Title
The role of AMPA receptor subunits in synaptic plasticity

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Publication Date
2013

Peer reviewed|Thesis/dissertation

The role of AMPA receptor subunits in synaptic plasticity

by

Adam J. Granger

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

[redacted]

For my parents

Acknowledgements

First and foremost, I would like to thank Roger Nicoll, my graduate advisor, for the invaluable mentorship he has provided during my thesis work. His unmatched integrity and passion for science set a standard that I will strive to meet for the rest of my career. Countless times during my graduate work, if I ever became discouraged or demoralized at the many confusing, contradictory, or unexpected results my experiments yielded, a short talk with Roger was all I needed to renew my confidence and refocus my efforts. It is only through his example and training that I now feel confident to critically assess research on synaptic physiology and to plan and execute rigorous and well-controlled experiments of my own.

I also owe a debt of gratitude to the lab environment fostered by Roger and maintained by my fellow lab members. When I first joined the lab, several senior lab members provided a model for knowledge and expertise that I have tried to replicate, including Anastassios Tzingounis, Aaron Milstein, and Carleton Goold. I would like to especially thank Wei Lu, who trained me when I first joined the lab and is largely responsible for passing on to me the project that would become my thesis. I also owe thanks to the post-docs and students who I worked in the lab, John Gray, Alex Jackson, Bruce Herring, Maya Yamazaki, Yun Shi, Nengyin Sheng, and Quynh-Anh Ngyuen for always being available for insightful discussions about our research, for help when my equipment or experiments were not working properly, and for generally making the lab a fun and exciting place to work. Kirsten Bjorgan and Manuel Cerpas provided crucial technical support, allowing me to focus more fully on planning and executing experiments.

My lab experience has been shaped most by my fellow graduate students, who I now consider among my closest friends. Seth Shipman originally convinced me to rotate in the lab, for which I am extremely grateful. His great skill and productivity with electrophysiology kept me motivated to be a better scientist. Jon Levy joined at the same time I did, and has become my last resort for technical issues and experimental problems. If I cannot figure out how to make a particular cloning work, or am having trouble with Igor, I know that Levy's problem solving-skills and generosity with his time will soon fix matters. I particularly want to thank Kate Lovero for tolerating working so close to me for the past five years. Sharing a work-space with her, more than anything else, is what makes coming into lab so consistently fun and rewarding.

Outside of the lab, I would like to thank my other mentors and advisors who have helped me throughout my academic career. The members of my thesis committee: Grae Davis, Rob Edwards, and Anatol Kreitzer all provided extremely valuable advice and guidance through-out my thesis work. Hillel Adesnik graciously served as my outside committee member, and through his previous work in Roger's lab and subsequent success as a post-doc and young faculty serves as a model for scientific rigor. During my graduate career, Louis Reichardt worked tirelessly to create a vibrant neuroscience community at UCSF, for which I am extremely grateful, as well as for the work of Pat Veitch, Carrie Huckaba, and Lucita Nacionales, who kept the trains running on time. I'd also like to thank my fellow classmates, especially Meg Younger, Sarah Fischbach,

I owe a large debt of gratitude to Sheri Holmen, who introduced me to my first experiences of working in a lab and publishing, and whose example of dedication and

hard work I still try to follow. I would also like to thank my professors from Kalamazoo College for giving me such a strong background in basic science, math, and neuroscience, especially Paul Sotherland, James Langeland, Micheal Tanoff, and Blaine Moore.

I also owe many thanks to my friends and family, both in San Francisco and across the globe. I have bounced around several apartments in the 6 years I've lived in San Francisco, and am lucky to be able to count many of my former roommates as close friends. In particular, I am grateful for the love and support of Chadé Severin, who has always been so supportive, encouraging, and understanding of my scientific work, and who has been such an important part of my life these past several years. I am grateful to my sister, Beth, who moved to the Bay area and started a family of her own, giving me a positive, grounding family-presence that I can rely on nearby.

Finally, I am grateful to my parents, Bill and Deb Granger. They have been such positive influences on my life, and without their constant encouragement and support, I know I never would have had the ability to succeed in graduate school. They have always pushed me to do my very best, even fighting throughout my early schooling to ensure that I was always in classes and environments where I could learn and achieve the most. To say I would not be here without them is an extreme understatement, and I will be forever thankful.

Contributions

The experiment in Figure 17 of Chapter 3 was done in collaboration with Yun Shi. All other expeirments were carried out by Adam Granger. The initial study aims in Chapter 3 were conceived in collaboration with Wei Lu and Roger Nicoll. In addition, Chapter 3 has been previously published in *Nature* and is reproduced with permission:

> Granger AJ, Shi Y, Lu W, Cerpas M & Nicoll RA. (2013). LTP requires a reserve pool of glutamate receptors independent of subunit type. *Nature* **493,** 495-500.

ABSTRACT OF THE DISSERTATION

The role of AMPA receptor subunits in synaptic plasticity

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Doctor of Philosophy in Neuroscience

University of California, San Francisco, 2013

Memories are stored in the brain via specific patterns of connectivity between individual neurons. Learning occurs through changes in this pattern of connectivity in response to activity, such that a synapse becomes more or less effective at influencing a postsynaptic neuron. This process, called synaptic plasticity, has been demonstrated at excitatory glutamatergic synapses of the hippocampus, where precise patterns of activity can either increase (long-term potentiation, LTP) or decrease (long-term depression, LTD) synaptic strength. Both LTP and LTD are carried out through changes in the number of postsynaptic AMPA-type glutamate receptors (AMPARs). Therefore, to understand synaptic plasticity, we must also understand the trafficking of AMPARs. In the case of LTP expression, the AMPAR subunit GluA1 is specifically required, and modifications of its cytoplasmic tail (C-tail) are thought to be particularly important for the activity-dependent recruitment of AMPARs. To identify the minimum region of the GluA1 C-tail required for LTP, I used a single-cell molecular replacement strategy where all endogenous AMPARs are replaced with transfected subunits. Surprisingly, I found no requirement for the GluA1 C-tail or for GluA1 generally for expression of LTP. Instead, molecular replacement with either GluA2 or the kainate receptor subunit GluK1 resulted in normal LTP. The only conditions under which LTP was impaired were those

with a dramatically decreased pool of receptors on the neuronal surface. Similar to LTP, I also found no specific AMPAR subunit requirement for LTD, which was expressed normally in neurons only expressing GluK1. These results suggest that synaptic plasticity is not necessarily a direct modification of the glutamate receptors subunits themselves, but a broader change in the ability of the synapse to anchor postsynaptic receptors.

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CHAPTER 1:

General Introduction

Learning and Synaptic Plasticity

A nervous system grants an organism with the ability to perceive and process information about its environment and rapidly produce an appropriate behavioral response. The adaptive advantage of a nervous system is greatly increased, however, if it can store relevant information and refine its output in response to experience. This may include being able to recall the location of good sources of food, to form distinct social relationships, or to develop new strategies to hunt and capture prey. Among all organisms, humans undoubtedly have the largest capacity for learning, and rely heavily on experience-dependent refinement of neuronal circuits for even basic functions such as motor coordination and perception. Understanding the neural mechanisms of memory formation is therefore a fundamental problem towards understanding the brain, and of particular importance for human thought and cognition.

Theories on the physiological basis of learning and memory can be crudely divided into two complementary theories. Learning may be mediated by persistent, reverberating activity in specific neuronal populations or by stable, structural changes in the synaptic connections between neurons (Seung, 2000). Though my thesis will deal exclusively with the latter theory, persistent reverberatory activity is still thought to be an important mechanism by which the brain can store information, though most likely only for very short times (Durstewitz *et al.*, 2000; Seung, 2000). The dominant theory in neuroscience for long-term learning, though, is through the stable and structural strengthening of neuronal pathways. This notion predates even the discovery of synapses, articulated by the psychologist Willam James in 1890 when he hypothesized that new behavioural habits may be formed when "a path once traversed by a nerve-

current might be expected to follow the law of most of the paths we know, and to be scooped out and made more permeable than before; and this ought to be repeated with each new passage of the current" (Berlucchi & Buchtel, 2009). Eugenio Tanzi and his student Ernesto Lugano extended this notion to the synapse, hypothesizing that learning involved a reduction in the distance between neuronal contacts, making it easier for the signal to cross between neurons. Of course, the famous neuroanatomist Santiago Ramon y Cajal also adopted this theory, and expanded on it by theorizing that it is insufficient to only strengthen pre-existing neural pathways, but that new ones must also be formed through the growth of dendrites and axons (Berlucchi & Buchtel, 2009). In modern neuroscience, It is Donald Hebb who is best recognized for laying down the theoretical framework for how memories might be encoded in synaptic strengths, to the extent that it is now referred to in shorthand as "Hebbian plasticity." He describes how memories may be stored in his textbook *Organization of Behaviour* (1949)*:*

> "Let us assume then that the persistence or repetition of a reverberatory activity (or 'trace') tends to induce lasting and cellular changes that add to its stability. When an axon of cell A is near enough to excite a cell B and repeatedly or persistently take part in firing it, some growth process or metabolic change takes places in one or both cells such that A's efficiency, as one of the cells firing B, is increased."

To this day, activity-dependent changes in synaptic strength, or synaptic plasticity, remain our best theory to explain how information is stored in the brain.

Long-term potentiation

Synaptic plasticity as a phenomenon remained purely theoretical until 1973, when Tim Bliss and Terje Lømo published a study on synaptic transmission in the hippocampus of anesthetized rabbits (Bliss & Lomo, 1973). They found that the amplitude excitatory postsynaptic potentials (EPSPs) recorded in the dentate gyrus following electrical stimulation of inputs from entorhinal cortex could be increased by a single burst of high frequency stimulation, called a tetanus. This increase was stable over long periods of time, lasting as much as 14 hours. Dubbed "long-term potentiation", or LTP, physiologists now had a phenomenon to study that, by virtue of its discovery in the hippocampus, which is required for memory formation, and its corroboration with previous theory, plausibly underlies learning. Though a variety of other forms of synaptic plasticity have since been described, LTP remains the gold standard of synaptic plasticity research.

At the time that LTP was discovered, relatively little was known about the basic mechanisms of synaptic transmission, much less how it might be altered during plasticity. After it became clear that glutamate was the main excitatory neurotransmitter throughout the brain, different classes of ionotropic glutamate receptors were described based on differential activation by artificial agonists – NMDA-type receptors and quisqualate-type receptors (Davies *et al.*, 1979). The quisqualate receptors were eventually identified as two separate classes of glutamate receptor, kainate receptors and AMPARs (Keinanen *et al.*, 1990; Pook *et al.*, 1993), the latter mediating the majority of fast, excitatory neurotransmission in the brain. The discovery of a highly selective antagonist against NMDARs (Davies *et al.*, 1981) allowed Collingridge and colleagues (1983) to demonstrate that specifically blocking NMDARs also blocked expression of

LTP following high frequency stimulation (Collingridge *et al.*, 1983). This finding has been so widely reproduced and accepted, that it is now commonly used as a mechanistic control in studies on LTP.

However, the requirement for NMDARs was initially puzzling, since current through NMDARs could not be recorded from neurons during normal synaptic stimulation (Collingridge *et al.*, 1983). This mystery was resolved by several concurrent discoveries. First, Wigstrom and colleagues (1982) showed that pairing depolarization with synaptic stimulation was sufficient to induce LTP (Wigstrom *et al.*, 1986). At the same time, NMDARs were shown to be blocked at resting membrane potentials by extracellular Mg^{2+} ions, which could be relieved by depolarizing the membrane, allowing NMDARs to pass current (Mayer *et al.*, 1984; Nowak *et al.*, 1984). NMDARs become active, then, when both the presynaptic axon terminal releases glutamate and the postsynaptic cell is depolarized, which occurs during high-frequency stimulation. With these remarkable findings, it is difficult to imagine a more straightforward means for physiology to confirm Hebb's postulate: NMDARs detect coincident activation of the axon of cell A by the presence of glutamate and the firing of cell B by depolarization, resulting in an increase in synaptic strength (LTP) such that cell A becomes more effective at firing cell B.

Beyond this convergence of theory and physiology, multiple lines of evidence also exist directly connecting LTP with behavioral learning. The hippocampus, where LTP was discovered and the brain region where it is most extensively studied, was already known to be required for memory formation, as demonstrated by the famous patient H.M., whose bilateral hippocampal lesions left him unable to form explicit

memories (Scoville & Milner, 2000). Subsequent experiments showed that similar hippocampal lesions in rats removes the ability to form spatial memories (Morris *et al.*, 1982). Manipulations of the NMDAR further provided experimental footholds connecting learning with LTP. For example, intracerebralventricular application of D-AP5, the selective antagonist of NMDARs, blocks spatial learning in mice (Morris *et al.*, 1986), as does specifically deleting the obligatory NMDAR subunit protein GluN1 in CA1 pyramidal neurons (Tsien *et al.*, 1996). It has been more difficult to demonstrate that the induction of LTP in the hippocampus follows learning, largely because the synapses altered by a single learning event are sparsely distributed and difficult to identify. One study, however, has shown a sparse increase in synaptic strength in CA1 neurons following an inhibitory avoidance task that occluded subsequent induction of LTP by high-frequency stimulation (Whitlock *et al.*, 2006). Changes in synaptic strength following learning have also been demonstrated in other brain areas such as the amygdala following cue-reward learning (Tye *et al.*, 2008) or fear conditioning (Sigurdsson *et al.*, 2007). Combined with the variety of LTP-inhibiting manipulations that also prevent learning tasks in experimental models, this strongly supports a model whereby memories are stored in the brain at least partially via LTP.

How does activation of NMDARs result in synaptic potentiation? The different biophysical properties of AMPA and NMDARs provide a clue: NMDARs in the hippocampus are permeable to Ca^{2+} ions, whereas AMPARs are not (MacDermott *et al.*, 1986; Jahr & Stevens, 1987; Mayer & Westbrook, 1987; Ascher & Nowak, 1988). Ca^{2+} is known to be a potent intracellular signaling molecule, and intracellular perfusion of EGTA, a Ca^{2+} chelator, prevents expression of LTP (Lynch *et al.*, 1983). Subsequent

experiments have shown that Ca^{2+} activates the Ca^{2+}/Calmodulin-dependent kinase CaMKII, which can be mutated to inhibit LTP (Giese *et al.*, 1998). Alternatively, expression of a constitutively active form of CaMKII increases synaptic transmission, occluding further potentiation by LTP (Pettit *et al.*, 1994; Lledo *et al.*, 1995). CaMKII's role in LTP induction is now widely accepted, rivaled only by NMDAR activation as a required signaling event. The downstream signaling steps following CaMKII activation remain unclear, but a large body of research is devoted to studying CaMKII signaling in dendritic spines and how it might regulate synaptic strength (Lisman *et al.*, 2012).

LTP expression: Presynaptic vs. Postsynaptic

By the mid-1990s, broad agreement had been reached on the developing mechanisms of LTP induction: coincident and persistent firing of pre and postsynaptic neurons results in the opening of NMDARs, influx of Ca^{2+}- ions, and activation of CaMKII. Despite this broad agreement, significant debate persisted as to the location of LTP expression. The main question was whether LTP was expressed presynaptically by some increase in the release of neurotransmitter, or postsynaptically by an increase in current through AMPARs. The crux of the disagreement was due to the observation that LTP decreased the trial-to-trial variation of synaptic transmission as well as synaptic failures, both of which had classically been attributed to an increase in the probability of releasing neurotransmitter, a presynaptic mechanism (Kerchner & Nicoll, 2008). However, a large body of evidence supported a specific postsynaptic increase in AMPARs, including the findings that current through AMPARs increases without changing NMDA-mediated currents (Kauer *et al.*, 1988; Muller *et al.*, 1988) and the lack of an observed increase in glutamate release by a large variety different assays (Nicoll,

2003). This debate was largely resolved with the discovery of silent synapses, which contain NMDARs but no AMPARs. These synapses pass no current at resting membrane potentials, but can still be potentiated by NMDAR activation. Following LTP induction, AMPARs are inserted into these synapses (Kullmann, 1994; Isaac *et al.*, 1995; Liao *et al.*, 1995), increasing the global amplitude of synaptic transmission in a way that both decreases synaptic failures and trial-to-trial variability.

Since the emergence of silent synapses, a broad consensus has emerged for a postsynaptic locus of LTP expression. However, at least one group of researchers continued to find evidence supporting a presynaptic locus of LTP, using a technique dubbed optical quantal analysis. (Emptage *et al.*, 1999; Emptage *et al.*, 2003; Ward *et al.*, 2006; Enoki *et al.*, 2009). In this technique, neurons are loaded with a Ca^{2+}-sensitive dye to optically identify activated synapses, with simultaneous monitoring of voltage by sharp electrodes. This method is designed to overcome the difficulty of monitoring unitary synaptic transmission, when stimulation of even a single afferent fiber may cause neurotransmitter release at multiple unique active zones. In these studies, synapses activated by electrical stimulation of Schaffer colatteral axons were identified by the presence of excitatory post-synaptic Ca^{2+} transients (EPSCaTs). Surprisingly, these EPSCaTs require AMPAR-mediated spine depolarization and Ca^{2+}-release from intracellular calcium stores (Emptage *et al.*, 1999). Though they were unable to detect Ca^{2+} entry through NMDARs directly, they do report that some NMDAR-mediated Ca^{2+} is required to induce release from intracellular stores. Nevertheless, they provide evidence that this indirect assay was capable of approximating the probability of presynaptic neurotransmitter release (P_r) by measuring the probability of eliciting an

EPSCaT (P_{Ca}) from electrical stimulation. In adult animals, LTP induction caused P_{Ca} to increase, leading to the conclusion that P_r has also increased (Emptage *et al.*, 2003; Enoki *et al.*, 2009), though in young animals, LTP expression was mediated entirely by AMPAR insertion into silent synapses (Ward *et al.*, 2006), i.e., a purely postsynaptic modification. While the presence of an EPSCaT at a single spine and a global EPSP are normally de-coupled in these studies due to limitations in imaging every spine in a neuron's dendritic arbor, Enoki *et al.* (2009) do report three cells where the presence of an EPSCaT correlates perfectly with an evoked EPSP. Based upon these three spines, and other experiments where they subtract the EPSP amplitude during EPSCaT failures from the EPSP recorded during EPSCaT successes, they conclude that the unitary EPSP amplitude does not increase during LTP, and therefore the increased global EPSP is due entirely to changes in P_r.

These conclusions rely on the assumption that EPSCaTs reliably indicate pre-synaptic release of neurotransmitter, instead of an increased coupling between neurotransmitter release and postsynaptic depolarization by AMPARs, resulting in Ca^{2+}-influx through NMDARs. Indeed, this possibility is suggested by the fact that the EPSCaT can be eliminated by blocking AMPARs or NMDARs (Emptage *et al.*, 1999). However, these papers provide several controls to explore this possibility, including using low concentrations of the AMPAR-antagonist CNQX to show that significant block of the EPSP does not decrease the frequency or amplitude of EPSCaTs (Emptage *et al.*, 2003), and by using CPA to deplete intracellular Ca^{2+} stores, demonstrating a reduction in EPSCaTs even in Mg^{2+}-free solution, ruling out NMDARs as the primary source of Ca^{2+} (Ward *et al.*, 2006). However, numerous other studies failed to find a

necessary role for intracellular stores in synaptically evoked calcium transients (Mainen *et al.*, 1999; Yuste *et al.*, 1999; Kovalchuk *et al.*, 2000), instead finding that the main source of Ca^{2+} is through NMDARs (Sobczyk *et al.*, 2005; Bloodgood & Sabatini, 2007). Because of the well-accepted block of NMDARs by Mg^{2+} at resting membrane potentials, other studies examining synaptically evoked Ca^{2+} transients therefore record either in Mg^{2+}-free solution (Mainen *et al.*, 1999) or at depolarized potentials to ensure reliable and direct coupling of glutamate release and a Ca^{2+}-evoked signal (Sobczyk *et al.*, 2005) . One study did record EPSCaTs in physiological conditions and saw dramatically decreased amplitude of the Ca^{2+}-transient at resting membrane potential due to block of NMDARs by Mg^{2+} (Sabatini *et al.*, 2002), in contrast to these papers looking at LTP, where the observed EPSCaT amplitudes are large at resting membrane potentials (Emptage *et al.*, 1999; Emptage *et al.*, 2003). Due to the lack of reproducibility from other groups, and the possibility of a postsynaptic mechanism to explain their data, these studies are not persuasive in arguing for presynaptic expression of LTP.

One way to conclusively demonstrate that LTP expression is postsynaptic would be to remove the contribution of the presynaptic terminal entirely. Technological advances in the past 10 years have allowed precisely that, such that experimenters can now apply glutamate with high enough spatial and temporal resolution to mimic release from single synaptic vesicles. This is done with an inert, caged derivative of glutamate, MNI-glutamate, that can undergo photolysis following 2-photon excitation, resulting in a release of active glutamate with 1 μm-spatial resolution at a submillisecond timescale. By adjusting the size and intensity of light excitation, uncaging glutamate onto a dendritic spine can produce uncaging EPSCs (uEPSCs) with the same amplitude and

kinetics as mEPSCs produced by neurotransmitter release (Matsuzaki *et al.*, 2001). Additionally, 2-photon uncaging can specifically activate synaptic receptors with minimal contamination from glutamate spillover onto extrasynaptic AMPARs, as demonstrated by experiments that specifically activated NMDARs with no AMPAR-mediated uEPSC in silent synapses (Beique *et al.*, 2006; Busetto *et al.*, 2008). Several studies have since demonstrated that pairing post-synaptic depolarization with repetitive glutamate uncaging causes an enhancement in AMPAR-mediated currents, with an associated increase in the volume of the spine (Matsuzaki *et al.*, 2004; Bagal *et al.*, 2005; Harvey & Svoboda, 2007; Lee *et al.*, 2009). This plasticity is blocked by NMDAR- and CaMKII-antagonists (Matsuzaki *et al.*, 2004) and is synapse-specific (Harvey & Svoboda, 2007), mechanistically identical to LTP. The magnitude of AMPAR potentiation is also similar to that seen in LTP, which combined with the complete removal of any contribution from the presynaptic terminal, leaves little doubt as to a major contribution to LTP expression from increased current through post-synaptic AMPARs. AMPARs are therefore a logical starting point for experiments wishing to solve the mechanism of LTP expression.

AMPA receptors

AMPARs are responsible for the majority of fast, excitatory synaptic transmission in the CNS. A functional AMPAR is a tetramer of four individual subunit proteins, each consisting of a large extracellular N-terminal domain, a glutamate-binding pocket, four transmembrane domains including a re-entrant loop that forms the pore of the receptor, and a short intracellular cytoplasmic carboxy terminus (C-tail) (Rosenmund *et al.*, 1998; Sobolevsky *et al.*, 2009). There are also four unique AMPAR

subunit proteins, each expressed from different genes: GluA1, GluA2, GluA3, and GluA4 (Keinanen *et al.*, 1990; Hollmann & Heinemann, 1994). In the brain and under certain conditions, the majority of AMPARs are heteromers containing two GluA2 subunits and two of the other subunits, GluA1, GluA3, or GluA4. In some parts of the brain, homomers of GluA1, GluA3, or GluA4 may also be expressed (Isaac *et al.*, 2007). In CA1 pyramidal neurons of the hippocampus, GluA1/2 heteromers dominate, comprising greater than 95% of the extrasynaptic surface pool of receptors, and ~80% of the synaptic pool. The remaining AMPARs are GluA2/3 heteromers, with little to no contribution from GluA1 homomers (Wenthold *et al.*, 1996; Lu *et al.*, 2009).

AMPARs undergo several important post-transcriptional modifications, including alternative splicing and RNA editing. Each of the subunit proteins has two alternatively spliced variants, flip or flop, which are developmentally regulated and can subtly alter their gating and desensitization properties (Sommer *et al.*, 1990; Monyer *et al.*, 1991). Throughout the brain, the flip isoform dominates, though the flop isoform receptors are heavily expressed in the hippocampus and cerebellum (Tonnes *et al.*, 1999). In addition to alternative splicing, the GluA2 subunit also undergoes RNA editing, which converts a glutamine (Q) in the pore of the receptors to an arginine (R) (Greger *et al.*, 2007). Arginine is larger and contains two positively charged amine groups, which significantly alters the permeability of the receptor. Because this editing only occurs with the GluA2 subunit, an AMPAR containing GluA2 has decreased single-channel conductance, is impermeable to Ca^{2+} ions, and has altered rectification properties (Greger *et al.*, 2003). GluA2-containing receptors have linear current-voltage relationships as would be predicted by Ohm's law, with a reversal potential around 0

mV. In contrast, GluA2-lacking receptors are blocked at positive potentials by intracellular spermine, which is itself positively charged and becomes stuck in the pore of the AMPAR. This results in inward rectification, and provides a convenient electrophysiological means to determine if the AMPAR is GluA2-containing; if the channel passes current at positive membrane potentials, it is GluA2-containing and Ca^{2+}-impermeable. In contrast, if the channel does not pass current at positive membrane potential, it is GluA2-lacking and Ca^{2+}-permeable (Panicker *et al.*, 2008).

AMPA receptor trafficking and LTP

The case for a postsynaptic locus of LTP expression by AMPAR insertion has been bolstered by several manipulations of the AMPAR complex itself. Mice with constitutive genetic deletion of GluA1 do not express LTP in the hippocampus, showing a lack of potentiation reminiscent of pharmacological blockade of NMDARs (Zamanillo *et al.*, 1999). Similarly, deletion of the TARP (transmembrane AMPAR regulatory protein) γ-8, an auxiliary subunit of AMPARs, inhibits LTP expression (Rouach *et al.*, 2005). Recently, a new member of the AMPAR complex, the cornichons (Schwenk *et al.*, 2009), were shown to be required for LTP following conditional deletion (Herring *et al.*, 2013). In what may have been a fatal blow to the notion that LTP is central to learning and memory, GluA1 knockout mice displayed normal spatial memory in the Morris water maze despite lacking LTP (Zamanillo *et al.*, 1999). Subsequent behavioral studies, however, have demonstrated significant defects in short-term spatial memory in these mice, as they failed to perform above chance in an alternating T-maze task (Reisel *et al.*, 2002; Sanderson *et al.*, 2010). Research into LTP and learning, and particularly the role for GluA1, has continued unabated.

This research was driven in large part by a convincing model developed by Malinow and colleagues describing subunit-specific differences in AMPAR trafficking. They found that overexpressed GluA1 subunits, which form homomeric channels, did not traffic to the synapse under baseline conditions, as assayed by synaptic rectification. However, either inducing LTP or co-expressing a constitutively active form of CaMKII could deliver GluA1 to the synapse (Hayashi *et al.*, 2000). In contrast, overexpressed GluA2 did traffic to the synapse under normal conditions. This difference was shown to depend entirely on their intracellular c-tails, as a GluA1 mutant containing the GluA2 C-tail trafficked to the synapse without requiring an LTP stimulus, but GluA2 with a GluA1 C-tail did not (Shi *et al.*, 2001). This led them to hypothesize that the GluA1 C-tail was integral for expression of LTP, supported by their finding that wash-in or expression a soluble form of the GluA1 C-tail inhibits LTP 30 minutes after induction (Shi *et al.*, 2001).

