation have been demonstrated *in vivo* and disruption of PSD-95 association by this modification has been verified by several biochemical techniques (Choi et al., 2002). Introduction of a T321 phospho-mimic stargazin construct into cultured hippocampal cells reduces the amplitude and frequency of AMPA receptor mEPSCs (Chetkovich et al., 2002), suggesting disruption of the stargazin/PSD-95 association is a potential mechanism for LTD-like processes.

Phosphorylation of multiple strongly conserved serine residues in the stargazin C-terminal tail selectively increases synaptic AMPA receptor responses (Tomita et al., 2005). Classic LTP involves depolarization of the postsynaptic membrane sufficient to

remove NMDA receptor channel block, allowing an influx of Ca^{2+} that subsequently activates various kinases, followed by rapid insertion of AMPA receptors into the synaptic membrane (Malinow and Malenka, 2002; Bredt and Nicoll, 2003). The downstream molecular events between kinase activation and receptor insertion, however, are unclear. Interestingly, phosphorylation and dephosphorylation of stargazin appear to be required for expression of hippocampal LTP and LTD, respectively. CaMKII activity is critical for induction of hippocampal LTP (Malenka et al., 1989; Malinow et al., 1989; Hayashi et al., 2000) while PKC activation is crucial in cerebellar LTD (Crepel and Krupa, 1988; Linden and Connor, 1991). Both kinases are capable of phosphorylating the nine serine residues in the stargazin C-terminus (Tomita et al., 2005). In light of the critical role stargazin plays in AMPA receptor trafficking, it is not surprising to find that this protein is involved in regulated insertion of receptors during activity-dependent synaptic plasticity. The presence of multiple phosphorylatable serines in the C-terminus further suggests graded responses to synaptic stimulation, a possibility which remains open to investigation.

1.9.3 Molecular Determinants of Stargazin/AMPA Receptor Binding

Unlike the association of GluR2 with PICK1 and GRIP1, the association of AMPA receptor subunits and stargazin is not mediated by PDZ domain interactions. The interaction of stargazin with individual AMPA receptor subunits has been reported to be non-specific (Chen et al., 2000; Tomita et al., 2004); however, research from this lab indicates this interaction is subunit-specific (Chapter 5). Co-expression of stargazin and kainate receptor subunits in *Xenopus* oocytes has been reported to have no effect on

glutamate-evoked currents (Chen et al., 2003); again, research from this lab has shown stargazin to increase surface trafficking of certain kainate receptor subunit combinations in heterologous cells.

Assessment of elevated stargazin-mediated AMPA surface trafficking has primarily used current recordings obtained during bath application of agonist. Interpretation of agonist-evoked responses obtained in this fashion is confounded by the profound steady-state desensitization of AMPA receptors (see Section 1.1.1). While an increase in current may be interpreted as increased surface expression (Schnell et al., 2002; Tomita et al., 2004), it may in fact represent altered functional properties, such as reduced receptor desensitization. The first extracellular loop and intracellular C-terminus of stargazin interact with the AMPA receptor and are important for stargazin-mediated enhancement of current responses to bath-applied glutamate (Tomita et al., 2004). While these regions of stargazin were interpreted to mediate AMPA receptor surface trafficking, research from this lab suggests the first extracellular loop alters AMPA receptor responses to the partial agonist kainate, while the C-terminal tail plays a role both in surface trafficking and altering the desensitization profile of the receptor (Chapter 5).

1.10 Modulation of AMPA Receptor Functional Properties by Stargazin/γ2

The effect of stargazin (γ2) on AMPA receptor trafficking in cerebellar granule cells is undisputed. The ability of stargazin to enhance currents in hippocampal neurons (Chen et al., 2000) suggests TARPS are involved in AMPA receptor membrane trafficking in brain regions beyond the cerebellum and are candidates for involvement in synaptic plasticity; however, the function of basal TARP association with synaptic

AMPA receptors is less clear (Vandenberghe et al., 2005b). Bredt and colleagues have concluded that the association of stargazin does not affect desensitization (Schnell et al., 2002); however, the resolution of evoked currents in their study was too gross to detect subtle changes in channel properties. Additionally, Bredt and colleagues did not examine other properties of the receptor altered by stargazin, such as rectification. Stargazin's homology to voltage-gated calcium channel subunits and its direct binding to AMPA receptors in the postsynaptic density suggests the fascinating possibility that TARPs alter synaptic channel function. The functional properties of voltage-gated sodium (Catterall, 2000), potassium (Nerbonne, 2000) and calcium channels (Arikkath and Campbell, 2003) are known to be modified by the presence of auxiliary subunits not involved in pore formation; however, stargazin is the first such auxiliary subunit defined for ionotropic glutamate receptors. Clarification of the role of TARPs at the plasma membrane will lead to a better understanding of native AMPA receptor function.

Chapter II

RESEARCH DESIGN AND METHODS

2.1 Neuronal Tissue Culture

2.1.1 Rat Hippocampal Neurons

Hippocampi dissected from the brains of newborn (P0-1) Sprague-Dawley rats were incubated with papain for 10 minutes in a 37°C water bath and further dissociated by trituration with a glass pipette. The cells were resuspended in 90% Dulbecco's modified Eagle's medium/10% FBS supplemented with 2 mM Glutamax (Life Technologies, Rockville, MD) and 1% penicillin/streptomycin at a density of 35-75 $x10^4$ per ml, plated on 35mm tissue culture dishes pre-coated with poly-L-lysine and maintained in a humidified 10% CO_2 incubator. These conditions inhibit neuronal survival and allow glial cell proliferation. After the glial monolayer became confluent, 33 μg/ml uridine and 13 μg/ml 5'-fluorodeoxyuridine were added to the culture medium to inhibit mitosis. Neurons from a second dissection were plated at a density of 75-500 $x10^4$ per ml onto the glial monolayer and maintained in 94% Dulbecco's modified Eagle's medium/5% horse serum/1% FBS, supplemented with 2 mM Glutamax, 5'-fluorodeoxyuridine, and 1% N3. Most experiments were performed at room temperature (23-25°C) using neurons 4-10 days in culture; recordings for hippocampal developmental studies were performed under the same conditions at DIV7, 14, and 21; recordings from transfected neurons were performed at DIV7.

2.1.2 Mouse Cerebellar Granule Cells

Primary cultures of cerebellar granule neurons were prepared as previously described (Bito et al., 2000). Briefly, cerebellae were dissected from P2 Swiss Webster mice, incubated in trypsin and dissociated by trituration with a glass pipette. Cells were resuspended in Minimal Essential Medium with Earle's salts (MEM, supplied without glutamine or phenol red), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 25 μg/ml insulin, and 2% B27 (Invitrogen, Grand Island, NY), and plated at a density of 1.6 cerebellae per 35mm dish. Dishes were coated with poly-D-lysine (100 ng/ml) and laminin (4 ng/ml) 24 hrs prior to the dissection. On the second day *in vitro* cultures were fed with MEM supplemented with 5% FBS, 0.5mM glutamine, 25 μg/ml insulin, 2% B27 and 10 μM cytosine arabinoside to inhibit glial division. Afterwards, cells were fed every 3 – 4 days with the 5% FBS medium, lacking Ara C. Cultures were maintained in a humidified 37°C, 5% CO_2 incubator. Note that these cultures are both plated and maintained in normionic K^+. All experiments were performed after 7 days *in vitro*.

2.1.3 Neuronal Transfection

Hippocampal neurons or cerebellar granule neurons were transfected on DIV5 using a Lipofectamine2000 (Invitrogen, Grand Island, NY) based protocol. For each 35mm dish to be transfected, 4 μg of DNA and 2 μl of lipid were diluted, separately, into 50 μl of DMEM and incubated for 5 min at room temperature. The diluted DNA and lipid were then combined and incubated for an additional 20 min at room temperature. During this

20 min incubation, 1 ml of media was removed from each dish (leaving ~1ml of media in the dish), and this conditioned media was reserved for later use. 100 μl of transfection solution was then added to each dish, the dishes were tilted to mix, and returned to their respective incubators. ~12 hrs after the initiation of transfection, 1 ml of conditioned media was returned to each dish, and the dishes were returned to their incubators. Electrophysiology experiments were conducted ~40 hrs after initiation of transfection. A trace amount of pEGFP (Clontech, Palo Alto, CA) was included in all transfections to allow identification of transfected cells during subsequent electrophysiology experiments. The stargazin (γ2) : green fluorescent protein (GFP) ratio used was 3 : 1 with control dishes receiving 4 μg of GFP.

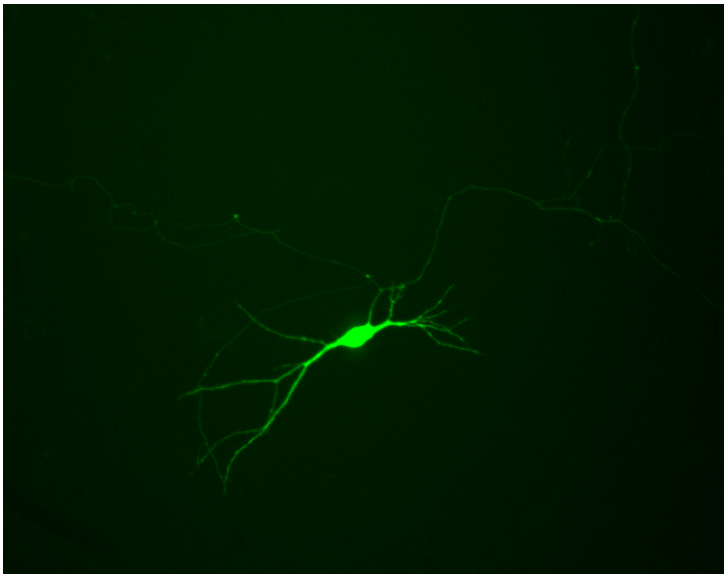


Diagram 9. Transfection of a hippocampal neuron with GFP. An example of a DIV7 rat hippocampal neuron expressing GFP is shown at approximately 40 hours post-transfection. Magnification is at 40X.

2.2 HEK293 Tissue Culture

For expression of recombinant AMPA receptors, human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas; VA) were grown at 37°C in a humidified 5% CO_2 incubator in 90% MEM plus Earle's salts/10% FBS in 25 cm^2 flasks. Once cells were 60-70% confluent, they were passaged and/or plated on 35 mm dishes for transfection and recording. Media was removed and cells were washed with MEM and then incubated for 2 minutes in trypsin-EDTA (0.02% trypsin, 0.53 mM EDTA). Media was added and the cells were centrifuged at 1500 rpm for 5 min, then resuspended in media. Cells were plated at 50-65 $x10^4$ per ml in 35 mm dishes for transfection or 1 ml of cells was combined with 5 ml media for passaging.

2.2.1 HEK293 Transient Transfection

HEK293 cells were transfected using a Lipofectamine™ Reagent-based protocol (Invitrogen Life Technologies, Grand Island, NY). Briefly, 2 μg of purified DNA and 8 μl Lipofectamine per 35 mm dish were diluted in OPTIMEM (room temperature, Invitrogen, Grand Island, NY), and incubated for 20 min at room temperature. Culture media was replaced with this transfection solution and cells were returned to 37°C incubator for 3 h. Afterwards, the DNA/lipid complexes were washed off the cells, and cultures were fed with MEM +10% fetal bovine serum or bovine growth serum containing 30 μM 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo[f]quinoxaline (NBQX) to block activation of expressed AMPA receptors by trace amounts of glutamate in the medium. Electrophysiology experiments were conducted at room temperature 40 to 48 h later.

GFP was included in all conditions to allow visualization of transected cells during electrophysiology experiments and excess GFP under a CMV promoter acted as a control in all other conditions. 2 μg total DNA per 35 mm dish was transfected for all HEK293 experiments. For experiments examining interactions of GRIP1, GRIP2 and GRASP-1 with AMPA receptors, the ratio of GluR-A_i : GluR-B_i was kept constant at 1 : 1.5 and the GRIPs were transfected at a 1 : 1 ratio with GluR-B_i. The transfection ratio of GRIP1/2 : GluR-B_i : GluR-A_i : GFP was 1.5 : 1.5 : 1 : 0.5. For experiments including GRASP-1, the ratio of GRIP1 : GluR-B_i : GluR-A_i : GRASP-1 : GFP was 1.5 : 1.5 : 1 : 1.5 : 0.5. For experiments examining PICK1 interactions, the ratio of GluR-A_i : GluR-B_i : PICK1 : GFP was 1 : 1.5 : 3 : 0.5. Because the PICK1 construct was driven by an SV40 promoter, a vector expressing the protein ranilla under an SV40 promoter was used as a control in these experiments.

For experiments examining stargazin (stg) interactions with homomeric AMPA receptors the ratio of GluR : stg : PSD-95 : GFP was 1 : 1 : 1 : 0.64, with conditions not containing stg and/or PSD-95 having additional GFP added to maintain a constant GluR : stg ratio in experiments without PSD-95. For heteromeric receptors a ratio of GluR-A : GluR-B : stg : GFP of 1 : 1.5 : 1.5 : 0.5 was employed. Kainate receptor subunits were transfected at a ratio of GluR-6 : KA2 : stg : GFP of 1 : 1 : 1 : 0.64, with conditions not containing stg and/or KA2 having additional GFP added to compensate.

Plasmids containing cDNA for the GluR subunits GluR-A, B, C, and D in both *flip (i)* and *flop (o)* isoforms were gifts from Dr. P. Seeburg (Heidelberg, Germany). Flag-tagged PICK1 in pFLAG under the control of an SV40 promoter was a gift from Dr. E. Olson (Dallas, TX). Dr. R. Huganir (Baltimore, MD) graciously provided GRIP1,

GRIP2, GRASP and PICK1 under the control of a CMV promoter. The plasmids were grown in *E. coli* and purified by 2X CsCl density centrifugation or using a plasmid kit (Qiagen, Inc., Valencia, CA). Restriction digests and gel electrophoresis were performed to verify the identity of the purifed construct. γ2, γ3, γ5 and various chimeric constructs were cloned by Dr. Dorothy Turetsky (Tulsa, OK). Cacng2 (γ2) was cloned from adult rat cerebellum, and Cacng3 (γ3) and Cacng5 (γ5) from adult rat forebrain total RNA using the ProSTAR Ultra HF RT-PCR system (Stratagene, La Jolla, CA), and the following primers: γ2forward 5' ATGGGGCTGTTTGATCGAGGTG 3', γ2reverse 5' TCATACGGGCGTGGTCCGGCG 3', γ3forward 5' ATGAGGATGTGTGACAGAGGTATCC 5', γ3reverse 5' TCAGACGGGCGTGGTGCGTCT 3', γ5forward 5' ATGAGCACCTGTGGGAGGAAGGC3', γ5reverse 5' TCAGCAGGGAGATGATGACATCTGG 3'. All three PCR products were subsequently subcloned into the expression vector pCMV-Script (Stratagene, La Jolla, CA) and sequenced for verification. The γ2/γ5 chimeras γ2(Δ26-103)/γ5(ins26-99) and γ2(Δ89-103)/γ5(ins85-99) were generated by taking advantage of unique *BsaB*I, *Bbs*I and *Xho*I sites in γ2 at nucleotide positions 72, 257 and 308 respectively. Both constructs were originally assembled in pGEM-T Easy (Promega, Madison, WI) and later transferred by PCR into pCMV-Script. The C-terminal truncation γ2(1-212) was generated by PCR using the γ2 forward primer (above) and the reverse primer: 5' TCAGGCCGTGGCCCGCAGCTGTTTG 3', and subcloned into pCMV-Script. Construct sequence was verified by sequencing at the Oklahoma State University Biochemistry Core Facility (Stillwater, OK).

2.3 Electrophysiology

2.3.1 Recording Solutions

Extracellular saline for neuronal experiments contained (in mM) 160 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$, and 0.01 mg/ml phenol red; the pH was titrated with NaOH to 7.3 and, when necessary, the osmolarity was adjusted to 325 mOsM with sucrose. Tetrodotoxin (400 nM; Calbiochem, La Jolla, CA), bicuculline (5 μM), and (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK-801; 5 μM) were added to block voltage-gated sodium channels, $GABA_A$ receptors, and NMDA receptors, respectively. The standard intracellular solution for neurons contained (in mM) 125 $CsMeSO_3$ (Aldrich Chemical Co., Milwaukee, WI), 15 CsCl, 10 HEPES, 5 Cs_4BAPTA (Molecular Probes, Eugene, OR), 0.5 $CaCl_2$, 3 $MgCl_2$ and 2 Na_2ATP. The osmolarity was adjusted to 295 mOsm when necessary and the pH was titrated to 7.2 using CsOH. Sucrose was added to standard neuronal intracellular solution to create a modified intracellular solution with higher osmolarity (345 mOsm) for peptide perfusion experiments. The pH was adjusted to 7.2 with CsOH.

Extracellular saline for HEK 293 cells contained (in mM) 145 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, 0.01 mg/ml phenol red. The pH was titrated to 7.3 with NaOH and the osmolarity was adjusted to 295 mOsm with sucrose when necessary. Patch pipettes were filled with intracellular solution containing (in mM) 135 CsCl, 10 CsF, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 Cs_4BAPTA, 10 HEPES, 2 Na_2ATP. Osmolarity was adjusted to 295 mOsm and pH to 7.2 with CsOH.

2.3.2 Whole Cell Voltage Clamp Recording Conditions and Drug Perfusion

The recording chamber was continuously perfused with control extracellular saline solution at ~0.4 ml/min. Bath solution for HEK 293 experiments contained 0.5 mM kynurenate, a non-selective antagonist of iontropic glutamate receptors, to protect against excitotoxicity and pre-exposure to agonists. Recording electrodes were pulled from borosilicate glass, coated with Sylgard, and fire-polished; typical electrode resistance was 2-7 MΩ when filled with intracellular solution and was compensated by 60-80% using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA). Recorded data was filtered at 1-3 kHz and sampled at 2-10 kHz using pClamp (Axon Instruments, Foster City, CA). See Ihle and Patnea, 2000 for further detail.

For whole-cell recording, rapid agonist application was achieved using a glass flowpipe array (10-12 parallel barrels, each 400 μm in diameter and a stepper-motor based system. The flowpipe array was placed near a voltage-clamped cell. Solutions were driven by a peristaltic pump (Minipuls3; Gilson, Middleton, WI and Dynamax; Rainin, Woburn, MA) through three-way Isolatch valves (Parker Hannifin, Fairfield, NJ) using a Warner Instrument Corporation SF-77B Perfusion Fast-Step system (Hamden, CT) to control the fast perfusion system and flowpipe movement, allowing millisecond solution exchange (τ = 6-8 ms) with highly repeatable time courses.

2.3.3 Peptide Perfusion

The short peptide pep2-SVKI (YNVYGIESVKI) has been used previously to disrupt interaction of the GluR-B C-terminal tail with GRIP, ABP, and PICK1. A more selective peptide, pep2-EVKI (YNVYGIRRVKI), blocks intereactions between the GluR-B C-

terminus and PICK1, exclusively (Li et al., 1999; Daw et al., 2000; Kim et al., 2001). pep2-SVKI and pep2-EVKI were obtained from Tocris (Ellisville, MO) and stored as 1 mM frozen stock (in H_2O). Shortly before electrophysiology commenced, the appropriate peptide, as well as the peptidase inhibitors leupeptin, bestatin and pepstatin A, were thawed and added to the intracellular solution for final concentrations of 100 μM, 100 μM, 100 μM, and 10 μM, respectively. Regular intracellular solution containing peptidase inhibitors alone was used as a control. Both solutions were kept on ice for the duration of the experiment. To compensate for the added volume of peptides and peptidase inhibitors, the osmolarity of the standard intracellular solution was increased from 295 mOsm to 345 mOsm with sucrose, as previously described.

Recordings from rat hippocampal or mouse cortical neurons voltage clamped at -60 mV were obtained immediately after whole cell configuration was achieved as intra-cell controls. Agonists and modulators were applied using fast perfusion techniques. After a 20 min interval allowing the peptide to fully disrupt GluR-B binding, a final set of recordings were obtained. Concentrations and perfusion times were based on the methods of Daw et al., 2000. Neurons were monitored by a brief glutamate pulse every 5 min. The recordings were analyzed for current density and kinetic differences. Initial recordings were obtained immediately after achievement of whole cell configuration and monitored by glutamate pulses every 5 minutes during the 20 min perfusion period. The initial protocol was repeated after peptide perfusion and both intra- and inter-cell analysis was performed.

Initial experiments examined the effect of perfusion of pep2-SVKI in DIV7 rat hippocampal neurons. A subsequent developmental study used the same protocol and

analysis on DIV4-5, 14-15, and 21-22 neurons. Recordings at each time point were obtained from a single dissection to control for variabilities in dissection and culture conditions. Mouse cortical neurons were also examined. Initial experiments were performed with pep2-SVKI. Because no significant differences in glutamate desensitization rate (Fig. 5B), absolute current density of glutamate- or kainate-evoked steady states or interactions the negative modulator GYKI 52466 (Fig. 5C) between control cells and those perfused with pep2-SVKI were seen during the 20 min incubation period (Fig. 5A), the lack of effect of pep2-EVKI was only confirmed in DIV 7 rat hippocampal neurons (data not shown).

2.4 Indirect Immunofluorescence

HEK293 cells were plated at $60x10^4$ per ml on a 24 well plate and transfected with various combinations of GluR-A_i, -B_i, PICK1 and flag-tagged GluR-D_i as a positive control 24 hr later. Cells were washed with neuronal extracellular recording solution to remove serum and then fixed for 30 min in 4°C 4% paraformaldehyde. Cells were washed with PBS and then either stored at 4°C or immediately processed. Some wells were permeabilized with 0.25% Triton-X 100 in PBS for 7 min at room temperature. All wells were blocked overnight at 4°C in 10% Normal Goat Serum (NGS) in PBS or 10% NGS in Flag TBS (to detect the Flag tagged epitope). Cells were incubated overnight at 4°C in primary antibody diluted in 2% NGS. The primary antibodies for each protein were diluted as follows: rabbit anti-GluR1 (-A) at 1:100, rabbit anti-GluR2/3 (-C/B) at 1:100, mouse anti-GluR2/4 (-B/D) at 1:500 and 1:250 (Chemicon International; Temecula, CA), and mouse anti-FLAG at 1:1000 (Sigma; St. Louis, MO). Anti-GluR2/4

was better visualized at the 1:250 dilution. Cells were washed in PBS or TBS to remove excess primary antibody and incubated in the dark at room temperature 30-60 min in secondary antibody diluted in 2% NGS in PBS or in TBS. Anti-Rabbit Cy^{TM}2 or Anti-Mouse Cy^{TM}3 (Jackson ImmunoResearch Laboratories; West Grove, PA) were used as appropriate at 1:100 dilution and visualized using a CoolSNAP Monochrome High Quality Camera (Photometrics; Tucson, AZ). Brightfield images were obtained using a CoolSNAP Color Camera (Photometrics; Tucson, AZ). Images were processed using Adobe Photoshop 7.0.

