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Metabotropic Receptor Modulation of Kainate Receptors in the Hippocampus

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METABOTROPIC RECEPTOR MODULATION OF KAINATE RECEPTORS IN THE
HIPPOCAMPUS

by

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Submitted in Partial Fulfillment of the Requirements

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Exercise Science

The Norman J. Arnold School of Public Health

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DEDICATION

I would like to dedicate this manuscript to my loving husband, Jonnifer L. Cooper, for started and ending this journey with me, and being my best friend and confidant the along the way. I pray that we continue to have a lifetime full of exciting journeys to conquer together.

I would also like to dedicate this manuscript to my loving parents, Charles E. and Pamela K. Barker, grandmothers, Catherine Gordon and Elizabeth Strickland, and grandfather, Robert Gordon, for believing and praying for me before I could even believe in myself. Grandma Cat, I will always remember how you never failed to end our phone conversations with, "I'm proud of you, C'iana". Those words are happily engrained into my head and heart.

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ABSTRACT

Kainate receptors (KARs) are glutamate-gated ion channels that mediate synaptic transmission, modulate transmitter release, and mediate excitation in the brain. Potentiation in their function predisposes the hippocampus to hyperexcitability and seizures. These receptors are widely expressed throughout the central nervous system as tetramers composed of various combinations of GluK1-5 subunits. In the hippocampus (a brain region commonly associated with seizure initiation), GluK2-, GluK4-, and GluK5-containing receptors are highly expressed in the CA3 pyramidal cell layer, whereas GluK1 is barely detectable. The lack of pharmacological tools hinders identifying the functional contribution of each kainate receptor subtype in normal CA3 synaptic transmission. To address this critical obstacle, we used whole cell patch clamp electrophysiology on HEK-T 293 cells transfected with GluK2 homomers, GluK2/K4 or GluK2/K5 heteromers. We found that the drug ACET selectively inhibits GluK5 and GluK4 subunits, whereas the drug kynurenate is an antagonist at all kainate subunits but was more potent at GluK2 subunits. Furthermore, we were able to discover that binding of glutamate to either the two GluK2 subunits or two GluK4/K5 subunits in the heteromeric tetramer was sufficient to open the kainate receptor, albeit to a non-desensitizing current. However, glutamate binding to three or more subunits in the tetramer was sufficient to enable kainate receptor desensitization. Lastly, using field

potential electrophysiology to stimulate and record KAR-mediated synaptic transmission (fEPSPs) at the mossy fiber – CA3 synapse, we found that perfusion of ACET was sufficient to entirely block KAR-mediated fEPSPs at the mossy fiber – CA3 synapse. These results suggest that 1) drugs ACET and kynureate can be used as pharmacological tools to delineate the functional contribution of specific receptor subtypes, 2) kainate receptor activation and desensitization depends on the number of subunits bound to an agonist, and 3) KAR-mediated synaptic transmission at the mossy fiber – CA3 is conducted through heteromeric GluK4- or GluK5-containing KARs.

Metabotropic receptors, such as muscarinic acetylcholine receptors (mAChRs) and dopamine receptors (DARs), can also alter the function of glutamate receptors and have been implicated in epilepsy. Muscarinic acetylcholine receptors also play a critical role in synaptic plasticity and neuronal excitability. There are five types of mAChR, M1-M5, all of which, except m5 mAChRs, are found at different levels of expression in area CA3 of the hippocampus where they are co-expressed with kainate receptors. Muscarinic receptors regulated the function of other glutamate receptors, but it is unknown whether they can interact with KARs. Dysfunctional interactions between KARs and muscarinic acetylcholine receptors (mAChRs) have been implied in neurological diseases, including temporal lobe epilepsy. For example, injection of a mAChR agonist (pilocarpine) in rodents induces prolonged seizures and epilepsy, which can be blocked by a KAR antagonist. Understanding how KARs and mAChRs interact may unlock novel therapies for epilepsy. Using field potential electrophysiology, we discovered that mAChR activation selectively depresses KAR-mediated fEPSPs at the mossy fiber – CA3 synapse. This mAChR depression of KAR fEPSPs is mediated through M1 mAChRs, but

cannot be totally explained through PKC phosphorylation. Interestingly, M1 mAChR depression of KAR fEPSP goes away with aging, suggesting this phenomenon is developmentally regulated.

Lastly, we investigated whether the dopaminergic system is altered in a chronic model of temporal lobe epilepsy. Similarly to mAChRs, DARs, specifically D1-like DARs, play a critical role in synaptic plasticity, neuronal excitability, and have been associated with seizure propagation. We demonstrated that D5 DARs, but not D1 DARs, expression is significantly depressed in the epileptic hippocampus. Furthermore, we found that dopamine clearance is reduced, while total dopamine content is unchanged in the epileptic brain compared to sham-treated controls.

Taken together, we demonstrate the first steps toward discovering a novel interaction between KARs and mAChRs in the brain. Furthermore, we identified compensatory changes that occur in the dopaminergic system as a result of chronic temporal lobe epilepsy. These findings will provide potential targets for therapeutic interventions for patients with epilepsy.

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LIST OF SYMBOLS

\pm	Plus/minus
α	Alpha
β	Beta
μ	Micro
n	Nano
Ω	Ohm
A	Amp
C°	Celcius
Hz	Hertz
M	Molar
m	Milli
n	number of samples
V	Volts
I	Current
G _q	Excitatory G protein coupled receptor
G _i	Inhibitory G protein coupled receptor
Ca ²⁺	Calcium
Cl ⁻	Chloride
CO ₂	Carbon Dioxide

K^+ Potassium

Mg^{2+} Magnesium

O_2 Oxygen

Na^+ Sodium

LIST OF ABBREVIATIONS

mAChR	Muscarinic Acetylcholine Receptor
ACh	Acetylcholine
A/C	Associational/commissural
AED	Antiepileptic drug
AMPA	α -amino-3-hydroxy-S-methylisoxazole-4-propionic acid
AMPA R	AMPA receptor
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DAB	Diaminobenzidine
D-APV	(2R)-amino-5-phosphonovaleric acid
DA	Dopamine
DAR	Dopamine receptor
DCG-IV	(2S,2'R,3'R)-2-(2'3'-Dicarboxycyclopropyl) glycine
DG	Dentate gyrus
DGCs	Dentate granule cells
DMSO	Dimethyl sulfoxide
DZP	Diazepam
EC	Entorhinal cortex

EC₅₀..... Effective concentration of 50% of subjects
 ECL..... Enhanced chemiluminescence
 EPSC..... Excitatory post-synaptic current
 ER Endoplasmic reticulum
 fEPSP Field excitatory post-synaptic potential
 GAD-67..... Glutamate decarboxylase 67
 GFAP Glial fibrillary acidic protein
 HPLC High-performance liquid chromatography
 I/V Current/Voltage
 KA..... Kainate
 KAR Kainate receptor
 KYN..... Kynurenic acid
 LTP Long Term Potentiation
 LTD..... Long Term Depression
 mGluR..... Metabotropic glutamate receptor
 MK801 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
 MF..... Mossy fiber
 mRNA..... messenger RNA
 NE Norepinephrine
 Neto1..... Neuropilin Tolloid-like 1
 Neto2..... Neuropilin Tolloid-like 2
 NMDA N-methyl-D-aspartate
 Osm..... Osmole

PB..... Phosphate buffer
PBS Phosphate buffered saline
PKA..... Protein kinase A
PKC..... Protein kinase C
PVDF Polyvinylidene fluoride
SDS/Page Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE..... Status epilepticus
SL..... Stratum lucidum
SLM Stratum lacunosm-moleculare
SO Stratum oriens
SP Stratum pyramidale
SR..... Stratum radiatum
TBS Tris-buffered saline
TBS-T Tris buffered saline with Tween20
TTX..... Tetrodotoxin

CHAPTER 1

GENERAL INTRODUCTION

1.1 SIGNIFICANCE

Glutamate receptors mediate fast excitatory transmission in the brain. Particularly, in the hippocampus they are required for synaptic plasticity that underlies learning and memory. There are three types of ionotropic glutamate receptors: α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA), and kainate receptors. Of these three types, kainate receptors are the least understood, although the most interesting of the bunch. Unlike the other receptors, kainate receptors are located at both presynaptic and postsynaptic sites, where they mediate synaptic transmission and neurotransmitter release through not only the classical ionotropic mechanism but also through a metabotropic G-protein mechanism. Additionally, kainate receptors have slow excitatory postsynaptic potentials (EPSPs), which allows for temporal summation. Furthermore, kainate receptor subtypes have unique pharmacology based upon the subunits that compose them, and can undergo posttranslational modification (i.e. phosphorylation), which allows kainate receptors to have diverse responses to the same ligand. Overactivation of kainate receptors have been linked to neurological diseases, such as epilepsy.

Neurotransmitters, like acetylcholine and dopamine, can modulate glutamate receptor function. In the hippocampus, dysfunctional interactions between these neurotransmitters and glutamate receptors have been linked to several neurological diseases, including epilepsy. For example, administering a muscarinic acetylcholine agonist reliably produces seizures in an animal model that remarkably resembles human temporal lobe epilepsy. These seizures no longer occur when animals are pretreated with a kainate receptor antagonist. Additionally, administering a dopamine receptor agonist can precipitate seizures, which can also be blocked by a kainate receptor antagonist. Interestingly, alternative therapies known to improve brain health and learning and memory, such as routine physical exercise, have been shown to modulate acetylcholine and dopamine content in the brain. This demonstrates a dynamic interaction between the glutamatergic system and other monoamines that are required for optimal brain health.

Our goal is to understand the interaction between the glutamatergic system and monoamines in the brain. Furthermore, we want to understand how disruption in these interactions could lead to neurological disease, such as in epilepsy, and how alternative therapies, such as physical exercise, can counteract this disruption. Several studies have observed how AMPA and NMDA ionotropic glutamate receptors are modulated by muscarinic and dopamine receptors. However, it is not known whether kainate receptors are altered by these metabotropic receptors. In this collection of studies we seek to 1) discover a pharmacological tool that will help us identify KAR subunits involved in synaptic transmission, 2) determine whether KAR-mediated synaptic transmission is modulated by muscarinic and/or dopamine receptors, and 3) determine how neurological

diseases, such as epilepsy, can alter monoamine content and infer how these changes can impact the hippocampal glutamatergic system.

1.2 THE HIPPOCAMPUS

1.2.1 HIPPOCAMPAL FUNCTION

The hippocampus is a group of millions of cells buried deep within the medial temporal lobe of the human and other mammalian brain. This structure resembles a seahorse, and was thus named after the Latin translation “hippocampus” by the Bolognese anatomist Giulio Cesare Aranzi (circa 1564). Since its discovery, the functional role of the hippocampus remained heavily debated until the late 1950’s when scientists William Scoville and Brenda Milner began excising the mesial temporal lobe from patients with brain damage. The most notable patient, known as H.M., suffered severely from uncontrollable seizures. He underwent surgery to remove the ‘epileptic core’, which included removing both hippocampi, in an effort to reduce his seizure frequency. The studies and observations of H.M. by William Scoville and Brenda Milner in 1957 (Scoville and Milner, 2000) concluded that while the surgery was successful in reducing the seizures, the patient was left incapable of developing new memories (anterograde amnesia) nor had he retained memory of events that occurred immediately before surgery. This was the first definitive link between memory and the hippocampus.

Since that initial clinical observation, decades of behavioral research have accumulated a large body of evidence confirming the hippocampus's role in acquiring, maintaining, and recalling memories. However, the hippocampus is not involved in every type of memory. It is generally accepted that the hippocampus is critical for both declarative memory and spatial memory (Burgess et al., 2002). Declarative memory refers to memories that can be consciously recalled, such as facts and verbal knowledge. These memories can be categorized into two subclasses: semantic memory, which is the capacity to remember factual knowledge, and episodic memory, which stores observational memories attached to specific personal experiences. In contrast, spatial memory is the ability to encode information regarding one's environment and spatial orientation, which is necessary for navigation. The hippocampus achieves this with "place cells". Place cells are neurons located in the hippocampus that activate in correspondence to a specific location independent of orientation (O'Keefe, 1976; Wilson and McNaughton, 1993; Muller et al., 1994). In addition to acquiring and storing memories, the hippocampus is also involved in memory retrieval. Although this hippocampal function is still heavily debated due to the complicated nature of experimental procedures and interpretation, it is believed that the hippocampus is required for retrieval of detailed contextual memories (generally recent memories) (Wiltgen et al., 2010). However, memories that lose precision (memories of our distant past) can be retrieved independently of the hippocampus (Wiltgen et al., 2010). This theory is also corroborated with clinical observations of patient H.M. and others with damaged hippocampi, whom found it difficult to recall events just prior to the surgery

removing their hippocampi, but did not experience deficits in recalling distant memories (Squire, 1992; Hodges, 1994; Squire and Alvarez, 1995).

In accordance to the hippocampus's central role in learning and memory, aging (Scahill et al., 2003; Raz et al., 2004; Rodrigue and Raz, 2004; Du et al., 2006) and diseases associated with learning and memory impairment, including Alzheimer's (Wang et al., 2006), epilepsy (Chang and Lowenstein, 2003) and schizophrenia (Heckers, 2001), are strongly associated with hippocampal atrophy and sclerosis. Additionally, therapies that are known to improve learning and memory performance, such as physical exercise, can reverse and/or delay hippocampal atrophy (Erickson et al., 2011). The ability to understand diseases that impair learning and memory and develop more effective treatments for these diseases likely lies within our understanding of the hippocampus. Fortunately, the anatomical structure and circuitry of this region in the healthy brain has been well characterized.

1.2.2 HIPPOCAMPAL ANATOMY

Humans and other mammals have two hippocampi, one located on either side of the brain – deep within the medial temporal lobe. The hippocampus is shaped like a curved tube, resembling a cashew nut. The hippocampus is intimately connected and function in coordination with other regions (dentate gyrus (DG), subiculum, presubiculum, parasubiculum, and entorhinal cortex (EC)), known collectively as the hippocampal formation. These accessory regions are required for funneling information from various brain regions into the hippocampus and redistributing information exiting

the hippocampus. Particularly, the hippocampus proper itself consists of three Cornu Ammonis fields (CA): CA1, CA2, and CA3. However, throughout this document and most commonly in peer reviewed literature, the hippocampus refers to regions CA1, CA2, CA3 and the DG.

Unlike discovering the function of the hippocampus, early investigations by neuroanatomists were remarkably accurate in their depictions of the structure and cellular organization of the hippocampus. In fact, illustrations of the hippocampus produced by Ramon y Cajal in his book *Histologie du Systeme Nerveux* (1911) are still widely used today. This ease in defining the hippocampal neuroanatomy was probably due to the unique laminar organization of the hippocampus. Principal cells are tightly packed into a single layer within each hippocampus region. This organization produces a highly organized unidirectional flow of information known as the ‘trisynaptic circuit’ (See Figure 1.1 for illustration). Briefly, the first synapse (perforant path) consists of axons from principal cells in the EC synapsing onto principal cells dendrites in the DG. The second synapse (mossy fiber pathway) consists of axons from the principal cells in the DG synapsing onto principal cells in CA3. Finally, the third synapse (Schaffer collateral path) consists of axons from CA3 principal cells (Schaffer collaterals) synapse onto CA1 principal cell dendrites. Information received by CA1 principal cells is then exited from the hippocampus proper to the subiculum. Although the ‘trisynaptic circuit’ accounts for general information flow through principal cells, diversions from this circuit and the inclusion of non-principal cells makes the hippocampus circuitry more complex and will be discussed in modest detail in the upcoming sections.

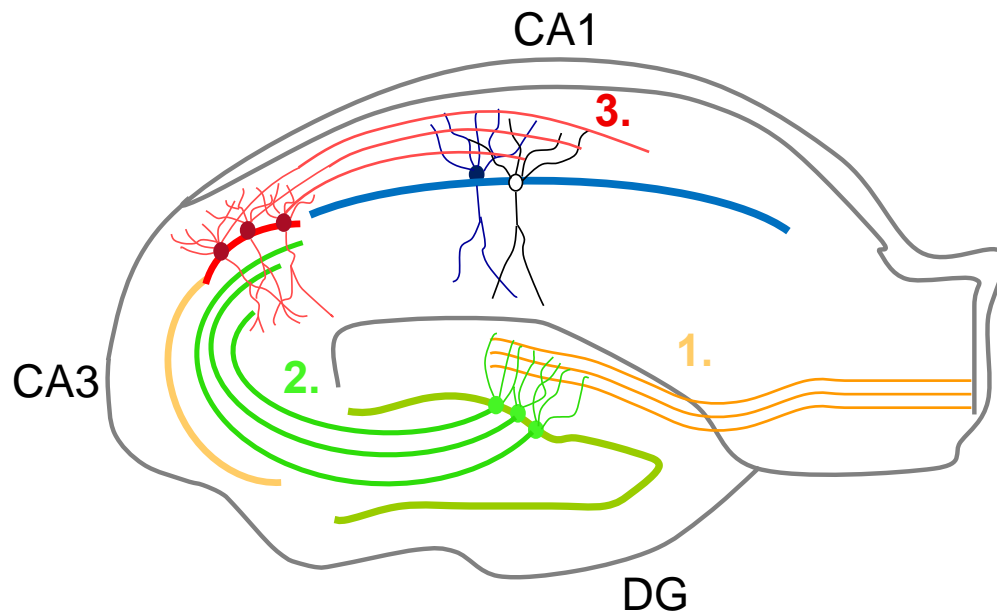


Figure 1.1 Illustration of the trisynaptic circuit: 1) perforant path axons synapse onto dentate granule cell dendrites, 2) dentate granule cell axons (mossy fibers) synapse onto CA3 pyramidal cell dendrites, and 3) CA3 pyramidal cell axons synapse onto CA1 pyramidal cell dendrites.

1.2.3 DENTATE GYRUS ANATOMY AND CIRCUITRY

The dentate gyrus (DG) is the first step in the hippocampus structure to receive information leading towards the processing and production of memories. The entorhinal cortex (EC), which accumulates cortical sensory information, projects unidirectionally mainly to the dentate gyrus through axons called the perforant path. The DG does not reciprocate axonal projections to the EC.

The DG has three distinct layers. The most superficial layer is called the molecular layer or *stratum moleculare*. This layer is relatively cell-free (albeit a small number of interneurons) and contains predominately dendrites from DG principal cells, called dentate granule cells (DGCs). The other major occupant of the molecular layer are the axon fibers projecting from the EC, which make up the perforant path. The layer in which the principal DGCs are tightly packed and aligned is called the granule cells layer or *stratum granulosum*. The cell body of interneurons, called pyramidal basket cells, are also located on the inner boundary of the granule cells layer and the third layer of the DG, called the polymorphic layer. The polymorphic layer, also called the hilus, is the most inner layer of the DG. Numerous cell types are located in the hilus, but the most prominent are the mossy cells. Mossy cell bodies and dendrites reside in the DG hilus, and their axons extend into the inner third of the molecular layer (associational/commissural projection) where they synapse onto both granule cells and GABAergic interneurons. Additionally, the hilus consists of axons from DGCs, called mossy fibers, projecting towards CA3.

1.2.4 CA3 ANATOMY AND CIRCUITRY

The CA3 region contains the second synapse in the trisynaptic pathway. This region can be subdivided into four distinct layers: stratum oriens, stratum pyramidale, stratum lucidum, and stratum radiatum. The most superficial region is the stratum oriens. This region contains the basal (downward extending) dendrites of CA3 pyramidal neurons. Additionally, this region contains axons projecting from CA3 pyramidal neurons and synapsing onto adjacent CA3 pyramidal neurons (recurrent collaterals), as well as CA3 pyramidal axons (Schaffer collateral) projecting to CA1 pyramidal neurons. Lastly, this region contains a plethora of inhibitory interneurons. Just deep of the SO layer, is the stratum pyramidale (SP), which contains the very densely packed pyramidal cell bodies. The stratum lucidum is located deep to SP and contains mossy fibers originating from the DG. Lastly, the stratum radiatum (SR) is the deepest layer and contains CA3 pyramidal neuron dendrites, EC axons projecting onto CA3 pyramidal dendrites (perforant path), and various interneuron subtypes.

1.2.5 CA1 & CA2 ANATOMY AND CIRCUITRY

The anatomical organization and nomenclature for regions CA1 and CA2 are identical. Densely packed pyramidal cells align to form a layer known as the *stratum pyramidale*. Superficial to this layer is the *stratum oriens*, which similarly to area CA3, contains both the apical dendrites of CA1/CA2 pyramidal cells and axons projecting from CA1/CA2 pyramidal neurons into mainly the subiculum. Deep to the *stratum pyramidale* is the *stratum radiatum*, which contains basal CA1/CA2 pyramidal neuron dendrites.

Additionally, this layer contains Schaffer collateral fibers synapsing onto CA1/CA2 pyramidal dendrites. Various types of interneurons are distributed throughout the *stratum oriens* and *stratum radiatum* and sparsely throughout the *stratum pyramidale*. Unlike the CA3 region, the CA1 contains very few recurrent connections, making it less likely to undergo feed-forward bursting.

1.3 SYNAPTIC PLASTICITY

1.3.1 GLUTAMATE RECEPTORS

Glutamate is the main excitatory neurotransmitter in the brain. At synapses, glutamate is stored in vesicles, where it is released from presynaptic neurons in response to nerve impulses. Released glutamate binds to glutamate receptors and produces neuronal activation, which is essential for brain function. Glutamate receptors come in two subclasses: ionotropic and metabotropic. Ionotropic receptors contain a channel pore that opens in response to bound glutamate, allowing the flow of ions and mediating fast synaptic transmission. Alternatively, metabotropic glutamate receptors couple to a G-protein and elicit a second-messenger signaling cascade when bound to glutamate. There are three primary categories of ionotropic glutamate receptors: *N*-methyl-D-aspartate receptor (NMDAR), α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic receptor (AMPA), and kainate receptor (KAR). Each receptor subtype was identified based on their relative affinity for agonists *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-

methyl-4-isoxazolepropionic acid (AMPA), kainate (KA) respectively. However, recent pharmacology studies have identified promiscuity among ionotropic glutamate receptors in their affinity for these agonists.

Ionotropic glutamate receptors are distributed throughout the hippocampus on principal and non-principal neurons, where they mediate the cellular form of learning and memory. It is believed that the cellular basis for learning and memory is intimately related to the ability of a synapse to strengthen or weaken. This phenomenon is called synaptic plasticity. There are several forms of synaptic plasticity that vary based on their persistence over time and the mechanisms by which they occur. Generally, synaptic plasticity is put into two categories: short-term plasticity, in which change in synaptic strength lasts seconds to minutes, and long-term plasticity, in which the change in synaptic strength lasts for an hour or more.

1.3.2 LONG-TERM POTENTIATION

Much experimental work has been performed on long-term plasticity, and specifically investigating mechanisms leading to persistent activity-dependent enhancement in synaptic strength, long-term potentiation (LTP). Long-term potentiation can be induced either by strongly stimulating the presynaptic neuron with a non-physiological relevant high frequency (100Hz, 1sec) train, stimulating the presynaptic neuron with an activation pattern that closely resembles learning in the awake brain (theta burst patterns), briefly pairing presynaptic and postsynaptic stimulation, or chemically

with pharmaceutical agents mimicking other neurotransmitters. All of these protocols for induction of LTP elicit a similar cascade of events.

Although similar mechanisms of LTP have been identified in several synapses throughout the brain, much of the studies have been conducted on Schaffer collaterals projecting to CA1 pyramidal neurons. Electrical stimulation of Schaffer collateral axons causes a release of stored glutamate from presynaptic vesicle. This released glutamate binds to various glutamate receptor subtypes located postsynaptically on CA1 pyramidal neurons. Specifically, AMPA receptors open when bound to glutamate allowing a net positive flow of Na^+ ions into the CA1 pyramidal cells. This causes the cell to become more depolarized (excitatory postsynaptic potential (EPSP)) and more likely to fire an action potential. NMDA receptors are also located on postsynaptic CA1 pyramidal neurons, where they can bind glutamate. However, the NMDAR channel pore is blocked by Mg^{2+} ions when the cell is near resting membrane potentials. Thus, if the Schaffer collaterals are stimulated at low frequencies, the EPSP amplitude will remain constant or slightly increase. However, if high frequency stimulation is applied to Schaffer collaterals, more glutamate is released from the presynaptic neuron and repeatedly activates AMPA receptors. The sustained AMPA receptor activation causes the CA1 pyramidal neuron to become highly depolarized. The Mg^{2+} block will then withdraw from the NMDA receptor and allow a high influx of Ca^{2+} and Na^{2+} into the CA1 pyramidal neuron. This increase in Ca^{2+} elicits signaling cascades with downstream effects of PKC and PKA phosphorylation of AMPA receptors to facilitate more AMPA receptor trafficking and insertion in the postsynaptic CA1 synapse. Now, the same stimulus will

elicit EPSP amplitudes that are increased and remain elevated for several hours. Thus, the CA1 pyramidal neuron is more likely to fire an action potential.

LTP at the perforant path to dentate gyrus granule cells and Schaffer collaterals to CA1 pyramidal neurons are NMDAR dependent. Also, LTP at the perforant path projected onto CA3 pyramidal neurons, and recurrent collaterals interconnecting CA3 pyramidal neurons are also NMDAR dependent. However, the mossy fiber pathway that synapses onto CA3 pyramidal neurons is not NMDAR dependent. Interestingly, KARs are highly expressed pre- and post-synaptic at this synapse, and is one of few regions in the hippocampus where there is a measurable KAR field potential.

1.3.3 MOSSY FIBER LONG-TERM POTENTIATION

Mossy fiber synapses show an unusual form of LTP. Unlike conventional synapses where LTP is NMDAR dependent, mossy fiber LTP can occur and persists even when NMDAR function is blocked (Harris and Cotman, 1986; Zalutsky and Nicoll, 1990). Thus, the mechanism by which the mossy fiber – CA3 synapse undergoes plasticity is fundamentally different than those at most synapses. Similar to classical (Hebbian) LTP, LTP of the mossy fiber – CA3 synapse can be evoked by various high frequency stimulation protocols (Zalutsky and Nicoll, 1990; Hirata et al., 1991; Castillo et al., 1994; Nicoll and Schmitz, 2005) or stimulation patterns that mimic natural granule cell firing patterns (Mistry et al., 2011). The mechanism contributing to MF-LTP is thought to occur predominantly presynaptic, by an increase in presynaptic calcium current causing enhanced glutamate release. Others have suggested that activation of

presynaptic KARs and/or an increase release of calcium from internal stores also contribute to MF-LTP. However, the involvement of postsynaptic mechanisms cannot be ruled out as other studies have demonstrated that a postsynaptic rise in Ca^{2+} (Kapur et al., 1998; Yeckel et al., 1999; Wang et al., 2004) and ephrin-mediated kinases (Contractor et al., 2002; Armstrong et al., 2006) are required for MF-LTP.

