Cell type selective expression of a slow AMPA/kainate EPSC in hippocampal interneurons

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A Dissertation

Presented to the Neuroscience Graduate Program
and the Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

December 2009

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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List of Common Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
AP2	Adaptor protein 2
ATPA	2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid
Bac	Baclofen
CA1	Cornu Ammonis1
CA3	Cornu Ammonis 3
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CNS	Central Nervous System
CTZ	Cyclothiazide
D-APV	D(-)-2-Amino-5-phosphonopentanoic acid
EAAT2	Excitatory amino acid transporter 2
EC50	half maximal excitatory concentration
eEPSC	Evoked excitatory postsynaptic current
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
GABA	γ-aminobutyric acid
γ-DGG	γ-D-Glutamylglycine
GRIP	Glutamate receptor interacting protein
GYKI	(±)-1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine
I	current
IC50	half maximal inhibitory concentration
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory postsynaptic potential
KA	Kainate
KAR	Kainate receptor
KRIP	Kainate receptor interacting protein

LTD	Long term depression
LTP	Long term potentiation
mEPSC	miniature excitatory postsynaptic current
mRNA	messenger ribonucleic acid
NBQX	2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline
NETO2	Neuropilin (NRP) and tolloid (TLL)-like 2
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NSF	n-ethylmaleimide sensitive factor
PKC	protein kinase C
P_R	Probability of release
PSD95	95 kDa postsynaptic density protein
RIL	Reversion induced LIM
SAP-90	synapse-associated protein 90
SAP97	synapse-associated protein 97
sEPSC	Spontaneous excitatory postsynaptic current
SLM	Stratum lacunosum moleculare
SNAP25	synaptosomal-associated protein 25
SO	Stratum oriens
SP	Stratum pyramidale
SR	Stratum radiatum
TARP	Transmembrane AMPAR regulatory protein
TBOA	(3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]me thoxy]-L-aspartic acid
UBP302	(S)-3-3(2-carboxybenzyl) willardiine

Acknowledgments

I would like to thank my mentor Matthew Frerking for his endless encouragement, much-needed optimism, scientific guidance, and whole-hearted support. He always managed to make frustrating and bewildering problems solvable and logical, and better yet, he gave successes and triumphs the credit and celebration they deserved. He was able to teach me how to trust the data and how to understand what it was truly saying, even when it seemed to be speaking a different language. His brilliant, analytical insight and friendly, understanding mentoring helped me through the rough patches. He always rooted for me and it was always appreciated.

I would also like to thank the members of my committee: Craig Jahr, David Rossi, Henrique von Gersdorff, John Adelman, and John Williams. Their feedback and suggestions along the way have been invaluable and critical to my progression as a scientist. I especially want to thank Craig Jahr and David Rossi for letting me join their lab meetings when I needed some extra help.

Of course I want to thank my friends and family for their love and support.

And Sean, 이 논문은 사랑하는 션 없이는 도저히 완성되지 못했을 것입니다. 비록 대학원 과정은 힘들었지만 당신이 나의 인생 안으로 들어올 수 있는 뜻 깊은 과정이였기도 합니다. 이 논문을 완성하기까지의 모든 역경을 우리가 함께 함으로써 극복 할 수 있어서 더욱 큰 의미가 느껴집니다. 나는 당신을 내가 가능하다고 믿었던 것 보다 더 사랑합니다.

Abstract

Kainate receptors (KARs) contribute to postsynaptic excitation in only a select subset of neurons. To define the parameters that specify the postsynaptic expression of KARs, I examined the contribution of KARs to EPSCs on hippocampal interneurons in area CA1. Activation of the somatodentritic KARs through bath agonist applications indicated that interneurons in stratum radiatum/lacunosum-moleculare (SR/SLM) express KARs both with and without the GluR5 subunit. However, activation of synaptic KARs through stimulus-evoked transmission indicated that only GluR5-containing KARs are targeted to the synapse.

Since I was able to pharmacologically silence the synaptic KARs, I was also able to isolate the AMPAR EPSC on these interneurons, and found that AMPARs also contribute to the slowly decaying tail current. Spontaneous EPSCs with a conventional AMPAR component did not have a resolvable contribution of KARs, suggesting that the KARs that contribute to the evoked EPSCs are at a distinct set of synapses. Similarly, since the AMPAR sEPSCs did not have a slow tail component, the AMPARs that contribute to this component of the eEPSC are either segregated to separate synapses or mediated by glutamate spillover. GluR5-containing KARs do not contribute substantially to the EPSC in stratum oriens interneurons, but are present somatodendritically. I conclude that KARs are localized to synapses by cell type-, synapse-, and subunit-selective mechanisms.

While the slow component of the AMPAR EPSC was preferentially recruited during block of glutamate transporters, it appears that the EPSC tail may actually reflect properties of the AMPARs rather than slow diffusion of glutamate out of the synapse.

Since the AMPAR tail current is not sensitive to high frequency stimulation, recording at high temperatures, reduced probability of release, or preferential block by the low affinity antagonist γ-DGG, it seems that the slow decay kinetics are not indicative of activation of extrasynaptic receptors by glutamate spillover. Surprisingly, the TBOA-recruited tail may also not reflect glutamate spillover as it was also not preferentially blocked by γ-DGG.

1. Introduction

1.1 Overview

Much of our understanding of synaptic transmission in the mammalian brain derives from research done in the hippocampus. This is largely due to its readily accessible tri-synaptic circuitry, which lends itself to easy experimental manipulations, and its behavioral and physiological relevance. Many of the fundamental aspects of glutamatergic transmission and synaptic plasticity have been studied and worked out at these synapses.

The hippocampus's involvement in temporal lobe epilepsy led to increased study of GABAergic-mediated inhibitory transmission in the circuit as well. Epileptiform activity results from an imbalance of excitation and inhibition, implicating an important role of interneurons in the etiology of the disorder. The function of interneurons and the inhibition they provide is much more intricate than a simple counterbalance for excitation. They play critical roles in the synchronization and fine-tuning of the hippocampal circuit during normal function and network processing (Freund and Buzsaki, 1996; McBain and Kauer, 2009). How they perform these tasks is not well understood, partly because hippocampal interneurons are heterogeneous, and different populations of interneurons likely have different characteristics (Freund and Buzsaki, 1996; Maccaferri and Lacaille, 2003; McBain and Fisahn, 2001). Studies of inhibition in the hippocampus have focused mainly on recording or manipulation of the inhibitory currents. Full understanding of interneurons and the roles they play will necessitate understanding what drives activation of these cells.

My dissertation research addresses this problem by studying and characterizing the excitatory synaptic transmission onto hippocampal interneurons. The glutamatergic synapses on these cells are unique in that they are known to contain kainate receptors (KARs). The roles and properties of kainate receptors are not well understood, especially compared to the other ionotropic glutamate receptors, NMDA receptors (NMDARs) and AMPA receptors (AMPARs). KARs and AMPARs are rarely differentiated because of their pharmacological similarities, yet they are known to have distinctive properties and roles in synaptic transmission. My research focuses on differentiating and classifying these glutamate receptor subtypes on CA1 hippocampal interneurons to better understand their functional significance.

This dissertation begins with a pharmacological classification of the interneuronal KARs and clarifies the subunit composition of these receptors. Starting with an investigation of SR/SLM interneurons in Chapter 3, I found that only a portion of the somatodendritic KARs contained the GluR5 subunit. However, the subpopulation of synaptic KARs selectively activated through evoked transmission was completely blocked by the GluR5 antagonist. The data also indicate that the synaptic KARs were tightly associated with the synapse and not accessed during glutamate spillover. Chapter 4 further examines these synaptic KARs and demonstrates that they are expressed in a synapse and cell-type dependent manner. KARs do not appear to contribute to synaptic transmission onto SO interneurons, though they are present on these cells. In characterizing the KAR-mediated EPSCs, I found that the SR/SLM AMPAR-mediated EPSCs have current kinetics distinct from canonical AMPAR-mediated transmission, and explored the possible mechanisms behind the observed slow decay kinetics in Chapter 5.

1.2 The Hippocampus

The hippocampus is composed of the subiculum, dentate gyrus, and cornu ammonis (CA) areas. The entorhinal cortex is the main input into the hippocampus with major contributions arising from layers II and III and minor inputs from layers IV and V. It sends projections from layer II and IV to the dentate gyrus via the perforant pathway, forming the first synapse of the tri-synaptic circuitry (Amaral and Witter, 1989; Freund and Buzsaki, 1996). The perforant path can be divided into two pathways, the lateral and medial perforant paths, depending on whether the fibers arise from the lateral or medial entorhinal cortex (Witter et al., 2000). The mossy fibers from the dentate granule cells project onto area CA3, the second synapse of the tri-synaptic circuit. Area CA3, which is extensively interconnected through recurrent connections, sends projections to ipsilateral CA1 via the Schaffer Collaterals, the final synapse of the circuit (Amaral and Witter, 1989). CA3 also sends projections to contralateral CA3 through the commissural pathway (Amaral and Witter, 1989) . Areas CA1 and CA3 receive direct projections from the entorhinal cortex; CA1 from layers III and V via the temporoammonic pathway, and area CA3 from layers II and IV via the perforant pathway (Witter et al., 2000). CA1 sends major projections to layers V and VI of the entorhinal cortex and to the subiculum, though there are also minor projections to other limbic areas, the nucleus accumbens, and the olfactory bulb (Freund and Buzsaki, 1996; Witter et al., 2000).

In addition to the entorhinal cortex and intrahippocampal projections, area CA1 also receives subcortical inputs. Thalamic inputs from the Reuniens nucleus have been shown to synapse onto CA1 interneurons (Dolleman-Van der Weel and Witter, 2000; Bokor et al., 2002; Vertes et al., 2006; Vertes et al., 2007). These projections are

possibly involved in theta rhythm generation and epilepsy (Cavdar et al., 2008), and disruption of their input has been shown to have effects on learning and memory (Davoodi et al., 2009). Another subcortical area shown to project to the hippocampus is the amygdala. While these projections are also thought to synapse onto interneurons, there is evidence that they mainly project to area CA3, with little input into area CA1 (Berretta et al., 2001; Berretta et al., 2004). The median raphe nucleus sends serotonergic and glutamatergic inputs to the area as well (Jackson et al., 2009) and has recently been shown to activate CA1 interneurons (Varga et al., 2009). Serotonergic and cholinergic projections seem to be particularly targeted to interneurons (Freund et al., 1990; Muller et al., 1992).

In cross-section, one can easily see the layers of the hippocampus, formed by the stratification of cell bodies and processes. Area CA1, the major output of the hippocampus and the focus of this dissertation, is comprised of stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), and stratum lacunosum moleculare (SLM) (Amaral and Witter, 1989; Freund and Buzsaki, 1996). Studying the circuitry of the hippocampus is simplified by the stratification and easy manipulation of the various afferent pathways. Activity-dependent long-term synaptic plasticity of hippocampal glutamatergic transmission is thought to be the major cellular correlate of learning and memory.

In addition to the major pathways described above, local inhibitory circuits also play major roles in shaping the patterns of activity in the hippocampus (Cobb et al., 1995; Buzsáki, 1997; Klausberger and Somogyi, 2008; McBain and Kauer, 2009). By adding inhibition to the circuit, these cells are critical in preventing hyperexcitability, such as seen during epileptiform activity (Freund and Buzsaki, 1996). The networks of

interneurons are also critically involved in the synchronization and precision of circuit's behavior (McBain et al., 1999; McBain and Fisahn, 2001; Pouille and Scanziani, 2004). They underlie the physiologically and behaviorally relevant oscillations of network activity (Buzsáki, 1997; Mann and Paulsen, 2007; Bartos et al., 2007).

1.3 Interneurons of the Hippocampus

The most commonly studied cells of the hippocampus are the pyramidal cells, with much less understood about interneuronal function and roles. Historically, this is due in large part to the fact that interneurons are not clustered into easily accessible layers, making them less available for field recordings and blind patch techniques. However, with technological advancements allowing for visually guided patching and recognition of their roles in the processes described above, interneurons are receiving much more attention. There are interneuron networks in the dentate gyrus, CA3 and CA1 areas (Freund and Buzsaki, 1996). While the interneurons in area CA1 are quite heterogeneous (see discussion below), for the purposes of this research they were divided into two major populations.

Interneurons are a heterogeneous group of cells, with variable ontogeny, neurochemistry, physiology, and anatomy (Freund and Buzsaki, 1996). How to parse this variability into distinct subclasses of interneurons is a subject of active debate in the field, with no clear consensus. While extensive classification systems have been formed around cellular anatomy, peptide expression, intrinsic electrophysiological properties, and connectivity, there is little correlation between such traits (Freund and Buzsaki, 1996; Parra et al., 1998; Maccaferri, 2005; McBain and Fisahn, 2001; Klausberger and Somogyi, 2008). Because this debate is ongoing and there is no clearly agreed upon

classification system or typology of these cells, I have opted to examine groups of interneurons that are located in clearly separated layers of area CA1 that receive distinct sets of inputs. My experiments examine interneurons near the alveus in SO and interneurons at the border of SR/SLM.

The majority of interneurons in SR/SLM have stellate dendritic trees that are contained mainly within these two layers (Kunkel et al., 1988; Lacaille and Schwartzkroin, 1988; Khazipov et al., 1995; Vida et al., 1998). The glutamatergic afferents formed onto these interneurons are mainly from CA3 pyramidal cells and layer III of the entorhinal cortex, with both groups of afferents being present on most SR/SLM interneurons (Kajiwara et al., 2008). Minor glutamatergic projections from the thalamus and raphe nuclei have also been reported in or near the SR/SLM border (Dolleman-Van der Weel and Witter, 2000; Somogyi et al., 2004). The axons of SR/SLM interneurons are distributed across all subfields; however, individual cells have projections that mainly overlap with Schaffer collateral afferents from CA3, temporoammonic afferents from layer III of entorhinal cortex, or both (reviewed in Freund and Buzsaki, 1996; Klausberger and Somogyi, 2008). Among the KAR subunits, SR/SLM interneurons express GluR5, GluR6, and KA2 robustly, with minimal expression of GluR7 and KA1 (Bureau et al., 1999; Lein et al., 2007).

In contrast, most SO interneurons have a dendritic organization that runs parallel to the alveus and is largely contained within SO; a major afferent projection onto these interneurons comes from CA1 pyramidal cells to form a local feedback circuit (Lacaille et al., 1987; McBain, 1994; Blasco-Ibáñez and Freund, 1995; Ali and Thomson, 1998; Ali et al., 1998). The axonal projections of SO interneurons extend through all layers of CA1, but a major subset of SO interneurons projects to SLM (O-LM interneurons) while most

of the other SO interneurons have axons that are restricted to more proximal layers and vary widely in their laminar specificity (reviewed in Maccaferri, 2005). In addition to their unusual axonal projections, O-LM interneurons can be distinguished from other common subtypes of SO interneurons on the basis of a pronounced short term facilitation of their excitatory inputs (Ali et al., 1998; Ali and Thomson, 1998; Losonczy et al., 2002; Wierenga and Wadman, 2003; Pouille and Scanziani, 2004). SO interneurons express GluR5, 6, 7 and KA2, but not KA1 (Bureau et al., 1999; Lein et al., 2007).

By only focusing my research on these two broad populations of interneurons, I do not mean to suggest that these groups cannot be further subdivided in meaningful and important ways. Indeed, evoked EPSC recordings from some SR/SLM interneurons facilitate, while others depress. Neuropeptide expression has been shown to differ between cells in both these areas, and has been linked to differences in presynaptic receptor expression (Sun and Dobrunz, 2006). The existing literature suggests several broad distinctions between these two groups of interneurons, with the caveat that none of these distinctions separates the two groups with absolute precision and sensitivity.

1.4 Glutamatergic neurotransmission

Glutamate is the most prevalent excitatory neurotransmitter in the central nervous system, accounting for the vast majority of excitatory synaptic transmission. The two major classes of glutamate receptors are metabotropic and ionotropic. Ionotropic glutamate receptors are divided into three main subtypes: AMPA receptors (AMPARs), NMDA receptors (NMDARs) and kainate receptors (KARs) (Bettler and Mulle, 1995; Lerma et al., 2001; Huettner, 2003; Mayer and Westbrook, 1987), the focus of this dissertation being AMPARs and KARs. AMPARs and KARs are often grouped

together due to the difficulty in pharmacologically isolating them (as discussed below) but are actually two separate classes of receptors. Historically there was some confusion regarding this point. KARs were first described as a separate subtype of receptors when it was discovered that kainate selectively depolarized dorsal root fibers (Davies et al., 1979; Watkins and Evans, 1981). Later binding studies with ^{3}H-labeled AMPA and kainate seemed to lend further credence to this division of receptor subtypes, as the two agonists appeared to selectively bind to different receptor populations. The discovery of domoate, which has even greater affinity for KARs than kainate, also seemed to indicate that there were separate AMPA- and KA-type recpetors (reviewed in Huettner, 2003; Lodge, 2009). Results showing rapidly desensitizing responses to AMPA and a mostly non-desensitizing response to kainate and domoate were interpreted to support the existence of two separate classes of receptors as well, as it was thought that the desensitizing responses were that of AMPARs and the non-desensitizing currents were due to KAR activation. However, later discoveries that kainate can effectively activate AMPARs, and does so with much less desensitization than glutamate or AMPA, brought uncertainty to the question of whether KARs were truly a unique subtype of receptor, or whether they were in fact AMPARs. It was not until the cloning of AMPARs and KARs that it was definitively proven that these were two separate subtypes of glutamate receptors (Lodge, 2009; Lerma et al., 2001).

AMPARs and KARs share many properties. While their subunits cannot coassemble, they do share a similar structure and channel forming properties. Each subunit has four membrane-spanning domains, with an extracellular N-terminus and intracellular C-terminus (Bettler and Mulle, 1995; Dingledine et al., 1999; Mayer, 2005). The second, pore-forming membrane domain does not actually cross the membrane, but

bends back and reenters the cytoplasmic side of the cell. A long extracellular loop (S2 domain) connects the 3rd and 4th transmembrane domains (Bettler and Mulle, 1995; Dingledine et al., 1999; Mayer, 2005; Lodge, 2009). Both channels are tetramers, formed by a dimer-dimer assembly: individual subunits form dimers via interactions of their N-terminal domains, and the subsequent dimerization of these units to form a tetramer is controlled by interactions of the membrane domains, likely through M2 (Ayalon and Stern-Bach, 2001; Greger and Esteban, 2007; Greger et al., 2007).

1.5 AMPA Receptors

AMPARs are the major contributors to glutamatergic transmission and are comprised of four subunits, GluR1-4. All AMPAR mRNAs may undergo alternative splicing in the S2 loop, leading to the flip and flop variants (Sommer et al., 1990; Partin et al., 1995; Dingledine et al., 1999; Mayer, 2005). Flip variants are dominant during pre- and early post-natal development, while flop is rare until postnatal week 8, after which it begins to appear at equal levels to flip (Greger et al., 2007). Flip undergoes slower and less complete desensitization compared to flop, and is more sensitive to cyclothiazide (Partin et al., 1995). GluR2 and GluR4 have alternative splice variants of their C-terminal domains. GluR1, 4, and the alternative splice of GluR2 have long cytoplasmic tails that are homologous whereas the dominant splice variant of GluR2, GluR3, and the alternatively spliced GluR4 have shorter homologous C-terminal domains (Malinow and Malenka, 2002). Almost all GluR2 subunits undergo a post-transcriptional modification where a glutamine is edited to an arginine (the Q/R edit) in the second transmembrane domain (Mayer, 2005; Isaac et al., 2007). The edited subunit confers

Ca^{2+} impermeability (noted by a linear current-voltage relation) and reduced single channel conductance to AMPA receptors (Isaac et al., 2007).

Protein-Protein Interactions: The N-terminal and membrane domains of the AMPAR subunits are fairly similar, while the C-terminal domains are more diverse (Mayer, 2005). The subunits interact with cytosolic proteins through these C-terminal tails, mostly through PDZ binding domains, which are well-characterized protein-protein binding motifs (Sheng and Sala, 2001). GluR1 is known to interact with SAP97, which is part of a family of proteins known to interact with NMDARs, and RIL, which may link to actin (Malinow and Malenka, 2002). GluR2, 3 and 4c, which form group II PDZ ligands, interact with PICK1 (protein interacting with C-kinase), and GluR2 interacts with GRIP (glutamate receptor-interacting protein) and the related ABP/GRIP2 (AMPAR binding protein) (Sheng and Sala, 2001; Malinow and Malenka, 2002; Isaac et al., 2007). GRIP/ABP and PICK1 bind to the same sites of the subunit, but their interactions with GluR2 can be regulated, as phosphorylation of serine 880 on GluR2 prevents interactions with GRIP/ABP but not PICK1 (Isaac et al., 2007; Malinow and Malenka, 2002). These three protein interactions may be linked to stabilization of AMPARs at the synapse, though there is also evidence that they work to keep AMPARs in intracellular pools. While we do not have a clear understanding of these proteins, it is likely that some of the confusion may rest in the possibility that these proteins have several functions in the delivery, endocytosis, and stabilization of AMPARs, and that such functions may differ in a cell-type and synapse-specific manner (Malinow and Malenka, 2002; Isaac et al., 2007).

GluR2 also interacts with NSF (NEM-Sensitive Factor) via a novel binding motif, a somewhat surprising finding, as NSF is an ATPase that mainly targets SNARE

proteins that induce exocytosis (Isaac et al., 2007). This interaction seems to play a role in AMPAR delivery to or maintenance at the synapse (Malinow and Malenka, 2002). AP2, a protein critical for clathrin-mediated endocytosis, also appears to interact with GluR2 in the same region, and may be important for both basal AMPAR function (Newpher and Ehlers, 2009) and LTD (Malinow and Malenka, 2002). There is some evidence that N-terminal interactions between GluR2 and proteins such as N-cadherin may be important for dendritic spine formation and stabilization (Saglietti et al., 2007).