2.5 Quantitative Western Blots

2.5.1 Biotinylation of Surface Proteins

HEK293 cells were plated and transfected according to previously described protocol. AMPA receptor surface expression was measured using the membrane impermeant biotinylation reagent NHS-SS-biotin (Pierce Biotechnology, Rockford, IL) (Mammen et al., 1997). Forty-eight hours after transfection, cells were biotinylated using eZ-link-sulfo-NHS-SS-biotin (Pierce; Rockford, IL). Briefly, cells were washed once in 37°C PBS/Ca^{2+}/Mg^{2+} (10 mM phosphate buffer, 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4), allowed to cool briefly and then washed twice in 4°C PBS/Ca^{2+}/Mg^{2+} and placed on a cold pack. Cells were incubated at 4°C on the cold pack in 2 ml of biotinylation reagent [1mg/ml EZ-link-NHS-SS-biotin (Pierce; Rockford, IL) in PBS/Ca^{2+}/Mg^{2+}] for 15 minutes, washed three times with cold PBS/Ca^{2+}/Mg^{2+} with 1% BSA, and thoroughly scraped using a 1 ml syringe plunger in 166 µl of room temperature precipitation buffer (PB; 10 mM $NaPO_4$, pH 7.4, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 10 mM sodium

pyrophosphate, 50 mM NaF, 1mM Na_3VO_4, 2µl/ml leupeptin, and 2µl/ml aprotinin) with 1% SDS (PB/SDS). For some experiments, the 166 µl PB with 1% SDS was used to scrape cells from an adjacent well with the same transfection conditions to maximize protein yield. The well was washed with 833 µl PB with 1% Triton X-100 (PB-Triton) and the solution was collected in a microfuge tube, sonicated and centrifuged at 1600g for 20 min at 4°C. The top 800 µl of the supernatant was removed to a new microfuge tube and 200 µl Ultra Link® Immobilized Streptavidin Plus beads (Pierce; Rockford, IL) were added. The supernatant was rotated at 4°C for 2 hr and then either stored at -80°C or immediately separated into membrane fractions.

For the total protein fraction, 200 µl of bead evenly suspended in the supernatant were removed and 100 µl 3X sample buffer containing β-mercaptoethanol (BME) was added. For the cytoplasmic fraction, the remaining beads were microfuged for 1 min and the supernatant added to 400 µl 3X sample buffer + BME. The beads were then washed first with PB-Triton, then PB-Triton with 600 mM NaCl, and finally PB alone. For the membrane fraction, 1 ml of 1X sample buffer was added to the beads. All three samples were boiled for 3 min, mixed thoroughly, and microfuged. The supernatant was removed to microfuge tubes. The protease inhibitors aprotinin and leupeptin were added at 2 µg/ml in detergent solutions and 1 µg/ml in wash solutions.

2.5.2 Western Blot and Quantification

Novex® NuPAGE™ 7% Tris-Acetate gels were loaded with 25 µl of total, membrane and cytoplasmic fractions of glutamate receptor subunits expressed with and without stargazin (γ2). A dilution series of 50, 20, and 10% of the total protein fraction was also

run on the same gel to verify quantification. Proteins were transferred to an Invitrolon PVDF membrane (Carlsbad, CA), which was then gently agitated for 2 hr at room temperature or overnight at 4°C with either 3% bovine serum albumin or 5% milk in TBS-tween to block. Blots were incubated with primary antibody in the appropriate block overnight at 4°C. The following primary antibodies were used: rabbit anti-GluR1 at 1:400, rabbit anti-GluR2/3 at 1:200 and mouse tubulin (MAB3804) at 1:1000 (Chemicon International; Temecula, CA). After three 5 min washes in TBS/Tween (TBS: 20 mM Tris Base, 137 mM NaCl; 1% Tween), blots were incubated for 1 hr at room temperature in secondary antibody at 1:10,000. HRP-conjugated mouse and rabbit secondary antibodies from Amersham Biosciences ECL^{TM} Plus Western blotting kit were used. After secondary antibody incubation, blots were washed 3X with TBS/Tween and incubated for 5 minutes with LumigenTM PS-3 detection reagent and imaged using a Storm PhosphorImager (Molecular Dynamics). ImageQuant was used to quantify bands.

Certain gels were stripped and then reprobed for tubulin. Blots were incubated for 30 min in a 50°C water bath with occasional agitation in stripping buffer (100mM BME, 2% SDS, 62.5 mM Tris-HCL, pH 6.7) and then 3X with TBS Tween for 10 min at room temperature. The blot was blocked at room temperature with gentle agitation for 1 hr and then incubated overnight in primary antibody. Secondary antibody incubation and imaging were as previously described.

2.6 Statistical Analysis

Peak and steady-state amplitude and kinetic measurements were based on single or the average of a few (2-5) agonist-evoked responses measured using Clampfit 8.1 or

Clampfit 9.2 (Axon Instruments, Foster City, CA). The kinetics of desensitization and onset and offset of modulation were determined using a standard Chebyshev fitting algorithm generated by Clampfit 8.1 or Clampfit 9.2. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA) or SPSS (SPSS, Inc.; Chicago, IL). For single comparisons, the data were evaluated using an unpaired Student's *t*-test. For multiple comparisons, the data were evaluated using 1-way ANOVA followed by Bonferroni's *post hoc* test to evalulate specific comparisons. The level of significance is set at $P<0.05$. Bar graphs, tables and all data in text are reported as mean ± SEM unless reported otherwise.

For experiments investigating the functional properties of stargazin (γ2) and other members of the TARP family, we calculated maximal current density by dividing peak current obtained by co-application of 3 mM glutamate and 500 μM trichlormethiazide by whole cell capacitance (pA/pF) as a functional assay for AMPA surface expression. Trichlormethiazide is a cyclothiazide analog with faster association and dissociation kinetics that reduces glutamate-evoked desensitization in hippocampal neurons to a similar degree as 100 μM cyclothiazide (Fig. 1). Time constants for onset of desensitization, association and dissociation of kainate, and onset of potentiation by TCM were determined by exponential fits using a Chebyshev algorithm. Time constants for TCM dissociation in hippocampal neurons were calculated from 50% decay times relative to glutamate steady-state response (τ = 50% decay time/0.69). The validity of this method was verified in selected cells in which longer recovery times were well fit with a single exponential function.

Because glutamate peaks in whole cell recordings can be affected by desensitization, both percent desensitization and current density were determined relative to maximum glutamate-evoked responses in the presence of the positive allosteric modulator trichlormethiazide. As an assay for inclusion of GluR-B in heteromeric GluR-A/B receptors in HEK293 cells +40/-60 rectification ratios for glutamate peak were calculated and cells with a rectification ratio of <0.5 were excluded from the data set.

Chapter III

STATE DEPENDENT INTERACTION OF THE BENZOTHIADIAZINE POSITIVE ALLOSTERIC MODULATORS WITH THE AMPA RECEPTOR

3.1 Introduction

Several drugs have been identified that slow or reduce AMPA receptor desensitization, most notably the benzothiadiazine cyclothiazide (Patneau et al., 1993; Yamada and Tang, 1993). Cyclothiazide exhibits marked selectivity for *flip* over *flop* AMPA receptor isoforms (Partin et al., 1994), with the selectivity being determined by a single residue in the *flip/flop* domain (Partin et al., 1995; Partin et al., 1996). Cyclothiazide almost fully blocks desensitization of *flip* isoforms in all four AMPA receptor subunits, but appears to merely slow the rate of onset of desensitization of *flop* isoforms. Its mechanism of action is frequently described as reduction or attenuation of desensitization (Nagarajan et al., 2001; Horning and Mayer, 2004), and the difference in its effects on *flip* versus *flop* receptors has been attributed to a difference in the affinity of cyclothiazide for the isoforms (Partin et al., 1994; Sun et al., 2002).

Previous work has shown that at saturating concentrations (100 μM) cyclothiazide blocks desensitization of AMPA receptor currents in most neurons (Patneau et al., 1993; Yamada and Tang, 1993). At subsaturating concentrations (1 μM, 3 μM), cyclothiazide potentiates glutamate steady-state currents but does not completely abolish desensitization. The rate of desensitization remains the same as that seen in the absence

of cyclothiazide (Diagram 9). The most straightforward interpretation of this data is the presence of two populations of receptors: one that is bound by agonist and exhibits normal desensitization kinetics and a second population of non-desensitizing receptors bound by both agonist and modulator. Similar effects with the AMPAkine CX546 have been interpreted as an effect on recovery from desensitization rather than an alteration in the kinetics of entry into the desensitizated state (Nagarajan et al., 2001).

In the dimeric crystal structure, cyclothiazide binds to residues in both subunits, suggesting that it acts as a bridge stabilizing the dimer interface (Sun et al., 2002), thus favoring the open, non-desensitized state of the receptor. This crystal structure makes several predictions regarding the mechanism of action of cyclothiazide that can be directly tested electrophysiologically in intact receptors. First, if cyclothiazide binds at the dimer interface, then the conformational change to the desensitized state disassembles the binding site, precluding the binding of cyclothiazide to desensitized receptors. Second, the rate at which cyclothiazide can bind in the presence of agonists should reflect the agonist-dependent rate at which the receptor recovers from desensitization. Third, receptors with cyclothiazide still bound cannot desensitize.

The aim of these studies is to verify and extend previous findings regarding the benzothiadiazines and their effects on receptor desensitization, specifically focusing on their mechanism of action at intact AMPA receptors. We have therefore examined binding of cyclothiazide and related benzothiadiazine modulators in the presence and absence of agonists using patch-clamp recording and rapid perfusion techniques, taking advantage of the fact that different AMPA receptor agonists have different affinities for

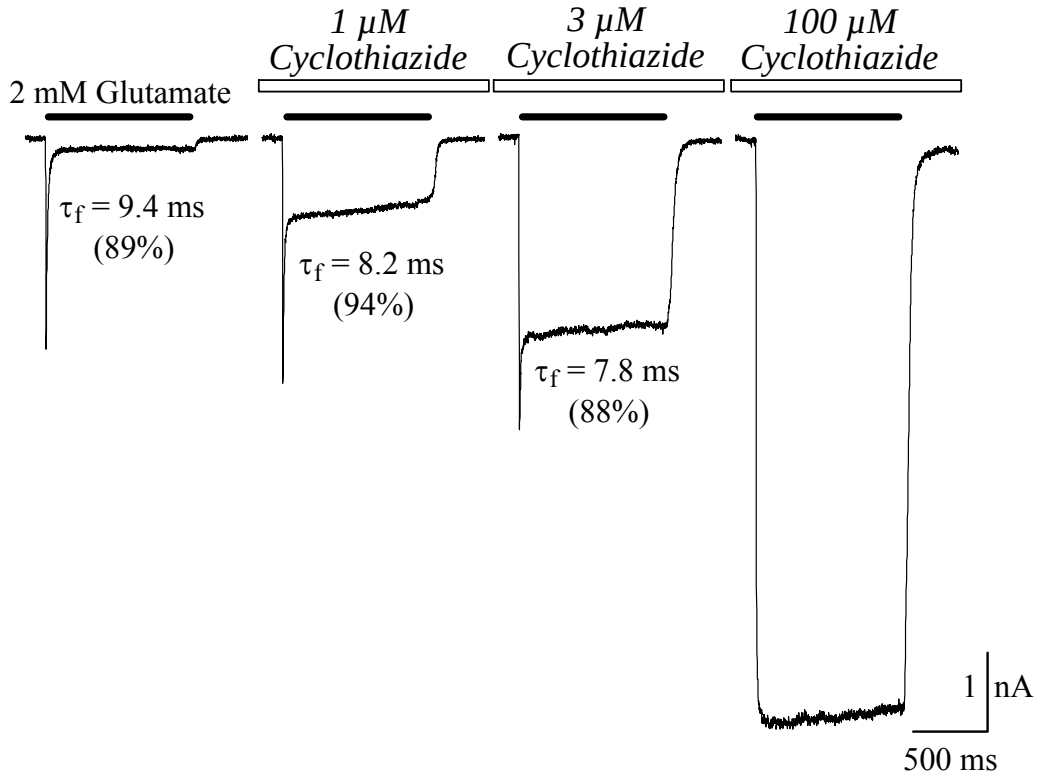


Diagram 10. Low concentrations of cyclothiazide reduce steady-state desensitization without slowing the rate of onset of desensitization. Illustrated are responses of a single hippocampal neuron to 2 mM glutamate alone and in combination with 3 concentrations of cyclothiazide. The onset of desensitization in the response to glutamate was fit with the sum of two exponentials, with the fast time constant and relative amplitude given next to each trace. The kinetics of onset of desensitization in response to glutamate are unaffected in the presence of 1 and 3 μM cyclothiazide, despite the substantial reduction in the amount of desensitization produced by glutamate. 100 μM cyclothiazide fully blocks desensitization. With concentrations of cyclothiazide that are not saturating, it appears that there are two populations of receptors present: one population of receptors which is unbound by cyclothiazide and desensitizing with normal rapid kinetics, and a second population bound by cyclothiazide which does not desensitize at all (unpublished data from the lab of Doris Patneau).

the desensitized state (Patneau and Mayer, 1991). The results demonstrate that agonist- and state-dependent interactions of the benzothiadiazines with their binding site reflect the inability of the modulator to bind the desensitized conformation of the receptor.

3.2 Statement of Hypothesis

Interaction of the benzothiadiazine class of positive allosteric modulators with AMPA receptors involves a state-dependent interaction in which the modulator cannot bind to the desensitized conformation of the receptor; conversely, the receptor cannot desensitize when modulator is bound.

3.3 Results

3.3.1 Benzothiadiazine Interaction with the AMPA Receptor Is Modulator-Dependent

The benzothiadiazines cyclothiazide (CTZ), trichlormethiazide (TCM) and hydroflumethiazide (HF) have dramatic effects on AMPA receptor desensitization (Fig. 1). Figure 1C compares the potency of block of desensitization by preapplication of HF and TCM with the amount of current elicited in the presence of 100 μM CTZ, a concentration shown to completely block desensitization (Patneau et al., 1993). Application of these modulators to a voltage-clamped hippocampal neuron during glutamate-evoked desensitization shows distinctly different rates of onset of modulation for each drug (Fig. 1D), which were well fit by the sum of two exponentials (Table 5). The rate of onset of modulation appears related to the size and hydrophobicity of the side chain at position three (Fig 1A, *), with CTZ exhibiting the slowest onset of modulation

and HF exhibiting the fastest. We chose to perform initial experiments with HF as this modulator significantly reduces desensitization, and, importantly, has binding kinetics that do not appear to be rate-limiting.

3.3.2 Agonist-Dependent Onset of Modulation by Hydroflumethiazide Suggests a State-Dependent Interaction

AMPA receptor agonists exhibit a distinct pattern of interaction with the three functional states of the native receptor: resting, conducting, and desensitized (Diagram 3 and 6). Because different AMPA receptor agonists have different affinities for the desensitized state (Patneau and Mayer, 1991), we expected to observe agonist-dependent differences in modulator binding. Representative traces of the onset of modulation by HF in the presence of three agonists with distinctly different desensitization profiles (Patneau and Mayer, 1991) are shown in Figure 2A: kainate, a low affinity, weakly desensitizing agonist; glutamate, the strongly desensitizing endogenous ligand; and quisqualate, a high-affinity, strongly desensitizing agonist. The onset of the modulation by HF was slowest in the presence of quisqualate. The onset of modulatory effects in the presence of the endogenous agonist glutamate and the selective ligand AMPA, which generate similar peak and steady-state amplitudes, was significantly faster ($p<0.001$) than the onset of modulation in the presence of quisqualate. The most rapid onset of modulation was seen in the presence of the weakly desensitizing partial agonist kainate (Fig. 2B, Table 1), with the rate of onset of modulation by HF more than 20 times faster than in the presence of the strongly desensitizing agonist quisqualate ($p<0.001$). In fact, binding of HF was almost as rapid in the presence as in the absence of kainate (data not shown).

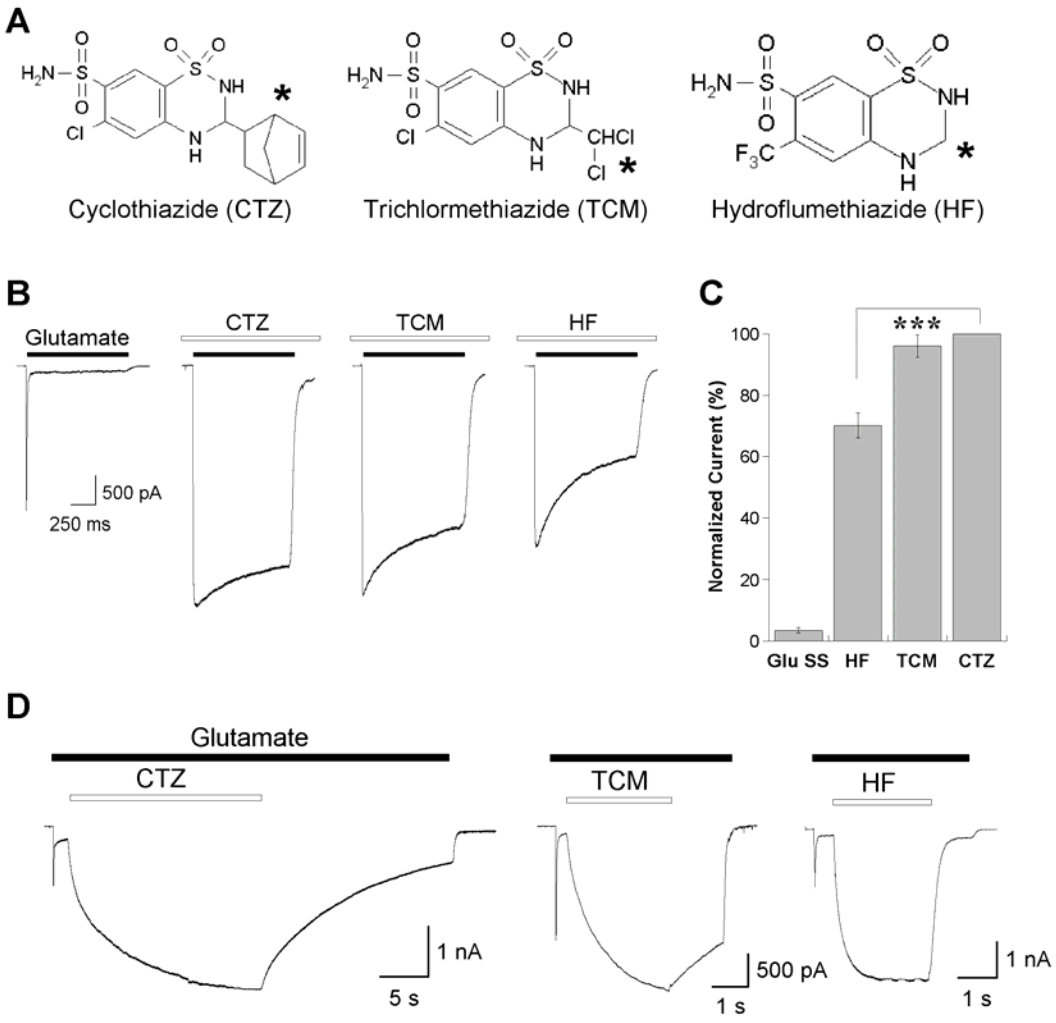


Figure 1. The onset of modulation by the benzothiadiazines is modulator dependent. A. Stuctures for the benzothiadiazines used in this study. The large constituent group found at position three (*) in trichlormethiazide (TCM) and cyclothiazide (CTZ) slows both association with and dissociation of the modulator from the AMPA receptor. B. Traces illustrating block of 3 mM glutamate-evoked desensitization in cultured hippocampal neurons by 1 mM HF, 500 μM TCM, and 100 μM CTZ. C. Summary of the relative degree of block of desensitization in cultured hippocampal neurons by HF and TCM relative to CTZ, which completely blocks AMPA receptor desensitization. Peak current measurements were obtained by pre-application of modulator to a hippocampal neuron followed by a 1 s co-application with 3 mM glutamate. All three modulators generated significantly larger currents than the peak glutamate response (*** $p<0.001$, 1-way ANOVA, Bonferroni's multiple comparison). Also shown relative to the CTZ peak response is the glutamate steady-state response, demonstrating how rapidly and extensively native receptors desensitize upon agonist binding. D. Representative traces obtained through whole-cell patch-clamp recording of the rate of onset of modulation of 1 mM hydroflumethiazide (HF) and 500 μM TCM in the presence of 1 mM glutamate, or 30 μM CTZ in the presence of 200 μM glutamate. Note the time scales for each trace.

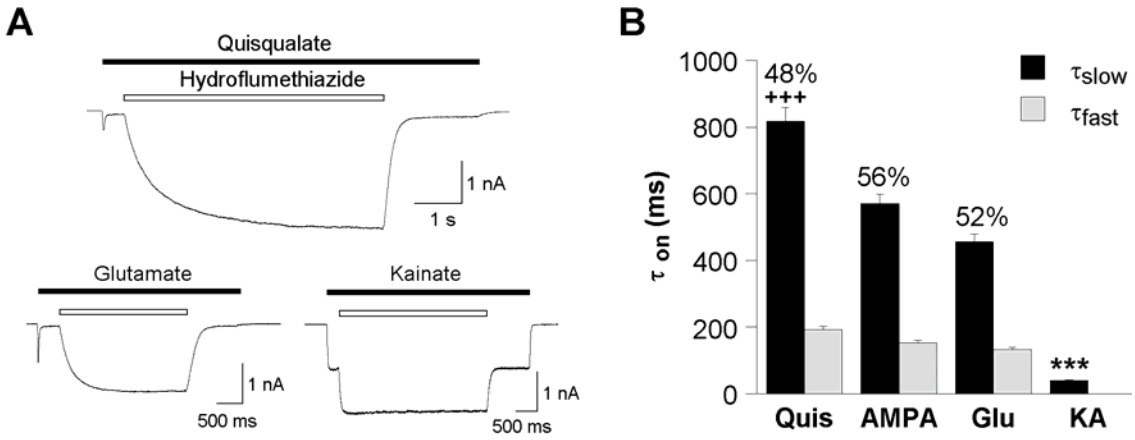


Figure 2: The onset of modulation by the benzothiadiazines is agonist-dependent. A. Representative traces show the agonist dependence of the rate of onset of modulation by HF (1 mM) in the presence of a low affinity, weakly desensitizing agonist (600 μM kainate), a strongly desensitizing endogenous agonist (200 μM glutamate) and a strongly desensitizing, high affinity agonist (10 μM quisqualate). B. The rates of onset of potentiation in the presence of quisqualate, AMPA (100 μM) and glutamate were best fit by the sum of two exponentials, while that in the presence of kainate was best fit by a single exponential. The amplitude of the slow component of the rate of onset of modulation is shown above as the percentage of the sum of the fast and slow components (n=24-28). The slow component of onset of modulation by HF in the presence of quisqualate is significantly different from the slow component of onset of modulation in the presence of AMPA, glutamate or kainate (+++, $p<0.001$, 1-way ANOVA, Bonferroni's multiple comparison). The rate constant describing the rate of onset of modulation of HF in the presence of kainate is significantly different from the slow component of onset of modulation in the presence of the other three agonists (***, $p<0.001$). The rate of onset of modulation of HF in the presence of AMPA versus glutamate is not significantly different ($p>0.05$).

TABLE IV

Summary of Onset Kinetics of the Benzothiadiazines

		Cyclothiazide			Trichlormethiazide			Hydroflumethiazide		
Agonist		τ (ms)	A_{slow}	n	τ (ms)	A_{slow}	n	τ (ms)	A_{slow}	n
Kainate	τ_{slow}	2306±464	68%	13	457±58	35%	10	40±1	--	24
	τ_{fast}	501±53			93±19			--		
Glutamate	τ_{slow}	5179±756	63%	10	1176±113	75%	9	457±43	52%	26
	τ_{fast}	751±102			237±6			133±16		
AMPA	τ_{slow}	--	--	--	1419±90	71%	23	571±42	56%	25
	τ_{fast}	--			261±20			153±15		
Quisqualate	τ_{slow}	6461±978	90%	3	--	--	--	1361±152	48%	28
	τ_{fast}	1711±125			--			316±51		

Table 4. Summary of the rates of onset of modulation by the benzothiadiazines in the presence of various agonists. The rate of onset of modulation by the benzothiadiazine modulators was obtained by whole-cell voltage clamp recording from cultured hippocampal neurons. Shown above are the rates of onset of modulation by 1 mM HF in the presence of 100 μM AMPA, 200 μM glutamate and 10 μM quisqualate and 500 μM HF in the presence of 600 μM kainate. Also shown are the rates of onset of modulation by 500 μM TCM in the presence of 300 μM AMPA, 600 μM kainate, and 1 mM glutamate, as well as the rates of onset of modulation by 30 μM CTZ in the presence of 1 mM glutamate, 600 μM kainate and 200 μM quisqualate. The amplitude of the slow component of the rate of onset of modulation is calculated according to the formula [$\tau_{slow}/(\tau_{slow} + \tau_{fast})$ X 100]. Time constants are shown as milliseconds ± SEM.