1.3.4 LONG TERM DEPRESSION

Long-term depression (LTD) is a persistent weakening in synaptic strength lasting for at least one hour. This form of plasticity ensures that the postsynaptic neuron is less likely to fire an action potential (depressed EPSPs). LTD occurs in several brain regions, including the hippocampus. The discovery of long-term depression (LTD) occurred much later (1980's) than LTP (1973) (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973; Ito and Kano, 1982), which may account for why slightly less is known about the mechanism of LTD. However, we do know that LTD can be induced by very low frequency stimulation patterns (1 to 5 Hz) given for prolonged periods of time (Braunewell and Manahan-Vaughan, 2001). This low stimulation intensity causes only a modest increase in NMDAR-mediated postsynaptic calcium concentrations, which subsequently activates calcium-dependent phosphatases (as opposed to protein kinases in LTP) to remove AMPAR from the postsynaptic cell surface (Lisman, 1989; Mulkey and Malenka, 1992; Mulkey et al., 1993, 1994). The threshold for determining whether Ca^{2+} levels will undergo LTD or LTP is dynamic and depends on the previous history of the synapse.

1.3.5 MOSSY FIBER LONG-TERM DEPRESSION

Low frequency stimulation of mossy fiber axons evokes long-term depression (LTD) at mossy fiber – CA3 synapses. This form of LTD is NMDAR-independent (Kobayashi et al., 1996) and involves activation of presynaptic metabotropic glutamate receptors (Manzoni et al., 1995; Yoshino et al., 1996), causing a reduction in cAMP and subsequent protein kinase A (PKA) activity (Tzounopoulos et al., 1998). The end result is a lower glutamate release probability. Interestingly, the mechanism of MF-LTD is age and developmentally dependent. Rats 2 – 3 weeks postnatal transiently express calcium-permeable AMPAR (CP-AMPA) (Pellegrini-Giampietro et al., 1992; Pickard et al., 2000). As the name suggests, activation of these receptors allows Ca^{2+} ions to flow into the cell. At MF synapses expressing CP-AMPA, the locus of LTD is postsynaptic, involving selective trafficking of CP-AMPA away from the CA3 pyramidal cell surface (Ho et al., 2007). Alternatively, rats older than 3 weeks postnatal express predominantly calcium-impermeable AMPAR (CI-AMPA) postsynaptically at mossy fiber – CA3 synapses, and do not undergo MF-LTD after low frequency depolarization of mossy fiber axons.

1.3.6 SHORT-TERM PLASTICITY

Changes in synaptic strengthening or weakening that lasts for less than 1 hour is considered short-term potentiation or short-term depression, respectively. Short-term plasticity is considered to occur predominantly presynaptic, by a change in the release

probability of glutamate from presynaptic terminals. An increase in glutamate release probability corresponds to short-term potentiation, whereas a decrease in glutamate release probability corresponds to short-term depression. The mechanism by which short-term potentiation occurs is coined the “residual Ca^{2+} hypothesis”. This theory states that enhanced transmitter release (facilitation) during a second stimulus is caused by remaining Ca^{2+} in the nerve terminal after the initial stimulus (Katz and Miledi, 1968; Rosenthal, 1969; Erulkar and Rahamimoff, 1978; Kretz et al., 1982).

There are several possible mechanisms for short-term depression. The most widespread mechanism is use-dependent depletion of release-ready neurotransmitter (glutamate) in the presynaptic terminal (Liley and North, 1953; Betz, 1970). In detail, when the presynaptic neuron is depolarized it releases glutamate from vesicles that are docked and ready to be released into the synapse. With low frequency stimulation, the released glutamate is re-uptaken back into the nerve terminal where it can be recycled and/or replaced with other glutamate-containing vesicles waiting “on deck”. However, with high frequency stimulation, this release-ready pool is not provided enough time to recycle (Simons-Weidenmaier et al., 2006). The result is less glutamate released and lower EPSPs recorded from the postsynaptic neuron. Another mechanism for short-term depression is the reduction of glutamate release mediated by autoreceptors located presynaptically. Each of the ionotropic and metabotropic glutamate receptors have been shown to be localized on presynaptic terminals where they regulate glutamate release after depolarization of the presynaptic neuron (Kerchner et al., 2001; Lee et al., 2002a; Bardoni et al., 2004). Alternatively, short-term depression can also occur through a postsynaptic mechanism. Exposure of ionotropic glutamate receptors to glutamate opens

the receptors and can also put some channels into a nonresponsive (desensitized) state. Desensitization of glutamate receptors located postsynaptically can make the target neuron less sensitive to glutamate (Mennerick and Zorumski, 1996; Larkman et al., 1997). The end result is transiently lower EPSPs.

1.3.7 SYNAPTIC PLASTICITY IN LEARNING AND MEMORY

Virtually every synapse in the brain is dynamically regulated. Mechanisms to either enhance or depress synaptic transmission can either be short-lived or long lasting. We previously described that the hippocampus is necessary for acquiring, retaining, and retrieving new memories. Furthermore, we described how ionotropic glutamate receptors are the key players in mediating several forms of synaptic plasticity, both long-term and short-term, in the hippocampus. However, the question prevails whether synaptic plasticity translates to learning and memory.

It is generally accepted that synaptic plasticity, especially LTP, is a cellular model for learning and memory. The first studies linking synaptic plasticity with learning and memory were correlational. Barnes (1979) and Barnes & McNaughton (1985) found that persistent LTP was statistically correlated with the rate of learning and/or memory retention (Barnes, 1979; Barnes and McNaughton, 1985). Subsequent studies accumulated over 20 years have found similar results. This correlation also occurs in the diseased brain. For example, in the murine model of Alzheimer's disease overexpressing mutant amyloid precursor protein, the decline in memory performance was also

statistically correlated with a decline in LTP, both in vivo and in vitro (Chapman et al., 1999).

In addition to correlation studies, pharmacological blockage of NMDA receptors impairs hippocampus-dependent learning. As described above, NMDAR are necessary for LTP and LTD to occur at most synapses, with the mossy fiber – CA3 synapse being an exception. Studies have shown that administering a potent NMDAR antagonist prevents LTP, and impairs hippocampal-dependent spatial memory, but not hippocampal-independent forms of memory (visual discrimination) (Morris et al., 1986). Several learning paradigms have been used to replicate these findings (Tonkiss et al., 1988; Danysz et al., 1988; Staubli et al., 1989; Tonkiss and Rawlins, 1991; Bolhuis and Reid, 1992; Shapiro and O'Connor, 1992; Cole et al., 1993; Lyford et al., 1993; Caramanos and Shapiro, 1994; Fanselow et al., 1994; Li et al., 1997). The impairment in learning performance is dose dependent and occurs at comparable drug concentrations that impair LTP in vivo and in vitro (Davis et al., 1992). Interestingly, blocking NMDA receptors with an antagonist prevents the development of new memories, but does not affect the retention of memories (Morris, 1989; Staubli et al., 1989; Morris et al., 1990). This suggests that the ability of a synapse to undergo LTP is necessary for the formation of new memories, as opposed to retaining or retrieving older memories. Also, drugs that activate ionotropic glutamate receptors can enhance both LTP and memory. One such class of drugs are the ampakines, which decrease the speed of AMPAR desensitization and activation (Arai et al., 1994, 1996). Interestingly, this drug shifts the AMPAR kinetics to those mimicking NMDA and KARs. Ampakines increase both the probability of LTP and enhance memory encoding (Stäubli et al., 1994; Lynch, 1998).

Synaptic plasticity also occurs naturally during learning. Spatial exploration causes a short-term modulation in perforant path EPSPs (Moser et al., 1993). This increase occurs rapidly at the onset of exploration and gradually declines to baseline over time (15 min). These observations present two interesting points. One, synaptic plasticity occurred in the time scale when learning is most likely taking place, and two, short-term plasticity also has important implications for learning and memory. Indeed, mice with genetically compromised presynaptic proteins (alpha CaMKII, synapsin II, and both synapsin I and synapsin II) but normal LTP, demonstrate impaired short-term plasticity and profound learning difficulties (Silva et al., 1996). However, there have been studies that also demonstrate natural occurring LTP during learning. For example, Ishihara et al. (1997) found a positive correlation between CA3 population spikes caused by mossy fiber stimulation and learning performance in radial arm maze (Ishihara et al., 1997). Furthermore, studies have proven post-hoc that adult animals raised in an enriched environment have enhanced perforant path – dentate EPSP compared to adult animals raised in normal cages (Green and Greenough, 1986). In conclusion, these results demonstrate that both short-term and long-term forms of synaptic plasticity are critical for the acquisition of new memories. Thus, any neuromodulator that would impact either form of synaptic plasticity would have profound effects on our ability to learning and form memories.

1.4 MUSCARINIC ACETYLCHOLINE RECEPTOR MODULATION OF GLUTAMATE RECEPTORS

1.4.1 ACETYLCHOLINE

Glutamate receptors, and consequently synaptic transmission, can be dynamically regulated by other neurotransmitters and their metabotropic receptors. One example is acetylcholine (ACh). Acetylcholine was the first neurotransmitter to be synthesized. Its discovery by Sir Henry Hallett Dale and Otto Loewi awarded them the Nobel Prize in 1936. Its name is derived from its chemical structure, an ester of acetic acid and choline. Acetylcholine has many functions throughout the body. In the brain, acetylcholine is used as a neurotransmitter and plays important roles in learning and memory, arousal, attention, and motivation. Aside from its effects in the brain, ACh is also a primary neurotransmitter at the neuromuscular junction where ACh activates muscles and is a major neurotransmitter in the autonomic nervous system.

The hippocampus receives acetylcholine from cholinergic fibers projecting from the medial septum of the basal forebrain (Dutar et al., 1995). In fact, the human hippocampus contains one of the highest densities of cholinergic fibers (Mesulam et al., 1992). Acetylcholine is synthesized from choline and acetyl coenzyme A by the enzyme choline acetyltransferase and becomes packaged into membrane bound vesicles in the axons of cholinergic fibers. After an action potential propagates down the cholinergic terminal, acetylcholine is released into the synaptic cleft. Released acetylcholine can then bind to acetylcholine receptors located postsynaptically. Alternatively, acetylcholine

is rapidly degraded by acetylcholinesterase into choline and acetate. The choline product can be re-uptaken into the presynaptic terminal and recycled into acetylcholine.

1.4.2 ACETYLCHOLINE RECEPTORS

Like other neurotransmitters ACh exerts its effects by binding to receptors. There are two categories of ACh receptors: nicotinic acetylcholine receptor (nAChR) and muscarinic acetylcholine receptor (mAChR). Similarly to glutamate, they were identified and named after their affinity for the selective agonists: nicotine and muscarine. The nicotinic receptors act as ligand-gated ionotropic receptors, meaning they undergo a conformation change and open a channel pore to allow the flow of ions (Na^+ , K^+ , and Ca^{2+}) when ACh is bound. Alternatively, mAChRs are G-protein coupled (metabotropic) receptors and have a slower response when activated. mAChRs can have either an inhibitory or excitatory effect based upon the mAChR activated and the G-protein in which the mAChR couples.

1.4.3 MUSCARINIC RECEPTORS

As stated earlier, mAChRs are metabotropic receptors that couple to G-proteins and elicit a second-messenger signaling cascade. mAChRs have seven transmembrane regions. There are five types of mAChRs (M1 – M5), each encoded by the gene with corresponding names (m1 – m5). M1, M3, and M5 couple to G_q proteins and stimulate a phospholipase C/ inositol trisphosphate/ intracellular calcium-signaling pathway

(Caulfield and Birdsall, 1998; Lanzafame et al., 2003). The M2 and M4 mAChRs couple $G_{i/o}$ protein and inhibits cAMP activity by downregulating adenylate cyclase. However, mAChRs occasionally vary in the G-protein in which they are bound.

Each of the mAChRs have distinct subcellular localization in the hippocampus (Levey et al., 1995). M1 mAChRs are the most abundantly expressed mAChRs in the human and rodent hippocampus (Cortes et al., 1984; Cortés et al., 1986; Rodríguez-Puertas et al., 1997; Scarr et al., 2007), where it is expressed widely in the cell body and dendrites of principal neurons (CA1, CA2 and CA3 pyramidal neuron and dentate granule neurons). M2 is expressed predominantly in non-principal neurons and discrete bands and puncta surrounding pyramidal neuron soma in CA3 more than CA1, but not expressed in the pyramidal neurons (Rouse et al., 1997). M3 is expressed in pyramidal neurons, neuropil in stratum lacunosum-moleculare, and the outer third of the molecular layer of dentate gyrus. M4 is enriched in non-principal neurons and the inner third of the molecular layer. Additionally, a subset of M2 and M4 mAChRs were localized presynaptically. In conclusion, only the M1 and M3 receptors are predominantly located on postsynaptic principal neurons, where they can modulate synaptic plasticity by directly interacting with postsynaptic ionotropic glutamate receptors, while M2 and M4 mAChRs are predominately located on interneurons. The M5 receptor is the least abundant of the muscarinic receptors and is expressed at low levels throughout brain. At the mossy fiber – CA3 synapse, mAChRs are located both pre- and postsynaptically where they alter synaptic neurotransmission.

1.4.3 FUNCTION OF MUSCARINIC RECEPTORS IN THE HIPPOCAMPUS

The cholinergic system is crucial for learning and memory. Perhaps the best behavioral illustration is in Alzheimer's disease, where degeneration of these cholinergic projections in the hippocampus is the hallmark symptom for deficits in learning and memory, working memory, and attention (Bartus et al., 1982). Additionally, scopolamine, a mAChR antagonist, induces memory deficits (delirium and amnesia) in healthy volunteer (Drachman and Leavitt, 1974). Still today, several pharmaceutical therapies for AD are aimed at inhibiting cholinesterase activity or enhancing cholinergic signaling (Lane et al., 2006). Indeed, enhanced cholinergic signaling has shown to improve LTP and memory deficits in vivo and in vitro (Caccamo et al., 2006; Chen et al., 2006). Furthermore, ablation or inhibition of M1 mAChRs impairs spatial memory encoding, demonstrating a critical role for mAChRs in hippocampal-dependent learning.

Muscarinic acetylcholine receptors are located at both presynaptic and postsynaptic sites where they modulate synaptic plasticity. Presynaptic mAChRs on principal neurons suppress glutamate release, and thereby reducing hippocampal activity (a form of short-term depression) (Valentino and Dingledine, 1981). Meanwhile, postsynaptic mAChR inhibit K^+ conductance, which enhances hippocampus activity (a form of short-term potentiation) (Cole and Nicoll, 1983). Thus, the same muscarinic agonist can have bidirectional affects on short-term synaptic plasticity. Additionally, presynaptic muscarinic receptors have been shown to suppress GABA release onto CA3 hippocampal pyramidal neurons, which is mediated predominantly through M2 mAChRs (Szabó et al., 2010).

Cholinergic activity can also facilitate LTP in various brain regions, including the hippocampus. Application of a cholinergic agonist (Blitzer et al., 1990; Auerbach and Segal, 1996) or stimulation of the medial septum in vivo can induce hippocampal LTP (Galey et al., 1994; Markevich et al., 1997). Indeed, when a muscarinic agonist is perfused onto a brain slice prior to a high frequency stimulus, it can enhance LTP in CA1 without affecting baseline EPSPs (Blitzer et al., 1990; Auerbach and Segal, 1994; Sokolov and Kleschevnikov, 1995; Adams et al., 2004; Shinoe et al., 2005). This phenomenon also occurs in the dentate gyrus and shown to be mediated through M1 mAChRs at both synapses. A recent study using newly developed M1 mAChR selective agonist and congenital knockout mice elegantly corroborates previous literature by demonstrating that M1 mAChR activation produces a robust potentiation of glutamatergic synaptic transmission onto CA1 pyramidal neurons and NMDA-dependent LTP (Dennis et al., 2015). However, M1 mAChRs seem not to be necessary for LTP to occur, since congenital M1 knockout mice demonstrates only mild deficits in CA1 LTP. Interestingly, these mice did experience significant memory deficits in tasks requiring both the cortex and hippocampus to interact (Anagnostaras et al., 2003). In conclusion, significant evidence supports that M1 mAChRs activation enhances synaptic plasticity at CA1 and the dentate gyrus and facilitates the ability to learn.

In contrast to its effects on tetanus-induced LTP (LTP induced by a high stimulus train), mAChR activation by itself can induce LTP (Auerbach and Segal, 1994, 1996) or LTD (McCutchen et al., 2006). Interestingly, the expression mAChR-induced LTP or LTD is dependent on the mAChR agonist concentration. A low dose of mAChR agonist induces mAChR-induced LTP. The mechanism mimics LTP induced by a high frequency

stimulus train, with postsynaptic increase in intracellular calcium and initiation of protein kinases activity is required (Auerbach and Segal, 1994, 1996). Whether mAChR LTP is NMDA-dependent is controversial. However, at higher concentrations muscarinic agonists can induce synaptic depression and LTD following prolonged exposure (Auerbach and Segal, 1996; McCutchen et al., 2006; Dickinson et al., 2009). The mechanism by which this effect is thought to occur is predominantly mediated through M1 and M3 receptors and involves the loss of surface AMPA receptors (NMDAR independent) via a calcium-independent signaling cascade involving dephosphorylation by protein tyrosine phosphatases (Dickinson et al., 2009).

Although significant studies have found that M1 mAChR play a major role in synaptic plasticity and learning and memory, M2/M4 mAChRs also have an important role in synaptic plasticity and learning and memory. An M2 receptor knockout demonstrates significant impairments in working memory, behavioral flexibility, and LTP (Seeger et al., 2004). Moreover, an M2/M4 specific antagonist can induce LTP in CA1 *in vivo*, albeit requires coactivation of M1/M3 receptors. These studies suggest that presynaptic and postsynaptic mAChRs, including specific mAChR subtypes, work in conjunction to modulate learning and synaptic plasticity.

1.4.4 MUSCARINIC MODULATION OF GLUTAMATE RECEPTORS

NMDA receptors

As described in the previous sections, neuronal excitability and synaptic transmission can be altered by interactions between the glutamatergic and cholinergic

systems. The mechanism for this interaction requires crosstalk between the two receptors. For example, NMDARs (specifically the NMDARs containing the GluN1 subunit) co-localize with M1 mAChRs on CA1 pyramidal neurons (Marino et al., 1998). Activation of M1 mAChRs elicits a protein kinase C (PKC) signaling cascade, which proceeds to phosphorylate PYK2 and in turn phosphorylates and activates Src kinase (Lu et al., 1999). Phosphorylation of ionotropic glutamate receptors is subunit and amino acid site specific. For example, the molecular targets of Src are postulated to be three tyrosines on the C-terminus of the NMDA subunit, GluN2A (Zheng et al., 1998). Phosphorylation of this subunit by the Src kinase increases NMDAR trafficking to the cell surface and thereby increased NMDA responses at CA1 synapses. In contrast to M1 receptor activation *enhancing* NMDAR responses at hippocampal CA1 synapse, M1 receptor activation *depresses* NMDAR responses at the mossy fiber – CA3 synapse (Grishin et al., 2005). The mechanism proposed involves calmodulin-activated tyrosine phosphatases. These findings demonstrate that GPCR, specifically mAChRs, can interact with NMDARs through second messenger signaling cascades and that phosphorylation of glutamate receptors are subunit and amino acid site specific.

AMPA Receptors

Muscarinic acetylcholine receptors have also been shown to modulate AMPAR activity. Muscarinic receptors (mainly M1) induce long-term depression (LTD) at CA1 in the hippocampus and cerebellum mainly through changes in AMPA receptor trafficking (Dickinson et al., 2009; Nomura et al., 2012). Interestingly, the mechanism eliciting M1 facilitation of NMDAR activity and M1 LTD of AMPAR activity require similar signaling cascades. mACh receptor-induced LTD at CA1 pyramidal neurons is mediated

by PKC activation leading to phosphorylation of the AMPAR specific GluA2 subunit (Dickinson et al., 2009). PKC phosphorylation of this subunit causes it to dissociate from glutamate receptor-interacting protein 1 (GRIP1), which is a protein involved in the regulation of glutamate receptor trafficking. Dissociation from GRIP1 allows the GluA2-containing AMPAR to diffuse laterally out of the synapse, and undergo endocytosis mediated by an AMPAR receptor auxiliary protein. These results show that mAChRs can also interact with AMPARs through phosphorylation cascades to alter their function at the synapse.

Alternatively, a study by Grishin et al. (2005) demonstrated that activation of M1 mAChRs had no effect on AMPAR current at CA3 pyramidal neurons, but reliably depressed NMDA receptor current at the same synapse (Grishin et al., 2005). This muscarine-induced depression of NMDA receptors was found to be mediated through a signaling cascade involving tyrosine phosphatase.

Kainate Receptors

While previous studies have demonstrated that mAChRs crosstalk with NMDAR and AMPAR, little is known about whether mAChRs can crosstalk with KARs. To date there has been one study directly investigating this interaction. Benveniste et al. (2010) demonstrated that activation of M1 and M3 mAChRs with pilocarpine potentiated GluK2-containing (GluK2/K4 and GluK2/K5) KAR heteromers, but not homomeric GluK2 KARs (Benveniste et al., 2010). This suggests that the subunits, GluK4 and GluK5, are necessary for mAChR potentiation of KARs. Benveniste et al. (2010) furthermore demonstrated that mAChR activation with pilocarpine increased kainate-

induced mossy fiber axon excitability. The mechanism responsible this interaction was not investigated.

This study presents a successful initial step in identifying that crosstalk between the mAChRs and KARs can potentially occur. However, it misses in demonstrating physiological relevance for this interaction. First, the experiments demonstrating mAChRs potentiating heteromeric KARs were performed in *Xenopus* oocytes. Although this model is ideal in deciphering the pharmacology of ligand-gated and voltage-gated ionotropic receptors, the relevance of this model diminishes when used to compare intracellular signaling mechanisms in oocytes to those in humans. Additionally, their experiments in brain slices that showed pilocarpine, a mAChR agonist, enhanced KAR-induced mossy fiber excitability were performed measuring antidromic spikes. An antidromic spike is an electrical impulse that originates in the axon and travels toward the cell body (soma), opposite the conventional action potential direction. Thus, the physiological relevance of these results is also left to be determined. More research is required to determine whether mAChRs can induce physiological relevant changes to KARs and to elucidate the corresponding signaling mechanism by which this interaction occurs.

1.5 DOPAMINERGIC MODULATION OF GLUTAMATE RECEPTORS

1.5.1 DOPAMINE

Dopamine (DA) in the central nervous system acts as a neurotransmitter and belongs to the catecholamine family (Carlsson et al., 1962). Its name is derived from its chemical structure, an amine formed by removing a carboxyl group from a molecule of L-DOPA. Dopamine is produced in several areas in the brain, including the substantia nigra and ventral tegmental area. DA neurons project to many areas throughout the brain through pathways categorized based upon the territories they innervate: mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular (Vallone et al., 2000). The hippocampus receives dopaminergic innervation from the mesolimbic pathway projecting from the VTA (Gasbarri et al., 1994a, 1994b, 1997). Some fibers from the substantia nigra also innervate the temporal (ventral and caudal) hippocampus.

DA neurons synthesize dopamine through a specific metabolic pathway. Briefly, phenylalanine is converted into tyrosine by the enzyme phenylalanine hydroxylase. Tyrosine is then converted into L-DOPA by the enzyme tyrosine hydroxylase (TH). In the final step, L-DOPA is converted into dopamine by the enzyme aromatic L-amino acid decarboxylase. Dopamine is then stored in vesicle in DA neurons terminals where it awaits release. When an action potential terminates at the axon terminal, dopamine is then released from the presynaptic vesicles into the synaptic cleft. Upon release, dopamine can either undergo 1) degradation by enzymes, such as catechol-O-methyl transferase (COMT), 2) reabsorption back into the presynaptic terminal by dopamine

transporter (DAT), or 3) binding to dopamine receptors (DAR) located on the postsynaptic neurons.

1.5.2 DOPAMINE RECEPTORS

Dopamine released from DA neuron terminals bind to receptors with a high affinity for dopamine. Dopamine receptors (DARs) are composed of seven transmembrane domains. They are metabotropic in function, meaning that they couple to G-proteins and elicit their action through second messenger signaling cascades. However, recent studies have demonstrated that dopamine receptors can directly bind (protein-protein interaction) to several other types of metabotropic and ionotropic receptors and alter their function (Liu et al., 2000; Lee et al., 2002b). There are five distinct types of dopamine receptors: D1, D2, D3, D4, and D5. D1 and D5 dopamine receptors (D1-like dopamine receptors) couple to the G_s protein, which stimulates adenylyl cyclase activity and increases cyclic adenosine monophosphate (cAMP) concentration. The D2, D3, and D4 dopamine receptors (D2-like dopamine receptors) couple to the G_i protein, which inhibits adenylyl cyclase activity and subsequently reduces cAMP production.

Increasing evidence suggests that dopamine, and specifically D1 and D5 DARs, play critical roles in learning and memory in the hippocampus. However, the anatomical data demonstrating the localization of specific dopamine subtypes are lacking. This gap in knowledge is due to the lack of antibodies that can convincingly distinguish between D1 and D5 DARs. In an effort to solve this problem, an elegant study performed by Gangarossa et al. (2012) used BAC transgenic mice expressing EGFP (a green

fluorescent protein) tagged under the promoter of either DRDI (D1 gene) or DRD2 (D2 gene) (Gangarossa et al., 2012). D1 dopamine receptors were found scattered on interneurons in the CA1 and CA3 regions. In the DG, D1 DARs were highly expressed throughout the outer and medial molecular layer. In contrast, D2 DARs were expressed almost exclusively in hilar mossy cells (Gangarossa et al., 2012; Etter and Krezel, 2014).