AMPAR trafficking and kinetic properties are also regulated by proteins that act as auxiliary subunits. The transmembrane AMPAR regulatory proteins (TARPs), consist of γ2 (also known as stargazin, the most extensively studied isoform) and the homologous γ3, γ4, and γ8. These proteins are known to play important roles in the trafficking of AMPARs to the cell surface and synapse (Chen et al., 2003), and also affect gating and functional properties of the receptors (Cho et al., 2007; Tomita et al., 2005; Milstein et al., 2007). These proteins may also couple AMPARs to the membrane associated guanylate kinases (MAGUKs), a family of proteins that includes PSD-95, the prototypical scaffolding protein of the post synaptic density and likely play roles in clustering AMPARs at the synapse and synaptic plasticity (Elias and Nicoll, 2007). The various TARP isoforms seem to have some distinct effects on AMPAR function (Milstein et al., 2007), though there is also considerable overlap and redundancy, likely due to their critical roles in AMPAR functions (Menuz et al., 2008). Recently, a new family of auxiliary AMPAR subunits, the cornichons, was discovered through proteomics analysis that also appears to have significant effects on channel gating and surface expression (Schwenk et al., 2009).

Subunit Composition and Receptor Trafficking: The exact subunit composition of synaptic and extrasynaptic AMPARs is not completely agreed upon, though it is known that most are heteromers that contain edited GluR2. The majority of AMPARs are GluR1/2 and GluR2/3 heteromers (Wenthold et al., 1996; Lu et al., 2009; Kessels and Malinow, 2009), with GluR2/4 being important in synaptic plasticity early in development (Ritter et al., 2002; Kessels and Malinow, 2009), on interneurons (Catania et al., 1998; Geiger et al., 1995), and possibly in the cerebellum (Zhu et al., 2000). GluR1 containing AMPARs (possibly including GluR1 homomers) are thought to be concentrated in extrasynaptic pools and are recruited to the synapse during LTP in a CAMKII-dependant manner (Kessels and Malinow, 2009), though there is also evidence that GluR1 is constitutively present in large numbers at the synapse, and may be present in the majority of synaptic AMPARs as well (Lu et al., 2009; Petralia and Wenthold, 1992). Phosphorylation of AMPARs can regulate channel localization and trafficking as well as channel properties. GluR1 has four known phosphorylation sites at serine 812 (S818), S831, S845, and threonine 840 (Boehm et al., 2006). The other AMPAR subunits are also subject to phosphorylation, but have not been as intensely studied.

GluR2/3 receptors are retained at the synapse in relatively constant numbers due to their ability to passively traffic in and out of the synapse without changes in synaptic strength, possibly due to a stable number of "slots" for these receptors, and are not thought to exist in large numbers extrasynaptically (Barry and Ziff, 2002; Kessels and Malinow, 2009). Stabilization of the increased synaptic strength may happen by the eventual replacement of actively recruited GluR1/2 receptors with GluR2/3 receptors. Weakening of synaptic strength during LTD involves removal of AMPARs from the synapse, which involves phosphorylation of GluR2 at S880 and/or dephosphorylation of

GluR1 at S845 or S831 and subsequent endocytosis (Malinow and Malenka, 2002; Kessels and Malinow, 2009).

Particle tracking studies have shed light on the roles of synaptic and extrasynaptic AMPAR pools (Petrini et al., 2009). Lateral diffusion is the likely mechanism for AMPAR movement in and out of the synapse, where extrasynaptic receptors are mobile, and when they reach the synapse, enter periods of immobility or confinement (Petrini et al., 2009; Newpher and Ehlers, 2009). Endocytic zones located near the synapses recycle the receptors to the recycling endosome inside the spine and then back out on the membrane acting to corral the receptors and preventing diffusion completely away from the synapse (Newpher and Ehlers, 2009).

Ca^{2+}-permeable AMPARs: While the majority of AMPARs in the adult brain contain GluR2, there are significant amounts of Ca^{2+}-permeable AMPARs in the developing brain (Petralia et al., 1997), some adult interneurons (Geiger 1995, Isaac & McBain), and possibly even small amounts in adult principal cells (Petralia et al., 1997). These AMPARs are not as Ca^{2+}-permeable as NMDARs (Isaac et al., 2007), but do play roles in long-term plasticity (Pelkey et al, 2005, Laezza et al 1999, Isaac & McBain 2007). As GluR2-lacking AMPARs are blocked by intracellular polyamines, they show outward rectification and use-dependant relief of the block resulting in unique short-term plasticity characteristics (Isaac & McBain 2007). GluR2-lacking receptors also have faster decay kinetics and larger single-channel conductance (Geiger et al 1995, Swanson et al 1997). On CA3 interneurons, Ca^{2+}-permeable AMPARs are found to be expressed in an input-specific manner, and can be selectively activated by mossy fiber stimulation (Toth & McBain 1998). Ca^{2+}-permeable AMPARs can also be found on the SR/SLM interneurons studied in the experiments of this dissertation (Buldakova et al

2007). In these cells expression of Ca^{2+}-permeable AMPARs was not specific to entorhinal or Schaffer collateral inputs, though this does not rule out the possibility of specific expression at other inputs, such as those from subcortical areas (Varga et al 2009).

1.6 Kainate Receptors

Basic properties of kainate receptors: There are five subunits that form kainate receptors, which are divided into two families (GluR5-7 and KA1-2) based on sequence homology and agonist binding properties. GluR5-7 have lower binding affinity for kainate than the KA1-2 subunits, and are able to form homomeric and heteromeric receptors when heterologously expressed (Lerma et al., 2001; Huettner, 2003). KA1 and KA2, on the other hand, have a higher affinity for kainate but are unable to form functional channels on their own. Rather, they are integrated into the receptors when co-expressed with other subunits (Lerma et al., 2001; Huettner, 2003). As is seen in AMPARs, subunits combine in a dimer-dimer interface (Ayalon and Stern-Bach, 2001). While AMPA receptors and kainate receptors show strong structural and pharmacological homology to one another, they show only 40% sequence homology, and the subunits of these different receptors are unable to coassemble (Lerma et al., 2001). All kainate receptor subunits show different levels of expression throughout development, with a general trend of peak expression around birth (Bahn et al., 1994; Ritter et al., 2002).

The properties of KARs are known to vary considerably depending on subunit composition, sometimes in an unpredictable manner. For example, while the KA2 subunit has a higher affinity for kainate, its integration into GluR6/KA2 heteromers in

vitro can have a seemingly paradoxical effect of lowering receptor affinity for kainate compared to GluR6 homomers (Howe, 1996; Huettner, 2003). These studies also demonstrate that the subunit composition affects single channel conductance, desensitization, recovery from desensitization, and agonist binding and unbinding rates (Howe, 1996; Huettner, 2003; Swanson et al., 1996; Lerma et al., 2001). The effects of subunit composition are complicated further by the presence of multiple splice variants of the GluR5, 6, and 7 subunits. GluR5 has splice variations in both its NH2- and COOH-terminal domains. GluR5-1 contains a 15 amino acid insert in its N-terminus. GluR5-2 has 3 different splice variants in its c-terminus, GluR5-2a, b, and c (the GluR5-1 variant has the 2b c-terminal domain). The 2a C-terminus has a premature stop codon, causing a 49 amino acid shorter tail than 2b, and 2c has a 29 amino acid insert. GluR6 and GluR7 both have 2 splice variants in the C-terminal domain (Lerma, 2003; Coussen, 2009). The functional effects of these subunit isoforms are not completely known, though it has been shown that the C-terminal differences have effects on protein interactions (Coussen, 2009). There are also reports of trafficking differences between the variants, with GluR6a being preferentially trafficked to the surface compared to GluR6b or the GluR5 variants (Jaskolski et al., 2004). KARs can also undergo phosphorylation and palmitoylation of the C-terminal domains that affect receptor function (Pickering et al., 1995; Huettner, 2003).

Kainate receptor subunits GluR5 and GluR6 undergo post-transcriptional modification involving a Q/R edit of a single amino acid residue in second transmembrane domain, just as the GluR2 subunit of AMPA receptors does (Sommer et al., 1991; Bettler and Mulle, 1995; Lerma et al., 2001; Huettner, 2003). AMPA receptors containing the edited GluR2 subunit are calcium (Ca^{2+}) impermeable and show outward

rectification, and virtually all GluR2 subunits are found in the edited form (Sommer et al., 1991; Swanson et al., 1996). However, editing of the GluR5 and GluR6 subunits is less stringent and developmentally regulated, with only 50-60% and 75-95% of the mRNAs respectively being found to be edited in adult rat whole brain homogenate (Lerma et al., 2001; Egebjerg and Heinemann, 1993). Furthermore, two additional sites on the first transmembrane domain of GluR6 are subject to editing and found to regulate Ca^{2+} permeability (Kohler et al., 1993). These findings suggest the possibility of a significant Ca^{2+} permeability of some kainate receptors, which could indicate a functional role of kainate receptors in Ca^{2+} signaling.

Post-transcriptional editing has been shown to reduce single channel conductance of kainate receptors (Swanson et al., 1996) and affects the anion permeability of these channels, further affecting their functional role and differentiating them from most cation channels (Burnashev et al., 1996; Swanson et al., 1996). Chloride not only passes through KARs, but also serves as an allosteric modulator, as does Na+, where the extracellular absence of these ions causes a novel inactivated state of the receptor (Wong et al., 2006). In addition to these unique ion-mediated modulations, KAR function and trafficking are affected by more classic protein interactions as well.

Protein-Protein Interactions: Interactions with cytosolic proteins have been demonstrated for the GluR5, 6, and KA2 subunits (Collingridge and Isaac, 2003; Garcia et al., 1998). GluR5 and GluR6 are known to bind PICK1 and GRIP. While both of these proteins are known to interact with AMPARs as well, their regulatory effects appear to be distinct between the two receptors (Hirbec et al., 2003; Collingridge and Isaac, 2003; Coussen, 2009). At mossy fiber to CA3 pyramidal cell synapses, disruption

of PICK1 interactions with AMPARs either had no effect or caused run up of the AMPAR EPSC, but disruption of interactions with KARs caused significant inhibition of the KAR-mediated transmission (Collingridge and Isaac, 2003; Hirbec et al., 2003). Also, interactions of the proteins with the two receptors appears to involve distinct PDZ domains, as peptide blockers that significantly blocked GRIP-GluR2 interactions had only very weak effects on GRIP-KAR interactions yet were able to disrupt PICK1-KAR binding (Collingridge and Isaac, 2003). It has been suggested that PICK1 and GRIP target PKC to phosphorylate KARs, stabilizing the receptor at the synapse (Hirbec et al., 2003; Collingridge and Isaac, 2003). PICK1 has also been proposed to increase peak current and desensitization of GluR6 (Laezza et al., 2008). KRIP6, a member of the BTB/kelch family of proteins, appears to interact with GluR6 at the same PDZ domain as PICK1, but with opposite effects on the receptor properties (Laezza et al 2008).

Interactions of postsynaptic KA2 and SNAP25 have been proposed to affect interactions with PICK1, reducing stability at the synapse and facilitating internalization (Selak et al 2009). KAR interactions with the SAP90/PSD95 family have also been observed, with SAP90/PSD95, SAP102, and SAP97 coimmunoprecipitating with GluR6, and SAP90, SAP102 coimmunoprecipitating with KA2. SAP90 has been shown to facilitate receptor clustering and reduce desensitization of GluR6 and KA2 receptors (Garcia et al 1998). Recently, a new KAR-binding protein was found through a functional proteomics approach, NETO2, which significantly slows the decay kinetics of KARs without affecting receptor trafficking (Zhang et al 2009).

Native subunit composition of hippocampal kainate receptors: The precise subunit composition of native hippocampal kainate receptors is not known with clarity due to contrasting findings in pharmacological, knock-out, and *in situ* hybridization

studies. GluR5 is thought to be of particular importance in interneurons, as mRNA expression data suggest that these cells contain most of the GluR5 found within the hippocampus (Bureau et al., 1999; Paternain et al., 2000). These experiments also indicate that GluR6 and KA2 are the most abundant subunits of the hippocampal formation with marked expression in principle cell layers, and that GluR7 is isolated to the granule cells of the dentate gyrus and a few interneurons. In situ data also suggests some overlap in GluR5 and GluR6 expression, with some GluR5 in pyramidal cells and some GluR6 in interneurons (Paternain et al., 2000). It has been proposed through pharmacological and in situ expression studies that GluR5 is especially enriched on CA1 interneurons (Bureau et al, 1999; Cossart et al., 1998; Mulle et al., 2000; Khalilov et al., 2002), and there is also evidence that kainate receptors of interneurons are heteromers of GluR5 and GluR6 (Mulle et al., 2000; Paternain et al., 2000). It has also been reported that the somatodendritic kainate receptors of interneurons are GluR6-KA2 heteromers while GluR5 kainate receptors are only located on the presynaptic terminals (Christensen et al., 2004). This last study also reports functional compensation by other kainate receptor subunits in subunit specific knock-out mice, limiting the utility of this genetic approach to understanding the native receptor makeup. Studies attempting to determine the subunit expression of postsynaptic kainate receptors on interneurons often do so by examining the effects of agonists and antagonists on IPSCs recorded in pyramidal cells. Rather than using such indirect methods for assessing receptor expression, I recorded directly from the interneurons of interest to address the question of interneuronal kainate receptor subunit composition.

1.7 Pharmacological manipulations of AMPA/kainate receptors

Kainate receptor agonists: The characterization and study of kainate receptors was significantly hindered for many years by a lack of specific agonists and antagonists for AMPA and kainate receptors. The agonist used to discover the channel, kainate, shows greater selectivity for kainate receptors over AMPA receptors (estimated between 5 to 30 fold higher affinity, see Table 1) (Schiffer et al., 1997; Perrais et al., 2009). However, it is very limited on its own as a selective ligand, as it effectively activates AMPA receptors at high concentrations and does not cause desensitization of these receptors (Lodge, 2009; Lerma et al., 2001). Furthermore, kainate has been shown to block glutamate transporter EAAT2 (Vandenberg et al., 1995). Domoate (domoic acid) shows greater specificity than kainate for activating kainate receptors over AMPA receptors, but also elicits non-desensitizing currents at AMPA receptors (Lodge, 2009; Lerma et al., 2001; Jane et al., 2009). ATPA, a GluR5 selective agonist, is by far the most selective kainate receptor agonist: it shows 500-fold greater selectivity for GluR5-containing receptors over AMPA receptors and has no apparent affinity for GluR6 homomers (Schiffer et al., 1997; Perrais et al., 2009). However, ATPA has been reported to have a weak affinity for GluR6/KA2 heteromers (EC_{50} = 84 μM) (Alt et al., 2004). AMPA has no effect on recombinant homomeric kainate receptor assemblies, does not activate kainate receptors in cultured hippocampal neurons, and only has small effects at high concentrations in dorsal root ganglion cells and heterologously expressed GluR6/KA2 heteromers in HEK293 cells, with significant currents elicited at concentrations of 1-5mM (Alt et al., 2004; Howe 1996).

Selective AMPA receptor and kainate receptor antagonists: The major breakthrough in the pharmacological distinction between AMPA and kainate receptors

came with the development of the highly selective non-competitive AMPA receptor antagonist, GYKI 53655 (Wilding and Huettner, 1995). The IC_{50} for GYKI 53655 is approximately 1 μM, with the drug reported to show little effect on kainate receptors at concentrations as high as 100 μM (Wilding and Huettner, 1995; Wilding and Huettner, 1997). A recent report has suggested that GluR7-containing KARs may be substantially blocked by GYKI 53655 at concentrations >10 μM (Perrais et al., 2009). Until recently, kainate receptor antagonists showed little utility in studying native receptors, as they only blocked GluR5 homomers or were unable to be used at high enough concentrations to be effective in slice or in vivo preparations (Mulle et al., 2000). The development of the GluR5-selective competitive antagonist UBP302 has allowed for blocking of heteromeric and homomeric GluR5-containing kainate receptors (Dolman et al., 2005; Dolman et al., 2006). There is evidence that this drug may also effectively block GluR7 homomers at similar concentrations (see Table 1) (Perrais et al., 2009), although previous reports did not find any sensitivity these receptors to the drug (Dolman et al., 2005; Dolman et al., 2006)

Use of Pharmacology in studying kainate receptors: While the pharmacological agents available for differentiating kainate and AMPA receptors and the various kainate receptor subunits are much better and plentiful than they were just a few years ago, there are several things to be kept in mind when designing experiments and interpreting data. Since kainate and domoate are effective activators of AMPA receptors, their use should be done in the presence of AMPA receptor blocker GYKI 53655, or at very low doses to ensure specific activation of kainate receptors. The specificities and affinities discussed above are known to vary slightly depending on the system they are being studied in, the subunit compositions and combinations of the receptors being

activated/blocked, the presence of other drugs and allosteric modulators, and possibly even the splice variants of the subunits.

1.8 Kainate receptors and synaptic transmission

Presynaptic kainate receptors: Recently, most research on KARs has focused on those located presynaptically. They are thought to regulate release at many synapses throughout the brain, a somewhat novel role for an ionotropic glutamate receptor especially since there are multiple reports of them doing so by directly interacting with G-proteins (Frerking et al., 2001; Rozas et al., 2003). As they are not the focus of this dissertation, I will only briefly discuss them here. Kainate receptors were first found to be directly involved in synaptic transmission by presynaptically inhibiting transmitter release at Schaffer collaterals to CA1 synapses (Chittajallu et al., 1996). Since this initial finding, investigations on presynaptic kainate receptors of the hippocampus have studied this synapse (Vignes et al., 1998; Kamiya and Ozawa, 1998), the kainate receptor mediated depression of both glutamate (Frerking et al., 2001) and GABA release from interneurons onto CA1 pyramidal cells (Bureau et al., 1999; Cossart et al., 1998; Clarke et al., 1997; Rodriguez-Moreno et al., 1997; but see Frerking et al., 1998), and kainate receptor modulation of glutamate release at the mossy fiber CA3 pyramidal cell synapse (Schmitz et al., 2000; Schmitz et al., 2001). Kainate receptors have been shown to affect transmitter release from a number of synapses in the CNS, either facilitating or depressing release depending on the synapse studied and the degree to which kainate receptors are activated (Schmitz et al., 2001; Delaney and Jahr, 2002; Lauri et al., 2001; Schmitz et al., 2000; Contractor et al., 2000; Chergui et al., 2000; Kerchner et al., 2001; Kerchner et al., 2001). Studies have also found activation

of presynaptic kainate receptors on Schaffer collaterals increases probability of release onto somatostatin (SOM) containing CA1 stratum radiatum interneurons but not onto other CA1 interneurons (Sun and Dobrunz, 2006).

Postsynaptic kainate receptors: In addition to their presynaptic mechanisms of action, kainate receptors have been shown to contribute to excitatory postsynaptic currents (EPSCs) at a significant number of areas including the retina (DeVries, 2000; DeVries and Schwartz, 1999), the cerebellum (Renard et al., 1995), the amygdala (Li and Rogawski, 1998), the spinal cord (Li et al., 1999), thalamocortical synapse (Kidd and Isaac, 1999; Kidd and Isaac, 2001), as well as on interneurons of the hippocampus (Frerking et al., 1998; Cossart et al., 1998), and mossy fiber/CA3 pyramidal cell synapses (Vignes and Collingridge, 1997; Castillo et al., 1997). Interestingly, while CA1 pyramidal cells express functional somatodendritic kainate receptors, they appear to lack a kainate receptor mediated EPSC (Clarke et al., 1997).

Kinetics: Heterologously expressed kainate receptor-mediated currents show large peak amplitude and rapid and complete desensitization to glutamate (Lerma et al., 2001; Huettner, 2003) and therefore resemble AMPA receptor-mediated currents. However, at most of the synapses studied (the retina being a notable exception) the kainate receptor EPSCs have a much smaller peak amplitude and much slower decay kinetics (Castillo et al., 1997; Frerking et al., 1998; Kidd and Isaac, 1999; Kidd and Isaac, 2001). One possible explanation for the observed kinetics of the kainate receptor EPSC is that the receptors are located extrasynaptically and activated by "spill-out" of glutamate from the synaptic cleft, causing the glutamate transient that activates them to be slowed by diffusion and the decay time of the current to be dependent on uptake. Arguing against this idea are the findings that kainate receptor and AMPA receptor

EPSCs respond in parallel to changes in glutamate release (Frerking et al., 1998; Frerking and Ohliger-Frerking, 2002), and that the kainate receptor EPSC is unaffected by glutamate uptake inhibitors at many synapses (Kidd and Isaac, 2001; Vignes and Collingridge, 1997; Castillo et al., 1997; Bureau et al., 2000). Instead, it is thought that interactions of native kainate receptors with accessory proteins (Garcia et al., 1998) or intracellular signals (Swanson and Heinemann, 1998) alter the kinetics of the receptors.

Temporal Summation: The slow kinetics of the kainate receptor EPSC seem to make it ideally suited for temporal integration of afferent inputs. Despite a small peak amplitude, total charge transfer via kainate receptors is thought to be comparable to that of AMPA receptors, estimated to be as large as 85% of AMPA receptor-mediated charge transfer (Frerking et al., 1998), indicating that kainate receptors could potentially generate a significant depolarization during physiologically-relevant levels of activity. Indeed, a study that modeled the AMPA receptor and kainate receptor EPSCs found that kainate receptors were able to generate large tonic depolarizations in response to asynchronous firing at physiological firing rates (Frerking and Ohliger-Frerking, 2002). At these slow firing rates, the kainate receptor tonic depolarization was able to exceed that generated by AMPA receptors, whereas AMPA receptors subserved transient or phasic depolarization, indicating that the receptors transmit different information about afferent spiking.

Colocalization with AMPA receptors: Kainate receptors and AMPA receptors have been shown to be segregated at other synapses in the CNS (DeVries and Schwartz, 1999; Li et al., 1999; Kidd and Isaac, 1999). In stratum oriens interneurons in the hippocampus, a portion of mEPSCs have been reported to be mediated by both AMPA receptors and kainate receptors indicating the possibility of some colocalization

(Cossart et al., 2002), though this data was not replicated in the experiments of this dissertation (see Chapter 4). Indirectly supporting colocalization of these receptors on stratum radiatum interneurons is the finding that similar numbers of release sites contribute to the AMPA receptor EPSC and kainate receptor EPSC (Frerking et al., 1998).