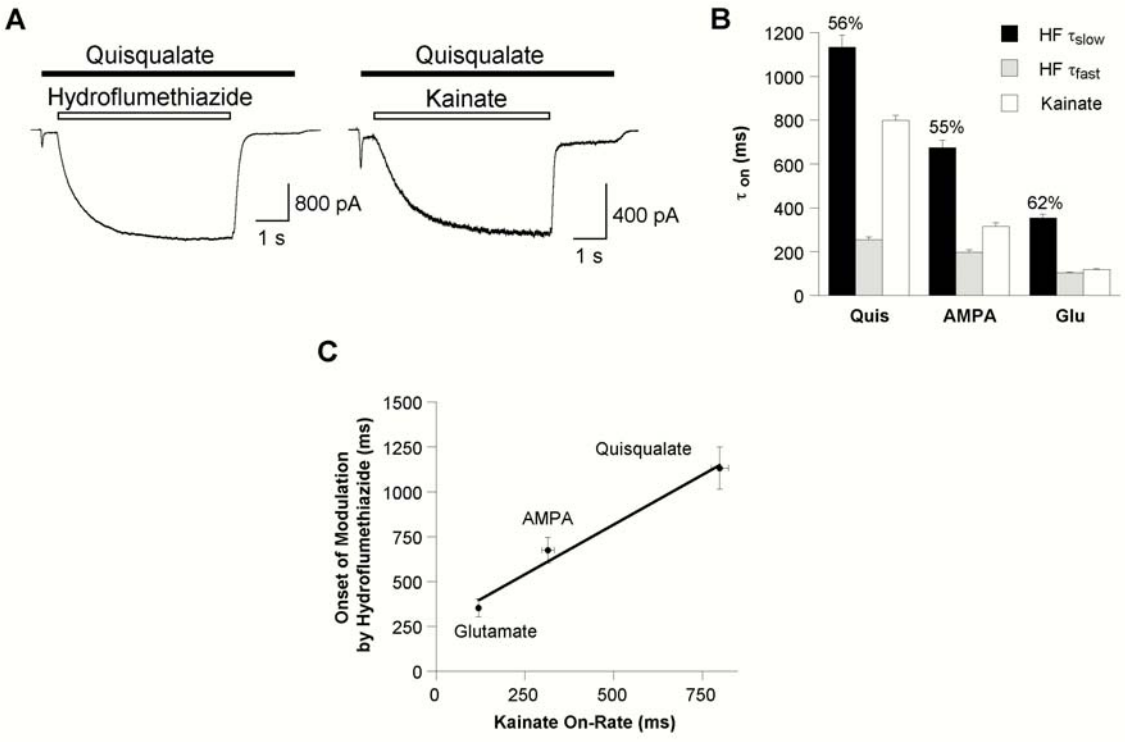


Figure 3. A correlation between the kinetics of recovery from desensitization and the onset of modulation by hydroflumethiazide. A. Representative traces showing the onset of modulation by HF (1 mM) and the on-rate of kainate (3 mM) in the presence of quisqualate (10 μM). B. Summary data for mean rate of onset of modulation of HF, best fit by the sum of two exponentials, and the mean on-rate of kainate, best fit by a single exponential function, in the presence of quisqualate, AMPA (100 μM) and glutamate (200 μM; n=9-12). The amplitude of the slow component of the rate of onset of modulation is represented as the percentage of the sum of the fast and slow components. C. A strong correlation exists between the slow component of the onset of modulation by the benzothiadiazine modulator and recovery from desensitization as measured by the on-rate of kainate in the cross-desensitization paradigm (r^2=0.98, error bars represent mean ± SEM).

These results, combined with the data for CTZ and TCM in Table 1, show that the binding of the benzothiadiazine modulators is agonist dependent. The profile of agonist-dependence is strikingly similar to the relative rates of recovery from desensitization for each agonist published by Patneau and Mayer (1991), suggesting a state-dependent interaction with the receptor in which the positive modulator is unable to bind the desensitized receptor. Alternatively, the modulator may bind to the desensitized state, but the conformation of this state precludes the molecular interactions between receptor and modulator necessary for stabilization of the conducting state.

3.3.3 The Rate of Onset of Modulation Examined Within a Cross-Desensitization Paradigm

If the benzothiadiazines can only bind or exert modulatory effects after the receptor exits the desensitized state, the rate of onset of modulation should reflect the rate at which the receptor recovers from desensitization. Previous studies have utilized a cross-desensitization paradigm in which the rate of binding of an agonist with weak affinity for the desensitized state (e.g., kainate) in the presence of a strongly desensitizing agonist can be used as an independent measure of the rate of AMPA receptor recovery from desensitization (Patneau and Mayer, 1991). We used within cell analysis to compare the on-rate of kainate with the onset of modulation by HF in the presence of quisqualate, AMPA, and glutamate (Fig. 3A). Although we could not directly compare the on-rate of kainate, which was best fit by a single exponential function, with the onset of modulation by HF, which was best fit by the sum of two exponentials, a strong correlation exists between the slow component of onset of modulation by HF and the on-rate of kainate

(r^2=0.98, Fig. 3C). A strong correlation also exists between the on-rate of kainate and the fast component of onset of modulation by HF (r^2=0.86, data not shown)

3.5 Discussion

Our exploration of the agonist-dependence of AMPA receptor modulation by cyclothiazide and other benzothiadiazine positive allosteric modulators reveals a state-dependent interaction that reflects the inability of modulator to bind the receptor in the desensitized state. In keeping with microscopic reversibility, the converse must also be true, that AMPA receptors cannot enter the desensitized state when cyclothiazide is bound. Our results confirm and extend molecular and crystallography work on the mechanism of AMPA receptor desensitization and its modulation by cyclothiazide (Stern-Bach et al., 1998; Partin, 2001; Sun et al., 2002; Horning and Mayer, 2004). As previously discussed (Section 1.3), in the structural model for activation and desensitization of AMPA receptors the agonist binding site is formed by two extracellular domains within a single subunit, domain 1 (D1) and domain 2 (D2), assembled into a "clamshell" conformation (Stern-Bach et al., 1994; Armstrong et al., 1998; Armstrong and Gouaux, 2000; Mayer et al., 2001; Hogner et al., 2002; Sun et al., 2002). In the resting state, two adjacent subunits form a dimer via interactions at the interface between the D1 domains (Figure 4A). Agonist binding at the ligand binding core near the hinge region of the clamshell produces movement of the D2 domains, which is communicated to the transmembrane domains to open the channel. Further closure of the "clamshell" destabilizes the D1-D1 interactions between adjacent subunits, facilitating transition into

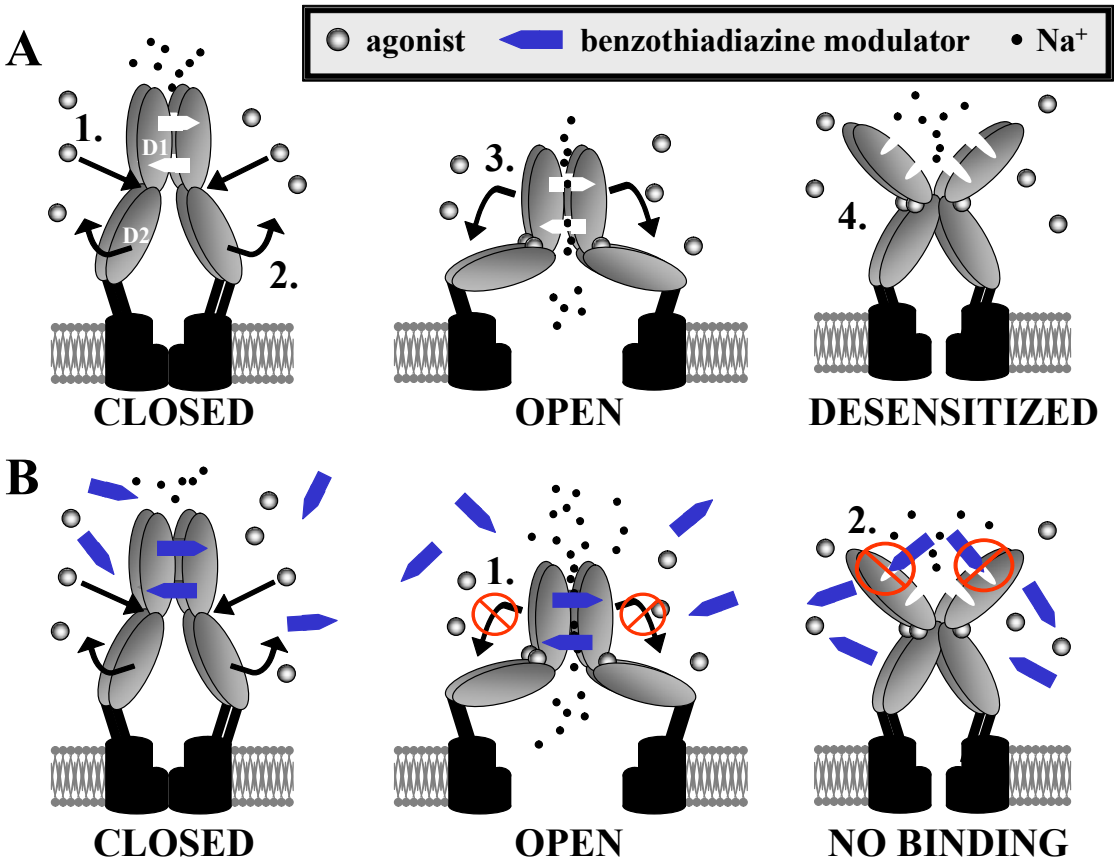


Figure 4. Model of channel activation and desensitization in the presence of agonist and modulator. A. In the absence of modulator, agonist binds the ligand binding core (1), interacting with residues from both domain 1 (D1) and domain 2 (D2) and inducing domain closure (2), a conformational change linked to channel activation (3). This is followed by domain rearrangement into the lower energy desensitized state (Armstrong and Gouaux, 2000). B. Mutations that stabilize the D1-D1 interface inhibit the conformational change into the desensitized state (Stern-Bach et al., 1998; Sun et al., 2002). Likewise, cyclothiazide and its benzothiadiazine family members stabilize the D1-D1 interface through interactions with both subunits forming the interface and prevent the transition into the desensitized state (1). The benzothiadizines clearly binds the receptor in the resting and conducting states; however, these compounds cannot bind when the receptor is in the desensitized state (2, see Appendix Fig. 2A). This implies that the benzothiadiazines require interaction with both portions of the binding site in order to modulate current at AMPA receptors.

the lower energy desensitized state and closing the channel (Armstrong et al., 1998; Sun et al., 2002; Horning and Mayer, 2004).

In the crystal structure of the soluble extracellular domains of GluR2*flop*N754S, two cyclothiazide molecules bind at and stabilize the dimer interface through interactions with residues near the agonist binding site and in the *flip/flop* region (Partin et al., 1996; Partin, 2001; Sun et al., 2002; Leever et al., 2003), shifting the K_D for dimerization to the left >1000-fold. Our data indicate that binding of cyclothiazide at this interface does not slow the rate of entry of the receptor into the desensitized state but rather prevents the conformational transition into the desensitized state (Fig. 4B). Because desensitization involves loss of interdomain contacts and separation of the dimer interface, the structural model predicts that cyclothiazide cannot interact with both sides of its binding site simultaneously in the desensitized state. Other data from our lab does not support the idea that cyclothiazide binds to one subunit in the desensitized state, and then, as the receptor recovers from desensitization, interacts with the other half of the dimer to hold the receptor in the non-desensitized state (Appendix Fig. 2A). Instead, the state- and agonist-dependence of modulation suggests that, without close apposition of adjacent D1 domains, the binding site for cyclothiazide is essentially abolished in the desensitized receptor; therefore, the benzothiadiazines bind only after the receptor recovers from desensitization and the interface is restored.

As predicted by the model, the onset of modulation by the rapidly binding drug hydroflumethiazide in the presence of agonist varied directly with the relative rates of recovery from desensitization for different agonists (Figure 2; Table 1). Unlike the on-rate of kainate, the onset of potentiation by HF in the presence of strongly desensitizing

agonists was best fit by the sum of two exponentials; also, the slow component was consistently slower than the on-rate of kainate in the cross-desensitization paradigm. We believe this discrepancy is a consequence of comparing two pharmacologically distinct mechanisms of action. HF binds allosterically to the non-desensitized receptor in the presence of agonist, while kainate competes with the more strongly desensitizing agonists for the binding cleft in both desensitized and non-desensitized states, albeit more effectively for the non-desensitized state (Patneau et al., 1993). In combination with evidence from this and other studies, we believe the strong correlation between the on-rate of kainate and τ_{slow} of the onset of modulation by HF supports the model for state-dependent binding of the benzothiadiazines.

The differential interaction of the benzothiadiazine modulators with the *flip* and *flop* isoforms of the AMPA receptor has been explained by weaker binding of CTZ in the presence of a bulkier asparagine versus a serine residue (N/S) at a critical position in the *flip/flop* region of the dimer interface (Partin et al., 1995; Sun et al., 2002). Data generated in this lab shows that cyclothiazide binds with similar affinity to both splice variants in the resting state (Appendix, Fig. 2), indicating this is an incomplete explanation. Differential modulation of *flip* and *flop* isoforms appears to be a consequence of allosteric interactions between ligand and modulator binding sites, with with negative cooperativity between agonist and benzothiadiazine binding sites accounting for the rapid decay of CTZ-mediated currents at *flop* AMPA receptor subunits. Chimera and crystallography studies indicate close proximity of residues involved directly in agonist binding with those involved in desensitization (Table 1) (Stern-Bach et al., 1998; Armstrong and Gouaux, 2000; Partin, 2001; Horning and

Mayer, 2004). Binding studies (Kessler et al., 1996) and dose-responses analyses (Patneau et al., 1993) show changes in agonist affinity and efficacy in the presence of CTZ. Thus, a functional link between the agonist binding site and the benzothiadiazine binding sites at the dimer interface is well supported.

In the context of previously published work, our data suggest that the nature of the allosteric interaction between agonist and modulator binding sites is defined by the identity of bound agonist as well as which subunits and splice isoforms comprise the dimer interface. Cyclothiazide slows AMPA receptor deactivation rates two- to three-fold in the presence of kainate, while increasing the apparent affinity of kainate to a similar degree (Patneau et al., 1993), reflecting positive cooperativity between binding of cyclothiazide and kainate. It is interesting to note that the shape of glutamate-evoked responses in the presence of another modulator presumed to share the same mechanism of action as cyclothiazide indicates positive cooperativity between the binding of glutamate and the modulator LY404187 for GluR2*flip* (steadily increasing currents with prolonged application), and negative cooperativity for GluR2*flop* (Quirk and Nisenbaum, 2003).

The time constants for dissociation of cyclothiazide from GluR-A_oB_o and GluR-A_iB_o receptors in the absence of agonist were almost identical (Appendix, Fig. 2C); however, cyclothiazide fully blocks desensitization of A_iB_o receptors at equilibrium, but has little effect on equilibrium responses of A_oB_o receptors (Partin et al., 1994). Thus the modulator binding sites at the dimer interface formed by these two tetramers have equivalent affinity in the resting state but differ in the conformational changes that occur upon agonist binding. Recent data showing that dimers are preferentially assembled from

one *flip* and one *flop* subunit (Brorson et al., 2004) suggests that each dimer interface in receptors comprised of *flip* and *flop* subunits will possess one cyclothiazide binding site with a serine in the critical position. It appears that a single cyclothiazide molecule bound at the dimer interface can confer complete block of desensitization. Therefore, maintenance of the interface by a single cyclothiazide molecule is sufficient to block the agonist-induced conformational change that disrupts the binding of cyclothiazide in pure *flop* receptors. We would also predict that crystallization of the GluR-B*flop* extracellular domains in the presence of cyclothiazide, but without glutamate, will show a substantive leftward shift in the K_D for dimerization, that will be lost in the presence of agonist.

Chapter IV
PERIPHERALLY ASSOCIATED AMPA RECEPTOR BINDING PROTEINS DO NOT ALTER AMPA RECEPTOR KINETICS

4.1 Disruption of GRIP and PICK1 Binding in Hippocampal Neurons

GluR-B (also termed GluR2) contains a type II PDZ domain at its extreme C-terminus that mediates binding to a variety of PDZ-domain containing proteins including GRIP1, GRIP2 and PICK1. PICK1 and the GRIPs are believed to play a role in regulating the removal, insertion and stabilization of AMPA receptors at synaptic and internal membrane sites during long term modifications of synaptic strength. The GRIPs appear to function primarily as membrane anchors while PICK1 plays a more dynamic role. Association of the PDZ domain containing protein PSD-95/SAP90 with kainate receptor subunits expressed in heterologous cells has been shown to reduce receptor desensitization (Garcia et al., 1998). These findings suggest that intracellular associations can modify channel function, prompting experiments to determine the effects of disrupting the association of GluR-B with GRIP1 and PICK1 on agonist-evoked responses in dissociated hippocampal neurons.

4.1.1 Statement of Hypothesis

The association of GRIP1, GRIP2 and PICK1 with the GluR2 subunit of the AMPA receptor alters channel properties in a manner that can be detected by whole-cell patch-clamp electrophysiology.

4.1.2 Results

Initial examination of the association of GRIP1 and PICK1 with endogenous AMPA receptors was examined using intracellular perfusion of short peptides that competitively disrupt GRIP1 and/or PICK1 association with GluR-B (see Section 2.3.3). We found no significant differences between control and experimental conditions in any of the parameters examined (Figure 5, data not shown). Studies using these inhibitory peptides have primarily focused on their effects on LTP and LTD (Daw et al., 2000; Kim et al., 2001). In our cultures, we examined only possible kinetic differences resulting from disruption of peptide binding; thus, in the absence of the synaptic rearrangements of LTP and LTD, kinetic differences may not be obvious. Secondly, our recordings were obtained from both synaptic and extrasynaptic receptors. Because synaptic receptors only comprise approximately 4-10% of surface receptors (Shi et al., 1999), extrasynaptic receptor currents may have masked changes in synaptic receptor kinetics.

4.2 Association of AMPA Receptor Subunits with GRIP or PICK1 in HEK293 Cells

Continued examination of the interaction of cytoskeletal proteins with the AMPA receptor was performed using transfected HEK 293 cells. The peripheral association of GRASP-1 was also examined. GRASP-1 is a neuron-specific guanine nucleotide

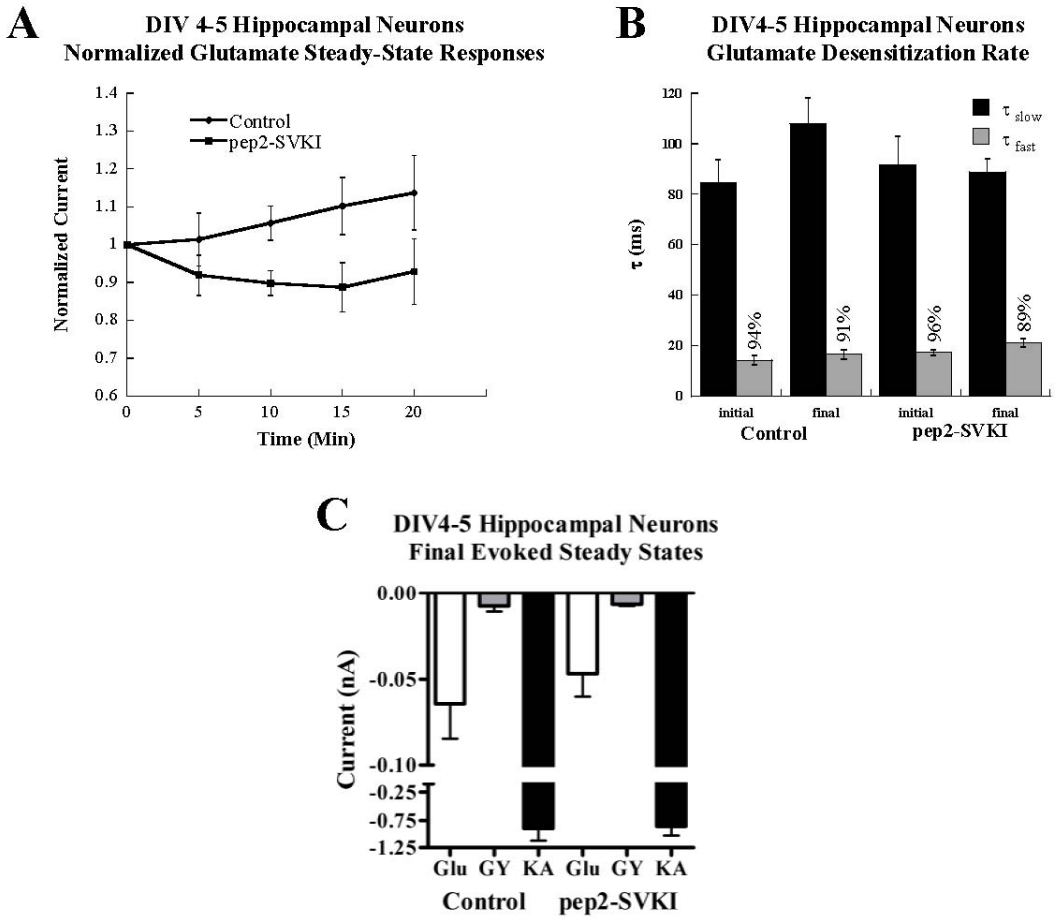


Figure 5: Disruption of interaction of GluR-B with GRIP1 and/or PICK1 interaction using pep2-SVKI perfusion of cultured hippocampal neurons. A. Responses to 3 mM glutamate were recorded immediately upon achieving whole-cell configuration and every 5 min during the perfusion period. Steady-state responses were normalized to the initial response. A slight, but not significant, decrease in current in cells perfused with pep2-SVKI versus a slight, non-significant increase with control intracellular solution is evident. B. The rate of desensitization of 3 mM glutamate, measured initially upon achieving whole-cell configuration and after a 20 min perfusion period, was not significantly different in cells perfused with pep2-SVKI *vs.* control intracellular solution. The relative proportion of the fast component of desensitization (τ_{fast}) is stated as a percentage of the sum of the slow and fast components. C. Steady-state currents evoked by 3 mM glutamate (Glu), 3 mM glutamate + 30 μM GYKI 52466 (GY), or 600 μM kainate (KA) were not significantly different between neurons perfused with control intracellular solution *vs.* pep2-SVKI. Bars represent mean ± SEM; Control, n=10; pep2-SVKI, n=10