D5 dopamine mRNA is highly abundant in the rat hippocampus (Tiberi et al., 1991), where they are located on principal cell (pyramidal and dentate granule cells) soma, spines, and apical dendrites (Ciliax et al., 2000; Khan et al., 2000; Medin et al., 2011, 2013). Interesting, D5 dopamine receptors have a tenfold higher affinity for dopamine than the D1 dopamine receptor (Sunahara et al., 1991). Thus, older studies using radioligands to investigate D1 DAR expression may have inadvertently detected both D1 and D5 dopamine receptors (Boyson et al., 1986; Dawson et al., 1986; Dubois et al., 1986; Mansour et al., 1990). In conclusion, D1 and D5 dopamine receptors have strikingly different expression patterns in the hippocampus that do not appear to co-localize.

1.5.3 FUNCTION OF DOPAMINE RECEPTORS IN THE HIPPOCAMPUS

DA has many functions in the brain, including regulating memory, mood, motivation, and motor activity. Due to its diverse roles in brain function, dysregulation of dopamine has been link to several neurological disorders, including Parkinson's disease (Ehringer and Hornykiewicz, 1960), drug addiction (Robinson and Berridge, 1993), schizophrenia (Goto and Grace, 2007; Lodge and Grace, 2011), and attention deficit

hyperactivity disorder (Del Campo et al., 2011). Specifically in the hippocampus, DA is released after novel (Ljungberg et al., 1992), salient (Ungless, 2004), aversive (Bromberg-Martin et al., 2010), or rewarding stimuli (Schultz et al., 1993), suggesting that dopamine is necessary for giving context to encoded information (Ihalainen et al., 1999; Bethus et al., 2010). In contrast to the basal ganglia, there is relatively limited DAergic input into the hippocampus. Thus, it is thought that DA's role in hippocampal signaling is more of a tonic nature (Shohamy and Adcock, 2010), although this has yet to be verified.

Both D1-like dopamine receptors and D2-like dopamine receptors mediate critical functions in the brain. However, D1-like dopamine receptors have gained increasing attention for their significant role in regulating both hippocampus-dependent memory and hippocampus-dependent synaptic plasticity (Huang and Kandel, 1995; Lemon and Manahan-Vaughan, 2006; Bethus et al., 2010; Clausen et al., 2011; da Silva et al., 2012). In the dentate gyrus, D1-like dopamine receptors are thought to regulate the threshold for LTP and consequently determine which encoded information becomes a lasting memory (Heinemann et al., 1992; Hamilton et al., 2010). Indeed, in freely moving animals, D1-like dopamine receptors activate during a “novel” or “rewarding” signal, and consequentially increases DG excitability (Hamilton et al., 2010). Furthermore, inhibition of D1-like dopamine receptors also inhibits LTP in the DG (Yanagihashi and Ishikawa, 1992; Kusuki et al., 1997; Swanson-Park et al., 1999). In the CA1 region, D1-like dopamine receptors have been shown to facilitate synaptic plasticity. Briefly, LTP at the Schaffer collateral – CA1 synapse is enhanced with D1-like receptor activation (Otmakhova and Lisman, 1996), while antagonizing D1-like receptors reduces or

prevents LTP both in vitro (Frey et al., 1991; Huang and Kandel, 1995; Otmakhova and Lisman, 1996; Swanson-Park et al., 1999) and in freely behaving rats (Lemon and Manahan-Vaughan, 2006). Interestingly, LTD is also facilitated by activation of D1-like dopamine receptors in CA1 (Chen et al., 1995; Liu et al., 2009) and hindered by D1-like dopamine receptor antagonism (Chen et al., 1995). These results are corroborated by an in vivo study (Lemon and Manahan-Vaughan, 2006).

Another form of synaptic plasticity in which D1-like dopamine has effects is depotentiation. Depotentiation occurs when a low frequency stimulation is delivered soon (maximum 30 minutes) after LTP has occurred, resulting in the ablation of LTP (Staubli and Lynch, 1990; Kulla et al., 1999). Depotentiation is suggested to be a mechanism for forgetting. Activation of D1-like dopamine receptors reduce depotentiation in the DG and CA1 (Otmakhova and Lisman, 1998; Kulla and Manahan-Vaughan, 2000), whereas antagonizing D1-like dopamine receptors prevented this effect (Kulla and Manahan-Vaughan, 2000).

In contrast to the plethora of research done in the CA1 and DG, relatively few studies have investigated the effect of D1-like receptors on synaptic plasticity in the CA3 region. More so than at any other hippocampal synapse, the mossy fiber – CA3 synapse is highly sensitive to intracellular cAMP concentrations (Weisskopf et al., 1994), and cAMP-dependent pathways have been implicated in its induction of LTP (Weisskopf et al., 1994) and LTD (Tzounopoulos et al., 1998). Also, as stated above, D1-like dopamine receptors are expressed on dentate granule cells and CA3 pyramidal neurons. Thus, it is conceivable that dopamine receptors, which modulate cAMP concentrations, would mediate plasticity at this synapse. Indeed, one study demonstrated that mossy fiber LTP

is reduced in guinea pigs in which dopamine is depleted (Ishihara et al., 1991). Also, another study by Kobayashi and Suzuki (2007) demonstrated that application of dopamine induced acute synaptic potentiation at the mossy fiber – CA3 synapse, which was mediated through a cAMP cascade by presynaptic D1-like receptors (Kobayashi and Suzuki, 2007). This potentiation was shown to affect AMPA and NMDA receptors. Although KARs play a critical role in synaptic plasticity at this synapse, KAR function was not investigated. Thus, more studies are required to elucidate the functional role of dopamine, specifically D1-like dopamine receptors, at the mossy fiber – CA3 synapse and corresponding KAR function.

1.5.4 DOPAMINERGIC MODULATION OF GLUTAMATE RECEPTORS

D1-like dopamine receptors alter synaptic transmission and learning and memory by interacting with glutamate receptors. Several studies have investigated the effects of D1-like dopamine receptor activation on NMDAR and AMPAR function and trafficking. However, no study has investigated whether D1 dopamine receptors can affect KAR function or trafficking.

D1-like dopamine receptors interact with NMDAR through both phosphorylation cascades and direct protein-protein interactions. D1-like dopamine activation enhances NMDAR surface expression (Hallett et al., 2006; Paoletti et al., 2008) and localization in the synapse through stimulation of the tyrosine kinase Fyn (Dunah et al., 2004; Tang et al., 2007). Notably, several studies have demonstrated that D1-like dopamine receptors can also interact with NMDAR through direct protein-protein receptor binding (Lee et al.,

2002b). Interestingly, this physical interaction is dynamic. Activation of D1 dopamine receptors reduces formation of this D1-like/NMDA receptor complex (Lee et al., 2002b; Luscher and Bellone, 2008). Interfering with the D1-like/NMDA receptor formation allows NMDAR to traffic (diffuse laterally) to the synapse, thereby increasing NMDAR mediated currents and facilitating long-term potentiation (Argilli et al., 2008; Ladepeche et al., 2013). Furthermore, formation of the D1/NMDAR receptor complex stabilizes D1-like dopamine receptors to the synapse. In conclusion, activation of D1-like dopamine receptors predominately enhances NMDAR currents.

Surprisingly few studies have investigated whether D1-like receptors can interact with AMPARs. However, we do know that D1-like dopamine receptors can also modulate AMPAR activity and trafficking. Early studies demonstrated that D1-like dopamine receptor activation both increased AMPAR phosphorylation through a PKA signaling cascade and potentiated AMPAR current amplitude (Price et al., 1999). Indeed, application of a D1-like agonist increases AMPAR surface expression (Snyder et al., 2000; Gao et al., 2006; Vastagh et al., 2012). These data suggests that D1-like dopamine receptors in general work to enhance AMPAR function.

No study to date has investigated whether D1 dopamine receptors can interact with KARs. Metabotropic receptors, such as muscarinic acetylcholine receptors and dopamine receptors have been shown to interact with both NMDA and AMPA receptors. However, KARs have been neglected. There are several reasons for why KARs should be investigated, including that they are unique to the other ionotropic receptors because not only do they mediate fast synaptic transmission, but they also modulate the amount of neurotransmitters released from presynaptic terminals, and alter neuronal excitability.

1.6 KAINATE RECEPTORS

Kainate receptors were the last of the three ionotropic glutamate receptors to be discovered. Like other ionotropic glutamate receptors, KARs are tetrameric, meaning that they are formed by the combination of four distinct subunits, and share a common subunit structure. Each subunit contains an extracellular N-terminus domain (NTD), followed by four membrane domains (M1, M2, M3, and M4) and a cytoplasmic C-terminus domain (CTD). M1, M3, and M4 are transmembrane domains, named TMD 1, TMD 2 and TMD 3 respectively. The M2 domain is a re-entrant pore loop, which penetrates and exits the cell membrane intracellularly. The ligand-binding domain (LBD) is composed of a segment of the NTD (deemed S1) and a segment of the extracellular loop between TMD2 and TMD3. KARs assemble by two subunits (dimers) binding to an identical dimer, which is referred to as ‘dimer of dimers’. There are five kainate receptor subtypes: GluK1, GluK2, GluK3, GluK4, and GluK5 (formerly GluR5 – GluR7, KA1 and KA2). Subunits GluK1, GluK2, and GluK3 can form functional homomers, meaning that each of the four subunits in the KAR can be entirely GluK1, GluK2, or GluK3. However, GluK4 and GluK5 subunits must combine with GluK1, GluK2, or GluK3 subunits to form a functional KAR. The structural repertoire of KAR subunits is further diversified by Q/R editing of the GluK1, GluK2, and GluK3 subunits, which alter the KAR permeability to Ca^{2+} , and alternative splicing of GluK1 – GluK3 subunits.

Evidently, the GluK4 nor GluK5 subunits do not undergo such post-transcriptional modifications.

1.6.1 LOCALIZATION

Determining the subcellular localization of KARs have been particularly elusive due to the absence of KAR subunit-specific antibodies. Several in situ hybridization studies have revealed tissue specific localization, but this method does not have the resolution to distinguish subcellular level localization (Wisden and Seeburg, 1993; Bureau et al., 1999). Currently, GluK2/K3 and GluK5 specific antibodies are available, although their reliability in immunohistochemistry is debatable. Nevertheless, corroborating evidence can be extracted from these results. The GluK2 and GluK5 subunits are the most robustly expressed subunits (Perrais et al., 2010). The GluK5 subunit is expressed abundantly throughout the brain on principal cells and interneurons (Wisden and Seeburg, 1993). Meanwhile, GluK2 subunits are mostly expressed on principal neurons throughout the brain, including pyramidal neurons and dentate granule cell in the hippocampus (Paternain et al., 2003). In contrast, GluK1 subunits are predominantly expressed on interneurons (Paternain et al., 2003). The GluK3 subunit is expressed at few synapses in the brain, including the hippocampal dentate gyrus (Wisden and Seeburg, 1993). However, the GluK4 subunit has the most restricted expression, mainly express in CA3 pyramidal neurons (Bahn et al., 1994).

1.6.2 PHARMACOLOGY

Each KAR subunit contains a binding site and contributes to the channel pore. The only known KAR agonist that is present in the brain is glutamate. Glutamate activates homomeric GluK1 and GluK2 receptors with a 50% effective concentration (EC_{50}) value in the 100 and 200 micromole (μM) range (Alt et al., 2004; Fisher and Fisher, 2014). However, the EC_{50} value for GluK3 is in the 5mM range (Schiffer et al., 1997; Perrais et al., 2009). Although GluK4 and GluK5 subunits are non-functional as homomers, when expressed in recombinant HEK-293 cells they bind to glutamate. In contrast, the EC_{50} glutamate concentrations for the GluK4 and GluK5 subunits are in the 30 μM and 10 μM range respectively (Fisher and Mott, 2011; Fisher and Fisher, 2014). Thus, the GluK4 and GluK5 subunits are given the distinction as being “high-affinity” subunits.

Kainate receptors were identified based upon their high binding affinity to the neurotoxin kainate acid, compared to the other ionotropic glutamate receptors (NMDA and AMPA receptors). Kainate acid (KA) is a naturally occurring analog of glutamate, which was isolated from the seaweed *Digenea simplex* (Nitta et al., 1958). Injection of kainate acid in the brain causes lesions in brain regions with abundant kainate receptors expression, such as the CA3 region. Similar to glutamate, KA activates the KARs subunits with diverse potency. The GluK1 – GluK3 subunits have a “lower” affinity for KA (K_{DS} near 50nM) than the “high-affinity” subunits GluK4 and GluK5 (K_{DS} 5 – 15nM) (Werner et al., 1991; Herb et al., 1992; Sommer et al., 1992).

The heteromeric kainate receptors predominate in the central nervous system. The abundant co-expression of the GluK2 and GluK5 subunits throughout the brain suggests that the GluK2/K5 heteromeric KARs compose majority of the expressed functional KARs. Interestingly, incorporation of the high-affinity subunits into KAR assemblies alters the pharmacology of the KAR. GluK2/K5 and GluK2/K4 heteromeric KARs are more sensitive to glutamate than GluK2 homomers due to the incorporation of the high-affinity subunits GluK5 and GluK4 (EC_{50} : GluK2/K5 = 10 μ M, GluK2/K4 = 50 μ M, GluK2 = 200 μ M) (Heckmann et al., 1996; Paternain et al., 1998; Barberis et al., 2008; Fisher and Mott, 2011, 2013). Additionally, the GluK4 and GluK5 subunits can also bind to ligands that the other KAR subunits cannot. For example, agonists AMPA, ATPA, and willardiine compounds do not activate GluK2 homomeric receptors, but can bind and activate heteromeric GluK2/K5 receptors (Alt et al., 2004). As expected, these compounds also have an affinity for AMPA receptors, making interpretation from earlier studies misleading.

The lack of KAR subunit specific antagonist has been the Achilles heel of the KAR field. The development and functional characterization of such compounds is essential in understanding the functional contribution of KARs subtypes. Recently, an intense effort was made to synthesize and characterize subunit-selective KAR antagonist against GluK1 (Dargan et al., 2009). They developed a series of KAR antagonist based upon the structure of the willardiine compound, which is thought to be a GluK1 specific agonist. Among this series of compounds were UBP310, UBP302, and UBP316 (also named ACET). However, recent evidence suggests that these compounds are not as specific as initially thought, as the drug UBP310 inhibits GluK2/K5 heteromeric receptor

current in recombinant cells and inhibits postsynaptic KAR transmission at mossy fiber – CA3 synapses where GluK1 and GluK3 expression is limited (Pinheiro et al., 2013).

Another antagonist distinguished as being subunit selective is kynurenic acid. Kynurenic acid is a product of normal metabolism and has neuroprotective properties (Chmiel-Perzyńska et al., 2014). Recent studies have shown that kynurenic acid can discriminate between homomeric GluK2 and heteromeric GluK2/K5 receptors. Kynurenic acid antagonizes the glutamate-induced current through GluK2 homomers at low concentrations ($IC_{50} = 0.3\text{mM}$), but virtually has no effect on GluK2/K5 heteromer current at concentrations up to 3mM (Alt et al., 2004; Fisher and Mott, 2011).

1.6.3 ACTIVATION AND DESENSITIZATION

When a ligand binds to an ionotropic receptor, it can either elicit a conformation change in the receptor that allows the receptor to open its channel pore (activation), or it can have no effect. A ligand that binds to the receptor and has an effect is an agonist, and a ligand that binds to a receptor but has no effect is called an antagonist. Once the ligand leaves the binding site, the receptor will undergo another conformational change to close the channel pore (deactivation). Alternatively, some receptors (including KARs) will decrease its response to the agonist even while the agonist is still bound (desensitization).

Each KAR subunit contains a binding site and contributes to the channel pore. Thus, there are four possible ligand-binding sites on each functional KAR. Previous studies demonstrate that, in heteromeric KARs receptors, glutamate binding to the higher affinity GluK4 or GluK5 subunits is sufficient to activate the channel, albeit to a non-

desensitizing current. Desensitization occurred when the glutamate concentration increased to allow binding to the lower affinity GluK2 subunit (Mott et al., 2010; Fisher and Mott, 2011). These papers concluded that the high-affinity subunits had the distinguished role in opening the KAR, while the low affinity subunit's role was to desensitize the receptor. However, these papers did not test whether binding to only the GluK2 subunit is sufficient to open the receptor, and does subsequent binding to the high affinity subunits ensue desensitization. More recent work with subunit-selective agonists or antagonist (Swanson et al., 2002; Fisher and Mott, 2011; Pinheiro et al., 2013; Fisher and Fisher, 2014) and tethered ligands (Reiner and Isacoff, 2014), suggest that partial occupancy of the KAR binding sites is sufficient to activate the receptor, and the onset of desensitization in heteromeric receptors is determined more by the number of subunits bound to the agonist than by the identity of those subunits.

1.6.4 INTERACTING PROTEINS

Kainate receptors interact with proteins on the plasma membrane that transiently regulate their location and function. For example, KARs can interact with PDZ motif-containing proteins such as postsynaptic density protein 95(PSD-95), protein interacting with C kinase-1 (PICK-1), and glutamate receptor interacting protein (GRIP), which regulate trafficking of KARs to the cell surface and stabilizing KARs to the synapse (Hirbec et al., 2003). It is important to note that these interacting proteins are not specific to KARs and also regulate the function of AMPARs. Interestingly, the apparent

regulation differs between KARs and AMPARs, as these proteins prevent AMPAR internalization, but facilitate KAR internalization (Hirbec et al., 2003).

Two proteins have been identified as true auxiliary proteins to KARs: neuropilin tolloid-like 1 and neuropilin tolloid-like 2 (Neto1 and Neto2) (Zhang et al., 2009; Straub et al., 2011a, 2011c; Tang et al., 2011). Neto1 and Neto2 are single transmembrane proteins that co-localize and associate with KAR in the brain. Neto1 is predominantly located in the hippocampus and is almost completely absent in the cerebellum, whereas Neto2 is almost completely absent in the hippocampus, but is abundantly expressed throughout the cerebellum (Straub et al., 2011b). Neto1 was initially discovered as an auxiliary protein to NMDA (Ng et al., 2009), although it arguably has a more pronounced effect on KAR function. Neither Neto1 nor Neto2 appear to associate with AMPA receptors. Co-expression of Neto1 or Neto2 with KARs in recombinant cells radically alters KAR gating. The most pronounced effect is the reduction in KAR receptor deactivation and desensitization, while recovery from desensitization accelerates (Copits et al., 2011; Straub et al., 2011a; Fisher and Mott, 2013). Thus, the glutamate-induced KAR current persists for longer periods in the presence of Neto. Indeed, this effect is conserved for all KAR subtypes (Fisher and Mott, 2013). Most importantly, the discovery of the Neto-KAR complex corroborates recombinant cell data with brain slice data demonstrating slow deactivation kinetics of KARs.

Current results are conflicting about whether Neto affects KAR trafficking to the synapse (Ng et al., 2009; Zhang et al., 2009; Copits et al., 2011; Straub et al., 2011a; Tang et al., 2011, 2012; Wyeth et al., 2014). Neto knockout mice demonstrate dramatically reduced localization of GluK2 and GluK5 subunits in the synapse (Tang et

al., 2011; Wyeth et al., 2014). Indeed, genetic ablation of Neto1 (but not Neto2) has effects on KAR EPSCs (Straub et al., 2011a; Tang et al., 2011) by showing faster decay and reduced amplitudes. However, KAR EPSCs can still occur. Thus, Neto1 may function more as a regulator of KAR kinetic properties in addition to targeting KARs to the synapse.

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1.6.5 PHOSPHORYLATION

Although relatively few studies have investigated whether KARs can be modulated by GPCRs mACh and dopamine receptors, several studies have shown that KARs can undergo protein kinase C (PKC), CaMKII, and PKA phosphorylation. Activation of PKC inhibits KAR currents in recombinant cells (Dildymayfield and Harris, 1994) and brain slices (Selak et al., 2009). Also in the perirhinal cortex, PKC and PICK1 interacts with KARs to cause KAR-EPSC LTD (Park et al., 2006). These studies suggest that the mechanism for this KAR LTD is that activation of KARs causes an increase in postsynaptic Ca^{2+} , which encourages the uncoupling of the PKC-PICK1-mediated maintenance of KAR EPSC (Staudinger et al., 1997; Park et al., 2006). In the hippocampus, PKC was also shown to induce KAR-EPSC LTD, albeit through a interacting with SNAP-25, PICK1, GRIP, and the KAR subunit GluK5 co-localized postsynaptically (Selak et al., 2009). Furthermore, blocking SNAP-25 activity causes a GluK5-dependent increase in KAR-EPSCs, suggesting a role for SNAP-25 in the PKC-dependent internalization of GluK5. However, these results are not without controversy,

as other studies demonstrate an PKC-dependent increase in KAR EPSCs (Cho et al., 2003).

In contrast to PKC, PKA phosphorylation potentiates kainate-evoked currents in recombinant cells (Raymond et al., 1993; Wang et al., 1993; Kornreich et al., 2007). Furthermore, the phosphatase calcineurin reverses PKA phosphorylation of GluK2 receptors (Traynelis and Wahl, 1997; Coussen et al., 2005), and causes an NMDA receptor and voltage-sensitive Ca²⁺ channel-dependent depression of KAR current (Ghetti and Heinemann, 2000; Rebola et al., 2007).

KARs activity can also be modulated by CaMKII phosphorylation. A stimulation protocol known to induce AMPAR LTP also induces CaMKII phosphorylation of GluK5-containing receptors and depression in KAR-mediated synaptic transmission at the mossy fiber – CA3 synapse (Caporale and Dan, 2008; Carta et al., 2013). However, instead of internalizing the KAR, CaMKII phosphorylation causes the GluK5-containing receptor to uncouple from PSD-95 and laterally diffuse out of the synapse (Carta et al., 2013).

1.6.6 KAINATE RECEPTOR FUNCTION

Postsynaptic

Unlike NMDA and AMPA receptors, KAR-mediated postsynaptic current is found at only few synapses in the hippocampus, including the mossy fiber – CA3 synapse (Castillo et al., 1997; Vignes and Collingridge, 1997) and the Schaffer collateral – CA1

hippocampal interneurons (Cossart et al., 1998; Frerking et al., 1998). At these synapses, KARs EPSCs are small in amplitude (Castillo et al., 1997) and have slow deactivation kinetics when compared to NMDA and AMPA receptors (Frerking and Ohliger-Frerking, 2002; Pinheiro et al., 2013). The slow deactivation kinetics allows the synaptic response to temporally summate and increase spike transmission, thereby facilitating LTP induction (Sachidhanandam et al., 2009).

Also unlike NMDA and AMPA receptors, KARs can influence neuronal excitability of postsynaptic neurons through metabotropic signaling by affecting the slow hyperpolarization (I_{sAHP}) of postsynaptic neurons (Melyan et al., 2002, 2004). The I_{sAHP} is activated in response to an action potential. Voltage sensitive- Ca^{2+} dependent K^+ channels open to hyperpolarize the neuron and prevent further bursting. Thus, the I_{sAHP} is instrumental in spike timing adaptation. Activation of kainate receptors suppresses the I_{sAHP} , thus increasing spike-firing frequency and neuron excitability. Evidence suggests that the mechanism for KAR inhibition of the I_{sAHP} is mediated through KARs directly coupling the $G_{i/o}$ protein, activating PKC (Melyan et al., 2002) and probably PKA, and downstream activation of MAP kinases (Grabauskas et al., 2007). Interestingly, KARs can simultaneously elicit both ionotropic and metabotropic actions at the mossy fiber – CA3 synapse (Fisahn et al., 2005; Ruiz et al., 2005).

Presynaptic

Kainate receptors are also localized both postsynaptic and presynaptic. Kainate receptors can act presynaptically to regulate neurotransmitter release at both inhibitory and excitatory synapses (Lerma, 2003). Presynaptic kainate receptors have been

extensively studied at the mossy fiber – CA3 synapse, where they have been implicated in the large paired-pulse facilitation characteristic of this synapse (Contractor et al., 2001; Lauri et al., 2001; Schmitz et al., 2001; Pinheiro et al., 2007). Indeed, a blocker of Ca²⁺ permeable KARs reduces this synaptic facilitation at the mossy fiber – CA3 synapse (Lauri et al., 2003; Scott et al., 2008). Interestingly, activation of presynaptic kainate receptors can have a bidirectional effect on transmitter release, either enhancing or inhibiting release, dependent upon the concentration of kainate receptor agonist used. Exogenous application of a low concentration of KAR agonist facilitates glutamate release (Kamiya and Ozawa, 2000; Schmitz et al., 2000), while a high dose of KAR agonist produces a depression in glutamate release at mossy fiber – CA3 synapse and Schaffer collateral – C1 pyramidal synapse (Chittajallu et al., 1996; Kamiya and Ozawa, 1998; Vignes et al., 1998; Frerking et al., 2001). Depression of glutamate release by a KAR agonist is sensitive to G proteins blockers, and is thus thought to be through a novel metabotropic function (Frerking et al., 2001; Negrete-Díaz et al., 2006; Salmen et al., 2012). Thus, these data suggest that KAR facilitation of glutamate release is mediated through ionotropic actions, whereas KAR depression of glutamate release is mediated through metabotropic G-protein actions.

1.7 IMPLICATIONS FOR EPILEPSY

1.7.1 KAR IN EPILEPSY

The disease most prominently associated with KARs is epilepsy. Overactivation of kainate receptors can cause seizures. In fact, intraperitoneal injection of kainate acid has served as a reliable animal model of temporal lobe epilepsy (TLE) that mimics several behavior and anatomical changes that occur in human epilepsy. Both human and rodent models of temporal lobe epilepsy demonstrate recurrent mossy fiber synapses onto dentate granule cells (Artinian et al., 2011). These synapses have aberrant kainate receptor EPSCs and thereby alter the activity pattern of dentate granule cells (Epsztein et al., 2005). It is thought that this positive feedback loop could be the epicenter for seizure generation in TLE patients. Indeed, Human tissue from patients with pharmaco-resistant TLE demonstrated an upregulation of GluK1 subunit expression, potentially contributed by the mossy fiber sprouting (Li et al., 2010). Additionally, genetic ablation of the GluK2 subunit prevents the development of seizures (Mulle et al., 1998). Conversely, overexpressing GluK2-containing KARs exhibit spontaneous seizures (Telfeian et al., 2000). As a result of the previous data demonstrating an association between KARs and epilepsy, significant effort has been attributed to demonstrating the therapeutic benefits of GluK1 antagonist to prevent the development of epileptiform bursting in the hippocampal CA3 region in vitro and in vivo (Khalilov et al., 2002; Smolders et al., 2002).