Since the discovery of this subunit-rules model of AMPAR trafficking, research on the GluA1 c-tail has proliferated, singling out multiple phosphorylation sites and protein-interactions as necessary for LTP expression. The first and perhaps most promising of these sites was a CaMKII phosphorylation site at serine 831 (S831) (Roche *et al.*, 1996; Barria *et al.*, 1997a). LTP causes an increase in phosphorylation at this site (Barria *et al.*, 1997b), and experiments in heterologous cells showed that S831 phosphorylation increases the single-channel conductance of homomeric GluA1 receptors in heterologous cells (Derkach *et al.*, 1999). If true, this would be an attractively simple model for LTP expression, directly connecting CaMKII activation to synaptic potentiation through an increase in the single-channel conductance of AMPARs. Alas,

whether S831 phosphorylation actually increases single channel conductance in neurons remains somewhat controversial, as S831 phosphorylation of heteromeric GluA1/GluA2 receptors does not result in increased channel conductance unless they are associated with TARPs (Oh & Derkach, 2005; Kristensen *et al.*, 2011). However, Andrasfalvy and Magee (2003) performed experiments where they pulled outside-out patches from the dendrites of potentiated neurons, finding no change in single-channel conductance, but rather an increase in the number of AMPARs (Andrasfalvy & Magee, 2004). Most problematic for this model, LTP expression is intact in phosphonull S831A GluA1 knock-in mice (Lee *et al.*, 2010). Whatever CaMKII's relevant downstream targets are, GluA1 does not appear to be one of them.

The PDZ-binding domain at the extreme GluA1 C-tail is another promising domain that was ultimately shown to be a mechanistic dead-end. The PDZ ligand is a four amino-acid stretch that can bind PDZ domains of postsynaptic scaffolding proteins such as the MAGUKs, and might explain how AMPARs are anchored to the PSD (Kim & Sheng, 2004). Overexpression experiments suggested a required role for the PDZ domain for synaptic trafficking and LTP expression (Hayashi *et al.*, 2000). Once again, however, a mouse with a knock-in mutation truncating the GluA1 c-tail immediately before the PDZ ligand demonstrated completely normal synaptic trafficking and LTP (Kim *et al.*, 2005). Since this finding, the focus on PDZ-binding domains has shifted to the TARPs, who have been shown to anchor AMPARs to the synapse through interactions with PSD-95 (Schnell *et al.*, 2002; Nicoll *et al.*, 2006; Jackson & Nicoll, 2011).

The next phosphorylation site of interest to be identified was a PKA site at S845 (Roche *et al.*, 1996). Though not as definitive as the findings for CaMKII, PKA

phosphorylation has also been implicated in LTP induction (Blitzer *et al.*, 1998; Yasuda *et al.*, 2003). Mutating this site in overexpressed GluA1 subunits did effect synaptic transmission and inhibit LTP (Esteban *et al.*, 2003), but its status as a requirement for LTP suffered the same fate as the CaMKII site and PDZ ligand – a knock-in mouse lacking this phosphorylation site demonstrated a normal LTP phenotype (Lee *et al.*, 2010). I should note, however, that a knock-in mouse with combined GluA1 S845A and S831A mutations did show decreased expression of LTP, so a role for these sites should not be completely ruled out (Lee *et al.*, 2003).

Most recently, the membrane proximal region (MPR) of the GluA1 C-tail, specifically the S818 PKC phosphorylation site, has been implicated in LTP expression. Wash-in of a small peptide mimicking only the MPR inhibited LTP in a very similar fashion to the full-length C-tail peptide, and overexpressed phosphomimic and phosphonull mutant subunits promoted and inhibited synaptic delivery, respectively (Boehm *et al.*, 2006). Furthermore, S818 phosphorylation controls interaction with the cytoskeletal adaptor protein 4.1N, resulting in a decreased rate of GluA1 exocytosis, suggesting a model whereby PKC phosphorylation of S818 results in binding to 4.1N and increased exocytosis, resulting in LTP. Indeed, shRNAs targeted against 4.1N also showed partial inhibition of LTP expression (Lin *et al.*, 2009b). However, genetic alterations of this region, which would be most definitive in demonstrating its requirement for LTP, have yet to be reported.

Clearly, a single, absolutely necessary region of the GluA1 c-tail has yet to be identified, with many of the putatively important interactions debunked through the use constitutive genetic mutations. The effects on LTP that are found are also often over

interpreted. Instead of resulting in a complete lack of potentiation seen with genetic deletion of GluA1 or pharmacological block by NMDARs, these manipulations result in largely normal initial potentiation, followed later by a gradual decrease in synaptic transmission towards the baseline amplitude. One can indeed argue that the "long-term" aspect of LTP is impaired, but we learn little about the actual mechanism of potentiation.

My goal at the outset of my graduate work was to define the minimal region of the GluA1 C-tail for LTP expression, and when found, use that knowledge to identify upstream molecules that bridge the gap between NMDAR activation and AMPAR insertion. The cleanest and most definitive method to conduct such experiments is targeted genetic knock-ins, replacing the endogenous GluA1 subunit with mutated subunits under control of the endogenous promoter. However, producing genetic knock-in mice is prohibitively time consuming and expensive when screening a number of different mutations. I therefore developed a single-cell molecular replacement technique where the endogenous receptors are deleted and replaced by transfected subunits in a mosaic, conditional fashion. To accomplish this, I used mice where the genes for GluA1, GluA2, and GluA3 were flanked by loxP sites, allowing for complete deletion of AMPARs by expression of Cre recombinase. These "triple-floxed" mice have been bred and characterized by Wei Lu in a previous study (Lu *et al.*, 2009). This provides a clean background upon which I can co-express recombinant GluA1 mutants and assess the effects on LTP (Lu *et al.*, 2010; Granger *et al.*, 2011).

In Ch. 3 of this thesis, I describe the experiments I performed to dissect the GluA1 C-tail and determine its role in AMPAR surface expression, baseline synaptic

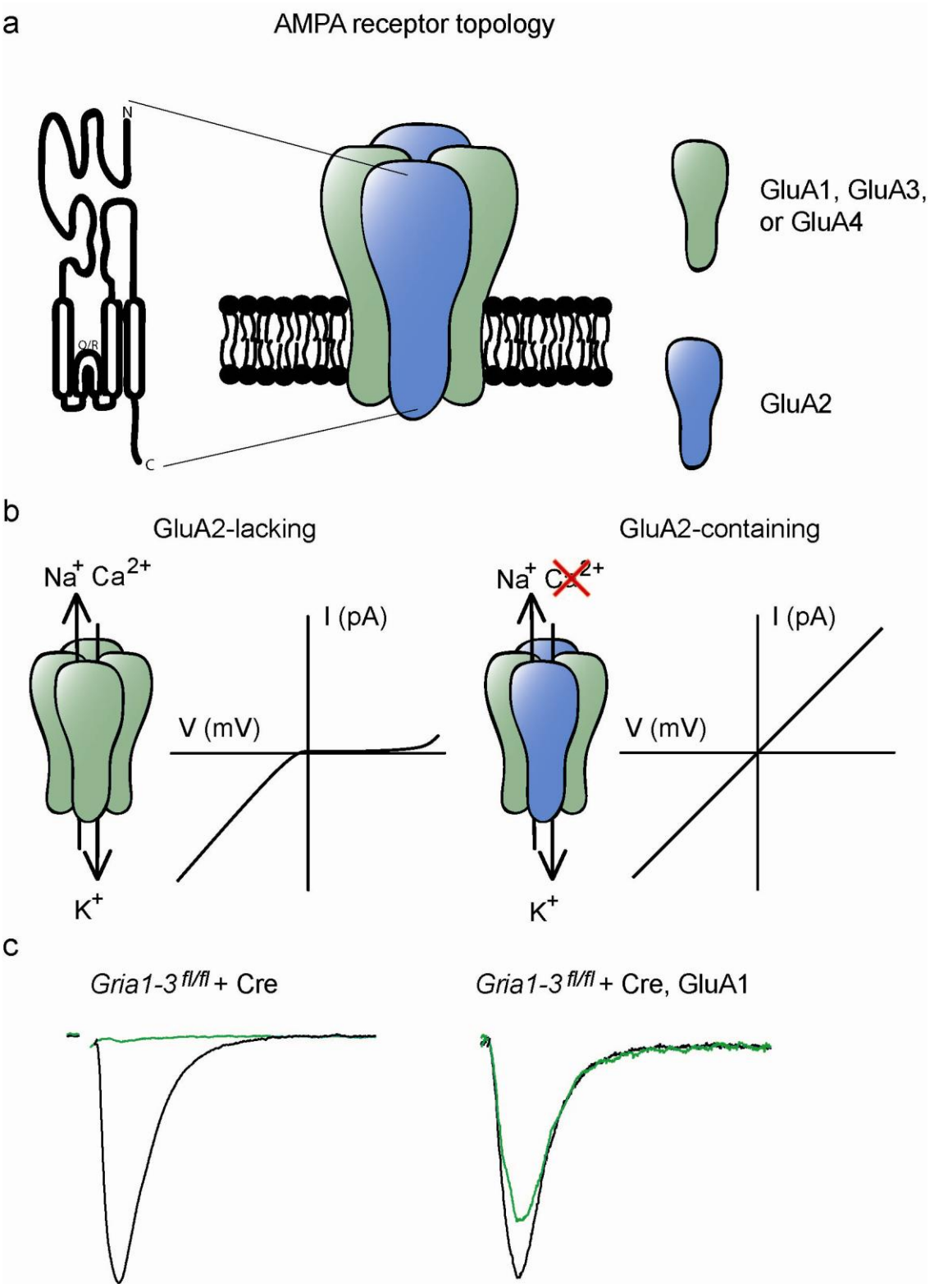
transmission, and LTP. Surprisingly, I was able to identify no required role for the GluA1 C-tail, or for GluA1 generally. Instead, I found that the GluA2 subunit, when slightly modified to enable it to express homomers on the neuronal surface, was sufficient for LTP expression. Even replacement with a foreign kainate-type glutamate receptor (KAR) that is not normally found in CA1 neurons resulted in normal expression of LTP. The only conditions under which LTP was impaired were those that lacked a large pool of glutamate receptors on the neuronal surface. Based on this, I suggest a revised model of LTP expression that focuses on increases in the size of the synapse and PSD, allowing them to anchor a greater number of AMPARs, which are normally abundantly expressed on the neuronal surface.

In Ch. 4, I describe similar experiments explore the AMPAR subunit requirement for long-term depression. LTD is the opposite phenomenon as LTP, the activity dependent decrease in synaptic strength based on persistent de-correlated firing of the presynaptic and postsynaptic neuron (Malenka & Bear, 2004). I find that no particular AMPAR subunit is required for LTD expression, and like LTP, is competently expressed with the KAR subunit GluK1. This again suggests that the ultimate effector of LTD is not the individual glutamate receptor subunits, but changes in the synapse itself.

Finally, in Chapter 4, I present some preliminary data on the subunit requirement of NMDARs, which are differentially expressed during development and during synaptic plasticity. My data suggests a specific requirement for the NMDAR subunit GluN2B, though much more work is required to confirm and extend that finding.

Figure 1. AMPA receptor properties and molecular replacement strategy

(a) A functional AMPAR is a tetramer of four individual subunit proteins, each of which contains a large extracellular N-terminal domain, four transmembrane regions including a reentrant loop that forms the pore of the receptor, and a short intracellular C-terminus. There are four separate subunit proteins, GluA1-4, each encoded by a different gene. (b) GluA2-lacking receptors show large single channel conductance, are permeable to Ca^{2+} ions, and do not pass any current (I) at positive membrane potentials (V). In contrast, GluA2- containing receptors, which dominate throughout the brain, are impermeable to Ca2+ and show a linear relationship between current and voltage. (c) Example traces of synaptically evoked AMPA-mediated EPSCs from a paired control (black) and Cre-expressiong (green) neuron where each of the GluA1, GluA2, and GluA3 subunits are flanked by loxP sites (*Gria1-3*$^{fl/fl}$). This completely removes all endogenous AMPARs (left), providing a clean background on which recombinant GluA1 subunits can be expressed to rescue the EPSC (right).


a
AMPA receptor topology
N
Q/R
C
GluA1, GluA3,
or GluA4
GluA2
b
GluA2-lacking
GluA2-containing
Na+ Ca2+
I (pA)
V (mV)
K+
Na+
I (pA)
V (mV)
K+
c
Gria1-3 fl/fl + Cre
Gria1-3 fl/fl + Cre, GluA1

CHAPTER 2:
Methods

Mouse Genetics

Animals were housed according to the IACUC guidelines at the University of California, San Francisco. Mice with the *Gria1*$^{flx/flx}$, *Gria2*$^{flx/flx}$, and *Gria3*$^{flx/flx}$ (*Gria1-3*$^{flx/flx}$) were generated and genotyped as previously described (Lu *et al.*, 2009).

Experimental Constructs

GluA1, GluA2(Q), and Cre:mCherry were cloned into the pFUGW expression plasmid by PCR and In-Fusion® HD Cloning System (Invitrogen). pFUGW-GluA1 and GluA2(Q) co-expressed with GFP behind an internal ribosomal entry site (IRES). GluA1 and GluA2(Q) truncations were generated by overlapping extension PCR. GluA1ΔC ended in amino acid 812, with the last 4 amino-acids being EFCY. GluA1Δ824 ended in amino acid 824, with the sequence MKGF. GluAΔ824-AA contained the C-tail sequence EFCYKSRAEAKRMKGF. GluA1ΔMPR had the following amino acids excised from the C-tail: KSRSESKRMKGFC, with the rest of the C-tail intact. GluA2(Q)ΔC also truncated in amino acids EFCY, and GluA2(Q)Δ847 ended in amino acids MKGF. GluK1 and Neto2 were cloned into the pCAGGs expression plasmid with GFP and mCherry, respectively, co-expressed behind an IRES.

Neuronal Transfection

Biolistic transfection of organotypic slice cultures were performed as previously described. Briefly, 80 ug total of mixed plasmid DNA was coated on 1 uM-diameter gold particles in 0.5 mM spermidine, precipitated with 0.1 mM $CaCl_2$, and washed 4x in pure ethanol. The gold particles were coated onto PVC tubing, dried using ultra-pure N_2 gas, and stored at 4 degrees in desiccant. DNA-coated bullets were shot with a Helios Gene

Gun (BioRad). Cre expression was confirmed by mCherry epifluorescence, and replacement AMPA/KAR subunits confirmed by GFP epifluorescence.

For *in utero* electroporations, pregnant E15.5 *GRIA1-3*$^{flx/flx}$ mice were anesthetized with 2.5% isoflurane in 0_2 and injected with buprenorphine for analgesic. Embryos within the uterus were temporarily removed from the abdomen and injected with 2 µl of mixed plasmid DNA into the left ventricle via a beveled micropipette. pFUGW-Cre:mCherry was typically diluted to approximately 0.5 µg/µl in 2-3 µg/µl of the replacement pFUGW AMPAR or pCAGGS GluK1 plasmid. Each embryo was electroporated with 5x50 msecond, 35 volt pulses. The positive electrode was placed in the lower right hemisphere and the negative electrode placed in the upper left hemisphere. Following electroporation, the embryos were sutured into the abdomen, and sacrificed on p17-20 for LTP recording. For further detail on electroporation, please see (Navarro-Quiroga *et al.*, 2007).

Electrophysiology

Voltage-clamp recordings were taken from CA1 pyramidal neurons in either acute hippocampal slices or organotypic slice cultures. For acute slices, 300 µM transverse slices were cut using aMicroslicer™ DTK-Zero1 (Ted Pella, Inc.) in chilled high sucrose cut solution containing (in mM): 2.5 KCl, 7 $MgSO_4$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 7 glucose, 210 sucrose, 1.3 ascorbic acid, 3 sodium pyruvate. The slices were then incubated for 30 minutes at 34 degrees in artificial cerebral spinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose. For acute slices, 2.5 mM $CaCl_2$ and 1.3 mM $MgSO_4$ were added to the aCSF, and 4 mM $CaCl_2$ and $MgSO_4$ were added for organotypic slice cultures. The aCSF was bubbled with 95%

O_2 and 5% CO_2 to maintain pH, and the acute slices allowed to recover at room temperature for 45 minutes to 1 hour. Slices cultures were prepared as previously described (see Schnell), and recorded between 7-24 DIV depending on the experiment. During recording, slices were transferred to a perfusion stage on an Olympus BX51WI upright microscope and perfused at 2.5 mL/min. with aCSF containing 0.1 mM pictrotoxin for acute slices experiments, and 0.01 mM gabazine, and 2-5 μM 2-Cl-adenosine for organotypic slice cultures. Synaptic responses were evoked by stimulating with a monopolar glass electrode filled with aCSF in stratum radiatum of CA1. To ensure stable recording, membrane holding current, input resistance, and pipette series resistance were monitored throughout recording. Data was gathered through a MultiClamp 700B amplifier (Axon Instruments), filtered at 2 kHz, digitized at 10 kHz.

Whole-cell synaptic recordings and LTP

Simultaneous dual whole-cell recordings were made between GFP and/or mCherry positive experimental cells as identified by epifluorescence, and neighboring non-transfected control cells. Internal recordings solution contained (in mM): 135 $CsMeSO_4$, 8 NaCl, 10 HEPES, 0.3 EGTA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP, and 0.1 spermine. Osmolarity was adjusted to 290-295 mOsm, and pH buffered at 7.3-7.4. AMPAR- and KAR- mediated responses were isolated by clamping the cell -70 mV, while NMDA responses were recorded at +40 mV, with amplitudes taken 100 msec following stimulation to avoid contamination by AMPAR current. Paired-pulse ratios of AMPAR EPSCs were taken by stimulating twice at a 40 ms interval. To examine AMPAR rectification, 0.1 mM D-AP5 was washed in to block NMDARs. LTP was induced by stimulating at 2 Hz for 90 sec while clamping the cell at 0 mV, after

recording a stable 3-5 minute baseline, but not more than 6 minutes after breaking into the cell. To minimize run-up of baseline responses during LTP, slices were stimulated for ~10 minutes prior to breaking in, and both cells held cell-attached for 2-5 minutes before breaking into the whole cell. Prior to breaking in, stimulation intensity was calibrated just below the threshold required to elicit an action potential from the wild-type control neuron. Rectification index was calculated as the ratio of the slopes of the lines connecting AMPA EPSC amplitude from 0 to +40 mV and from -70 mV to 0 mV. This calculation can be taken as follows: R.I. = $70(I_{40} - I_0)/40(I_0-I_{70})$ where I_x represent EPSC amplitude at x mV.

Long-term depression

LTD was induced in field EPSP recordings by 15 minutes of 1 Hz stimulation. A variety of induction protocols were attempted for induction of whole-cell LTD, summarized in Table I. Ultimately, whole-cell LTD was best induced using 0.25 mM EGTA in the intracellular solution, 100 µM 7-CK in the aCSF, with an induction protocol similar to that used for field LTD, 15 minutes of 1 Hz stimulation while holding the cell at -40 mV.

Outside-out patches

Outside-out patches were taken from CA1 cells by obtaining whole-cell access to CA1 pyramidal neurons at -70 mV with a 4-5 MΩ patch pipette, then slowly pulling the pipette away from the soma until a high-resistance seal reformed. HEPES-aCSF containing (in mM): 150 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 1 $MgCl_2$, 2 CaCl1, 0.1 D-AP5, 0.1 picrotoxin, 0.1 cyclothiazide, and 0.5 µM TTX was then perfused over the tip of

the pipette. Glutamate and kainate currents were evoked by perfusion of HEPES-ACSF containing 1 mM L-glutamic acid and 1 mM kainic acid, respectively. A ValveLink 8 (AutoMate Scientific Inc.) was used for fast perfusion of control, glutamate, and kainite containing HEPES-aCSF. During outside-out patch experiments, experimental cells were interleaved with non-transfected control cells. Rectification Index was calculated as in synaptic experiments.

Statistics

For all experiments involving un-paired data, including all outside-out patch data, a Mann-Whitney U-test with Bonferonni correction for multiple comparisons was used between the wild-type control group and experimental group. For all experiments using paired whole-cell data, including all synaptic replacement and synaptic overexpression a two-tailed Wilcoxon signed-rank test was used. LTP data was gathered as pairs of control and experimental neurons, but occasionally during experiments, one of the cells would be lost. Comparisons were therefore made using the Mann-Whitney U-test, and the reported n-values represent that number of cells at the end of each experiment. Data analysis was carried out in Igor Pro (Wavemetrics), Excel (Microsoft), and R (The R Project for Statistical Computing, http://www.r-project.org/).

CHAPTER 3:
AMPA receptor subtypes and LTP

Introduction

Information storage in the brain is widely accepted to involve the rapid increase in synaptic strength between two neurons that can persist over long periods of time. This phenomenon, known as long-term potentiation (LTP), has been well described at glutamatergic synapses in the hippocampus, a region of the brain that is required for formation of new memories. At these synapses, LTP is expressed by the immediate increase in post-synaptic AMPA-type glutamate receptors (AMPARs) following coincident activation of pre- and post-synaptic neurons. However, the exact mechanism that allows AMPARs to be brought to the synapse so quickly are not fully understood.

AMPARs are responsible for the large majority of fast, excitatory synaptic transmission in the brain. A functional AMPAR is a tetramer of individual subunit proteins, of which there are four unique isoforms, GluA1 – 4 (Wisden & Seeburg, 1993; Hollmann & Heinemann, 1994). In CA1 pyramidal neurons, most receptors exist as GluA1/A2 heteromers, with a minor contribution from GluA2/A3 receptors (Wenthold *et al.*, 1996; Lu *et al.*, 2009). Over the past decade, a large body of research has focused on understanding how individual AMPAR subunits are trafficked. A widely held model posits that GluA1 receptors are excluded from synapses unless an LTP-stimulus is provided, whereas GluA2 receptors traffic to the synapse constitutively. This difference in trafficking behavior is mediated by the cytoplasmic tails (C-tails) of the individual subunit proteins (Hayashi *et al.*, 2000; Shi *et al.*, 2001; Boehm *et al.*, 2006). Supporting this model is the finding that LTP is impaired in GluA1 knock-out mice (Zamanillo *et al.*, 1999), but normal in GluA2/A3 double knock-out (Meng *et al.*, 2003). Based on these findings, a broad consensus has emerged that LTP is mediated by synaptic insertion of

GluA1 via its C-tail interactions (Malinow & Malenka, 2002; Bredt & Nicoll, 2003; Malenka, 2003; Collingridge *et al.*, 2004; Malenka & Bear, 2004; Shepherd & Huganir, 2007; Kessels & Malinow, 2009; Anggono & Huganir, 2011).

Despite the consensus that GluA1 is required for LTP, no single phosphorylation site or protein-protein interaction in the GluA1 C-tail has been shown to be absolutely required for synaptic potentiation. My goal was to find the minimum requirement of the GluA1 C-tail for LTP, and, if found, use that region to identify crucial protein interactions that mediate synaptic AMPAR potentiation. To accomplish this, I used a single-cell molecular replacement strategy to replace all endogenous AMPARs with transfected subunits (Lu *et al.*, 2010; Granger *et al.*, 2011). Using this approach, I systematically mutated the GluA1 C-tail and examined their effects on three stages of AMPAR trafficking: surface expression, synaptic transmission, and LTP. We failed to identify any region in the GluA1 C-tail that was essential either for basal synaptic incorporation or for LTP. In fact, homomeric GluA2(Q) receptors exhibited normal LTP. Most surprisingly, hippocampal synapses in which AMPARs had been replaced with kainate receptors were found to express normal LTP. Only manipulations that severely compromised the extrasynaptic pool of receptors showed defects in potentiation.

Results

The role of the GluA1 C-tail in surface expression

AMPAR trafficking can be thought to occur in three distinct steps: surface expression, basal synaptic targeting, and activity-dependent synaptic insertion. It is a general property of GluA1 that it is abundantly expressed on the neuronal surface (Andrasfalvy *et al.*, 2003; Lu *et al.*, 2009). As an initial screen, we therefore first characterized the surface expression of various GluA1 C-tail truncations in wild-type neurons using somatic outside-out patches. We were able to discriminate between overexpressed and endogenous receptors based on a difference in their channel properties. Specifically, the overexpressed subunits form homomeric receptors that are inwardly rectifying at positive membrane potentials (Hayashi *et al.*, 2000), whereas native receptors demonstrate linear current-voltage (I-V) relationships (Panicker *et al.*, 2008) (Fig. 2a). Overexpression of full-length GluA1 by biolistic transfection into CA1 pyramidal neurons significantly decreased the rectification index by approximately 40% compared to wild-type controls (Fig. 2b), indicating the presence of surface homomers. In contrast, overexpressing a GluA1 subunit with a full C-tail truncation (ΔC) showed a rectification index similar to wild-type neurons, indicating an impairment in trafficking to the surface. To narrow down the necessary C-tail region for surface expression, we expressed a less severe truncation, up to amino acid 824 (GluA1Δ824), which removes the S845 and S831 phosphorylation sites and the PDZ-binding domain. This decreased the rectification index to a similar degree as full-length GluA1. However, selective excision of the remaining membrane proximal region (ΔMPR), which contains a well-characterized binding site of the protein 4.1N (Shen *et al.*, 2000; Coleman *et al.*, 2003), also

significantly decreased rectification (Fig. 2b). Combined, these two modified subunits represent complementary truncations of the entire C-tail, ruling out a necessary role for any single part of the C-tail for steady-state surface expression.

To further explore whether the most membrane proximal amino acids of the GluA1 C-tail were required for surface expression, we tested a variety of mutations where those amino acids were deleted (GluA1Δ809-25, Δ809-13, Δ811-25, Δ813-25). Overexpression of none of these mutants increased rectification (Fig. 3a), suggesting that the receptor was not being properly expressed. However, it was unclear whether this impairment was due to the loss of a sequence-specific protein interaction, or due to some structural impairment caused by mutating the subunit so close to the transmembrane region. We therefore tested several mutations that leave the C-tail structurally intact, but changed the specific amino acid sequence. In particular, we examined the importance of palmitoylation at amino acid C811, which has been implicated in activity-dependent exocytosis of GluA1 (Lin *et al.*, 2009a). A palmitoylation-null mutatation at that site (C811S) had no effect on steady-state surface expression, as indicated by the significantly increased surface rectification following overexpression (Fig 3a). A more significant mutation of the four most membrane proximal amino acids (809-812) to alanines (4A) also did not impair the receptor from trafficking to the surface (Fig. 3a). Additionally, mutations that contained only the MPR region of the GluA1 c-tail, but with a scrambled amino acid sequence (Scram), or with that region replaced with the hydrophobic Myc-tag (Myc) both abundantly expressed on the surface (Fig. 2a). However, the combining the 4A mutation with the Myc-tag C-tail swap mutation did impair GluA1 surface expression (Fig. 3a). In sum, these mutations rule out any necessary sequence-specific

protein interaction, and argue strongly that there is a structural requirement for the C-tail, explaining the surface trafficking defect of GluA1ΔC.