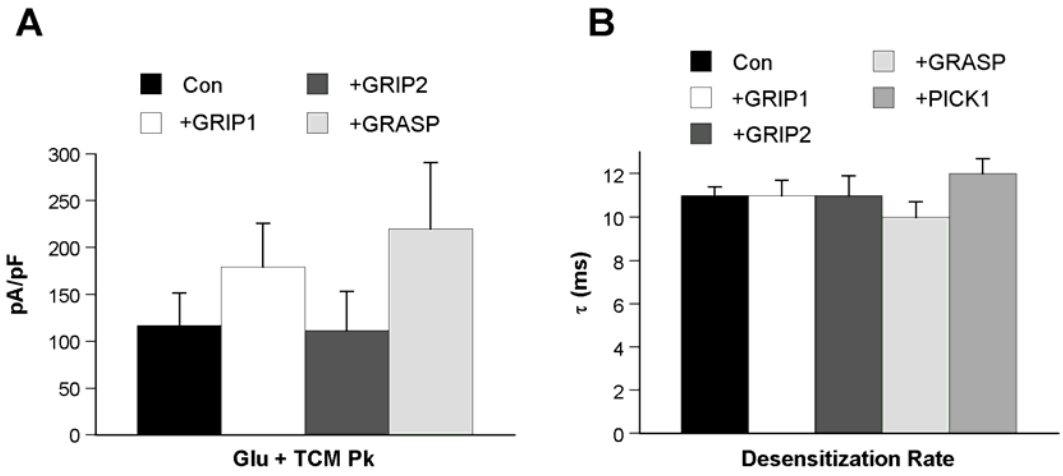


Figure 6: Co-expression of GRIP1, GRIP2, GRIP1 and GRASP-1, or PICK1 with GluR-A_iB_i in HEK293 cells. A. As a measure of surface receptor expression, peak currents elicited by 3 mM glutamate + 500 μM trichlormethiazide were normalized to whole cell capacitance (pA/pF). B. The glutamate-evoked (3 mM) desensitization rate was not significantly different from control for any of the conditions examined (1-way ANOVA, p=0.47). The rate of desensitization was best fit by a single exponential function. Bars represent mean ± SEM, Control, n=12-28; GRIP1, n=7; GRIP2, n=9; GRIP1 + GRASP-1, n=6, PICK1, n=17

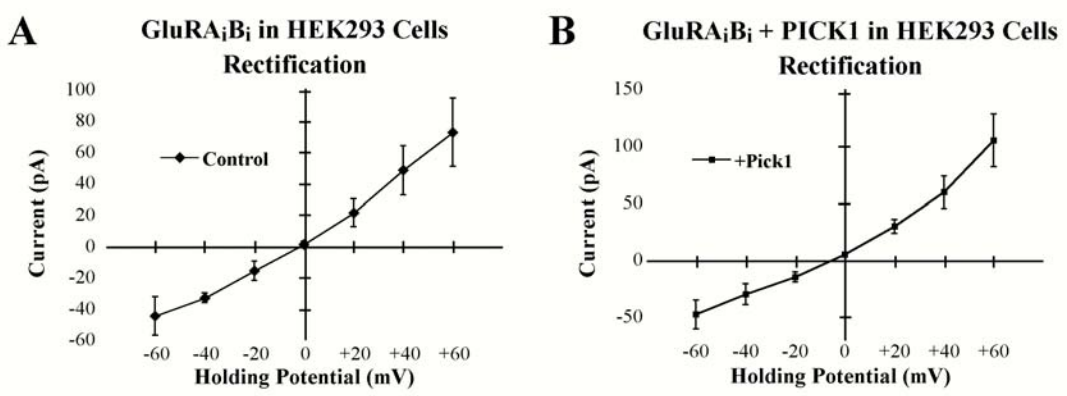


Figure 7: Rectification of GluR-A_iB_i receptors in the presence and absence of PICK1. A. Rectification of steady-state current elicited by 3 mM glutamate. Voltage was briefly ramped from -60mV (holding potential) to the indicated voltage. Note the slight outward rectification due to a cesium rather than a potassium-based intracellular solution (see Section 2.3.1). Near linear rectification indicates GluR-B_i is incorporated into the expressed receptor. Bars represent mean ± SEM; Control, n=11; Pick1, n=7

TABLE V

Summary Data for GRIP and PICK1 Experiments

	Glu SS (pA)	Glu Pk (pA)	KA SS (pA)	GY SS (pA)	TCM Pk/Wcc (pA/pF)	% Desens	Rate Desens (ms)
Control	66±14 28	1222±225 28	110±23 28	26±6 27	117±35 10	94.3±0.4 28	11±0.4 28
+GRIP1	138±43 7	1672±491 7	189±54 7	60±20 7	180±46 6	92.2±0.6 7	11±0.7 7
+GRIP2	71±27 9	1352±575 9	100±35 9	33±14 9	112±42 8	93.7±0.6 9	11±0.9 9
+GRASP1	87±33 6	1474±533 6	115±43 6	39±15 6	220±71 6	94.2±0.8 6	10±0.7 6
+PICK1	87±25 17	1659±567 17	154±43 17	31±11 16	--	92.6±1.9 17	12±0.7 17

Table 5: Summary Data for GRIP and PICK1 Experiments. HEK293 cells were transfected with GluR-A_iB_i alone (Control) or with GRIP1, GRIP2, GRIP1 and GRASP1, or PICK1. Peak (Glu Pk) and steady-state (Glu SS) responses to 3 mM glutamate in the presence and absence of 30 μM GYKI 52466 (GY SS) as well as steady-state responses to 600 μM kainate (KA SS) were assayed. Surface AMPA receptor expression was assayed by normalizing the steady-state current elicited by 3 mM glutamate in the presence of 500 μM trichlormethiazide to the whole cell capacitance (TCM Pk/Wcc) and is expressed as pA/pF. % Desensitization is calculated with the formula [(1-(Glu steady-state/Glu peak)) x 100]. Values are expressed as mean ± SEM; n is given below the corresponding mean. Analysis was performed using a 1-way ANOVA; $p>0.05$ for all comparisons.

exchange factor associated indirectly with AMPA receptors via PDZ-domain interactions with GRIP1 in rat brain (Ye et al., 2000). The constructs were co-expressed with GluR-A_iB_i heteromers to most readily detect subtle changes in desensitization properties.

4.2.1 Results and Discussion

In various models of GRIP function this protein either stabilizes AMPA receptors at a cytoplasmic (Daw et al., 2000) or surface membrane location (Osten et al., 2000; Iwakura et al., 2001; Kim et al., 2001; Perez et al., 2001; Seidenman et al., 2003). According to these models, cotransfection of GRIP1 with the receptor should result in either a decrease or an increase, respectively, in AMPA receptor surface expression. There were no significant differences between control and experimental conditions in currents elicited by the full agonist glutamate (Fig. 6A), by the partial agonist kainate, or in glutamate-evoked responses in the presence of the positive and negative allosteric modulators trichlormethiazide and GYKI 52466, respectively (Table 5). There appeared to be a non-significant trend towards greater current density in the presence of GRIP1, but not GRIP2 (Fig. 6A, Table 5; 1-way ANOVA, $p=0.34$). This is consistent with the second model of GRIP function, in which GRIP1 stabilizes AMPA receptors in neuronal surface membranes. Graphical representation of the data additionally suggests that GRIP2 may be less able to stabilize AMPA receptors at the membrane surface than GRIP1 (Fig. 6A). Because palmitoylation of GRIP2 modulates its ability to mediate surface expression of AMPA receptors (deSouza et al., 2002), it is possible that expression of this construct in a non-neuronal cell prevents this posttranslational modification and therefore its function as a membrane anchor. Co-expression of GRIP1

with GRASP-1 resulted in a slight, but again not significant, increase in current density (Fig. 6A, Table 5). Ye et al (2000) found that over-expression of GRASP-1 partially blocked GRIP-mediated AMPA receptor surface localization; however, this function of GRASP-1 is dependent on NMDA receptor activity, suggesting this interaction may not be intact in a heterologous system lacking NMDA receptors. Co-expression of GRIP1, GRIP2, or GRIP1 and GRASP-1 with GluR-A_iB_i did not significantly change either the magnitude of steady-state glutamate-evoked desensitization or the rate of desensitization (Fig. 6B, Table 5).

PICK1 appears to play a more dynamic role in AMPA receptor trafficking. One model presents PICK1 as a recruitment protein for activated PCKα, clustering the enzyme with the GluR-B C-terminus to facilitate phosphorylation at serine 880 of GluR-B, which disrupts GRIP binding and allows receptor internalization and long term synaptic depression (Iwakura et al., 2001; Kim et al., 2001; Perez et al., 2001; Chung et al., 2003). There was no significant difference in agonist-evoked responses or glutamate desensitization rate between control and PICK1 transfected cells (Table 5). There were also no significant differences in rectification properties of GluR-A_iB_i heteromers in the presence of PICK1 (Fig. 7).

The presence of a FLAG tag in the expression vector allowed us to use immunofluoresence to detect PICK1 protein levels. The HEK293 cells in Fig. 8A and B are transfected with GluR-B and show detectable levels of receptor expression. Parts C and D verify binding of the FLAG antibody to the FLAG epitope: these cells are expressing a FLAG-tagged GluR-D protein. Parts E and F show an undectable level of

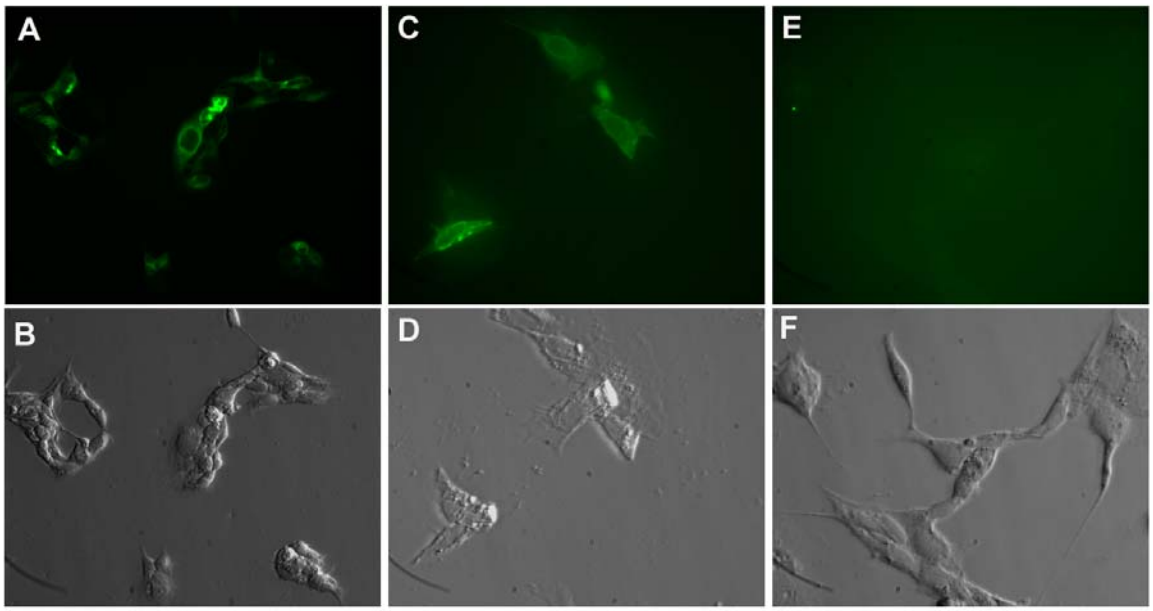


Figure 8: Immunofluorescent detection of GluR-B and PICK1 in transfected HEK293 cells. A. & B. Fluorescent and phase images, respectively, of HEK293 cells transfected with GluR-B at 20X magnification. C. & D. Fluorescent and phase images, respectively, of HEK293 cells transfected with FLAG-tagged GluR-D at 40X. E. & F. Fluorescent and phase images, respectively, of HEK293 cells transfected with GluR-B and FLAG-tagged PICK1 at 40X. Expression of the PICK1 protein was undetectable.

FLAG-tagged PICK1 expression, explaining our negative results in HEK293 cells and indicating the SV40 promoter driving the PICK1 gene is too weak for electrophysiological examination of the interaction of PICK1 with the AMPA receptor. Our attempts to subclone the PICK1 construct into a pcDNA3.1 vector with a CMV promoter were unsuccessful. A recent study detailed decreased GluR-B surface expression during PICK1 over-expression in hippocampal slice cultures (Terashima et al., 2004; see Section 1.7.4). Poor expression of the PICK1 construct prevented our ability to detect this change, which would have been evident as a decrease in outward current (increased inward rectification) in the presence of PICK1 (Fig. 7B). Because our initial examination of the effects of PICK1 and the GRIPs on AMPA receptor functional properties suggests that these proteins have no additional effects on channel function, we chose to focus our efforts on the substantial changes in receptor function elicited by the transmembrane protein stargazin as presented in Chapter V.

Chapter V

STARGAZIN/γ2 REDUCES AMPA RECEPTOR DESENSITIZATION BY DESTABILIZING THE DESENSITIZED STATE AND ALSO INCREASES KAINATE EFFICACY

5.1 Introduction

While several cytosolic AMPA receptor associated proteins have been described that are involved in controlling insertion, removal and stabilization of AMPA receptors at the synapse (reviewed by Bredt and Nicoll, 2003), none have been demonstrated to directly change receptor biophysical properties. However, evidence that the glutamate receptor binding protein PSD-95/SAP90 decreases kainate receptor desensitization (Garcia et al., 1998) and increases NMDA receptor open channel probability suggests an associated protein could alter AMPA receptor function.

Stargazin, which is disrupted in the *stargazer* mutant mouse, was initially characterized by homology as the second member (γ2) of the voltage-gated calcium channel γ subunit family (Letts et al., 1998). *Stargazer* mice exhibit seizures characterized by upward tilting of the head, thus the term "stargazer". Stargazin/γ2 is specifically expressed in the brain and, like the skeletal muscle γ1 subunit, produces a hyperpolarizing shift in inactivation of the channel-forming VGCC α subunit (Letts et al., 1998). This defect in neuronal VGCC inactivation in the absence of stargazin would be predicted to increase excitability, providing an appealing explanation for the spike-wave

seizures observed in *stargazer* mice. Of greater functional significance is the profound deficit in AMPA receptor-mediated EPSCs in *stargazer* cerebellar granule cells (Hashimoto et al., 1999), a phenotype that can be rescued by over-expression of γ2 (Chen et al., 2000). γ2 has three apparent functions in AMPA receptor trafficking: facilitating surface receptor delivery as a molecular chaperone involved in correct folding and ER processing of AMPA receptors (Tomita et al., 2003; Vandenberghe et al., 2005a), stabilizing AMPA receptors on the cell surface (Tomita et al., 2004) and, via interactions with PSD-95 and n-PIST, targeting AMPA receptors to the synapse (Chen et al., 2000; Cuadra et al., 2004). Three closely related isoforms, γ3, γ4 and γ8, show distinct patterns of expression, and have also been identified as transmembrane AMPA receptor regulatory proteins (TARPs) based on their ability to restore AMPA receptor-mediated currents in *stargazer* cerebellar granule cells (Tomita et al., 2003).

TARPs have recently been shown to co-purify with neuronal AMPA receptors (Nakagawa et al., 2005; Vandenberghe et al., 2005b), suggesting that they may function as auxiliary subunits of the receptor. Because auxiliary subunits in voltage-gated channels can affect channel properties, we examined the effects of stargazin on channel function of neuronal and recombinant AMPA receptors. We find that stargazin reduces glutamate-evoked desensitization of native hippocampal AMPA receptors through an allosteric interaction that destabilizes the desensitized state, and also substantially increases the efficacy of the partial agonist kainate. Experiments in HEK293 cells demonstrate the relative roles of two domains of stargazin in mediating these functional effects. Both trafficking and functional effects of the TARPs γ2 and γ3 vary with

receptor composition, suggesting that inclusion of TARPs as auxiliary subunits affects AMPA receptor heterogeneity *in vivo*.

5.2 Statement of Hypothesis

Enhancement of AMPA receptor currents in the presence of stargazin is a consequence not only of enhanced receptor trafficking but also altered receptor properties, including destabilization of the desensitized state and increased efficacy of the partial agonist kainate.

5.3 Results

5.3.1 Stargazin/γ2 Decreases Desensitization and Increases Kainate Efficacy at Hippocampal AMPA Receptors

While stargazin-mediated increases in AMPA receptor currents have traditionally been interpreted as increased AMPA receptor surface density, it also seemed possible, given the potential role of stargazin/γ2 as an auxiliary subunit, that γ2 could increase AMPA receptor currents by altering receptor functional properties. We therefore examined the effects of exogenous γ2 expression on AMPA receptor function in hippocampal neurons using fast perfusion techniques during whole-cell voltage-clamp recording. Dissociated cultures were prepared from newborn rat pups, and recordings were done at 7 days *in vitro* to minimize the contribution of endogenous neuronal TARPs (γ2, γ3 and γ8), which increase their expression during the 2nd and 3rd postnatal weeks (Tomita et al., 2003). Exogenous γ2 increased normalized steady-state glutamate responses more than 6-fold relative to controls (Fig. 9A, B), decreasing desensitization

from 98.3 ± 0.2 to 87.8 ± 1.5% (mean ± SEM, n = 21 for each group). Additionally, exogenous γ2 more than doubled the relative size of currents evoked by the weakly desensitizing partial agonist kainate. It is striking that, while kainate currents in hippocampal neurons are typically only 40% as large as peak glutamate responses (Fig. 9A, C), they approached parity in cells over-expressing γ2.

Glutamate currents evoked in the presence of trichlormethiazide (TCM), a cyclothiazide analog that blocks receptor desensitization (Fig. 9A; see Section 2.6), provide a functional assay for surface AMPA receptor expression. Exogenous γ2 had no significant effect on AMPA receptor current density in hippocampal neurons (Fig. 9D). Thus, in a system where exogenous γ2 did not increase surface expression of native AMPA receptors, it nonetheless clearly altered receptor functional properties as evidenced by both reduced desensitization of glutamate responses and increased apparent efficacy of kainate.

Co-expression of γ2 significantly decreased steady-state potentiation by TCM (Fig. 9D) and significantly increased glutamate-evoked steady-state inhibition by the 2,3-benzodiazepine GYKI 52466 (GYKI, Fig. 9E). Both modulators act in a state-dependent manner; TCM selectively binds and holds the receptor in the non-desensitized conformation (see Chapter 3), while GYKI binds and preferentially holds the receptor in the desensitized state (Turetsky et al., 2004). For both drugs, altered modulation in the presence of γ2 is a consequence of a larger proportion of receptors in the non-desensitized state at equilibrium (Fig. 9F). The 6-fold reduction of steady-state potentiation upon γ2 co-expression (Fig. 9B) reflects a similar degree of reduction in

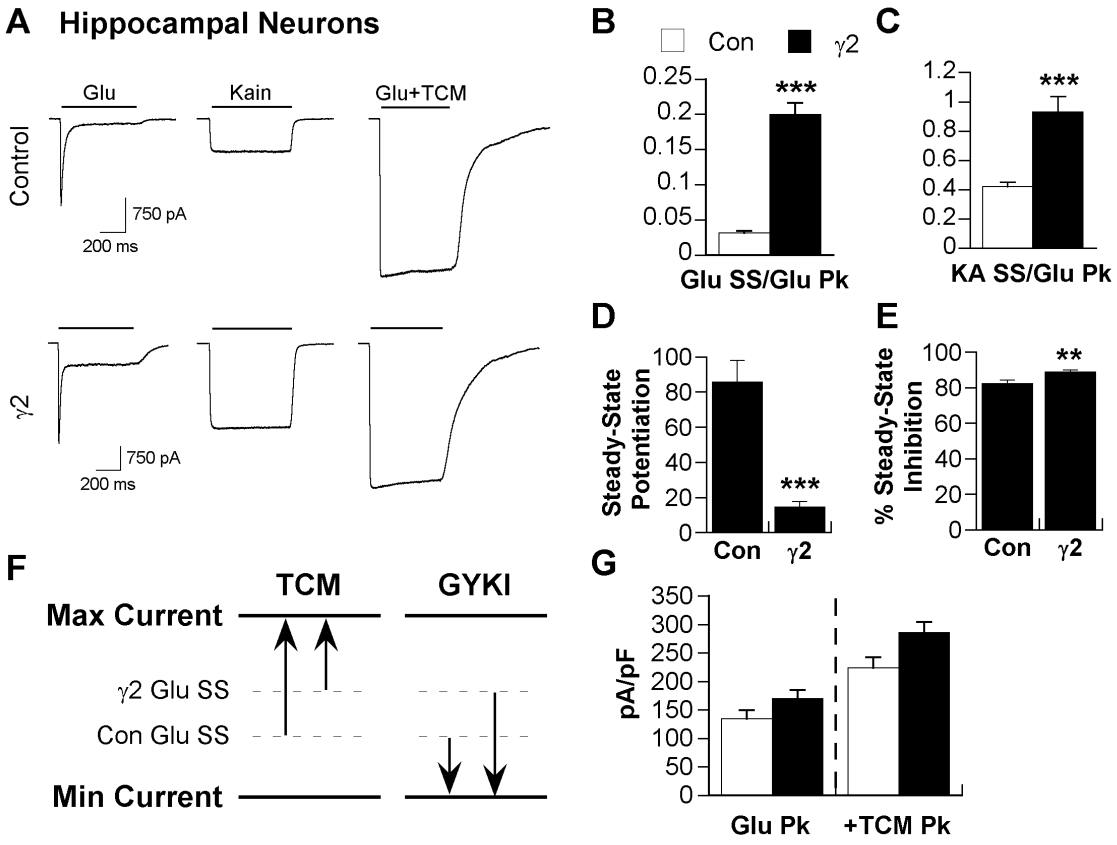


Figure 9. γ2 selectively potentiates steady-state glutamate and kainate-evoked currents in hippocampal neurons. See accompanying page for legend.

Figure 9. γ2 selectively potentiates steady-state glutamate and kainate-evoked currents in hippocampal neurons. (A) Responses of representative hippocampal neurons transfected with GFP alone (Control) or γ2+GFP to 3 mM glutamate (Glu), 600 μM kainate (Kain) and glutamate in the presence of 500 μM trichlormethiazide (Glu+TCM). Steady-state currents evoked by glutamate (B) or kainate (C) in hippocampal neurons were normalized to the peak glutamate response to assess changes in AMPA receptor functional properties. The ratio of Glu SS/Glu Pk is a relative measure of the proportion of receptors in the desensitized state; the inverse of this ratio describes the relative occupancy by the receptor of open, non-desensitized states. In control cells, 1.7 ± 0.2% of AMPA receptors occupy the open state at equilibrium while 12.2 ± 1.5% of receptors occupy the open state at equilibrium when γ2 is present. (D) Potentiation of glutamate-evoked steady-state responses by 500 μM TCM was calculated by (Glu+TCM SS/Glu SS). (E) Inhibition of glutamate-evoked steady-state responses by GYKI 52466 was calculated by [1-(Glu+GYKI SS/Glu SS)]. (F) Schematic demonstrating the relative decrease in potentiation by TCM and increase in inhibition by GYKI in the presence of γ2. A larger percent of receptors associated with γ2 occupy the open, non-desensitized state than receptor alone (γ2 Glu SS *vs.* Con Glu SS). Simply put, the decreased efficacy of TCM reflects a smaller step towards full occupancy of the open state of the receptor (Max Current) from a steady-state current with less desensitization than control currents, while increased efficacy of GYKI reflects a larger step towards full occupancy of the desensitized state. (G) Current density was determined by normalizing peak glutamate currents in the presence of TCM to whole-cell capacitance as a functional measure of AMPA receptor surface expression. For comparison, normalized glutamate peak currents are also graphed. Bars in (B-D) represent mean ± SEM (n = 21 for each group). Asterisks (*) indicate a significant difference from corresponding control (*** $P<0.001$).

glutamate-evoked steady-state desensitization (Fig. 9B, see figure legend). Because GYKI increases the proportion of receptors in the desensitized state, the relative increase in GYKI inhibition reflects a larger shift between normal and modulator-induced levels of steady-state desensitization (Fig. 9F).