1.7.2 MUSCARINE AND EPILEPSY

Several lines of evidence support a role for mAChRs in epilepsy. For example, pirenzepine, a mAChR antagonist, significantly retards the development of kindled seizures (Eşkazan et al., 1999). Alternatively, in hippocampal slices, when inhibition is removed, the anticholinesterase, eserine, evokes spontaneous epileptiform discharges that are blocked by pirenzepine (Gruslin et al., 1999; Potier and Psarropoulou, 2004). Perhaps the strongest evidence for an association between epilepsy and mAChRs is the epileptogenic effect of the muscarinic agonist, pilocarpine (Cavalheiro et al., 1991). Pilocarpine produces sustained seizures (status epilepticus, SE) by acting on M1 mAChRs (Maslanski et al., 1994; Hamilton et al., 1997; Bymaster et al., 2003). However, the role played by the M1 mAChRs in producing SE in the pilocarpine model, the subsequent neurodegeneration and the development of spontaneous seizures, is not known.

Interestingly, the role played by M1 mAChRs in the induction of SE appears to be critical but limited. Thus, the muscarinic antagonist, atropine suppresses the induction of lithium-pilocarpine-induced SE in rats if injected before pilocarpine, but will not block ongoing lithium-pilocarpine-induced SE (Jope et al., 1986; Morrisett et al., 1987). Given the role of KARs in this model, this observation suggests that mAChR activation induced a KAR-dependent process required for the induction and maintenance of SE. It is further suggested that M1 mAChR activation stimulates KARs by both directly depolarizing pyramidal cells causing glutamate release and by directly potentiating KAR function.

Several studies have demonstrated a synergistic effect of subconvulsant doses of pilocarpine and kainate on the induction of seizures. For example, Millan et al. (1988) reported that a subconvulsant dose of kainate induces motor limbic seizures only when injected into the prepiriform cortex after a subconvulsant dose of pilocarpine (Millan et al., 1988). Similar results were found by De Sarro et al. (1992) in the lateral habenula and pedunculopontine nucleus and by Patel et al. (1988) in the entopeduncular nucleus and dorsal striatum (Patel et al., 1988; De Sarro et al., 1992). These epilepsy models are thought to be predictive of partial complex seizures, suggesting that the interaction between muscarinic and KARs may contribute to the evolution of seizures of this type. In contrast, KARs do not appear to be involved in models of more generalized seizures such as picrotoxin or bicuculline, since KAR antagonists do not block epileptiform activity in these models.

1.7.3 DOPAMINE AND EPILEPSY

The dopaminergic system has been associated with epilepsy and seizures for over a century. The basal ganglia (primary source of dopamine in the brain) are significantly involved in seizure initiation and determining seizure threshold (Trimble, 1977; Toone, 1991). Forty years ago, epilepsy was thought to be a disease of dopaminergic hypoactivity (Lamprecht, 1977; Starr, 1996). This theory was conceived as a result of studies demonstrating that application of a general dopamine receptor agonist reduces seizure susceptibility (Lamprecht, 1977; Lal, 1988), whereas blockage of dopamine receptors induced seizures (Laird et al., 1984; Jann et al., 1993). Indeed, it was

discovered that a class of antipsychotic drugs that were known to precipitate seizures were general dopamine receptor antagonist (Seeman, 1981). We now know that there are two subtypes of dopamine receptors: D1-like and D2-like. These dopamine receptors have opposite effects on seizure susceptibility. D1-like dopamine receptor activation precipitates seizures (Starr et al., 1987; al-Tajir et al., 1990a, 1990b), while D2-like dopamine receptors reduce seizure susceptibility (al-Tajir et al., 1990a; Al-Tajir and Starr, 1991).

These studies demonstrate a long withstanding association between the dopaminergic system and epilepsy. Although we have now identified two subclasses of dopamine receptors and their opposing roles in seizure generation, the mechanism for how the interaction occurs is not known, nor is it known how the dopaminergic system is altered in chronic models of temporal lobe epilepsy. Further studies are needed to elucidate these mechanisms.

1.8 IMPLICATIONS FOR EXERCISE

1.8.1 EPILEPSY AND PHYSICAL EXERCISE

Over the past decade, physical activity has been noted as a powerful tool in the battle against cognitive impairment. In healthy adults, physical activity has been shown to improve performance on memory test and counterbalance the age-related decline in hippocampal volume and learning and memory performance tasks (Erickson et al., 2011).

These results are indiscriminant on the type of physical activity, but are predicated on the intensity of the exercise, with greater cognitive benefits occurring following acute or repetitive (chronic) moderate to vigorous physical activity.

Exercise has also been shown to prevent the development and reduce the progression of neurological diseases. Several reviews exist outlining the beneficial effects of exercise on Alzheimer's disease and Parkinson's disease (Paillard et al., 2015). However, in this document we will focus our attention on exercise's benefits on epilepsy.

The stigma of exercise being harmful for people with epilepsy was prevalent until recent years. It was recommended by the American Medical Association in 1968, that people with uncontrolled epilepsy should not be allowed to participate in physical activity for the fear of inducing seizure and increased risk of injury for the person with seizures and others within the vicinity (American Medical Association Committee on the Medical Aspects of Sports, 1968). Due to much controversy, this recommendation was relaxed six years later to allow the participation of exercise when it was deemed beneficial for social integration (Corbitt et al., 1974).

Recent evidence in human and animal models demonstrates that physical activity can prevent the development in seizures. In animal models, prior exposure to exercise can reduce brain cell loss and neuronal damage secondary to brain insults (Arida et al., 2013). One study investigating the effects of physical exercise during post-natal development showed that when submitted to daily exercise for forty days, rats presented delayed onset and reduced intensity of pilocarpine-induced seizure during midlife (Gomes da Silva et al., 2011). Additionally, another study demonstrated that animals exposed to acute and

chronic physical exercise required more electrical stimulus to the limbic brain region (kindling) to provoke seizures than controls (Arida et al., 2008). Although there are several studies in animal models demonstrating physical activity can prevent seizures and the development of epilepsy, the possible preventative effects of exercise on epilepsy in humans are still uncertain. There is only one study that has tested this question directly. According to a Swedish population-based cohort of 6,796 individuals examined for 40 years, participants who presented low cardiovascular fitness at the age of 18 had an increased risk of developing epilepsy later in life (Nyberg et al., 2013). Thus, this study suggests that engaging in physical activity early in life has protective effects on the development of epilepsy later in life. Although more studies in humans are needed to answer the question whether physical activity can protect the brain from developing epilepsy, the consensus among several animal studies and one human study suggest that physical exercise can be used as a powerful tool to protect the brain against the development of repetitive seizures.

As opposed to the development of epilepsy, more human studies have sought to answer the question whether exercise can reduce seizure frequency in individuals who have already been diagnosed with epilepsy. People who practice regular physical exercise report fewer seizures than inactive individuals (Roth et al., 1994). However, human intervention studies have rendered mixed results. One study examining fifteen women with pharmacologically intractable epilepsy were administered aerobic activity for fifteen weeks and presented a decrease in seizure frequency (Eriksen et al., 1994). However, another study including twenty-one patients with uncontrolled epilepsy experienced no change in seizure frequency two weeks before, during, or after a four-week exercise

program (Nakken et al., 1990). Although these studies provide contradictory results about whether exercise reduces seizure frequency in people with epilepsy, they both support the notion that engaging in physical exercise is not a seizure precipitant. Furthermore, other studies examining acute bouts of maximal and sub-maximal physical exercise in people diagnosed with epilepsy demonstrated reduced epileptiform discharges after physical exertion compared to before the exercise bout (Camilo et al., 2009; Vancini et al., 2010; de Lima et al., 2011). Animal studies demonstrate a more definitive role of epilepsy reducing seizure frequency. Rats with pilocarpine-induced epilepsy who submitted to a physical training program presented a lower frequency of spontaneous seizures than controls (Westerberg et al., 1984; Arida et al., 1999, 2004, 2009a, 2013). Other animal models of epilepsy have demonstrated similar results (Rambo et al., 2009; Reiss et al., 2009; Tutkun et al., 2010).

Lastly, physical activity can also reduce the comorbid mental disorders associated with epilepsy. As stated earlier, people with epilepsy are more like to suffer from other mental and psychosocial disorders, such as mood and personality disorders and behavioral problems. Depression, deficits in learning and memory, stress and anxiety are among the most common. In physically healthy adults, engaging in regular physical exercise has shown to consistently improve emotional well-being (Roth et al., 1994). Similarly, people with epilepsy who practice physical exercise present lower levels of depression independently of other factors like age, gender, seizure frequency or stressful life experience (Eriksen et al., 1994; Roth et al., 1994; de Lima et al., 2013). Other studies have reported positive effects of physical exercise training on mental state, self-esteem, social integration, several anxiety scales, fatigue, sleep problems, and overall

quality of life (Nakken et al., 1990; Eriksen et al., 1994; McAuley et al., 2001; Arida et al., 2009b). Thus, physical exercise can improve overall mental health and emotional wellbeing in people with epilepsy. In individuals with managed epilepsy, physical activity's benefits of improving mental health may prove more beneficial than the possible reduction of seizure frequency.

Mental health disorders in people with epilepsy may result from the complicated interactions between the antiepileptic medications, epilepsy surgery, and social interventions (such as restricted license to drive and fear of seizure in public). Interestingly, epilepsy and mood disorders share dysfunctions in neurotransmitter systems (dopamine, acetylcholine, glutamate, GABA, etc.), which might contribute to their coexistence and bidirectional relationship (Kanner, 2007; Thapar et al., 2009; Arida et al., 2012).

Several theories for how exercise mediates benefits on cognition in the healthy and diseased brain exist. The most prevalent theory is that an increase in cardiovascular blood flow that occurs during physical activity initiates the synthesis of several growth neurotrophic factors and neurotransmitters, which ultimately results in increased neurogenesis, angiogenesis, and synaptogenesis. The alteration of the human brain caused by acute and chronic exercise leads to a change in the fundamental network and structure in the brain compared to the sedentary brain. Thus, this new brain is efficient at preventing the development of seizure, reducing seizure frequency, and ameliorating psychosocial comorbidities associated with epilepsy (such as depression, learning and memory deficits, anxiety, stress, and schizophrenia).

1.8.2 ACETYLCHOLINE AND EXERCISE

The cholinergic system and exercise have a close interconnected relationship. First, exercise alters the cholinergic system in the brain. Particularly in the hippocampus, acetylcholine release increases 2-fold during moderate intensity walking in young (Nakajima et al., 2003) and aged adult rats (Uchida et al., 2006). Because depletion of acetylcholine is causal for memory impairment, this increase can be beneficial when applied to diseased brains. In addition to an increase in acetylcholine release, exercise can also reduce degradation of released acetylcholine. One study demonstrated that acetylcholinesterase was inhibited in the whole brain by 30 – 50% 2hr to 5hr post forced swimming exercise (Tsakiris et al., 2006). Thus, these studies show that exercise can modulate the amount of acetylcholine in the extrasynaptic space by both increasing the amount of acetylcholine release and inhibiting the amount of acetylcholine degraded, which seems to have beneficial effects in the diseased brain.

A theoretical mechanism for how exercise mediates its beneficial effects in the brain is thought to be triggered by an increase in cerebral blood flow. Several studies have shown that acetylcholine is necessary for the exercise-induced increase in cerebral blood flow. For example, acetylcholine released in the cerebral cortex and hippocampus can increase regional blood flow in the cerebral cortex and hippocampus in anesthetized rats (Biesold et al., 1989; Cao et al., 1989; Kurosawa et al., 1989; Adachi et al., 1992; Sato and Sato, 1992, 1995; Sato et al., 2004). Thus, independent of periphery stimulation, an increase in acetylcholine can increase cerebral blood flow. Furthermore, walking at

moderate speed can increase cerebral blood flow in the hippocampus and cerebral cortex independent of systemic arterial pressure via activation of intracranial cholinergic fibers and release of ACh from these nerves. Thus, physical exercise stimulates the central cholinergic system to increase cerebral blood flow independent of periphery stimulation.

Lastly, the cholinergic system is involved in the regulation of adult hippocampus neurogenesis. Selective lesioning of the medial septum system negatively affects the proliferation of neural stem cells (NSCs) and neuronal progenitor cells (Cooper-Kuhn et al., 2004; Mohapel et al., 2005; Van Der Borght et al., 2005), whereas the administration of acetylcholine esterase (AChE) inhibitor promotes NSC/neuronal progenitor cell proliferation (Mohapel et al., 2005; Narimatsu et al., 2009). Specifically, M1 muscarinic receptors are necessary for cholinergic-induced NSCs proliferation. Furthermore, exercise-induced promotion of aged NSC proliferation was abolished by specific lesioning of the septal cholinergic system, demonstrating the cholinergic system is necessary for exercise-induced neurogenesis (Itou et al., 2011). Thus, the cholinergic system also mediates exercise-induced increase in neurogenesis.

1.8.3 DOPAMINE AND EXERCISE

The effects of exercise on dopamine levels in the hippocampus have rendered mixed results. Some studies find that exercise significantly increases dopaminergic content (Chaouloff et al., 1986). For example, De Castro *et al.* confirmed an increase in dopaminergic metabolism in the whole brain of rats sacrificed 48 hours after an 8-week running training period (de Castro and Duncan, 1985). A related study used a 1-week

training model to examine brain dopamine metabolism and found that the sum of DOPAC and homovanillic acid was increased with running and remained elevated throughout the first hour of recovery (Chaouloff et al., 1987). Indeed, more recent studies have demonstrated increases in dopamine concentration and dopamine receptor binding (Greenwood et al., 2011). These results may be a result of increased bursting/activation of dopamine neuron in the VTA due to wheel running (Wang and Tsien, 2011). Additionally, chronic voluntary and forced wheel running demonstrate increase tyrosine hydroxylase and well as dopamine in several brain areas (Dishman et al., 1997; Sutoo and Akiyama, 2003; Droste et al., 2006; Foley and Fleshner, 2008; Greenwood et al., 2011). Thus, exercise seems to mediate an increase in dopamine production and release in the brain.

Other studies have found no change or a decrease in dopamine levels after acute or chronic physical exercise (Brown and Van Huss, 1973; Lukaszyk et al., 1983; Sudo, 1983; Acworth et al., 1986). Notably, no study has found a significant decrease in dopaminergic content specifically in the hippocampus after an acute or chronic physical exercise-training plan, although several studies report a significant increase in dopamine content in the hippocampus (Chaouloff et al., 1987; Bailey et al., 1992). Thus, it seems that exercise, when it does have an effect, tends toward increasing dopaminergic content in the hippocampus.

1.9 SIGNIFICANCE REVISITED

As discussed above, interplay between the glutamatergic system and cholinergic/dopaminergic system in the hippocampus are required for learning and memory. Specifically, kainate receptors play an important role in the physiology and pathophysiology of synaptic transmission. However, there have been no studies that have systematically examined whether mAChRs or D1 DARs in the brain alters kainate receptor function. To fill this significant knowledge gap, we first needed to identify pharmacological tools that can be used to identify specific kainate receptor subtypes. This tool will be useful to differentiate which kainate receptor subtypes are responsible for mediating KAR synaptic transmission in the normal hippocampus. After identifying which kainate receptor subtypes are responsible for KAR synaptic transmission, we must determine whether mAChRs and/or D1-like DARs can alter KAR synaptic transmission in the normal hippocampus. Several lines of evidence suggest that interaction between KARs and muscarinic/dopaminergic receptor may occur; however, this will be the first directly tested proof of concept. Understanding whether this interaction occurs and identifying which receptors are responsible, will reveal a novel mechanism by which the normal brain undergoes synaptic plasticity. Additionally, it will provide newfound mechanisms by which neurological disorders, such as epilepsy, and neurological therapies, such as exercise, may illicit their effects. Lastly, we will determine how the dopaminergic system is altered in the chronically epileptic brain. Understanding long-term changes in dopaminergic content in the epileptic brain will provide us with a

comprehensive view of where the dysfunctional interaction between the glutamatergic and dopaminergic system exists. These studies are important, because they can shed light on a novel mechanism that occurs in the brain, which are necessary for brain health. Furthermore, understanding these mechanisms can unlock targets for therapies for neurological diseases.

CHAPTER 2

GENERAL METHODS

2.1 INTRODUCTION TO ELECTROPHYSIOLOGY

Electrophysiology is the study of the flow of ions through a cell or group of cells, and it also refers to the specific techniques required to measure this flow of ion. Every thought, every nerve impulse, every movement, and every heartbeat is controlled by highly specific and precisely timed flow of ions through cell membranes. Interfering with this flow could have a wide range of effects, from beneficial (as in exercise) to fatal (such as a prolonged seizure). Thus, having a detailed understanding of the ions and the transporters/receptors that mediate their passage through the cell membrane is indispensable when deciphering neurological disorders and developing novel therapies to remedy them.

Electrophysiologists use the basic physical laws of electricity to understand the electrical activity of cells and the receptors inhabiting them. By far, the most useful law of electricity in electrophysiology is Ohm's law. Ohm's law states that voltage (V) or electrical potential (ΔV) is equal to current (I) multiplied by resistance (R): $V=IR$. In a cell, voltage is a measure the electrical potential difference across the cell membrane, which in most cells is negative at rest. The current is a measure of the amount of charged particles flowing through the cell membrane. Last, but certainly not least, resistance is a

measure of the friction or resistance on ion flow, which is primarily a function of technical access to the cell. Electrophysiology equipment allows us to measure and modify eACh electrical component of the cell, as the experimental procedure sees fit.

2.1.1 ELECTRICAL POTENTIAL

Ions flow through plasma membranes because there is a difference in electrical potential (volts, V or ΔV) between the interior and exterior of cells. The ability of every cell to maintain an electrical potential is derived from two main characteristics. First, the distribution of ions inside the cell differs from those outside the cell. Most cells have a higher concentration of potassium (K^+) and a lower concentration of sodium (Na^+) and calcium (Ca^{2+}) inside the cell than in the extracellular space. Ion specific pumps that require energy maintain this ionic gradient. Furthermore, the cell's interior contains negatively charged proteins that are impermeable to the cell membrane. Taken together, the interior electrical potential of most cells at rest is negative, compared to the extracellular space.

Secondly, the plasma membrane itself has selective ion permeability. Every cell is enveloped by a plasma membrane consisting of lipids and proteins. Lipids are hydrophobic (repel water), which causes them to arrange into a phospholipid bilayer that is impermeable to ions. Ions are transported through this otherwise impermeable layer through specialized proteins, ion channels and transporters. The selective permeability of the cell membrane for each ion depends on the amount and states of its varied ion channels. During rest, most cells are highly permeable to K^+ , moderately permeable to

Cl⁻, and only slightly permeable to Na⁺ and Ca²⁺. These two properties of the cell, the varied distribution of ions and the selective permeability of ions, are necessary for a cell to maintain its electrical potential.

2.1.2 VOLTAGE CLAMP

Voltage clamp is an experimental paradigm used by electrophysiologists to measure ion currents through a cell membrane by holding (“clamping”) the electrical potential inside the cell constant. In theory, when ionotropic channels, such as kainate receptor, bind to glutamate, they open and allow Na⁺ ions to flow into the cells and K⁺ ions to flow out of the cell, and thereby mediating an inward current of positive ions. By holding voltage constant, a change in current is directly related to the function of the receptors and channels expressed on the cell. Thus, we can reliably measure and confidently interpret KAR kinetics by measuring the current produced by ions flowing across the cell membrane at a given voltage.

2.2 WHOLE CELL PATCH-CLAMP ELECTROPHYSIOLOGY

In patch clamp electrophysiology, we are measuring the flow of ions across a single cell or single channel. This technique was first demonstrated by Erwin Neher and Bert Sakmann (Neher and Sakmann, 1976) in 1976, and later won the Nobel Prize in Physiology or Medicine in 1991. Changes in current and voltage are sensed by an electrode. Several different configurations of this technique exist, one of which is whole-

cell recording. To acquire a whole-cell configuration a glass electrode is placed on the surface of a cell and mild suction is applied to rupture the membrane, allowing for the contents of the cell cytoplasm to diffuse and exchange with the internal solution inside the glass pipette. The whole-cell configuration produces a convenient method to directly apply substances into the cell in order to study the electrical currents and properties from the entire cell, or we may perfuse agonist or antagonist on the extracellular space of the cell to measure changes in ions flow (current) through receptors.

Whole-cell electrophysiology is used to study the movement of electrically charged ions, commonly through voltage-gated or ligand-gated channels. Glutamatergic receptors, such as kainate receptors, are permeable to Na^+ and K^+ ions. When these receptors open, Na^+ enters the cell and creates a more positive intracellular environment (depolarizes). In an effort to maintain the cell electrical potential at -70mV (in voltage clamp), a negative current is applied to the cell. Thus, a KAR response from voltage-clamped data is negative.

2.2.1 EXPERIMENTAL PROTOCOL FOR WHOLE-CELL PATCH CLAMP RECORDINGS

HEK-293T cells (GenHunter, Nashville, TN, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin. Cells were passaged with a 0.025% trypsin/0.01% EDTA solution in phosphate-buffered saline (10 mM Na_2HPO_4 , 150 mM NaCl, pH 7.3). Full length cDNAs for the GluK or Neto1 subunits in mammalian expression vectors were transfected into cells using calcium phosphate precipitation as

previously described (Mott et al. 2010). Plasmids encoding rat GluK2 (Q), GluK4 or GluK5 were provided by S. Heinemann (Salk Institute, San Diego, CA, USA). Human Neto1 was provided by S. Tomita (Yale University, New Haven, CN, USA). Plasmids were transfected at ratios of 1:3:4 (GluK2: GluK4 – GluK5: Neto1), previously shown to optimize formation of heteromeric receptors (Barberis et al. 2008) and Neto1 assembly with KARs (Fisher & Mott, 2012). To identify transfected cells, we co-transfected 1 μ g of a cDNA encoding a single-chain antibody recognizing the hapten 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx; Chesnut et al. 1996). Positively transfected cells were isolated using phOx-coated beads 18 – 28 hr after transfection and plated onto glass coverslips treated with poly-L-lysine and collagen.

Whole-cell recordings were performed on isolated HEK-293T cells 40 – 52 h after transfection under voltage-clamp conditions. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota, FL, USA) on a two-stage puller (Narishige, Japan) to a resistance of 5 – 10 M Ω and filled with a solution containing (in mM): 130 CsGluconate, 5 CsCl, 10 Hepes, 5 CsBAPTA, 2 MgCl₂, 2 MgATP, 0.3 NaGTP (pH 7.3, 290-300 mosmol l⁻¹). Cells were continually perfused with an external solution containing (in mM): 150 NaCl, 3 KCL, 10 Hepes, 1 CaCl₂, 0.4 MgCl₂ (pH 7.4, 295-305 mosmol l⁻¹). For whole-cell recordings glutamate and antagonists (ACET and kynurenate) were applied to cells using a stepper solution changer (SF-77B, Warner Instruments, Hamden, CT, USA). The time course of drug application was <50 ms in the whole-cell recording configuration as determined using a diluted external solution applied to the electrode. Rise times (10 – 90%) of the junction potential at the open tip were consistently faster than 400 μ s and were tested using a

diluted external solution. All antagonists were applied to cells before glutamate per sweep. Current recordings were amplified (Axopatch 200B; Molecular Devices, Foster City, CA, USA), filtered (1kHz) and digitized at 10 kHz using a Digidata 1320 analog to digital board (Molecular Devices) and stored on a computer hard drive for off-line analysis.

2.3 FIELD POTENTIAL ELECTROPHYSIOLOGY

Field-potential electrophysiology is performed extracellularly, as opposed to whole cell record which is recorded intracellularly. Once the tissue is prepared a stimulating electrode is placed into the tissue to depolarize a population of neurons. Field potential electrophysiology allows electrophysiologists to record activity of a population of neurons by placing the recording electrode within a cell body region or within the dendrites or axons of the neuronal population. Activation of these neurons results in a summation of the extracellular potentials creating either a field inhibitory postsynaptic potential (fIPSP) or a field excitatory postsynaptic potential (fEPSP).

Because these recordings are extracellular and measures collective cell activity, the placement of the recording electrode and stimulation electrode is critical to ensure proper interpretation of the data. Relative to the electrode, neuronal activation can create what is denoted as an extracellular sink or source. The flow of positively charged ions into a cell or dendrite leaves the extracellular space more negative, creating an extracellular sink, while a positive waveform (source) is created by the current that leaves the cell (generally at the cell body) to complete the local circuit. Field potential

recordings in the mossy fiber – CA3 synapse are at particular risk for improper electrode placement, as the CA3 region contains mossy fibers input as well as the associational/commissural fibers. Pure stimulation of the mossy fibers, elicited through activation of dentate granule cells, would evoke an extracellular sink in area CA3 stratum lucidum. However, if the placement of the recording electrode was not precise an extracellular source evoked from the CA3 A/C fibers would contaminate the response. The overall response would be a fEPSP comprised of both a downward sink and upward source. Thus, in experiments involving the mossy fiber – CA3 synapse, steps are taken to ensure pure mossy fiber stimulation and recording.

2.3.1 EXPERIMENTAL PROTOCOL FOR FIELD POTENTIAL RECORDINGS

Hippocampal slices were made from postnatal day (P) 21 – P28 and P45 – P60 Sprague-Dawley rats as described previously (Iyengar and Mott, 2008). Briefly, animals were deeply anesthetized with isoflurane and decapitated with a guillotine. The brain was removed under ice-cold (4°C), sucrose-based ‘cutting’ artificial cerebrospinal fluid (aCSF) that contained (in mM): 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 248 sucrose, 0.5 CaCl₂, and 5 MgSO₄ (350mOsm). The use of this solution during the cutting process enhances neuronal survival by limiting excessive excitation. Transverse slices were made with a vibratome (Leica VT1000S, Nussloch, Germany) at 500 μm and incubated for at least one hour at room temperature in aCSF containing (in mM): 125 NaCl, 2.7 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 glucose, 0.5 CaCl₂, 5 MgSO₄, 20 μM D-

AP-5, and 1 mM kynurenatate (305 mOsm; pH=7.4). Both the sucrose-based and standard aCSF were oxygenated with 95% O₂/ 5% CO₂.