Because competition with endogenous receptors may have hindered GluA1ΔC trafficking, we wished to study surface expression in the absence of native AMPARs. To accomplish this, we used mice with the genes for GluA1, GluA2, and GluA3 flanked by loxP sites (*Gria1-3*$^{fl/fl}$). A previous study has shown that expression of Cre into *Gria1-3*$^{fl/fl}$ neurons results in a complete absence of AMPARs within 12-15 days (Lu *et al.*, 2009), providing an effective AMPAR-null background onto which mutant GluA1 subunits can be expressed. We confirmed that Cre expression eliminated all glutamate-evoked current from somatic outside-out patches of *Gria1-3*$^{fl/fl}$ CA1 neurons, which can be rescued to control amplitudes by co-expression with full-length GluA1 (Fig. 4a,d), indicating complete rescue of surface expression. Consistent with overexpression, molecular replacement with GluA1ΔC showed significantly decreased glutamate-evoked currents (Fig. 4a,d). This trafficking defect was not due to decreased association with TARPs, auxiliary subunits important for AMPAR trafficking (Tomita *et al.*, 2006; Jackson & Nicoll, 2011), as both full-length and GluA1ΔC subunits had KA/Glu ratios similar to control (Fig. 4b). Also, both GluA1 and GluA1ΔC replacement subunits showed strong inward rectification, confirming the absence of endogenous receptors (Fig. 4c). Since both GluA1ΔMPR and GluA1Δ824 showed normal surface trafficking, the GluA1ΔC subunit must be impaired solely due to its severe truncation so close to the transmembrane region, which may inhibit proper protein folding.

In these molecular replacement experiments, we are expressing homomeric GluA1 receptors. However, endogenous AMPARs are primarily heteromers of GluA1

and GluA2 (Lu *et al.*, 2009). GluA1 homomers are Ca^{2+}-permeable, which may confound our results. In order to test the surface expression of more natural GluA1, GluA2 heteromers, we co-expressed Cre with both GluA1ΔC and GluA2. This results in complete rescue of the glutamate-evoked current amplitude from outside-out patches (Fig. 5a), indicating that co-expression with GluA2 is able to rescue the surface trafficking defects of GluA1ΔC. Additionally, we observed no change in the KA/Glu ratio compared to control (Fig. 5b), suggesting normal association with TARPs. In contrast to molecular replacement with GluA1 homomers, surface rectification is only slightly increased (Fig. 5c). The fact that surface rectification is not as linear as control cells must be due to some residual expression of GluA1ΔC homomers. Regardless, we have demonstrated that GluA1ΔC and GluA2 form functional, TARP-associated heteromers that abundantly express on the neuronal surface.

Basal synaptic transmission does not require the GluA1 C-tail

Given the decreased surface expression caused by complete truncation of the GluA1 C-tail, we next examined whether it would also impair basal synaptic targeting. Similar to surface currents, we assessed baseline synaptic transmission by transfecting *Gria1-3*$^{fl/fl}$ organotypic slice cultures with Cre and a replacement GluA1 subunit. After 17 days, we recorded evoked AMPAR excitatory post-synaptic currents (EPSCs) simultaneously from control and neighboring GluA1-replacement CA1 neurons. Similar to previously described results (Lu *et al.*, 2010), full-length GluA1 rescued AMPAR EPSC amplitudes to ~68% of control cells, while leaving NMDAR EPSCs unchanged (Fig. 6a,b). We also observed no change in paired-pulse ratio, indicating that GluA1 molecular replacement did not affect presynaptic release probability (Fig. 6c). Synaptic

EPSCs were strongly inwardly-rectifying compared to control, confirming the absence of endogenous receptors (Fig. 6d). Surprisingly, replacement with GluA1ΔC rescued AMPAR EPSCs to the same degree as full-length GluA1, with no effect on the NMDA EPSC or PPR and the expected dramatic decrease in rectification of synaptic currents (Fig. 5e). Replacement with GluA1Δ824 produced similar results (Fig. 6e). This demonstrates that despite having dramatically decreased somatic expression owing to its severe truncation, GluA1ΔC homomers manage to effectively rescue basal synaptic transmission.

Similar to molecular replacement with GluA1 homomers, GluA1ΔC/GluA2 heteromers also effectively trafficked to the synapse, resulting in approximately 85% rescue of the average AMPA EPSC (Fig. 7a), with no change in the NMDA EPSC or PPR (Fig. 7b,c). In contrast to surface expression, GluA1ΔC/GluA2 heteromers completely rescue synaptic rectification to that of control neurons (Fig. 7d). Overall, we report effective synaptic trafficking of GluA1 subunits under baseline conditions, either as homomers or heteromers with GluA2.

However, these results contrast with previous studies showing that GluA1 only traffics to synapses after an LTP stimulus (Hayashi *et al.*, 2000; Shi *et al.*, 2001). To explore this discrepancy, we turned to acute overexpression of GluA1 subunits in wild-type rat CA1 neurons, and assayed synaptic trafficking by changes in rectification, in order to most closely mimic the methods of these previous studies. Both full-length GluA1 and GluA1Δ824 trafficked to the synapses following acute overexpression, as indicated by the increase in synaptic rectification compared to paired untransfected control neurons (Fig. 8a). In contrast, GluA1ΔC did not traffic to the synapse either

acutely or after 16 days of overexpression (Fig. 8a). This is presumably attributable to its impaired surface trafficking and competition with endogenous receptors, indicating the utility of our molecular replacement approach. However, these results still differ from the findings of Malinow and colleagues that established the subunit-specific rules governing AMPAR trafficking (Hayashi *et al.*, 2000; Shi *et al.*, 2001). One possibility is that our slice culture conditions are especially over-active, which may induce delivery of GluA1 to the synapse. To control for this possibility, we cultured neurons in 10 mM Mg^{2+} following transfection, and saw comparable delivery of GluA1 to synapses (Fig. 8b). The other significant difference between our experimental set-up and that of the previous studies is that we are using un-tagged GluA1 subunits of the flip isoform, whereas they had used GFP-tagged GluA1 subunits of the flop isoform. We therefore tried overexpressing the different combinations of GFP-tagged or un-tagged, flip or flop GluA1 subunits to determine if one of these variables contributed to this discrepancy. We found no difference in synaptic trafficking between the flip or flop GluA1 isoforms, but we did find that GFP-tagged GluA1 was impaired at trafficking to the synapse (Fig. 8b). Our results indicate that GFP-tagging GluA1 can impair synaptic trafficking, explaining the experimental difference between our findings and previously studies. Analysis of the site of GFP insertion in these mutants showed that it disrupted a protein motif that has been implicated in AMPAR surface expression (Xia *et al.*, 2002). We therefore assayed surface expression of GFP-tagged GluA1, and saw the same level of steady-state surface expression between untagged and GFP-tagged GluA1 (Fig. 8c), indicating that GFP-tagging specifically prevents GluA1 from entering the synapse following delivery to the surface. While this result helps clarify why we consistently observed trafficking of GluA1 into synapses under baseline conditions, it is unclear why

the GFP-tag would impair GluA1 in a way that could be rescued by LTP induction or by specific C-tail manipulations, as reported in a variety of studies (Hayashi *et al.*, 2000; Shi *et al.*, 2001; Esteban *et al.*, 2003; Boehm *et al.*, 2006).

No GluA1 C-tail domains are required for LTP

To assess how GluA1 C-tail truncations affect LTP, we transfected Cre and GluA1 into the hippocampus of ~E15.5 *Gria1-3*$^{fl/fl}$ mouse embryos by electroporation. This allowed us to record from acute hippocampal slices, where LTP can be more reliably induced, and to replace the AMPAR subunits at an earlier developmental timepoint. Like biolistic transfection, electroporation results in sparse expression of transfected cells. To ensure that electroporation did not significantly increase the time-course of endogenous AMPAR removal, we transfected Cre alone and saw complete absence of the AMPAR EPSC as early as p10 with no effect on NMDAR EPSCs (Fig. 9a,b). Additionally, we ruled out the unlikely situation that some protected population of AMPARs could enter the synapse following LTP, potentially confounding our results, by showing that no AMPAR EPSC appeared following an LTP stimulus (Fig. 9c). In p17-20 acute hippocampal slices, we induced LTP after recording stable (3-5 minute) baseline AMPAR EPSCs simultaneously from control and GluA1-replacement neurons. Molecular replacement with full-length GluA1 exhibited normal LTP (Fig. 10a), confirming that the GluA1 subunit is sufficient. To avoid the confounding effect of decreased surface expression seen by GluA1ΔC, we next assessed the competence of GluA1Δ824 and GluA1ΔMPR subunits, which represent overlapping truncations of the entire C-tail. Both expressed LTP comparable to control (Fig. 10b,c). LTP was also expressed in neurons replaced with a truncated GluA1Δ824 subunit with S816A and

S818A (GluA1Δ824-AA) mutations, which specifically prevent 4.1N binding (Lin *et al.*, 2009b) (Fig. 11a,b). The GluA1Δ824-AA mutant is also identical to GluA2 in that region, effectively making it a GluA1 subunit with a truncated GluA2 C-tail. Finally, LTP was fully rescued by replacement with GluA1ΔC/GluA2 heteromers (Fig. 10d). This manipulation most closely mimics the endogenous situation where GluA1/GluA2 heteromers dominate, differing only in the presence of the GluA1 C-tail. Combined, these data show that the GluA1 C-tail is not in fact required for LTP.

GluA2 surface expression, synaptic targeting, and LTP

Given that no individual portion of the GluA1 C-tail was necessary for LTP, we hypothesized that expression of an alternative AMPAR subunit might also rescue LTP. GluA2 is another such subunit with limited C-tail homology to GluA1 (Malinow & Malenka, 2002) that is normally highly expressed in CA1 neurons, but is ineffective at forming homomers and trafficking to the cell surface (Andrasfalvy *et al.*, 2003; Lu *et al.*, 2009). This is attributable to Q/R RNA editing in the pore of the receptor, which severely limits channel permeability and may make formation of homomers energetically unfavorable (Greger *et al.*, 2007). Expression of un-edited GluA2(Q) resulted in abundant appearance of homomers on the neuronal surface, as observed by increased rectification (Fig. 12a). Truncation of the GluA2 c-tail up to amino acid 847 (GluA2(Q)Δ847), which includes all identified GluA2 protein interactions such as GRIP, PICK, NSF, and AP2 also abundantly expressed on the neuronal surface (Fig. 12a). Like GluA1ΔC, GluA2(Q)ΔC showed impaired surface expression (Fig. 12a), also most likely due to an impairment in proper protein folding from truncating so close to the transmembrane region.

The GluA2 C-tail has been shown to promote synaptic delivery of AMPARs (Shi *et al.*, 2001), so I explored whether truncation of the C-tail impaired synaptic delivery of GluA2(Q) during molecular replacement. Full-length GluA2(Q) rescued AMPA EPSCs to ~75% of control, with no change in NMDA EPSCs or PPR (Fig. 12a). As expected, synaptic rectification was significantly increased (Fig. 13a), indicating complete replacement of endogenous receptors. We observed similar levels of AMPA EPSC rescue and increase in rectification with molecular replacement of GluA2(Q)ΔC and GluA2(Q)Δ824, with no change in NMDA EPSCs or PPR (Fig. 13b,c). The GluA2 C-tail is therefore not required for basal delivery of GluA2(Q) to synapses, consistent with previous results published from our lab (Panicker *et al.*, 2008).

Finally, we assayed LTP expression in GluA2(Q) molecular replacement neurons. LTP in *Gria1-3*$^{fl/fl}$ neurons that expressed only GluA2(Q) was indistinguishable from control cells (Fig. 14a), despite lacking any of the intracellular phosphorylation sites and protein-protein binding sites of GluA1. Similarly intact LTP was seen in a GluA2(Q) truncation that lacks the majority of its C-tail and known protein-interaction sites (Fig. 14b). These results demonstrate that GluA2(Q) is sufficient to support LTP expression, independent of any particular C-tail interaction.

LTP requires a reserve pool of AMPARs.

Previous studies have shown that LTP is impaired in mice with constitutive deletion of GluA1, but not GluA2 or GluA3 (Zamanillo *et al.*, 1999; Meng *et al.*, 2003), demonstrating that GluA1 is both necessary and sufficient for LTP. These findings appear to be at odds with our data showing that GluA2(Q) homomers readily express LTP. We therefore reexamined the requirement for GluA1 in single-cell conditional

knockouts and found that conditional deletion of GluA1 alone did indeed impair LTP (Fig. 15a). Furthermore, deletion of GluA2 or GluA3 separately (Fig. 16a,b) or in combination (Fig. 15b) had no effect. How can this data be reconciled with our previous experiments? One profound difference between deleting GluA1 and deleting GluA2/GluA3 is that in the former condition there is an absence of extrasynaptic receptors (Zamanillo *et al.*, 1999; Andrasfalvy *et al.*, 2003; Lu *et al.*, 2009), whereas in the latter condition this pool remains entirely intact (Lu *et al.*, 2009). Also, unlike endogenous GluA2, our replacement GluA2(Q) showed abundant surface expression. We reasoned then that perhaps it is the depletion of this pool that accounts for the loss of LTP in the GluA1 knockout. To test this possibility, we returned to the extreme C-tail truncations of both GluA1 and GluA2(Q), in which surface expression is impaired, but synaptic targeting is maintained (Fig. 15e,f). Indeed, LTP was substantially impaired in both GluA1ΔC and GluA2(Q)ΔC replacement neurons (Fig. 15c,d). These findings suggest that the minimum requirement for LTP is a reserve pool of extrasynaptic AMPARs, regardless of the subunit type.

GluK1 is sufficient for mediating LTP

Having failed to identify any specific domains in the C-tails that are important for LTP, we wondered whether other domains in the AMPAR are required. In search of a null condition to conduct domain-swapping experiments, we turned to kainate receptors (KARs), a separate class of fast, ionotropic glutamate receptor which differs in fundamental ways from AMPARs. They bind to different auxiliary subunits and share little to no sequence homology (Contractor *et al.*, 2011). We therefore set out to replace all endogenous AMPARs with KARs at CA1 synapses. CA1 pyramidal neurons do not

normally express synaptic KARs, as shown by the absence of synaptic currents in the presence of the AMPAR-selective antagonist GYKI (Fig. 17c). However, co-expression of the KAR subunit GluK1 with the auxiliary subunit Neto2 (Copits *et al.*; Zhang *et al.*, 2009) in wild type CA1 neurons results in an increase in basal synaptic currents at -70 mV, with no change seen to the NMDA EPSC (Fig. 17a,b). In addition, GluK1 overexpression generated a GYKI-resistant current that was blocked by NBQX, an antagonist that blocks both KARs and AMPARs (Fig. 17c). This indicates that overexpressed KARs are capable of being targeted to the synapse and contribute to EPSCs. The mechanism by which KARs anchor to synapses appears to be independent of that used by AMPARs, as GluK1 increases the amplitude of the EPSC at -70 mV, which is not observed when GluA1 is overexpressed.

To examine KAR currents in isolation, we co-expressed Cre with GluK1 and Neto2 in *Gria1-3*$^{fl/fl}$ CA1 neurons. In this case, we recorded a population of pure KARs on the surface that desensitize to glutamate even in the presence of cyclothiazide (Fig. 19a). Unlike other KAR or AMPAR subunits, GluK1 is highly sensitive to the antagonist ACET, which saturates GluK1 at concentrations as low as 200 nM (Dargan *et al.*, 2009). In contrast, 1 mM ACET had no effect on the AMPA EPSC in wild-type neurons, though increasing the concentration to 10 mM does show an off-target decrease in the AMPA EPSC (Fig. 18a). Indeed, 1 mM ACET completely blocked the glutamate evoked currents in GluK1 replacement neurons (Fig. 19a). Furthermore, these neurons exhibit EPSCs that are entirely blocked by ACET, while the EPSCs in neighboring controls neurons are unaffected (Fig. 19b), further demonstrating that exogenous KARs are expressed on the surface and targeted to synapses. As with AMPAR replacement, NMDA EPSCs were

unaffected (Fig 19b). Finally, we tested whether neurons expressing only KARs could express LTP. To our surprise, we found that the KAR EPSC showed potentiation indistinguishable from that recorded simultaneously from neighboring control neurons (Fig. 19c,d). The LTP expressed in GluK1-replacement neurons was partially insensitive to treatment of GYKI, which is selective for AMPARs (Fig. 19c), but was still decreased due to the non-specific effects of GYKI. To ensure that the EPSC in the KAR expressing neuron was, in fact, mediated entirely by KARs, we applied ACET at the end of the experiments instead and found that it abolished the EPSC in the GluK1-replacement neuron, but had no effect on neighboring control neurons (Fig. 19d). Thus, GluK1, a completely foreign ionotropic glutamate receptor subunit that is not normally expressed in CA1 neurons and is of an entirely different receptor class as AMPARs, is sufficient to express LTP. These experiments demonstrate that even neurons completely lacking AMPARs can undergo LTP, so long as they are provided with an alternative fast, ionotropic glutamate receptor.

Is LTP mediated by KARs just an artifact of the complete lack of AMPARs available to the neuron? As suggested by Sheng *et al.* (2013), one explanation for this data is that LTP normally does require a specific manipulation of AMPARs, but can be expressed by KARs only when AMPARs are absent. To test this possibility, I induced LTP into wild-type mouse slices that had been electroporated with GluK1 and Neto2 without deleting endogenous AMPARs. This resulted in a level of potentiation slightly higher than in neighboring control neurons. Wash-in of ACET decrease the amplitude of LTP in GluK1-expressing CA1 to the level of paired control neurons (Fig. 20a). Because the concentration of ACET used in this experiment was too high, both the control and

GluK1-expressing cells saw decreased EPSCs. However, the level of EPSC block was greater in GluK1-expressing cells, bringing those EPSCs down to the level of control neurons. If LTP preferentially increased AMPARs at the expense of KARs, one would expect the amplitude of LTP to be decreased, not increased. This therefore suggests that LTP potentiates KARs as well as AMPARs when both are present, and argues against an AMPAR-specific effect. An important caveat to this experiment, however, is that we observed little to no increase in the baseline EPSC amplitude (Fig. 20b), in contrast to that seen using biolistic transfection in organotypic slice culture (Fig. 17a). Electroporation tends to produce lower levels of expression than biolistic transfection, and therefore the average potentiation was lower. This experiment therefore warrants repeating, but with increased GluK1 expression, either by using higher concentration DNA during electroporation, or by selecting cell with especially bright GFP fluorescence.

Run-up of EPSC amplitude is not dependent on NMDARs or on LTP induction

We also wished to confirm that LTP mediated by KARs, which are also Ca^{2+}-permeable, was not induced by a fundamentally different mechanism than wild-type LTP. We therefore tried inducing LTP in the presence of NMDAR-antagonist APV, and saw no significant potentiation (Fig. 21a). However, we did notice a run-up in the amplitude of the EPSC to approximately 150% of baseline over the duration of the experiment. To test if this run-up depended on the LTP induction protocol, we recorded from neurons for an hour without providing an LTP stimulus. Indeed, we saw the EPSC amplitude increase to ~150% of baseline, indistinguishable from the run-up seen when an LTP stimulus was given in the presence of APV (Fig. 21b,c).

We hypothesized that this run-up of EPSCs could be caused be several factors: gradual increase in the input resistance of the neuron due to wash-in of Cs^+ ions, wash-out of intracellular solution that had been blown around the neuron while approaching with the patch pipette, or some unknown increase in synaptic transmission during the life of the recording. To test these possibilities, we made paired recordings from two cells, one of which was broken into immediately to obtain whole-cell access, while the other was held in cell-attached configuration. The cell-attached patches were then ruptured to gain whole-cell access after ten minutes, except in those cases where the patch broke into the neuron spontaneously. We saw substantial run-up of EPSC amplitude recorded in cells with immediate whole-cell access, which subsided around 10 minutes into the recording. In contrast, the level of EPSC run-up in the cell-attached cells seemed to depend on the time of break-in (Fig. 22a). Recordings where whole-cell access was obtained relatively quickly saw significant run-up of the EPSC, whereas cells that remained cell-attached for the entire 10 minutes saw little or no run-up of the EPSC. Therefore, the majority of the run-up of the EPSC seems to be a results of intracellular solution that was expelled out of the recording pipette prior to patching, which can alter ionic driving forces and prevent presynaptic neurotransmitter release. This experiment was performed early in my graduate career, and subsequent LTP experiments were performed by beginning synaptic stimulation prior to patching, and holding neurons in a cell-attached configuration for 5 minutes prior to breaking into the whole cell. This allowed the intracellular solution bathing the neurons to be washed out while postponing whole-cell break-in, ensuring that a brief, stable EPSC baseline could be obtained before dialysis of the cytoplasm of the recorded neurons prevented LTP induction. Despite this precaution, some EPSC run-up remained in future experiments

(such as that seen in Fig. 21) that lasted anywhere from 20 to 40 minutes. However, the level of EPSC run-up was easily distinguishable from the large levels of potentiation seen with LTP, and do not interfere with the interpretation of our results.

Discussion

Using a single-cell molecular replacement approach that allowed us to exert complete control over the complement of expressed AMPARs, we found no requirement for the GluA1 C-tail for basal synaptic transmission or for LTP. In fact, we found no requirement for the GluA1 subunit generally, as both GluA2(Q), another AMPAR subunit, and GluK1, an entirely separate class of glutamate receptor, exhibited normal levels of LTP. Previous studies that have implicated the GluA1 C-tail in LTP demonstrated phenotypes with a largely normal initial stage of potentiation, followed by a gradual decrease in EPSC amplitude towards baseline (Shi *et al.*, 2001; Lee *et al.*, 2003; Boehm *et al.*, 2006; Lin *et al.*, 2009b). In contrast, we saw impaired potentiation immediately in GluA1 conditional KO cells and with GluA1ΔC and GluA2ΔC replacement, which more closely mimics the absence of LTP seen with pharmacological blockade of NMDARs. With all three of these manipulations, there was a profound decrease in the pool of extrasynaptic receptors, indicating that the main requirement for LTP is an adequate reserve pool of glutamate receptors. This conclusion is consistent with data from the TARP γ-8 and cornichon 2/3 knockout mice, where the extrasynaptic pool is markedly decreased and LTP is impaired (Rouach *et al.*, 2005; Herring *et al.*, 2013). In addition, these manipulations all show relatively normal baseline synaptic transmission despite having little or no extrasynaptic surface receptors, suggesting that neurons normally express surface AMPARs in great excess of what is required to maintain synaptic transmission. A concise visual description of the trafficking behavior of the relevant replacement AMPAR and KAR subunits is summarized in Fig. 23.

It is worth noting that several previously published papers had also disputed the necessity of GluA1 for LTP. In one case, LTP expression was intact in juvenile GluA1 knockout mice, which may been attributed to early expression of GluA4 (Jensen *et al.*, 2003). Other studies saw potentiation of synaptic transmission with alternative induction protocols, which could be mechanistically different from the NMDA-dependent pairing protocol most commonly used (Hoffman *et al.*, 2002; Romberg *et al.*, 2009). Regardless, it seems clear that GluA1 is not required for synaptic plasticity in all cases.

One model of LTP proposes that exocytosis of AMPAR-containing recycling endosomes is required to replenish peri-synaptic receptors for potentiation (Opazo & Choquet, 2011). Our experiments measured somatic surface expression, which is extrasynaptic by virtue of the lack of somatic excitatory synapses (Megias *et al.*, 2001), but we cannot rule out a role for a large pool of AMPARs in recycling endosomes, which may be depleted under the same conditions that deplete surface extrasynaptic receptors. Still, given that blockade of exocytosis typically only impairs LTP at later stages (Lledo *et al.*, 1998), pre-existing surface receptors are most likely used to initially mediate potentiation.

Fundamentally, our results suggest an ability of the synapse to cluster a broad variety of receptors following LTP, shifting the focus of LTP expression from the receptor to the synapse itself and specifically the post-synaptic density (PSD). In this model, AMPARs freely diffuse on the neuronal surface, and are trapped by the PSD for synaptic transmission (Opazo & Choquet, 2011). LTP, then, can be understood as an immediate increase in the ability of the PSD to trap receptors that relies on a large pool

of freely diffusing surface receptors (Fig. 24). This model is consistent with evidence from 2-photon glutamate uncaging experiments, which show an immediate increase in the volume of post-synaptic spines following LTP induction (Matsuzaki *et al.*, 2004; Patterson & Yasuda, 2011; Murakoshi & Yasuda, 2012), suggesting significant alterations to the synapse and PSD. Despite this shift of focus, research on AMPARs and their auxiliary subunits, such as TARPs, remain important for identifying LTP-related PSD proteins. In the absence of a role for the GluA1 C-tail, the question remains exactly how AMPARs are clustered at the synapse both basally and during plasticity. Identification of this interaction will be crucial to understanding the synaptic modifications that underlie learning in the brain.

Figure 2. Requirement of the GluA1 C-tail for neuronal surface expression

(a) Experimental protocol and example trace showing voltage ramps applied to outside-out patches of control (black) and GluA1-overexpressing (green) CA1 neurons. Rectification Index (R.I.) was measured as the normalized glutamate-evoked current at +40 mV over -70 mV. (b) Full-length GluA1, GluA1Δ824 and GluA1ΔMPR significantly increased rectification of surface currents compared to control. Overexpression GluA1ΔC slightly decreased the rectification index (Control, n= 47; GluA1, n= 10, $p < 0.001$; GluA1Δ824; n = 13, $p< 0.001$; GluA1ΔMPR, n = 18, $p < 0.001$; GluA1ΔC, n = 8, $p < 0.05$).

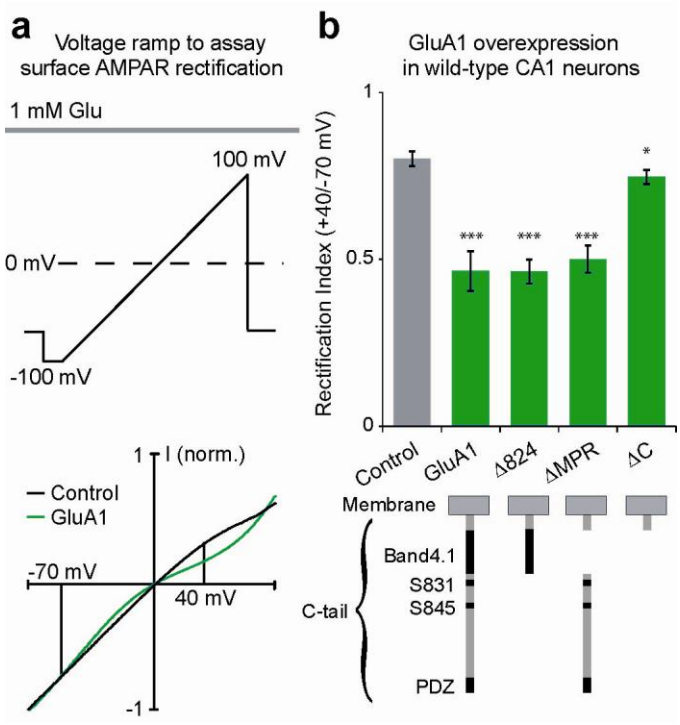

a
Voltage ramp to assay
surface AMPAR rectification
1 mM Glu
100 mV
0 mV
-100 mV
1
I (norm.)
Control
GluA1
-70 mV
40 mV
-1
b
GluA1 overexpression
in wild-type CA1 neurons
Rectification Index (+40/-70 mV)
1
0.5
0
***
***
***
*
Control
GluA1
Δ824
ΔMPR
ΔC
Membrane
Band4.1
S831
S845
C-tail
PDZ

Figure 3. An arbitrary GluA1 C-tail sequence is sufficient for surface expression.

(a) Rectification index was measured as the normalized glutamate-evoked current at +40 mV over -70 mV following overexpression of various GluA1 c-tail mutants. Any deletion or truncation too close to the transmembranre region impaired did not increase rectification (Control, n = 47; Δ809-25, n = 5; Δ809-13, n = 4; Δ811-825, n = 5; Δ813-25, n = 18; all $p > 0.05$). Mutation of the C811 palmitoylation site significantly increased rectification (C811S, n = 13, $p < 0.001$), as did mutation of the four most membrane proximal amino acids (EFCY) to alanines (4A, n = 10, $p < 0.001$), a scrambled amino acid sequence (AAARSKREKGMSKSF; Scram, n = 9, $p < 0.001$), or a myc-tag replacement c-tail (EFCYEQKLISEEDL; Myc, n = 6, $p = 0.000052$). A combined 4A-Myc mutant did not significantly increase rectification (4A-Myc, n = 5, $p = 0.055$).