In contrast to TARP expression in hippocampal neurons, mouse cerebellar granule neurons exclusively express γ2, which is crucial for AMPA receptor surface expression in these cells (Chen et al., 2000; Tomita et al., 2003). Cultures were prepared from P2 mouse pups, and recordings were performed at 7 days *in vitro*, a time when endogenous γ2 is expected to be present but not yet at maximal levels. Although γ2 over-expression significantly increased current density, which reflects increased AMPA receptor surface expression (Fig. 10D), it did not significantly increase normalized steady-state glutamate currents (Fig. 10A, B) and no significant change in desensitization was observed (control 97.5 ± 0.4% versus γ2 96.4 ± 0.4%, mean ± SEM, n = 12-14). There was a small (17%) but significant increase in relative kainate responses (Fig. 10A, C). Interestingly, control kainate responses in cerebellar granule cells averaged 50% larger than peak glutamate currents (Fig. 10C), a percentage only approached in certain hippocampal neurons with exogenous γ2 expression. Thus, in a system where most surface receptors are associated with γ2, over-expression of γ2 increases trafficking of receptors to the membrane but has little additional effect on AMPA receptor functional properties.

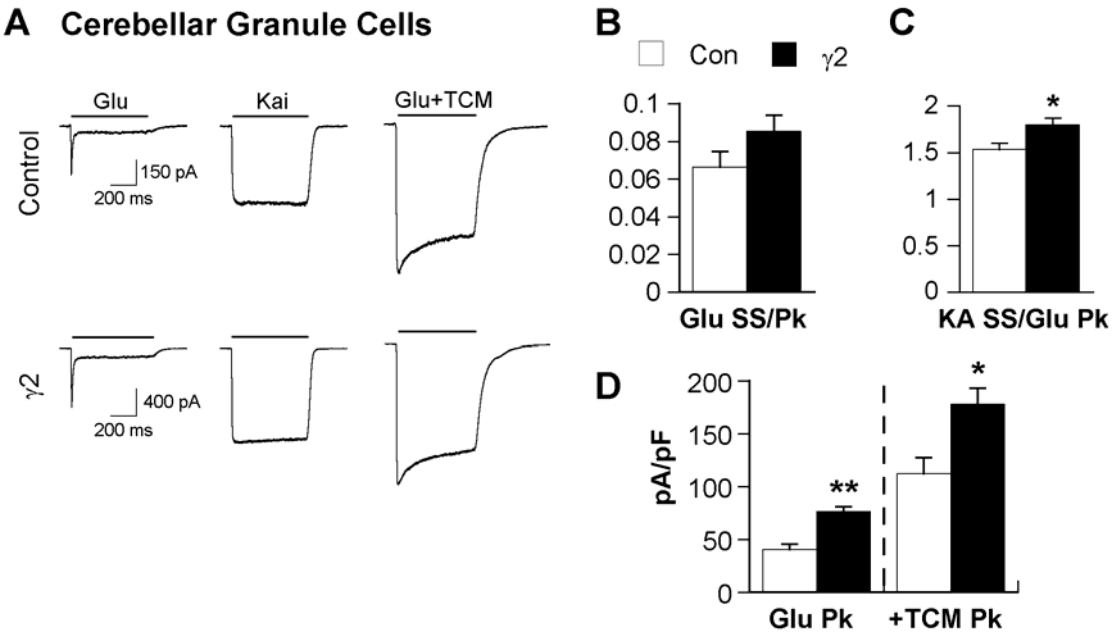


Figure 10. γ2 increases receptor surface expression in cerebellar granule neurons with minimal functional effects. (A) Traces from representative cerebellar granule cells transfected with GFP or γ2+GFP illustrate the large kainate responses characteristic of these neurons and the relatively smaller functional effects of exogenous γ2 expression. Steady-state currents evoked by glutamate (3 mM, B) or kainate (600 μM, C) were normalized to the peak glutamate response to assess changes in AMPA receptor functional properties. (D) Current density was determined by normalizing peak glutamate currents in the presence of TCM to whole-cell capacitance as a functional measure of AMPA receptor surface expression. Bars in (B-D) represent mean ± SEM (n = 12–15). Asterisks (*) signify a difference for γ2 relative to the respective control (* $P<0.05$, ** $P<0.01$).

5.3.2 Stargazin/γ2 Destabilizes the Desensitized State of the AMPA Receptor

To begin to address the mechanisms underlying γ2's functional effects on native AMPA receptors, we first examined the relative increase in kainate efficacy. In hippocampal neurons, γ2 over-expression shifted the kainate dose-response curve to the left ~3 fold (Fig. 11A). This is similar to the shift produced by the positive modulator cyclothiazide (Patneau et al., 1993) and could reflect decreased receptor desensitization. However, a mutation that allows kainate to induce greater domain closure, and thus to more effectively gate the channel, also shifts the dose-response curve ~3-fold to the left. Alternately, γ2 could produce a conformational change in the ligand binding pocket that directly affects kainate's affinity. In order to distinguish between these possibilities, we examined the association and dissociation rates of kainate applied in the presence of glutamate. Because glutamate has a higher affinity for the desensitized state, kainate can only compete glutamate from non-desensitized receptors (Patneau and Mayer, 1991). Thus, this protocol allowed us to assess both the rate of recovery from glutamate-evoked desensitization and the rate of dissociation of kainate. γ2 over-expression significantly increased the rate of recovery from glutamate-evoked desensitization as determined by faster kainate association (Fig. 11B, C). However, there was no change in dissociation kinetics (Fig. 11B, C), indicating that γ2 does not alter the affinity of kainate for the receptor.

A faster rate of recovery from desensitization and reduced steady-state desensitization in hippocampal neurons suggest γ2 alters the equilibrium between desensitized and non-desensitized states of the receptor. One way to obtain direct information about the

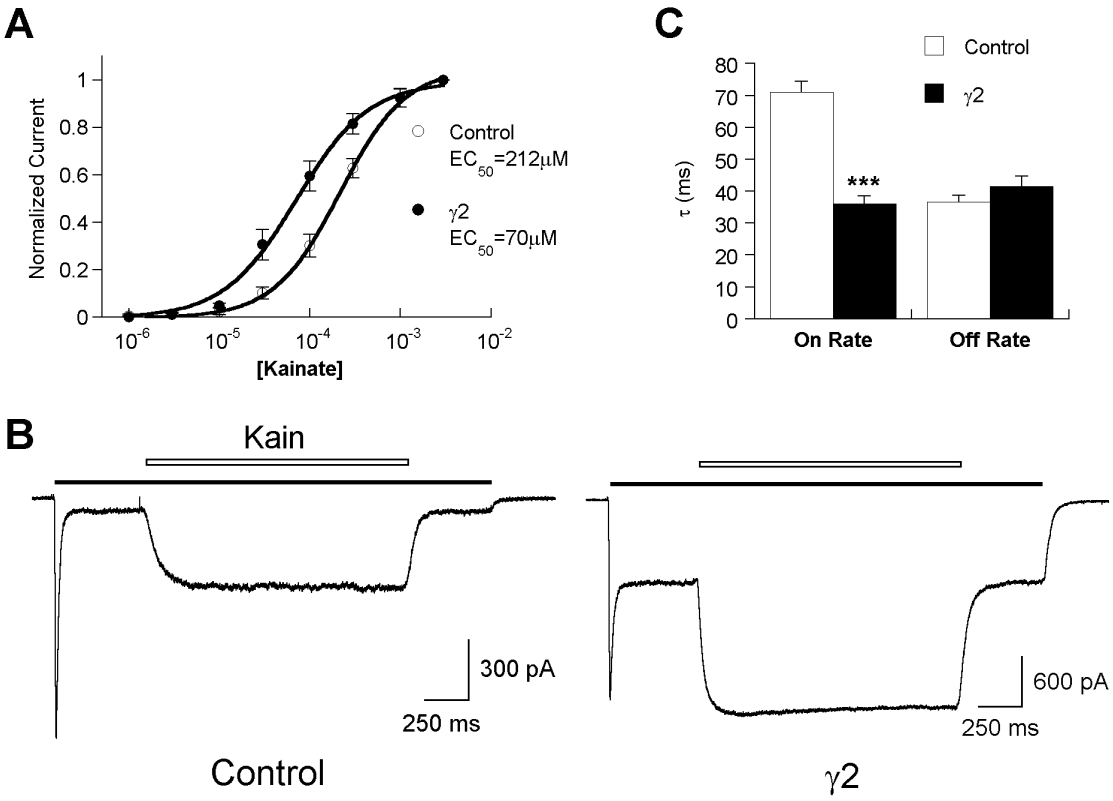


Figure 11. γ2 increases kainate efficacy and speeds recovery from desensitization in hippocampal neurons. (A) Dose-response curves for kainate-evoked currents in hippocampal neurons transfected with GFP (Control) or γ2+GFP. Currents were normalized relative to 3 mM kainate. Points represent mean ± SEM (n = 4 -11, depending on dose). (B) Representative traces from neurons transfected with GFP or γ2+GFP illustrate the association and dissociation of kainate (600 μM) in the presence of glutamate (200 μM). (C) Both association (τ_{on}) and dissociation (τ_{off}) of kainate in the presence of glutamate were well fit by a single exponential function. Bars represent mean ± SEM (control, n = 16; γ2, n = 15). Asterisks (*) signify a difference for γ2 relative to the respective control (*** P<0.001).

conformational state of the receptor is to examine the association kinetics of the positive modulator trichlormethiazide (TCM). Crystallization of the ligand-binding cores of GluR-B has shown that the interface between two adjacent AMPA receptor subunits is closely apposed in both the resting and open states but separates during the conformational transition to the desensitized state (Sun et al., 2002). Positive allosteric modulators such as cyclothiazide bind at and stabilize this dimer interface, reducing desensitization. Implicit in this model is the loss of the binding site for the positive modulators in the desensitized conformation, with substantially slowed modulator binding in the presence of strongly desensitizing agonists (unpublished data). In hippocampal neurons over-expressing γ2, the time constants for onset of TCM modulation were more than 5 times faster than in control neurons, consistent with a faster rate of recovery from desensitization (Fig. 12A, B). The time constant of dissociation was unaffected (Fig. 12C), indicating that the affinity of TCM for the non-desensitized state is unchanged, and by implication, that γ2 does not substantially alter the conformation of the dimer interface in the open state. These data therefore suggest that γ2 association shifts the equilibrium between desensitized and open conformations of the AMPA receptor by destabilizing the desensitized state.

5.3.3 The Functional Effects of Stargazin/γ2 Are Specific for AMPA Receptors

Because both hippocampal and cerebellar granule neurons express heterogeneous receptor populations and have the complication of endogenous TARP expression, we

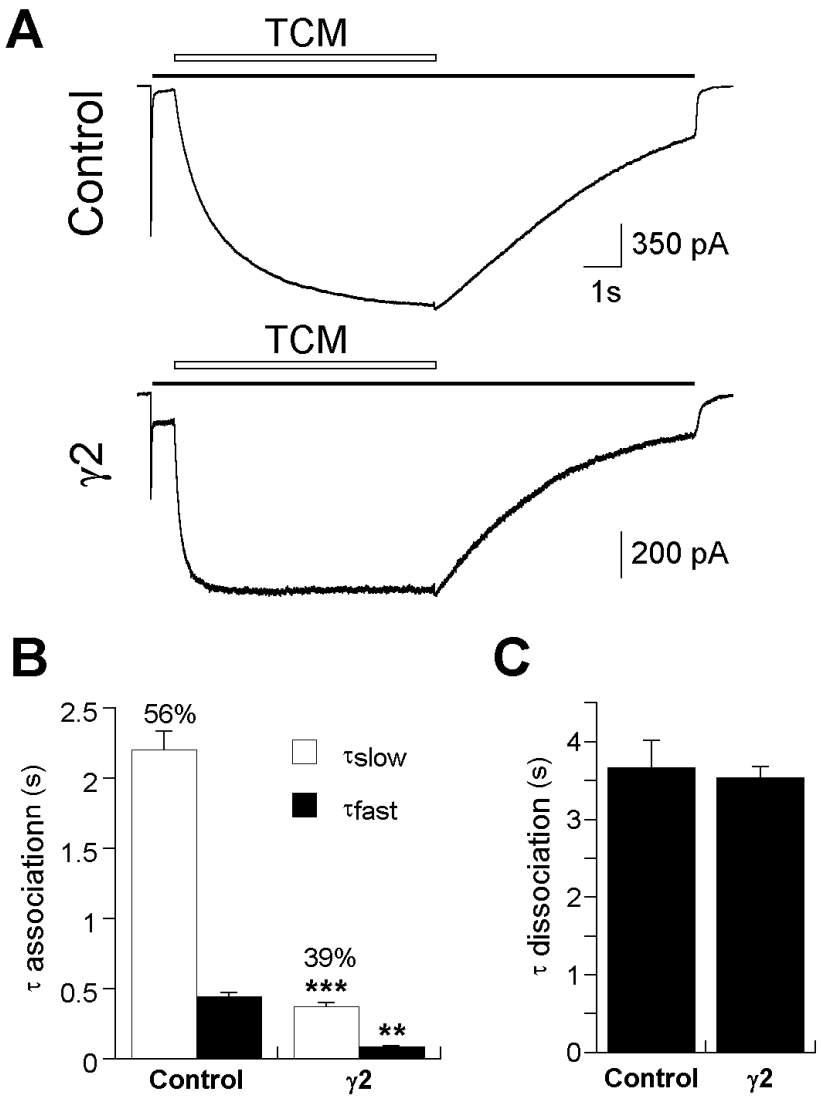


Figure 12. The onset of modulation by trichlormethiazide is faster in neurons transfected with γ2. (A) Traces from representative hippocampal neurons transfected with GFP or γ2+GFP show the association and dissociation kinetics of TCM (500 μM) in the presence of glutamate (3 mM). Time constants for the onset (B) and offset (C) of TCM modulation in hippocampal neurons transfected with GFP or γ2. Onset kinetics were fit with the sum of two exponentials (% contribution of τ_{slow} is shown), while dissociation kinetics were best described by a single exponential. Bars represent mean ± SEM (control, n = 19; γ2, n = 17). Asterisks (*) signify a difference for γ2 relative to the respective control (** $P<0.01$, *** $P<0.001$).

moved to a heterologous expression system to further explore the effects of γ2 on AMPA receptor function. AMPA receptor subunits occur naturally as one of two alternatively spliced isoforms, *flip* (*i*) or *flop* (*o*), which differ in their desensitization properties (Sommer et al., 1990). AMPA receptors are preferentially assembled as heteromers containing both *flip* and *flop* isoforms (Brorson et al., 2004). HEK293 cells were therefore co-transfected with GluR-A_i and B_o to form heteromeric receptors that reproduce some characteristics of native hippocampal AMPA receptors. γ2 co-expression had substantially larger effects on kainate efficacy in recombinant AMPA receptors than in hippocampal neurons, with both normalized steady-state glutamate and kainate currents potentiated approximately 10-fold over control (Fig. 13A, B, C). We also observed a pronounced slowing in the rate of glutamate desensitization (control $\tau = 4.4 \pm 0.2$ ms, n = 14; γ2 $\tau = 7.1 \pm 0.5$ ms, n = 17, mean ± SEM; $P < 0.001$, *t*-test). Note that relative glutamate and kainate steady-state responses in control HEK293 cells expressing GluR-A_iB_o are much smaller (1 and 7% of peak glutamate, respectively) than those of native receptors in hippocampal neurons (Fig. 9). It is intriguing that the characteristics of recombinant receptors co-expressed with γ2 more accurately reproduce those observed in native receptors.

In contrast to effects in hippocampal neurons, γ2 co-expression increased current density 3.4-fold in HEK293 cells expressing A_iB_o heteromers (Fig. 13D). To corroborate this functional assay for effects on AMPA receptor trafficking, surface biotinylation experiments were performed in sister A_iB_o cultures. Quantitative western blots revealed an average 3.2-fold increase in GluR-A, and a 2.9-fold increase in GluR-B surface protein with γ2 co-expression (Fig. 13E, F). Thus, the increase in AMPA receptor

surface expression can account for the potentiation of glutamate peak responses; however, when this increase in surface expression is factored out, a 10-fold increase in relative glutamate steady-state responses remains. To determine whether γ2 differentially affected *flip* and *flop* splice variants, we also examined A_iB_i and A_oB_o heteromers. We observed trafficking and functional effects for all heteromeric combinations; however, the magnitude of these effects were dependent on receptor composition (Table 6).

Three calcium channel γ subunit family members closely related to stargazin, γ3, γ4 and γ8, have also been identified as TARPs based on their ability to restore AMPA receptor-mediated currents in *stargazer* cerebellar granule cells, while the more distantly related family members γ1, γ5, γ6 and γ7 cannot (Tomita et al., 2003). To determine whether other TARPs share the functional effects of γ2, HEK293 cells were co-transfected with A_iB_o and γ3 or γ5. γ3 potentiated both steady-state glutamate and kainate currents, while γ5 had no significant effect on receptor function (Fig. 13B, C). Similar results were obtained with γ3 and γ5 in hippocampal neurons (data not shown). γ3, but not γ5, also significantly increased current density in HEK293 cells expressing A_iB_o heteromers (Fig. 13D). It is particularly interesting that although γ3 significantly decreased receptor desensitization, the effect was only half that observed with γ2, indicating that various TARPs may differentially associate and/or have different functional effects on a given AMPA receptor subtype.

Chen and colleagues (2003) reported that stargazin could increase AMPA receptor surface expression, but had no effect on kainate receptor trafficking. To determine if γ2's effects on receptor functional properties were also AMPA receptor specific, we co-expressed γ2 with GluR6 homomers or GluR6/KA2 heteromers in HEK293 cells.

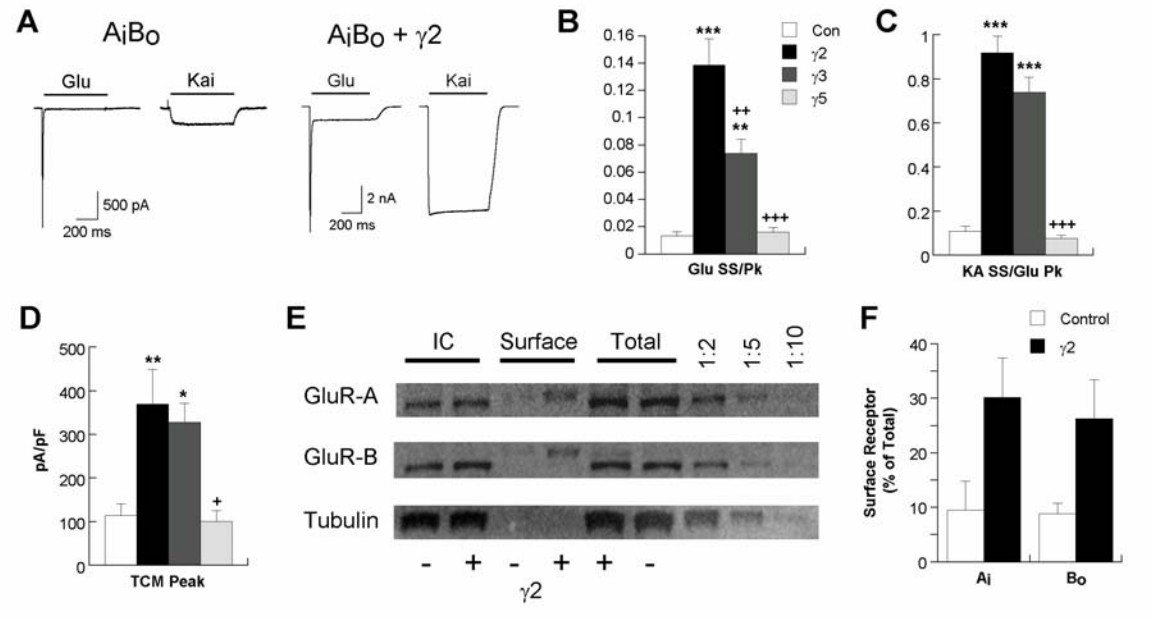


Figure 13. Dissociation of the effects of TARPs on steady-state agonist-evoked currents and AMPA receptor surface expression in heteromeric recombinant receptors. (A) Responses evoked by glutamate (3mM) and kainate (600 μM) for representative HEK293 cells expressing heteromeric GluR-A_iB_o receptors or GluR-A_iB_o + γ2. Note the different scale bars for control (500 pA) and γ2 cells (2 nA). Steady-state glutamate (B) or kainate currents (C) normalized to peak glutamate responses in HEK293 cells transfected with GluR-A_iB_o alone or GluR-A_iB_o + γ2, γ3 or γ5. (D) Current density was calculated by normalizing peak glutamate + TCM responses to whole cell capacitance. Bars in (B-D) represent mean ± SEM (n = 12–17). Asterisks (*) indicate significant difference relative to respective control (* $P<0.05$, ** $P<0.01$, *** $P<0.001$), and pluses (+) indicate significant difference from γ2 ($^{+}$ $P<0.05$; $^{++}$ $P<0.01$, $^{+++}$ $P<0.001$). (E) Surface biotinylation experiments were performed in HEK293 cells transfected with GluR-A_iB_o alone or GluR-A_iB_o + γ2. Western blots from a single experiment probed with antibodies for GluR-A or GluR-B are shown. The GluR-B blot was reprobed with tubulin as a control. The three lanes on the right are 1:2, 1:5, and 1:10 dilutions of the GluR-A_iB_o total protein sample. (F) Mean data from surface biotinylation of 3 independent transfections (± SEM).