1.9 Table 1: Subunit-dependent pharmacological profiles of KARs

	GluR5	GluR6	GluR7
Agonists (EC_{50})			
Domoate	0.05-.36	0.07-.12	>100
Kainate	1-10	0.6-7	>100
ATPA	0.3-20	≥80	nd
Glutamate	600	300-800	6000
Antagonists (IC_{50})			
GYKI 53655	>100	>100	30-60
UBP302	2*	>100	4, >100**

(concentrations listed in μM)

* UBP302 leads to a complete blockade of not only GluR5 homomers, but also GluR5-containing heteromers (Alt et al., 2004).

** Perrais et al., 2009 reported that GluR7 homomers were antagonized by 4 μM UBP302; GluR6/7 heteromers were much less sensitive (IC_{50} >100 μM). In marked contrast, Dolman et al. (2005, 2006) found that GluR7 homomers were largely insensitive to UBP 302, and did not bind to the receptor at concentrations exceeding 100 μM. The reason for the discrepancy between datasets is unclear and so the effect of UBP302 on GluR7 homomers remains unresolved; regardless, two factors make it unlikely that GluR7 homomers interfere with our conclusions. First, GluR7 homomers have such a low affinity for the KAR agonists that they are certainly not active in response to our exogenous agonist applications, and unlikely to contribute substantially to the EPSC; second, in situ hybridization suggests that GluR7 is minimally expressed in SR/SLM interneurons, and we were unable to demonstrate any effect of UBP302 on the EPSCs in the SO interneurons where GluR7 expression is more clearly evident.

Data in Supplemental Table 1 is summarized from {Sommer et al., 1992; Paternain et al., 1995; Wilding and Huettner, 1995; Clarke et al., 1997; Wilding and Huettner, 1997; Paternain et al., 1998; Donevan et al., 1998; Paternain et al., 2000; Brehm et al., 2003; Alt et al., 2004; More et al., 2004; Dolman et al., 2005; Dolman et al., 2006; Pe

rrais et al., 2008. (Paternain et al., 1995; Wilding and Huettner, 1995; Clarke et al., 1997; Wilding and Huettner, 1997; Paternain et al., 1998; Donevan et al., 1998; Paternain et al., 2000; Brehm et al., 2003; Alt et al., 2004; More et al., 2004; Dolman et al., 2005; Dolman et al., 2006; Perrais et al., 2009)

2. Materials and Methods

Experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at OHSU and NIH guidelines. Unless otherwise noted, experiments were conducted in 2-3 week old Sprague-Dawley rats or 2-3 week old mice. Animals were deeply anesthetized with halothane or isoflourane, the level of anesthesia was determined using toe pinch, and when it had been determined that the rat was fully anesthetized, and it no longer responded to toe pinch, it was rapidly decapitated. The hippocampi were bilaterally dissected out and sliced at 300-500μM thick using a Vibratome, in choline chloride solution (contained in mM: 110 choline chloride, 7 $MgCl_2$, 2.5 KCl, 1.25 KH_2PO_4, 0.5 $CaCl_2$, 25 $NaHCO_3$, 1.3 Na-ascorbate, and 10 glucose, saturated with 95% O_2/5% CO_2).

The slices were incubated for 30 minutes at near physiological temperature (31-35°C) and then an additional 30 minutes at room temperature in artificial cerebrospinal fluid (acsf) containing (in mM): 119 NaCl, 26.2 $NaHCO_3$, 11 glucose, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.0 NaH_2PO_4, bubbled with 95% O_2-5% CO_2, and kept in a perfusion chamber until the experiment, at which time they were transferred to a recording chamber. Unless otherwise noted, recordings were performed at room temperature with a subset done at physiological temperature (32-37°C) to ensure temperature independence of the results.

Patch electrodes were pulled from borosilicate glass to have a final resistance of 3-9 MΩ, and were filled with an internal solution adjusted to pH 7.2-7.4, 270-290 mOsm, containing (in mM) 100 CsOH, 100 gluconic acid, 2.5 CsCl, 10 TEA Cl, 5 QX314 Cl, 8 NaCl, 10 HEPES, 10 CsBAPTA, 4 Mg ATP, 0.3 Na_3GTP, 0.1 spermine. A high

concentration of CsBAPTA was used in the internal solution to block slow Ca^{+2}-gated conductances during the EPSC that might contaminate the measured tail currents. Recording solutions contained 20μM CPP or 100μM APV to block NMDA receptors, 100μM picrotoxin to block $GABA_A$ receptors, and 100μM NBQX was applied at the end of experiments to ensure that the measured EPSCs were mediated by AMPAR/KAR. In order to reduce hyperexcitability of the slice I performed a subset of experiments in a high divalent acsf solution, in which Ca^{2+} and Mg^{2+} concentrations were both raised to 4mM in order to reduce release probability. I did not note any obvious dependence of the results described here on the divalent concentration.

Recordings from interneurons were made by visual identification of these cells using infrared differential interference contrast (IR DIC) microscopy. Cells with a characteristic pyramidal shape or large dendritic branches sent out toward stratum lacunosum were avoided, as they might be displaced pyramidal cells. Interneurons of area CA1 were clustered around the border of stratum radiatum and stratum lacunosum moleculare, as is also evident from in situ data as well (Allen Brain Atlas), and were the targeted population when studying SR/SLM interneurons. Interneurons near the alveus in stratum oriens, which are also evident by visual identification and in situ data, were the target when studying SO interneurons.

Whole cell patch clamp recordings were done in voltage clamp, with the cells held at -70mV. Electrophysiological sweeps were collected at 5-10 kHz and filtered at 2 kHz. I either measured changes in holding current in response to agonist application, which activated all somatodendritic kainate receptors, or postsynaptic responses that were evoked by extracellular stimulation of afferent processes with a bipolar electrode. EPSCs were evoked once every 15 seconds, except when noted, and stimuli were

delivered either singly, or in brief trains of 2-5 stimuli at 20-100 Hz. For experiments in area CA1, area CA3 was removed by microdissection in order to prevent recurrent excitation. Experiments were only accepted if the input and series resistances remained stable (<25% change). Cells were also excluded if the observed result could be explained by an associated change in either parameter, even if the magnitude of the change was less than 25%. Series resistances were generally between 10 and 30 MΩ, and input resistances were 300-1200 MΩ. Electrophysiological recordings were obtained using an Axopatch 200B or 700A amplifier and IgorPro Software, filtered at 2 kHz and digitized at 10 kHz.

Bath application of agonists was done in voltage clamp in the presence of the sodium channel blocker tetrodotoxin (TTX; 1μM) to prevent activity-dependent release of neurotransmitters, picrotoxin (100 μM), to block $GABA_A$ receptors, and the AMPA receptor antagonist GYKI 53655 (100 μM) and the NMDAR antagonist APV (100 μM) in order to isolate kainate receptors. Rapid application of agonists was done with PV830 Pneumatic PicoPump attached to a patch pipette placed near the cell; agonist delivery was driven by brief (10-20 msec) application of pressure (5-25 psi) to the pipette. For pressure-evoked application of agonists, a high concentration of agonist in the pipette was used (100 μM), as is generally the case for this type of experiment; the concentration of agonist seen by receptors on the cell is unknown but presumably far smaller, due to dilution as the agonist diffuses through the tissue. Series and input resistances were monitored, and since in these experiments changes in input resistance were being experimentally induced, recordings were accepted based on stability before and after these changes.

Data analysis was performed using IgorPro and SigmaPlot software; sEPSCs detected and analyzed using MiniAnalysis or Neuromatica software. Our threshold for resolution of sEPSCs was routinely around 3-5 pA. Spontaneous events were then aligned by rise time and averaged. All data are presented as mean ± SEM. Data were compared using the Student's t-test when appropriate, and paired t-tests were used when making same-cell comparisons, with significance assessed as $P<0.05$. EPSC amplitudes were calculated by subtracting a baseline period preceding stimulation from a region of 5-10ms during the peak of the EPSC. Charge transfer of the EPSC was calculated as the integral of NBQX-sensitive current.

3. Subcellular kainate receptor localization on SR/SLM interneurons

3.1 Introduction

Within the hippocampus, KARs play a variety of roles and are expressed both on excitatory principal neurons and inhibitory interneurons. Interneurons limit circuit excitability, coordinate network oscillations, and mediate release of several neuropeptides (Freund and Buzsaki, 1996; McBain and Fisahn, 2001; Baraban and Tallent, 2004). It has been widely proposed that interneuronal KARs may be a key site at which interneurons can be regulated to affect circuit excitability (Frerking and Nicoll, 2000; Khalilov et al., 2002; Christensen et al., 2004), but the roles of KARs on interneurons remain unclear.

One unresolved issue surrounding the functional roles of KARs is that the kinetics of KAR EPSCs fall into two distinct categories, with some cells expressing a large and rapid KAR EPSC that is similar to the conventional AMPAR EPSC seen throughout the CNS (Cossart et al., 2002; DeVries et al., 2006; DeVries and Schwartz, 1999; Goldin et al., 2007) while others express a small and very slow KAR EPSC that lasts for more than 100 msec (Bureau et al., 2000; Castillo et al., 1997; Cossart et al., 1998; Frerking et al., 1998; Kidd and Isaac, 2001). The kinetics of the slow EPSC allow temporal summation over such a wide range that the excitation mediated by this EPSC can dominate the synaptic depolarization during asynchronous activation of afferents at modest firing rates (Frerking and Ohliger-Frerking, 2002). This lab previously reported that the KAR EPSC on SR/SLM hippocampal interneurons is small and slow (Frerking et

al., 1998), while others have found that the KAR EPSC on SO hippocampal interneurons is large and fast (Cossart et al., 2002; Goldin et al., 2007).

There are several possible causes for these observed differences in receptor kinetics, including accessory protein interactions (Zhang et al., 2009), subunit composition (Howe, 1996; Huettner, 2003; Swanson et al., 1996) and localization of the receptors relative to the synapse (Min et al., 1998), with the latter two mechanisms examined in this chapter. In heterologously expressed KARs, definitive differences have been observed in the affinity and desensitization properties depending on the subunits present, the splice variants of the subunits, and post-transcriptional editing of GluR5 and 6 (reviewed in Lerma et al., 2001). Identifying the subunit composition of KARs in situ has been quite difficult due to a scarcity of reliable subunit-specific pharmaceutical agents and antibodies. Indeed, there has been some debate regarding the subunit composition of KARs on hippocampal interneurons.

KARs are composed of 5 subunits (GluR5-7, KA1-2) (Huettner, 2003), and GluR5 is thought to be of particular importance in interneurons, as these cells express most of the GluR5 found within the hippocampus (Bureau et al., 1999; Paternain et al., 2000). KAR-mediated currents can be elicited by GluR5-selective agonists (Maingret et al., 2005) and KAR currents elicited by exogenous agonists are abolished in mice that simultaneously lack both the GluR5 and GluR6 KAR subunits (Mulle et al., 2000). These findings suggest that GluR5-containing KARs contribute to interneuronal excitation and activation. However, other studies report that KAR currents are abolished in mice lacking only GluR6 (Fisahn et al., 2004) and are resistant to GluR5 antagonists (Christensen et al., 2004); on this basis, it has been proposed that GluR5-containing KARs are excluded from the somatodendritic compartment and are instead located presynaptically, where they would regulate GABA release rather than interneuronal excitation.

The location of KARs relative to the synapse could also have profound effects on their current kinetics. Classic synaptic transmission involves activation of receptors located in the synaptic cleft with fast, concentrated glutamate transients. However, it has been shown that glutamate spillover can sometimes also activate peri- or extrasynaptic receptors (Carter and Regehr, 2000; Kullmann, 2000; Szapiro and Barbour, 2007). The kinetics of such receptor activation is shaped by the slow diffusion and smaller amounts of glutamate, leading to small amplitude, slowly decaying EPSCs, leading us to wonder if this mechanism could possibly explain the unique properties of the KAR EPSCs. However, it may not explain the diversity of all KAR-mediated EPSCs, as many are presumed to be located in the synaptic cleft (Kidd and Isaac, 2001; Frerking and Nicoll, 2000). To better define the roles of KARs in the interneuronal EPSC, I used whole-cell patch clamp recordings to directly examine and compare the properties of SR/SLM interneuronal KARs during activation by exogenous agonists and by synaptically released glutamate.

3.2 Results

3.2.1 GluR5 is present in a subset of SR/SLM interneuronal kainate receptors

To define the somatodendritic population of KARs, I examined agonist-induced currents on SR/SLM interneurons. These interneurons have been shown in previous studies to express the pore-forming subunits GluR5, GluR6, and the accessory subunit KA2 (Bureau et al., 1999; Lein et al., 2007). Changes in the holding current were measured in response to bath application of kainate receptor agonists and antagonists, with an extracellular solution containing D-APV (100 μM), picrotoxin (100 μM), and GYKI

53655 (100 μM) to block NMDA, $GABA_A$, and AMPA receptors, respectively. To activate all KAR subunits I used the nonselective agonists kainate and domoate. Low doses of domoate (50 nM) and kainate (3 μM) elicited average inward currents of 103±26 pA, n=5, and 81±14 pA, n=7 respectively, that were subsequently blocked with NBQX (100 μM), a nonselective AMPAR/KAR antagonist.

To assess the contribution of GluR5-containing KARs to agonist-evoked currents, I used the GluR5-selective antagonist UBP 302 (10 μM). I first established that this antagonist was selective for KARs, as it did not block currents induced by bath-applied AMPA (100 nM + 100 μM cyclothiazide in the absence of GYKI 53655) (2±2% block, n=6) (Fig. 1A, D); I then examined whether UBP302 blocked the currents evoked by kainate and domoate. UBP 302 partially blocked the domoate (15±9% block, n=5) and kainate (21±9%, n=7) currents, which indicates that these currents are mediated mainly by kainate receptors that lack GluR5 (Fig. 1B, D). However, it is also possible that 10μM UBP 302 is not effective at fully blocking GluR5 KARs. To address this possibility, I bath applied the KAR agonist ATPA (3 μM), which selectively activates GluR5-containing KARs at concentrations < 10 μM (Paternain et al., 2000; Alt et al., 2004). ATPA elicited KAR currents (126±42 pA, n=5), and these currents were almost completely blocked by UBP 302 (91±4% block, n=5) (Fig. 1C, D). Thus, KARs containing GluR5 are indeed present on these cells, can be recruited by exogenous agonists, and can be effectively blocked by UBP 302. Thus, while GluR5-containing KARs were present and could be selectively engaged by ATPA, the majority of the KAR current was mediated by GluR5-lacking KARs when this current was elicited by the nonselective KAR agonists domoate and kainate.

To ensure that these conclusions were not confounded by steady state activation of the receptors during the prolonged bath application of agonists, I repeated a subset of

these experiments with rapid pressure ejection of kainate (Fig. 1E). Rapid application (10-20 msec) of kainate (100 μM) elicited average inward currents of 29±7 pA (n=5) that was only modestly blocked (31±6%, n=5) by UBP 302. There was no significant difference in effectiveness of UBP302 whether the agonist was delivered by bath application or pressure ejection (P>0.4), so the data have been pooled from the two conditions (Fig 1 D).

3.2.2 GluR5-containing KARs are located at the synapse

Agonist application activates all of the KARs on the cell, precluding differentiation between postsynaptic and extrasynaptic receptors. We felt it necessary to not draw broad conclusions about KARs that contribute to interneuronal excitation based on these data as extrasynaptic pools of receptors have been shown to have distinct subunit composition (Newpher and Ehlers, 2009; Kessels and Malinow, 2009) and can serve very different functions than their synaptically located versions (Melyan et al., 2002). In order to determine whether the KARs activated through agonist application were representative of the receptor population activated during synaptic transmission, I examined the effects of UBP 302 on stimulus-evoked EPSCs (eEPSCs) recorded from SR/SLM interneurons. Dual KAR/AMPAR-mediated EPSCs recorded from these cells are biphasic, with a large peak amplitude that decays rapidly, and a small tail component that decays slowly. Work done previously by the Nicoll lab found that addition of GYKI 53655 blocked almost all of the fast peak of the biphasic EPSC; in contrast, the slow tail of the EPSC was largely, although not entirely, resistant to GYKI 53655 (Frerking et al., 1998).

To examine the effects of UBP 302 on these components of the EPSC, synaptic responses were elicited by extracellular stimulation in stratum radiatum, using either single stimuli or a brief train of 5 stimuli for better resolution of the small tail current. On average, UBP 302 blocked the peak of the eEPSC by 9±2% and the slowly decaying tail of the eEPSC by 40±5% (n=9) (Fig. 2A-B).

The observation that the tail current was only partially blocked by UBP 302 suggests that only a fraction of the receptors generating the tail current contain GluR5. We thought it likely that the UBP-insensitive tail current was mediated by KARs that lack GluR5, as these KARs generate the majority of the currents seen during domoate and kainate application. However, because AMPARs were not blocked in this experiment, it remained possible that this remaining tail current was mediated by AMPARs. To differentiate between these possibilities, I recorded pharmacologically isolated KAR eEPSCs in the presence of 50 μM GYKI 53655. Surprisingly, UBP 302 blocked the KAR eEPSC in the presence of GYKI 53655 almost entirely (peak 79±7% block, tail 79±10% block, n=8) (Fig. 2C, D), indicating that the UBP-insensitive tail current of the mixed AMPAR/KAR eEPSCs was in fact mediated by AMPARs. In fact, the fractional inhibition of UBP302 on the KAR EPSC was not significantly different from the fractional inhibition of UBP302 on currents elicited by the GluR5-selective agonist ATPA.

These results suggested that KARs at the synapse are composed mainly, if not entirely, of GluR5-containing KARs, and that the UBP302-insensitive tail current of the EPSC is in fact mediated by AMPARs. However, one potential concern with this interpretation is that a recent report has suggested that GluR7-containing KARs may be substantially blocked by GYKI 53655 at concentrations >10 μM (Perrais et al., 2009). In contrast to GluR5 and GluR6, GluR7 expression is minimal in SR/SLM interneurons, so we think it unlikely that GluR7-containing KARs are a significant factor in these cells;

however, to ensure that the UBP-insensitive component of the EPSC is not mediated by GluR7-containing KARs, I examined the effects of 2 μM GYKI 53655 on the EPSC in the presence of UBP302. This dose of GYKI 53655 is similar to the published IC_{50} of GYKI 53655 for AMPARs (1-2 μM), but is well below the IC_{50} for GluR7-containing KARs (30-60 μM;Perrais et al., 2009). I found that 2 μM GYKI 53655 blocked the UBP-insensitive EPSC by 67±5% (n=6; Figure 2E), consistent with the inhibition expected if AMPARs mediate the overwhelming majority of the UBP-insensitive EPSC. Importantly, this low dose of GYKI 53655 blocked the peak and tail of the UBP 302-insensitive EPSC equally, and had no effect on the time-course of the eEPSC (Figure 2E-F; P>0.3); this rules out the idea that the slow component of the UBP302-insensitive EPSC is selectively mediated by GluR7-containing KARs.

Thus, GluR5-containing KARs are preferentially targeted to the synapse. In contrast, GluR5-lacking KARs are largely if not entirely excluded from the synapse, even though they are the major contributor to KAR currents elicited by domoate and kainate. Additionally, these data reveal a slow tail current that is mediated by AMPARs.

3.2.3 Interneuronal kainate receptors are localized at the synapse more precisely than AMPA receptors

Studies of KARs at several synapses have suggested that the small amplitude and slow kinetics of the KAR EPSC may be due to inherent properties of these receptors (Bureau et al., 2000; Castillo et al., 1997; Kidd and Isaac, 2001; Vignes and Collingridge, 1997). However, KARs at other synapses (Cossart et al., 2002; DeVries, 2000; DeVries et al., 2006) and those that are heterologously expressed (Lerma et al., 2001; Paternain et al., 1998; Swanson and Heinemann, 1998) have large peak amplitudes and rapidly

desensitize, similar to the kinetics of AMPARs. What accounts for these striking differences in receptor kinetics remains unknown, but possibilities include post-transcriptional editing, and protein interactions. Another possibility with some functional support is that the kinetics of KARs may be slowed by the expression of the KA1 and KA2 subunits (Contractor et al., 2003; Barberis et al., 2008). The presence of a similarly slow AMPAR-mediated tail current raised the possibility that these KAR and AMPAR-mediated tail currents are due to activation of extrasynaptic receptors resulting from glutamate spillover.

As an initial test of this idea, I examined the effects of brief stimulus trains on the tail current relative to the peak current, being careful to examine both the KAR and AMPAR-mediated tail currents. If glutamate accesses extrasynaptic KARs and AMPARs through spillover out of the cleft, then the successive release events during a brief stimulus train might be expected to more effectively overwhelm uptake and lead to extrasynaptic accumulation of spillover, thereby disproportionately enhancing the slow tail currents.

I did same-cell comparisons of responses to single stimuli and those elicited by a high frequency train of 5 stimuli (Fig. 3). Due to the slow decay kinetics of these EPSCs, each successive stimulus during the train occurs before the current returns to baseline, and there is summation of the tail current. To determine whether this summation can be explained by linear summation of the EPSC in response to individual stimuli, I scaled and summated the eEPSC in response to single stimuli and compared this to the eEPSC in response to the stimulus train. The tail current in response to high frequency stimulation was not disproportionately larger than that expected based on the summation of EPSCs in response to individual stimuli (Fig. 3A). UBP 302 blocked an identical proportion of the charge transfer of the single pulse and train of 5 eEPSCs (Fig. 3B),

indicating that the KAR to AMPAR ratio remains unchanged in response to high frequency stimulation. Thus, KAR tail currents during the EPSC are not selectively affected by high-frequency activity.

One possible complication in interpreting the amount of block produced by UBP 302 on single versus high frequency eEPSCs is that the high-frequency train might activate presynaptic KARs in addition to postsynaptic KARs, as has been shown to occur in a subset of somatostatin-containing interneurons (Sun and Dobrunz, 2006). A block of presynaptic KARs could conceivably affect Pr during the train, which would alter short-term plasticity and complicate a comparison of the train-evoked EPSCs with those evoked by single stimuli. However, application of UBP 302 had no effect on short-term plasticity during the train, as assessed by measuring the ratio of peak amplitudes during the fifth and first stimuli (the p5/p1 ratio; Fig. 3C). This argues against an effect of UBP 302 on Pr.