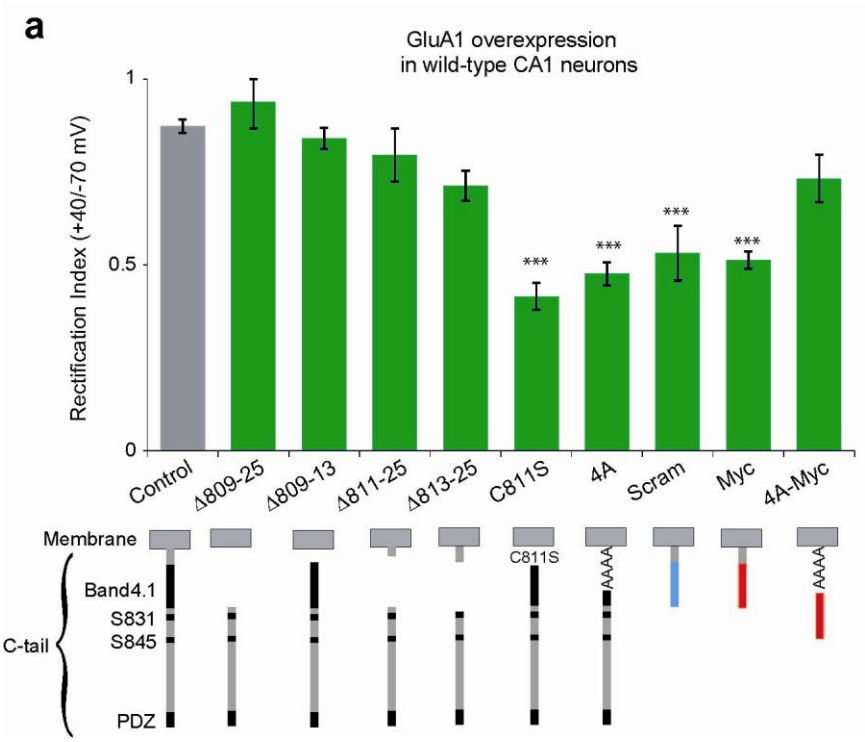

a
GluA1 overexpression
in wild-type CA1 neurons
Rectification Index (+40/-70 mV)
1
0.5
0
***
***
***
***
Control
Δ809-25
Δ809-13
Δ811-25
Δ813-25
C811S
4A
Scram
Myc
4A-Myc
Membrane
C811S
AAAA
AAAA
Band4.1
S831
S845
C-tail
PDZ

Figure 4. Molecular replacement shows impaired GluA1ΔCsurface expression.

(a) Glutamate-evoked currents from outside-out patches show complete absence of surface AMPARs following Cre expression in *Gria1-3*$^{fl/fl}$ CA1 neurons that is fully rescued to control by co-expression with full-length GluA1. GluA1ΔC replacement has significantly decreased current amplitude compared to control (Control, n = 28; Cre, n = 9, $p < 0.001$; GluA1, n = 11, $p > 0.05$; GluA1ΔC, n = 15, $p < 0.001$). (b) Kainate/glutamate (KA/Glu) ratios are similar between full-length GluA1 and control, while GluA1ΔC is slightly, but significantly decreased (Control, n = 28; GluA1, n = 11, $p > 0.05$; GluA1ΔC, n = 15, $p < 0.01$). (c) R.I. of both replacement receptors are strongly inwardly rectifying compared to control cells (Control, n = 24; GluA1, n = 10; GluA1ΔC, n = 13, both $p < 0.001$). (f) Example traces of glutamate-evoked current (d1) and kainate/glutamate ratios (d2) from *Gria1-3*$^{fl/fl}$ control neurons, Cre-expressing neurons, GluA1, and GluA1ΔC replacement neurons. Scale bars: 1 sec, 100 pA. Error bars represent mean ± s.e.m.

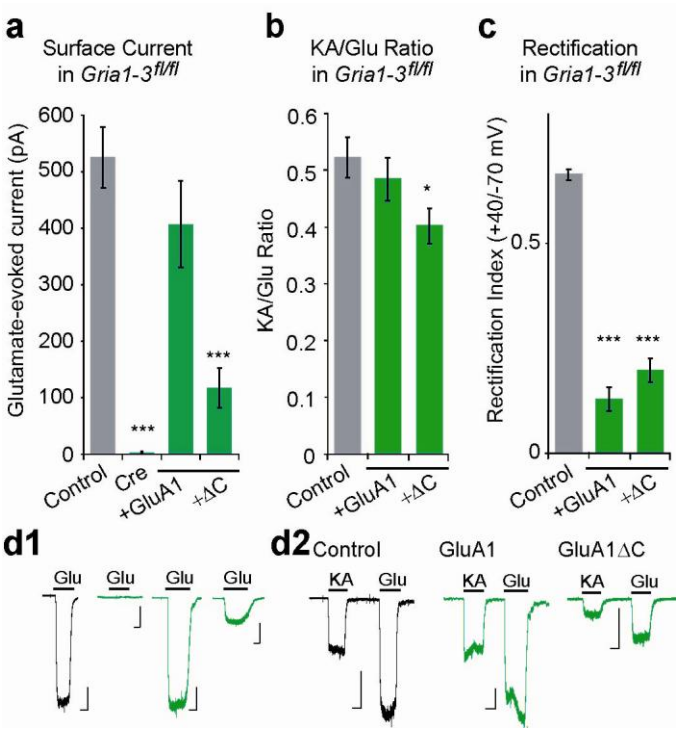

a
Surface Current
in Gria1-3fl/fl
Glutamate-evoked current (pA)
600
500
400
300
200
100
0
***
***
Control
Cre
+GluA1
+ΔC
b
KA/Glu Ratio
in Gria1-3fl/fl
KA/Glu Ratio
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
+GluA1
+ΔC
c
Rectification
in Gria1-3fl/fl
Rectification Index (+40/-70 mV)
0.5
0
***
***
Control
+GluA1
+ΔC
d1
Glu
Glu
Glu
Glu
d2
Control
GluA1
GluA1ΔC
KA
Glu
KA
Glu
KA
Glu

Figure 5. Replacement of GluA1ΔC/GluA2 heteromers shows normal surface expression.

(a) Co-expression of Cre with GluA1ΔC and GluA2 (A1ΔC/A2) in *Gria1-3*$^{fl/fl}$ CA1 neurons fully rescues glutamate-evoked surface currents to control levels (Control, n = 10; GluA1ΔC + GluA2, n = 13, $p > 0.05$). (b) KA/Glu ratios are similar between control and A1ΔC/A2 replacement neurons (Control, n = 10; A1ΔC/A2, n = 12, $n > 0.05$). (c) Rectification index of glutamate-evoked surface currents are significantly decreased in A1ΔC/A2 replacement neurons compared to control (Control, n = 10; A1ΔC/A2, n = 11, $p < 0.01$). (d) Example traces of glutamate-evoked current, KA/Glu ratios, and surface rectification from control (black traces) and A1ΔC/A2 repalcement (green traces) neurons. Scale bars: 1 sec and 100 pA. Error bars represent mean ± s.e.m.

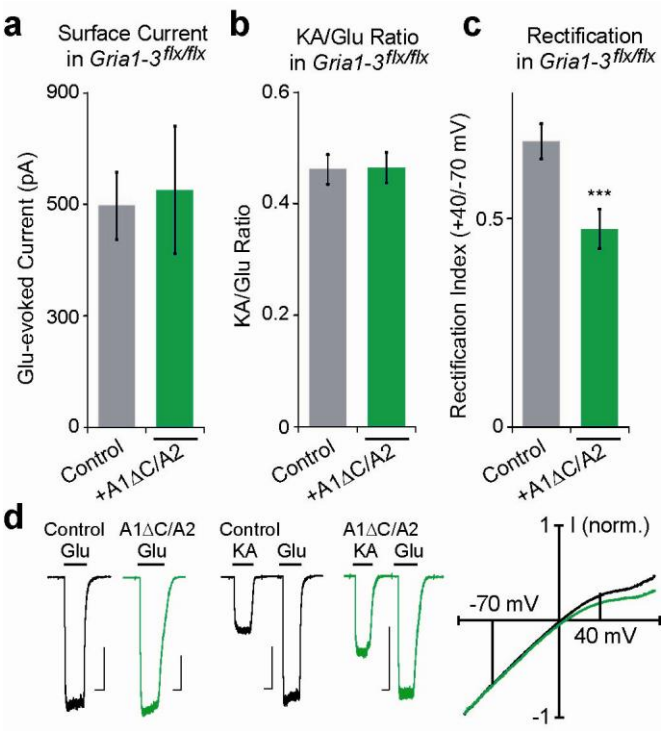

a
Surface Current
in Gria1-3flx/flx
Glu-evoked Current (pA)
900
500
300
0
Control
+A1ΔC/A2
b
KA/Glu Ratio
in Gria1-3flx/flx
KA/Glu Ratio
0.6
0.4
0.2
0
Control
+A1ΔC/A2
c
Rectification
in Gria1-3flx/flx
Rectification Index (+40/-70 mV)
***
0.5
0
Control
+A1ΔC/A2
d
Control
Glu
A1ΔC/A2
Glu
Control
KA
Glu
A1ΔC/A2
KA
Glu
1
I (norm.)
-70 mV
40 mV
-1

Figure 6. GluA1Δ824 and **GluA1ΔC have normal synaptic targeting**.

Paired whole-cell recordings from control and Cre + GluA1, Cre + GluA1Δ824, or Cre + GluA1ΔC-expressing CA1 neurons in *Gria1-3*$^{fl/fl}$ organotypic slice cultures. (a-d) Full-length GluA1 rescued synaptic AMPARs to 71% of control cells (n = 13 $p > 0.05$), while NMDA EPSCs remained unchanged between control and transfected cells ($p > 0.05$). Paired-pulse ratio is unchanged between control and GluA1 replacement neurons ($p > 0.05$), and rectification is significantly increased ($p < 0.001$). (e) Repalcement with GluA1Δ824 rescues ~75% of AMPA EPSCS with no change in the NMDA EPSC or PPR (n = 15, all $p > 0.05$), while rectification is significantly increast ($p < 0.001$). (f) Replacement with GluA1ΔC results in 75% rescue of AMPA EPSCs without a change in the NMDA EPSC or PPR (n = 15, all $p > 0.05$), and rectification is significantly increase ($p < 0.001$). (g) Summary graph showing comparable levels of AMPA and NMDA EPSC rescue between GluA1, GluA1Δ824, and GluA1ΔC. Example traces show average EPSCs for paired control (black) and replacement (green) neurons. Scale bars: 20 msec (AMPA), 100 msec (NMDA), 50 pA. Error bars represent mean ± s.e.m.

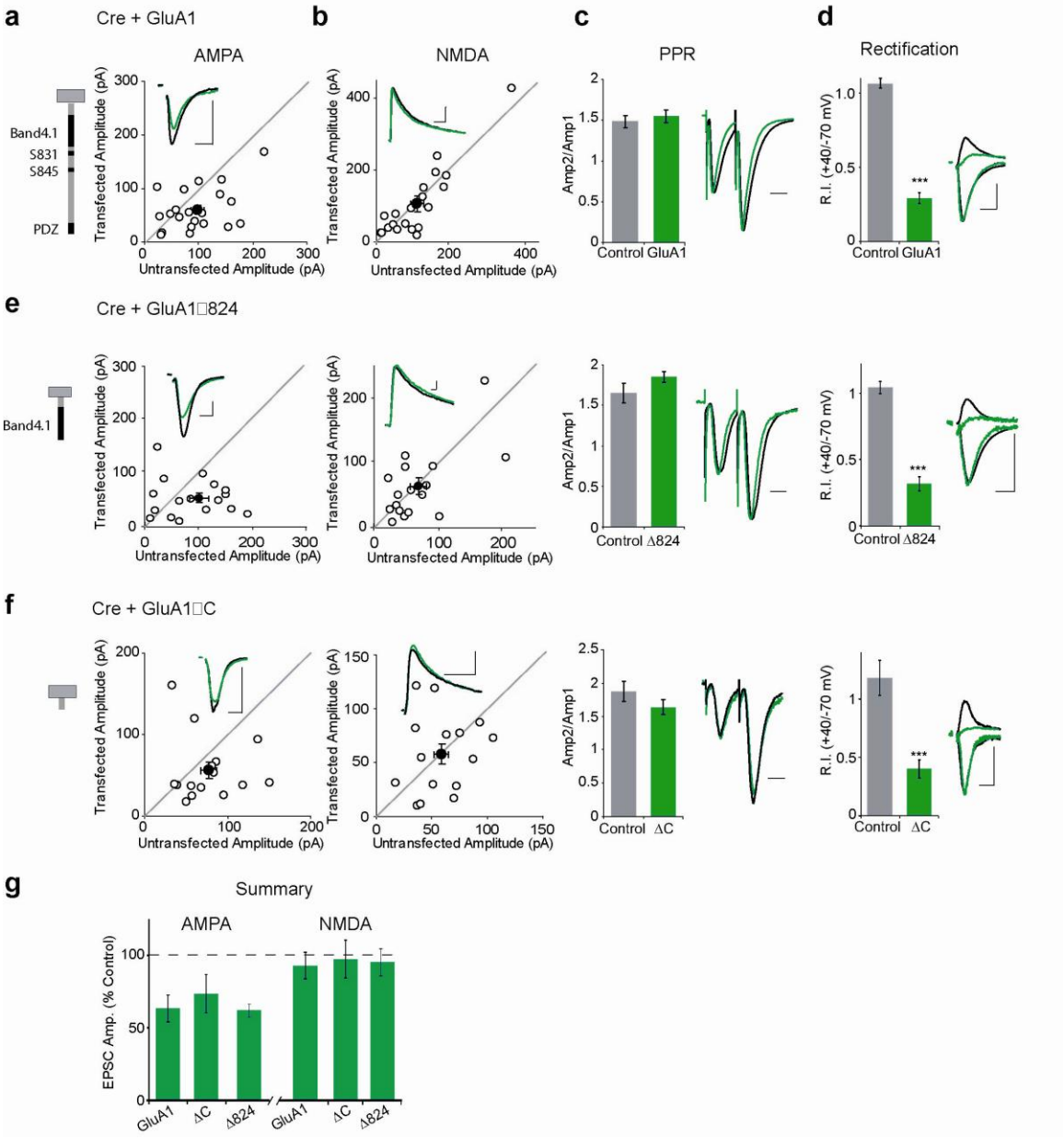

a
Cre + GluA1
AMPA
b
NMDA
c
PPR
d
Rectification
Band4.1
S831
S845
PDZ
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
Amp2/Amp1
R.I. (+40/-70 mV)
Control
GluA1
***
e
Cre + GluA1□824
Band4.1
Control
Δ824
f
Cre + GluA1□C
Control
ΔC
g
Summary
AMPA
NMDA
EPSC Amp. (% Control)
GluA1
ΔC
Δ824

Figure 7. GluA1ΔC/GluA2 heteromers traffic to the synapse.

(a-d) Paired whole-cell recordings from control and GluA1A1ΔC/GLuA2 replacement CA1 neurons show comparable evoked AMPA and NMDA EPSC amplitudes, as well as paired-pulse ratio (PPR) and synaptic rectification (AMPA ,n = 12; NMDA, n = 8; Rectification, n = 6; PPR, n = 6, all $p > 0.05$). Example traces show average EPSCs for paired control (black) and A1ΔC/A2 replacement (green) CA1 neurons. Scale bars: 20 ms (AMPA, PPR, Rectification), 100 ms (NMDA) and 50 pA. Error bars represent mean ± s.e.m.

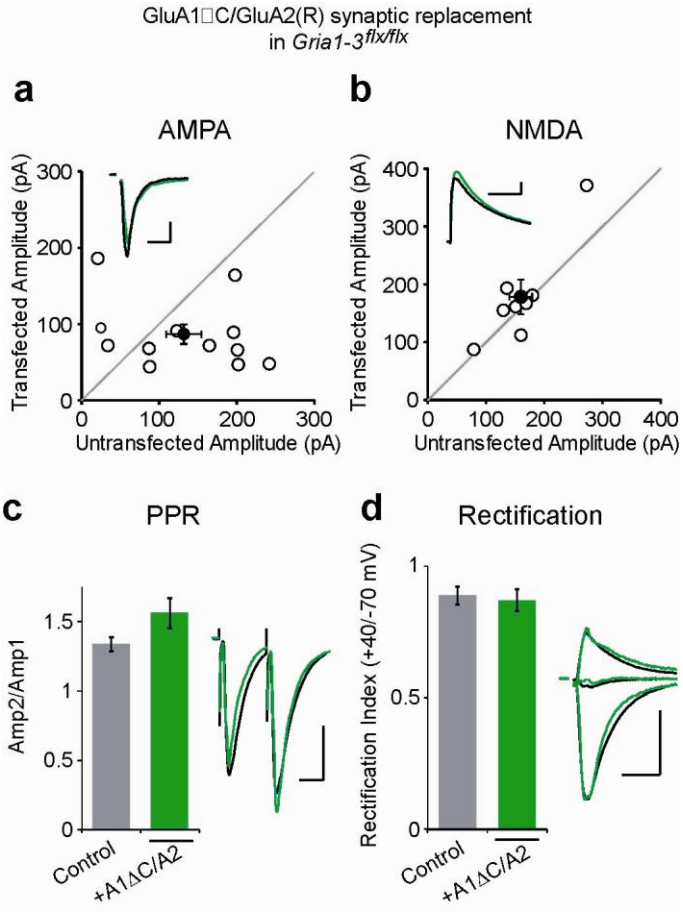

GluA1□C/GluA2(R) synaptic replacement
in Gria1-3flx/flx
a
AMPA
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
b
NMDA
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
c
PPR
Amp2/Amp1
Control
+A1ΔC/A2
d
Rectification
Rectification Index (+40/-70 mV)
Control
+A1ΔC/A2

Figure 8. GluA1 synaptic delivery with acute overexpression.

(a) Paired whole-cell recordings were taken from GluA1-overexpressing and neighboring control CA1 pyramidal neurons in rat organotypic slice cultures. GluA1 and GluA1Δ824 both significantly reduced rectification index compared to control (GluA1, n = 15; GluA1Δ824, n = 9, both $p < 0.01$), while neither short-term nor long-term overexpression of GluA1ΔC did (GluA1ΔC 2 days, n=7; GluA1ΔC 16 days, n=8, both $p > 0.05$). (b) Acute overexpression of neither GFP-tagged GluA1 flop (GFP-A1(o), n = 22) nor flip (GFP-A1(i), n = 12) increased synaptic rectification compared to paired control CA1 neurons. In contrast, both GFP-tagged GluA1 flop (A1(o), n = 7, p = 0.012) and GluA1 flip (A1(i), n = 15, p = 0.015) overexpression increased synaptic rectification. Culturing GluA1 flip expressing slices in 10 mM Mg^{2+} to decrease activity also increased synaptic rectification (A1(i) + 10 mM Mg, n = 13, p = 0.0037). (c) Both untagged and GFP-tagged GluA1 flip increase surface rectification from outside-out patches (GluA1, n = 10, p = 0.000001; GFP-A1(i), n = 12, p = 0.0000025). All example traces show scaled EPSCs from paired control (black) and experimental (green) CA1 neurons to show rectification. Scale Bars: 20 msec, Error bars represent mean ± s.e.m.

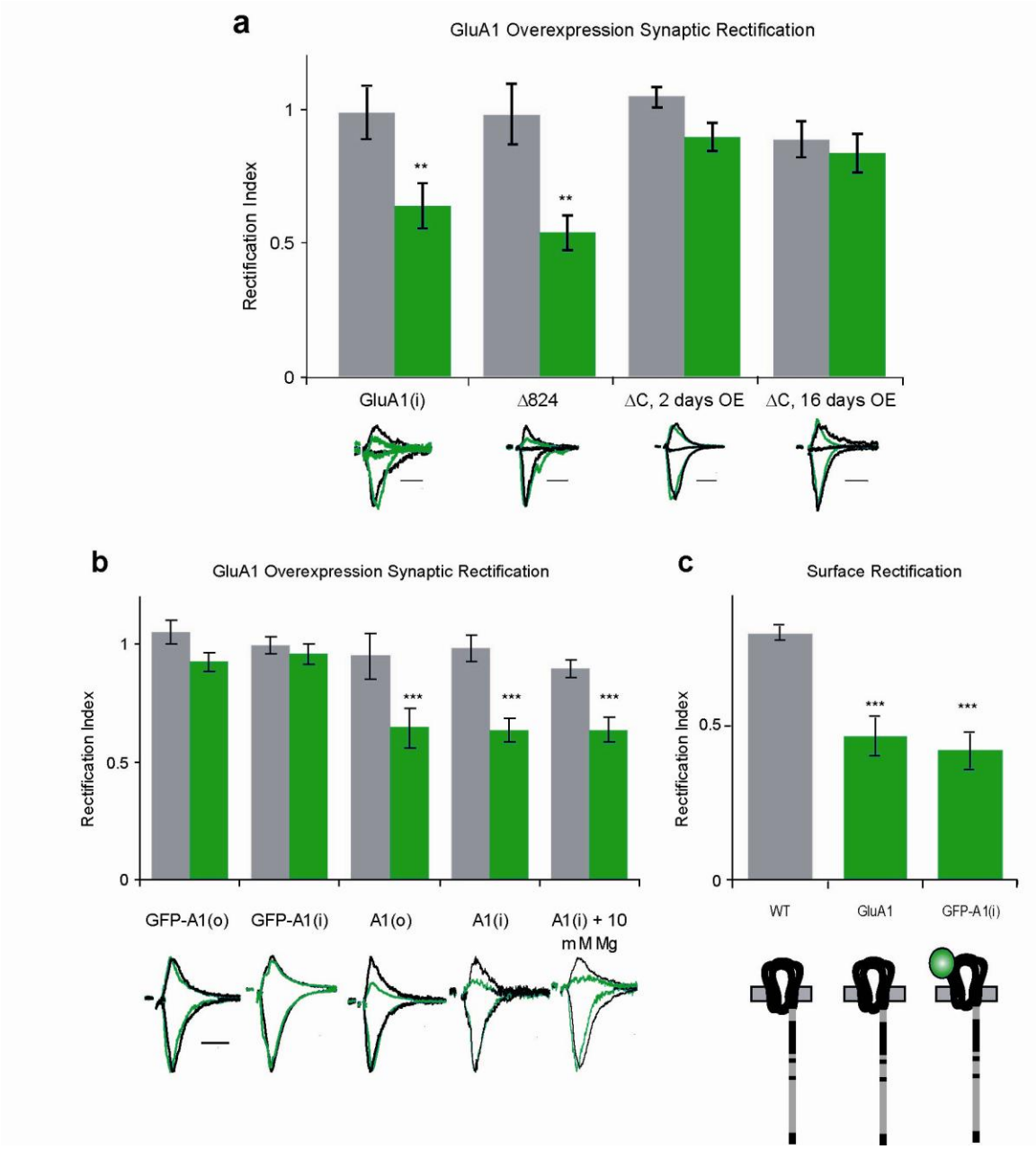

a
GluA1 Overexpression Synaptic Rectification
Rectification Index
1
0.5
0
**
**
GluA1(i)
Δ824
ΔC, 2 days OE
ΔC, 16 days OE
b
GluA1 Overexpression Synaptic Rectification
Rectification Index
1
0.5
0
***
***
***
GFP-A1(o)
GFP-A1(i)
A1(o)
A1(i)
A1(i) + 10 mM Mg
c
Surface Rectification
Rectification Index
0.5
0
***
***
WT
GluA1
GFP-A1(i)

Figure 9. Cre transfection in *Gria1-3*$^{fl/fl}$ neurons results in complete deletion of endogenous AMPARs.

(a,b) Paired whole-cell recordings from Cre-expressing and neighboring control neurons in P10 hippocampal slices shows a complete loss of endogenous AMPAR EPSC and no significant change versus control cells with the NMDA EPSC. ($n = 16$; AMPA, $p < 0.001$; NMDA, $p > 0.05$). (c) LTP induction cannot recruit AMPARs in Cre-expressing *Gria1-3*$^{fl/fl}$ neurons ($n = 3$, $p > 0.05$). All example traces show average EPSCs from paired control (black) and experimental (green) CA1 neurons. In (c), average EPSCs are shown before and after LTP induction. Scale bars: 20 msec, 50 pA. Error bars represent mean ± s.e.m.

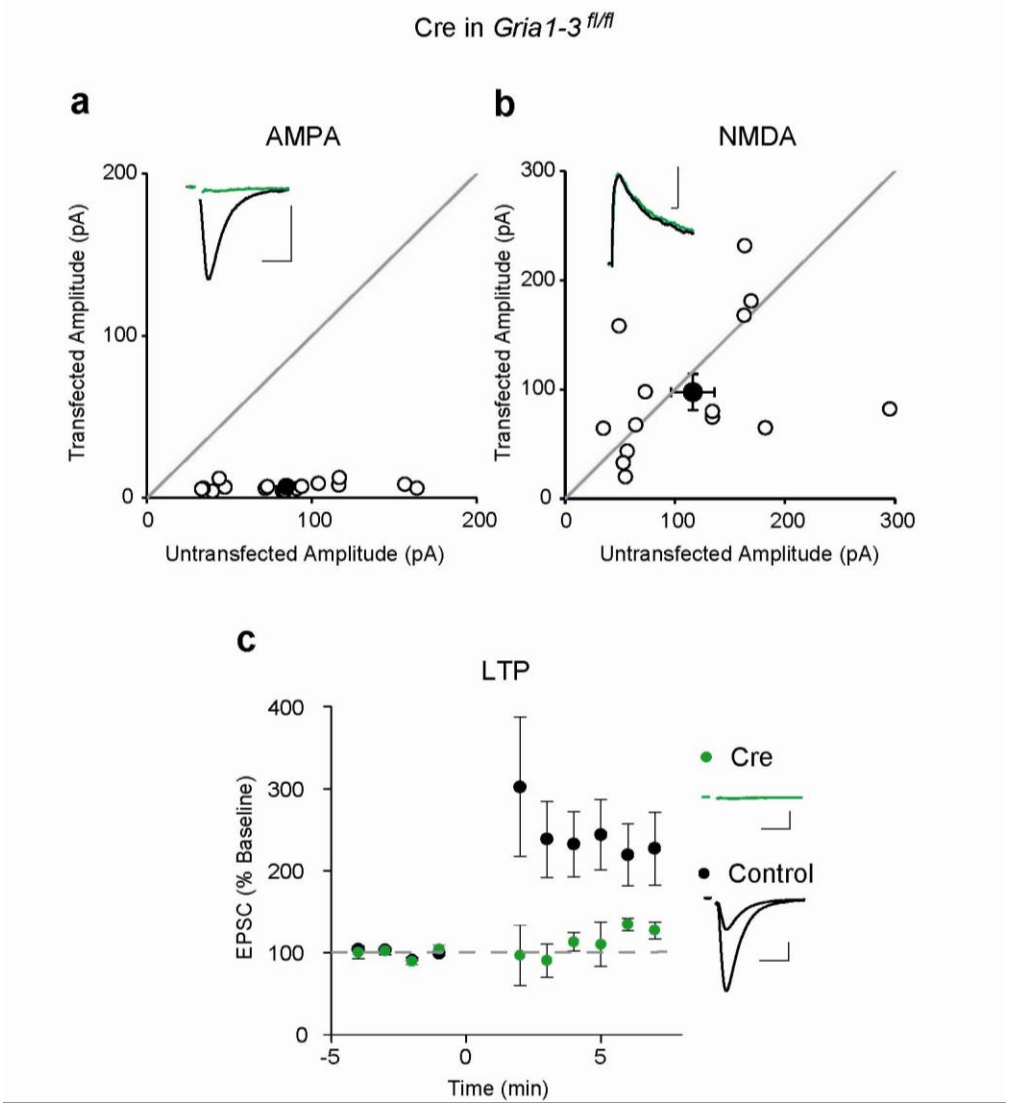

Cre in Gria1-3 fl/fl
a
AMPA
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
0
100
200
b
NMDA
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
0
100
200
300
c
LTP
EPSC (% Baseline)
Time (min)
0
100
200
300
400
-5
0
5
Cre
Control

Figure 10. LTP requires no single portion of the GluA1 C-tail.