After 1 hour, individual slices were transferred to a submersion chamber maintained at 32 – 34 °C, held in place by a bent piece of platinum wire resting on the surface of the slice, and perfused continuously at 3 – 4 ml/min with recording aCSF containing (in mM): 125 NaCl, 2.01 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 glucose, 2 CaCl₂, 1 MgSO₄. Glass electrodes were pulled from borosilicate glass to achieve 2 – 3 MΩ and filled with recording aCSF. Extracellular dendritic field potentials recording were made from the stratum lucidum of the CA3 region of the hippocampus, which was visually identified with a light microscope (See Figure 2.1). Synaptic currents were evoked with a monopolar platinum-iridium glass electrode positioned in the stratum lucidum. For AMPA-fEPSPs recordings, we recorded composite MF-fEPSPs, which were primarily composed of AMPA-fEPSPs (~ 93%) (Contractor et al., 2003), with aCSF containing 10 μM MK-801, 50 μM picrotoxin, and 1 μM CGP 55845 to block NMDA, GABA_A, and GABA_B receptors, respectively. We defined synaptic currents as MF-fEPSPs if they showed characteristically large paired-pulse facilitation (Salin et al., 1996), had a rapid rise time and short latency, and were inhibited by >70% by the group II-selective metabotropic glutamate receptor (mGluR) agonist (2S,2'R,3'R)-2-(2'3'-Dicarboxycyclopropyl) glycine (DCG-IV, 1 μM), which was bath applied at the end of some experiments. To isolate KA-fEPSPs recordings, we recorded from CA3 pyramidal cells in the presence of the aCSF containing the AMPA antagonist GYKI53655 (50 μM) in addition to the GABA_A, GABA_B and NMDA receptor antagonist cocktail. Once KAR-fEPSPs were isolated, a four pulse train stimuli (100 Hz, 0.1 ms/stimulus, cathodal,

monophasic, rectangular constant current pulses) were delivered every 30 sec through monopolar, platinum-iridium stimulating electrodes (FHC Inc., Bowdoin, ME) referenced to the bath ground. KAR-fEPSPs were stable for a minimum of 20 min. prior to pilocarpine (300 nM) perfusion onto the slice for 10 – 15 min. to activate predominately M1 mAChRs. In experiments where M1 mAChRs or PKC were inhibited, telenzepine (100 nM) or chelerythrine (5 μM) respectively were applied concurrently with AMPA, GABA_A, GABA_B, and NMDA antagonists. To resolve small KAR-fEPSPs that were obscured by stimulation artifacts, we digitally subtracted a “template” stimulation trace that was acquired after application of an AMPA/KAR antagonist (CNQX, 50 μM) at the end of the recordings.

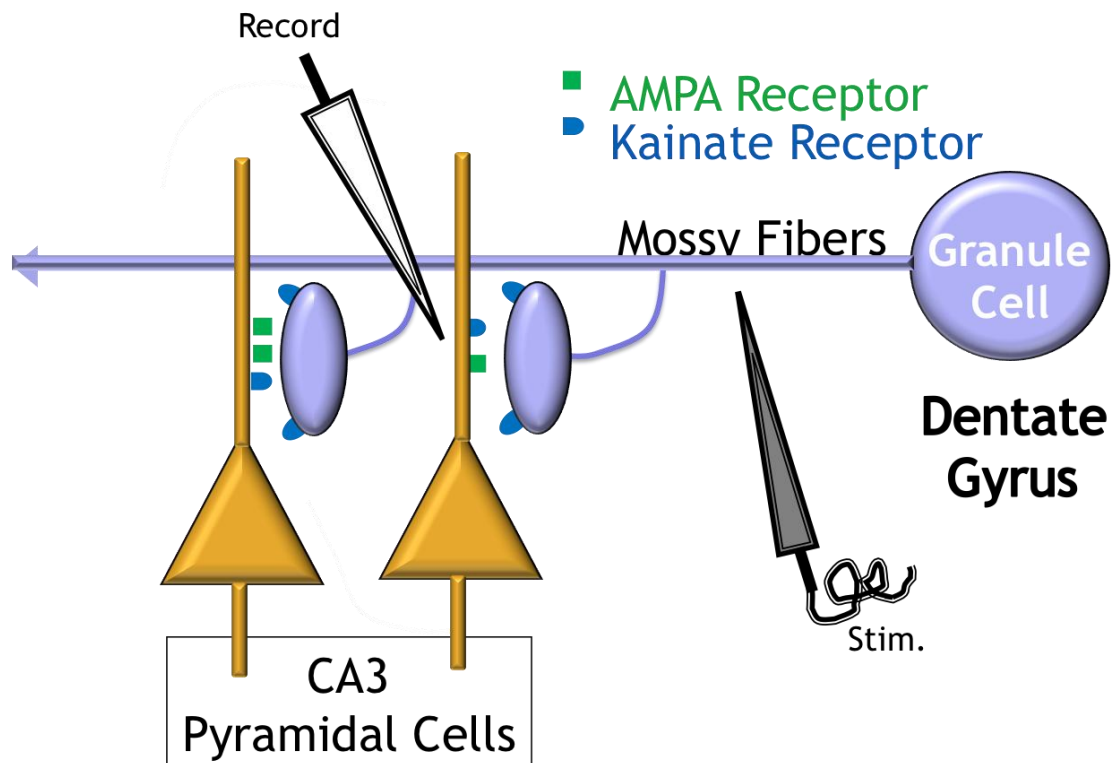


Figure 2.1 Illustration of the mossy fiber – CA3 synapse and field recording configuration.

2.4 PILOCARPINE MODEL OF SE

The use of animal models in investigation potential therapies for epilepsy is indispensable. These models, and specifically the pilocarpine model, closely resembles distinct anatomical and physiological alterations seen in the human disease, including sclerosis and cell loss and mossy fiber sprouting. Additionally, the pilocarpine model develops spontaneous seizures, chronic hyperexcitability, appropriate responsiveness to AEDs, and develop comorbidities, such as anxiety and learning and memory deficits, that are strongly associated with chronically epileptic patients. Without these models would not be able to elucidate the mechanism precipitation or preventing seizures. The current study will utilize a pilocarpine to initiate status epilepticus followed by a period of epileptogenesis (30 days) and eventually the development of spontaneously recurring seizures.

2.4.1 ANIMAL CARE AND USE

All animal care and use procedures were carried out in accordance with protocols written under the guidance of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional Animal Care and Use Committee at the University of South Carolina. Male Sprague-Dawley rats were purchased from Harlan, and housed at 1 – 3 rats per cage with *ab libitum* access to food and water. Rats were housed in a climate-controlled facility with a light/dark cycle of 12/12 hours.

2.4.2 PILOCARPINE MODEL OF STATUS EPILEPTICUS

Adult male Sprague-Dawley rats (42 – 50 days; Harlan, Indianapolis, IN) received intraperitoneal (i.p.) injections of scopolamine methyl bromide, to block peripheral muscarinic receptors, and terbutaline hemisulfate (2 mg/kg) to activate peripheral β 2-adrenergic receptors to act as a bronchodilator and prevent respirator depression associated with SE. Pilocarpine hydrochloride (390 mg/kg) was intraperitoneally injected 30 – 40 minutes after scopolamine and terbutaline injection. Following pilocarpine injection, rats were observed continuously for the occurrence of behavioral seizures and were scored on the Racine scale (Racine, 1972) (Table 2.1). Approximately 70% of rats experienced class V seizures. Animals that did not exhibit SE after 45 minutes were given an additional ‘booster’ of pilocarpine (200 – 300 mg/kg). Nearly 90% of animals receiving a pilocarpine ‘booster’ experienced sufficient SE. Animals that successfully had a stage V seizure were allowed to seize 2 hours (status epilepticus) before the seizures were suppressed by intraperitoneally injected diazepam (DZP, 25 mg/kg). This group of animals served as the ‘pilo’ group. In order to limit physical distress and any peripheral effects of pilocarpine, animals received additional doses of the scopolamine/terbutaline cocktail approximately one-hour into SE as well as upon termination of SE with DZP. A separate group of animals were treated identically, but received saline (390 mg/kg) instead of pilocarpine hydrochloride. This group of animals served as the ‘sham’ group. Approximately 1 – 2 hours after seizures were terminated, animals received a subcutaneous dose of the scopolamine/terbutaline cocktail (2 mg/kg), lactate Ringer’s solution (2 mL), and rat chow that had been softened and

sweetened in high sucrose water. Animals that did not enter SE were excluded from the study.

All animals were housed overnight in the animal facility and their health status was checked as early as possible on the following day to determine if they successfully recovered from treatment. Animals still experiencing side effects from treatment (lethargy, immobility) were given additional subcutaneous injections of lactated Ringer’s solution and the scopolamine/terbutaline cocktail and placed on a heating pad. For animals who endured the first night post-treatment the survival rate was nearly 100%. Animals in our “pilo” group were video monitored a minimum of 30 days post-treatment to ensure the appearance of spontaneously occurring seizures. This experimental protocol was used in Chapter 5 to explore alterations in the dopaminergic system in the epileptic brain.

Table 2.1 Racine scale of epileptic seizures

Score	Observed Rat Motor Behavior
0	Normal behavior (exploring, walking, grooming, etc.)
1	Immobility, staring ‘curled up’ posture
2	Automatisms (Chewing, head bobbing, twitching, wet-dog shakes)
3	Forelimb/hindlimb myoclonic jerking, head tremor
4	Rearing and whole body clonus
5	Rearing and falling over; loss of posture
6	Tonic-clonus seizures with tonic forelimb flexion/extension, whole-body clonus
7	Wild-running with bouncing; death

2.5 WESTERN BLOT ANALYSIS

Western blotting is a widely used tool to detect the presence and measure the abundance of specific proteins (MacPhee, 2010). Western blotting begins with successful extraction and isolation of a particular lysate. Proteins are then separated by molecular weight through electrophoresis onto a polyacrylamide gel. The proteins are then transferred onto a membrane and probed with antisera to detect the presence of the specific protein. During application of the appropriate primary and secondary antibody, the membrane undergoes blocking to prevent non-specific binding between antibody and membrane. Blocking usually consists of submerging the membrane in a non-fat milk or bovine serum albumin solution. Once the appropriate antisera is bound to the protein, its relative expression can be determined through process such as enhanced chemiluminescence (ECL). The peroxidase conjugated secondary antisera serves to oxidize the luminol substrate within the ECL to emit light. This light is then captured onto film and measured using densitometry. The following sections describe in detail the western blotting protocol utilized in Chapter 5 to determine differences in dopamine D1/D5 receptor, DAT, and COMT protein abundance within specific brain regions in the hippocampus following pilocarpine-induced temporal lobe epilepsy.

2.5.1 MEMBRANE PREPARATION

Immunoblot analysis was performed as described in a previous study (Grillo et al., 2011). Briefly, the hippocampus was rapidly extracted and processed ‘whole’ or microdissected into CA1, CA3, and dentate gyrus. Tissue was homogenized in ice cold

homogenization buffer (mM: 2 EDTA, 2 EGTA, 20 HEPES, 0.32M sucrose, 1% phosphatase inhibitor, and 1% protease inhibitor). The samples were then homogenized and centrifuged for 10 min at 500 x g at 4C to remove nuclear fraction. The supernatant containing the total membrane fraction was centrifuged at 31,000 x g for 30 min at 4C. The resulting crude plasma membrane fraction was resuspended in phosphate buffered saline (PBS) and stored at -80°C until used for analysis. A Bradford protein assay was used as a simple and accurate way to measure protein concentration in the crude plasma membrane, and used to ensure equal protein is loaded across all lanes.

2.5.2 SDS-PAGE AND ANTIBODY DEVELOPMENT

Aliquots of 20 µg of crude plasma membrane proteins were denatured by diluting them in a 1:1 ratio with BioRad Laemmli Sample Buffer with 5% β-mercaptoethanol. Samples were then heated at 95C for 5 min and loaded into pre-cast polyacrylamide gels (4 – 15%). Running buffer (in mM: 25 Tris, 240 Glycine, 0.1% SDS) was added to the appropriate tank, BioRad mini PROTEAN-Tetra, and ran using the BioRad Power Pack HC for at least 30 min at 175 – 200 V. Following sodium dodecyl sulfate polyacrylamide gel polyvinylidene fluoride (PVDF) membranes using the BioRad Trans-Blot SD Semi-Dry Transfer Cell. Protein transfer was completed using the BioRad Power Pack HC for 30 min at 25V. Transfer buffer contained (in mM): 25 Tris, 192 Glycine, and 20% methanol. All equipment was acquired from Bio-Rad Laboratories, Inc.

PVDF blocked in TBS plus 5% nonfat dry milk for 60 min. PVDF membranes were incubated with primary antisera in TBS/5% nonfat dry milk. After an

overnight incubation at 4°C, blots were washed with TBS plus 0.05% Tween 20 (TBS-T) and incubated with peroxidase labeled species-specific secondary antibodies. PVDF membranes were then washed with TBS-T and developed using enhanced chemiluminescence reagents (ECL, Amersham) as described by the manufacturer. Normalization for protein loading was performed using a mouse monoclonal primary antibody selective for actin (Sigma Chemical Company).

Table 2.2 Primary antibodies for western blot analysis

Ligand	Manufacturer; Catalog number	Primary Concentration
D1	Millipore; AB1765P	1:200
D5	Santa Cruz; sc-1441	1:250
TH	Millipore; AB152	1:1,000
COMT	Millipore AB5873	1:1,500
DAT	Millipore: AB2231	1:2,000

2.6 IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a power tool used to visualize the localization and distribution of specific protein the fixed tissue. Thus, much care should be taken to preserve the original architecture and connectivity in the brain. Immunostaining techniques use a combination of immunology and histology. Immunology is used to produce protein-specific antibodies from the serum of animals. Antibodies bind to corresponding antigens presented on the cell surface. We use an indirect method of immunohistochemistry to amplify the labeled signal. Antibodies for the specific protein is applied to the tissue, where they bind to corresponding antigen, while excess and

unbound antibody is washed off (primary). To identify the location of the antibody and consequently the location of the antigen, an additional antibody is applied to tissue. This antibody was developed in another species to recognize and bind to the species from which the primary antibody was developed (secondary). Lastly, another antibody conjugated with a peroxidase enzyme will bind to the antibody complex. This peroxidase enzyme recognizes peroxide and allows us to visualize the location of the antibodies and consequently the location of the antigen.

Histology is used because we observe the structure and preservation of tissue. Generally, brains are fixed with formaldehyde and can either be sectioned immediately or undergo cryoprotecting, which allows longer preservation of the tissue morphology in the freezer. In many cases, this fixative can prevent sufficient antibody/antigen binding. Thus, a popular detergent, Triton X can be used to improve penetration of the antibody and improve labeling. We employed such techniques in this study. The following sections describe in detail the immunohistochemistry protocol used in Chapter 5 to determine alterations in dopamine D1/D5 receptor expression within the hippocampus following pilocarpine-induced chronic temporal lobe epilepsy.

Free-floating sections were incubated with a mouse anti-glutamic acid decarboxylase 67 (GAD-67, 1:1000; Millipore; product No. MAB5406), goat anti-dopamine D5 receptor (D5, 1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; product No. sc-1441), rat anti-dopamine D1 receptor (D1, 1:100; Sigma, St. Louis, MO, USA; product No. D2944), or rabbit anti-tyrosine hydroxylase (TH, 1:2000; Millipore; product No. AB152) antibodies for 24 hours at room temperature (RT) or 48 hours at 4°C. The GAD-67 antibody was used for cell body labeling as well as

quantification of GABAergic neurons. These steps were followed by secondary antibody incubation with either biotinylated donkey anti-mouse, anti-rabbit, anti-goat, or anti-rat (1:400, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; product Nos. 715-065-151, 711-065-152, 705-065-003, 712-065-150) for 1.5 hours at RT, and horseradish peroxidase conjugated streptavidin (1:1600; Jackson ImmunoResearch Laboratories, Inc.; product No. 016-030-084) for 1 hour at RT. Immunoreactivity was developed using nickel sulfate-cobalt chloride intensified diaminobenzidine with hydrogen peroxide, yielding blue-black immunopositive cells.

2.6.1 PERFUSION OF TISSUE FIXATION

All tissue was processed according to previously described protocols (Stanley et al., 2012). Briefly, rats were deeply anesthetized using isoflurane and transcardially perfused with phosphate-buffered saline and 4% paraformaldehyde. Briefly, once anesthetized, a lateral incision to the lower abdomen was made which extended upwards to expose the chest cavity and the heart. A hemostatic clamp was placed on the descending vena cava to prevent perfusate flow to the lower extremities. A 20 x ½ gauge needle attached to the perfusion pump was inserted through the apex of the left ventricle and held firmly in place with a hemostat. Once inserted, the right atrium was clipped to allow perfusate flow. Ice cold, oxygenated (95% O₂ / 5% CO₂) 0.05 M PBS (pH 7.4) was perfused at approximately 60 mL/min for 4 – 5 min, followed by the ice cold fixative (0.1 M phosphate buffer / 4% paraformaldehyde), for approximately 20 min. Whole

brains were removed and postfixed overnight followed by cryoprotection in 30% sucrose. Tissue was coronally sectioned at a 45 μm thickness on a cryostat.

2.6.2 IMMUNOPEROXIDASE

Free-floating sections were incubated with primary antibodies (Table 2.2) antibodies for 24 hours at room temperature (RT) or 48 hours at 4°C. The GAD-67 antibody was used for cell body labeling as well as quantification of GABAergic neurons. These steps were followed by secondary antibody incubation for 1.5 hours at RT, and horseradish peroxidase conjugated streptavidin (1:1600; Jackson ImmunoResearch Laboratories, Inc.; product No. 016-030-084) for 1 hour at RT. Immunoreactivity was developed using nickel sulfate-cobalt chloride intensified diaminobenzidine with hydrogen peroxide, yielding blue-black immunopositive cells.

Table 2.3 Primary antibodies for immunohistochemistry analysis

Ligand	Manufacturer; Catalog Number	Primary Concentration
GAD-67	MAB5406	1:1,000
D5	sc-1441	1:500
D1	D2944	1:100
TH	AB152	1:2,000

2.6.3 SLIDE PREPARATION AND ANALYSIS

Stained and free floating tissue sections were mounted on slides with a 0.15% gelatin solution and allowed to dry overnight. Once dry, tissue were serial dehydration step with increasing concentrations of ethanol (50%, 70%, 95%, 100%, 100%; 3 min/bath) followed by two, 10 min incubations in xylene. Slides were immediately cover-slipped using cover glass and DPX mounting solution (Sigma, St. Louis, MO) and allowed several days to dry prior to analysis and long-term storage. Analysis on immunohistochemical data was performed using cell counts.

2.6.4 CELL COUNTS & PHOTOMICROGRAPHS

Immunopositive cells were counted using a Nikon ECLIPSE 80i microscope equipped with a CX900 camera (Nikon; Tokyo, Japan) using NeuroLucida software (v.10; MicroBrightField, Inc.; Williston; VT, USA). Minor adjustments to color, contrast, and brightness were made using Adobe Photoshop 7.0 (San Jose, CA, USA).

2.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

High performance liquid chromatography (HPLC) is a technique that can be used to identify, quantify and purify individual analytes of a given solution. Typically, HPLC involves a high pressure pump used to carry analytes suspended in a mobile phase through an analytical column. The column contains densely packed silica beads that facilitate the separation of analytes based on polar interactions. After being separated,

analytes are detected by an electrochemical detector. Retention times can be varied based on mobile phase flow rate, composition or pH, as well as ambient temperature in some cases. All HPLC products were purchase from BASi.

Rats were deeply anesthetized with isoflurane. Their hippocampi were rapidly extracted and immediately weighted and chilled. Hippocampal tissue was homogenized using individual plastic disposable pestles and 1.5mL centrifuge tubes. The tissue was homogenized in a 0.1M perchloric acid (HClO₄) solution containing 0.02% sodium metabisulfite (Na₂S₂O₅) and dihydroxybenzylamine (DHBA, 146.5 ng/mL, as an internal standard for monoamines) in a proportion of 15 µL solution for each milligram of tissue. The homogenized tissue was then centrifuges at 11,000g at 4°C for 40 min. After centrifugation, the sample supernatant was filtered using 0.2 µM nylon disposable syringe filters and stored at -80°C until analyzed. Centrifuged tubes containing the tissue pellet was also stored at -80°C to use as a post hoc control to normalize for protein concentration. We analyzed hippocampal tissue homogenates from “control” and “epileptic” rats using high performance liquid chromatography (HPLC) with electrochemical detection for the analytes dopamine, DOPAC, and norepinephrine (NE). Elution times for these monoamines and their metabolites are displaying in Table 2.2 (Cavalheiro et al., 1994).

Table 2.4 Chromatographic parameters for monoamines and monoamine metabolites

Neurotransmitter	Retention time (min)
4-hydroxy-3-methoxy-mandelic acid (VMA)	3.60

4-hydroxy-3-methoxyphenyl glycol (MPHG)	4.20
Norepinephrine (NE)	4.60
Dihydroxybenzylamine (DHBA)	7.30
Dihydroxyphenylacetic acid (DOPAC)	8.00
Dopamine (DA)	11.00
4-hydroxy-3-methoxy-phenylacetic acid (HVA)	20.00

2.7.1 DOPAMINE CHROMATOGRAPHY

Dopamine, DOPAC, and NE were separated by a C18 carbon polymer column using a mobile phase (pH 3.4) containing 14.5 mM NaH₂PO₄. A post-column immobilized enzyme reactor containing covalently bound dopamine, DOPAC, and NE oxidase generated stoichiometric quantities of hydrogen peroxide, which were detected by a “peroxidase wired” glassy carbon electrode (Huang et al., 1995; Fadel et al., 2001). Dopamine peak areas were quantified by comparison with a daily three-point standard curve defining the range of dopamine values. Dopamine, DOPAC, and NE levels in epileptic hippocampi were normalized as a percent of the average control hippocampi dopamine, DOPAC, and NE content.

2.8 STATISTICAL ANALYSIS

Electrophysiological data was analyzed using pClamp10 (Molecular Devices, Sunnyvale, CA) and Origin7.5 (OriginLab, Northampton, MA). Data collected from western blot analysis and immunohistochemistry was analyzed using ImageJ 1.47V

(National Institute of Health, USA) or cell counts. Data depicting changes in DAR, DAT, and COMT protein expression were obtained by averaging data collected from a minimum of two separate western blots.

All cell counts, electrophysiological data, and immunoblot data were analyzed using independent samples Student's t-test for significant main effects. All values are expressed as the mean \pm standard error of the mean (SEM). $P < 0.05$ defined significant main effects.

CHAPTER 3

SUBUNIT DEPENDENT PHARMACOLOGY OF KAINATE RECEPTORS

3.1 INTRODUCTION

Kainate receptors (KARs) are ionotropic glutamate receptors composed of tetrameric assemblies of GluK1-5 subunits (Pinheiro and Mulle, 2006). GluK2, GluK4, and GluK5 are thought to compose postsynaptic KARs at mossy fiber (MF) to CA3 pyramidal cell synapses (mossy fiber-CA3 synapses) (Mulle et al., 1998; Contractor et al., 2003; Fernandes et al., 2009), where they contribute with small amplitude, slowly decaying currents (Castillo et al., 1997; Vignes and Collingridge, 1997). Despite their small amplitude, KAR-excitatory postsynaptic currents (EPSCs) were hypothesized to be important for the integration of information because they summate temporally (Frerking and Ohliger-Frerking, 2002). Furthermore, kainate receptors are composed of subunits that demonstrate distinct pharmacology, kinetics, and localization, thus, providing a higher level of complexity to deciphering the role of kainate receptor subunits in the brain. Although we know that each subunit can bind to glutamate and open the receptor, we do not know whether each subunit contributes equally to receptor activation, desensitization, and subsequently the KAR current at postsynaptic MF-CA3 synapses.

The lack of pharmacological tools that selectively block KAR subunits known to be located on CA3 pyramidal cells hampers clarifying the physiological relevance of postsynaptic KARs at mf-CA3 synapses. Recently, an intense effort was made to synthesize and characterize subunit-selective KAR antagonists, mainly against GluK1 (Jane et al., 2009). However, no selective antagonist of GluK5 or GluK4 subunits yet exists, although they likely represent a major population of KARs in the brain (Wenthold et al., 1994). Previous papers hint at how other GluK1 antagonists can be promiscuous in also inhibiting heteromeric GluK2/K5 receptors (Pinheiro et al., 2013). The implication of KAR-EPSCs at hippocampal MF synapses has only been addressed by comparing wild-type and GluK2^{-/-} mice, which display loss of postsynaptic KARs (Mulle et al., 1998; Sachidhanandam et al., 2009). This was followed by demonstrations showing that KAR-EPSCs are eliminated when GluK4 and GluK5 subunits are knocked out (Fernandes et al., 2009). Thus, these two experiments can be explained by the latter, suggesting that GluK2 is necessary for integration of GluK4 and GluK5 into the synapse. However, it is the GluK2/GluK4 and/or the GluK2/GluK5 heteromers that are necessary for KAR-EPSCs at the MF-CA3 synapses. Interestingly, the GluK4^{-/-} demonstrated the most diminished KAR-EPSCs at MF-CA3 synapses compared with the GluK5^{-/-} (Contractor et al., 2003; Fernandes et al., 2009). However, these results are not conclusive that the GluK4 subunit is more important for postsynaptic KAR transmission than the GluK5 subunit, because receptor compensation from congenital knockouts cannot be ruled out. In addition to the previous research demonstrating a need for the GluK4 and GluK5 subunits to produce postsynaptic KAR current, new research has implicated a novel role for the auxiliary protein Neto1 in trafficking of KARs to the

synapse and altering the properties KAR postsynaptic current at mf-CA3 synapses (Copits et al., 2011; Straub et al., 2011a).