The UBP 302-insensitive tail also showed no significant increase in response to a train of stimuli. This suggests that neither the AMPAR tail currents nor the KAR tail currents during these brief trains are due to spillover, although we cannot exclude the possibility that the increased glutamate release during a brief train is not sufficient to elicit a resolvable change in spillover relative to a single stimulus. We also note that this result is difficult to reconcile with preferential saturation of KARs. The frequency-dependent depression at these synapses implies a high initial release probability, so the train is likely to evoke repetitive activation of the same synapses. If KARs but not AMPARs were saturated by glutamate, then AMPARs would be able to respond during repetitive activation but KARs would not. This would lead to an increase in the AMPAR contribution to the charge transfer during trains relative to single stimuli, which we did not observe.

It remains possible that repetitive stimulation is not effective enough at increasing spillover to cause detectable differences in recruitment of the tail current. To address this limitation and more robustly manipulate glutamate spillover, I evoked EPSCs in the presence of TBOA (100μM), an inhibitor of glutamate transporters (Fig. 4). The dual KAR/AMPAR-mediated eEPSC showed a dramatic and selective potentiation of the slow tail component in the presence of the TBOA (Fig. 4A, C), indicating that the inhibition of glutamate uptake could lead to substantial recruitment of AMPARs/KARs due to glutamate spillover. The increase in synaptic charge transfer during TBOA application was profound (910±146% charge transfer in TBOA relative to baseline, n=11; Fig. 4A, E). However, UBP 302 had a very modest effect on this potentiated tail current, arguing that GluR5-containing KARs were not substantially recruited by TBOA (8±6% block, n=10; Fig. 4B, D). The tail current elicited by TBOA could be due to extrasynaptic AMPARs, or GluR5-lacking KARs that are not accessed by synaptic glutamate release when uptake is intact. To block AMPARs and examine the effects of spillover on KARs in isolation, I repeated the experiment in GYKI 53655; the KAR eEPSC recorded under these conditions showed no significant enhancement in response to TBOA application (104±21% charge transfer in TBOA relative to control, n=6; Fig. 4C, E). I also found that the tail current elicited by TBOA was completely blocked by UBP 302 and GYKI 53655 in combination (100±2% inhibition, n=4, Fig. 4D). The effects of TBOA were not associated with a significant change in the holding current (n=10; Fig. 4F).

These results both indicate that the receptors recruited by TBOA are AMPARs and not GluR5-lacking KARs. Thus, TBOA can lead to a large and slow tail current, indicating that it is effective in eliciting glutamate spill-over. However, the tail current recruited by this spillover is mediated by AMPARs and not KARs, indicating that KARs are localized at the synapse more precisely than AMPARs.

3.3 Discussion

3.3.1 Subunit composition regulates the synaptic localization of KARs

These results demonstrate the existence of two distinct subtypes of KARs on hippocampal SR/SLM interneurons that differ in their subunit composition. GluR5-containing KARs appear to be very precisely targeted to synapses and generate a small, slow EPSC. GluR5-lacking KARs, in contrast, are the predominant KAR subtype activated by exogenous agonists but do not respond appreciably to synaptically released glutamate—even when the spread of glutamate is enhanced by inhibiting glutamate uptake. This suggests that GluR5-lacking KARs are excluded from the area surrounding the synapse, and their function remains unclear. One possibility is that they act like the non-synaptic KARs on pyramidal cells which are located on the soma and regulate neuronal excitability via metabotropic effects on the afterhyperpolarization following spiking (Melyan et al., 2002; Fisahn et al., 2005); another is that they play a protective role by detecting ambient glutamate, driving the interneuron to fire and release GABA when extracellular glutamate rises to pathological levels during events like ischemia.

UBP302 was far more effective at inhibiting synaptic currents than it was on currents evoked by kainate or domoate, so the current evoked by non-selective agonists is dominated by GluR5-lacking KARs that are distinct from the GluR5-containing KARs at the synapse. This dominance of GluR5-lacking KARs in response to exogenous agonists compared to synaptic glutamate is unlikely to be due to different subunit-preferences for the different agonists, as none of them have a substantial preference for GluR6 over GluR5 (see Table 1). It is also unlikely that the difference stems from

activation of distinct subunits within heteromeric receptors, as UBP302 can fully antagonize currents elicited in GluR5-containing heteromers (More et al., 2004; Alt et al., 2004).

To my knowledge, these results are the first direct demonstration that native KARs are differentially incorporated into the postsynaptic site or excluded from it based on their subunit composition. Similar subunit-dependent targeting of AMPARs is the subject of intense interest as the mechanism underlying long-term plasticity (Lüscher and Frerking, 2001; Bredt and Nicoll, 2003), and it seems likely that similar mechanisms will be involved in targeting of the structurally homologous KARs. Consistent with this, the C-terminal sequences of both GluR6 and some of the splice variants of GluR5 contain PDZ-binding motifs and can interact with the PDZ domains of several scaffolding proteins (Garcia et al., 1998; Hirbec et al., 2003). However, it should be noted that most of our current understanding of AMPAR trafficking is based on results from pyramidal neurons; it remains to be seen whether interneurons, which target a distinct set of AMPARs to the synapse (Geiger et al., 1995), use the same mechanisms. Further elucidation of the mechanisms underlying AMPAR/KAR localization in interneurons will be of interest.

3.3.2 Glutamate spillover recruits AMPARs but not KARs

In previous studies, Dr. Frerking noted that the EPSC on SR/SLM interneurons was biphasic and proposed that the slow component was mediated by KARs (Frerking et al., 1998). In those studies, he found that a substantial fraction of the slow EPSC was blocked by GYKI 53655, but he thought it likely at that time that this was due to a non-selective partial blockade of KARs by GYKI 53655. However, my data with UBP 302

renders this explanation unlikely, and instead supports the surprising conclusion that a substantial fraction of the slow component of the EPSC is mediated by AMPARs. The fact that both AMPARs and KARs can support a slow tail current lasting for hundreds of milliseconds led us to carefully evaluate the possibility that the EPSC is generated by glutamate spillover onto extrasynaptic receptors. Attempts to elicit spillover by blocking uptake did not enhance the KAR EPSC. For AMPARs, a large spillover-mediated EPSC was recruited by inhibiting glutamate uptake, but not by comparing brief stimulus trains to individual stimuli.

The finding that glutamate transporters profoundly limit the activation of AMPARs but not KARs during synaptic glutamate release is surprising, as it suggests that KARs are located opposite the glutamate release site more precisely than AMPARs. Thus, the slow kinetics of the KAR EPSC are unlikely to reflect glutamate spillover, and likely reflect intrinsic receptor properties. It remains unclear why the kinetics of synaptic KARs on SR/SLM interneurons are so distinct from KARs in heterologous systems or even from synaptic KARs in other systems; possible explanations include effects based on the subunit composition of the receptor (Contractor et al., 2000), interactions with accessory proteins (Garcia et al., 1998), or possibly even cytosolic messengers, as the decay kinetics of KAR-mediated currents have been found to shift during long recordings, perhaps due to dialysis of the intracellular solutions into the recording pipette (Swanson and Heinemann, 1998).

The results with TBOA clearly indicate that a slow AMPAR EPSC can be recruited when glutamate uptake is blocked. This effect is useful in the current context as a control, because it seems to indicate that TBOA can elicit spillover even though it has no significant effect on the KAR EPSC. More generally, however, the effect of TBOA on AMPARs at this synapse is massive in comparison to other effects attributed to spillover

(Arnth-Jensen et al., 2002; Takayasu et al., 2004), and is very surprising when one considers that AMPARs are very poorly suited to detect spillover as they have a low affinity for glutamate and rapidly desensitize. Nevertheless, detection of spillover with AMPARs is not unheard of (DiGregorio et al., 2002; DeVries et al., 2006; Szapiro and Barbour, 2007). This finding lends some credence to the idea that the slow AMPAR component of the tail current during the EPSC might be mediated by spillover, even during single stimuli under normal conditions. It might be imagined, for example, that the synchronous activation of multiple fibers by extracellular stimulation could drive glutamate release from adjacent synapses and overwhelm uptake to recruit the slow AMPAR EPSC. However, it cannot be ruled out that this slow AMPAR current is mediated by inherent properties of the receptors or accessory protein interactions, as we feel is the case with KARs. These theories are explored experimentally in greater detail in Chapter 5.

3.4 Figures and figure legends

3.4.1 Figure 1

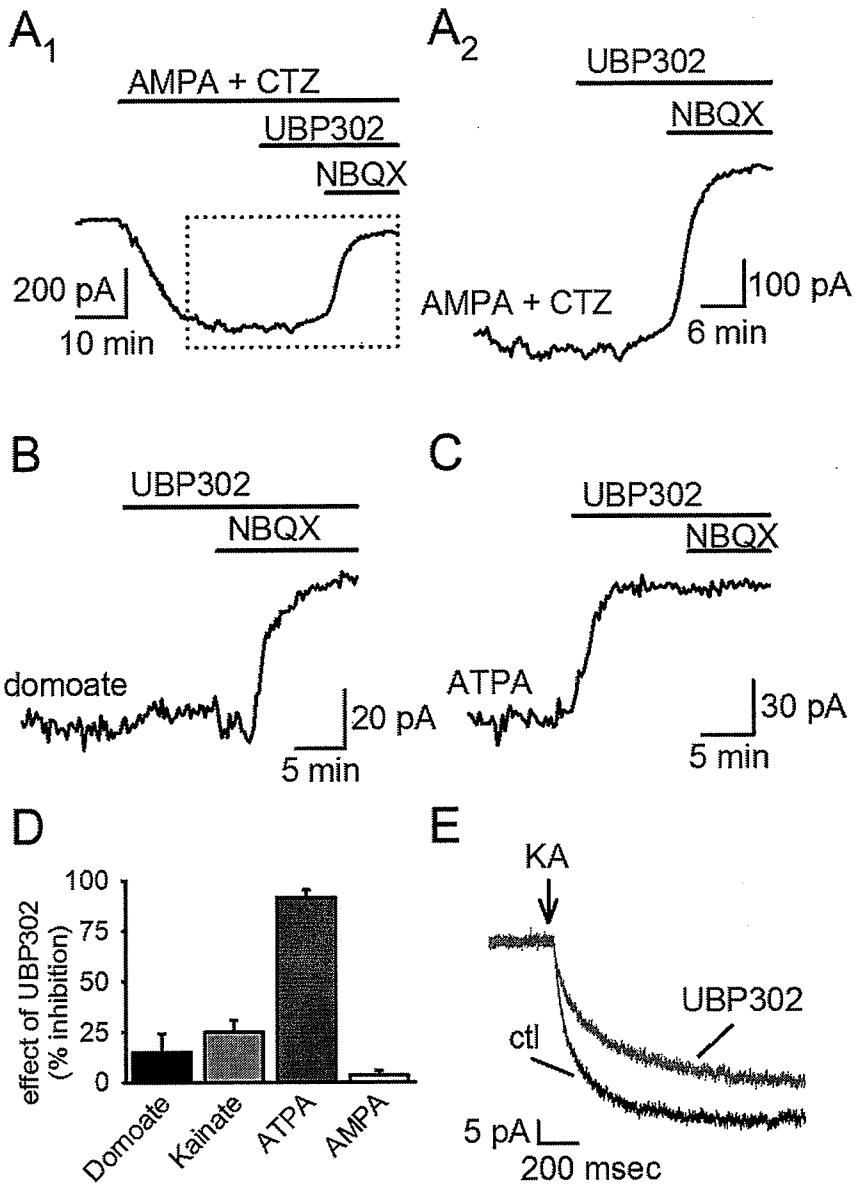

Figure 1: GluR5 is present in a subset of interneuronal kainate receptors. Effects of UBP 302 on agonist-induced currents were examined. UBP 302 had no effect on AMPA-induced currents shown over the entire application of AMPA (A_1) and at higher

temporal resolution around the antagonist application (A_2). (B) The nonselective KAR agonist domoate induced an inward current that was largely insensitive to the GluR5-selective antagonist UBP 302. (C) ATPA, an agonist that selectively activates GluR5-containing KARs, evoked an inward current that was completely blocked by UBP 302. (D) A summary of the effect of UBP 302 on various agonist induced currents. (E) Effects of UBP 302 on agonist-induced currents were also examined using rapid application methods. A brief puff (10-20 msec) of 100 μM kainate was applied to the cell and induced an inward current in the absence (black) and presence (gray) of UBP 302.

3.4.2 Figure 2

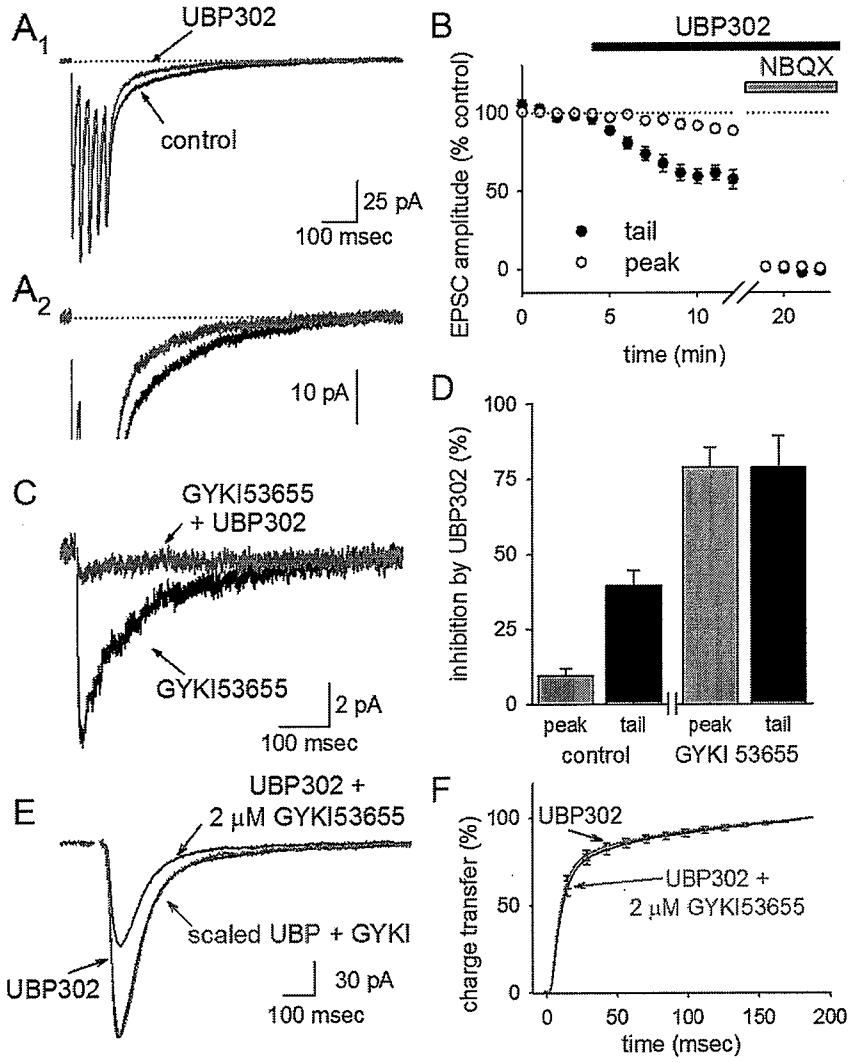

Figure 2: GluR5-containing KARs are selectively activated in response to synaptic glutamate. (A) EPSCs elicited by a brief train of 5 stimuli were recorded from a SR/SLM interneuron, as shown at a low (A_1) and a high (A_2) gain. Traces were averaged from 5-25 sweeps in control conditions, and again after bath application of 10 μM UBP 302. Here and throughout the figures, averaged traces recorded in NBQX have been subtracted from the data, and the stimulus artifact has been removed for clarity. (B) The peak of the EPSC is largely unaffected by UBP 302 and the late, slow

component of the tail is significantly, but incompletely, blocked by UBP 302. (C) The averaged KAR-mediated EPSC recorded in the presence of GYKI 53655 is almost completely blocked by UBP302. (D) A summary of the amount of inhibition elicited by UBP 302 in the absence and presence of GYKI 53655 is shown. (E) The UBP302-insensitive component of the eEPSCs (recorded in the presence of 10 μM UBP 302) was partially blocked by 2 μM GYKI 53655. The kinetics of the partially blocked eEPSC were no different from that of the unblocked eEPSC, as shown by scaling the trace in 2 μM GYKI 53655 (gray trace) to the same peak amplitude as the EPSC in the absence of GYKI 53655. (F) The same finding was observed in 6 cells, as displayed by a comparison of the cumulative charge transfer of the averaged EPSCs in both conditions. Cumulative charge transfers under each condition were normalized to their own maximum value, to facilitate a direct comparison of the kinetics of the EPSCs in each condition.

3.4.3 Figure 3

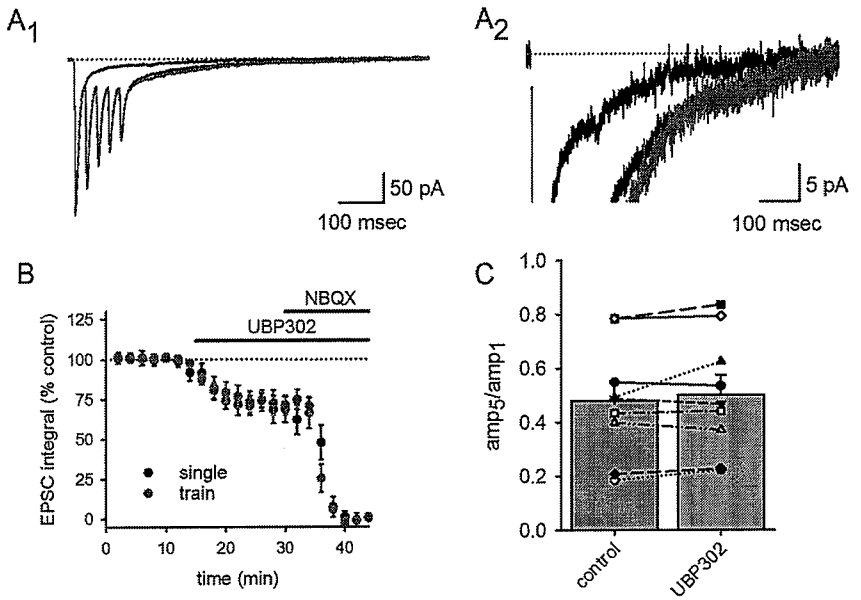

Figure 3. The contribution of KARs to the EPSC is unchanged in response to high frequency stimulation. High frequency stimulation was compared to single pulse stimulation in the same cell (A_1 low gain, A_2 high gain) to assess whether the tail component of the eEPSC was enhanced during bursts of activity. eEPSCs in response to single stimuli were scaled and summated (gray trace) and compared to eEPSCs recorded during high frequency stimulation (black trace). (B) Increasing stimulus frequency did not affect the relative contribution of KARs and AMPARs to charge transfer during the evoked EPSC. (C) Application of UBP 302 had no effect on the ratio of the fifth EPSC relative to the first EPSC during the train of EPSCs, indicating that UBP 302 did not affect short-term plasticity during the train.

3.4.4 Figure 4

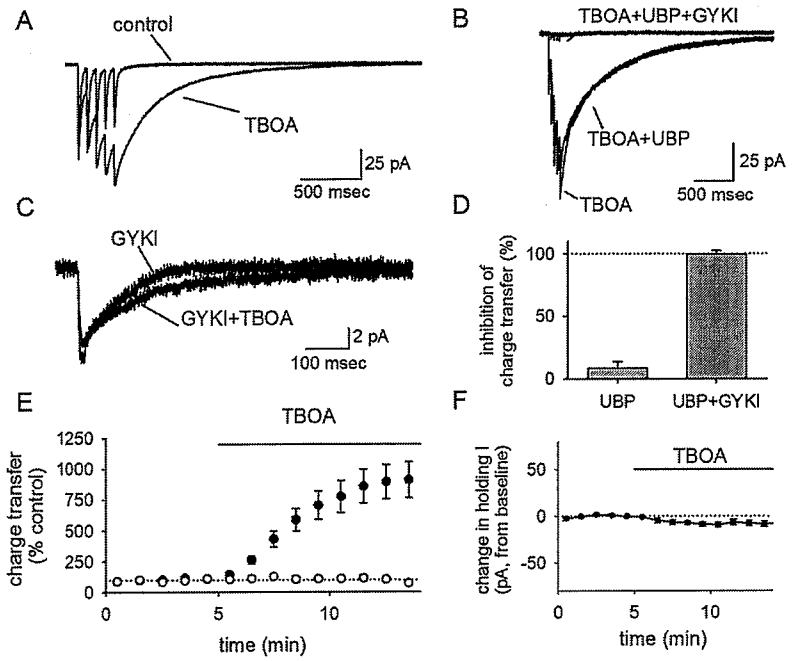

Figure 4. Interneuronal KARs are not accessed by glutamate spill-over. (A) The averaged EPSCs evoked before and after TBOA application are shown for a representative experiment. The tail current is dramatically potentiated by TBOA. (B) UBP 302 had a minimal effect on the TBOA-potentiated tail current, indicating that it is not mediated by GluR5-containing KARs. (C) TBOA had no significant effect on EPSCs evoked in the presence of GYKI 53655, indicating that the large TBOA-induced tail current seen in (A) is not mediated by KARs, but by AMPARs. (D) UBP 302 has little effect on the charge transfer in the presence of TBOA, but UBP 302 and GYKI 53655 together block the charge transfer entirely. (E) The TBOA-induced potentiation of charge transfer seen in the absence of GYKI 53655 (filled symbols) is not seen in the presence of GYKI 53655 (open symbols). (F) TBOA had no significant effect on the holding current.