Paired whole cell recordings from control CA1 neurons and neighboring Cre/GluA1-expressing neurons in p17-20 *Gria1-3*$^{fl/fl}$ acute slices. LTP is similar to control in GluA1 (a), GluAΔ824 (b), GluA1ΔMPR (c), and GluA1ΔC/ GluA2 (d) replacement neurons (GluA1, n = 11; GluA1Δ824, n = 11;. GluA1ΔMPR, n = 20; GluA1ΔC/GluA2, n = 11; all $p > 0.05$). Example traces show EPSC before and 45 minutes after LTP induction in paired control (black) and GluA1-replacement neurons (green). Scale bars: 20 ms, 100 pA. Error bars represent mean ± s.e.m.

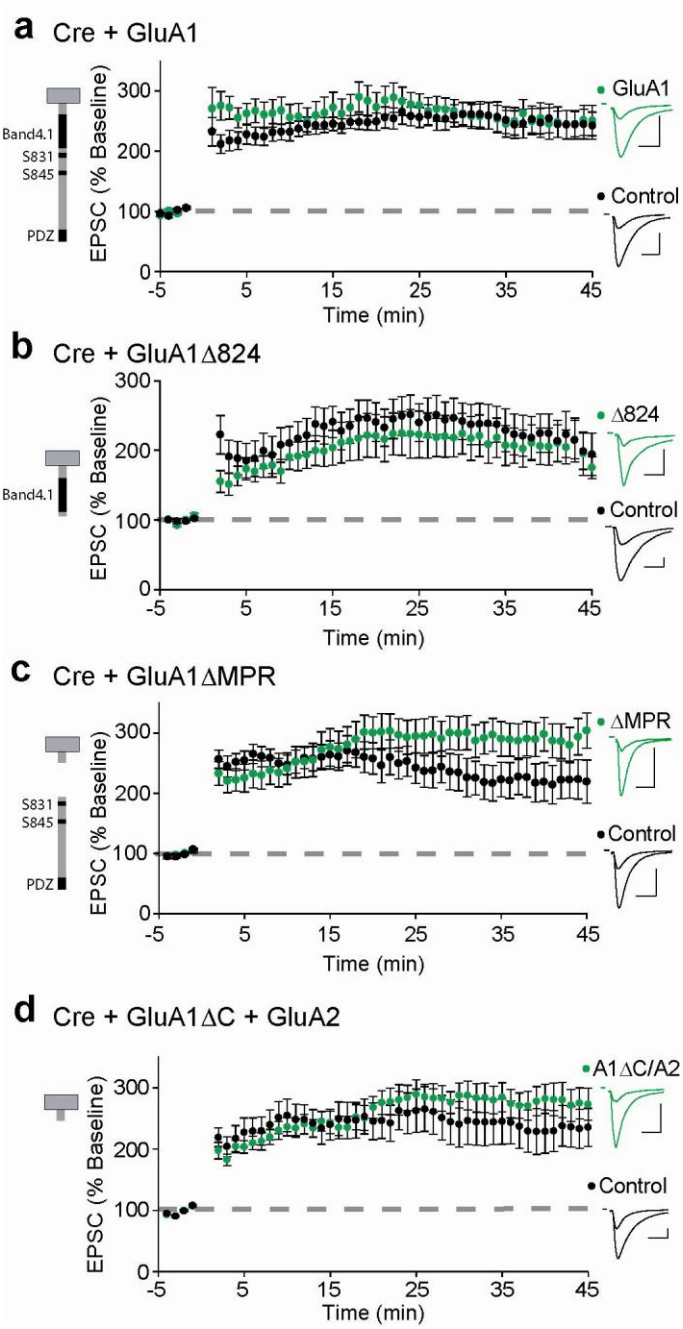

a
Cre + GluA1
Band4.1
S831
S845
PDZ
EPSC (% Baseline)
300
200
100
0
-5
5
15
25
35
45
Time (min)
GluA1
Control
b
Cre + GluA1Δ824
Band4.1
EPSC (% Baseline)
Time (min)
Δ824
Control
c
Cre + GluA1ΔMPR
S831
S845
PDZ
EPSC (% Baseline)
Time (min)
ΔMPR
Control
d
Cre + GluA1ΔC + GluA2
EPSC (% Baseline)
Time (min)
A1ΔC/A2
Control

Figure 11. S818 or S816 phosphorylation is not required for surface expression or LTP.

(a) Overexpression of GluA1Δ824-AA significantly decreases rectification index of glutamate-evoked surface currents compared to control in somatic outside-out patches from CA1 neurons. (Control, n = 47, GluA1Δ824-AA , n = 15, $p < 0.001$). (b,c) Paired whole cell recordings from control and Cre/GluA1d824-AA-expressing CA1 neurons from *Gria1-3*$^{fl/fl}$ acute hippocampal slices show LTP comparable to control (n = 8, $p > 0.05$, minute 45). Example traces show average EPSCs before and 40 minutes following LTP in control (black) and GluA1Δ824-AA-replacement neurons (green). Scale bar: 20 ms and 100 pA. Error bars represent mean ± s.e.m.

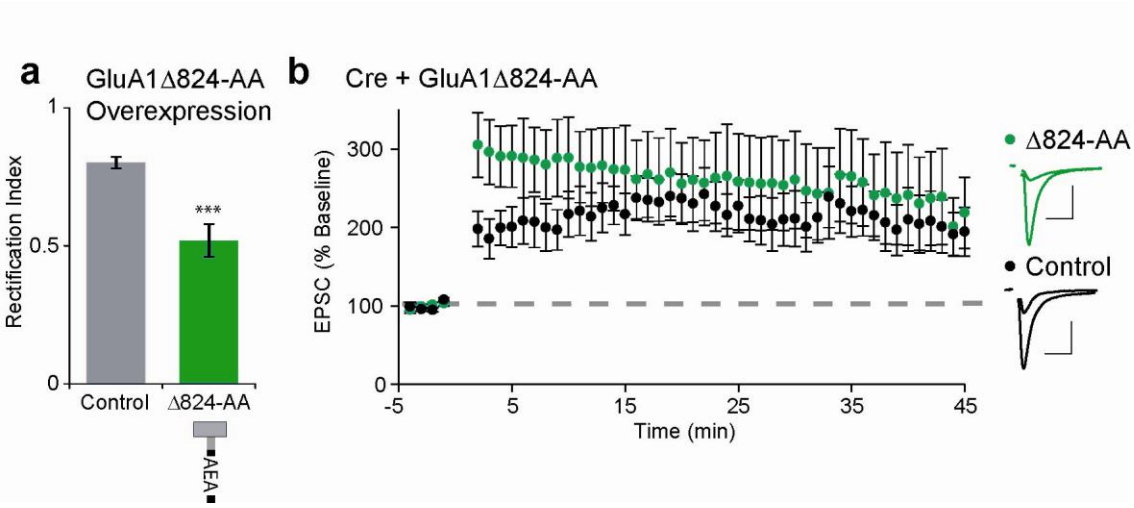

a
GluA1Δ824-AA
Overexpression
Rectification Index
1
0.5
0
***
Control
Δ824-AA
AEA
b
Cre + GluA1Δ824-AA
EPSC (% Baseline)
300
200
100
0
-5
5
15
25
35
45
Time (min)
Δ824-AA
Control

Figure 12. Requirement of the GluA2(Q) C-tail for neuronal surface expression.

Voltage ramps were applied to outside-out patches from GluA2(Q)-expressing and control CA1 neurons in the presence of glutamate. Rectification index (R.I.) was measured as the normalized glutamate-evoked current at +40 mV over -70 mV. (a) Full-length GluA2(Q) and GluA2(Q)Δ847 significantly increased rectification of surface currents compared to control. Overexpression GluA1ΔC slightly decreased the rectification index (Control, n= 9; GluA2(Q), n= 14 , p = 0.000017; GluA2Δ847; n = 5, p = 0.0001; GluA2(Q)ΔC, n = 4, p = 0.011).

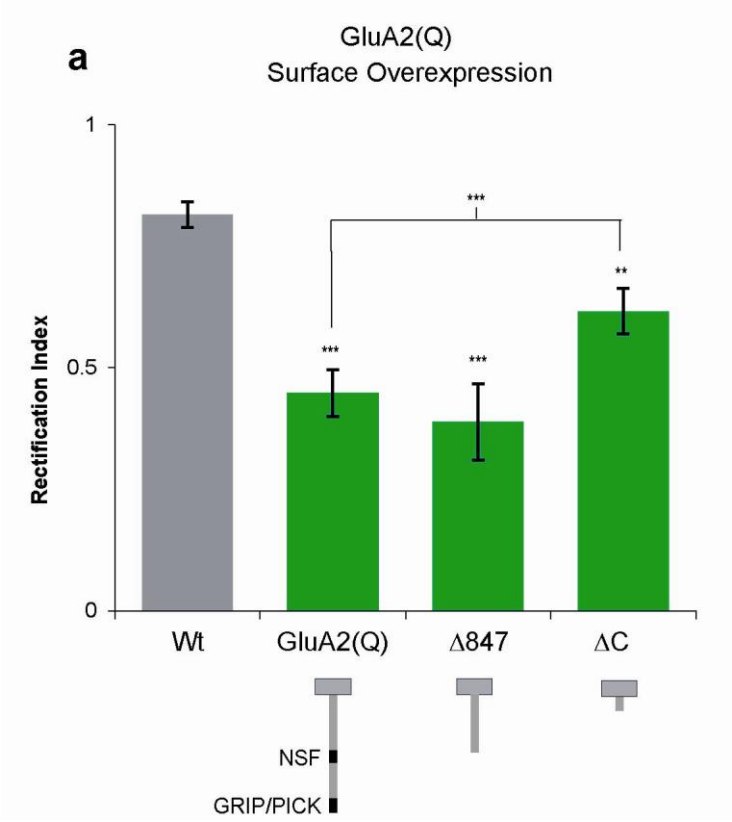

a
GluA2(Q)
Surface Overexpression
Rectification Index
1
0.5
0
***
**
***
***
Wt
GluA2(Q)
Δ847
ΔC
NSF
GRIP/PICK

Figure 13. The GluA2 C-tail is not required for trafficking to the synapse.

(a-c) Synaptic replacement with GluA2(Q), GluA2(Q)ΔC, or GluA2(Q)Δ847 results in comparable rescue of AMPA and NMDA currents, with no change in paired-pulse ratio (GluA2(Q), n = 16; GluA2(Q)ΔC, n = 21; GluA2(Q)Δ847, n = 9; all $p > 0.05$). Rectification is significantly increased, indicating complete replacement of native receptors (all $p < 0.01$). Example traces are average EPSCs from paired control (black) and experimental neurons (green). Scale bars: 20 msec, 25 pA (a-c). Error bars represent mean ± s.e.m.

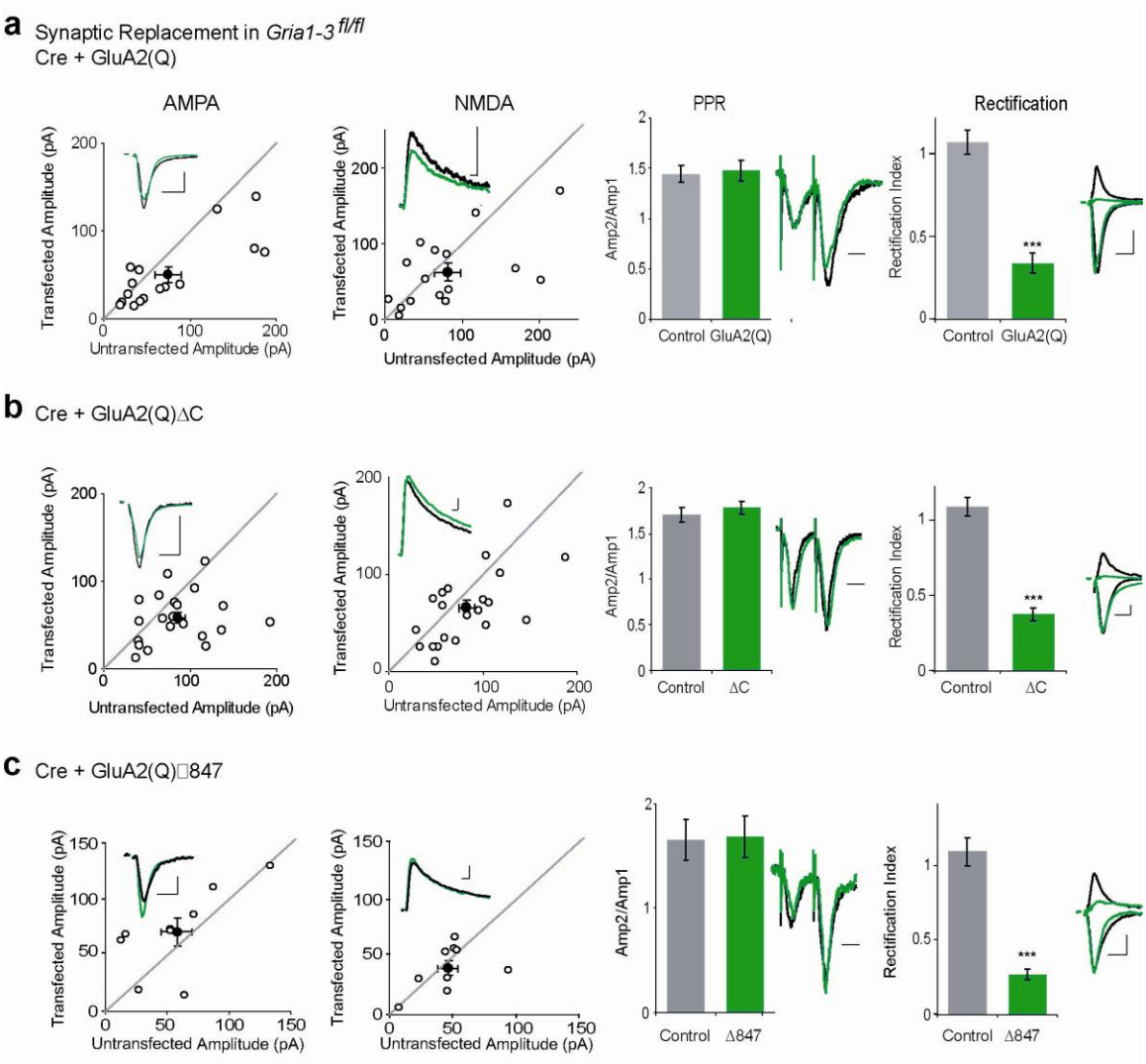

a
Synaptic Replacement in Gria1-3 fl/fl
Cre + GluA2(Q)
AMPA
NMDA
PPR
Rectification
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
Amp2/Amp1
Rectification Index
Control
GluA2(Q)
***
b
Cre + GluA2(Q)ΔC
ΔC
c
Cre + GluA2(Q)Δ847
Δ847

Figure 14. GluA2(Q) alone is sufficient to express LTP.

(a,b) Paired whole-cell recordings between control and Cre + GluA2(Q) or Cre + GluA2(Q)Δ847-expressing *Gria1-3*$^{fl/fl}$ CA1 neurons show similar expression of LTP compared to control (GluA2(Q), n = 14, p > 0.05, minute 45; GLuA2(Q)Δ847, n = 5, p > 0.05, minute 45). Example traces show average AMPA EPSCs before and 45 minutes after LTP induction. Scale bars: 20 ms and 50 pA. Error bars represent mean ± s.e.m.

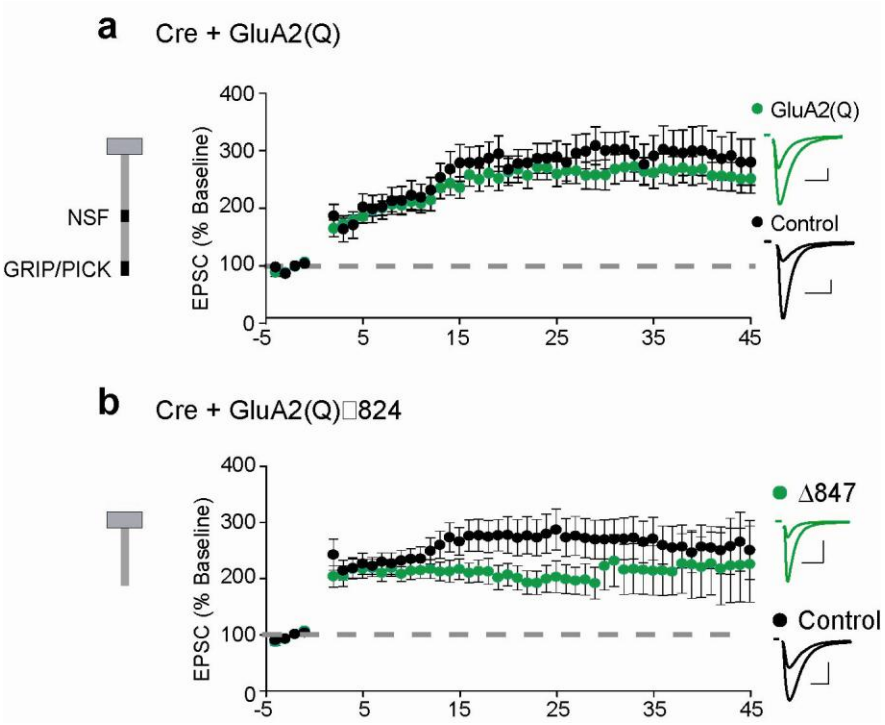

a
Cre + GluA2(Q)
NSF
GRIP/PICK
EPSC (% Baseline)
400
300
200
100
0
-5
5
15
25
35
45
GluA2(Q)
Control
b
Cre + GluA2(Q)□824
Δ847
Control

Figure 15. Lack of surface expression corresponds with loss of LTP in GluA1 conditional knock-outs, and GluA1ΔC and GluA2ΔC replacement neurons.

(a) GluA1 knock-out cells (*Gria1*$^{fl/fl}$ + Cre) demonstrate impaired LTP compared to control (n = 13, p < 0.001, 45 min). (b) Double GluA2/3 knock-out cells demonstrate comparable LTP in control and Cre-expressing neurons (n = 6, p > 0.05, minute 45). (c,d) Molecular replacement with either GluA1ΔC or GluA2ΔC results in reduced expression of LTP (GluA1ΔC, n = 16, p < 0.05; GluA2(Q)ΔC, n = 10, p < 0.05, both at 45 min). (e,f) Overexpressed GluA1ΔC and GluA2(Q)ΔC are both impaired at expressing on the surface, as indicated by their decreased rectification compared to overexpression of full length GluA1 and GluA2(Q) (GluA1, n = 10; GluA1ΔC, n = 8, p = 0.00032; GluA2(Q), n = 14, GluA2(Q)ΔC, n = 4, p = 0.03). Example traces show averaged AMPA EPSCs before and 45 minutes following induction of LTP in paired experimental neurons (green) and control cells (black). Scale bars: 20 msec, 50 pA. Error bars represent mean ± s.e.m.

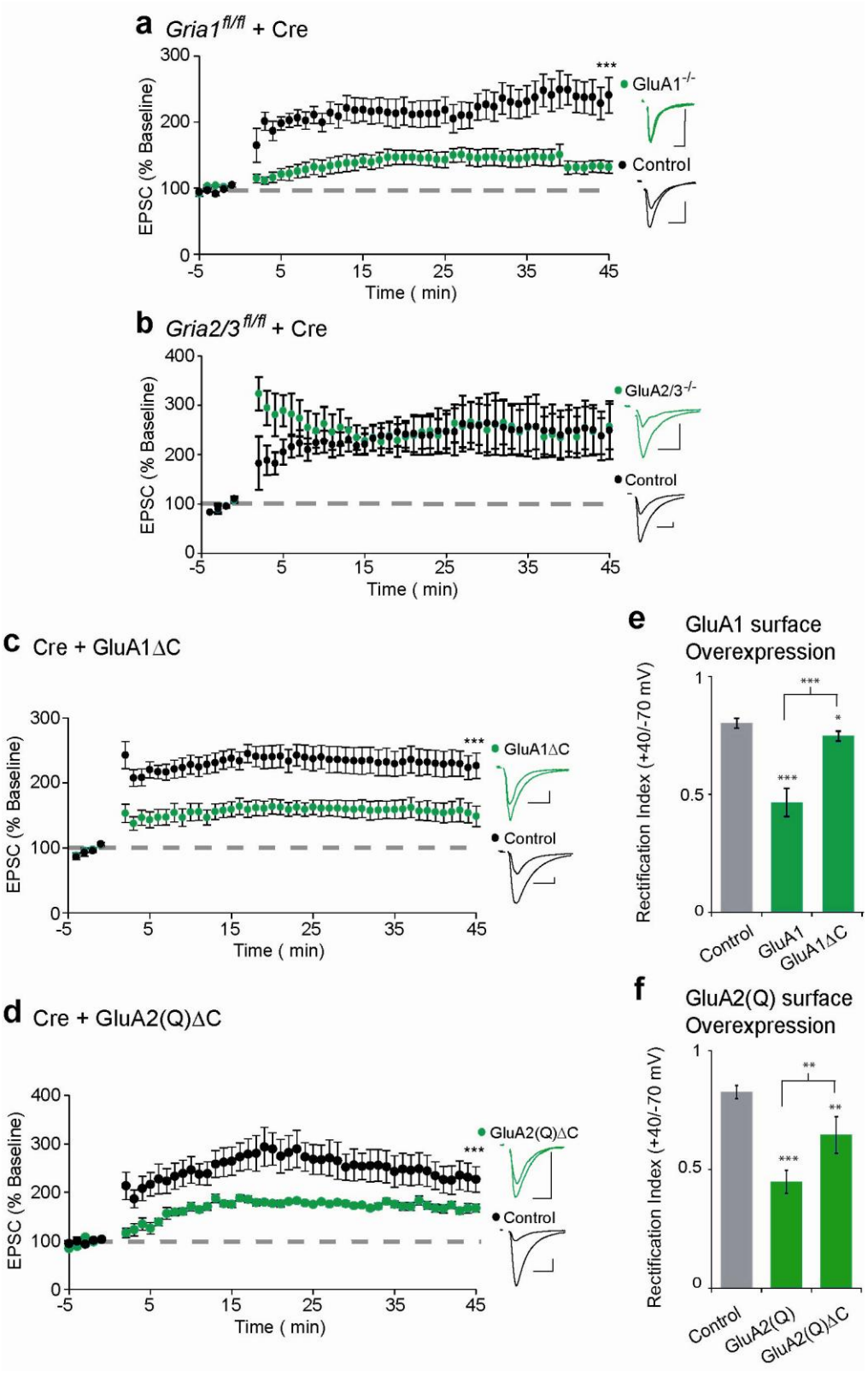

a Gria1fl/fl + Cre
EPSC (% Baseline)
Time (min)
GluA1-/-
Control
b Gria2/3fl/fl + Cre
GluA2/3-/-
Control
c Cre + GluA1ΔC
GluA1ΔC
Control
d Cre + GluA2(Q)ΔC
GluA2(Q)ΔC
Control
e GluA1 surface Overexpression
Rectification Index (+40/-70 mV)
Control
GluA1
GluA1ΔC
f GluA2(Q) surface Overexpression
Rectification Index (+40/-70 mV)
Control
GluA2(Q)
GluA2(Q)ΔC

Figure 16. GluA2 and GluA3 single-cell conditional knock-outs demonstrate normal LTP.

(a,b) Paired whole-cell recordings between control and neighboring Cre-expressing CA1 neurons show no defect in LTP in *Gria2*$^{fl/fl}$or *Gria3*$^{fl/fl}$ single-cell knock-outs. (GluA2, n =8; GluA3, n = 4; both $p > 0.05$). Example traces show paired average EPSCs from control (black) and experimental neurons (green). Scale bars: 20 msec, 100 pA. Error bars represent mean ± s.e.m.

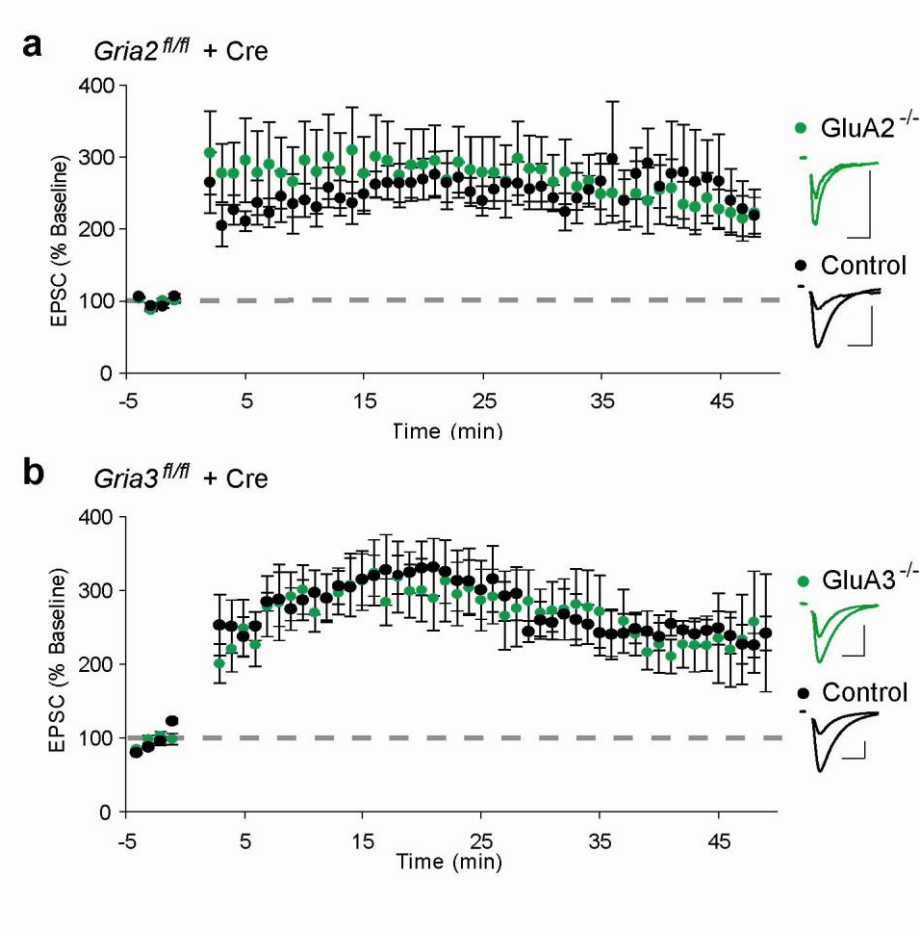

a
Gria2 fl/fl + Cre
400
300
200
100
0
EPSC (% Baseline)
-5
5
15
25
35
45
Time (min)
GluA2 -/-
Control
b
Gria3 fl/fl + Cre
400
300
200
100
0
EPSC (% Baseline)
-5
5
15
25
35
45
Time (min)
GluA3 -/-
Control

Figure 17. GluK1 traffics to the synapse in CA1 pyramidal neurons.