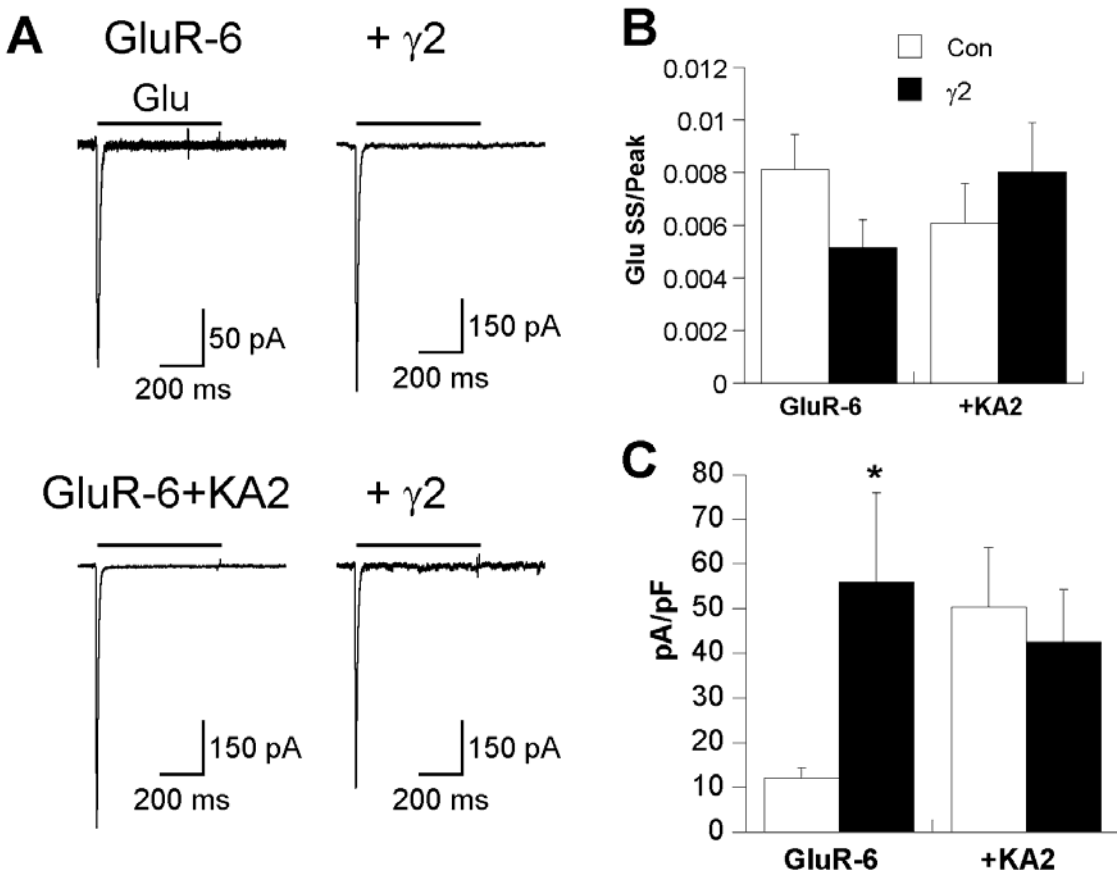


Figure 14. The functional effects of γ2 are AMPA receptor specific. (A) Representative responses evoked by 3 mM glutamate in HEK293 cells expressing homomeric GluR6 (top) or heteromeric GluR6/KA2 receptors (bottom) with or without γ2. Note the different scale bars for control (50 pA) and γ2 (150 pA) cells expressing GluR6. (B) Steady-state glutamate currents normalized to peak glutamate responses in HEK293 cells transfected with GluR6 or GluR6/KA2 ± γ2. (C) Because the benzothiadiazines are inactive at kainate receptors, current density in HEK293 cells transfected with GluR6 or GluR6/KA2 ± γ2 was determined by normalizing peak glutamate responses to whole cell capacitance. Bars in (B) and (C) represent mean ± SEM (n = 11–13). Asterisks (*) signify a difference for γ2 relative to the respective control (* $P<0.05$).

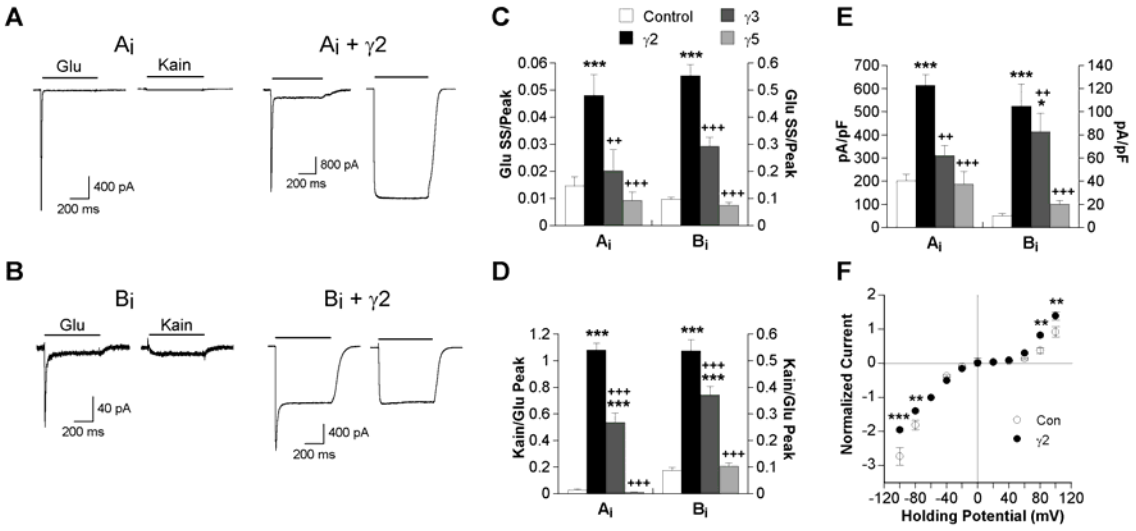


Figure 15. The functional effects of γ2 are subunit-dependent. Agonist-evoked responses of representative HEK293 cells transfected with GluR-A_i (A) or GluR-B_i (B) alone or with γ2. Note the different pA scale bars for control and γ2 cells. Normalized glutamate (C) and kainate (D) steady-state currents in HEK293 cells expressing homomeric A_i or B_i receptors alone (control) or with γ2, γ3, or γ5. (E) Current density was determined by normalizing peak glutamate + TCM responses to whole-cell capacitance. Note the different ordinate scales for A_i (left) and B_i (right) for each bar graph (C-E). Values represent mean ± SEM (n = 7-44). (E) Current-voltage relationship for peak glutamate (3 mM) currents in HEK293 cells transfected with GluR-A_i alone or GluR-A_i+γ2. Points represent mean ± SEM, (n = 12-41). Asterisks (*) indicate a significant difference relative to the corresponding control (* $P<0.05$, ** $P<0.01$, *** $P<0.001$), and pluses (+) indicate significant difference from γ2 condition ($^{++}$ $P<0.01$; $^{+++}$ $P<0.001$).

Surprisingly, γ2 did increase current density in cells expressing GluR6 homomers (Fig. 14C), however, it had no significant effect on normalized steady-state glutamate current for either GluR6 homomers or GluR6/KA2 heteromers (Fig. 14A, B). Thus the functional effects of γ2 are specific for AMPA receptors.

5.3.4 Stargazin/γ2 Differentially Affects Individual AMPA Receptor Subunits

To rule out the possibility that γ2 affected AMPA receptor functional properties by altering subunit composition in native or heteromeric recombinant receptors, we also examined the effects of γ2 expression on homomeric GluR-A_i, B_i, C_i, and D_i receptors. In HEK293 cells expressing GluR-A_i homomers, γ2 co-expression potentiated normalized steady-state glutamate and kainate currents (3.3- and 37-fold, respectively; Fig. 15A, C, D), while increasing current density 3-fold (Fig. 15E). Although γ2 significantly altered functional properties of all four homomers, subunit-specific effects were apparent. Most strikingly, effects of γ2 on kainate and glutamate steady-state were similar for GluR-B_i (Fig. 15A-D), while kainate responses were much more strongly affected in the other three subunits (Table 7). The functional effects of γ3 also differed between subunits, with effects on GluR-B_i homomers more closely resembling those seen in GluR-A_iB_o heteromers (compare Fig. 13B, C and Fig. 15C, D). As expected, γ5 had no functional or trafficking effects on GluR-A_i or B_i. Surprisingly, γ2 exhibited subunit-specific trafficking effects, producing a 12-fold increase in current density for GluR-B, a 2-4 fold increase for GluR-A and D, and no significant change for GluR-C (Table 7). Taken together these results suggest that the functional effects of γ2 are mediated by association

at the cell surface rather than by effects on receptor assembly, and that the affinity and/or efficacy of the γ2 / AMPA receptor association differs for individual subunits.

γ2's effect on peak glutamate-evoked currents in GluR-A_i homomers was voltage-dependent, with co-expression resulting in significantly less inward current at holding potentials ≤ -80 mV and significantly more outward current at potentials ≥ +80 mV (normalized relative to -60 mV, Fig.15F). We also observed a significant increase in outward rectification for GluR-D_i (data not shown). Given the voltage-dependence of AMPA receptor gating and/or desensitization (Patneau et al., 1993), it is unclear whether this reflects an additional effect of γ2 on channel function or is a consequence of changes in other voltage-dependent channel properties.

Because binding of γ2 to PSD-95 via its C-terminal PDZ-binding motif is important for synaptic delivery of AMPA receptors (Chen et al., 2000), we examined co-expression of PSD-95 on γ2's functional effects in homomeric GluR-A_i receptors. The effects of γ2/PSD-95 co-expression were statistically indistinguishable from γ2 alone for normalized glutamate (Fig. 16A) and kainate steady-state currents (Fig. 16B) as well as whole-cell current density (Fig. 16C). Although the effects of γ2 relative to control on glutamate steady-state were not significant for this subset of experiments (Fig. 16A), we nonetheless observed a significant slowing of the onset of desensitization in the presence of γ2, which was independent of PSD-95 expression (Fig. 16D). As expected, PSD-95 co-expression alone did not alter either the functional properties or levels of surface expression for GluR-A_i receptors. Thus PSD-95 is not necessary for, and does not significantly alter, γ2's functional effects on AMPA receptors.

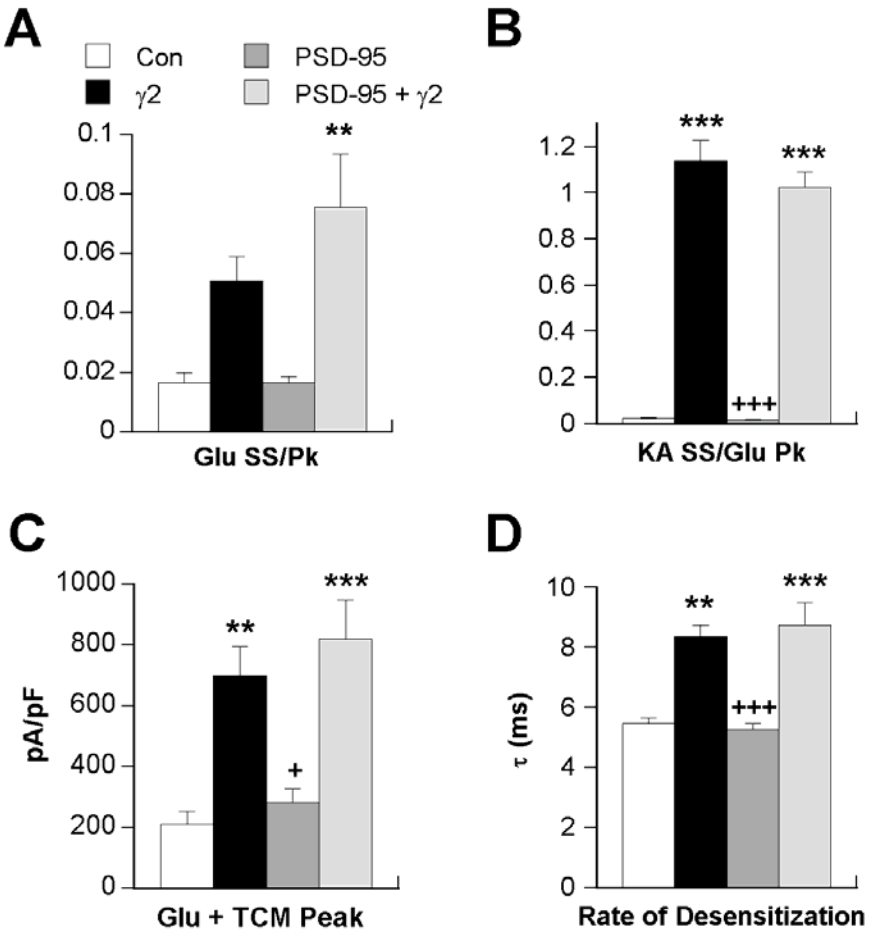


Figure 16. PSD-95 co-expression does not alter the effects of γ2 on AMPA receptor functional properties. Normalized glutamate (A) and kainate (B) steady-state currents in HEK293 cells transfected with GluR-A_i alone or with γ2, PSD-95, or PSD-95 + γ2. (C) Current density was determined by normalizing peak glutamate + TCM responses to whole cell capacitance. (D) Time constants determined by single exponential fits to the onset of desensitization for glutamate-evoked responses in HEK cells expressing GluR-A_i alone or with γ2, PSD-95, or PSD-95 + γ2. Bars in (A-D) represent mean ± SEM, (n = 11-19). Asterisks (*) indicate a significant difference relative to the corresponding control (** $P<0.01$, *** $P<0.001$) and pluses ($^{+}$) indicate significant difference from γ2 condition ($^{+}$ $P<0.05$; $^{+++}$ $P< 0.001$).

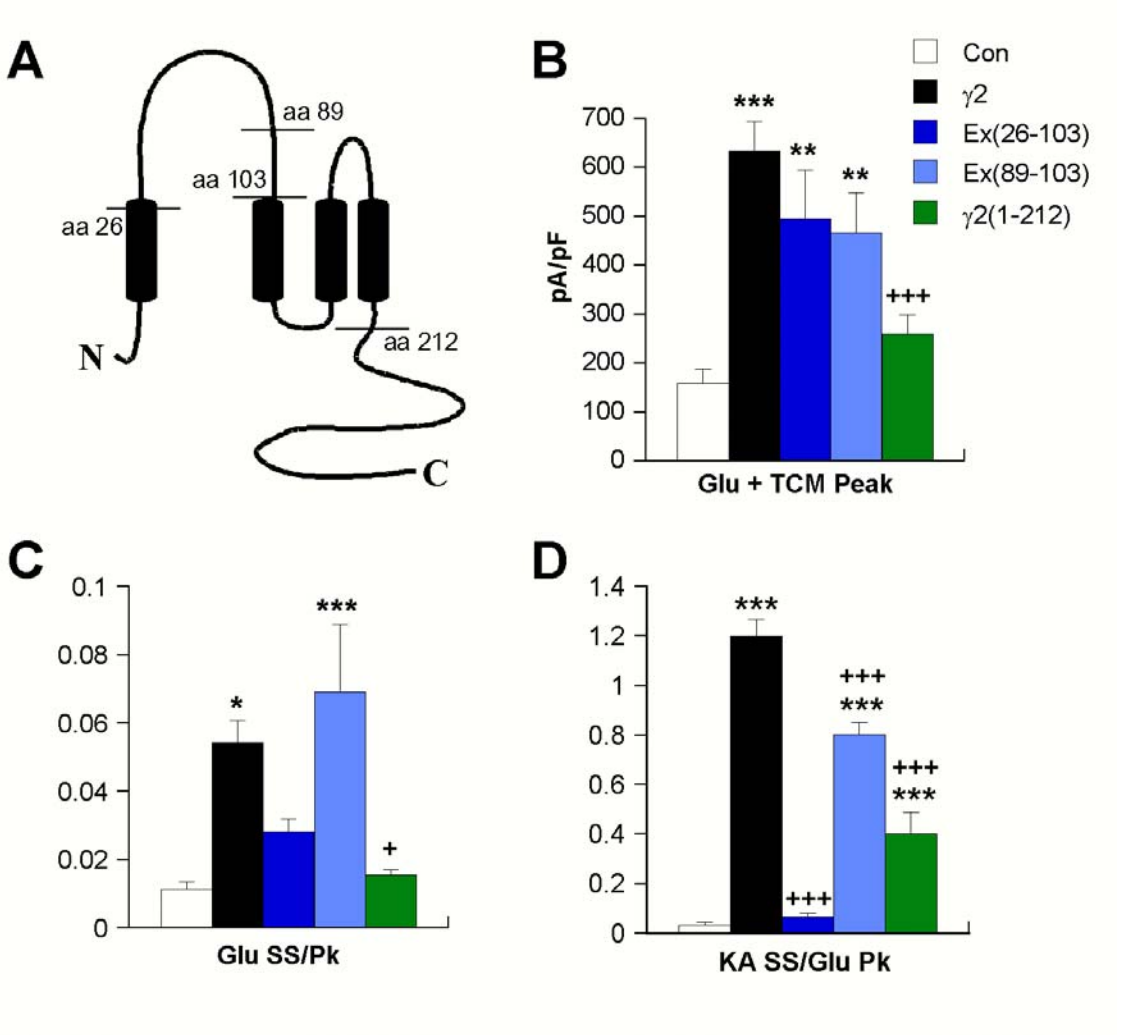


Figure 17. Both intracellular and extracellular regions of γ2 mediate functional changes in AMPA receptor properties. (A) Schematic depiction of the transmembrane topology of γ2. Amino acids demarcating boundaries for the chimeras and the C-terminal truncation are noted. Current density (B), normalized glutamate steady-state currents (C) and normalized kainate currents (D) in HEK293 cells transfected with GluR-A_i alone or GluR-A_i + γ2, γ2(Δ26-103)/γ5(ins26-99), γ2(Δ89-103)/γ5(ins85-99) or γ2(1-212). Bars in (B-D) represent mean ± SEM (n = 9-46). Asterisks (*) indicate a significant difference relative to the corresponding control (* $P<0.05$, ** $P<0.01$, *** $P<0.001$) and pluses (+) indicate significant difference from γ2 condition ($^{+}$ $P<0.05$; $^{+++}$ $P<0.001$).

5.3.5 Extracellular and Intracellular Domains of Stargazin/γ2 Differentially Mediate Trafficking and Functional Effects

Tomita and colleagues (2004) demonstrated that two regions in γ2, an area in the first extracellular loop and a region in the cytoplasmic tail, mediated association with GluR-B and were necessary for GluR-A trafficking in *Xenopus* oocytes. It was therefore of interest whether these same regions mediated the functional effects of γ2. A γ2 construct lacking most of the cytoplasmic tail [γ2(1-212), truncated 10 amino acids after the 4th transmembrane domain, see Fig. 17A] produced a significant decrement in current density relative to the effects of γ2 on GluR-A_i (Fig. 17B), but not quite to control level. This suggests that the γ2 cytoplasmic tail is the major mediator of AMPA receptor trafficking. Removal of the C-terminal tail of γ2 also abolished the effect on glutamate steady-state responses for GluR-A_i (Fig. 17C). Kainate-evoked currents in cells co-expressing γ2(1-212), although reduced by two-thirds relative to γ2, were significantly potentiated over controls (Fig. 17D). Interestingly, γ3, which shares substantial homology (84% amino acid identity) with γ2 through the end of the 4th transmembrane region but has greater divergence (56% identity) in the C-terminus, has effects on both trafficking and AMPA receptor function that were statistically indistinguishable from those of γ2(1-212) (P>0.89).

Because γ5 had no significant effects on AMPA receptor trafficking or function, we constructed a γ2/γ5 chimera, γ2(Δ26-103)/γ5(ins26-99), that replaced the first extracellular loop of γ2 with the corresponding sequence from γ5 (Fig. 17A). Co-expressing γ2(Δ26-103)/γ5(ins26-99) with GluR-A_i homomeric receptors produced effects that were almost the inverse of those observed with γ2(1-212). Although current

density was unaffected relative to γ2 (Fig. 17B), the shift in kainate efficacy was totally eliminated (Fig. 17D). While normalized steady-state glutamate responses in cells co-expressing γ2(Δ26-103)/γ5(ins26-99) were approximately half of those observed with γ2, they were not significantly different from either γ2 or control (Fig. 17C). A second γ2/γ5 chimera, γ2(Δ89-103)/γ5(ins85-99), which exchanged only 15 aa just prior to TM2, was statistically indistinguishable from γ2 on steady-state glutamate-evoked current, but still showed a significant decrement in the shift of kainate efficacy (Fig. 17C, D). Taken together these data suggest that functional effects are mediated by multiple regions on γ2. While both the intracellular C-terminal domain and first extracellular loop of γ2 appear to contribute to each functional effect, the cytoplasmic tail plays a larger role in reducing glutamate steady-state desensitization, and the first extracellular loop plays a larger role in increasing kainate efficacy.

5.3.6 Subunit-Dependent Differences in Functional Effects Support the Role of Extracellular and Intracellular Domains in Altered Kainate Efficacy and Desensitization, Respectively

The trafficking and functional effects of γ2 differed between AMPA receptor subunits. Co-expression of γ2 significantly increased surface trafficking of GluR-A_i, B_i and D_i but not C_i (Fig. 18B, Table 7). Cells co-expressing γ2 and GluR-B_i exhibited a distribution distinct from that of control responses, while with GluR-A_i and D_i the two populations prominently overlapped (Fig. 18B). Co-expression of γ2 with GluR-A_i and D_i also exhibited considerable overlap in desensitization profiles with control responses, while co-expression with GluR-B_i and C_i generated responses distinct from subunit alone

(horizontal lines, Fig. 18C). Note the striking variability in desensitization profiles of glutamate-evoked responses of GluR-A_i co-expressed with γ2 (Fig. 17A). The significant increase of the relative kainate response seen upon γ2 co-expression with every subunit (vertical lines, Fig. 18C, Table 7) suggests the extracellular portions of the AMPA receptor mediating this effect exhibit strong amino acid sequence identity between GluR-A, B, C and D. Interestingly, while γ2 did not effectively traffic GluR-C_i, it maintained both functional effects (Table 7, Fig. 18C), suggesting that in the absence of trafficking, γ2 maintains a functionally effective association with the AMPA receptor.

While the first extracellular loop of γ2 is essential for increased kainate efficacy (Fig. 17D), effects on desensitization, and especially effects on trafficking, are mediated through intracellular interactions (Fig. 17B, C). Comparison of the intracellular sequences of the four AMPA receptor subunits suggests three candidate areas as sites of γ2 interaction. The downstream portion of the first intracellular loop of GluR-C contains six proline residues, two of which are not present in the other subunits (Fig. 17D). GluR-C is unique from GluR-A, B and D in that it is not trafficked by γ2, suggesting the possibility that these prolines introduce tertiary changes in the first intracellular loop that block the association with γ2 necessary for surface trafficking. It is interesting that GluR-D, which is trafficked less effectively than GluR-A and B (Fig. 17B, Table 7), also contains several proline residues in the downstream portion of its first intracellular loop. Secondly, GluR-B and C have short C-terminal tails, while GluR-A and D have long C-termini (Fig. 17D). The overlap of γ2-mediated GluR-A_i and D_i desensitization profiles with control responses and the lack of overlap between control and γ2-mediated GluR-B_i and C_i responses suggest a long C-terminal tail interferes to varying degrees with

destabilization of the desensitized state by γ2. While the sequence of the second intracellular loop is identical between the four subunits (Fig 17D), it is ideally placed to influence channel properties due to its proximity to the pore-lining re-entrant loop (M2, Diagram 1). Steric hindrance by long C-terminal tails of the interaction of γ2 with this region may underlie the variability in desensitization profiles seen with GluR-A and D.

Figure 18 presented on page 109.

Figure 18. Subunit-specific effects of γ2 isolate intracellular regions of interaction. (A) Representative responses evoked by 3 mM glutamate or 600 μM kainate in cells transfected with GluR-A_i alone or with γ2. In Example 1, trafficking, altered desensitization and kainate efficacy are evident. In Example 2 trafficking and altered kainate efficacy are apparent (see pA scale bar, compare kainate steady-state and glutamate peak) while the desensitization profile is similar to that of the control glutamate response. (B) Scatter plots of the trafficking effects of γ2 on GluR-A_i, B_i, C_i and D_i homomers. Means are represented as a horizontal line. (C) Scatter plots comparing the extent of altered desensitization (Glu SS/Glu PK) and kainate efficacy (KA SS/Glu Pk) for each subunit. Vertical lines highlight a clear separation in the magnitude of kainate-evoked currents between control and γ2 cells, while horizontal lines separate populations of receptor co-expressed with γ2 that have distinct effects on desensitization. (D) Amino acid sequence of the first and second intracellular loops and C-terminal tail of GluR-A, B, C, and D. Note the proline residues in the first intracellular loop (*) as well as 100% amino acid sequence conservation in the second intracellular loop. GluR-B and C have short C-terminal tails, while GluR-A and D have long C-terminal tails. See also Diagram 7.