4. Kainate receptor expression is input- and cell type-specific

4.1 Introduction

KARs have multiple roles in transmitting and regulating information both within the hippocampus and throughout the nervous system. Within the hippocampus, KARs are located on multiple cell types, pre-, post-, and extra-synaptically. Recently, much effort has been directed towards the study of the presynaptic KARs which are thought to regulate release of both glutamate (Chittajallu et al., 1996; Vignes et al., 1998; Kamiya and Ozawa, 1998; Frerking et al., 2001) and GABA (Bureau et al., 1999; Cossart et al., 1998; Frerking et al., 1998; Clarke et al., 1997; Rodriguez-Moreno et al., 1997). The extrasynaptic KARs on CA1 (Melyan et al., 2002) and CA3 (Fisahn et al., 2005) pyramidal cells are thought to affect excitability by regulation of the I_{AHP}. With both the extrasynaptic and some of the presynaptic KARs, there is some evidence to suggest that these receptors exert their effects through metabotropic mechanisms, with some even postulating through direct interactions of the receptors and G-proteins (Frerking et al., 2001; Rodriguez-Moreno and Lerma, 1998; Rozas et al., 2003).

However, KARs also contribute to synaptic transmission post-synaptically (DeVries and Schwartz, 1999; Kidd and Isaac, 1999; Renard et al., 1995; Frerking et al., 1998; Cossart et al., 1998; Vignes and Collingridge, 1997; Castillo et al., 1997), a more conventional role for an ionotropic receptor, though their properties at these synapses may suggest somewhat unconventional roles in information processing. Within the hippocampus, they are known to contribute to excitatory transmission at mossy fiber to CA3 pyramidal cell synapses (Vignes and Collingridge, 1997; Castillo et al., 1997) and at

excitatory inputs onto CA1 interneurons (Frerking et al., 1998; Cossart et al., 1998). Outside the hippocampus, KARs are known to contribute to synaptic transmission at thalamocortical synapses (Kidd and Isaac, 1999), the amygdala (Li et al., 1998), and the spinal cord (Li et al., 1999). At many of these synapses KAR-mediated EPSCs are known to have small peak amplitudes and slow kinetics, though at other synapses their properties mirror those of AMPARs (DeVries and Schwartz, 1999; DeVries, 2000).

In most regions where synaptic KARs are known to contribute to transmission, AMPARs are also found, with the notable exception of the spinal cord. A key question has been whether these two receptors are colocalized or whether they are segregated to separate individual synapses. They are known to segregated to separate synapses in the retina and cortex. At the cone to off-bipolar cell synapses of the retina KARs encode separate channels of information from AMPARs (DeVries, 2000), while at thalamo-cortical synapses the switch from KAR to AMPAR marks an activity and developmental-dependent shift (Kidd and Isaac, 1999). Colocalization of KARs and AMPARs with strikingly different kinetics could suggest that they encode different information from the same sources of input.

Data from the previous chapter indicate that GluR5- containing KARs are located precisely at the synapse, where they contribute a small amplitude and slowly decaying excitatory current on CA1 SR/SLM interneurons. The role of such small and slow currents is not immediately obvious as interneurons are known for their temporal precision and synchrony (Pouille and Scanziani, 2001; Assisi et al., 2007; Bartos et al., 2007; Mann and Paulsen, 2007). An important step in understanding their purpose is to understand the source of inputs driving the excitation of these receptors. Are they colocalized with AMPARs or segregated to their own synapses? Here I look at this issue

and expand my examination of hippocampal interneurons to those located in stratum oriens to address whether the role of KARs is consistent across interneurons.

4.2 Results

4.2.1 Kainate receptors are segregated from AMPARs in at least a subset of synapses

My results described in the previous chapter show that KARs are not recruited by glutamate spillover, even when spillover is substantially enhanced by blocking glutamate uptake. Thus, KARs appear to be located directly at the synapse. These experiments examined the synaptic receptors through stimulus evoked transmission, which caused synchronous activation of multiple afferent inputs onto the cell. This technique precludes differentiation between colocalization and segregation of the AMPA and kainate type receptors at individual synapses. I therefore examined spontaneous EPSCs (sEPSCs), which are not subject to this limitation. sEPSCs are events that occur without a stimulus to evoke them and are a combination of action potential-independent events (mEPSCs) and spontaneous activity-mediated events.

Colocalization of AMPARs and KARs should lead to biphasic sEPSCs, with an AMPAR-mediated peak, and a tail mediated by KARs and AMPARs, while segregation of the two receptors should lead to separate fast and slow sEPSCs (Fig. 5). One would not necessarily expect to resolve the tail currents of individual sEPSCs, which would be very small in the presence of substantial baseline noise, but the signal-to-noise resolution can be improved several-fold by averaging together many sEPSCs (>50) and examining the kinetics of the averaged sEPSC. A slow tail current was not discernable in

the average sEPSCs, nor was there a significant residual transfer of charge 100 msec following the peak of the sEPSC. There was also no effect of UBP302 on the sEPSC during the region where a tail might be expected 100 msec following the peak (Fig. 6A-B, n=10). To ensure that the absence of a tail current in the sEPSC was not being artificially depressed by 4 mM Ca^{2+}/4 mM Mg^{2+} external solution used in these experiments, I repeated these experiments in another set of cells with 2.5 mM Ca^{2+}/1.3 mM Mg^{2+} (n=9). I observed identical results in both cases, and therefore pooled the data together.

To assess our limits of resolution for the kinetics of the averaged sEPSCs, I examined the cumulative charge transfer over the course of the averaged sEPSC and compared it to the cumulative charge transfer during the eEPSC. The charge transfer during averaged sEPSCs was obviously complete in a much shorter period of time than eEPSCs and the two charge transfer curves were significantly different (Fig. 6B, $P<0.001$), indicating that we should have been able to resolve a tail current in the averaged sEPSCs if it were there to a comparable degree as it is seen in the eEPSCs. The residual charge transfer 100 msec after the eEPSC onset was 24±4% (n=10), while the residual charge transfer 100 msec after the sEPSC onset was negligible (-1±2%; n=19). Of course, not all of the tail of the eEPSC is mediated by KARs. Given the 40±5% inhibition of the tail by UBP302, we calculate that the residual KAR-mediated charge transfer 100 msec after the onset of the eEPSC is 10±2%; this is still clearly much larger than the entire charge transfer during a comparable time window following the sEPSC ($P<0.002$). Thus, the KARs activated during the eEPSC cannot be explained by KAR colocalization with AMPARs at the synapses that generate conventional AMPAR sEPSCs.

This led us to the prediction that there should be a separate population of synapses with a smaller contribution of AMPARs but a larger contribution of KARs. However, we would not expect to be able to resolve them, as the peak amplitude of the KAR eEPSC relative to the AMPAR eEPSCs (see Figure 2) would predict an average KAR sEPSC with <1pA peak amplitude and very slow decay kinetics. This is well below our limit of resolution for individual events and contrasts markedly to previous reports (Cossart et al., 2002; Goldin et al., 2007), which have found that KARs on interneurons in SO generate miniature EPSCs (mEPSCs) that are large, rapid, and readily detectable; these KAR mEPSCs are similar to AMPAR-mediated currents but persist when AMPARs are blocked. To see if I could similarly resolve large and fast KAR sEPSCs in SR/SLM interneurons, I first examined whether UBP 302 had any effect on the frequency or peak amplitude of sEPSCs. I was unable to detect an effect of UBP 302 on either parameter (Fig. 6C; amplitude: 6±9% inhibition; frequency: 8±11% inhibition; n=10). I next examined the effects of GYKI 53655 on sEPSCs recorded from SR/SLM interneurons, to see if I could resolve any events that were resistant to GYKI 53655. Addition of GYKI 53655 blocked all detectable events (Fig. 6C, D; n=7), consistent with our initial expectations based on observations of the eEPSC. These results argue that AMPARs and KARs are not colocalized at the same synapses, but are instead segregated to separate synapses. However, this data does not rule out the possibility that AMPARs and KARs are colocalized at small numbers of synapses or at synapses with very low probability of release, and therefore did not contribute to the sEPSCs at great enough levels to be detected in the average.

4.2.2 Attempts to measure kainate receptor-mediated sEPSCs

There are several methods to study sEPSCs, and since I felt it necessary to try and study KAR-mediated events on their own, I attempted to use many of them. One method I used was application of α-latrotoxin. This toxin from the black widow spider is known to cause activity-independent release of vesicles and has been used to study exocytosis (Silva et al., 2009; Ushkaryov et al., 2008). One way in which this toxin induces vesicle release is through the formation of cation-permeable pores in the cell membrane through homotetrameric assemblies, causing depolarization and calcium entry (Henkel and Sankaranarayanan, 1999; Silva et al., 2009). The toxin must bind to a receptor to exert an effect; known receptors for the toxin are neurexin Iα, latrophilin 1, and receptor-like protein tyrosine phosphatase σ (Ushkaryov et al., 2008). Binding to the receptor causes both pore-formation and separate receptor-mediated effects. These subsequent receptor mediated actions are receptor-specific and can be calcium-dependant (modulation of Ca channels, release of Ca stores) (Ushkaryov et al., 2008; Deák et al., 2009) or calcium-independent (modulation of K channels) (Capogna et al., 1996; Silva et al., 2009).

I attempted to use this toxin to study KAR mediated events, as they were undetectable studying standard sEPSCs. I first assessed the toxin's effects by monitoring AMPAR-mediated events. I was able to obtain toxin-mediated sEPSCs, however individual events were not readily distinguished from the noise. Titrating the concentration of the toxin to get smaller amount of vesicle release was unsuccessful. Eventually, at low enough concentrations, no observable affect was seen, with no concentration leading to easily distinguished spontaneous events. I then attempted to

see if I could measure KAR-mediated events using the toxin, by recording in the presence of 20-50μM GYKI 53655. Addition of the toxin eventually led to what looked like a very large increase in noise of my recordings, and, again, I was not able to titrate the effect. This could have been the result of activation of many KAR-containing synapses, with the concomitant reduction of membrane resistance leading to very noisy recordings. However, this could also be the result of pore formation of the toxin. To distinguish between these possibilities, I recorded in the presence of 100 μM NBQX, and once again found a very large increase in the noise. In all conditions, the cells usually died within 5-10 minutes of toxin application (data not shown).

Another method used to study single-synaptic events is through the use of strontium. When Ca^{2+} is replaced with Sr^{2+} in the extracellular recording solution, extracellular stimulation can be used to evoke both a synchronized EPSC and a flurry of mEPSCs for up to 2 seconds after the stimulus (Goda and Stevens, 1994; Bannister et al., 2005). I first tried to use this method to get evoked minis with AMPAR-containing synapses. Attempts with multiple solution combinations (replacing 2.5 mM Ca^{2+} with 4mM Sr^{2+}, 0mM Ca^{2+}, and 2mM EGTA; 2.5 mM Ca^{2+} with 4mM Sr^{2+} and 0.5 mM Ca^{2+}; 2.5 mM Ca^{2+} with 4mM Sr^{2+} and 2.5 mM Ca^{2+}, 2.5 mM Ca^{2+} with 2.5mM Sr^{2+}, 0mM Ca^{2+}, and 2mM EGTA) led to the elimination of the evoked AMPAR EPSC and no discernable sEPSCs after the stimulus (data not shown).

Other attempts to study the KAR sEPSCs were also unsuccessful. Increasing the probability of release (P_R) with higher extracellular Ca^{2+} or high frequency stimulation were not robust enough to lead to discernable events or even readily distinguishable changes in baseline noise that might reflect activation of KARs (data not shown).

4.4.3 Kainate receptors are not selectively activated by multivesicular release

While we did not observe a KAR-mediated tail in sEPSCs, one final possibility regarding the colocalization of these receptors with AMPARs is that the KARs are located perisynaptically in an annulus surrounding a synapse containing AMPARs. In this scenario, the KARs would be located at a distance where they can be engaged by multivesicular release but not univesicular release. This might lead to a selective activation of KARs during stimulus-evoked transmission but not spontaneous transmission because multivesicular release requires the high probability of release (P_R) that occurs during the calcium transient elicited by a presynaptic spike but not during spontaneous vesicle fusion (Christie and Jahr, 2006; Tong and Jahr, 1994).

If this scenario is correct, then multivesicular release should occur at high P_R, and lead to a higher concentration of glutamate in the synaptic cleft. One can test whether multivesicular release occurs at excitatory synapses onto interneurons by examining whether the concentration of glutamate in the synaptic cleft is sensitive to P_R, using low affinity competitive antagonists to assess glutamate concentration. Briefly, low affinity competitive antagonists are sensitive to the concentration of synaptically released glutamate because they unbind from the receptor so rapidly that glutamate has a chance to compete with the antagonist for the ligand binding site. The antagonist can more effectively compete against low concentrations of glutamate than high concentrations. If multivesicular release occurs, we would expect that a low affinity competitive antagonist should be less effective during high P_R, where multivesicular release is dominant, and more effective at low P_R, where univesicular release is dominant.

To test this hypothesis, I examined the partial block of the eEPSC caused by the low affinity AMPAR/KAR antagonist, γ-DGG (500 μM). In control conditions, γ-DGG

blocked the peak amplitude of the eEPSC by 40±2% (n=10; Fig 7A-C). I then bath applied the $GABA_BR$ agonist baclofen (5 μM) to engage presynaptic inhibition and lower Pr, and reapplied γ-DGG. Baclofen reduced the size of the EPSC by 57±6% (n=10 ; Fig. 7A-B); in the presence of baclofen, γ-DGG was not significantly more effective than in control conditions (41±3% n=10 ; Fig. 7C). Thus, multivesicular release does not occur at these synapses, precluding a model in which perisynaptic KARs are selectively recruited during evoked transmission but not spontaneous transmission.

4.2.4 A comparison of synaptic kainate receptors on SR/SLM and SO interneurons

These observations of SR/SLM interneurons contrast markedly with previous studies of SO interneurons, which found that KARs produce clearly resolvable spontaneous synaptic currents in these cells. In these previous studies, even miniature KARs in SO interneurons were found to be large (averaging ~10 pA), fast (decay time constant ~10 msec), and frequent (~2 Hz); these KAR-mediated events were a sizeable fraction of the total population of spontaneous synaptic currents observed (>30% under a wide range of conditions and across all identified subsets of SO interneurons) (Cossart et al., 2002; Goldin et al., 2007). Interneurons are heterogeneous, so one possible resolution to these conflicting results is that the properties of KARs in SR/SLM interneurons and SO interneurons differ so that the KAR EPSC in SR/SLM interneurons is small and slow, while the KAR EPSC in SO interneurons is large and fast.

To test this idea, I recorded eEPSCs from SO interneurons. The slowly decaying tail current was much less robust in SO interneurons than in SR/SLM interneurons

(Figure 8 A-B); in most SO interneurons, there was no detectable tail current at all. The difference in the average magnitude of the tail current between interneurons in SO and those in SR/SLM was clearly evident in averaged charge transfer traces in response to brief stimulus trains (Fig. 8C; SO: n=11, SR/SLM: n=9). SO interneurons can be divided into subpopulations with distinct physiological characteristics (Freund and Buzsaki, 1996; Pouille and Scanziani, 2004); cells with physiological features typical of a common subtype, the O-LM interneuron, were readily identified and accounted for more than half of the SO interneurons recorded (7 out of 11; Fig. 9). None of these putative O-LM interneurons expressed a detectable tail current.

I next examined whether GluR5-containing KARs contribute substantially to the evoked EPSCs on SO interneurons. I was unable to detect any significant effect of UBP 302 on the cumulative charge transfer for eEPSCs on SO interneurons, again in marked contrast to what I found on SR/SLM interneurons (Fig. 8D, E; SR/SLM: n=9; SO: n=11). These results indicate that GluR5 is absent from synapses onto SO interneurons.

One possible explanation for this finding is that SO interneurons might not express GluR5. To assess the contribution of GluR5 to KARs on SO interneurons, we examined the effect of UBP 302 on currents elicited by domoate (120±34 pA, n=3) and kainate (105±52 pA, n=4) (Fig. 10A, C). On average, UBP 302 was substantially more effective on currents elicited by these nonselective agonists in SO interneurons than in SR/SLM interneurons (SO: 57±14% inhibition; SR/SLM: 18±6% inhibition; Fig. 10D). Rapid application of ATPA also elicited a current that was almost entirely blocked by UBP302 (I_{ATPA}=19±3 pA; block by UBP302=80±7%; n=4; Figure 10B). Thus, GluR5 is not only present on SO interneurons, but present in a greater proportion of KARs than on SR/SLM interneurons.

These results suggest that GluR5 is selectively localized to synapses onto SR/SLM interneurons but not SO interneurons, confirming that the properties of KARs differ across different classes of interneuron. We reasoned that the large and rapid spontaneous KAR EPSCs found in prior studies of SO interneurons were likely due to KARs that lack the GluR5 subunit, which would not be sensitive to UBP 302. To address this possibility, I examined whether a population of sEPSCs could be isolated in the presence of 50 μM GYKI 53655, as reported previously. Surprisingly, however, I was unable to detect any GYKI 53655-resistant sEPSCs in SO interneurons (Fig. 11A; n=13).

To address the possibility that GluR7 might confer a higher GYKI sensitivity to KARs on SO interneurons, I repeated these experiments using 10 μM GYKI 53655. This dose should strongly block AMPARs (~90% inhibition) with only modest effects on GluR7-containing KARs (~25% inhibition) (Perrais et al., 2009). Even under these conditions, GYKI 53655 led to an almost complete inhibition of sEPSCs (97±3% reduction in frequency, n=3; see Supplemental Figure 5). The rare remaining events in 10 μM GYKI 53655 are likely AMPAR-mediated EPSCs that were still within our limits of resolution during the strong—but incomplete—blockade by this concentration of GYKI 53655. However, I cannot exclude the alternative that they could be mediated by GluR7-containing KARs. In either case, I conclude that the contribution of postsynaptic KARs to the EPSC is minimal in SO interneurons, and clearly distinct from the role of KARs in generating the slow EPSC in SR/SLM interneurons. A recent study was also unable to detect GYKI-resistant sEPSCs in SO interneurons (Oren et al., 2009).

4.3 Discussion

These experiments demonstrate that KARs are expressed in a synapse- and cell-type specific manner. KARs do not contribute a resolvable tail component to sEPSCs identified by the presence of a conventional rapid AMPAR component, indicating that the synapses that generate these large, rapid sEPSCs cannot account for the KAR currents in the evoked EPSC. This would suggest that KARs are expressed in an input specific manner, and are not ubiquitously co-expressed at AMPAR-containing synapses. In contrast to these findings in SR/SLM interneurons, I was unable to demonstrate any substantial contribution of postsynaptic KARs to the EPSC in SO interneurons, though GluR5-containing KARs are present on these cells.

4.3.1 KAR expression at synapses underlying the rapid AMPAR sEPSCs is negligible

The absence of a resolvable tail current in averaged sEPSCs from SR/SLM interneurons indicates that at least some of the synapses that mediate the fast component of the evoked EPSC have AMPARs without a substantial complement of KARs. For the same reason, the AMPARs that contribute to the tail current of the evoked EPSC are also unlikely to be present at these synapses. Because the KAR component of the evoked EPSC is not a result of spillover, we infer that a separate population of synapses must have KARs without a substantial complement of AMPARs. We were unable to directly resolve sEPSCs with these properties; however, given the small size of the KAR EPSC even when evoked by extracellular stimulation, we would

expect that these events are too small for us to detect. A comparison of the charge transfer curves for averaged sEPSCs and eEPSCs demonstrates that we likely should have been able to resolve a tail current in the average sEPSC if it were present. An alternate explanation is that the KAR-enriched synapses might have an exceptionally low spontaneous release rate, so that they simply do not generate sEPSCs.

While the focus of this chapter is on KARs, it is of interest to note that I was unable to detect any AMPAR-mediated tail current on the averaged sEPSCs either. One possible explanation is that the AMPAR component of the tail current in evoked EPSCs is due to spillover even when uptake is present. An alternative is that the AMPARs underlying the slow component of the AMPAR EPSC are segregated to distinct synapses from the AMPARs underlying the fast component of the EPSC. If this alternative is correct, selective targeting of AMPARs with distinct properties could be achieved by subunit selective-targeting to distinct population of synapses, as has been observed for AMPARs on hilar interneurons (Tóth and McBain, 1998); alternatively, it might be the case that the scaffolding proteins at each type of synapse have distinct effects on AMPAR kinetics.

If KARs (and possibly also the AMPARs mediating the slow tail of the AMPAR eEPSC) are segregated to distinct synapses from the AMPARs mediating the fast EPSC, what functional role would this imply? One obvious possibility is that the interneuron integrates different types of information from the distinct afferent sources. Inputs generating the fast EPSC would possibly be important in temporal precision, while inputs generating the slow EPSC would likely be important in temporal summation (König et al., 1996; Frerking and Ohliger-Frerking, 2002). In this context, it is of interest to note that SR/SLM interneurons receive input not only from the Schaffer collaterals, but

also from the entorhinal cortex and the thalamic nucleus reuneins (Freund and Buzsaki, 1996; Somogyi and Klausberger, 2005; Cavdar et al., 2008).

Another possibility is that the synapses underlying slow and fast EPSCs come from the same population of afferents but encode different features, such as developmental state. In support of this possibility, thalamocortical synapses undergo a maturational shift from a slow KAR EPSC to a fast AMPAR EPSC that is due to activity-dependent plasticity (Kidd and Isaac, 1999; Bannister et al., 2005).

4.3.2 GluR5-containing KARs are a substantial contributor to the EPSC on SR/SLM interneurons but not SO interneurons

In contrast to these results in SR/SLM interneurons, most SO interneurons had little or no tail current in the evoked EPSC, and we were unable to detect any effect of UBP 302 on the eEPSCs in these cells. Thus, the slow KAR EPSC mediated by GluR5-containing KARs in SR/SLM interneurons is differentially expressed across distinct subsets of interneurons. GluR5-containing KARs on SO interneurons were activated by exogenous agonists, consistent with prior reports (Yang et al., 2006; Yang et al., 2007); in fact, GluR5-containing KARs generated a larger fraction of the agonist-elicited currents in SO interneurons than in SR/SLM interneurons. Thus, the absence of synaptic GluR5-containing KARs in SO interneurons is not due to a lack of GluR5, but presumably reflects an inability to target or retain these KARs at the synapse.