In search for antagonists of KARs at mf-CA3 synapses, we reevaluated the efficacy of willardiine derivatives (Jane et al., 2009) on native KARs. ACET was originally developed as a GluK1 selective antagonist (Dolman et al., 2007). We found that ACET blocks postsynaptic KARs at mf-CA3 synapses. We further show that ACET is also an antagonist of recombinant GluK5 and GluK4 subunits, the subunits required for postsynaptic KAR current. Using this tool and the drug kynurebate, which was previously demonstrated to antagonize the GluK2 subunit, we directly investigated the relative contribution of the GluK2 and GluK5 subunits to KAR current in recombinant cells and the brain.

3.2 MATERIALS AND METHODS

3.2.1 CULTURE AND TRANSFECTION OF HEK-293T CELLS

HEK-293T cells (GenHunter, Nashville, TN, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. Cells were passaged with a 0.025% trypsin/0.01% EDTA solution in phosphate-buffered saline (10 mM Na₂HPO₄, 150 mM NaCl, pH 7.3). Full-length cDNAs for the GluK or Neto1 subunits in mammalian expression vectors were transfected into cells using calcium phosphate precipitation as

previously described (Mott et al. 2010). Plasmids encoding rat GluK2 (Q), GluK4 or GluK5 were provided by S. Heinemann (Salk Institute, San Diego, CA, USA). Human Neto1 was provided by S. Tomita (Yale University, New Haven, CN, USA). Plasmids were transfected at ratios of 1:3:4 (GluK2: GluK4 – GluK5: Neto1), previously shown to optimize formation of heteromeric receptors (Barberis et al. 2008) and Neto1 assembly with KARs (Fisher & Mott, 2012). To identify transfected cells, we co-transfected 1 μ g of a cDNA encoding a single-chain antibody recognizing the hapten 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx; Chesnut et al. 1996). Positively transfected cells were isolated using phOx-coated beads 18 – 28 hr after transfection and plated onto glass coverslips treated with poly-L-lysine and collagen.

3.2.2 WHOLE-CELL RECORDING

Whole-cell recordings were performed on isolated HEK-293T cells 40 – 52 h after transfection under voltage-clamp conditions. Patch pipettes were pulled from borosilicate glass with an internal filament (World Precision Instruments, Sarasota, FL, USA) on a two-stage puller (Narishige, Japan) to a resistance of 5 – 10 M Ω and filled with a solution containing (in mM): 130 CsGluconate, 5 CsCl, 10 Hepes, 5 CsBAPTA, 2 MgCl₂, 2 MgATP, 0.3 NaGTP (pH 7.3, 290-300 mosmol l⁻¹). Cells were continually perfused with an external solution containing (in mM): 150 NaCl, 3 KCL, 10 Hepes, 1 CaCl₂, 0.4 MgCl₂ (pH 7.4, 295-305 mosmol l⁻¹). For whole-cell recordings glutamate and antagonists (ACET and kynurenate) were applied to cells using a stepper solution changer (SF-77B, Warner Instruments, Hamden, CT, USA). The time course of drug

application was <50 ms in the whole-cell recording configuration as determined using a diluted external solution applied to the electrode. Rise times (10 – 90%) of the junction potential at the open tip were consistently faster than 400 μ s and were tested using a diluted external solution. All antagonists were applied to cells before glutamate per sweep. Current recordings were amplified (Axopatch 200B; Molecular Devices, Foster City, CA, USA), filtered (1kHz) and digitized at 10 kHz using a Digidata 1320 analog to digital board (Molecular Devices) and stored on a computer hard drive for off-line analysis.

3.2.3 ANALYSIS OF WHOLE-CELL CURRENTS

Whole-cell currents were analyzed using the programs Clampfit (pClamp9.2 suite, Molecular Devices, Foster City, CA, USA) and Origin (MicroCal, Northampton, MA, USA). Concentration-response data were fit with a four-parameter logistic equation: $\text{Current} = [\text{Minimum current} + (\text{Maximum current} - \text{Minimum current}) / (1 + 10^{-(\log \text{EC}_{50} - \log [\text{Glutamate}]) \times n})]$, where n represents the Hill number. All fits were made to normalized data with current expressed as a percentage of the maximum response to glutamate for each cell. Peak currents and $\log \text{EC}_{50}$ values were compared using unpaired Student's t tests with a significance level of $P = 0.05$.

3.3 RESULTS

3.3.1 ACET INHIBITION OF HETEROMERIC KAINATE RECEPTORS

ACET is a williardine compound previously described to be a GluK1 antagonist (Dargan et al., 2009; Jane et al., 2009). Studies have confirmed that ACET does not inhibit the activation of GluK2 homomeric currents (Dargan et al., 2009). However, doubts about the specificity of ACET to inhibit only GluK1 receptors were raised when it was demonstrated that ACET could inhibit KAR currents at mossy fiber-CA3 synapses (Dargan et al., 2009), a synapse in which few if any postsynaptic GluK1 receptors are located (Paternain et al., 2003). To examine the selectivity of ACET, we transfected HEK-293T cells with heteromeric and homomeric kainate receptors known to be predominantly expressed on CA3 pyramidal cells (GluK2, GluK2/K4 and GluK2/K5) and evaluated the efficacy of ACET to inhibit their glutamate-induced current.

Using whole cell patch-clamping electrophysiology, we gained access to the internal milieu of the cell and clamped the voltage inside the cell at -70 mV. An EC₅₀ glutamate dose was applied to each cell as a reference for maximum current activation. Glutamate was applied at the beginning and end of each experiment to 1) receive a reference for maximum current activation, and 2) as a way to determine receptor rundown throughout the experiment. In cells transfected with GluK2/K4 KAR subtypes, a 5 sec application of 30 μ M glutamate produced a rapid on and fast desensitizing current (Figure 3.1A). These receptor kinetics are similar to those reported in other papers (Fisher and Fisher, 2014). Co-application of 1 μ M ACET with 30 μ M glutamate reliably reduced

the peak current amplitude by $54 \pm 6.3\%$ (Figure 3.1A). In cells transfected with GluK2/K5 KAR subtypes, an EC_{50} glutamate dose of $10 \mu\text{M}$ produced a rapidly activated but slowly desensitizing current (Figure 3.1A). This receptor kinetic was similar to those reported in other papers (Fisher and Mott, 2011; Fisher and Fisher, 2014). Co-application of $1 \mu\text{M}$ ACET with $10 \mu\text{M}$ glutamate reliably reduced the peak current by $92 \pm 0.85\%$ (Figure 3.1A). However, in cell transfected with GluK2 KAR subtypes, and EC_{50} glutamate dose of $300 \mu\text{M}$ glutamate produced a rapidly activating and desensitizing current (Figure 3.1B). Co-application of $1 \mu\text{M}$ ACET with $300 \mu\text{M}$ glutamate did not inhibit the peak current amplitude (Figure 3.1B).

Interestingly, our results demonstrate that $1 \mu\text{M}$ ACET is not sufficient to inhibit GluK2 when expressed alone. However, $1 \mu\text{M}$ ACET is sufficient to inhibit peak currents when GluK2 is expressed with either GluK4 or GluK5 subunits. Thus, we designed further experiments to test whether the GluK4 or GluK5 subunits contribute to the appearance of ACET inhibition. To test whether GluK4 or GluK5 subunits contribute to ACET inhibition of heteromeric KAR currents, we co-transfected GluK4 or GluK5 subunits with a mutated GluK2, in which the 738th glutamate (E) amino acid was replaced with a aspartate (D) amino acid. This mutation reduces the GluK2 subunit sensitivity to glutamate 1,000 fold (Fisher and Mott, 2011), resulting in the lack of GluK2 activation at glutamate concentrations less than 1 mM . Now, when GluK4 and GluK5 is expressed with the mutated GluK2 subunit (GluK2_{E738D}), the receptor kinetics change from a rapid on and desensitizing current to a non-desensitizing current. This was reported and elucidated in other papers, where they described this phenomenon was due to activation of only two identical subunits in the tetramer (Fisher and Mott, 2011).

Desensitization requires that activation of at least three or more subunits. By removing the sensitivity of the GluK2 subunit to glutamate, only the GluK4 or GluK5 subunits in the tetramer can bind to glutamate, and thus produces a non-desensitizing current. Application of 1 μM ACET was sufficient to block activation induced by EC_{50} dose of glutamate (Figure 3.1B)

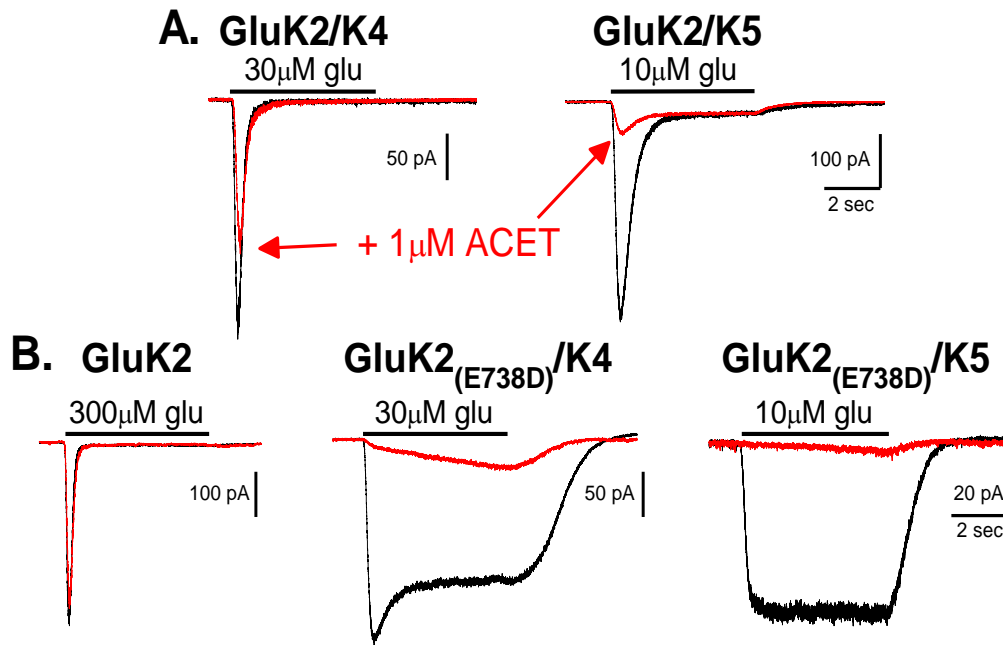


Figure 3.1 ACET inhibits current at GluK2/4 and GluK2/5 heteromers by antagonizing the GluK4 and GluK5 subunit, respectively.

- A. Sample waveforms showing the effect of ACET (1 μM , red trace) compared to glutamate alone (black trace). ACET inhibits the glutamate-induced current in HEK-293T cell transfected with wildtype GluK2/K4 or GluK2/K5 kainate receptors.
- B. Sample waveforms showing the effect of ACET (1 μM , red trace) compared to glutamate alone (black trace). Similarly to previously reported, ACET did not inhibit current at cells transfected with wildtype GluK2 homomeric KAR receptors. However, in cells transfected with heteromeric KARs in which the GluK2 subunit was mutated so that it was 1,000 fold less sensitive to glutamate (GluK2(E738D)/K4 and GluK2(E738D)/K5), ACET almost completely inhibits the glutamate-induced current.

Desensitization requires that activation of at least three or more subunits. By removing the sensitivity of the GluK2 subunit to glutamate, only the GluK4 or GluK5 subunits in the tetramer can bind to glutamate, and thus produces a non-desensitizing current. Application of 1 μ M ACET was sufficient to block activation induced by EC_{50} dose of glutamate (Figure 3.1B)

3.3.2 ACET SIMILAR INHIBITS THE GLUK4 AND GLUK5 SUBUNITS

The GluK4 and GluK5 subunits can have drastically different pharmacology from the other KAR subtypes. They can be activated by ligands, such as AMPA, that GluK1 – GluK3 subunits cannot bind (Alt et al., 2004). Also, GluK4 and GluK5 are more sensitive to glutamate, given them the name of ‘high affinity’ KAR subunits (Heckmann et al., 1996; Paternain et al., 1998; Barberis et al., 2008; Fisher and Mott, 2011, 2013). Thus, their incorporation into KAR subtypes can have drastic effects on the pharmacology of the whole KAR receptor. We previously, demonstrated that 1 μ M of ACET was sufficient to reduce EC_{50} glutamate activation of GluK2-containing heteromers, although the inhibition of GluK2/K5 wildtype KARs current was more than in GluK2/K4 KARs. This difference in current inhibition in GluK4 versus K5 KARs was occluded when co-expressed with a mutant GluK2, which lacked sensitivity to the EC_{50} glutamate dose. Next, we sought to determine whether the sensitivity of the GluK4- and GluK5-containing KARs to ACET was similar. To test this, we conducted an ACET concentration curve, where increasing concentration of ACET were co-applied with an EC_{50} concentration of glutamate. Peak current responses were recorded for each

concentration in each cell. All data was normalized to the EC₅₀ glutamate concentration alone. Results are reported as a measure of how much a particular drug (ACET or kynurenate) is needed to inhibit current amplitude by half (IC₅₀). Analyses of wildtype GluK2, GluK2/4 and GluK2/5 receptors demonstrated that the IC₅₀ of ACET was very different across the KAR subtypes (GluK2, IC₅₀ = 126 ± 3 nM, n = 4; GluK2/K4, IC₅₀ = 71 ± 5 nM, n = 8; and GluK2/K5, IC₅₀ = 29 ± 7 nM, n = 10, respectively) (Figure 3.2A). As shown above, wildtype GluK2/K5 heteromers have the highest sensitivity to ACET inhibition, followed by wildtype GluK2/K4 heteromers. As expected, GluK2 homomers were non-responsive to ACET inhibition until concentrations exceeding 1 μM. It is also important to note that ACET inhibition of the GluK2/K4 peak current plateaued at ~45% of the maximum glutamate response irrespective of increased ACET concentrations (Figure 3.2A). Thus, the difference in wildtypes GluK2/K4 and GluK2/5 sensitivity to ACET inhibition is more pronounced with higher concentrations of ACET (Figure 3.2A).

Interestingly, the difference between GluK2/K4 and GluK2/K5 sensitivity to ACET inhibition was abolished when co-expressed with the mutated GluK2 subunit (GluK2(E738D)). ACET concentration-response curves for GluK2(E738D)/K4 and GluK2(E738D)/K5 were not statistically different. This was also supported by the similarities of their ACET IC₅₀s: GluK2(E738D)/K4, IC₅₀ = 112 ± 1 nM, n = 6; GluK2(E738D)/K5, IC₅₀ = 64 ± 1 nM, n = 7 (Figure 3.2C).

Steady-state current is achieved when there is equilibrium between the amount of receptors in the desensitized state and the open state. Thus, the amount of current remains constant in the presence of glutamate. ACET did not inhibit the steady-state current in wild-type GluK2/K4 and GluK2/K5 KARs (Figure 3.2B). However, the steady-state

current in GluK2(E738D)-containing heteromers was sensitive to ACET inhibition (Figure 3.1B).

GluK2 homomeric KARs with a glutamine (Q) residue in the pore domain are subject to voltage dependent block by intracellular polyamines, which cause inward rectification of their current – voltage relationship. The ending result is that positive ions are hindered from leaving the cell even when the cell is depolarized to higher voltages. Cell that express heteromeric KARs do not experience inward rectification and allow ions to flow freely in or out of the cell in response to hyperpolarization or depolarization, respectively. To ensure that heteromeric receptors were expressed when we transfected with GluK2 and GluK4 or GluK5, we test for the lack of inward rectification. To test for the presence of rectification, we took the cell through a range of voltage steps (-90 mV - +70 mV) and measured the peak current in response to the EC₅₀ glutamate. Results were plotted on a current (I) – volts (V) curve, where peak current for each step is averaged and normalized to the peak current at -90 mV. The presence of positive current indicates the lack of inward rectification and the successful expression of functional heteromeric KARs. In cell transfected with GluK2/K5 and GluK2/K4, we saw the presence of positive current when the cells were depolarized above 0 mV (Figure 3.2C). However, we saw a lack of positive current at higher voltage potential in cell transfected with GluK2 homomers (Figure 3.2C).

The glutamate EC₅₀ for GluK2-containing homomers and heteromers and GluK2(E738D) have been previously described (Fisher and Fisher, 2014). Our glutamate concentration curve agrees with this published data (Figure 3.2D).

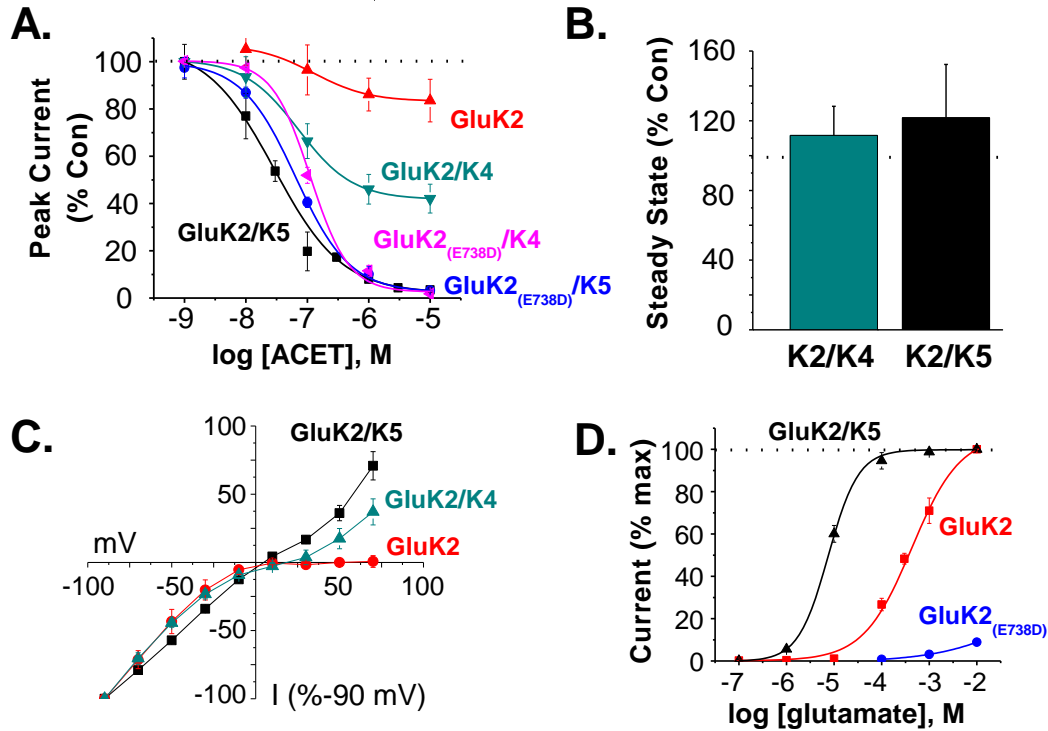


Figure 3.2 The efficacy of ACET is similar at GluK4 and GluK5 KAR subunits. However this similarity is occluded when these subunits are incorporated with the GluK2 subunit.

- Graph illustrating an ACET concentration curve. Peak amplitude of each current to concentration of ACET was normalized to the peak current response to glutamate application alone. The difference in ACET inhibition of wildtype GluK2/K4 to GluK2/K5 was abolished when the GluK2 subunit was mutated to be 1,000-fold less sensitive to glutamate.
- ACET (1 μ M) does not inhibit steady state currents at wildtype GluK2/K4 or GluK2/K5 KAR receptors.
- Graph illustrating the I-V curve for cells transfected with GluK2 homomers and GluK2/K4 and GluK2/K5 heteromers. Cells transfected with heteromers GluK2/K4 and GluK2/K5 demonstrated reduced inward rectification compared to GluK2 homomers, and thus, confirms expression of heteromeric receptors.
- Glutamate EC_{50} s are 330 μ M (GluK2) and 8 μ M (GluK2/K5). GluK2(E738D) is less sensitive to glutamate than wildtype (GluK2).

3.3.3 NETO1 ALTERS ACET INHIBITION

Neto1 is an auxiliary protein to KARs. Neto1 alters the kinetics of GluK2-containing receptors (Copits et al., 2011; Straub et al., 2011a; Fisher and Mott, 2013). As previously described, co-expressing Neto1 with GluK2/K5 KARs removes desensitization of the glutamate-induced current (Figure 3.3A). Co-application of ACET inhibited peak current amplitude in GluK2/K5 cells expressing Neto1 similarly to those

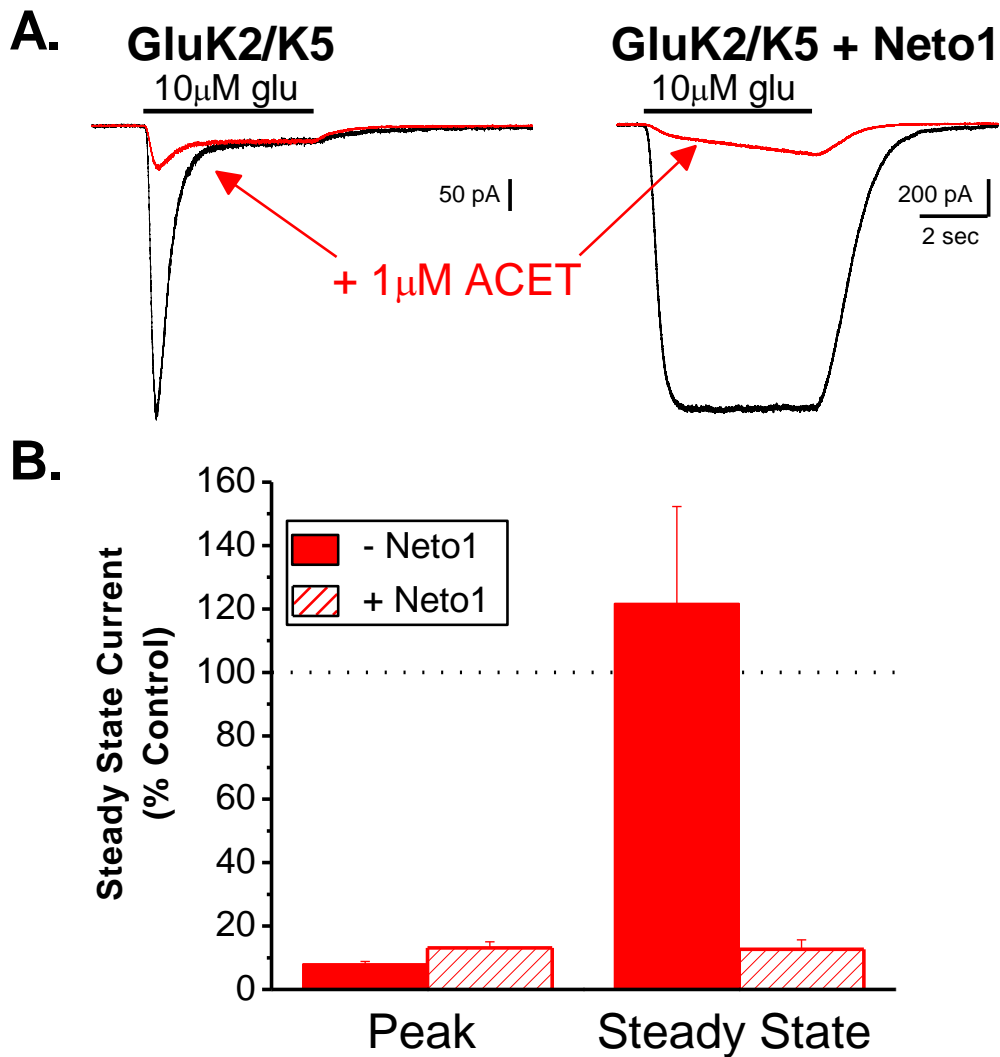


Figure 3.3 Neto1 enhances ACET inhibition of steady state GluK2/K5 currents

- A. Sample waveforms demonstrating that ACET (1 μ M) inhibits steady state current at GluK2/K5 receptors only when Neto1 is present.
- B. Averaged data demonstrating that co-transfecting with Neto1 enhances ACET inhibition of steady state, but not peak current at GluK2/K5 receptors. We suggest that the steady state current remaining in the presence of ACET is mediated by activated GluK2 subunits.

not expressing Neto1. Alternatively, we previously demonstrated that ACET did not inhibit the steady-state current in cells not expressing Neto1. However, when Neto1 is co-expressed with GluK2/K5 receptors, ACET now inhibits the steady-state current (Figure 3.3A, B).