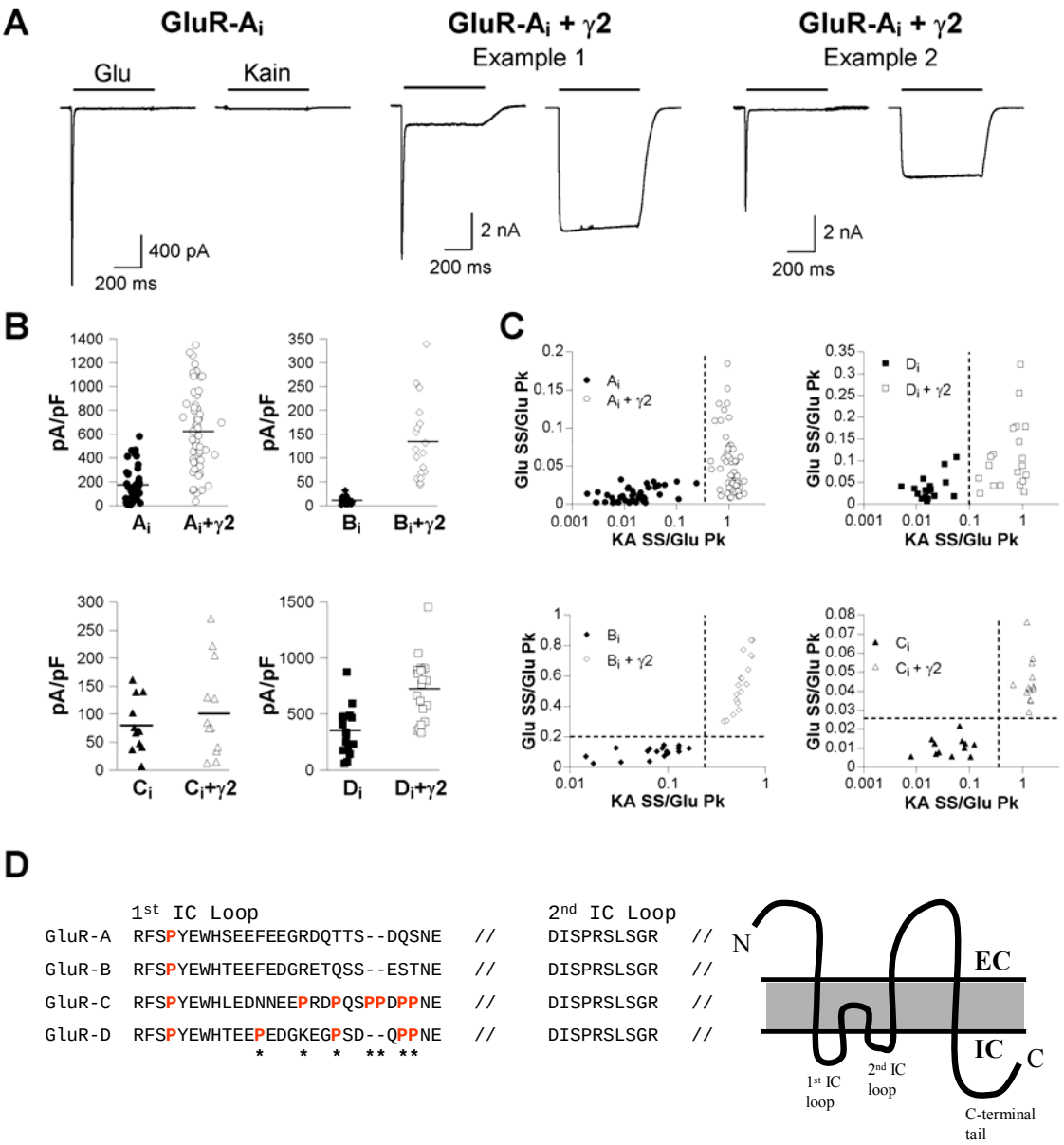


Figure 18. Subunit-specific effects of γ2 isolate intracellular regions of interaction. See Legend on accompanying page.

TABLE VI

Effects of γ2 Co-Expression on Heteromeric AMPA Receptors in Heterologous Cells

		Glu Pk (pA)	Glu SS (pA)	KA SS (pA)	KA/Glu Pk (%)	TPk/WCC (pA/pF)	Desensitization (%)
A_iB_o	Con	745±168	6±1	55±12	11±2	114±27	99.4 ±0.2
	γ2	**4242±646*** **	**550±107*** **	**3625±516*** **	**92±8*** **	**350±65***	**89.3±1.7*** **
A_iB_i	Con	1349±442	64±19	147±48	12±1	195±48	97.7±0.4
	γ2	**3894±592** **	**989±174*** **	**2398±426*** **	**61±6*** **	**414±66***	**84.0±3.0*** **
A_oB_o	Con	165±48	3±1	56±15	35±5	22±5	99.3±0.1
	γ2	**1843±339*** **	**205±53** **	**3079±537*** **	**170±11*** **	**214±42** **	**93.4±1.1*** **

Table 6: Effects of γ2 co-expression on heteromeric AMPA receptors in heterologous cells. HEK293 cells were transfected with the AMPA receptor combinations shown with or without γ2 co-expression. Relative kainate efficacy (600 μM) is expressed as a percent of the peak response to 3 mM glutamate. Current density, as an assay for surface receptor expression, was determined by normalizing Glu+TCM Peak to whole-cell capacitance (Tpk/WCC; 500 μM). Desensitization was calculated relative to maximal currents in the presence of TCM (% desensitization = [1- (GluSS / Glu+TCM Peak)]*100). A_iB_o n = 15-17, A_iB_i n = 12-14, A_oB_o n= 10-14. Asterisks (*) indicate γ2 condition is significantly different from receptor alone (* $P<0.05$; *** $P<0.001$, *t*-test).

TABLE VII

Effects of γ2 Co-Expression on Homomeric AMPA Receptors in Heterologous Cells

	Glu Pk (pA)	Glu SS (pA)	KA SS (pA)	KA/Glu Pk (%)	KA/Glu SS	TPk/WCC (pA/pF)	Desens (%)
A_i Con	739±124	9±2	9±1	3±1	3±1	173±23	99.6±0.1
γ2	**5530±421*** **	**350±52*** **	**5682±409*** **	**112±4*** **	**42±5*** **	**621±42*** **	**96.3±0.7*** **
B_i Con	78±13	9±2	7±1	8±1	1±0.1	11± 2	96.4±0.4
γ2	**1841±298*** **	**1090±220*** **	**1070±219*** **	**55±3*** **	**1±0.1**	**134±20*** **	**44.7±4.0*** **
C_i Con	324±57	4±1	19±5	6±1	5±1	80±14	99.7±0.1
γ2	**726±269**	**40±21**	**973±335***	**138±7*** **	**32±3*** **	**102±24**	**98.5±0.2*** **
D_i Con	1995±347	100±37	48±19	2±0.4	1±0.2	354±51	98.6±0.4
γ2	**6924±458*** **	**786±148*** **	**4926±648*** **	**70±8*** **	**9±2*** **	**728±63*** **	**92.2±1.8** **

Table 7: Effects of γ2 co-expression on homomeric AMPA receptors in heterologous cells. HEK293 cells were transfected with GluR-A, -B, -C or -D *flip* isoforms with or without γ2 co-expression. Relative kainate efficacy (600 μM) is expressed as a percent of the peak response to 3 mM glutamate. Current density, as an assay for surface receptor expression, was determined by normalizing Glu+TCM Peak to whole-cell capacitance (TPk/WCC; 500 μM). Desensitization was calculated relative to maximal currents in the presence of TCM (% desensitization = [1- (GluSS / Glu+TCM Peak)]*100). A_i n = 42-62, B_i n = 18, C_i n= 12-13, D_i n = 17-20. Asterisks (*) indicate γ2 condition is significantly different from receptor alone (* $P < 0.05$; *** $P < 0.001$, t-test).

5.4 Discussion

5.4.1 A Role for TARPs in Functional Heterogeneity of AMPA Receptors

The data presented here provide evidence that stargazin/γ2 alters the biophysical properties of native AMPA receptors in two distinct ways, one causing decreased receptor desensitization and the other increased kainate efficacy. Although reliable expression of functional AMPA receptors in heterologous systems demonstrates that association with TARPs is not obligatory for AMPA receptor surface expression, the more robust surface trafficking upon co-expression with stargazin indicates this protein can substantially affect trafficking of native AMPA receptors; however, the extent of native receptor TARP association is unclear. Our data suggest that surface AMPA receptors in P7 cerebellar granule cells already contain saturating levels of stargazin, but receptors in P7 hippocampal neurons can accommodate additional stargazin association. It is not clear whether this is because few AMPA receptors are associated with TARPs in immature hippocampus, or because stargazin can displace other TARPs from the receptor complex; however, it is clear that TARPs must now be considered a factor in generating AMPA receptor functional heterogeneity. Our observation that individual subunits are differentially modulated by stargazin and γ3 further suggests that a given TARP will have distinct effects on native receptors with specific subunit compositions.

During the course of our work two short reports appeared that addressed effects of stargazin on recombinant AMPA receptors. Yamazake and colleagues reported that stargazin enhanced glutamate-evoked currents in HEK293 cells and *Xenopus* oocytes expressing GluR-A or GluR-B homomeric receptors, and that other TARPs had a similar effect. More recently, Priel et al. showed that stargazin slowed both desensitization and

deactivation of glutamate-evoked responses. Differences in the biophysical characteristics of native AMPA receptors, including desensitization and deactivation, have primarily been attributed to differences in subunit and isoform composition. Our data and that of Priel et al. (2005) indicate that association with TARPs provides an additional mechanism controlling channel properties. Whether TARPs are present, and which TARPs are included in a receptor are both expected to make significant contributions to the functional heterogeneity of neuronal AMPA receptors.

5.4.2 Differential Contribution of Intra- and Extracellular Domains to the Functional Effects of Stargazin

Based on recordings from *Xenopus* oocytes, Tomita and colleagues (2004) found that both the first extracellular domain of stargazin and a region in the cytoplasmic tail were necessary for increasing AMPA receptor currents, a finding interpreted as being exclusively due to trafficking. However, because this conclusion was based upon whole-cell currents obtained by bath application of agonists, this study could not discriminate between trafficking and potential functional effects of stargazin. Using whole-cell current density as a measure of total cell surface receptors, we found that increased receptor surface expression was mediated primarily by the C-terminal intracellular domain of stargazin. In contrast, both the first extracellular domain and the C-terminal tail were required for stargazin to exert maximal functional effects on AMPA receptors. Interestingly, the functional effects of stargazin can be partially dissociated, with the decrease in receptor desensitization being more dependent on an intact cytoplasmic tail, and the increase in kainate efficacy being more dependent on the first extracellular loop.

Priel and colleagues' (2005) observations that deletions of the cytoplasmic tail of stargazin up to aa 244 did not alter stargazin's effect on glutamate-evoked desensitization, combined with our observation of almost complete loss of effect with a truncation at aa 212, suggest that the region between aa 212 and 244 is important for modulating steady-state AMPA receptor desensitization. Conversely, our observation that exchange of the entire first loop abolishes the effect of γ2 on kainate efficacy indicates that interactions between the extracellular domains of stargazin and AMPA receptor subunits mediate the increase in kainate efficacy. The physical dissociation of regions important for the two principal functional effects of stargazin suggests they are mediated by distinct local conformational changes in the AMPA receptor protein.

The decrease in macroscopic glutamate-evoked desensitization observed with stargazin co-expression indicates that stargazin shifts the equilibrium between desensitized and open conformations of the AMPA receptor toward open states. This could be achieved either by stabilizing the dimer interface, and thus hindering the transition into the desensitized state, or by destabilizing the desensitized conformation so that the receptor more readily cycles back to an open state. Based upon reduced potentiation of glutamate-evoked currents by cyclothiazide in the presence of stargazin, Priel and colleagues (2005) suggest that, similar to cyclothiazide, stargazin affects desensitization by stabilizing the dimer interface. While the observation that stargazin slows the onset of desensitization is consistent with stabilization of the dimer interface, it can also result from the slowed rate of deactivation documented by Priel and colleagues (2005), as well as rapid recovery from the desensitized state. The speeding of both kainate and TCM association rates in the presence of glutamate, without a change in

affinity for kainate or TCM, suggests rather that stargazin primarily affects microscopic receptor desensitization by destabilizing the desensitized state. While numerous manipulations have been described that alter the stability of the dimer interface and thus the stability of the open state (Stern-Bach et al., 1998; Sun et al., 2002; Horning and Mayer, 2004), our results with stargazin indicate consideration should be given to interactions that alter the stability of the desensitized state.

5.4.3 Increased Efficacy of Kainate

The observed increase in kainate efficacy also suggests specific conformational changes in the receptor. Rosenmund et al. (1998) demonstrated that sequential binding of agonist to single AMPA receptor channels elicits three unique subconductance states; however, the precise mechanism by which agonist-induced conformational changes in the extracellular ligand binding domains are coupled to various conductance states is not known. Partial agonists induce a lesser degree of domain closure between the extracellular D1 and D2 domains in the GluR2/GluR-B crystal structure than do full agonists, and the amount of domain closure has been correlated with a specific agonist-dependent, electrophysiological profile of subconductance states (Armstrong et al., 2003; Jin et al., 2003). A binding site mutation that allows kainate to induce greater domain closure increases kainate efficacy and shifts the dose-response curve ~3-fold to the left (GluR2 L650T, Jin et al., 2003), which is very similar to the effect we see with stargazin. It is also possible that rather than affecting the degree of domain closure, stargazin alters the efficacy by which the same amount of domain closure is coupled to channel opening. Either mechanism would be predicted to result in a change in the distribution of

subconductance states, with more frequent and/or longer higher-conductance openings with kainate bound.

Given that the maximal increases in glutamate peak current can be attributed to stargazin's effects on trafficking, stargazin does not appear to alter maximum glutamate-evoked single-channel conductance. Normalization of kainate-evoked responses to glutamate peak responses (Fig. 18C) suggests that the effect of stargazin on kainate-evoked currents reaches a relative maximum defined by the highest conducting state achievable by the receptor.

5.4.4 Stoichiometry

Electron microscopy of native receptor complexes indicates TARPs contribute to the transmembrane portion of AMPA receptors (Nakagawa et al., 2005); however, the number of TARPs associated with a single native AMPA receptor is unknown. Stargazin-mediated responses of GluR-A_i (Fig. 18C) appear to partition into three groups, each with a similar shift in kainate efficacy, but with varying degrees of altered desensitization. Similar to the model for agonist binding and channel opening proposed by Rosenmund et al. (1998), the presence of two stargazin proteins per GluR-A_i tetramer may produce the minimum effect of increasing kainate efficacy and slightly altering glutamate-evoked desensitization, while association of three and then four stargazin proteins with the receptor could alter desensitization in a graded manner to a maximal level. The range of stargazin-mediated desensitization is greater for GluR-A_i and D_i than for GluR-B_i and C_i, possibly reflecting the distinct effects of stoichiometry on long-tailed rather than short-tailed subunits. Alternatively, assembly of stargazin with short-tailed

subunits may result in a less variable stoichiometry. The ratio of stargazin : GluR-A_i was varied in an attempt to manipulate the distribution of glutamate-evoked desensitization (1:1, 0.25:1, 2:1, data not shown); however, the results were inconclusive.

5.4.5 Differential Trafficking of AMPA and Kainate Receptor Subunit Combinations

The recent finding that stargazin acts as an AMPA receptor specific chaperone in the endoplasmic reticulum (Vandenberghe et al., 2005a) places it in an ideal location to affect AMPA receptor subunit assembly. The observation of decreased desensitization and increased kainate efficacy in homomeric receptors expressed in HEK293 cells indicates that, rather than altering subunit composition, stargazin affects AMPA receptor functional properties via a direct interaction with the receptor on the cell surface. Stargazin most dramatically affects trafficking of GluR-B_i (12-fold increase in surface expression) with intermediate effects on GluR-A_i and D_i (2-4-fold) and no significant alteration of GluR-C_i surface expression (Table 7), suggesting the possibility of a higher affinity interaction between stargazin and GluR-B_i. Stargazin-mediated surface trafficking of heteromers composed of GluR-A_i with either *flip* or *flop* isoforms of GluR-B resembles that of GluR-A_i rather than B_i homomers (Table 6). Interestingly, surface expression of GluR-A_oB_o heteromers is enhanced 10-fold, which may indicate that the surface trafficking function of stargazin is more vital for receptors composed predominantly of *flop* isoforms. Thus, our data suggest that stargazin may preferentially traffic native receptors of certain subunit combinations.

It is unclear why we observed stargazin-mediated trafficking of GluR6 when others have not (Chen et al., 2003), but this may reflect differences in expression systems

(HEK293 cells vs. *Xenopus* oocytes). Chen and colleagues also reported no increase in kainate currents in cerebellar granule neurons, and we concur that stargazin does not increase trafficking of GluR6/KA2 heteromers, the predominant kainate receptor in granule cells. It is, however, quite interesting that co-expression of KA2 apparently breaks the association of stargazin and GluR6. KA2 is known to contain an ER-retention signal in its C-terminal cytoplasmic tail that is masked when KA2 associates with GluR6, allowing the GluR6/KA2 receptor to exit the ER (Hayes et al., 2003). It is tempting to speculate that the association of GluR6 and KA2 cytoplasmic tails blocks the cytoplasmic interaction of stargazin and GluR6.

5.4.6 Physiological Consequences of AMPA Receptor Association with TARPs

The contribution of glutamate deactivation versus desensitization to synaptic responses varies with receptor type, quantal content, and structure of the synapse (Trussell et al., 1993; Kinney et al., 1997; Conti and Weinberg, 1999; Wall et al., 2002; Lawrence et al., 2003). Slowed deactivation, that is, dissociation of ligand from receptor, would be expected to prolong the time course of most synaptic responses. In contrast, reduction of and faster recovery from desensitization will have the greatest impact at synapses with high-frequency or prolonged activation (Trussell et al., 1993; Otis et al., 1996; Eliasof and Jahr, 1997). Certain native receptors exhibit extremely rapid recovery from desensitization (16 ms, Raman and Trussell, 1995) that is not observed in recombinant receptors. The demonstration of TARPs as components of native AMPA receptor complexes (Nakagawa et al., 2005; Vandenberghe et al., 2005b) and the ability of stargazin to destabilize the desensitized state (Fig. 11, 12) suggests that rapid recovery

from desensitization in native receptors may be due to the functional effects of TARP association.

Accelerating the rate of recovery from desensitization will affect synaptic responses during high frequency stimulation, such as that seen during plasticity-related events. Although PSD-95 limits the incorporation of TARP-associated receptors into the synapse (Chen et al., 2000; Schnell et al., 2002), our data indicates PSD-95 is not necessary for stargazin to alter functional properties of the receptor. While phosphorylation of stargazin plays an important role in AMPA receptor trafficking during bidirectional synaptic plasticity (Tomita et al., 2005), the functional effects of association of synaptic AMPA receptors with stargazin are also likely to contribute to activity-dependent events.

The substantial effects of stargazin on steady-state glutamate responses additionally suggest that TARP association may influence pathological events. Principle neurons in the hippocampus, striatum and cerebellum are selectively vulnerable to ischemia, while interneurons in these same regions may be spared (Cervos-Navarro and Diemer, 1991). This selective vulnerability has previously been attributed to the specific subtypes of AMPA receptors expressed in particular neurons. For example, cortical pyramidal cells exhibit slower and less extensive desensitization than cortical interneurons (Hestrin, 1993), which has been correlated with expression of *flip* AMPA receptor subunit isoforms (Lambolez et al., 1996). Similarly, AMPA receptor-mediated responses of Purkinje cells, which are among those cell types most vulnerable to ischemia, exhibit less desensitization than those of other cerebellar neurons (Brorson et al., 1995), which has also been related to greater expression of *flip* isoforms (Tomiyama et al., 1999).

The assumption that AMPA receptors exhibiting less steady-state desensitization (e.g., *flip*-containing receptors) will disproportionately contribute to the neuronal depolarization resulting from tonic glutamate release is well established. Our data indicate that TARP expression may make an equal or larger contribution to glutamate-evoked steady-state currents than does AMPA receptor subunit composition. While GluR-B mediated calcium-impermeability has been presumed to be neuroprotective, the dramatic decrease in desensitization of GluR-B_i homomers co-expressed with stargazin suggests that, when associated with stargazin, this subunit may exacerbate postsynaptic depolarization during excitotoxic events. In general, neurons expressing AMPA receptors associated with stargazin would be predicted to be more vulnerable to glutamate-mediated excitotoxicity during ischemic events. The lesser effect of γ3 on glutamate desensitization (Fig. 13, 15) also suggests that the particular TARP complement of the receptor could influence the susceptibility of neuronal subtypes to excitotoxicity.

5.4.7 Future Directions

Guided by the subunit-specific effects of stargazin on AMPA receptor trafficking and functional properties, we are generating several AMPA receptor subunit chimeras to localize domains in the AMPA receptor influenced by stargazin. Table 8 lists these chimeras and predicts their effects on stargazin-mediated alterations in trafficking and glutamate-evoked desensitization. We predict the first intracellular loop of AMPA receptor subunits is important in trafficking and that the presence of multiple proline

residues in this region of GluR-C disrupts stargazin association in a manner that interferes with its trafficking effects.

Conservation of the sequence of the second intracellular loop between AMPA receptor subunits suggests this is a region of functional importance. This loop is ideally placed near the re-entrant channel-lining membrane domain to influence channel properties such as desensitization; therefore, we predict that the second intracellular loop mediates effects on glutamate desensitization. We postulate that the variable effects on desensitization are due to interference by a long C-terminal with stargazin's interaction with this region.

TABLE VIII

Intracellular Domain AMPA Receptor Subunit Chimeras

Exchange	From	Into	Rational	Interpretation
1st IC loop	GluR-B →	GluR-C	GluR-C is not trafficked by Stg; GluR-B exhibits the greatest effect on trafficking	If trafficking is mediated by this region, this exchange should result in significant GluR-C surface trafficking
1st IC loop	GluR-C →	GluR-D	GluR-C is not trafficked by Stg; GluR-D has greatest sequence identity to GluR-C	This exchange should diminish surface trafficking of the chimera
2nd IC loop	GluR5 →	GluR-B	Stg has no functional effects on GluR5; Stg dramatically reduces GluR-B glutamate-evoked desensitization	If altered desensitization is mediated by this region, the chimera should be effectively trafficked but have no changes in desensitization
C-term tail	GluR-C →	GluR-A	Stg consistently affects GluR-C desensitization; Stg effects on GluR-A overlap with controls	A short C-terminal tail in GluR-A should eliminate overlap of Stg-mediated glutamate-evoked desensitization with control responses

Increased kainate efficacy is mediated by an extracellular interaction between the latter portion of the first extracellular loop of stargazin and the AMPA receptor. The similarity of the shift in kainate efficacy between AMPA receptor homomers, combined with the size and complexity of the extracellular domains, makes isolation of AMPA receptor residues involved in this effect more complicated than isolation of residues involved in intracellularly-mediated effects. Because the binding site mutant GluR-B L650T exhibits a nearly identical shift in kainate efficacy (Armstrong et al., 2003), we will examine the effects stargazin co-expression with this mutant. Occlusion of the shift in the dose response curve would suggest a similar mechanism of action, that is, an increased degree of kainate-evoked domain closure between D1 and D2 in the presence of stargazin.