While these findings did support the idea that the synaptic expression of KARs is different for SR/SLM interneurons and SO interneurons, this does not completely explain the discrepancy between our own findings and those of previous reports (Cossart et al., 2002; Goldin et al., 2007) because even in SO interneurons, we were unable to detect

any sEPSCs in the presence of GYKI 53655. However, another lab has also reported being unable to detect GYKI-resistant KAR events (Oren et al., 2009). Even miniature KAR EPSCs reported by Cossart and colleagues were frequent (~ 2 Hz on average) and large (~10 pA on average), so we should have been able to easily detect these events if they were present in our recordings. Similarly, we would not expect the size or frequency of miniature EPSCs to drop below our limit of resolution due to any of the minor experimental variables that often vary between labs. The reason for this difference in results is unclear. It may still be the case that we inadvertently recorded from a distinct subset of SO interneurons from those examined by Cossart and colleagues; however, we think this is unlikely, as a significant fraction of the cells studied by that group are O-LM interneurons. These interneurons express unusual physiological characteristics, most notably a pronounced short-term facilitation (Pouille and Scanziani, 2004), and this was readily observed in many of our recordings of SO interneurons.

Is there a functional significance to the selective expression of the slow KAR EPSC on SR/SLM interneurons but not SO interneurons? There are a number of differences between SR/SLM interneurons and SO interneurons; a notable distinction is that SO interneurons are the major source of feed-back inhibition, while SR/SLM interneurons are major contributors to feed-forward inhibition (Freund and Buzsaki, 1996; McBain, 2000; McBain and Fisahn, 2001). Research on feed-forward inhibition has focused on temporal precision and synchrony (Pouille and Scanziani, 2001; Assisi et al., 2007; Bartos et al., 2007; Mann and Paulsen, 2007) which is likely mediated by fast AMPAR inputs on SR/SLM interneurons. Synapses that express KARs would more likely be an activity-dependent mechanism for slow, reliable changes in the background membrane potential that would regulate the degree of synchrony needed by the fast AMPAR EPSCs to cause interneuronal firing.

In summary, the synaptic expression of KARs in hippocampal interneurons is finely tuned, through subunit-dependent localization of KARs to a subset of synapses on SR/SLM interneurons but not SO interneurons. The precision of KAR targeting and the unusual kinetics of the KAR EPSC suggest that these receptors play a specific and distinct role in signal processing during the transfer of information through the hippocampal circuit.

4.4 Figure and Figure Legends

4.4.1 Figure 5

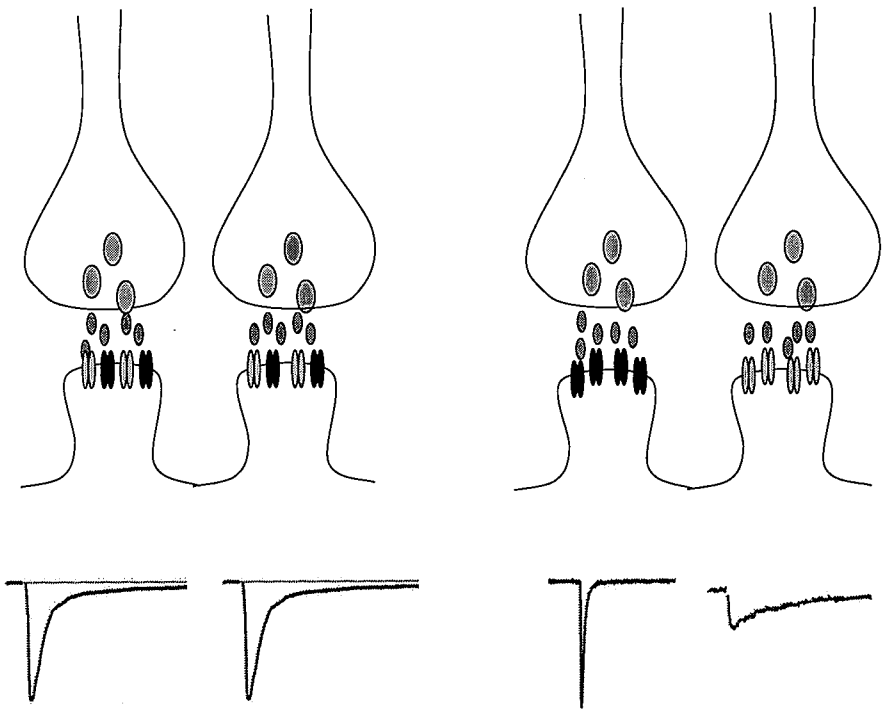

Figure 5. Are slow kainate receptors and fast AMPA receptors colocalized or segregated to separate synapses?

Bulk stimulation of the afferent fibers during stimulus evoked transmission precludes differentiation of colocalization (left) of the large amplitude, fast AMPA receptors and small, slow kainate receptors from segregation (right) of the two receptor types at separate synapses.

4.4.2 Figure 6

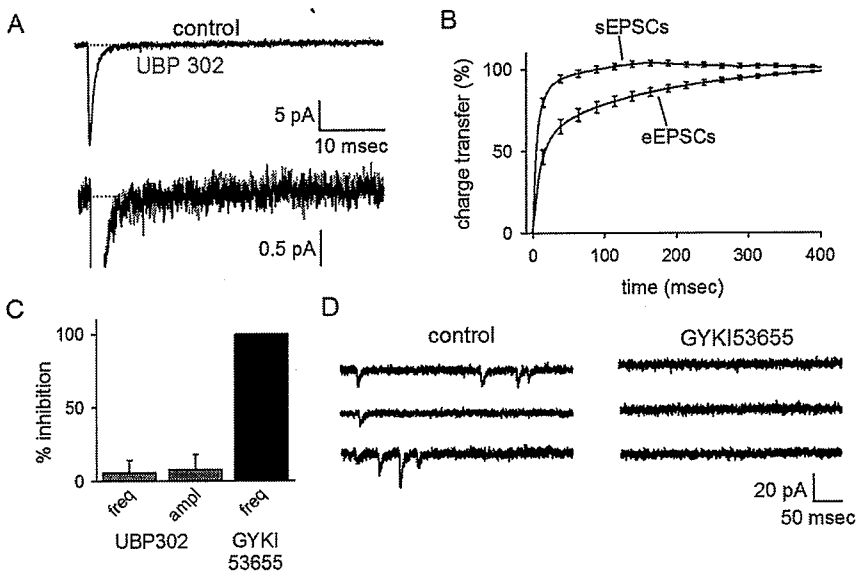

Figure 6. Synapses that generate fast AMPAR sEPSCs cannot account for the tail current of the evoked EPSC. Spontaneous EPSCs were recorded in the presence of picrotoxin and D-APV. (A) Averaged sEPSCs were recorded in control conditions (black) and in the presence of UBP 302 (gray). (B) The cumulative charge transfer of sEPSCs reaches its maximum in <100 msec, in contrast to that of the evoked EPSC. (C) UBP302 had no effect on the frequency or amplitude of sEPSCs recorded in control conditions. The sEPSCs were completely blocked by GYKI 53655, indicating that these events were mediated solely by AMPARs. (D) GYKI 53655 blocked all resolvable sEPSCs, as shown in a representative cell.

4.4.3 Figure 7

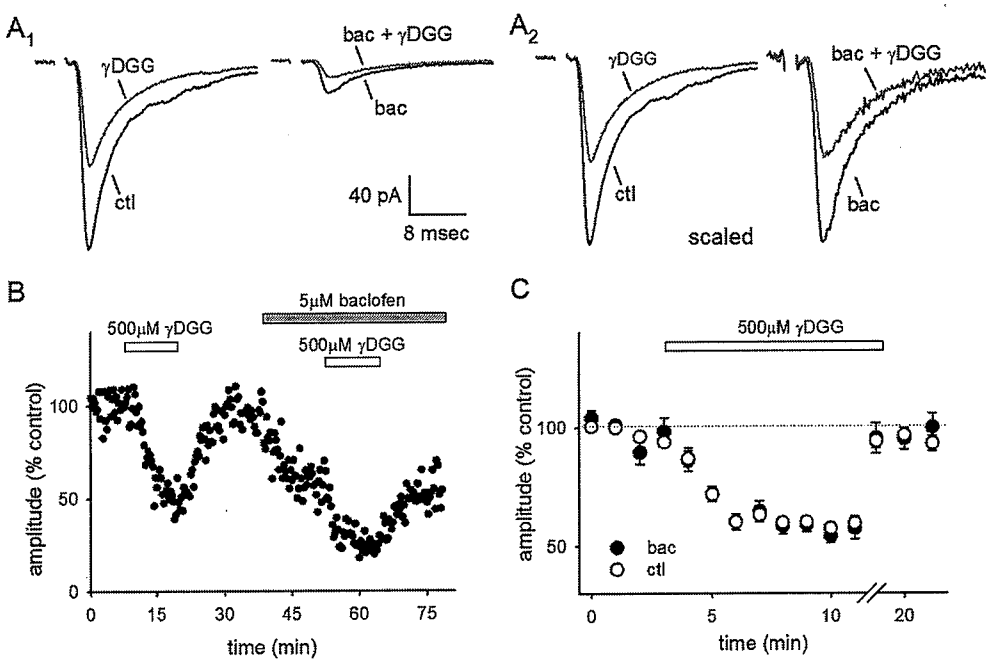

Figure 7. Multivesicular release does not contribute to the interneuronal EPSC. AMPAR EPSCs were evoked in the presence of APV, picrotoxin, and UBP 302. (A_1) (Left) Averaged traces of a representative cell in control conditions (black) and in the presence of γ-DGG (gray). (Right) Averaged traces of EPSCs evoked in the presence of 5 μM baclofen (black) and in the presence of both baclofen and γ-DGG (gray) (A_2) The same traces in (A_1) scaled so that the control trace and the trace in baclofen, both prior to the addition of γDGG, are amplitude-matched. (B) EPSC amplitudes (% control) were recorded from a representative cell. γ-DGG caused a reversible partial block of the AMPAR EPSCs that was similar in control conditions and in baclofen. (C) A summary is shown of the block elicited by γ-DGG in the presence (black) and absence (white) of baclofen, averaged across all experiments.

4.4.4 Figure 8

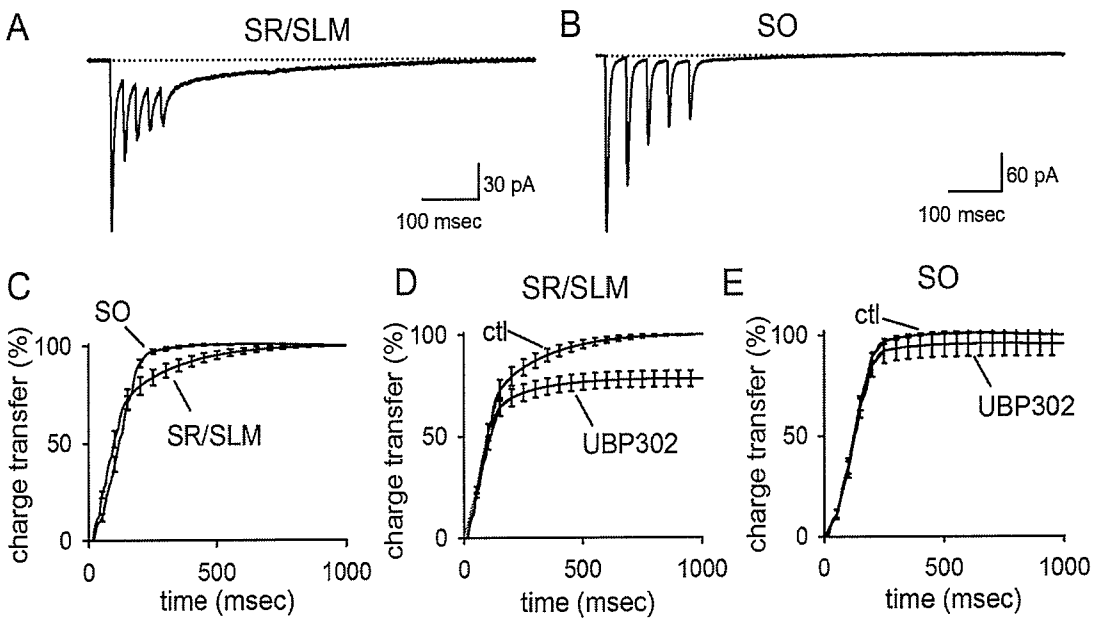

Figure 8. GluR5 KARs are excluded from the synapse at stratum oriens interneurons. (A) Averaged eEPSCs recorded from a stratum radiatum/lacunosum moleculare (SR/SLM) interneuron have a distinct, slowly decaying tail current. (B) Averaged eEPSCs recorded in stratum oriens (SO) have notably smaller tail currents, when detected at all. (C) The slow tail current contributes significantly to the charge transfer of the EPSCs recorded from SR/SLM cells, but not for EPSCs recorded from SO interneurons. (D) In SR/SLM cells, the late, slow component of the tail is significantly blocked by UBP 302. The total charge transfer during the EPSCs are normalized with respect to control conditions; thus, the fact that the charge transfer in the presence of UBP302 reaches an asymptote at significantly less than 100% in SR/SLM interneurons indicates that the total charge transfer is significantly reduced by UBP302 in these cells. (E) UBP302 has no significant effect on the cumulative charge transfer of eEPSCs in SO interneurons.

4.4.5 Figure 9

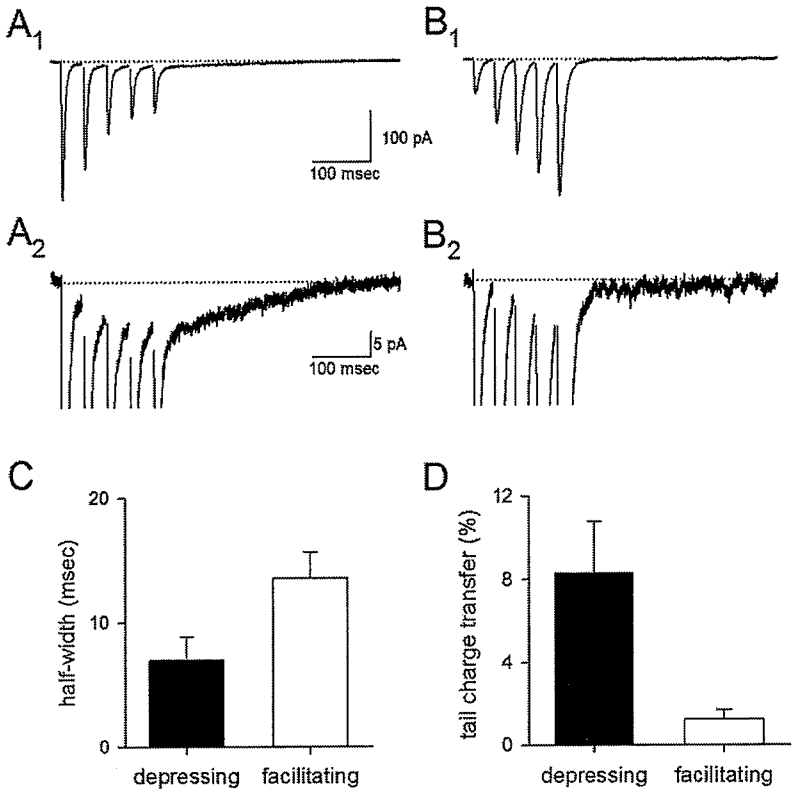

Figure 9. Correlated physiological properties in different subsets of SO interneurons. Averaged eEPSCs were elicited by a brief train of 5 stimuli and recorded from stratum oriens interneurons. (A) eEPSCs that depress shown in low gain (A_1) and high gain (A_2) on average show a small tail current. In contrast, eEPSCs that facilitate show no tail current (B) Facilitating responses are reported to correlate well with the O-LM subtype of SO interneuron; O-LM interneurons also have been reported to have EPSCs that decay with a slower time constant. Consistent with this, interneurons that show short-term depression have EPSCs with a shorter half-width than that seen in interneurons that show short-term facilitation (C). (D) The tail current of putative O-LM interneurons contributes to the overall charge transfer less than in other SO interneurons.

4.4.6 Figure 10

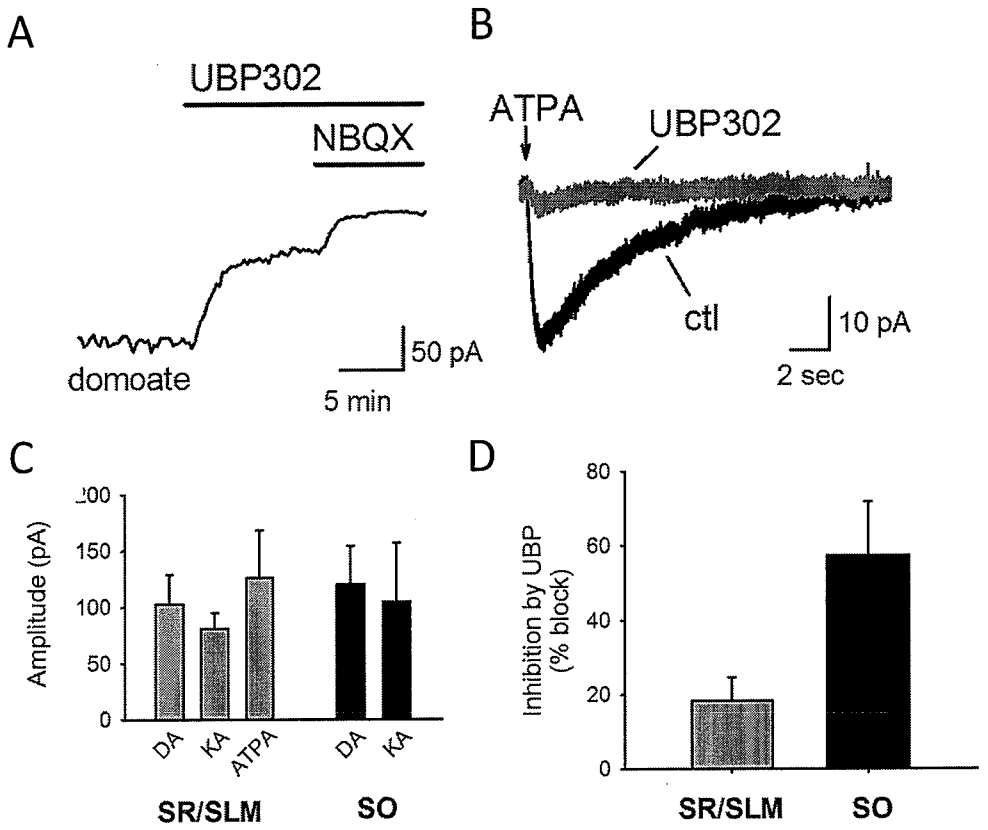

Figure 10. GluR5 is present on stratum oriens interneurons.

Effects of UBP 302 on agonist induced currents were examined in SO interneurons as they previously were in SR/SLM interneurons. UBP302 substantially blocked currents induced by non-selective KAR agonists, as shown by the effect of UBP 302 on currents elicited by 50 nM domoate (A) or by the GluR5-selective agonist ATPA (B). C). Agonist application appeared to activate comparable amplitude KAR currents. While GluR5-containing KARs do not contribute to synaptic transmission in these cells, they do appear to make up a larger percentage of overall somatodendritic KARs compared to SR/SLM interneurons (D).

4.4.7 Figure 11

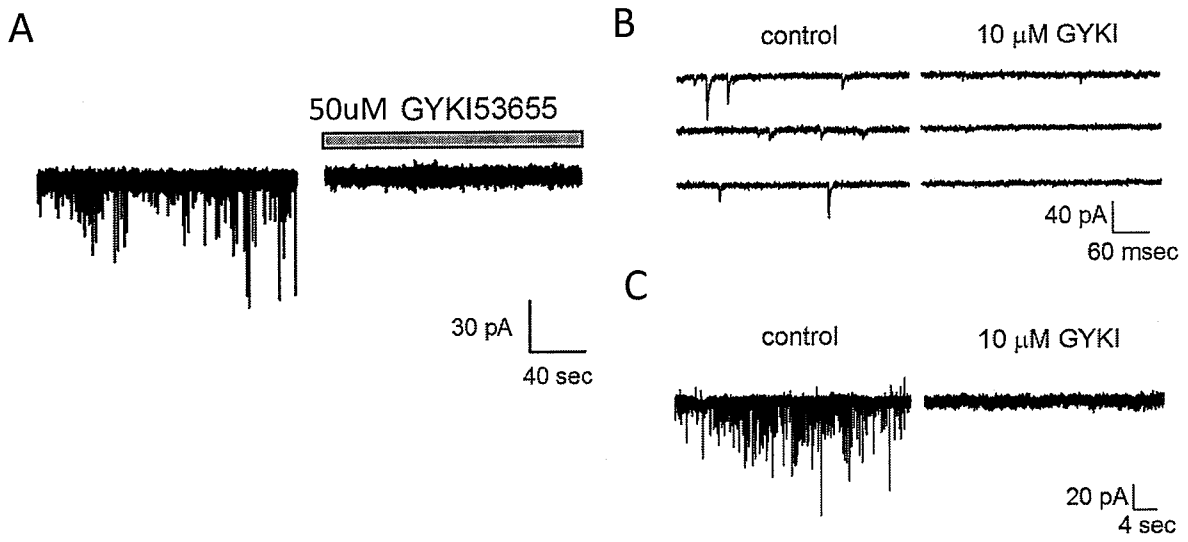

Figure 11. GYKI-resistant KAR sEPSCs were not seen in SO interneurons.

A) All resolvable sEPSCs were blocked by 50μM GYKI 53655 in SO interneurons as shown in a representative cell. 10 μM GYKI 53655 also blocked all resolvable sEPSCs indicating that GluR7 homomers were not inadvertently blocked in (A) shown in a representative cell at high (B) and low (C) gain.

5. Is the slow AMPA receptor EPSC mediated by glutamate spillover?

5.1 Introduction

AMPAR current properties can vary depending on the synapse and cell type where they are expressed. Reasons for such variance include differences in subcellular localization, subunit composition, and even auxiliary subunit interactions. Other factors affecting AMPAR currents are not related to the receptors themselves, but to the glutamate transient that activates them. Glutamate concentration inside the cleft rapidly reaches a peak of 1-10mM, then decays to very low concentrations, possibly on a sub-millisecond timescale (Clements et al., 1992). Further away from the synapse, glutamate concentration is much lower and the time course of clearance is slower (Rusakov and Kullmann, 1998; Szapiro and Barbour, 2007), though this depends on a number of factors such as structural elements and density of synapses. Another factor significantly affecting the spread of glutamate is uptake by glutamate transporters. The relative contributions of diffusion and uptake also differ across synapses and cell-types, as glutamate spillover has been shown to activate extrasynaptic receptors and nearby synapses (Carter and Regehr, 2000; DiGregorio et al., 2002; Kullmann, 2000), and even receptors on nearby cells (Szapiro and Barbour, 2007).