3.3.4 KYNURENATE INHIBITION OF GLUK2-CONTAINING KAINATE RECEPTORS

Kynurenate (KYN) is a non-selective glutamate antagonist. Among GluK2-containing KARs, kynurenate preferentially inhibits the GluK2 subunit (Fisher and Mott, 2011). Thus, when co-expressed with GluK2/K5 kainate receptors, coapplication of kynurenate with an EC₅₀ concentration of glutamate yielded a non-desensitizing current. Representative waveforms illustrate that KYN dose-dependently inhibits the peak current amplitude of GluK2, GluK2/K4, and GluK2/K5 KARs induced by an EC₅₀ dose of glutamate. In GluK2 homomers, the rapidly activating and desensitizing current is progressively inhibited by increasing concentrations of KYN (Figure 3.4A). GluK2/K4 currents, although desensitizing slight slower, responded to KYN inhibition similarly to GluK2 homomers (Figure 3.4A). A difference remained in that at higher KYN

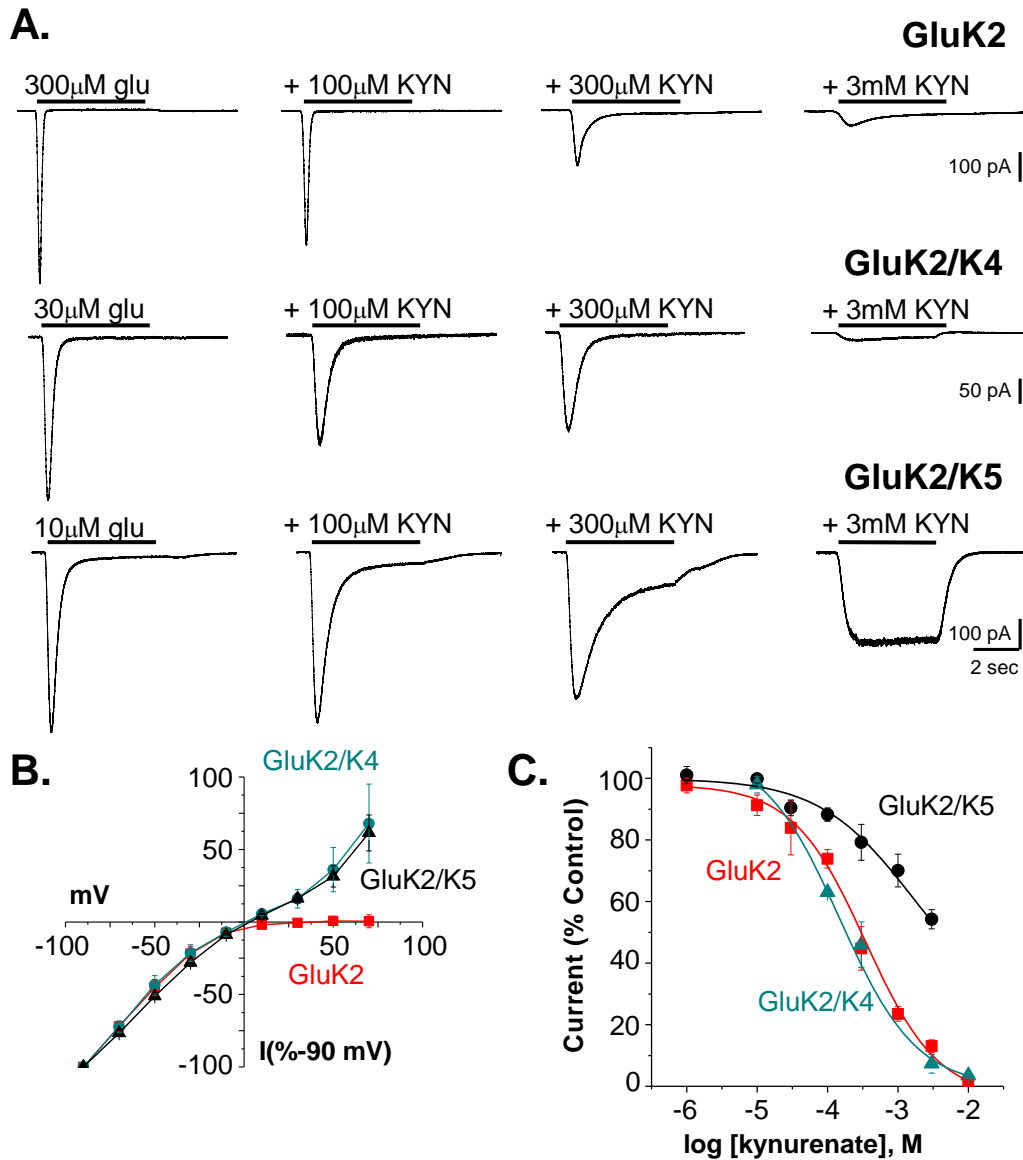


Figure 3.4 Kynureate has distinct effects on GluK2/K4 and GluK2/K5 receptors

- A. Sample waveforms demonstrating that with increasing kynureate (KYN) concentration, glutamate-activated current at GluK2 homomeric receptors is progressively inhibited. Increasing concentrations of kynureate had a similar effect on GluK2/K4 receptors. Alternatively, at GluK2/K5 receptors, kynureate inhibits peak current and strongly removes desensitization.
- B. Reduced inward rectification in IV-curves confirms expression of heteromeric GluK2/K4 and GluK2/K5 receptors.
- C. Graph illustrating a kynureate concentration curve. Kynureate inhibition of glutamate-induced currents was normalized to the peak current amplitude of glutamate application alone. Peak glutamate current at GluK2/K5 receptors is less sensitive to kynureate inhibition than GluK2 or GluK2/K4 receptors.

concentrations, GluK2/K4 currents adopted a non-desensitizing receptor kinetic (Figure 3.4A). KYN application had a distinguishing different effect on GluK2/K5 KAR kinetics. Increasing concentrations of KYN did inhibit the peak current amplitude, however, it more so removed desensitization (Figure 3.4A). A KYN concentration-curve, further demonstrates that GluK2 ($IC_{50} = 345 \pm 50 \mu\text{M}$, $n = 8$) and GluK2/K4 ($IC_{50} = 162 \pm 15 \mu\text{M}$, $n = 5$) have similar sensitivities to KYN inhibition (Figure 3.4C). However, GluK2/K5 KARs ($IC_{50} = 3857 \pm 163 \mu\text{M}$, $n = 9$) were less sensitive to KYN inhibition than the other GluK2-containing KARs (Figure 3.4C). These results agree with previously published data (Fisher and Mott, 2011). Additionally, the presence of heteromers in GluK2/K4 and GluK2/K5 transfected cells was confirmed by the lack of inward rectification (Figure 3.4B).

3.3.5 NETO1 ALTERS KYNURENATE INHIBITION

As described previously, incorporation of Neto1 into GluK2/K5 preparations removes desensitization of the glutamate-induced current. In wildtype GluK2/K5 receptors, coapplication of 1 mM KYN with an EC_{50} dose of glutamate moderately suppresses the peak current amplitude (Figure 3.5A, B). However, the steady-state current is significantly increased (Figure 3.5A, C). When Neto1 is expressed with GluK2/K5 KARs, KYN inhibits the peak current amplitude similarly to preparations without Neto1 (Figure 3.5A, B). However, the steady-state current is now inhibited by 3 mM KYN. Thus, KYN reduces desensitization of GluK2/K5 receptors not expressed with Neto1 and thereby alters the ratio of the steady-state amplitude to the peak amplitude. However,

KYN does not alter this steady-state/peak amplitude ratio when applied to GluK2/K5 receptors with coexpressed Neto1 (Figure 3.5A, C).

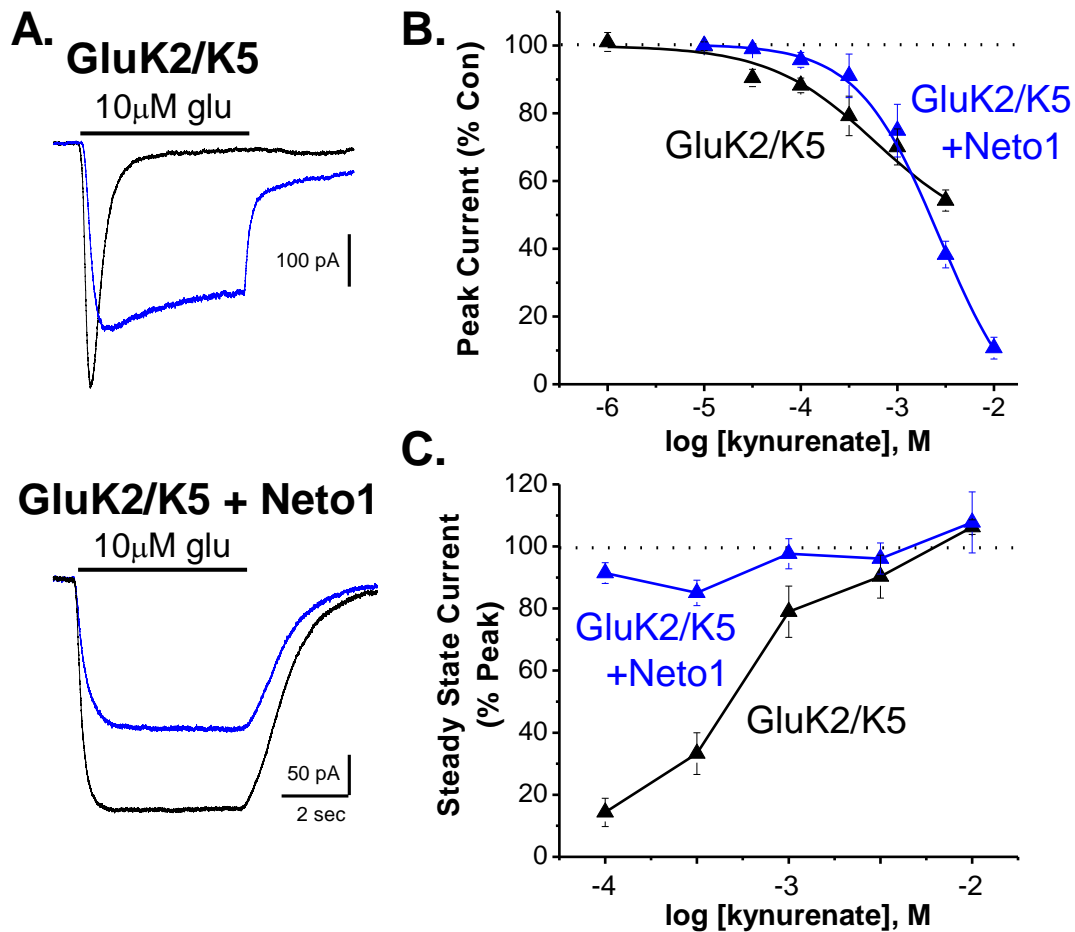


Figure 3.5 Neto1 occludes the effect of kynureneate on desensitization, but not inhibition

- Representative traces illustrating that co-transfected cells containing GluK2/K5 with Neto1 occludes kynureneate removal of desensitization and allows inhibition of the steady-state current.
- Concentration curve comparing the relative kynureneate inhibition of GluK2/K5 ± Neto1 peak current amplitude. Co-transfection with Neto1 does not alter inhibition of GluK2/K5 peak current by kynureneate.
- Concentration curve illustrating the relative inhibition of GluK2/K5 ± Neto1 steady state current. Co-transfection with Neto1 prevents inhibition of GluK2/K5 steady state current by kynureneate.

3.3.6 USING ACET AND KYNURENATE AS PHARMACOLOGICAL TOOLS

In the previous sections, we demonstrated that ACET inhibits the GluK5 and GluK4 KAR subunits in heteromers. Additionally, we confirmed that KYN preferentially does not inhibit the GluK5 KAR subunit in heteromers. Thus, we can use these drugs as pharmacologic tools to selectively activate or inhibit specific receptors subunits in the GluK2/K5 heteromer. To test the effectiveness of these tools, we sought to verify the receptor occupancy model for KARs. The receptor occupancy model states that desensitization occurs when three or more subunits are activated. If only two or less KARs are activated, the receptor can open, but will not desensitize (Fisher and Mott, 2011; Fisher and Fisher, 2014; Reiner and Isacoff, 2014). Application of an EC₅₀ glutamate dose allowed GluK2/K5 receptors to rapidly open and desensitize, as illustrated previously. Co-application with 3 mM kynurenate, preferentially inhibits the GluK2 subunits, leaving the GluK5 subunits to open the receptor to a non-desensitizing current. Consecutively, co-applying 1 μM ACET inhibited the remaining predominately GluK5-mediated current (Figure 3.6A). Steady-state was measured across conditions (10 μM glutamate alone, 3 mM KYN, and 3 mM KYN + 1 μM ACET) and normalized to the condition with the maximum steady-state current (KYN). Averaged data demonstrates that the steady-state current was significant reduced in the glutamate alone ($26 \pm 1\%$, $n = 5$) and KYN + ACET (1 μM: $23 \pm 4.5\%$, $n = 5$; 10 μM: $8.3 \pm 1.7\%$, $n = 5$; $p < 0.05$) condition compared to the KYN condition.

We determined that activation of the GluK5 subunit is sufficient to open the receptor. Next, we wanted to determine whether activation of the GluK2 subunit is sufficient to open the heteromeric receptor. We applied the EC₅₀ dose of glutamate (300

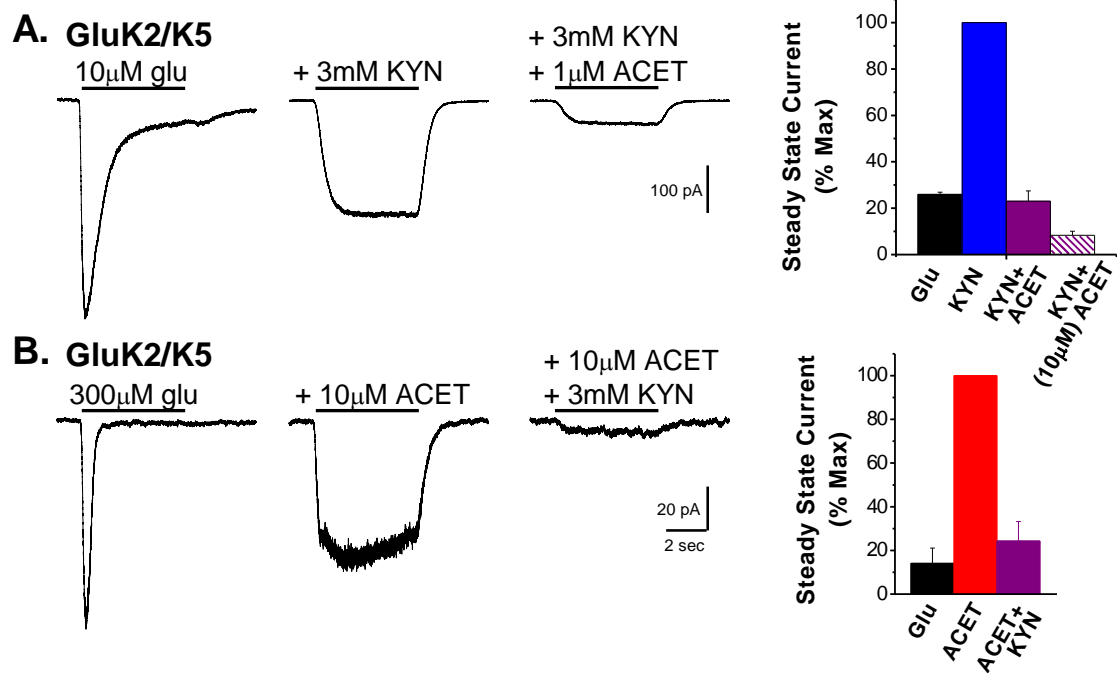


Figure 3.6 Occupancy of either the GluK2 or GluK5 subunits can activate GluK2/K5 receptors

- A. Sample waveforms (left) and bar graph (right) illustrating that kynureate (3 mM) removed desensitization at GluK2/K5 receptors, and consequently enhances the steady state current. ACET (1 μ M) inhibits this steady state current. Bar graph (right) illustrates the amplitude of the steady state current for each condition normalized to the condition with the maximum steady state amplitude (3mM kynureate alone).
- B. Sample waveform (left) and bar graph (right) illustrating that co-application of ACET (10 μ M) with glutamate (300 μ M) enhances the steady state current and the addition of KYN (3 mM) inhibits this steady state current. Bar graph (right) illustrates the steady state current amplitude for each condition normalized to the condition with the maximum steady state current (10 μ M ACET).

μ M) to ensure sufficient activation of the GluK2 subunits. The GluK2 subunit is less sensitive to glutamate activation than the GluK2/K5 subunit. Thus, more glutamate is needed to ensure sufficient binding of the GluK2 subunit. Perfusion of 300 μ M glutamate opened and rapidly desensitized the GluK2/K5 KARs. Co-application of 10 μ M ACET (a

dose chosen to maximally inhibit the GluK5 subunits with minimal inhibition of the GluK2 subunits), open the GluK2/K5 KAR to a non-desensitizing current. Consecutive application of 3 mM KYN with 10 μ M ACET completely inhibited GluK2/K5 current. The steady-state current was significantly reduced in the 300 μ M glutamate alone ($14 \pm 7\%$, $n = 5$, $p < 0.05$) and 10 μ M ACET + 3 mM KYN condition ($26 \pm 1\%$, $n = 5$, $p < 0.05$) compared to the ACET condition.

3.3.7 ACET INHIBITS KAINATE – MEDIATED SYNAPTIC TRANSMISSION

The GluK4 and GluK5 subunits are necessary for kainate receptor synaptic transmission at the mossy fiber – CA3 synapse (Fernandes et al., 2009). Thus, we hypothesized that ACET will inhibit kainate receptor fEPSPs at this synapse. Field EPSPs were evoked by stimulating mossy fibers extending from the dentate gyrus pyramidal cell layer and recording synaptic field potentials in CA3 stratum lucidum. The KAR component of the fEPSP was isolated with an antagonist cocktail: 50 μ M GYKI 53655, 10 μ M MK801, 50 μ M picrotoxin, and 1 μ M CGP 5585, and resembled the slow fEPSPs as previously described (Castillo et al., 1997) (black trace, Figure 3.7A,B). Concurrent perfusion of 1 μ M ACET significantly and completely blocked the KAR fEPSP (red trace, $6.6 \pm 4.8\%$, $n = 8$ $p < 0.05$), Figure 3.7A,B). Averaged data was represented as a bar graph, where the peak amplitude was normalized to the fEPSPs amplitude with the antagonist cocktail alone.

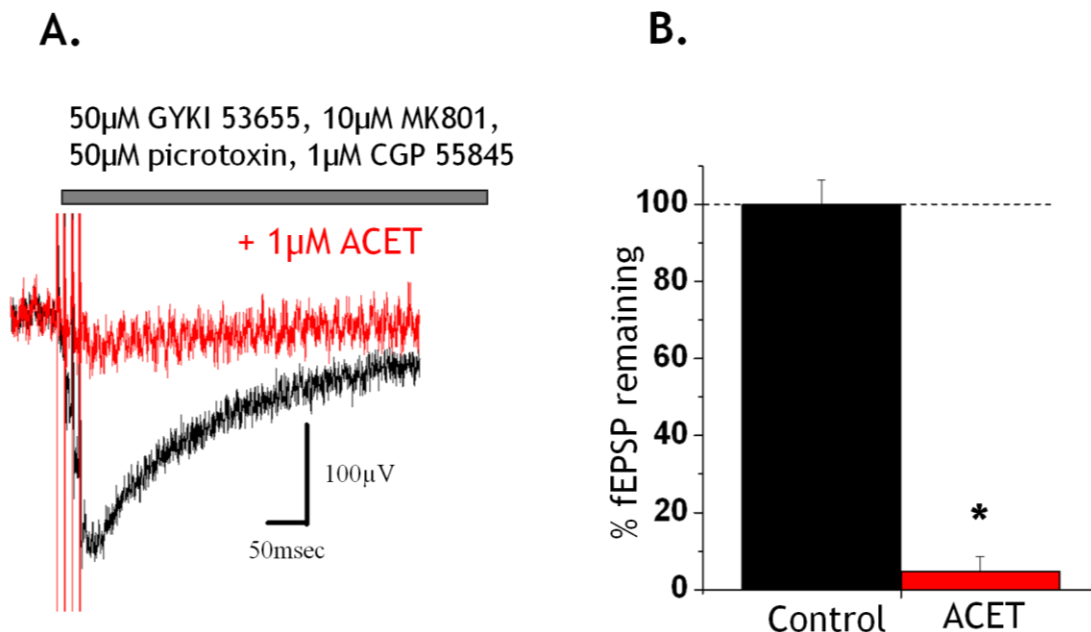


Figure 3.7 ACET blocks kainate receptor synaptic transmission at mossy fiber – CA3 synapses.

- A. Sample waveform of KAR fEPSPs (black trace) isolated by perfusing an antagonist cocktail (50 μ M GYKI 53655, 10 μ M MK801, 50 μ M picrotoxin, and 1 μ M CGP 55845). Adding 1 μ M ACET to the perfusing antagonist cocktail completely inhibited the KAR EPSPs.
- B. Bar graph of averaged data normalized to the KAR fEPSPs. ACET significantly inhibited the KAR fEPSPs.

3.4 DISCUSSION

In the present study, we used whole cell electrophysiology on cells transfected with various GluK2-containing KARs to determine whether ACET and/or kynurenate can be used as pharmacological tools to delineate specific subunit contribution to KAR current. We found that ACET inhibits current at heteromeric KARs by binding to the

GluK4 and GluK5 subunits, and KYN preferentially does not inhibit the GluK5 subunit. Furthermore, we demonstrated that ACET inhibits KAR – mediated synaptic current in the brain, specifically the mossy fiber – CA3 synapse. These drugs can be used as pharmacological tools to distinguish GluK2 subunit contribution from KAR current from the GluK5 subunit.

3.4.1 INHIBITION OF GLUK2-CONTAINING KAINATE RECEPTORS BY ACET

ACET is a willidarine compound originally characterized as a GluK1 antagonist (Jane et al., 2009). However, doubts about ACET being a selective drug for GluK1 has surfaced. One reason for recent suspicion is that other compounds within the ACET family and with a similar structure, have demonstrated a more promiscuous ligand affinity than for the GluK1 subunit alone. One such compound was the drug UBP310 (Pinheiro et al., 2013). Pinheiro et al., described quite convincingly that UBP310 can inhibit current at GluK2/K5 currents in cultured cells and acute brain slices (Pinheiro et al., 2013). Here we describe that ACET, similar to UBP310, also has a more promiscuous ligand affinity than previously thought and inhibits glutamate current at GluK2/K5 receptors. We took it a step further and characterized another GluK2-containing heteromer, GluK2/K4. Our results demonstrated that not only is the GluK2/K5 receptor sensitive to ACET inhibition, but also GluK2/K4 receptors. Furthermore, by mutating the GluK2 subunit, we determined that ACET inhibition of GluK2-containing heteromers was due to ACET inhibiting specifically the GluK4 and GluK5 subunits.

Interestingly, ACET inhibition at GluK2/K4 wildtype receptors plateaued around 45% of the maximum glutamate response, such that higher concentrations of ACET did not produce additional inhibition of the GluK2/K4 current. The reason for the plateau could be that the GluK4 is fully inhibited (as indicated by the GluK2(E738D)/K4 concentration-curve), however the remaining current is mediated by the GluK2 subunit. An alternative explanation is that ACET may act as a partial agonist. Partial agonists have partial efficacy at the receptor relative to a full agonist. They are often confused with competitive antagonists because partial agonists can compete with full agonists for receptor binding sites and produce a net reduction in receptor activation. Future studies are needed to determine whether ACET is in fact a partial agonist. Our results suggest that ACET is not a specific GluK1 antagonist, but should be considered more of an inhibitor of most KARs (there is no data available on ACET inhibition of GluK3 KARs) *except* at GluK2 subunits.

We also examine the impact of the auxiliary subunit Neto1 on the ACET inhibition of GluK2/K5 current. Neto1 is an auxiliary protein known to bind to KARs in the brain and recombinant cells (Tang et al., 2011). Neto1 has a role in incorporation of KARs to the synapse and alters for KAR synaptic transmission (Copits et al., 2011; Straub et al., 2011a). In recombinant cells, Neto1 has little impact on surface expression of recombinant KARs. However, Neto1 does change receptor kinetics (Fisher and Mott, 2012, 2013). Co-expression of Neto1 shifts the concentration dependent for the onset of desensitization towards higher levels of glutamate, but does not change the glutamate EC₅₀ for GluK5-containing subunits (Fisher and Mott, 2013). Thus, at the glutamate EC₅₀ for GluK2/K5 (10 μ M) the presence of Neto1 changed the receptor kinetics from a

rapidly desensitizing current to a non-desensitizing current, as previously reported (Fisher and Mott, 2013). In the present study, we demonstrate that the presence of Neto1 does not significantly change ACET inhibition of GluK2/K5 peak current. However, the presence of Neto1 did alter how ACET inhibited the heteromeric current. ACET did not inhibit the remaining steady-state current in the absence of Neto1. However, in the presence of Neto1, ACET inhibited the steady-state current. It is our suggestion that ACET does not bind to the desensitized conformation of GluK2/K5 heteromeric receptor. Thus, in preparations when Neto1 is absent and the GluK2/K5 receptors are allowed to undergo a desensitized conformation, ACET will inhibit the GluK2/K5 peak current when the receptors are predominantly in an open/activated conformation. However, ACET did not inhibit the steady state GluK2/K5 current when majority of the GluK2/K5 receptors are in the desensitized conformation. Alternatively, when Neto1 is co-expressed with GluK2/K5 receptors, the GluK2/K5 receptor is prevented from undertaking a desensitized conformation, and now ACET can inhibit both the peak and steady state current. These results suggest that ACET may have bias towards the physical conformation of GluK5-containing kainate receptors.

Another cause for suspicion on the subunit selectivity of ACET, is that ACET produced inhibition of kainate mediated mossy fiber – CA3 EPSPs (Dargan et al., 2009). Interestingly, GluK1 is not expressed at detectably levels on CA3 pyramidal cells, and thus, does not contribute to kainate mediated mossy fiber – CA3 EPSPs (Bahn et al., 1994; Bureau et al., 1999; Paternain et al., 2000). These results produced confusion about the function of KARs in the brain. We submit that the previous confusion was stemmed from the misunderstanding about the subunit selectivity of ACET. Our results from

recombinant cells demonstrate that ACET inhibits current at heteromeric receptors containing either GluK4 or GluK5 subunits. Both GluK4 and GluK5 subunits are expressed at high level postsynaptically on CA3 pyramidal cells (Bahn et al., 1994), and they are required for kainate-mediate current at that synapse (Fernandes et al., 2009). Thus, our results provide further reasoning that postsynaptic KARs located on CA3 pyramidal neurons are composed of GluK4 and GluK5 subunits.

Additionally, we discovered in our own hands that ACET completely blocks kainate mediated EPSPs at mossy fiber – CA3 synapses. This was quite interesting to us, because although we expected ACET to reduce KAR EPSPs at this synapse, we did not expect a complete blockage of this KAR EPSPs. Glutamate released from presynaptic terminals can reach concentrations of ~ 1mM before rapidly declining (Clements et al., 1992; Clements, 1996; Diamond and Jahr, 1997). Majority of our data in this paper used the EC₅₀ dose of 10 μ M glutamate. This concentration is sufficient to activate the GluK5 subunits; however, it is 30 fold less than the EC₅₀ glutamate concentration for the GluK2 subunit. Thus, most of the current is produced as a result of glutamate binding to the GluK5 subunit, in which 1 μ M ACET produced a pronounced inhibition of the current. However, when glutamate concentration was increased (300 μ M glutamate) equivalent to the EC₅₀ for the GluK2 subunit and allowing sufficient activation of the GluK2 subunit, 1 μ M ACET produced much less inhibition of the peak GluK2/K5 current (Figure 3.6B). We suggest that these seemingly contradictory data between ACET inhibition of the recombinant and brain slice KAR responses is an extension of ACET bias for the open/activation conformation over the desensitized conformation. At the synapse, released glutamate is rapidly removed before kainate receptors can undergo a

desensitized conformation (deactivation). However, in our preparations the onset and duration (5 seconds) of applied glutamate is slow compared to the synapse, and allows sufficient time for KAR desensitization. Indeed, other studies have demonstrated with similar antagonist to ACET (UBP310), that rapidly applying glutamate allows UBP310 to inhibit the GluK2/K5 response significantly more than with prolonged application of glutamate (Pinheiro et al., 2013). Another potential explanation is that higher-affinity GluK5 subunits contribute to the rapid activation of GluK2/K5 heteromeric receptors even at high glutamate concentrations, while during prolonged glutamate application the binding of lower-affinity GluK2 subunits (which are insensitive to ACET and UBP310) to glutamate contributes more to the GluK2/K5 heteromeric current.