(a,b) Paired whole-cell recordings between CA1 neurons expressing GluK1 and Neto2 show increased EPSC amplitude at -70 mV compared to control ($n = 9$, $p > 0.05$), with no change in the NMDA receptor EPSC ($p > 0.05$). (c) While WT neurons do not express a GYKI-resistant EPSC, GluK1/Neto2 overexpressing rat CA1 neurons do express a GYKI-resistant EPSC (Control, $n = 13$, GluK1/Neto2, $n = 13$, $p < 0.001$). Example traces show an average EPSC before wash-in of 100 μM GYKI (black), after (green), and following addition of 50 μM NBQX (red).

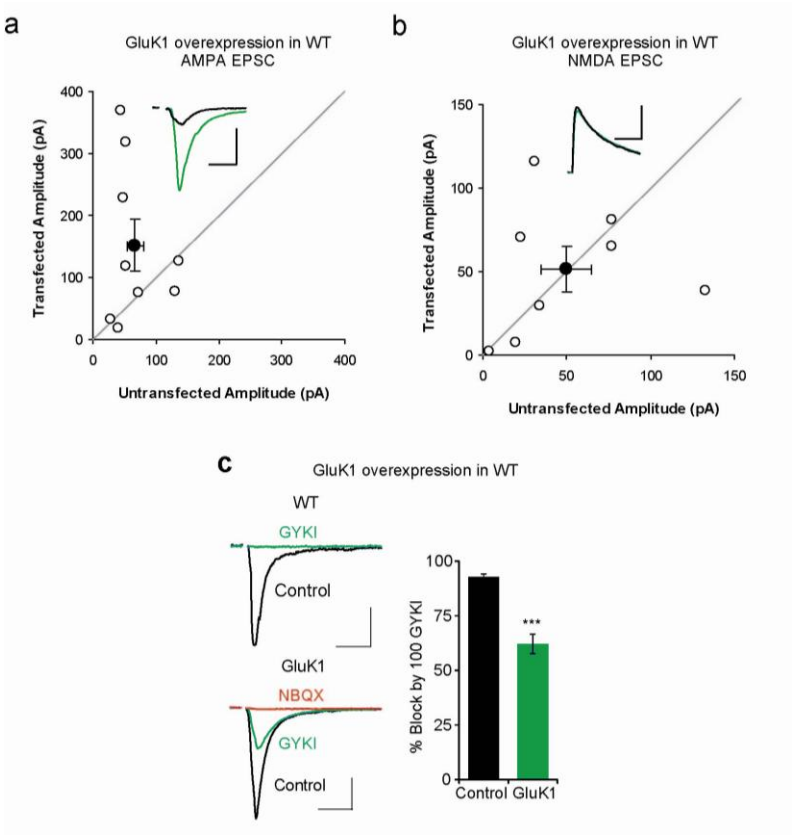

a
GluK1 overexpression in WT
AMPA EPSC
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
0
100
200
300
400
b
GluK1 overexpression in WT
NMDA EPSC
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
0
50
100
150
c
GluK1 overexpression in WT
WT
GYKI
Control
GluK1
NBQX
GYKI
Control
% Block by 100 GYKI
***
Control
GluK1

Figure 18. 1 μM ACET does not block AMPARs in control CA1 neurons.

(a) 1 μM ACET did not decrease synaptic transmission in wild-type CA1 neurons from rat organotypic slice cultures compared to control neurons that did were not exposed to ACET (Control, n = 5; 1 μM ACET, n = 4, $p > 0.05$). 10 μM ACET decreased the amplitude of recorded EPSCs by ~50%, though not yet significantly decreased compared to control (10 μM ACET, n = 4, $p = 0.1143$).

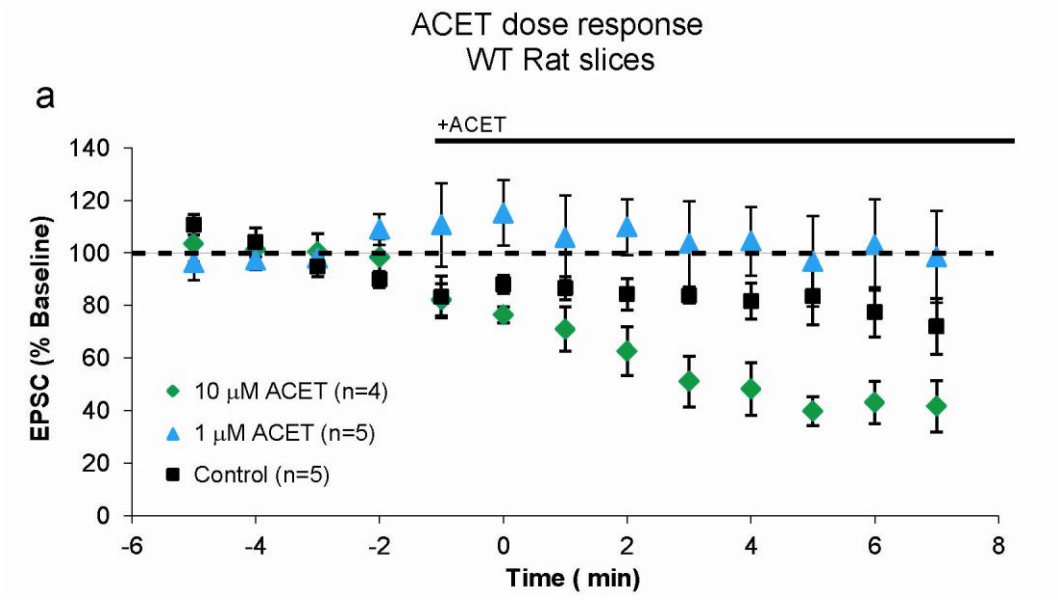

ACET dose response
WT Rat slices
a
+ACET
EPSC (% Baseline)
140
120
100
80
60
40
20
0
-6
-4
-2
0
2
4
6
8
Time (min)
10 μM ACET (n=4)
1 μM ACET (n=5)
Control (n=5)

Figure 19. GluK1 expresses on the neuronal surface, targets to synapses, and mediates LTP.

(a) Co-expression of Cre, GluK1, and Neto2 (n = 10) in *Gria1-3*$^{fl/fl}$ neurons results in robust glutamate-evoked currents from somatic outside-out patches. The current desensitizes in the presence of 100 μM cyclothiazide (CTZ), and is completely blocked by 1 uM ACET. (b) Paired recordings from Cre/GluK1/Neto2-expressing and neighboring control CA1 neurons resulted in a 33% rescue of synaptic EPSCs (n = 20, $p < 0.001$) with no change in NMDA EPSCs (n = 13, $p>0.05$). Example trace (inset) shows paired control (black) and GluK1-replacement (green) EPSCs. Application of 1 μM ACET resulted in little to no block of control cell EPSCs (black example traces, lower middle), but complete block of GluK1 ESPCs (green example traces, upper middle, n = 14, $p < 0.001$). (c) Paired whole-cell recording from control and Cre/GluK1/Neto2-expressing *Gria1-3*$^{fl/fl}$ CA1 neurons show similar levels of LTP (n =12, $p > 0.05$, minute 45). Wash-in of 100 mM GYKI completely blocked synaptic tranmsision in control neurons, but only blocked 50% of EPSC amplitude in GluK1 replacement neurons (n = 9, p = 0.0000265 , minute 60). (d) LTP produced in GluK1 replacement neurons that was similar to control (n = 12 , $p > 0.05$, minute 45) was completely blocked by 1 μM ACET, but not in control (n = 11, $p < 0.001$, minute 60). Example traces show average EPSCs before and 45 minutes following LTP induction in control (black) and GluK1-replacement neurons (green). Scale bars:

20 msec (AMPA), 100 msec (NMDA), and 50 pA in (a,b), 100 pA (e). Error bars represent mean ± s.e.m.

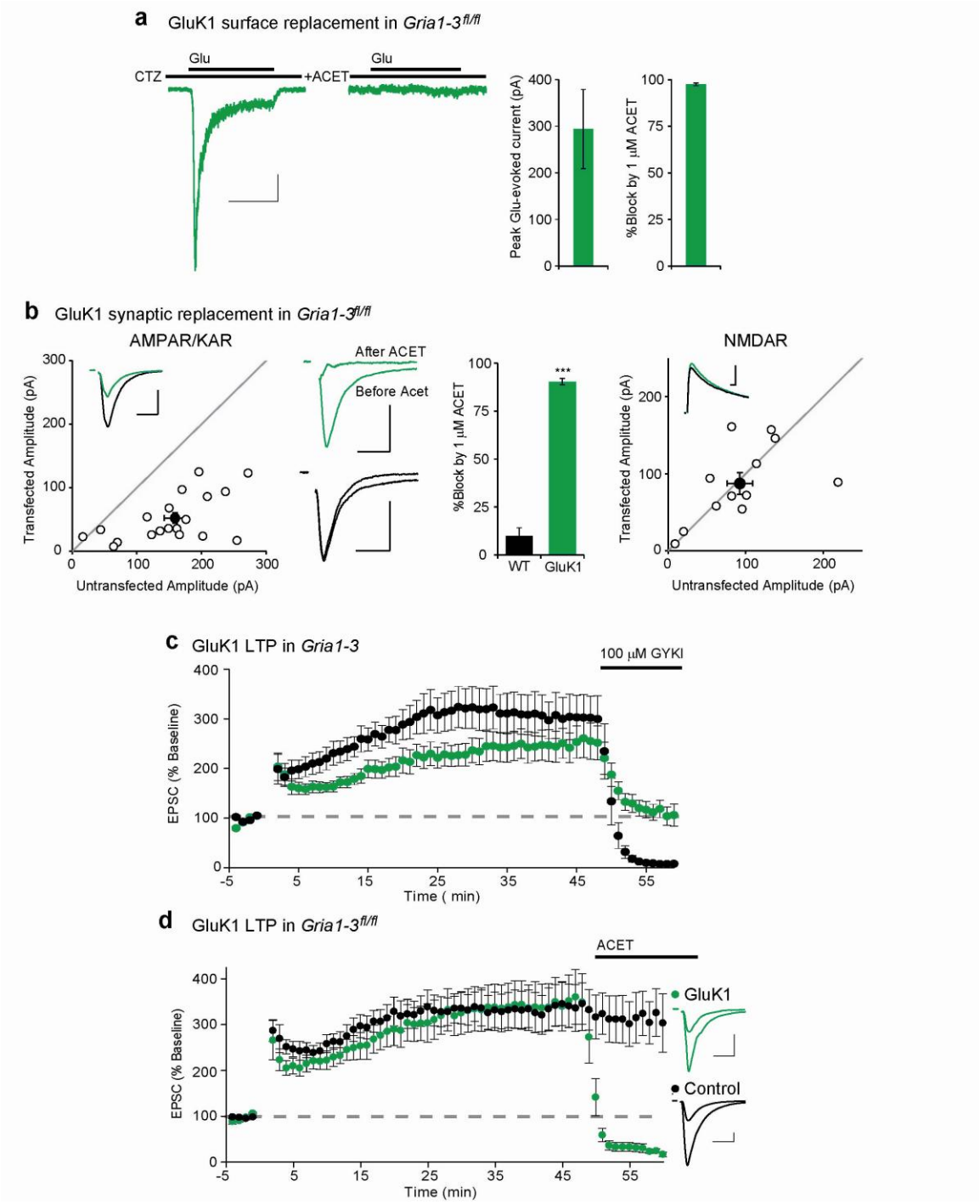

a
GluK1 surface replacement in Gria1-3fl/fl
Glu
Glu
CTZ
+ACET
Peak Glu-evoked current (pA)
%Block by 1 μM ACET
b
GluK1 synaptic replacement in Gria1-3fl/fl
AMPAR/KAR
After ACET
Before Acet
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
%Block by 1 μM ACET
***
WT
GluK1
NMDAR
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
c
GluK1 LTP in Gria1-3
100 μM GYKI
EPSC (% Baseline)
Time (min)
d
GluK1 LTP in Gria1-3fl/fl
ACET
GluK1
Control
EPSC (% Baseline)
Time (min)

Figure 20. Overexpressed GluK1 does not compete with endogenous AMPARs during LTP.

(a) Paired whole-cell recordings between CA1 neurons expressing GluK1 and Neto2 show increased LTP compared to untrasnfected control neurons in acute slices ($n = 7$, $p > 0.05$, minute 30). 10 μM ACET decreases the GluK1-expressing and control neuron to the same level ($p > 0.05$, minute 45). (b) Electroporation of GluK1 and Neto2 results in modest increase in the amplitude of the EPSC in transfected CA1 neurons compared to control ($n = 8$, $p > 0.05$).

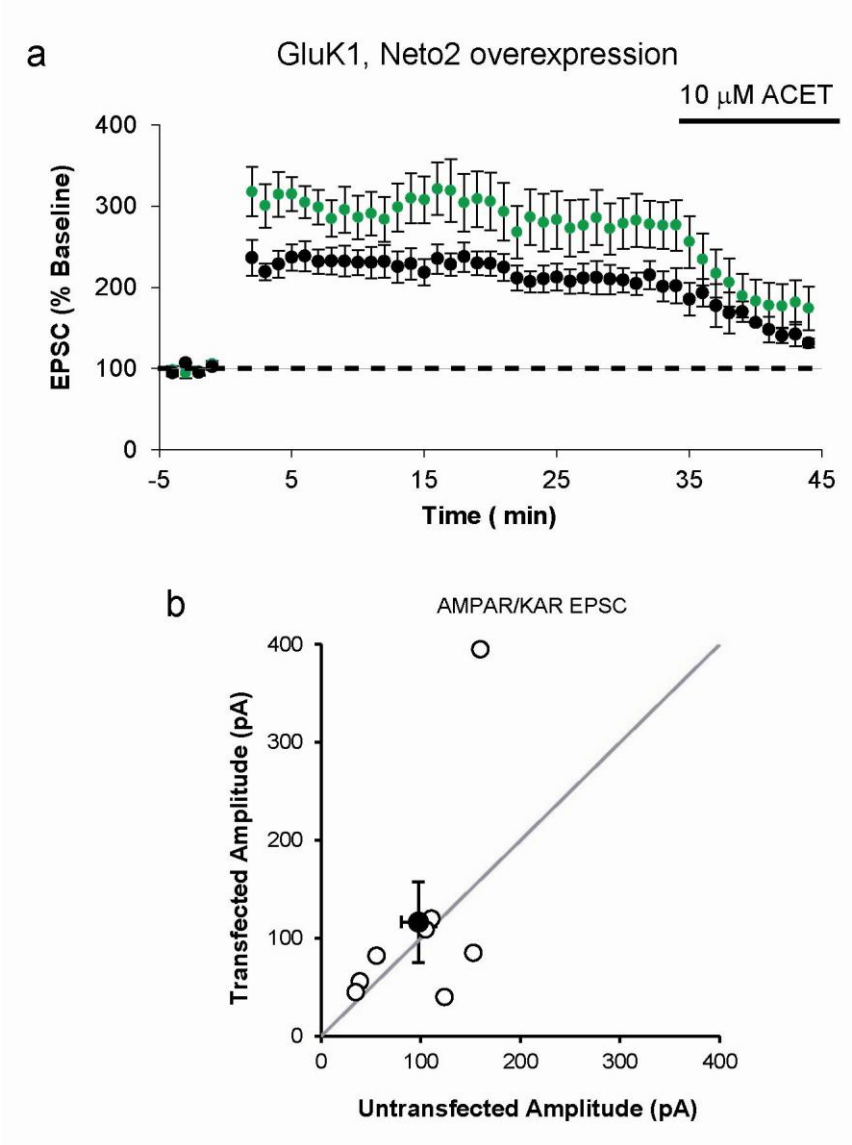

a
GluK1, Neto2 overexpression
10 μM ACET
EPSC (% Baseline)
400
300
200
100
0
-5
5
15
25
35
45
Time (min)
b
AMPAR/KAR EPSC
Transfected Amplitude (pA)
400
300
200
100
0
0
100
200
300
400
Untransfected Amplitude (pA)

Figure 21. GluK1 molecular replacement results in LTP that is blocked by APV.

(a) APV prevents potentiation in both control and GluK1-replacement neurons (n = 10, $p > 0.05$, 45 minutes). Subsequent wash-in of 1 uM ACET results in complete block of the GluK1-replacement EPSC versus control ($p = 0.00031$). (b) Recordings were made exactly as shown in (a), but with no APV and no pairing. This data shows that the increase in EPSCs in (a) is due to a modest run up in the EPSCs over time. (c) Superimposition for data in (a) and (b). Error bars represent mean ± s.e.m.

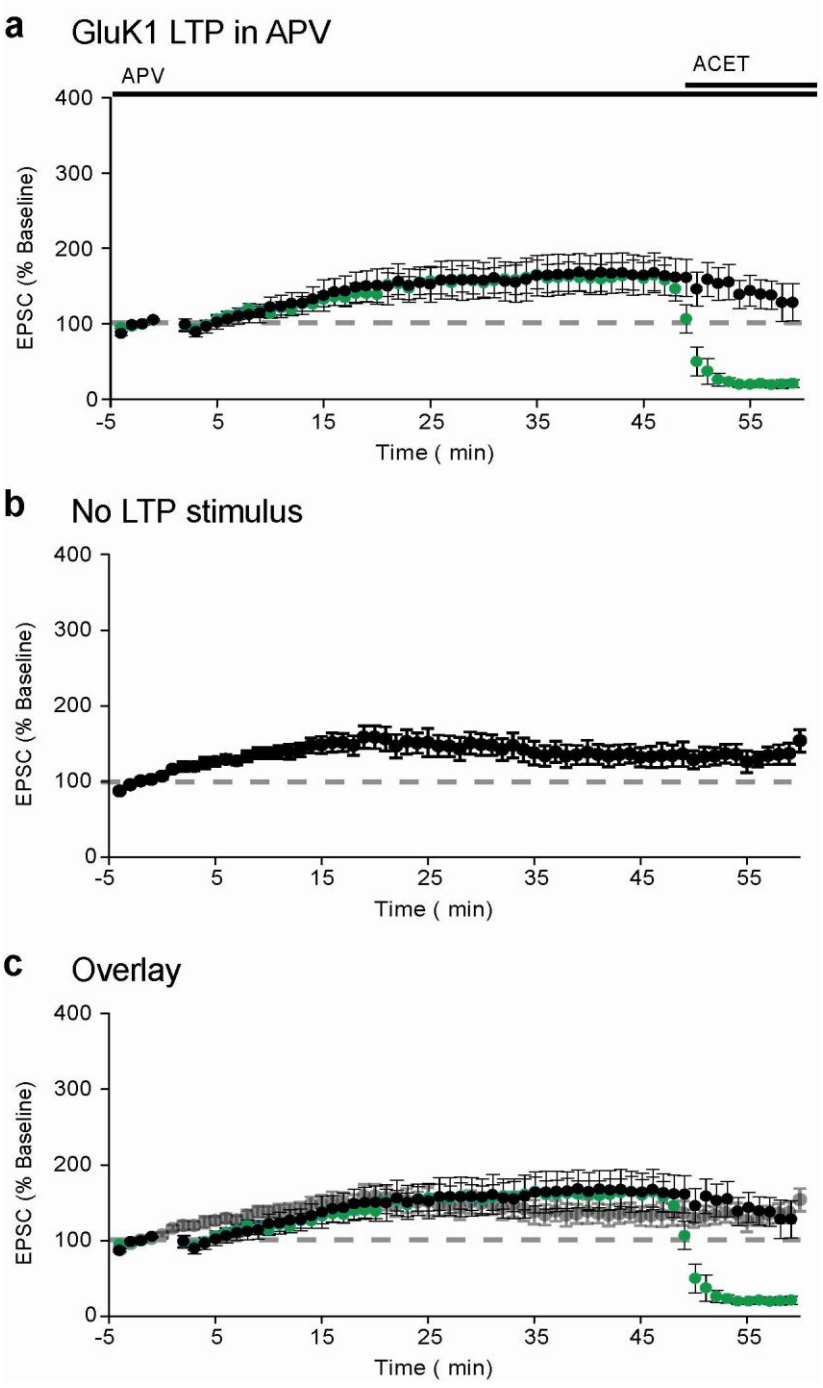

a
GluK1 LTP in APV
APV
ACET
EPSC (% Baseline)
400
300
200
100
0
-5
5
15
25
35
45
55
Time (min)
b
No LTP stimulus
EPSC (% Baseline)
Time (min)
c
Overlay
EPSC (% Baseline)
Time (min)

Figure 22. Run-up of EPSC amplitude is attenuated by maintaining a cell-attached configuration 10 minutes prior to breaking in.

(a) Paired recordings were made between two control neurons in acute slice culture from wild-type mice. One neuron was maintained in a cell-attached configuration, while whole-cell access was made immediately into the control neuron. EPSC amplitude was normalized to that of the control cell 20 minutes following break-in. Average control amplitude over time is represented by the solid black trace, with the dotted lines representing the 95% confidence interval. A smoothed running average of EPSC amplitude for each individual cell-attached neuron (colored traces) is plotted following cell break-in, either spontaneously or 10 minutes following the control cell. EPSC amplitude increases overtime in the control neurons, where whole-cell access is obtained immediately. EPSC run-up depends on time of whole-cell access in each of the experimental cells, and seems to follow the average rate of run-up in the control neurons.

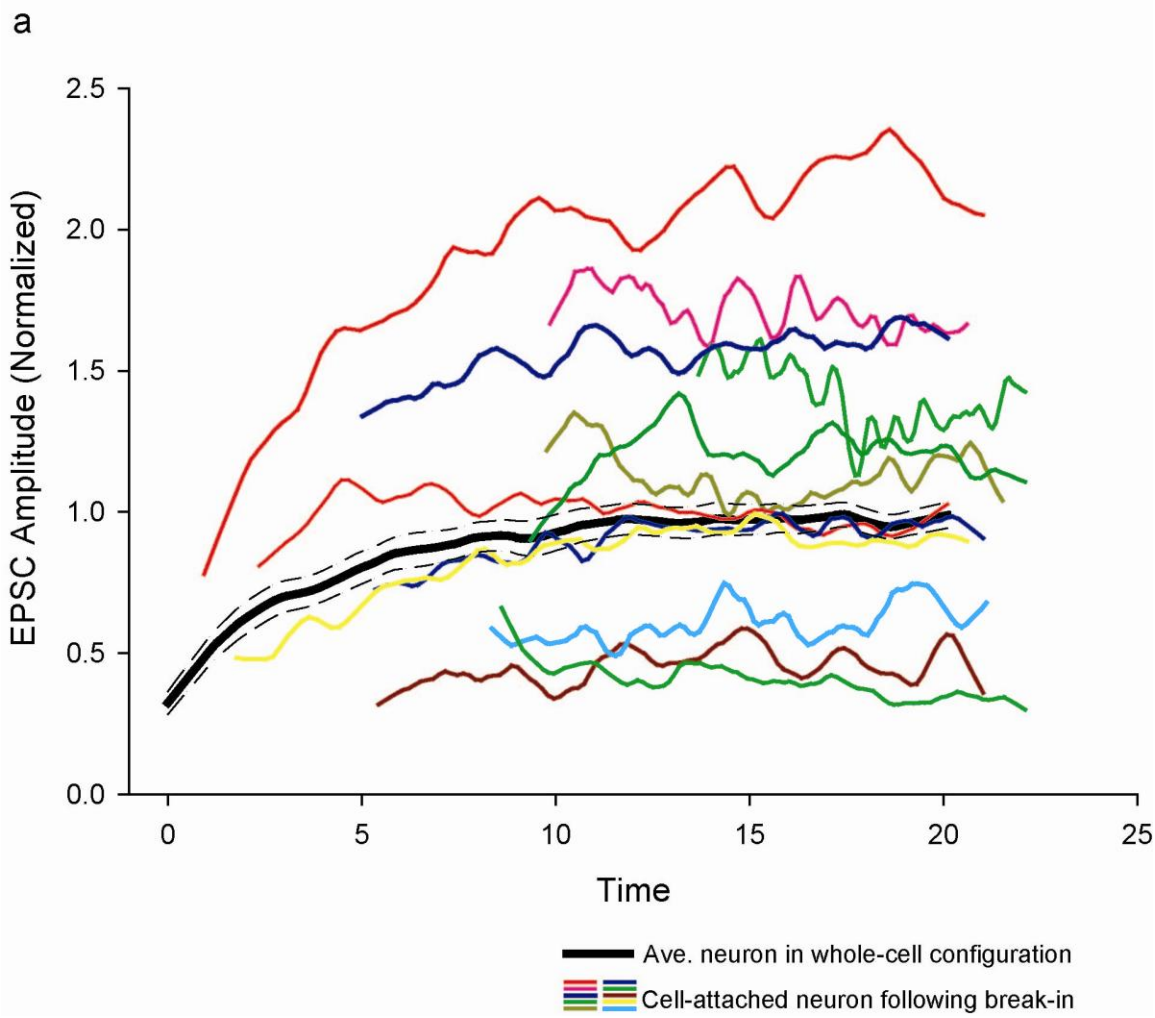

a
EPSC Amplitude (Normalized)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Time
Ave. neuron in whole-cell configuration
Cell-attached neuron following break-in

Figure 23. Summary of trafficking behavior of different GluA1, GluA2, and GluK1 C-tail mutants.