The experiments of chapter 1 demonstrate that AMPARs contribute to a slowly decaying EPSC on hippocampal SR/SLM interneurons, but not on SO interneurons. However, the mechanisms underlying the observed slow decay kinetics were unclear. The glutamate transporter blocker TBOA caused a large potentiation of the evoked

AMPAR tail current, suggesting that the slow component of the AMPAR EPSC was being selectively recruited during reduced glutamate uptake. The effect of TBOA on AMPARs at this synapse is substantial in comparison to effects of blocking glutamate uptake at other synapses (Arnth-Jensen et al., 2002; Chen and Diamond, 2002; Diamond, 2002; DiGregorio et al., 2002; Takayasu et al., 2004; DeVries et al., 2006). Recruitment of a slow AMPAR EPSC by TBOA lends some support to the idea that the slow AMPAR component of the eEPSC might be mediated by spillover.

The data, however, are not completely compatible with this idea: if single stimuli were sufficient to overwhelm uptake and elicit spillover lasting for hundreds of milliseconds, it would also seem likely that this spillover could be facilitated during brief high-frequency trains, which should more effectively overwhelm uptake than single stimuli. I did not observe a facilitation of the tail current during trains, nor was there an activity-dependent change in the relative contribution of AMPARs to the EPSC. It is possible that the slow AMPAR component of the EPSC recorded when uptake is intact reflects activation of a distinct subset of synaptic AMPARs with unusual properties, possibly conferred by accessory proteins (Cho et al., 2007; Milstein et al., 2007). In this chapter, I begin to examine the possible causes and mechanisms underlying the slow decay kinetics of the AMPAR EPSC, both while uptake is intact and in the presence of TBOA.

5.2 Results

5.2.1 Glutamate spillover does not mediate the kinetics of the slow AMPA receptor tail current

The experiments of Chapter 1 compared the decay kinetics of the AMPAR EPSC elicited through a single stimulus and through a high frequency train and found that

repeated stimulation did not preferentially recruit a slow component of the EPSC, suggesting that spillover of glutamate does not cause this tail current. However, TBOA caused substantial potentiation of the AMPAR tail, which indicates that reducing glutamate clearance selectively increases activation of the receptors underlying this part of the EPSC. To better understand the underlying mechanism of the slow AMPAR-mediated current, we further tested the role of glutamate spillover on shaping the decay kinetics of the AMPAR EPSC. Since experimentally attempting to increase spillover had given seemingly conflicting results, we decided to examine the effects of manipulations that could, in theory, reduce spillover. One method to manipulate glutamate transporter efficiency is to record at different temperatures, as the glutamate clearance is steeply temperature sensitive, with higher levels of transport at higher temperatures (Asztely et al., 1997). In pharmacologically isolated AMPAR eEPSCS (recorded in the presence of 10μM UBP302, 100μM picrotoxin, and 100μM APV to block KARs, $GABA_A$Rs, and NMDARs respectively) at near physiological temperatures (32-37°C), a large tail current was still readily detected, indicating that even at increased transporter efficiency, the slow decay kinetics are intact (Fig. 12A). The cumulative charge transfer averaged across cells shows a clear slow component that accounts for approximately 25% of the total charge transfer (Fig. 12B). These results indicate that the slow AMPAR EPSC is prominent even when uptake is increased, and that the slow tail current is not an artifact of recording at room temperature.

Since temperature affects multiple processes, and recording temperature-dependent changes in kinetics from the same cell is very difficult, I pursued a more direct manipulation of glutamate spillover that would lend itself to same-cell comparisons. If a single stimulus is enough to overwhelm the glutamate clearance mechanisms and cause a spillover-induced tail current, then substantially reducing the probability of release and

minimizing synaptic transmission should reduce spillover and cause a disproportionate reduction of the tail compared to the peak. I examined differences in the isolated AMPAR kinetics after reducing the probability of release with the $GABA_BR$ agonist baclofen (5-15μM). Baclofen reduced the size of the single stimulus eEPSC by 40±9% (Fig. 13A, n=5), with no significant difference in the reduction of the peak and tail (Fig. 13B, C). This argues that the AMPAR decay kinetics are not sensitive to reductions of glutamate release and likely reflect inherent properties of the receptors.

The above data, which argue against a spillover mechanism behind the slow tail current, are difficult to resolve with the TBOA results described in chapter 1. If the properties of this current reflect smaller amounts of glutamate slowly escaping from the synapse, then the tail current would indicate activation of receptors by smaller concentrations of glutamate compared to the EPSC peak, and should therefore be more effectively blocked by γ-DGG. When I partially blocked the AMPAR EPSC with 500μM γ-DGG (Fig. 14A, n=5), a concentration high enough to cause a substantial block of the fast component of the EPSC, I found no difference in the block of the peak and tail, arguing they reflect activation of receptors by a similar concentration of glutamate, and ruling out spillover as the mechanism for the slow decay kinetics (Fig. 14B). The cumulative charge transfer averaged across all cells reveals no selective effect of γ-DGG on the slow tail (Fig. 14C).

5.2.2 The TBOA-mediated recruitment of a slow AMPA receptor EPSC is not due to glutamate spillover

In light of these data, we concluded that the TBOA-induced potentiation of the slow AMPAR tail represented a spillover-mediated tail current that was not related to the tail current seen when glutamate uptake was intact. Still, the presence of such a large potentiation by TBOA was surprising, as blocking uptake does not cause such large increases in AMPAR EPSCs at other synapses (Arnth-Jensen et al., 2002; Chen and Diamond, 2002; Diamond, 2002; DiGregorio et al., 2002; Takayasu et al., 2004; DeVries et al., 2006). Since it is known that extrasynaptic pools of AMPARs exist at these synapses as well, it is curious that TBOA would cause such distinctly different changes in the EPSC kinetics at different synapses. Variation in synaptic structure could contribute to these differences, as may relative numbers and proximity of extrasynaptic receptors. It is also possible that during TBOA-induced block of transporters, glutamate spillover is activating nearby synapses, indicating widespread glutamate diffusion away from the primary synapse and/or high densities of synapses on these cells. Another possibility is that the TBOA-mediated potentiation does not reflect spillover, but continued activation of synaptic AMPARs. We initially didn't consider this possibility, as AMPARs are known to rapidly desensitize. Consistent with this property of AMPARs, we did note a small decrease of the AMPAR EPSC peak during TBOA application. However, since desensitization properties are modulated by subunit composition (Hansen et al., 2007; Mayer, 2005) and auxiliary protein interactions (Milstein et al., 2007; Milstein and Nicoll, 2008), it remains possible that the receptors mediating the slow AMPAR EPSC are not subject to rapid desensitization.

To see if the TBOA-mediated potentiation of the slow AMPAR EPSC was indeed caused by spillover-mediated activation of extrasynaptic or nearby synaptic receptors, I tested whether the slow tail recruited by TBOA was susceptible to preferential block by γ-DGG. TBOA caused a significant potentiation of the slow tail current of the single stimulus evoked AMPAR EPSC (527% ± 106%, n=5) (Fig. 15A, C). The cumulative charge transfer of the cells indicates a significant increase in the contribution of the slow tail current in the presence of TBOA (Fig. 15B). The EPSCs recorded in TBOA were then partially blocked with 500μM γ-DGG and peak scaled to the unblocked TBOA EPSC (Fig. 15D). Surprisingly, I found no detectable difference in the block of the TBOA-potentiated tail compared to the peak (Fig. 15D, E), arguing that the TBOA potentiation is not mediated by spillover. One possible explanation is that the fast AMPAR EPSCs are limited by clearance so that in the presence of TBOA the glutamate transient is profoundly prolonged and can lead to continuous activation of synaptic AMPARs. If this were the case, then one might expect an increase in the sEPSC decay time when recorded in the presence of TBOA. I averaged sEPSCs recorded in control conditions and compared them to the averaged sEPSCs recorded in TBOA. I was unable to detect any differences in the decay of the averaged sEPSCs (Fig, 15F, G). A small increase in the amplitude of averaged sEPSCs is likely due to a decreased ability to differentiate smaller spontaneous events from the increased noise of the baseline recordings in the presence of TBOA.

These results agree with the data presented in chapter 1 which showed that the slow AMPAR tail was not detectable in sEPSCs, and that slow and fast AMPARs are not colocalized at the same synapses. As I showed with KARs, any small, slow AMPAR-mediated sEPSCs would likely be undetectable. Taken together, these date indicate the

possibility of two distinct types of synaptic AMPARs: those that mediate the well-known large and fast AMPAR EPSCs and cannot be reactivated due to rapid desensitization, and those that underlie the small, slow AMPAR tail and possibly are able to be continually activated by lingering glutamate. If TBOA block of glutamate uptake causes reactivation of the synaptic slow AMPARs, this should cause potentiation of slow sEPSCs. Unfortunately, potentiation of any small, slow AMPAR sEPSCs may still be undetectable, because their unique kinetics elude most mini-analysis programs and are difficult to distinguish from noise. TBOA increased the noise of my recordings in general and decreased my ability to detect sEPSCs (especially smaller amplitude events), though it is unknown whether this is due to potentiation of slow sEPSCs or to the general effects of decreased membrane resistance and cell viability resulting from excess glutamate.

These results seem to indicate that there are slow and fast AMPARs segregated to separate synapses. When glutamate transport is blocked with TBOA, it may be that instead of causing spillover-mediated activation of extrasynaptic receptors, the decrease in uptake results in glutamate lingering in the synaptic cleft. This lingering glutamate has little effect on the fast AMPAR EPSC, since they rapidly desensitize. However, if the AMPARs underlying the small, slow EPSC do not desensitize, then they may be continuously activated by high concentrations of glutamate, causing the large potentiation we see in TBOA. These conclusions are preliminary, and not fully supported by the data presented. Indeed, I was unable to detect small, slow AMPAR sEPSCs that were selectively potentiated during TBOA application. Since the small, slow KAR sEPSCs were undetectable, it seems likely that AMPAR sEPSCs with similar properties will also be elusive.

5.3 Discussion

The experiments of this chapter examine whether glutamate spillover mediates the slowly decaying tail current of the AMPAR eEPSCs. Our previous findings that blocking glutamate uptake substantially recruits the slow component of the EPSC suggested that this may be the mechanism underlying the unique kinetics of these currents. However, all other experiments that attempted to manipulate glutamate spillover did not affect AMPAR EPSC kinetics, and γ-DGG did not preferentially block the slow tail, arguing against a spillover mechanism. Indeed, even the TBOA-recruited tail was not preferentially blocked by a low affinity antagonist indicating that we were possibly looking at continued excitation of synaptic AMPARs when glutamate uptake was blocked. My attempt to support this possibility by looking for potentiation of slow AMPAR sEPSCs was unsuccessful, possibly due to the difficulty of detecting such small, slow events out of the noise.

Further experiments are necessary to fully understand the mechanisms causing the slowly decaying AMPAR EPSC recorded in SR/SLM hippocampal interneurons. While many experiments seem to support the conclusion that glutamate spillover is not causing the small, slow tail, there is still some uncertainty. Experimental manipulations that should serve to reduce any existing spillover, such as increasing transporter efficiency with higher temperatures and reducing the probability of release with baclofen, did not selectively reduce the slowly decaying tail currents. The lack of any effect of γ-DGG on EPSC decay kinetics argues that these slow currents are not being activated by smaller amounts of glutamate, as would be assumed with a spillover mechanism. This interpretation is contingent on γ-DGG more effectively blocking lower concentrations of

glutamate. We know it is acting as a low affinity antagonist as it only partially blocks the EPSCs, and it is known to act as a competitive antagonist (Liu et al., 1999; Watkins, 1991). There are no immediately obvious reasons that such a drug should not be able to more effectively compete with smaller amounts of transmitter than larger amounts. Since γ-DGG has been effectively used by multiple labs to differentiate between different glutamate concentrations (Watkins et al., 1990; Liu et al., 1999; Wadiche and Jahr, 2001; Christie and Jahr, 2006), it stands to reason that this drug should act as we expect it to. However, a positive control that demonstrates conclusively that the drug does act in this way will be necessary to confidently interpret these experiments. Examining the amount of block of currents elicited by different known concentrations of glutamate to excised patches may help in assessing the capabilities of this drug.

Another possible complication in interpreting these γ-DGG results is that different subtypes of AMPARs could have different glutamate affinities. An extrasynaptic receptor that is activated by smaller concentrations but that has a lower affinity could appear to be blocked by the same amount as the synaptic receptors. Such an explanation would seem to require such an exact compensation of affinity relative to concentration, that this seems somewhat unlikely, though cannot be ruled out.

The finding that the TBOA-recruited tail was not preferentially blocked by γ-DGG was quite surprising, and leads us to presume that the TBOA potentiation is due to selective activation of the slow AMPARs mediating the tail current recorded when uptake is intact. Such activation may be due to glutamate lingering in the synapse for much longer periods of time, causing rapid desensitization of the fast AMPARs and continuous activation of slow, desensitization-resistant AMPARs. A good test for this theory will be to test the effects of TBOA on nearby SO interneurons. As described in chapter 2,

these interneurons do not appear to have the slowly decaying tail currents that we observe in SR/SLM interneurons. Since these cells don't appear to have the slow AMPARs, then we would expect that TBOA should not cause the large potentiation we see in SR/SLM interneurons. If we do see a large tail current appear in these cells when uptake is blocked, and it is not preferentially blocked by γDGG again, we will have to reevaluate our working hypothesis.

The existence of synaptic AMPARs that underlie the slow tail current is difficult to directly verify without being able to selectively evoke or block the slow AMPAR EPSC. The subunit composition and subunit-specific protein interactions can have major effects on the biophysical properties and localization of AMPARs, though the exact nature of these relationships is poorly defined. The subunit composition of AMPARs on hippocampal interneurons is known to be unique, as these cells express much greater numbers of GluR2-lacking, Ca^{2+} permeable AMPARs than seen in principle cells (Geiger et al., 1995; Petralia et al., 1997), as well as higher levels of GluR3 and 4 (Catania et al., 1998; Moga et al., 2003). The Ca^{2+} permeable AMPARs can be targeted to separate synapses than GluR2-containing AMPARs and may be preferentially trafficked to different subcellular regions (Tóth and McBain, 1998). Experiments elucidating the subunit composition of these receptors may help in understanding the mechanisms underlying the slow kinetics, or at least provide valuable tools to better manipulate these currents. Furthermore, Ca^{2+} permeability of these receptors could indicate important roles of these receptors in Ca^{2+} signaling and plasticity. It will be important to test whether subunit specific antagonists such as NASPM and joro spider toxin for GluR2 and philanthotoxin for GluR1/2 heteromers preferentially block either the fast or slow component of the AMPAR EPSC. GluR3 and GluR4 knockout mice may also be of use in determining the possible contribution of these subunits.

5.4 Figures and Figure Legends

5.4.1 Figure 12

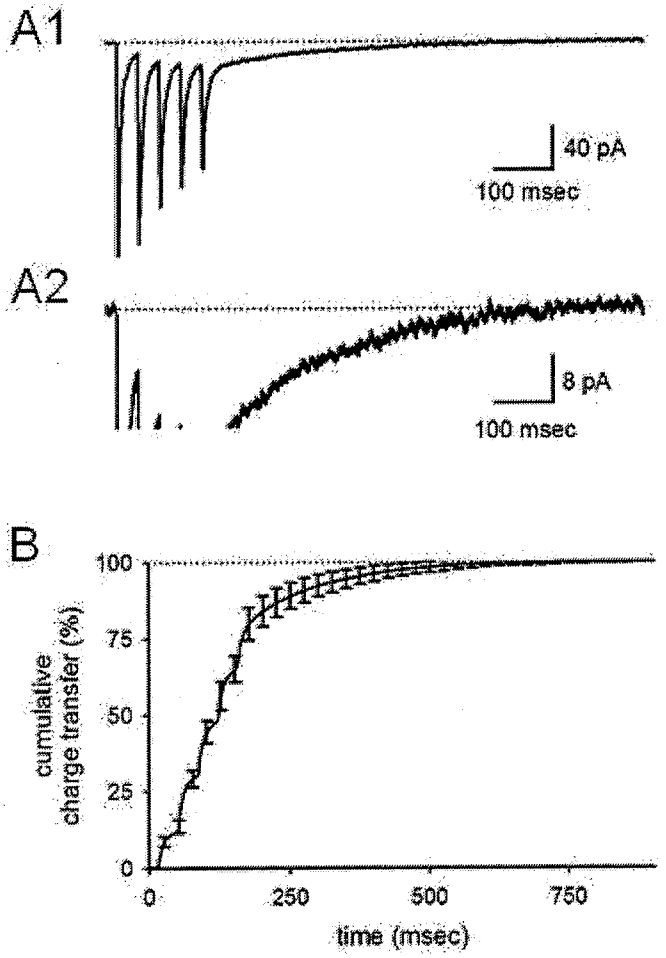

Figure 12: The slow AMPAR-mediated tail is still present in EPSCS recorded at physiological temperatures. (A) The pharmacologically-isolated AMPAR EPSC was elicited by a brief train of 5 stimuli at 32-37°C. A prominent slow component of the EPSC was still readily detected, and lasted for up to several hundred milliseconds, shown at low (A1) and high (A2) gain. (B) The cumulative charge transfer was averaged across cells (n=5) and demonstrates the slow decay kinetics and significant charge transfer contributed by the tail current.

5.4.2 Figure 13

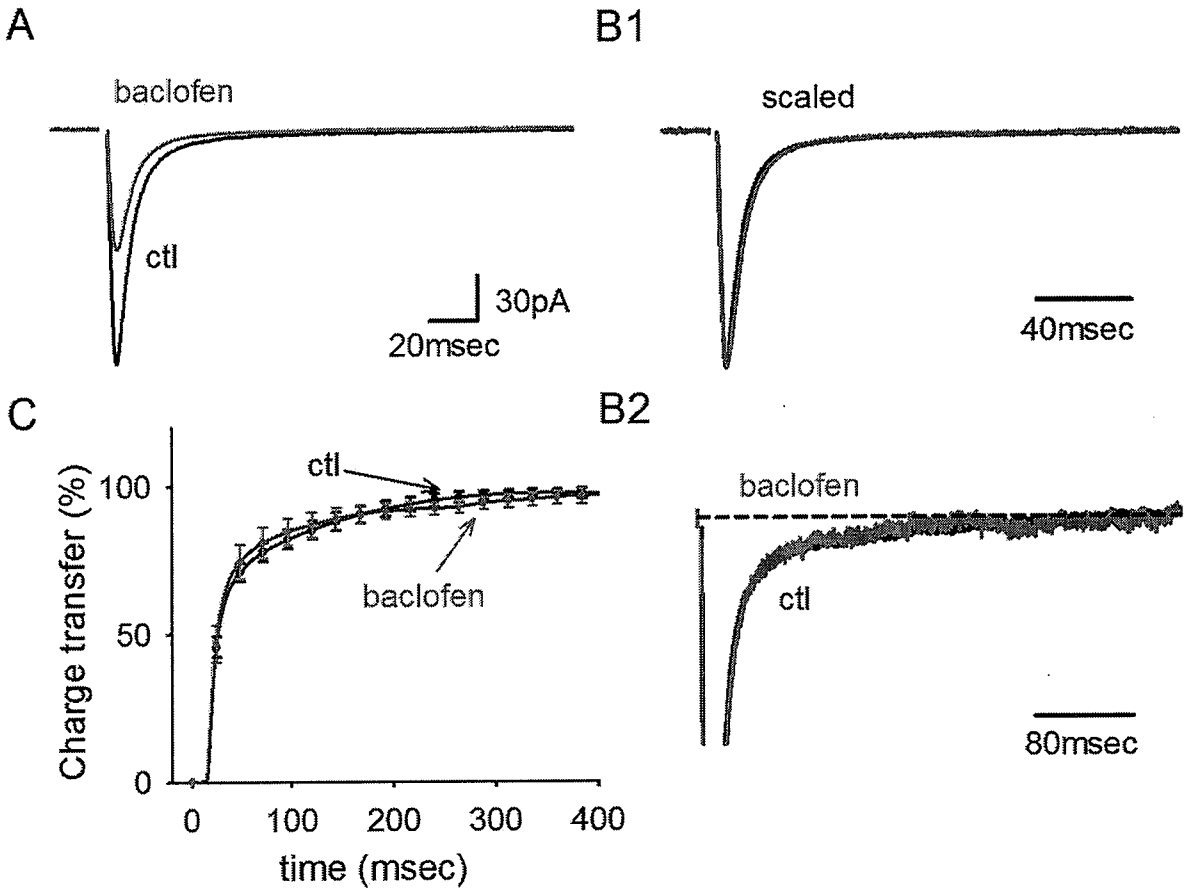

Figure 13: Reducing the probability of release does not affect the decay kinetics of the AMPAR EPSC. The AMPAR EPSC was recorded before (black) and after (grey) the addition of baclofen, a $GABA_AR$ agonist that lowers the P_R. (B) The averaged EPSCs recorded in the two conditions were scaled for comparison and showed no difference in their decay kinetics, shown at a low gain (B1) and high gain (B2). (C) The averaged cumulative charge transfer of the EPSCs recorded in the absence (black) and presence (grey) of baclofen also shows no affect of baclofen on the EPSC kinetics.