The crystal structure of the extracellular portions of the AMPA receptor suggests a loop structure in domain 1 (loop 1) may be an extracellular site of interaction between the receptor and stargazin. The effects of stargazin on both kainate efficacy and glutamate-evoked desensitization are primarily or in part mediated by residues between aa 26 and aa 89 of stargazin's first extracellular loop (Fig. 17A, C, D). The secondary structure of stargazin has not yet been resolved; therefore, the distance stargazin's first extracellular domain extends from the membrane is unknown. It is possible that the extracellular residues mediating the effects of stargazin on the AMPA receptor are clustered near the apex of the first extracellular loop and interact with loop 1 on the AMPA receptor (Diagram 11). While chimeric AMPA receptor subunits with loop 1 exchanges may have significantly altered receptor kinetics in the absence of stargazin, this mutant may disrupt

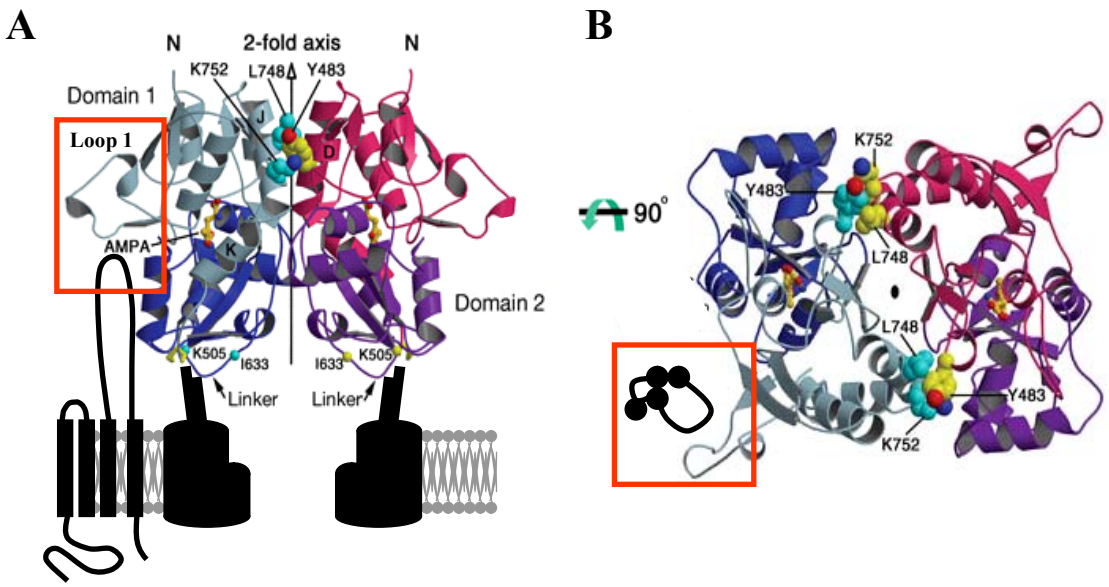


Diagram 11. Loop 1 is a possible extracellular region of interaction between stargazin/γ2 and the AMPA receptor. A. The crystal structure of the extracellular domains is shown with the relative location of the channel portions of the receptor and the possible location of stargazin. The red box outlines regions of possible interaction between stargazin and the AMPA receptor. B. A 90° rotation of stargazin and the crystallized domains of the AMPA receptor depicts a bird's eye view of the possible extracellular regions of interaction between stargazin and loop 1 of the AMPA receptor (red box; modified from Sun et al., 2002).

the extracellular interactions between stargazin and the AMPA receptor necessary for alterations in kainate efficacy and glutamate-evoked desensitization.

While all four subunits exhibit a distinct increase in kainate efficacy, this shift is relatively smaller for GluR-B and of a similar magnitude as the effect of γ2 on desensitization. Decreased desensitization at AMPA receptors has been shown to left-shift the kainate dose-response curve (Patneau et al., 1993), suggesting the kainate effect

in GluR-B may reflect reduced desensitization. mRNA editing of GluR-B at the Q/R site in the channel-lining re-entrant loop severely decreases channel conductance (Swanson et al., 1997). While stargazin appears to increase the amount of time the kainate-bound receptor resides in higher conductance states, GluR-B may less effectively transduce stargazin-mediated allosteric changes to the channel portion of the receptor than GluR-A, -C, and -D. The heteromeric receptors examined in these studies exhibited shifts in kainate efficacy resembling that seen with GluR-B rather than GluR-A, suggesting that, similar to the dominance of GluR-B in Ca^{2+} permeability (Hollmann et al., 1991; Sommer et al., 1991), the presence of the GluR-B subunit differentially affects the responsiveness of the channel to stargazin-mediated conformational changes in the extracellular domains of the AMPA receptor. A shift in kainate efficacy comparable to that of GluR-A, -C and -D upon co-expression of stargazin with unedited GluR-B homomers would indicate the presence of an arginine in the pore constrains the allosteric effects of stargazin on kainate efficacy.

Co-expression of GluR-A with γ2(Δ89-103)/γ5(ins85-99), a chimera with a small region in the latter portion of the first extracellular loop of stargazin exchanged with γ5, decreases the effect of stargazin on kainate efficacy but not glutamate-evoked desensitization (Fig. 17). If the shift in kainate efficacy in GluR-B is primarily a consequence of altered desensitization, expression of GluR-B with this construct should result in kainate efficacy indistinguishable from that of wild-type stargazin.

While stargazin is involved in hippocampal LTP (Tomita et al., 2005), γ8 is the primary TARP expressed in hippocampus (Tomita et al., 2003). γ8 both rescues AMPA receptor surface expression in *stg/stg* cerebellar granule cells (Tomita et al., 2003) and

selectively enhances glutamate-evoked currents in heterologous cells (Yamazaki et al., 2004); however, the specific functional effects identified in this study have not been characterized for γ8. We plan to clone and express this protein in both hippocampal neurons and heterologous cells to determine the functional effects of γ8 on native and recombinant AMPA receptors.

Chapter VI

SUMMARY AND SIGNIFICANCE

6.1 Disassembly of the Benzothiadiazine Binding Site in the Desensitized State of the AMPA Receptor

Crystallization of the extracellular domains of the GluR-B/GluR2 subunit of the AMPA receptor at rest and in complex with various agonists has greatly enhanced our understanding of the mechanism of glutamate receptor desensitization. The strong correlation between enhanced dimerization of AMPA receptor extracellular domains with certain mutations at the dimer interface and reduced desensitization seen electrophysiologically in heterologous systems expressing AMPA receptor subunits with the same mutations suggest that the stability of this interaction is crucial in determining the extent of desensitization at native receptors. Binding of the benzothiadiazine positive allosteric modulator cyclothiazide at the dimer interface also significantly stabilizes the dimer interface (Sun et al., 2002) and greatly decreases desensitization of recombinant and native AMPA receptors (Patneau et al., 1993; Yamada and Tang, 1993).

The initial portion of this dissertation focused on confirmation and extension of the model of AMPA receptor desensitization in native hippocampal AMPA receptors. Crystallization of the extracellular domains of GluR2 has generated great insight into the structure of the non-desensitized state; however, the separation of the dimer interface has

thus far prevented crystallization of the extracellular domains in the desensitized conformation. Our results indicate the binding site for the benzothiadiazines is available in both the resting and non-desensitized state; however, the conformational change into the desensitized state disassembles the binding site for the modulator. Our findings in native hippocampal AMPA receptors reveal that the benzothiadiazines exhibit a state-dependent interaction with the receptor in which the conformation of residues forming the dimer interface is altered in the desensitized state in a manner that precludes modulator binding.

6.2 Stargazin/γ2 Decreases Glutamate-Evoked Desensitization and Increases Kainate Efficacy at AMPA Receptors

Unlike the associated proteins GRIP1 and PICK1, stargazin dramatically affects AMPA receptor functional properties. This is the first demonstration of an associated protein directly altering AMPA receptor functional properties, adding significant support to the argument that TARPs act as the first demonstrated auxiliary subunit of an ionotropic glutamate receptor. While the findings that stargazin selectively enhances glutamate-evoked steady-state currents at AMPA receptors (Yamazaki et al., 2004) by reducing desensitization (Priel et al., 2005) have recently been published, our lab has further identified novel effects of stargazin on kainate efficacy and rectification properties of certain AMPA receptor subunits. We have also presented strong evidence that endogenous stargazin contributes to the functional properties of native receptors in cerebellar granule cells. Additionally, we have determined that PSD-95, which is known

to be necessary for stargazin-mediated synaptic trafficking of AMPA receptors, is not required for stargazin's effects on AMPA receptor function.

The identification of domains in stargazin involved in mediating both functional effects and AMPA receptor trafficking is a novel finding with the potential of great impact in the AMPA receptor field. Domain swaps between stargazin and the non-TARP γ5 revealed that the latter portion of the first extracellular loop of stargazin primarily mediates altered kainate efficacy, while truncation of the C-terminal tail suggests that trafficking and effects on desensitization are primarily mediated by this portion of stargazin. We have also shown that γ3, another member of the TARP family, affects both function and trafficking of the AMPA receptor. Interestingly, the trafficking and altered desensitization seen with γ3 are very similar to that observed with the C-terminal truncation mutant. Stargazin and γ3 have greatest sequence divergence in their C-terminal tails, further corroborating the importance of the stargazin C-terminus in AMPA receptor trafficking and desensitization.

Because our initial studies identified the state-dependent mechanism of interaction of the benzothiadiazine modulators with the AMPA receptor, we were able to use this tool to define the mechanism by which stargazin decreases glutamate-evoked desensitization at the AMPA receptor. Unlike mutations and pharmacological compounds that act at the dimer interface and stabilize the non-desensitized state, stargazin is unique in that it destabilizes the otherwise energetically favorable desensitized state. Destabilization of, and therefore faster recovery from, the desensitized state would decrease the latency at which a synapse could produce a full amplitude response during periods of high frequency stimulation such as occur during plasticity-related events.

While molecular manipulation of various domains of stargazin identified areas of functional significance in stargazin, subunit-specific differences in trafficking and functional effects suggest areas of interaction within the AMPA receptor. The variable effects of stargazin on desensitization seen in AMPA receptor subunits with long C-terminal tails versus the more consistent effects with short-tailed subunits further supports the idea of an intracellular locus playing a substantial role in altered glutamate-evoked desensitization.

6.3 Stargazin Differentially Traffics AMPA and Kainate Receptor Subunits

Although the well-established trafficking function of stargazin was not our primary focus, we identified several novel aspects of this interaction. In contrast to the results of Chen et al (2003), we found stargazin traffics certain homomeric kainate receptors, indicating the trafficking effects of stargazin are not AMPA receptor-specific. Additionally, stargazin differentially traffics both homomeric and heteromeric AMPA receptors. Homomeric GluR-B exhibits the most dramatic increases in surface expression, suggesting a higher affinity interaction of stargazin with GluR-B. Surprisingly, stargazin does not appear to increase trafficking of GluR-C, although both functional effects are evident, indicating that in the absence of trafficking, stargazin remains capable of a functionally significant association with AMPA receptors at the cell surface. Interestingly, while GluR-A_iB_o and GluR-A_iB_i heteromers are trafficked similarly to GluR-A_i, stargazin-mediated surface expression of GluR-A_oB_o is more like that of GluR-B_i, suggesting stargazin preferentially traffics certain heteromeric subunit combinations.

6.4 TARPs as Auxiliary Subunits of the AMPA Receptor

Our data add to the argument for designation of TARPs as auxiliary AMPA receptor subunits. Three criteria have typically been considered as evidence that a protein acts as an ion channel auxiliary subunit: demonstration of a direct and stable interaction with the pore-forming subunit, direct modulation of the biophysical properties, and trafficking of the pore-forming subunit (Arikkath and Campbell, 2003). In addition to the considerable literature documenting the role of stargazin in AMPA receptor trafficking (Chen et al., 2000; Schnell et al., 2002; Cuadra et al., 2004; Vandenberghe et al., 2005a), two recent papers report the co-purification of TARPs with native AMPA receptors (Nakagawa et al., 2005; Vandenberghe et al., 2005b). The observation that stargazin/γ2 alters AMPA receptor channel properties in hippocampal neurons completes these three criteria. The distinct effects of stargazin and the TARP family member γ3 on the function of various AMPA receptor subunit combinations suggests that incorporation of TARPs as auxiliary subunits increases the functional heterogeneity of native AMPA receptors. The ability of TARPs to alter AMPA receptor functional properties further implies these proteins play significant but as yet undefined roles in basal neurotransmission, activity-dependent synaptic plasticity and excitotoxic events.

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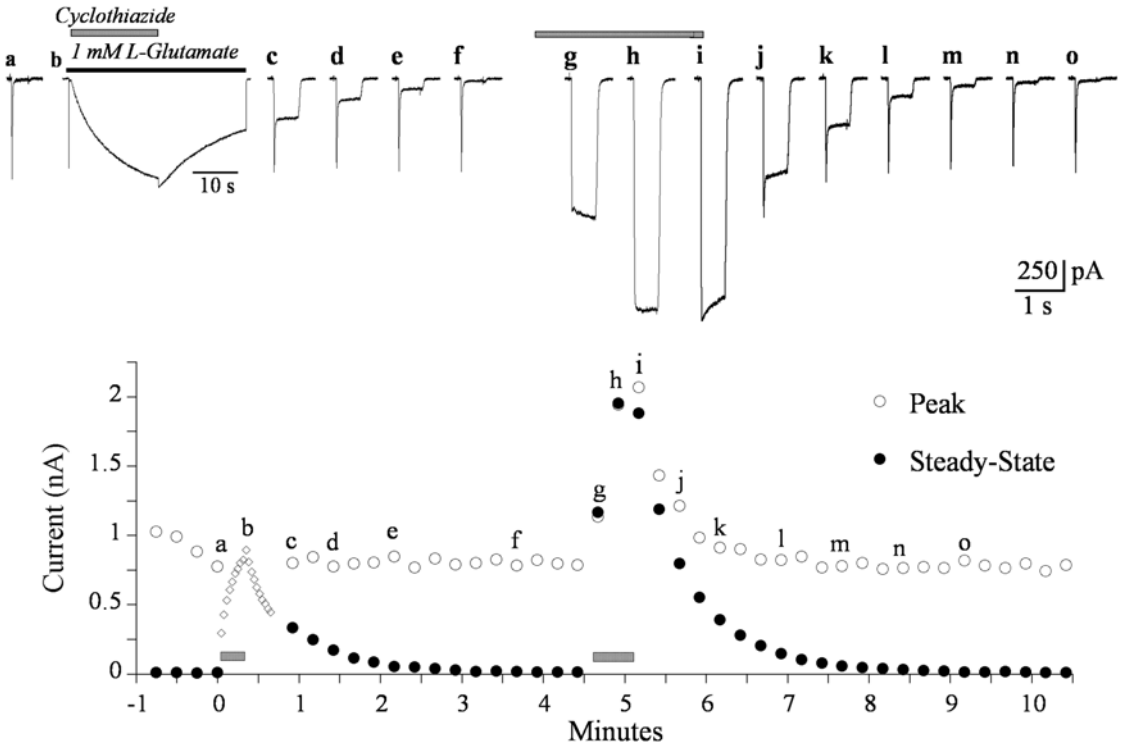
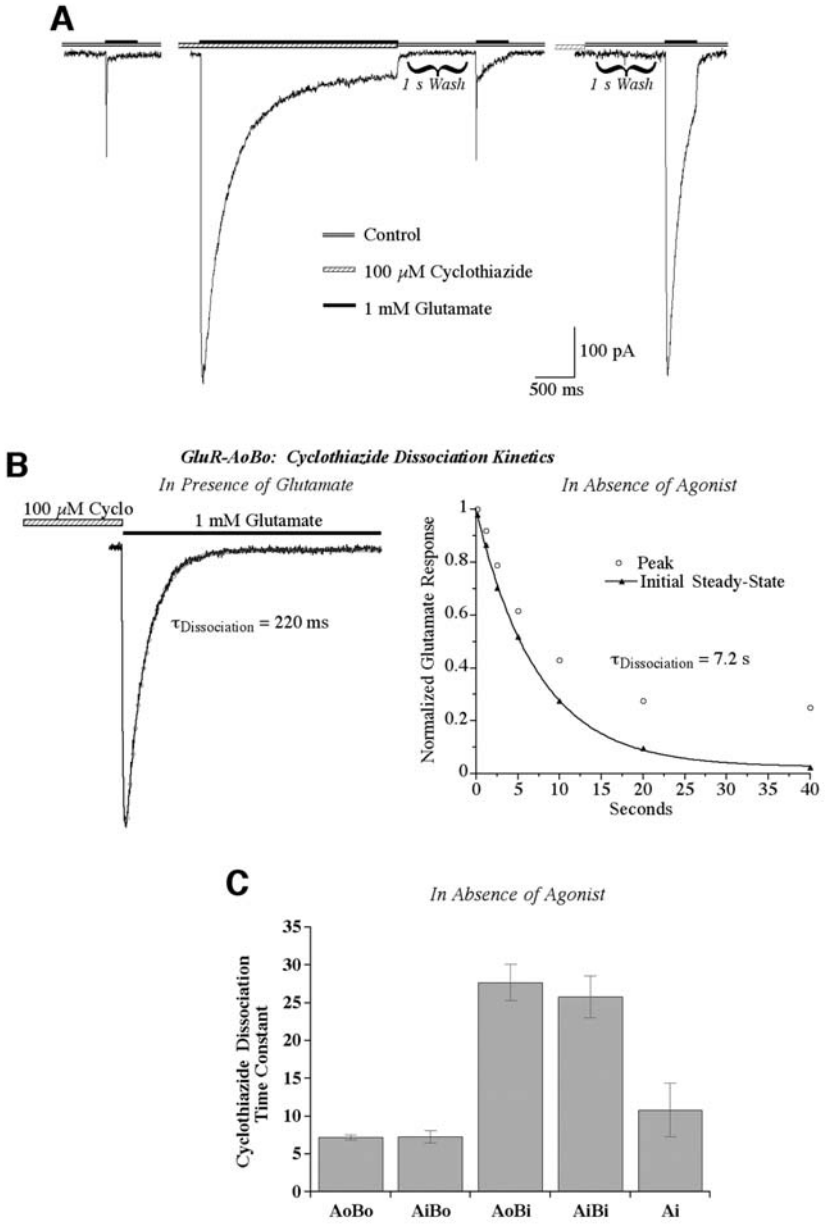
APPENDIX 1

Supplemental Figures



Appendix Figure 1. The Efficacy and Onset Kinetics of Modulation by Cyclothiazide (CTZ) Are Dependent on the State of the Receptor. Responses to 500 ms applications of 1 mM glutamate (top) correspond to the single data points as indicated on the graph below (bottom, **a, c-o**). **a** is the response to glutamate alone before modulator application. **b** shows the response to a 40 s application of glutamate (filled bar; see separate time scale), with CTZ co-applied for 20 s as indicated by the grey bar. Note the very slow onset of potentiation, rapid increase in current immediately after removal of CTZ, and the slow kinetics of recovery from potentiation, which was complete in approximately 3 minutes (**f**). CTZ was then applied in the absence of glutamate, and the onset of potentiation developed much more rapidly, with greater potentiation observed

after 2 s pretreatment with CTZ (**g**) than with a 20 s coapplication with glutamate (compare to **b**), and was complete within 17 s (**h**). The response in **i** is 2 s after removal of CTZ. The rate of recovery from potentiation by CTZ is well described by a single time constant of approximately 40 s. Note that the kinetics of desensitization in response to glutamate as the block of desensitization by CTZ decreases (e.g., **k-n**) are similar to those for the control response (**a**) before CTZ treatment. These results are consistent with the interpretation that CTZ either cannot bind to or has very low affinity for the desensitized state of the receptor. Also, the similarity of the rate of dissociation in both the presence and absence of agonist indicates CTZ has similar affinity for the receptor in the presence and absence of agonist.



Appendix Figure 2. Strong Negative Cooperativity Between Binding of Cyclothiazide and Agonist Underlies Differential Modulation of AMPA Receptor Isoforms. Although cyclothiazide dramatically reduces or fully blocks desensitization of most native AMPA receptors (Patneau et al., 1993; Fleck et al., 1996), in experiments with recombinant receptors cyclothiazide appears to slow, but not block, desensitization of *flop* splice variants (Partin et al., 1994). Because our data (Appendix Fig. 1, Chapter 3) strongly

suggest that cyclothiazide cannot bind to desensitized receptors, and that receptors with cyclothiazide bound cannot desensitize, we hypothesized that this "desensitization" of responses in the presence of cyclothiazide might instead reflect the dissociation of cyclothiazide from the receptor. To test this hypothesis, we recorded responses to glutamate from HEK293 cells transfected with GluR-A_o and -B_o before (A, trace at left), during, and after application of 100 μM cyclothiazide. Note that the percent of receptors with cyclothiazide still bound following a 1 s wash with control solution was dependent on whether cyclothiazide was applied in the presence (middle traces) or absence (trace at right) of glutamate. This indicates that the apparent desensitization of agonist responses in the presence of cyclothiazide reflects negative cooperativity between the binding of agonist and cyclothiazide, which results in the rapid dissociation of cyclothiazide from the receptor in the presence of glutamate, rather than receptor desensitization with cyclothiazide still bound. (B) When cyclothiazide was removed just prior to the glutamate pulse, the response “desensitized” even faster; we interpret this as the time constant of dissociation of cyclothiazide in the presence of glutamate. (C) In comparison, the rate of dissociation of cyclothiazide for A_oB_o receptors in the absence of agonist was 30-fold slower, and was similar to rate of dissociation from A_iB_o receptors, a composition that has a fully non-desensitized response to glutamate in the presence of cyclothiazide. This indicates that the apparent selectivity of cyclothiazide for *flip* isoforms is not due to inherent differences in affinity (Partin et al., 1994), but rather due to differences in the allosteric interactions between modulator and glutamate binding sites, such that binding of glutamate lowers the affinity for cyclothiazide 30-fold in *flop* receptors.

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ABSTRACT:

AMPA receptors play a central role in basal excitatory synaptic transmission as well as synaptic maturation and plasticity. An important characteristic of AMPA receptors (AMPARs) is the rapid and profound desensitization that occurs within milliseconds of glutamate binding; however, the mechanisms controlling desensitization of native AMPARs are incompletely understood. We have applied patch-clamp recording techniques in cultured neurons and heterologous cells to (1) test a model of AMPAR desensitization based on the crystal structure of the extracellular domains and (2) determine whether proteins associated with synaptic AMPARs affect desensitization and/or other channel properties. A key finding from crystallization of the extracellular domains of the receptor is that the benzothiadiazine positive modulator cyclothiazide, which blocks AMPAR desensitization, binds at and stabilizes the dimer interface formed by adjacent subunits. This and other data suggested a model in which desensitization involves a conformational change disrupting the dimer interface. Our data from intact hippocampal AMPAR indicate the benzothiadiazines prevent the conformational change to the desensitized state, and, conversely, cannot bind desensitized AMPARs.

The transmembrane AMPAR regulatory protein (TARP) stargazin (γ2) serves multiple roles in trafficking and stabilizing synaptic AMPARs and may be incorporated as an auxiliary subunit. Transfection of hippocampal neurons with stargazin produced two distinct effects on AMPAR functional properties without affecting surface receptor density: reduced glutamate-evoked desensitization and increased efficacy of the partial agonist kainate. Kinetic and dose-response analyses suggest that stargazin affects glutamate desensitization through an allosteric interaction that destabilizes the desensitized state of the receptor, and that potentiation of kainate responses reflects increased efficacy rather than altered affinity. Stargazin's effects were also observed in HEK 293 cells transfected with various heteromeric and homomeric AMPARs, with subunit-dependent effects on glutamate desensitization, kainate efficacy, and trafficking. We show that two regions of stargazin mediate its functional effects: the C-terminal intracellular domain appears more important for effects on glutamate-evoked desensitization and receptor trafficking, while the first extracellular domain makes a larger contribution to altered kainate efficacy. These data indicate that TARPs directly modulate channel function and, as auxiliary subunits of AMPARs, contribute to functional heterogeneity of the desensitization properties of neuronal AMPARs.

ADVISER'S APPROVAL: Doris K. Patneau