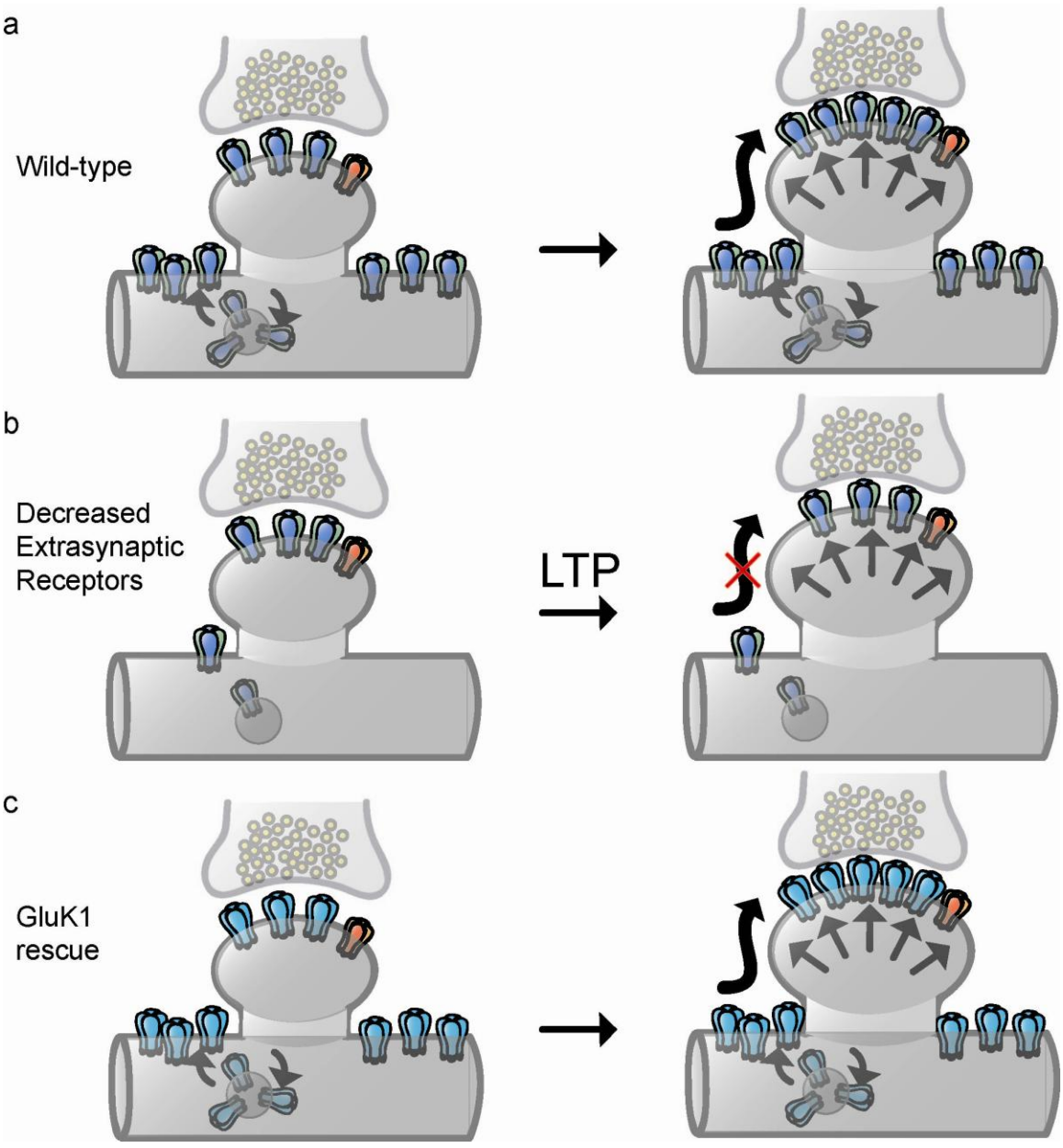
The amino acid sequence of different replacement glutamate receptor subunits is shown, indicating whether it showed expression to the surface, synapse, or LTP. In each case where the receptor subunit shows surface expression, LTP is also expressed, even in cases where surface expression is mediated by a foreign receptor (GluA2(Q), GluK1). In contrast, wherever surface expression is impaired, so is LTP.

Molecular Replacement Summary

	C-tail sequence	Surface	Synaptic	LTP
GluA1 -/-	EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL	-	+	-
GluA1	EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL	+	+	+
GluA1Δ824	EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL	+	+	+
GluA1Δ824-AA	EFCYKSRAEAKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL	+	+	+
GluA1ΔMPR	EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL	+	+	+
GluA1ΔC	EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL	-	+	-
GluA2 -/-	EFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI	+	+	+
GluA2(Q)	EFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI	+	+	+
GluA2(Q)Δ847	EFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI	+	+	+
GluA2(Q)ΔC	EFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI	-	+	-
GluA1ΔC +GluA2(R)	EFCYKSRSESKRMKGFCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL EFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI	+	+	+
GluK1	KSRKNNDIEQKGKSSRLRFYFRNKVRFKHSKTESLGVEKCLSFNAIMEELGISLKNQKKIKKKSRTKGKSSFTSILTCHQRRTQKETVA	+	+	+

Figure 24. Model for glutamate receptor trafficking during LTP expression.

(a) In the wild-type case, LTP expression causes a global increase in the size of the synapse and in its capacity to cluster glutamate receptors, primarily GluA1/GluA2 heteromers, either through lateral diffusion from the extrasynaptic membrane, or from exocytosis from internal stores. (b) In conditions where the surface pool of receptors is severely depleted, LTP expression is blocked, because there are not enough additional receptors to enter the newly enlarged synapse. (c) Rescue of extrasynaptic receptors by a foreign glutamate receptor subtype, such as GluK1, is sufficient to rescue LTP expression.


a
Wild-type
b
Decreased
Extrasynaptic
Receptors
LTP
c
GluK1
rescue

CHAPTER 4:
Glutamate receptor subtypes and LTD

Introduction

Excitatory synapses in the brain modify their strength to store information in response to specific patterns of activity, either by getting stronger through long-term potentiation (LTP) or weaker through long-term depression (LTD) (Malenka & Bear, 2004). In the case of LTP, coincident activation of the presynaptic terminal and postsynaptic neuron induces synaptic strengthening, whereas LTD is induced by persistent asynchronous firing of a postsynaptic neuron and activation of presynaptic terminals. However, both LTP and LTD are known to require the differential activation of NMDA-type glutamate receptors (Luscher *et al.*, 1999; Malinow & Malenka, 2002; Kessels & Malinow, 2009). The difference between induction of LTP or LTD through NMDARs has been attributed to the magnitude and duration of Ca^{2+} signaling, with a relatively low level of persistent Ca^{2+} signaling inducing LTD, whereas a rapid, high-level of Ca^{2+} influx inducing LTP (Malenka & Bear, 2004). However, a recent study cast doubt on this model, showing that while NMDAR activation is required for LTD, channel-opening and Ca^{2+}-influx is not (Nabavi *et al.*, 2013). Such a "metabotropic" function of NMDARs has not been previously described, and may aid experimenters in differentiating between LTP and LTD.

Regardless of the means of induction, both LTP and LTD are expressed through the insertion or removal, respectively, of AMPA-type glutamate receptors. AMPARs are expressed as heterotetramers of different subunit proteins, GluA1-4. In CA1 pyramidal neurons, where GluA1/GluA2 heteromers dominate, evidence exists for specific roles for both GluA1 or GluA2 subunits in mediating synaptic removal and endocytosis of AMPARs. In the case of GluA2, phosphorylation at amino acid S880 causes increased

AMPAR internalization in cultured neurons (Chung *et al.*, 2000), and increased phosphorylation is observed following LTD induction in hippocampal slices (Kim *et al.*, 2001). More convincingly, intracellular perfusion of a peptide mimicking the C-tail of GluA1 inhibited LTD expression (Daw *et al.*, 2000; Kim *et al.*, 2001). Finally, overexpression of a GluA2 S880 phosphomimic mutant decreased synaptic transmission and occluded LTD, while overexpression of the phosphonull mutant partially blocked LTD expression (Seidenman *et al.*, 2003). However, the requirement for GluA2 has been cast in serious doubt by the discovery that LTD is intact in GluA2 knockout mice (Meng *et al.*, 2003). GluA1, in contrast, has not typically been implicated in LTD expression, except by the finding that the GluA1 S845A knock-in mouse does not express LTD (Lee *et al.*, 2003; Lee *et al.*, 2010). To date, no one has examined whether LTD can be expressed after genetic deletion of GluA1.

To assess whether LTD requires a particular AMPAR subunit, we tested for LTD expression in constitutive knockouts of both GluA2 and GluA1. We found normal LTD in both of these knockouts, indicating that no particular AMPAR subunit is necessary. To more rigorously test this notion, we turned to molecular replacement of all endogenous AMPARs with the KA receptor subunit GluK1. Indeed, we again observed normal LTD. This strongly argues that LTD does not require a specific manipulation of the glutamate receptor itself, but a more general decrease in the capacity of the synapse or global non-specific endocytosis of synaptic receptors. While carrying out these experiments, , we found that 7-CK, a glycine-site antagonist of the NMDAR (Kemp *et al.*, 1988), aided expression of whole-cell LTD, confirming a potential metabotropic role for NMDARs (Nabavi *et al.*, 2013) .

Results

To test for any AMPAR subunit specificity of LTD expression, we recorded from acute hippocampal slices taken from GluA1 and GluA2 constitutive knockout mice (*Gria1*$^{-/-}$ and *Gria2*$^{-/-}$, respectively). Slices from wild-type littermates were used as a control. After recording a stable 10-minute baseline of field EPSPs from stratum radiatum, LTD was induced by stimulating at a low frequency (1 Hz) for 15 minutes. Surprisingly, LTD expression was intact in GluA1 knockout slices, indistinguishable from LTD recorded from control slices (Fig. 25a), suggesting that GluA1 is not absolutely required for LTD expression. Normal LTD expression was also observed in GluA2 knockout slices (Fig. 25b), in agreement with previously published results (Meng *et al.*, 2003). Given that the majority of AMPARs in CA1 pyramidal neurons are GluA1/GluA2 heteromers, this argues strongly against any AMPAR subunit-specificity for LTD.

If no specific AMPAR subunit is required for LTD, we wondered if AMPARs generally are required, or if LTD occurs through a generic decrease in the size of the synapse (Zhou *et al.*, 2004), or non-specific endocytosis of all synaptic receptors. To answer this question, we turned to molecular replacement of endogenous AMPARs with the KA receptor subunit GluK1. The specific KAR subunit GluK1 also allows us to use the highly specific antagonist ACET, which potently inhibits GluK1 without affecting AMPARs or other KAR subunits (Dargan *et al.*, 2009). To achieve molecular replacement, we co-transfected Cre-recombinase in CA1 pyramidal neurons of *Gria1-3*$^{fl/fl}$ mice, resulting in complete deletion of endogenous AMPARs, along with a replacement GluK1 subunit and Neto2, a KAR auxiliary subunit (Copits *et al.*). Paired whole-cell

recordings were made between GluK1-replacement CA1 neurons and neighboring untransfected control, and LTD induced by stimulating at 1 Hz for 15 minutes while depolarizing both cells to -40 mV. We observed comparable expression of LTD between the GluK1 replacement neuron and control (Fig. 26a). To ensure that the LTD expressed in GluK1 replacement neurons used the same mechanism as control neurons, we tried expressing LTD in the presence of the NMDAR antagonist APV. In both GluK1 replacement and control cells, LTD expression was blocked by APV (Fig. 26b). Based on these experiments, NMDAR-dependent LTD does not appear to require AMPARs.

While conducting whole-cell LTD experiments, we found a high degree of variability with LTD expression and were unable to induce LTD with multiple previously published induction protocols (Morishita *et al.*, 2001; Morishita *et al.*, 2005). Ultimately we found that using an induction protocol similar to that used during field recordings was most successful. A recently published study suggested that LTD induction requires metabotropic activation of NMDARs, and not channel opening or Ca^{2+}-influx (Nabavi *et al.*, 2013). To test for a metabotropic role for NMDARs, we induced LTD during whole-cell recordings from CA1 pyramidal neurons in the presence of 7-chlorokynurenic acid (7-CK). 7-CK is a glycine-site antagonist, which prevents NMDAR channel opening, but not binding to glutamate. Surprisingly, 7-CK did not inhibit LTD expression, but actually appeared to make it more reliable (Fig. 27a). Combining 7-CK with APV did block LTD expression, indicating that NMDAR activation is still required (Fig. 27a). 7-CK still permits binding of glutamate to the NMDAR, but prevents the channel from opening and allowing Ca^{2+} influx (Fig. 27b). This evidence implies that the NMDAR has some metabotropic role in inducing LTD.

With a more reliable means of expressing LTD, we returned to GluK1-replacement neurons. We recorded robust expression of LTD in both control and GluK1-replacement neurons (Fig. 28a), confirming that KAR receptors are sufficient. To confirm that the LTD in these experiments is indeed mediated by GluK1, we washed-in 1 μM ACET and observed potent inhibition of synaptic transmission in the GluK1-replacement neuron, with no change in the paired control cell (Fig. 28a). LTD is therefore not specific to a particular AMPAR subunit or even glutamate receptor subtype, but can be expressed with a variety of different fast, ionotropic glutamate receptors.

Discussion

Our results indicate that long-term depression does not require a specific AMPAR subunit, or even AMPARs generally. Indeed, molecular replacement with the KAR subunit GluK1 fully supported expression of LTD. However, several past studies have demonstrated that LTD expression can require specific phosphorylation sites on different AMPAR subunits. Overexpressing GluA2 with mutations to the S880 phosphorylation site (Seidenman *et al.*, 2003) or germline mutations of the S845 site on GluA1(Lee *et al.*, 2010) both impair LTD expression, yet complete genetic deletion of either subunit leaves LTD expression intact (Meng *et al.*, 2003, Fig. 25). These results are not easily reconciled, though it is possible that each subunit requires a unique mechanism to promote receptor endocytosis during LTD, disruption of which can impede LTD expression.

It is clear from these results that the presence of LTD in GluK1-replacement neurons indicates that the mechanism of LTD expression is more general than modifications to specific AMPAR subunits. Instead, LTD likely involves a broader reorganization of the synapse as a whole, or perhaps an increased rate of bulk endocytosis. Indeed, several studies have demonstrated that chemical or electrical induction of LTD both caused a decrease in the volume of existing spines, and in some cases, complete removal of dendritic spines (Zhou *et al.*, 2004; Wang *et al.*, 2007). This decrease has been directly linked to the destabilization of actin in dendritic spines (Okamoto *et al.*, 2004), which could impair the integrity of the structural scaffold that anchors AMPARs, or in the case of our experiments, KARs, in the postsynaptic density. However, this decrease in spine size can be dissociated from a decrease in the EPSC, as

observed in mice with the S845A mutation on GluA1, where the structural decrease in dendritic spine size occurs without a change in the EPSC amplitude (He *et al.*, 2011). It therefore remains unclear how synaptic transmission is maintained when dendritic spines are shrinking, and what the exact relationship is between spine size and glutamate-receptor content.

We also confirmed the findings of a previous study that demonstrated a metabotropic, as opposed to ionotropic, role for NMDAR activation in inducing LTD. Indeed, we saw robust LTD expression even when the NMDARs were not passing current. This could also explain why we found the expression of whole-cell LTD so unreliable – persistent activation of NMDARs may have been activating both the ionotropic, Ca^{2+}-dependent pathway that promotes synaptic strengthening, as well as the metabotropic pathway that promotes synaptic depression, cancelling the two phenomena out. By blocking any flow of Ca^{2+} through the NMDARs, we are therefore biasing the synapse towards depression.

A metabotropic function of NMDARs for LTD makes considerable conceptual sense. In the case of LTP, NMDARs act as simple coincidence detectors that permit the induction of Hebbian plasticity. That is, when the postsynaptic neuron is consistently firing while the presynaptic neuron is releasing glutamte, NMDARs will open, and the resulting Ca^{2+} influx will induce LTP. With a metabrotropic NMDAR function, one can now imagine NMDARs as an ideal means to induce anti-Hebbian plasticity as well. If the postsynaptic neuron is consistently silent or hyperpolarized during presynaptic glutamate release, the NMDARs will bind to glutamate, but not pass current, which can then induce synaptic depression. Separating these two functions would require that the

Ca^{2+}-induced synaptic strengthening overrides the metabotropic signal for synaptic depression, but it at least removes the necessity of a model where differing levels of Ca^{2+} result in opposite effects on regulation of synaptic strength (Malenka & Bear, 2004).

This also opens a new and potentially fruitful avenue of research into exploring NMDAR function beyond the ionotropic role they play. It has been demonstrated that NMDARs play a critical structural role in anchoring activated CaMKII to synapses following LTP expression, allowing local regulation of synaptic strength (Barria & Malinow, 2005; Halt *et al.*, 2012). We now have evidence that NMDARs may also have some metabotropic signaling mechanism based on conformational change following binding to glutamate, but what downstream effectors are involved are completely unknown. Future experiments will be required to understand how this signal is transduced, which accessory and signaling molecules are involved, and what regions of the extensive intracellular portion of the NMDAR are involved.

Figure 25. GluA1 and GluA2 constitutive knockouts demonstrate normal expression of LTD.

(a,b) Interleaved recordings of field EPSPs show normal induction of LTD in either *Gria1*$^{-/-}$ or *Gria2-/-* hippocampal slices compared to control slices. (both $p > 0.05$). Example traces show paired average field EPSPs from control (black) and experimental neurons (green). Scale bars: 10 msec, 0.5 mV. Error bars represent mean ± s.e.m.

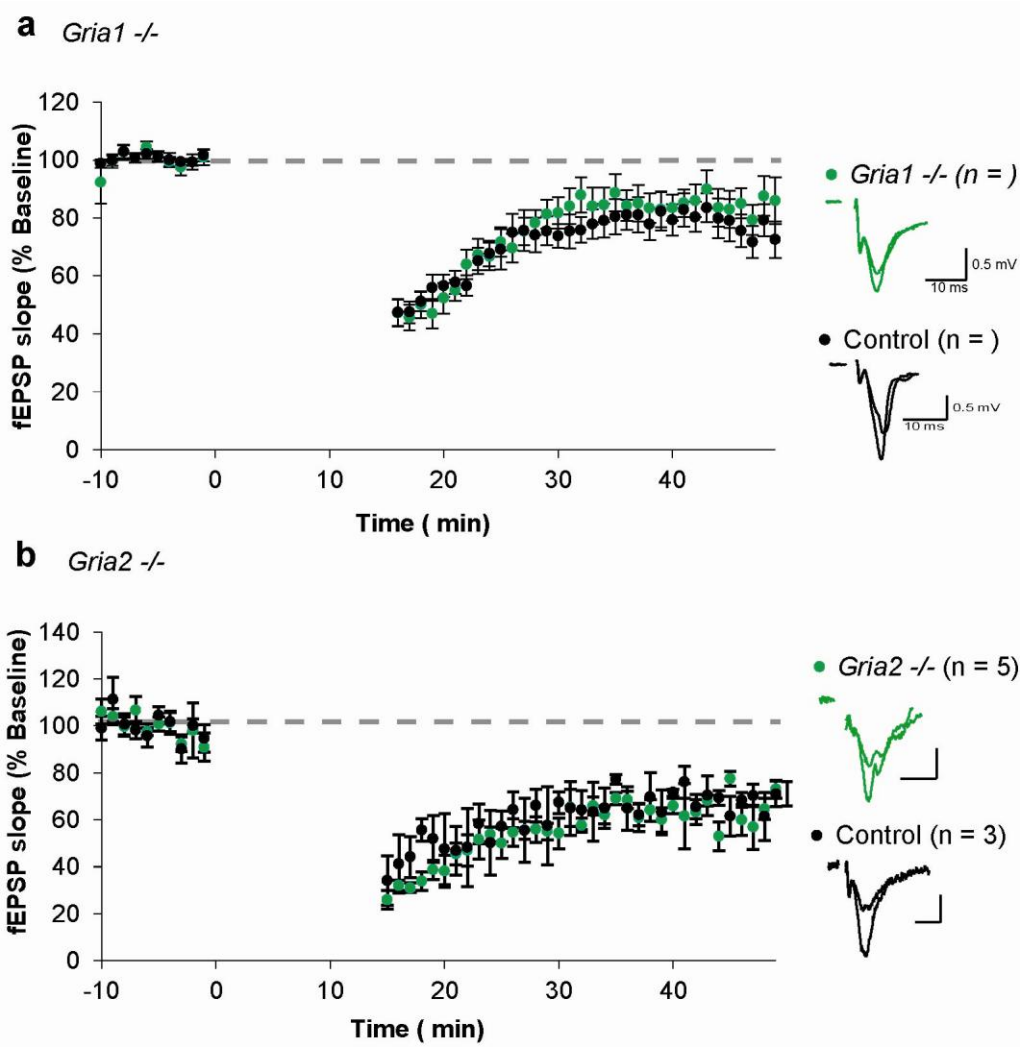

a
Gria1 -/-
fEPSP slope (% Baseline)
120
100
80
60
40
20
0
-10
0
10
20
30
40
Time (min)
Gria1 -/- (n =)
0.5 mV
10 ms
Control (n =)
0.5 mV
10 ms
b
Gria2 -/-
fEPSP slope (% Baseline)
140
120
100
80
60
40
20
0
-10
0
10
20
30
40
Time (min)
Gria2 -/- (n = 5)
Control (n = 3)

Figure 26. GluK1 replacement neurons support normal expression of LTD.

(a) Paired whole-cell recordings between Cre + GluK1, Neto2- expressing CA1 neurons and neighboring untransfected control cells in acute slices from *Gria1-3*$^{fl/fl}$ mice show comparable expression of LTD between GluK1 replacement neurons and control ($n = 4$, $p > 0.05$). LTD was induced following a minimum of 10 minutes of baseline recording by 1 Hz stimulation for 15 minutes while holding the postsynaptic cells to -40 mV. (b) LTD expression was blocked in both control neurons and paired GluK1-repalcement neurons by 100 µM APV ($n = 5$, $p > 0.05$). Example traces show averaged EPSCs from GluK1 repalcement (green) and control (black) neurons both before LTD induction (Pre) and after 15-45 minutes (Post). Scale bars: 20 msec, 100 pA. Error bars represent mean ± s.e.m.

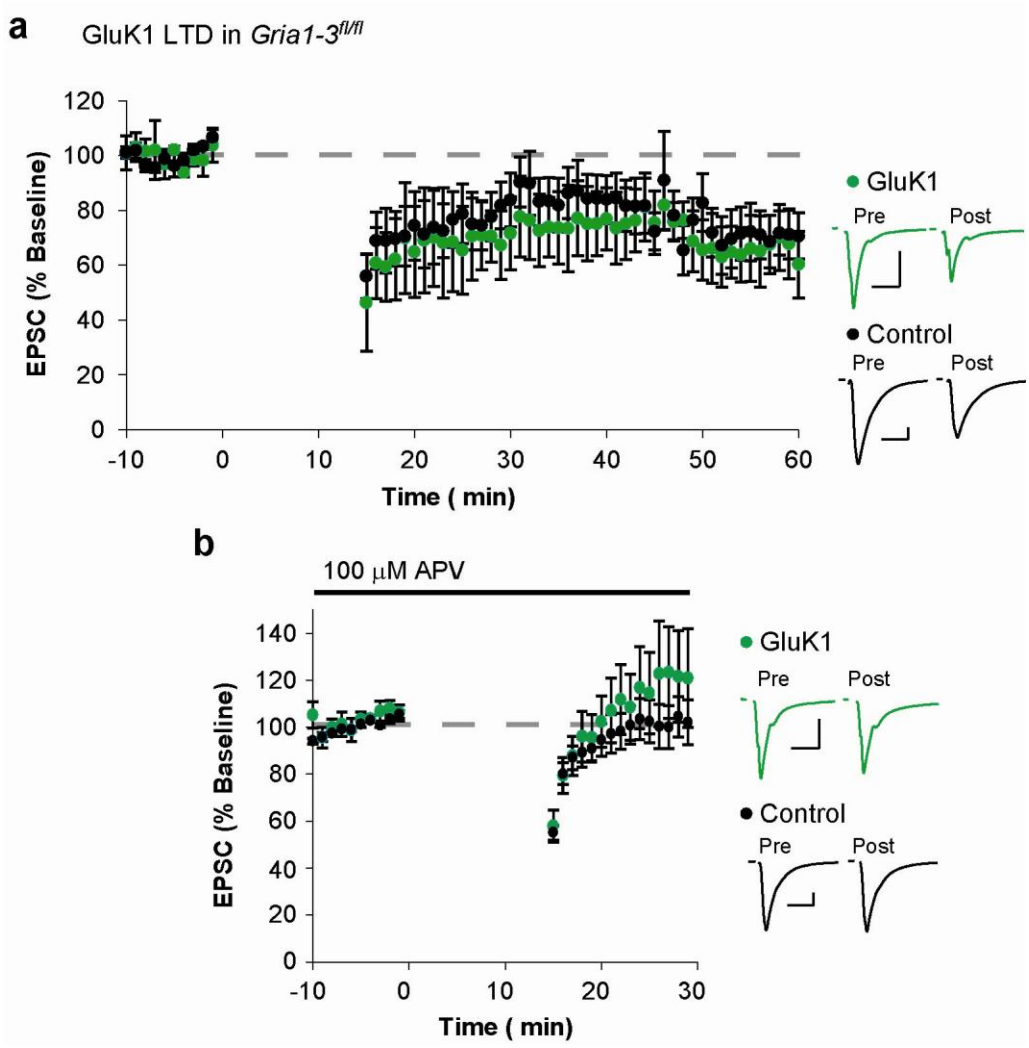

a
GluK1 LTD in Gria1-3fl/fl
EPSC (% Baseline)
Time (min)
GluK1
Pre
Post
Control
Pre
Post
b
100 μM APV
EPSC (% Baseline)
Time (min)
GluK1
Pre
Post
Control
Pre
Post

Figure 27. Metabotropic action of the NMDA receptor for LTD induction.

(a) Whole-cell LTD was induced in acute slice cultures from wild-type mice by 900 pulses of 1 Hz stimulation at -40 mV. LTD was blocked by 50 μM APV (n = 12), but not by 100 μM 7-chlorokynurenic acid (n = 14, $p = 0.048$). Example traces show average EPSC amplitude in APV-treated (blue) and 7-CK-treated (black) cells both before (Pre) and 40 minutes after (Post) induction of LTD. Scale bars: 20 msec, 100 pA. Error bars represent mean ± s.e.m.