5.4.3 Figure 14

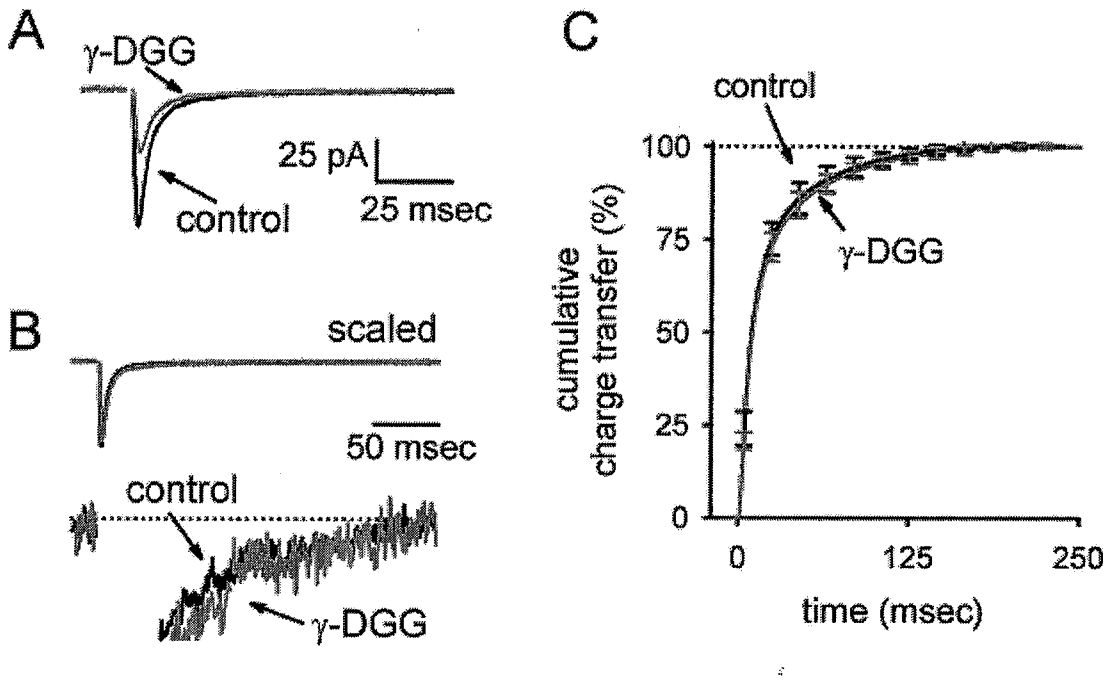

Figure 14: The slow component of the AMPAR EPSC is not preferentially blocked by γ-DGG. (A) The AMPAR EPSC recorded in the presence of UBP302 (black trace) in a representative cell was partially blocked by the low affinity competitive antagonist γ-DGG (grey trace). (B) Averages of the EPSCs recorded in the two conditions were scaled for comparison shown at low (top) and high (bottom) gain. No differential block of γ-DGG on the fast and slow components was observed. (C) The averaged cumulative charge transfer of all cells shows that γ-DGG had no effect on the decay kinetics of the AMPAR EPSC.

5.4.4 Figure 15

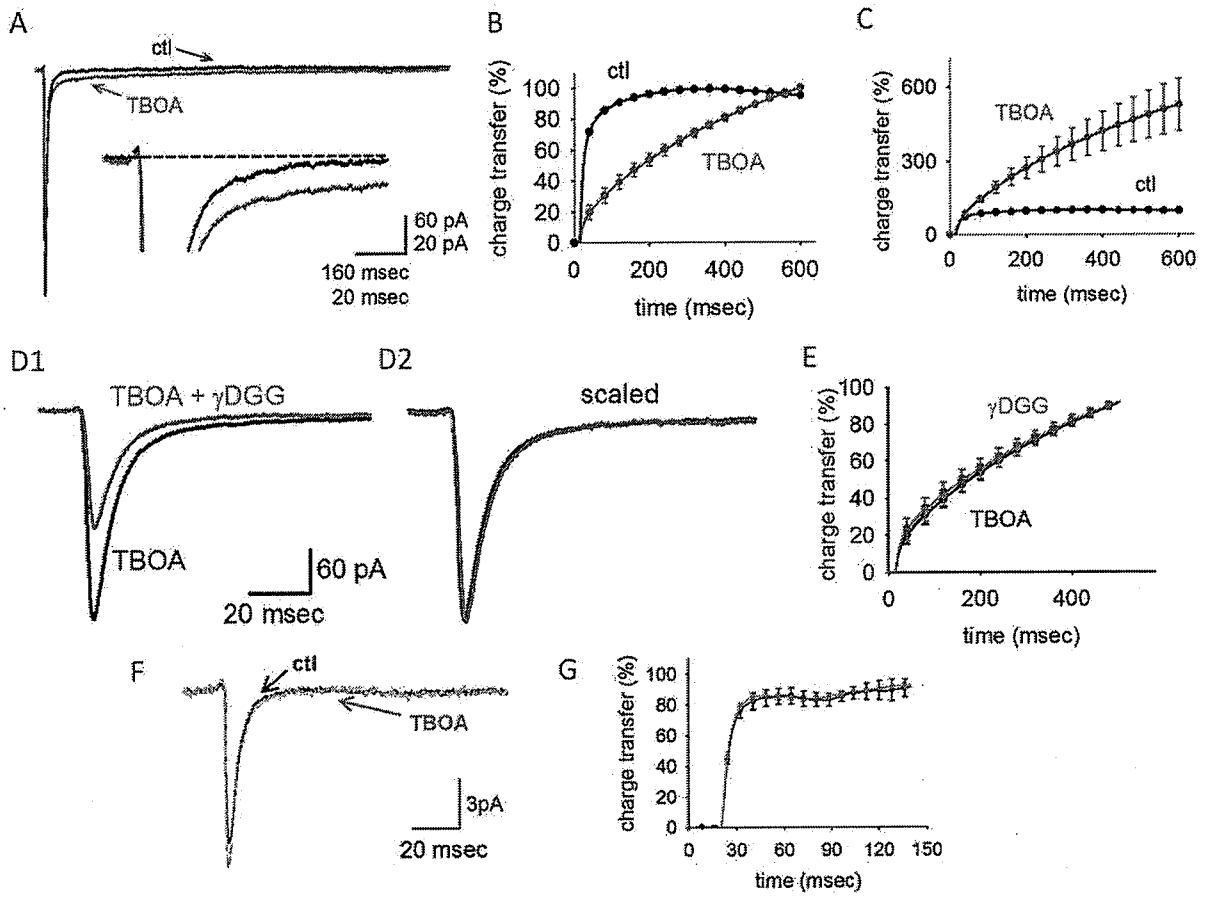

Figure 15: The TBOA-induced potentiation of the slow AMPAR EPSC is not preferentially blocked by γ-DGG. (A) Averaged AMPAR EPSCs recorded before (black trace) and after (grey trace) blocking glutamate transporters with 100μM TBOA, shown at high gain in inset. (B) Averaged cumulative charge transfer before and after blocking uptake shows that TBOA caused a significant slowing of the AMPAR EPSC due to a large potentiation of the slow tail current. (C) The average cumulative charge transfer of the AMPAR EPSC in control conditions and in the presence of TBOA are shown normalized to the control average. TBOA caused a substantial increase in the

charge transfer of the AMPAR EPSC compared to control conditions (527% ± 106%, n=5). (D) The AMPAR EPSC recorded in TBOA was partially blocked with γ-DGG (D1) and scaled to the TBOA peak (D2). γ-DGG did not preferentially block the TBOA-potentiated tail current. (E) The average cumulative charge transfer of the cells shows no difference in the decay kinetics after partial block with γ-DGG. (F, G) Averaged sEPSCs recorded in the absence (black trace) and presence (grey trace) of TBOA show no difference in the decay kinetics between the two conditions. The slightly bigger amplitude of the averaged sEPSCs detected in TBOA is likely due to increased noise that decreases our ability to detect small events.

6. Summary and Discussion

6.1 Subunit-specific subcellular targeting of kainate receptors

Several conclusions can be drawn from the work presented here that expand our understanding of KAR and AMPAR function on hippocampal interneurons. Two pharmacologically distinct subsets of KARs exist on SR/SLM interneurons, UBP-sensitive and UBP-insensitive, which presumably corresponds to the inclusion and exclusion of the GluR5 subunit respectively. When activating all the surface receptors during agonist applications, the GluR5-lacking KARs were the dominant contributors, though these findings cannot be taken to demonstrate relative numbers of the receptor subtypes, as we know nothing of single channel conductance and desensitization differences. Conversely, when examining just the synaptic receptors through stimulus evoked EPSCs, I found that the currents were mediated mainly by GluR5-containing receptors, arguing that GLuR5-lacking KARs are preferentially excluded from the synapse. Interestingly, I also found that KARs only partially contribute to the slowly decaying tail current of the eEPSCs, with AMPAR EPSCs also contributing a small, slow current.

Experiments of Chapter 3 indicate that the GluR5-KARs appear to be precisely localized to the synapse. KARs did not appear to be extrasynaptically located as they were not recruited during repetitive stimulation or when glutamate uptake was blocked. However, the experiments of Chapter 5 do bring some uncertainty to this interpretation. Our conclusions about KAR localization at the synapse in Chapter 3 were based, in part, on the TBOA potentiation of the AMPAR currents serving as a control to show that

increasing spillover recruits extrasynaptic AMPARs but not KARs. Since the TBOA-induced tail was not preferentially blocked by γ-DGG, we are uncertain whether TBOA was able to induce spillover. If control experiments confirm that the γ-DGG should be able to detect whether spillover is occurring, then we may want to further test the synaptic location of KARs, but overall the conclusion that these receptors are synaptically located is still supported. While we were unable to detect spillover with AMPARs or KARs during block of uptake, this likely indicates that both receptors are located fairly precisely at the synapse, as does the high frequency stimulation experiment. Indeed, the experiments of Chapter 5 support a synaptic location for the slow AMPARs. One possible mechanism for the slow kinetics that may also explain the kinetic differences seen among KARs is an interaction with an auxiliary subunit. Indeed, there is evidence that the transmembrane protein NETO2 modulates KAR channel properties, and may be playing a role in the KAR currents on these interneurons (Zhang et al., 2009).

The results of chapter 3 suggest that GluR5-lacking KARs are excluded from the area surrounding the synapse, and their function remains unclear. One possibility is that they act like the non-synaptic KARs on pyramidal cells which are located on the soma and regulate neuronal excitability via metabotropic effects on the afterhyperpolarization following spiking (Melyan et al., 2002; Fisahn et al., 2005); another is that they play a protective role by detecting ambient glutamate, driving the interneuron to fire and release GABA when extracellular glutamate rises to pathological levels during events like ischemia.

A subunit-selective mechanism for inclusion or exclusion of KARs at the synapse is consistent with what we see with AMPARs at pyramidal cell synapses. With AMPARs,

there is much interest in understanding the subunit composition of synaptic and extrasynaptic pools of receptors and the processes that determine this subcellular specificity. It is thought that recruitment of the extrasynaptic AMPARs plays a role in plasticity, and may be regulated by interactions with scaffolding proteins (Malinow 2002; Lu 2009}. Similar processes may be involved in the targeting of KARs.

6.2 Are kainate receptors activated by distinct afferent inputs?

The experiments of Chapter 4 demonstrate that KARs are expressed in an input- and cell-type specific manner. The absence of a resolvable tail current in averaged sEPSCs from SR/SLM interneurons indicates that most if not all of the synapses that mediate the fast component of the evoked EPSC have fast AMPARs but no KARs or slow AMPARs. Because the KAR component of the evoked EPSC is not a result of spillover, I infer that a separate population of synapses must have KARs without a substantial complement of AMPARs. I was unable to directly resolve sEPSCs with these properties; however, given the small size of the KAR EPSC even when evoked by extracellular stimulation, I would expect that these events are too small for me to detect. A comparison of the charge transfer curves for averaged sEPSCs and eEPSCs demonstrates that I likely should have been able to resolve a tail current in the average sEPSC if it were present. An alternate explanation is that the KAR-enriched synapses might have an exceptionally low spontaneous release rate, so that they simply do not generate sEPSCs.

One possible reason we observe a much lower rate of spontaneous events is that the cell bodies projecting to the KAR-enriched synapses are absent. While the major inputs to CA1 interneurons are present in transverse hippocampal sections, the

sources of subcortical inputs are removed. Thalamic, amygdala, and raphe nuclei all send projections to the hippocampal interneurons. Thalamic inputs from the Reuniens nucleus have been shown to synapse onto CA1 interneurons (Dolleman-Van der Weel and Witter, 2000; Bokor et al., 2002). These projections are possibly involved in theta rhythm generation and epilepsy (Cavdar et al., 2008), and disruption of their input has been shown to have affects on learning and memory (Davoodi et al., 2009). Another subcortical area shown to project to the hippocampus is the amygdala. While these projections are also thought to synapse onto interneurons, there is evidence that there is not much input into area CA1 (Berretta et al., 2001; Berretta et al., 2004). The median raphe nucleus sends serotonergic and glutamatergic inputs to the area as well (Jackson et al., 2009) and has recently been shown to activate CA1 interneurons (Varga et al., 2009).

Usually, subcortical monoamine modulation is thought to work on slow time scales due to metabotropic signaling, though there is also evidence that these ascending pathways, many of which also act through glutamatergic transmission can act through rapid time scale signaling mechanisms (Varga et al., 2009). The possibility of KAR expression at such glutamatergic inputs could indicate multiple slow mechanisms for subcortical modulation of the hippocampus.

Since intra- and extra-hippocampal inputs into area CA1 are intermingled, selectively activating any one input with conventional stimulation will not be possible. A recent study of the raphe inputs utilized channelrhodopsin to selectively stimulate these fibers. Expressing this light activated channel in these subcortical areas in vivo and then activating the fibers with light activation in the slice would allow us to selectively activate these various inputs and determine if any of them selectively or preferentially activate the slow KAR or AMPAR synapses. Another way to potentially target some of these

subcortical inputs would be to record from horizontal slices, in which some of these connecting projections may still be intact allowing for direct stimulation and further analysis of spontaneous events.

Of course, input specificity to the KAR and/or slow AMPAR synapses may arise from within the hippocampus as well. CA1 interneurons receive projections from both the Schaffer collaterals and the entorhinal cortex (Freund and Buzsaki, 1996). While I was targeting Schaffer collaterals during evoked transmission, the bipolar electrode could conceivably activate the temporoammonic fibers as well. A simple test of whether these synapses are preferentially innervated from these fibers would be to examine the effects of dopamine on the peak to tail ratio of the eEPSCs, as dopamine has been shown to depress the temporoammonic inputs onto CA1 interneurons (Ito and Schuman, 2007).

6.3 Current kinetic differences between SR/SLM and SO interneurons

In contrast to the results in SR/SLM interneurons, most SO interneurons had little or no tail current in the evoked EPSC, and I was unable to detect any effect of UBP 302 on these cells. I was also unable to detect any GYKI 53655-resistant sEPSCs, arguing that there are also not any KARs at these synapses with kinetics and conductance properties similar to AMPARs. Thus, the slow KAR EPSC mediated by GluR5-containing KARs in SR/SLM interneurons is differentially expressed across distinct subsets of interneurons. GluR5-containing KARs on SO interneurons were activated by exogenous agonists, consistent with prior reports (Yang et al., 2006, 2007); in fact, GluR5-containing KARs generated a larger fraction of the agonist-elicited currents in SO interneurons than in SR/SLM interneurons. So the absence of synaptic GluR5-containing

KARs in SO interneurons is not due to a lack of GluR5, but presumably reflects an inability to target or retain these KARs at the synapse. Once again, the question of function for these non-synaptic receptors arises. Are these receptors meant to sense ambient glutamate? Do they regulate the I_{AHP} as seen in pyramidal cells (Melyan et al., 2002)? Are they accessible during glutamate spillover? While I have done numerous experiments to examine the effects of spillover on SR/SLM interneurons, I have not performed any on these SO interneurons. However, high frequency stimulation did not appear to have any effect on the decay kinetics of the SO eEPSCs, indicating that these somatodendritic KARs are not easily accessed.

While these findings did support the idea that the synaptic expression of KARs is different for SR/SLM interneurons and SO interneurons, this does not completely explain the discrepancy between our own findings and those of previous reports (Cossart et al., 2002; Goldin et al., 2007) because even in SO interneurons, we were unable to detect any sEPSCs in the presence of GYKI 53655. However, another lab has also reported being unable to detect GYKI-resistant KAR events (Oren et al., 2009). Even miniature KAR EPSCs reported by Cossart and colleagues were frequent (~ 2 Hz on average) and large (~10 pA on average), so we should have been able to easily detect these events if they were present in our recordings. Similarly, we would not expect the size or frequency of miniature EPSCs to drop below our limit of resolution due to any of the minor experimental variables that often vary between labs. The reason for this difference in results is unclear. It may still be the case that we inadvertently recorded from a distinct subset of SO interneurons from those examined by Cossart and colleagues; however, we think this is unlikely, as a significant fraction of the cells studied by that group are O-LM interneurons. These interneurons express unusual physiological characteristics,

most notably a pronounced short-term facilitation (Pouille and Scanziani, 2004), which was readily observed in many of our recordings of SO interneurons.

Is there a functional significance to the selective expression of the slow KAR and AMPAR EPSCs on SR/SLM interneurons but not SO interneurons? There are a number of differences between SR/SLM interneurons and SO interneurons; a notable distinction is that SO interneurons are the major source of feedback inhibition, while SR/SLM interneurons are major contributors to feed-forward inhibition (Freund and Buzsaki, 1996; McBain, 2000; McBain and Fisahn, 2001). Research on feed-forward inhibition has focused on temporal precision and synchrony (Pouille and Scanziani, 2001; Assisi et al., 2007; Bartos et al., 2007; Mann and Paulsen, 2007), which is likely mediated by fast AMPAR inputs on SR/SLM interneurons.

However, as mentioned above, there is also evidence of slow, diffuse modulation of these interneurons from subcortical inputs. While the synchronization and oscillatory rhythmic activity of the hippocampus have understood behavioral and functional relevance, the significance for such slow regulation is not known. While it has been proposed that the slow subcortical inputs may have roles in processing motivational or state-dependent information, the functions of slow signals in interneurons are not known, and are likely diverse. Synapses that express KARs would likely be an activity-dependent mechanism for slow, reliable changes in the background membrane potential that would regulate the degree of synchrony needed by the fast AMPAR EPSCs to cause interneuronal firing.

Testing the contribution of KARs to interneuronal excitability will be an important step in understanding their roles in synaptic transmission. Recording from the SR/SLM interneurons in current clamp would allow us to explore the contributions of KARs to excitability and firing rates of the cell. While blocking AMPARs with GYKI53655 would

likely block almost all firing, we may be able to detect differences in excitability when blocking synaptic KARs with UBP 302. Since basal firing rates are likely to be quite low and KARs are less likely to be critically important during coordinated stimulation of large numbers of inputs, we would want to stimulate release onto the cell in a more asynchronous manner that perhaps better mimics *in vivo* processes. One manipulation that has been used to induce asynchronous release is the application of hypertonic sucrose solution that causes random vesicle fusion. This technique would have the advantage of equally inducing release from all inputs, allowing for activation of all projections.

Another experiment could couple holographic photolysis with light activated channels to allow for more intricate stimulation patterns within the hippocampus. Recent technologies that allow for holographic patterns of illumination can be used to photolyze caged compounds or stimulate light-activated channels with spots of variable size that can be arranged in experimenter-defined patterns (Lutz et al., 2008). This technique could be coupled with expression of channelrhodopsin or use of a "photoswitchable affinity label" (PAL) that allows photoactivation of K+ channels to asynchronously activate a wide array of inputs onto individual interneurons (Fortin et al., 2008).

6.4 A slow AMPA receptor EPSC

Chapter 5 examined whether glutamate spillover mediates the slowly decaying tail current of the AMPAR eEPSCs. My findings from Chapter 3 that showed that blocking glutamate uptake substantially recruits the slow component of the EPSC which suggests that this may be the mechanism underlying the unique kinetics of these currents. However, all the experiments that attempted to manipulate glutamate spillover

did not affect AMPAR EPSC kinetics, and γ-DGG did not preferentially block the slow tail, arguing against a spillover mechanism. Indeed, even the TBOA-recruited tail was not preferentially blocked by a low affinity antagonist, indicating that we were possibly looking at continued activation of synaptic AMPARs when glutamate uptake was blocked. My attempt to support this possibility by looking for potentiation of slow AMPAR sEPSCs was unsuccessful, possibly due to the difficulty of detecting such small, slow events out of the noise.

Further experiments are necessary to fully understand the mechanisms causing the slowly decaying AMPAR EPSC recorded in SR/SLM hippocampal interneurons. While many experiments seem to support the conclusion that glutamate spillover is not causing the small, slow tail, there is still some uncertainty. The issue of whether γ-DGG is able to more effectively block lower concentrations of glutamate must be addressed with experiments that indicate we are able to differentiate the concentrations of glutamate involved. Examining the ability of the drug to block currents elicited by different known concentrations of glutamate to excised patches may help in assessing the capabilities of this drug.

The subunit composition and subunit-specific protein interactions can have major effects on the biophysical properties and localization of AMPARs, though the exact nature of these relationships is poorly defined. The subunit composition of AMPARs on hippocampal interneurons is known to be unique, as these cells express much greater numbers of GluR2-lacking, Ca^{2+} permeable AMPARs than seen in principle cells (Geiger et al., 1995; Petralia et al., 1997), as well as higher levels of GluR3 and 4 (Catania et al., 1998; Moga et al., 2003). Experiments elucidating the subunit composition of these receptors may help in understanding the mechanisms underlying the slow kinetics, or at

least provide valuable tools to better manipulate these currents. Furthermore, Ca^{2+} permeability of these receptors could indicate important roles of these receptors in Ca^{2+} signaling and plasticity. It will be important to test whether subunit specific antagonists such as NASPM and joro spider toxin for GluR2 and philanthotoxin for GluR1/2 heteromers preferentially block either the fast or slow component of the AMPAR EPSC. GluR3 and GluR4 knockout mice will also be useful in determining the possible contribution of these subunits.

There has been recent interest in the affects of auxiliary subunits that interact with AMPARs. Several groups have shown that these protein interactions can have profound effects on the trafficking and kinetics of AMPARs, with the described AMPAR currents looking very similar to the currents I have recorded (Milstein et al., 2007; Cho et al., 2007; Schwenk et al., 2009). Examining the AMPAR EPSCs in knockout mice may allow us to better understand the underlying mechanisms behind these uniquely small and slow AMPAR currents.

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