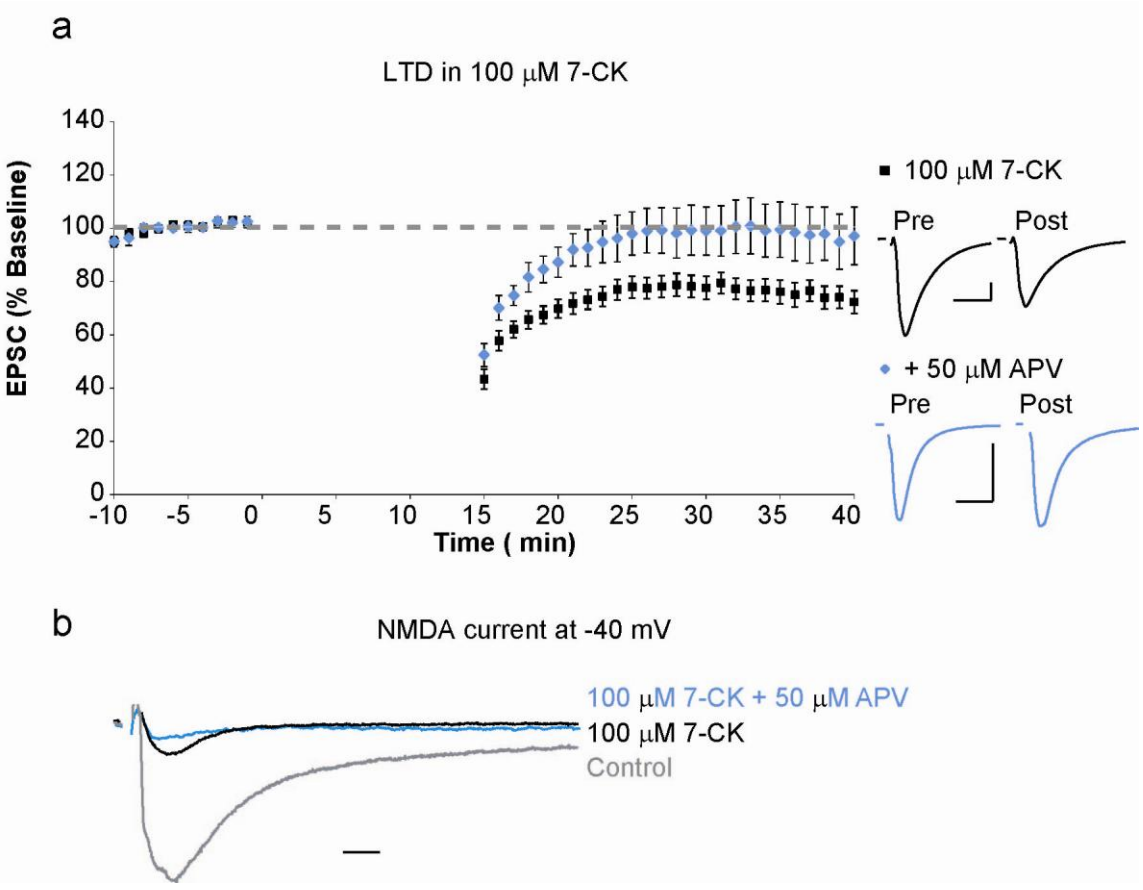
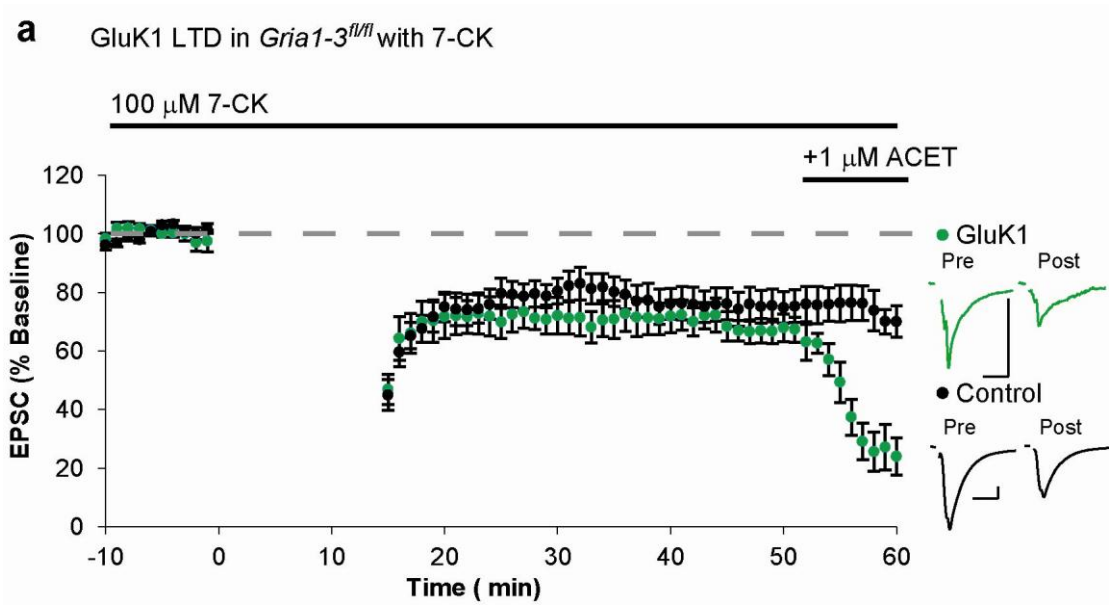

a
LTD in 100 μM 7-CK
EPSC (% Baseline)
140
120
100
80
60
40
20
0
-10
-5
0
5
10
15
20
25
30
35
40
Time (min)
100 μM 7-CK
Pre
Post
+ 50 μM APV
Pre
Post
b
NMDA current at -40 mV
100 μM 7-CK + 50 μM APV
100 μM 7-CK
Control

Figure 28. GluK1 replacement neurons support LTD in the presence of 7-CK.

(a) Paired whole-cell recordings between Cre + GluK1, Neto2- expressing CA1 neurons and neighboring untransfected control cells in acute slices from *Gria1-3*$^{fl/fl}$ mice show comparable expression of LTD between GluK1 replacement neurons and control ($n = 7$, $p > 0.05$). LTD was induced as before, now in the presence of the 100 µM 7-CK, an NMDA receptor glycine-site antagonist. Wash-in of 1 µM ACET blocked the EPSC in GluK1 replacement neurons, with no change in control ($n = 7$, $p = 0.00117$). Example traces show averaged EPSCs from GluK1 repalcement (green) and control (black) neurons both before LTD induction (Pre) and after 45 minutes (Post). Scale bars: 20 msec, 100 pA. Error bars represent mean ± s.e.m.


a
GluK1 LTD in Gria1-3fl/fl with 7-CK
100 μM 7-CK
+1 μM ACET
EPSC (% Baseline)
Time (min)
GluK1
Pre
Post
Control
Pre
Post

CHAPTER 5:
NMDA receptor subunits and LTP

Introduction

The unique biophysical properties of the NMDAR allow it to act as a simple detector of coincident activation of the presynaptic nerve terminal and postsynaptic neuron. It passes current only when it is both bound to glutamate and depolarized, which relieves block of the pore by extracellular Mg^{2+} ions. Subsequent Ca^{2+} influx through the NMDAR then acts as an intracellular signaling molecule to trigger induction of long-term potentiation. NMDARs are therefore crucial proteins involved in the induction of synaptic plasticity.

In CA1 pyramidal neurons, a functional NMDAR is comprised of two obligatory GluN1 subunits that heteromize with two GluN2 subunits, either GluN2A or GluN2B (Monyer *et al.*, 1994). These two GluN2 subunits confer different biophysical properties to the NMDAR, with the GluN2A containing receptors showing fast deactivation kinetics and relatively small total charge transfer, and GluN2B-containing receptors showing much slower deactivation and greater transfer of ions, including Ca^{2+} (Vicini *et al.*, 1998). The subunit composition of NMDARs is regulated both developmentally and by plasticity, with GluN2B-containing receptors dominating at younger ages, which are replaced by GluN2A-containing receptors during development and following induction of LTP (Sheng *et al.*, 1994; Sans *et al.*, 2000; Bellone & Nicoll, 2007). Some evidence suggests that this subunit switch may alter the ability of the synapse to undergo subsequent plasticity. GluN2B receptors bias the synapse towards induction of LTP, either because it passes a greater amount of Ca^{2+}, or because of unique interactions with its intracellular C-tail (Barria & Malinow, 2005; Gardoni *et al.*, 2009; Foster *et al.*, 2010). Our goal was to test the requirement of NMDAR subunits for LTP using conditional

knockouts for GluN2A and GluN2B. If either GluN2A or GluN2B are specifically required for LTP induction, this provides an ideal genetic background on which to perform molecular replacement experiments that could determine whether subunit-specific protein interactions or Ca^{2+} signaling alone are required.

Results

Conditional deletion of GluN2A and GluN2B

To test the requirement for different NMDAR subunits, we injected AAV-Cre:GFP into the hippocampus of *Grin2a*$^{fl/fl}$ and *Grin2b*$^{fl/fl}$ mice, respectively. After allowing 17 days for complete removal of any remaining subunits, we cut acute hippocampal slices and made paired recordings between GFP-positive, Cre-expressing CA1 neurons and neighboring untransfected controls. Conditional deletion of GluN2A results in a modest increase in average AMPA EPSC and no change in the NMDA EPSC amplitude, though our sample size is too small to draw definitive conclusions (Fig. 29a). As expected, the decay kinetics of the NMDA EPSC appeared slower in the GluN2A knockout neurons. Additionally, LTP expression was identical between control neurons and the GluN2A knockouts (Fig. 28a), indicating that GluN2A is not specifically required. This has been demonstrated previously, and given the subunit switch from GluN2B to GluN2A following LTP, is not entirely surprising.

In contrast, conditional deletions of GluN2B resulted in significantly decreased NMDAR amplitude and noticeably faster decay kinetics, with little to no change in the AMPAR EPSC (Fig. 29b). This is in contrast to previously published effects on synaptic transmission, where NMDAR deletion typically results in an increase in synaptic AMPARs (Adesnik *et al.*, 2008; Gray *et al.*, 2011; Lu *et al.*, 2011). However, this discrepancy is most likely due to the small sample size reported here, and a more thorough experiment may well confirm these previous findings. LTP expression appeared impaired in the GluN2B knockout neurons, though it did run-up to almost the same levels as control over the subsequent 20 minutes (Fig. 28b). However, these results

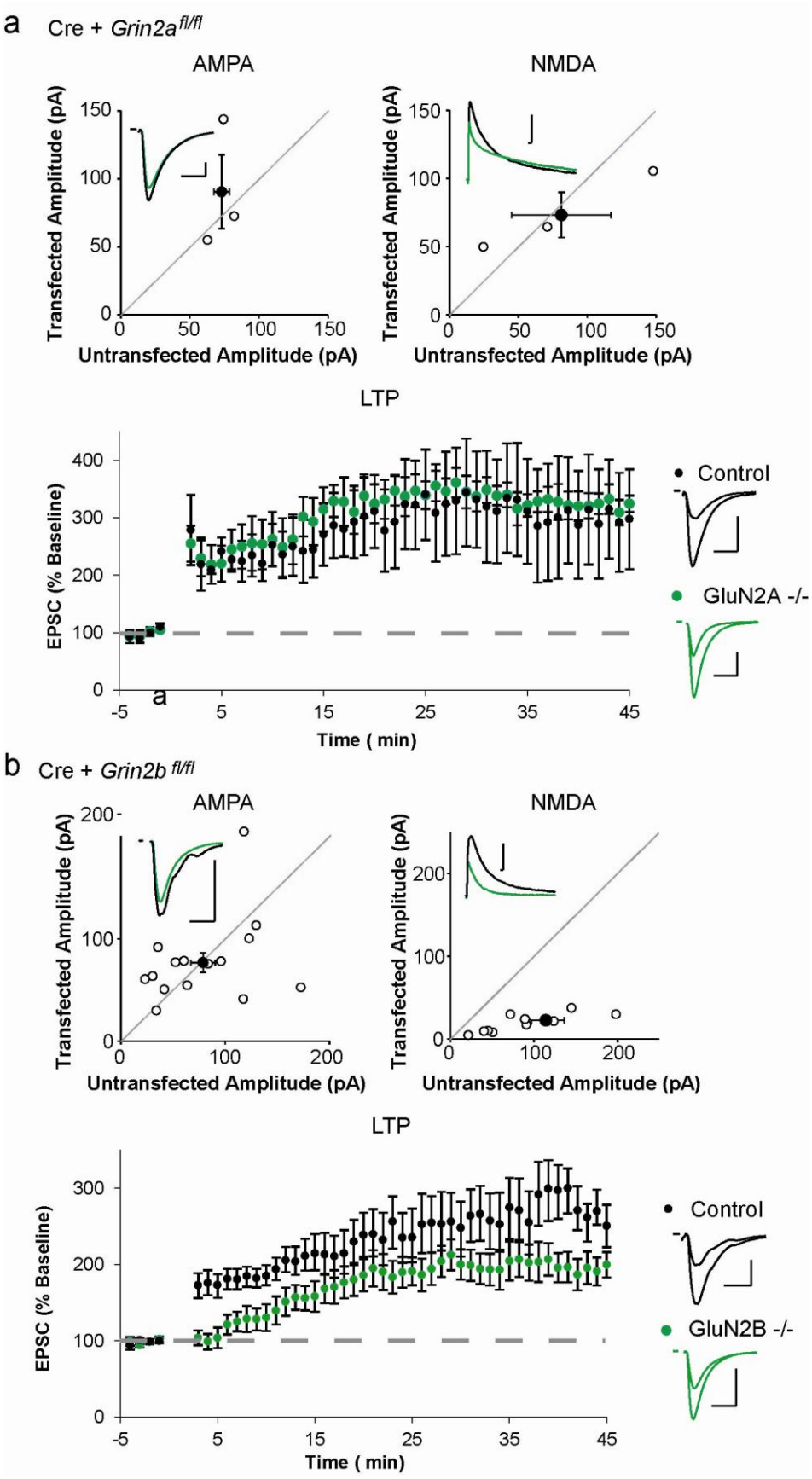
need to be interpreted with caution. The number of cells recorded from is small, and it is unclear whether there is a specific, early phenotype to LTP induction or if the GluN2B knockout experiment contained significant contamination by the run-up of EPSC amplitude, entirely separate from NMDAR dependent LTP. Further experiments are required to clarify this difference, as well as to determine if any LTP defect seen with GluN2B deletion is a specific result of lacking the GluN2B subunit, or due to the overall decreased Ca^{2+} current, which is considerable.

Discussion

Significant evidence exists in the literature for a specific requirement, or at least a bias, for GluN2B-mediated induction of LTP. Though the data presented here hints at that result, it is far from conclusive and further experiments are required. If GluN2B does impair LTP, the question remains as to whether this is due to decreased Ca^{2+} signaling or a loss of some specific protein interaction with GluN2B, such as CaMKII. This could easily be delineated by the combined use of sub-saturating levels of D-APV to mimic the decrease in NMDA-mediate Ca^{2+}-influx and molecular replacement with modified GluN2B subunits. The later technique also provides an ideal means to identify the different GluN2B-interactions that may be involved in LTP induction.

Figure 29. Conditional deletion of GluN2B impairs LTP expression.

(a) Paired whole-cell recordings from Cre-expressing and neighboring control neurons in acute hippocampal slices from *Grin2a*$^{fl/fl}$ mice show no significant change in AMPA or NMDA currents (100 msec following stimulation), though NMDA kinetics appear altered (n = 3, both $p > 0.05$). LTP expression is similar between control and cre-expressing CA1 neurons. (n = 5, $p > 0.05$). (b) AMPA EPSC amplitude is similar between control and cre-expressing CA1 neurons (n = 15, $p > 0.05$), and NMDA EPSCs are significantly decreased in cre-expressing neurons *Grin2b*$^{fl/fl}$ (n = 14, $p = 0.00024$). LTP is significantly decrease immediately following LTP induction in cre-expressing neurons compared to control (n = 6, $p = 0.0303$). Example traces show averaged EPSCS from cre-expressing (green) and control (block) neurons before and 45 minutes following induction of LTP. Scale bars: 20 msec, 100 pA. Error bars represent mean ± s.e.m.


a
Cre + Grin2a fl/fl
AMPA
NMDA
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
LTP
EPSC (% Baseline)
Time (min)
Control
GluN2A -/-
b
Cre + Grin2b fl/fl
AMPA
NMDA
Transfected Amplitude (pA)
Untransfected Amplitude (pA)
LTP
EPSC (% Baseline)
Time (min)
Control
GluN2B -/-

CHAPTER 6:
General Conclusions

LTP requires a reserve pool of glutamate receptors independent of subunit type

The results of my thesis suggest a model for synaptic plasticity that extends beyond modification to the glutamate receptors themselves to broader changes in the structure and organization of the synapse. I found that no single GluA1 C-tail interaction or AMPAR subunit was required for expression of LTP. Furthermore, I have found a remarkable and unexpected ability for LTP to be expressed with a variety of different glutamate receptor subtypes, be it the AMPAR subunits GluA1, GluA2, or the KAR subunit GluK1. Instead, the only glutamate receptor requirement for LTP expression is a large pool of extrasynaptic receptors, regardless of their subtype (Granger *et al.*, 2013). Finally, I extended these findings to LTD, showing normal LTD expression following molecular replacement with GluK1, arguing that LTD does not require a specific glutamate receptor subtype.

The fact that GluK1, a KAR, also supports LTP expression when all previous evidence suggests that AMPAR insertion was specifically required for LTP (Malinow & Malenka, 2002; Bredt & Nicoll, 2003; Nicoll, 2003; Anggono & Huganir, 2011; Nicoll & Roche, 2013) may at first glance revive the notion that LTP is expressed presynaptically. However, this would be in disagreement with the large majority of LTP research supporting a postsynaptic locus of LTP expression mediated by an increase in glutamate receptors, as discussed in the introduction. In fact, compelling evidence for postsynaptic expression of LTP can be seen in several of my own experiments, specifically the GluA1 conditional knockout and molecular replacement with GluA1ΔC and GluA2ΔC. In each of these cases, there is a fully competent population of synaptic receptors, with no change in NMDARs, so induction of LTP remains intact. If LTP was expressed

presynaptically by an increase in neurotransmitter release, that would have been detected by the existing synaptic AMPARs.

Instead, LTP expression specifically requires a large pool of extrasynaptic receptors, perhaps so that they can be readily inserted or captured by the synapse following LTP induction. This is demonstrated both by the lack of LTP in several conditions where synaptic transmission is largely normal, and by the rescue of LTP in conditions with an abnormal pool of extrasynaptic receptors, such as molecular replacement with GluA2(Q) or GluK1. Evidence supporting this model also exists throughout the literature. The GluA1 knockout mouse, the first AMPAR specific manipulation shown to lack LTP, also has a dramatic decrease in surface expression, with relatively little impairment in synaptic transmission (Zamanillo *et al.*, 1999; Andrasfalvy *et al.*, 2003). It is therefore more accurate to describe a subunit-specific role for GluA1 not for LTP, but for delivery of AMPARs to the neuronal surface. Likewise, knockout of the TARP γ-8 results in no LTP expression, despite normal NMDA-mediated EPSCs and only 30% decreased AMPAR-mediated EPSC. This is most likely because the total amount of AMPAR protein in these neurons is decreased by 90% (Rouach *et al.*, 2005). The same appears to be the case for conditional knockout of CNIH-2 and CNIH-3, where LTP expression is blocked with only a moderate decrease in baseline synaptic transmission and a dramatic decrease in surface AMPARs (Herring *et al.*, 2013).

In each or the above conditions, there is a common theme where the extrasynaptic surface receptors are significantly decreased, with little or no effect on the synaptic transmission. This suggests that AMPARs are not the limiting factor to

determining synaptic strength, but are present in excess in order to allow the cell to rapidly increase synaptic transmission on demand. A variety of manipulations show that synaptic transmission can also be dramatically increased, such as overexpression of different MAGUK proteins (Schnell *et al.*, 2002; Stein *et al.*, 2003; Elias *et al.*, 2006), or overexpression of synaptogenic proteins such as the neuroligins (Shipman *et al.*, 2011) or LRRTMs (Linhoff *et al.*, 2009). These manipulations increase the number of synapses presumably without a corresponding increase in AMPAR transcription, translation, or surface expression, though that is certainly a formal possibility. Instead, it seems that the pre-existing population of AMPARs is more than sufficient to support an overwhelming increase in total synaptic transmission.

Exocytosis and LTP

If AMPARs are not the limiting factor for synaptic transmission, this presents an interesting conundrum with respect to LTP expression and exocytosis. There are several studies demonstrating that postsynaptic exocytosis is required for LTP expression (Lledo *et al.*, 1998), and it has widely been accepted in the literature that the relevant endosomal cargo is AMPARs (Oh *et al.*, 2006; Park *et al.*, 2006; Kennedy *et al.*, 2010; Jurado *et al.*, 2013). My experiments only deal with the steady-state amount of AMPARs at the somatic surface, so I cannot extend my findings to perisynaptic AMPARs or the rate of exocytosis in dendrites. It is completely possible that the conditions under which I see decreased surface expression are the same in which the pool of endosomal receptors are depleted. Indeed, analysis of photoinactivatable AMPARs suggests that the majority of fast endosomal recycling occurs at the soma, with those receptors supplying the dendrites and synapses by lateral diffusion (Adesnik *et al.*, 2005).

If AMPARs are already present in abundance on the surface of the neuron, how would the delivery of more result in increased synaptic transmission or be necessary for this increase during LTP? One possibility is that synaptic and extrasynaptic AMPARs exist in equilibrium, such that increasing the concentration of extrasynaptic receptors will in turn drive more AMPARs into the synapse. Another possibility is that exocytosis is not required for immediate potentiation, but to replenish those receptors that diffused laterally from the perisynaptic space into the synapse. In fact, limiting exocytosis has been seen to block LTP only after the initial potentiation (Lledo *et al.*, 1998), and LTP-induced AMPAR exocytosis has been observed in spines at time points beyond the initial potentiation (Makino & Malinow, 2009). Additionally, one study analyzing the trafficking of photobleached receptors tagged to pH-sensitive GFP found only a ~10% contribution of newly exocytosed AMPARs to LTP, with the rest coming from lateral diffusion (Patterson & Yasuda, 2011). Finally, it may be the case that it is not specifically AMPARs that are being delivered to the surface, but some unknown soluble or transmembrane protein that can increase the capacity of the PSD. Given the apparent abundance of AMPARs on the surface, I support a model where the main source of AMPARs during LTP is from diffiusion along the lateral surface (Opazo & Choquet, 2011), with exocytosis necessary either to replenish the perisynaptic receptors or to deliver as yet un-identified cargo.

Re-examining subunit-specific expression of LTP

My findings are at odds with the pre-existing subunit-rules model of LTP, which posits that LTP expression depends on specific protein interactions with the GluA1 C-tail. How can the body of literature supporting this model be reconciled with my data?

The evidence for the subunit-rules model is based on three main findings: First, LTP is absent in GluA1 knockout neurons. As explained above, this can be attributed to the lack surface receptors, and is consistent with my findings. Second, LTP expression can be disrupted by manipulations involving the GluA1 C-tail. Finally, overexpressed GluA1 does not traffic to the synapse until an LTP-like stimulus is provided. These other two findings will require more in-depth discussion.

First, I would argue that the experiments showing a disruption of LTP expression by manipulating the GluA1 C-tail have been interpreted beyond what the data will support. The first such experiments relied on expression of a soluble peptide that mimics the GluA1 C-tail (Shi *et al.*, 2001; Boehm *et al.*, 2006). This resulted in fully normal LTP expression for the first 20 minutes, followed by a gradual decrease in synaptic transmission back down to pre-LTP levels. These experiments have been replicated once in behavioral experiments where this peptide blocks expression of a fear-conditioning when expressed in small subset of amygdala neurons and again by wash-in in a smaller portion of the C-tail that contains only the MPR. However, soluble peptides may be prone to exhibiting off-target effects. This is best exemplified in the LTP literature by several studies showing that a zeta inhibitory peptide (ZIP), a soluble peptide previously shown to inhibit LTP and prevent learning by blocking the constitutive activation of PKM-zeta (Ling *et al.*, 2002; Serrano *et al.*, 2005), has non-specific interactions that effect LTP expression (Lee *et al.*, 2013; Volk *et al.*, 2013). The other main manipulation of the GluA1 C-tail demonstrating impaired LTP is genetic knock-in of phosphonull mutations at S831 and S845 (Lee *et al.*, 2003). Again, the potentiation shown is largely normal, and slowly decreases back down to baseline over

the course of two hours. Follow up studies trying to separate these two phosphorylation sites found that either mutation alone had no phenotype (Lee *et al.*, 2003). In all of these experiments, only the "long-term" aspect of LTP is disrupted - the initial potentiation is largely intact. Therefore, any model that posits that the initial increase in AMPARs during LTP depends on the GluA1 C-tail is experimentally unfounded.

Basal synaptic targeting of AMPA receptors

Finally, it is important to point out the fundamental difference between my experiments and those of Malinow and colleagues that established the subunit-rules model. I observed delivery of overexpressed GluA1 to the synapse under baseline conditions, whereas they required some LTP-like stimulus to observe synaptic delivery of overexpressed GluA1. To explore this discrepancy, I attempted to mimic exactly the experimental conditions of these original studies. Only when I expressed their GFP-tagged GluA1 construct did I fail to see GluA1 delivered to the synapse. Un-tagged GluA1, of either flip or flop isoforms, readily trafficked to the synapse, even when activity was decreased by culturing in 10 mM Mg^{2+}. Though initially it seemed as if the GFP-tag might impair surface expression, it now appears that it specifically impairs synaptic targeting in a way that can be rescued by LTP or certain C-tail manipulations. Further experiments may be warranted to explain how the GFP tag is adversely effecting AMPAR trafficking. Certainly, given the bulk of evidence showing changes in synaptic delivery, the GluA1 C-tail must play some modulatory role in synaptic transmission. However, I would argue it plays no necessary role in LTP and tells us little about the fundamental mechanism of learning and memory.

I am also reluctant to conclude that there is no subunit difference with regard to synaptic trafficking. After all, molecular replacement with either GluA1 or GluA2 alone only rescued at most 75% of the AMPA-mediated EPSC while fully rescuing surface receptors. In contrast, rescue with both GluA1 and GluA2 achieves a more complete rescue (Lu *et al.*, 2011). This would suggest that they have partially non-overlapping roles in anchoring AMPARs to the synapse. Indeed, overexpression of GluK1 in wild-type neurons resulting in an increase in synaptic transmission, whereas such an increase is never seen by overexpression of GluA1 or GluA2. GluK1 must therefore occupy synapses in a way that is not limited by the number of synaptic AMPARs. This evidence does argue for some AMPAR subunit-specific, and even glutamate receptor subtype-specific synaptic interactions, perhaps the same interactions that play a modulatory role in determining synaptic strength.

AMPAR surface expression

I also found no specific role for either the GluA1 or GluA2 C-tails in mediating surface expression. Some C-tail amino acids were required, but only to provide a purely structural role, as opposed to mediating any specific protein-protein interaction. Indeed, GluA1 with a scrambled C-tail sequence, or with only a myc-tag as an artificial C-tail both expressed on the surface. However, my experiments only assayed steady-state surface expression on the soma, and previous studies have shown necessary roles for both GluA1 and GluA2 C-tail sites in the rate of exocytosis (Lin *et al.*, 2009a; Araki *et al.*, 2010).

Potential Caveats

In the time since my main findings have been published (Granger *et al.*, 2013), a couple of critiques have surfaced. One criticism of our study is that our manipulations are "too artificial," and that by completely deleting all endogenous receptors, we are likely fundamentally altering the normal signaling mechanisms used by the cell to regulate synaptic strength (Bassani *et al.*, 2013; Sheng *et al.*, 2013). We argue that our manipulations are no more artificial than over-expression on a wild-type background, and that at no time during our experiments are the synapses devoid of AMPARs. This is because the exogenous receptor is expressed many days before the loss of the endogenous receptors. In particular, we report one condition where we expressed GluA1/A2 heteromers in which the GluA1 subunit lacked its C-tail (GluA1ΔC), and saw normal expression of LTP. This condition most closely mimics the endogenous situation, where surface and synaptic transmission are dominated by GluA1/A2 heteromers (Lu *et al.*, 2009), differing only in the absence of the GluA1 C-tail.

Future Directions

Given that much of my thesis work yielded negative results, I feel compelled to offer some positive model to guide future research. The ability to image individual spines while inducing LTP has led to the discovery that synaptic potentiation is coupled with a structural increase in the size of dendritic spines (Matsuzaki *et al.*, 2004; Harvey & Svoboda, 2007; Lee *et al.*, 2009). Combined with my data, I believe this argues for a model whereby LTP causes a general increase in the size of the postsynaptic density and the number of glutamate receptor "slots" that can be filled by lateral diffusion or exocytosis. This immediately suggests two lines of research. First, it would be valuable to understand better what mediates the structural plasticity, be it increased actin

polymerization (Okamoto *et al.*, 2009) or increased cluster of PSD proteins (Kim & Sheng, 2004). Second, we need to understand better how AMPARs are anchored to the synapse, and how that changes during LTP. We already know that TARP-MAGUK interactions may play a crucial role (Schnell *et al.*, 2002). Molecular replacement of TARP-tethered AMPARs (Shi *et al.*, 2009) may provide a means of identifying this interaction more definitively and indicate how it is altered during plasticity. Previous studies have suggested that phosphorylation of the TARP itself is required for LTP (Tomita *et al.*, 2005), which may explain the lack of effect from truncating the GluA1 C-tail. Some mechanism must cluster AMPARs to the synapse, further understsanding of which will undoubtedly expand our knowledge of LTP as well.

CHAPTER 7:
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