3.4.2 INHIBITION OF GLUK2-CONTAINING KAINATE RECEPTORS BY KYNURENATE

Kynurenate is a nonselective competitive antagonist of glutamate receptors. Previous studies have reported that kynurenate has a substantially higher affinity for GluK2 than GluK5 subunits (Fisher and Mott, 2011). However, it is not known whether kynurenate can inhibit currents at GluK2/K4 heteromers nor whether the presence of Neto1 alters kynurenate inhibition of KAR current. At a concentration that blocked current at GluK2 homomers, our findings demonstrate that kynurenate produced little inhibition of peak current and removed desensitization from GluK2/K5 receptors. These results are consistent with previously reported data (Fisher and Mott, 2011). We further characterized kynurenate inhibition of GluK2/K4 receptors. Kynurenate dose dependently inhibited GluK2/K4 heteromers. Unlike kynurenate inhibition of GluK2/K5

receptors, kynurenate did not substantially increase the steady-state current. The resulting kinetics of kynurenate inhibition resembled kynurenate inhibition of GluK2 homomers. We do not know why there is such qualitative difference in receptor kinetics between the GluK2/K4 and GluK2/K5 receptors when kynurenate is applied. One suggestion is that the GluK4 subunit appears to make greater contribution to desensitization (Mott et al., 2010), and can desensitize to glutamate activation in and of itself (Fisher and Fisher, 2014).

We also examined whether co-expression with Neto1 alters kynurenate inhibition of KAR current. Similarly to our previous data with ACET, the presence of Neto1 did not alter GluK2/K5 sensitivity to kynurenate inhibition. However, it did alter the kinetics of the receptor once kynurenate was applied. As reported earlier, kynurenate potentiated the GluK2/K5 steady-state current (Fisher and Mott, 2011). Co-expression of Neto1 removed desensitization and allowed kynurenate to inhibit the steady-state current.

3.4.3 FUNCTIONAL SIGNIFICANCE OF PHARMACOLOGICAL TOOLS FOR KAINATE RECEPTORS

KARs contribute to synaptic integration at glutamate synapses (Frerking and Ohliger-Frerking, 2002). Specifically, GluK2/K5 receptors are known to constitute a major population of KARs in the brain: both subunits are abundantly co-expressed in the cerebellum, neocortex, striatum, amygdala, and hippocampus, at higher levels than other KAR subunits (Bureau et al., 1999). The lack of pharmacological tools has hindered our understanding of KARs and their function in the brain. Recent studies, using congenital

knockout animals, have shown that GluK5 subunits are required for proper function of synaptic KARs and contribute to their slow kinetics (Contractor et al., 2003; Barberis et al., 2008; Fernandes et al., 2009). However, congenital knockouts are not site or region specific and remove presynaptic KARs. Additionally, congenital knockouts may undergo receptor compensation and not represent KAR function in the normal brain. It would therefore be of great value to be able to directly test the implication of KARs in synaptic integration and information transfer in CA3 pyramidal cells.

Based on our results we identified ACET and kynureate as potential pharmacological tools to elucidate the relative function of GluK2 and GluK5 receptors at GluK2/K5 heteromers. Previous studies observed that glutamate binding of GluK5 subunits activated GluK2/K5 channels and subsequent activation of the GluK2 subunit desensitizes the receptor (Fisher and Mott, 2011). They concluded that the GluK2 subunit intrinsically desensitized the receptor. However, it is not known whether the GluK2 subunit can open the GluK2/K5 receptor to a non-desensitizing current. We identified ACET as a drug selective for the GluK4 and GluK5 subunits in GluK2-containing heteromers. Additionally, we identified kynureate as a glutamate antagonist that preferentially inhibits the GluK2 subunits. Thus, we used both ACET and kynureate to determine whether the GluK2 or the GluK5 subunits are intrinsically unique in their functional role in desensitizing the receptor. Applying 10 μ M ACET inhibited glutamate binding to the GluK5 subunits, and thus glutamate binding to only the GluK2 subunits was sufficient to open the heteromeric receptor to a non-desensitizing current. The remaining current was sensitive to 3 mM kynureate inhibition, suggesting the remaining current was predominantly mediated by glutamate binding to the GluK2 subunit.

Furthermore, applying 3 mM kynurenate to GluK2/K5 receptors exposed to 300 μ M glutamate (an EC_{50} for the GluK2 subunit) produced a non-desensitizing current as previously described in this study and other publications (Fisher and Mott, 2011). Concurrent application of 1 μ M ACET inhibited the remaining current, suggesting that the remaining current was mediated predominantly by glutamate binding to the GluK5 subunit. Our results indicate that either GluK2 or GluK5 subunits can open the KAR channel, while binding of the other can initiate desensitization. Thus, we propose that KAR desensitization requires three or more subunits to bind to glutamate regardless of the subunits activated. Additionally, we observed that glutamate binding to either the GluK2 or the GluK5 subunits is sufficient to open KAR receptors. Thus, ACET and kynurenate can be used as pharmacological tool to differentiate subunit contribution to the KAR current.

CHAPTER 4

MUSCARINIC AND DOPAMINERGIC RECEPTOR REGULATION OF KAINATE RECEPTOR NEUROTRANSMISSION AT THE MOSSY FIBER – CA3 SYNAPSE

4.1 INTRODUCTION

Muscarinic acetylcholine receptors (mAChRs) play a critical role in synaptic plasticity and neuronal excitability (Dodd et al., 1981). In the hippocampus, they are important for modulating learning and memory in the hippocampus (Power et al., 2003). There are five types of mAChR, M1 – M5 (Bonner, 1989), all of which, except M5 mAChRs, can be found at different levels of expression in area CA3 in the hippocampus (Volpicelli and Levey, 2004) where they are co-expressed with kainate receptors. One way mAChRs influence the synaptic network is by modulating the function of glutamate receptors. Both NMDA and AMPA receptors have been shown to be susceptible to mAChR modulation (Marino et al., 1998; Lu et al., 1999; Grishin et al., 2005; Dickinson et al., 2009; Nomura et al., 2012). Specifically, NMDA receptor currents in CA3 pyramidal neurons are reduced by activation of M1 mAChRs through a mechanism proposed to involve a calmodulin-activated tyrosine phosphatase (Grishin et al., 2005). Additionally, M1 mAChRs induce long-term depression of hippocampal AMPA receptor activity through protein kinase C (PKC) mediated phosphorylation of specific AMPAR subunits, which cause AMPAR dissociation from GRIP1 (a postsynaptic domain

localization protein) and subsequent removal of AMPARs from the synapse (Dickinson et al., 2009). Although we know that NMDAR and AMPARs can be modulated by M1 mAChRs, it is not known whether M1 mAChRs can alter KAR activity in the brain.

There are several lines of evidence to suggest that M1 mAChRs can modulate KAR activity in the brain. Dysfunctional interactions between KARs and mAChRs have been demonstrated in a number of neurological diseases, including temporal lobe epilepsy (Frucht et al., 2000). For example, injection of a mAChR agonist (pilocarpine) in rodents induces prolonged seizures and epilepsy (Cavalheiro et al., 1991), which can be blocked by a KAR antagonist (Smolders et al., 2002). Also, previous studies have reported that M1 mAChRs can increase KAR current in recombinant cells (Benveniste et al., 2010). Although these studies were not conducted in brain tissue, it does validate the potential for KAR and M1 mAChR interaction. As stated earlier, M1 mAChRs are GPCRs and elicits modulatory effects through phosphorylation cascades and second messenger systems (Felder, 1995). KAR subunits can be phosphorylated, which can affect both KAR channel conductance and KAR trafficking to and away from the synapse (Cho et al., 2003; Park et al., 2006; Selak et al., 2009; Rojas et al., 2013). Phosphorylation sites for PKC have been found on the GluK2 and GluK5 subunits (Rojas et al., 2013). However, it remains unclear whether M1 mAChRs can alter KAR activity in the hippocampus or whether this can be mediated through a PKC phosphorylation-signaling cascade.

In this study we sought to determine whether M1 mAChRs alters KAR activity in the hippocampus. We evaluated this question at the mossy fiber – CA3 synapse because it contains a reliable and well-characterized postsynaptic KAR activity. Our hypothesis is

that, similarly to NMDAR at the same synapse, activation of M1 mAChRs will depress KARs at the mossy fiber – CA3 synapse. Furthermore, we suspect this M1 mAChR mediated depression will be mediated through a PKC phosphorylation signaling cascade.

4.2 MATERIALS AND METHODS

4.2.1 BRAIN SLICES PREPARATION

Hippocampal slices were made from postnatal day (P) 21 – P28 and P45 – P60 Sprague-Dawley rats as described previously (Iyengar and Mott, 2008). Briefly, animals were deeply anesthetized with isoflurane and decapitated with a guillotine. The brain was removed under ice-cold (4°C), sucrose-based ‘cutting’ artificial cerebrospinal fluid (aCSF) that contained (in mM): 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 248 sucrose, 0.5 CaCl₂, and 5 MgSO₄ (350mOsm). The use of this solution during the cutting process enhances neuronal survival by limiting excessive excitation. Transverse slices were made with a vibratome (Leica VT1000S, Nussloch, Germany) at 500 μm and incubated for at least one hour at room temperature in aCSF containing (in mM): 125 NaCl, 2.7 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 glucose, 0.5 CaCl₂, 5 MgSO₄, 20 μM D-AP-5, and 1 mM kynureate (305 mOsm; pH=7.4). Both the sucrose-based and standard aCSF were oxygenated with 95% O₂/ 5% CO₂.

4.2.2 FIELD POTENTIAL ELECTROPHYSIOLOGY

After 1 hour, individual slices were transferred to a submersion chamber maintained at 32 – 34 °C, held in place by a bent piece of platinum wire resting on the surface of the slice, and perfused continuously at 3 – 4 ml/min with recording aCSF containing (in mM): 125 NaCl, 2.01 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 glucose, 2 CaCl₂, 1 MgSO₄. Glass electrodes were pulled from borosilicate glass to achieve 2 – 3 MΩ and filled with recording aCSF. Extracellular dendritic field potentials recording were made from the stratum lucidum of the CA3 region of the hippocampus, which was visually identified with a light microscope. Synaptic currents were evoked with a monopolar platinum-iridium glass electrode positioned in the stratum lucidum. For AMPA-fEPSPs recordings, we recorded composite MF-fEPSPs, which were primarily composed of AMPA-fEPSPs (~ 93%) (Contractor et al., 2003), with aCSF containing 10 μM MK-801, 50 μM picrotoxin, and 1 μM CGP 55845 to block NMDA, GABA_A, and GABA_B receptors, respectively. We defined synaptic currents as MF-fEPSPs if they showed characteristically large paired-pulse facilitation (Salin et al., 1996), had a rapid rise time and short latency, and were inhibited by >70% by the group II-selective metabotropic glutamate receptor (mGluR) agonist (2S,2'R,3'R)-2-(2'3'-Dicarboxycyclopropyl) glycine (DCG-IV, 1 μM), which was bath applied at the end of some experiments. To isolate KA-fEPSPs recordings, we recorded from CA3 pyramidal cells in the presence of the aCSF containing the AMPA antagonist GYKI53655 (50 μM) in addition to the GABA_A, GABA_B and NMDA receptor antagonist cocktail. Once KAR-fEPSPs were isolated, a four pulse train stimuli (100 Hz, 0.1 ms/stimulus, cathodal, monophasic, rectangular constant current pulses) were delivered every 30 sec through

monopolar, platinum-iridium stimulating electrodes (FHC Inc., Bowdoin, ME) referenced to the bath ground. KAR-fEPSPs were stable for a minimum of 20 min. prior to pilocarpine (300 nM) perfusion onto the slice for 10 – 15 min. to activate predominately M1 mAChRs. In experiments where M1 mAChRs or PKC were inhibited, telenzepine (100 nM) or chelerythrine (5 μM) respectively were applied concurrently with AMPA, GABA_A, GABA_B, and NMDA antagonists. To resolve small KAR-fEPSPs that were obscured by stimulation artifacts, we digitally subtracted a “template” stimulation trace that was acquired after application of an AMPA/KAR antagonist (CNQX, 50 μM) at the end of the recordings.

4.3 RESULTS

4.3.1 KAINATE RECEPTOR SYNAPTIC TRANSMISSION IS DEPRESSED BY mAChR ACTIVATION

Muscarinic receptors have been shown to affect the function of AMPA and NMDA glutamate receptors. However, it is not known whether muscarinic receptors can regulate the function of kainate receptors. To test this question, we used the drug pilocarpine. Pilocarpine is a M1 preferring mAChR agonist. Previous studies have demonstrated that mAChR activation with pilocarpine can alter KAR currents in oocytes and KAR-dependent excitability in mossy fibers (Benveniste et al., 2010). Here, we demonstrate that pilocarpine (300 nM), a muscarinic acetylcholine receptor (mAChR)

agonist that preferentially binds to M1 mAChRs, produced a depression in KAR fEPSPs at the mossy fiber – CA3 synapse (Pilo: $63.6 \pm 6.9\%$ of baseline fEPSP amplitude, $n = 8$, $p < 0.05$) (Figure 4.1A). We previously demonstrated that ACET (1 μM), a previously characterized GluK1 KAR subunit – selective antagonist, inhibits current at heteromeric receptors containing either the GluK4 or GluK5 subunits (Chapter 3). Additionally, we demonstrated that ACET (1 μM) was sufficient at completely inhibiting KAR current at the mossy fiber – CA3 synapse (Chapter 3). Thus, we applied ACET (1 μM) after each experiment as a control to verify that we were measuring the KAR fEPSP. Any further depression induced by ACET application indicated the remaining KAR fEPSPs. After pilocarpine administration, ACET (1 μM) application completely blocked the KAR fEPSP, such that a competitive AMPA/KAR antagonist (CNQX, 50 μM) did not produce any additional inhibition of the KAR fEPSP current (Figure 4.1A). Thus, by using both controls (ACET and CNQX) we are confident that we measured the KAR fEPSP in its entirety.

There are several ways mAChRs can modulate KAR fEPSPs. Muscarinic acetylcholine receptors, specifically M1, co-localize with KARs on CA3 pyramidal neurons. There are two potential ways these receptors can interact. Activation of mAChRs can either 1) initiate a G-protein to begin a signaling cascade that would eventually cause phosphorylation of the KARs, or 2) directly binding to KARs. Both of these scenarios are a direct postsynaptic mechanism and require mAChRs and KARs to be co-localized. Alternatively, mAChRs could affect KAR fEPSPs by affecting the amount of glutamate released from presynaptic terminals. If activation of mAChRs reduced glutamate release from mossy – fiber terminals, then the KAR fEPSPs would be

reduced because less glutamate is binding to KARs postsynaptically. This scenario does not require mAChRs to be co-localized with KARs, and thus, is an indirect presynaptic mechanism by which activation of mAChRs can affect KAR fEPSPs. To determine whether mAChR depression of fEPSPs at the mossy fiber – CA3 synapse is postsynaptic

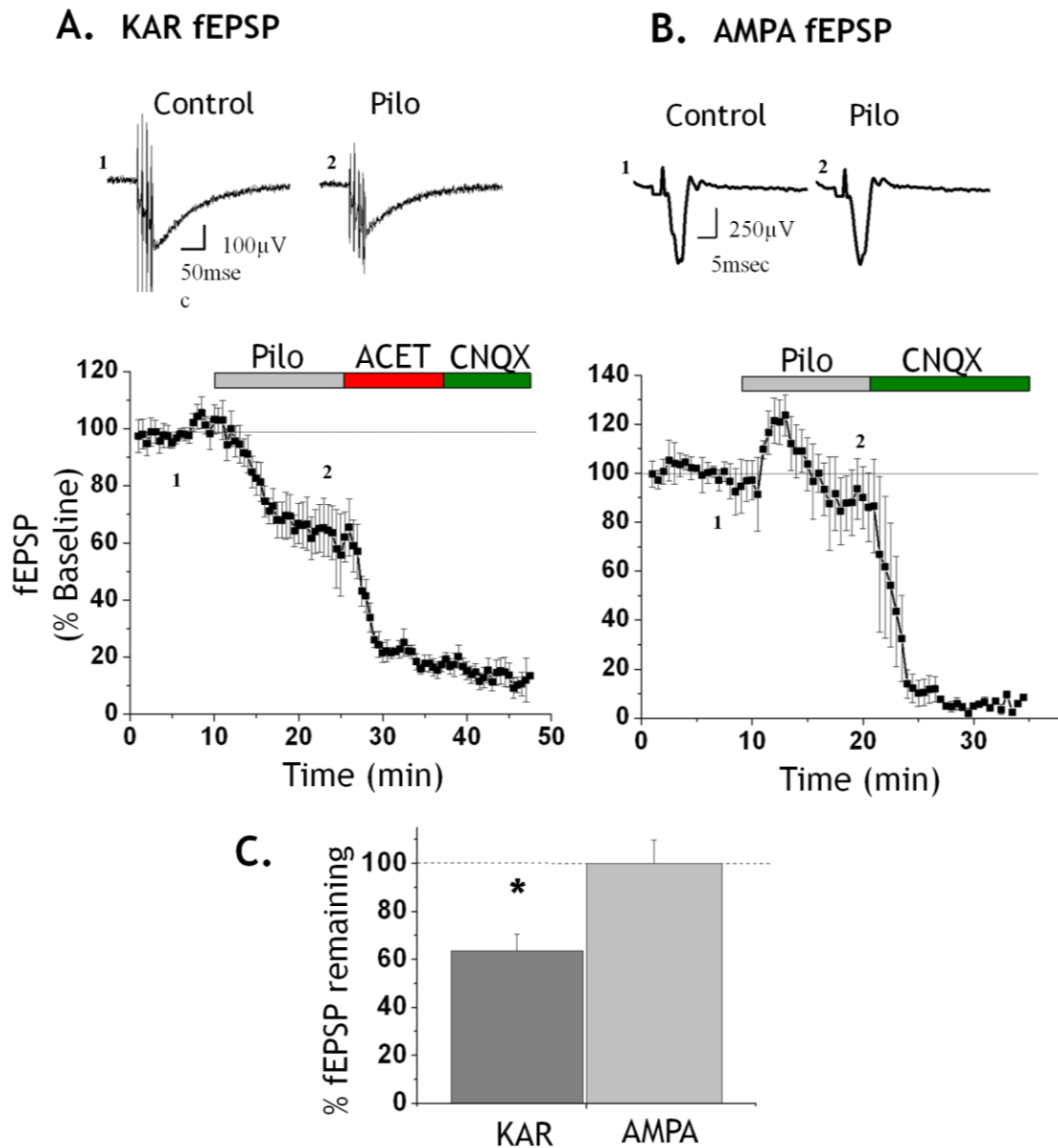


Figure 4.1 mAChR activation depresses kainate, but not AMPAR-mediated fEPSPs

- A. A graph illustrating the peak amplitude of KAR fEPSP as it changes over time as a result of drug application. Trains were evoked by stimulation of the mossy fiber – CA3 pathway. Application of pilocarpine (300 nM) depresses the kainate fEPSP. ACET (1 μ M), a drug we previously characterized as an antagonist at GluK4 and GluK5 receptors, completely inhibited the KAR-fEPSP, as verified by an AMPA/KAR antagonist (CNQX, 50 μ M)
- B. Pilocarpine (300 nM) does not depress the AMPA fEPSP. Subsequent application of CNQX (50 μ M) completely block synaptic transmission, confirming the presence of AMPA receptors.
- C. Bar graph illustrating peak amplitude for KAR and AMPA fEPSP normalized to the peak amplitude recorded during baseline. Pilocarpine significantly depresses the kainate, but not the AMPA fEPSP measured after 10 min. of pilocarpine application.

or presynaptic, we tested whether the AMPA fEPSP is also affected by mAChR activation. To isolate the AMPA fEPSPs, we perfused an antagonist cocktail (same as Chapter 3) to block NMDA, GABA_A, GABA_B, and KAR synaptic transmission throughout the experiment (Figure 4.1B). Application of pilocarpine (300 nM) did not inhibit the AMPA fEPSP (Pilo: $100.9 \pm 9.7\%$ of baseline fEPSP amplitude, $n = 7$, $p < 0.05$). Subsequent application of CNQX (50 μ M) served as a control and completely inhibited the AMPA fEPSP. The inhibition of KAR fEPSPs by pilocarpine (300 nM) was significantly more than AMPA fEPSPs.

4.3.2 mAChR DEPRESSION OF KARs IS A FORM OF SHORT-TERM PLASTICITY

Next we wanted to determine whether this mAChR – induced depression of KAR fEPSPs was a form of short-term plasticity or long-term plasticity. Plasticity is a change

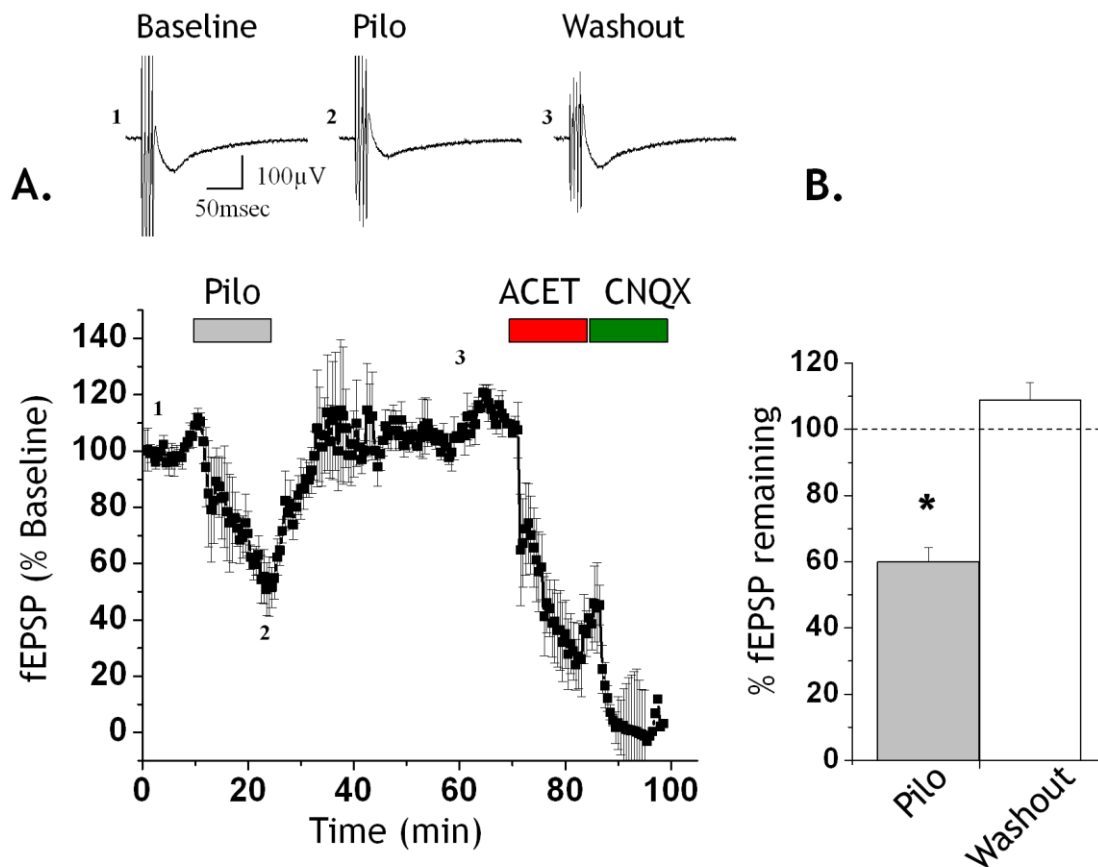


Figure 4.2 mAChR depression of KAR is short term

- A. Pilocarpine (300 nM) transiently depresses the kainate fEPSP.
- B. Pilocarpine (300 nM) significantly depresses the kainate fEPSP, but not 45 minutes after pilocarpine is washed out.

in the way one neuron communicates with another neuron. This change in communication can persist longer than 1 hour (long-term plasticity) or less than 1 hour (short-term plasticity). Both forms of plasticity have important implication in the brain (Martin et al., 2000). Inhibition of either short-term or long-term plasticity in the

hippocampus causes significant deficits in learning and memory (Morris et al., 1986; Silva et al., 1996). To test which form of synaptic plasticity is exemplified, we applied pilocarpine (300 nM) as previously described. Then, we washed out the pilocarpine for 45 minutes and recorded the KAR fEPSP amplitude as a percent to the baseline amplitude (Figure 4.2A). Pilocarpine (300 nM) produced a similar depression to KAR fEPSPs as we reported previously (Pilo: $60.0 \pm 4.3\%$ of baseline fEPSP amplitude, $n = 5$, $p < 0.05$). However, this depression went away as pilocarpine (300 nM) was washed out (Washout: $108.7 \pm 5.5\%$ of baseline fEPSP amplitude, $n = 5$, $p < 0.05$). Thus, the mAChR depression of the KAR fEPSP did not last longer than 1hour, and resembles a short-term plasticity. Subsequent application of ACET (1 μ M) and CNQX (50 μ M) produced complete inhibition of the measured KAR fEPSP. As stated previously, pilocarpine (300 nM) induced a significant depression of KAR fEPSPs. However, the KAR fEPSP amplitude was not significantly different from baseline 45 min. after pilocarpine (300 nM) application (washout) (Figure 4.2B).

4.3.3 ACTIVATION M1 mAChRS DEPRESS KAR fEPSPS AT MOSSY FIBER – CA3 SYNAPSES

Our previous data demonstrated that activation of mAChRs transiently depresses KAR fEPSP at mossy fiber – CA3 synapses through a direct postsynaptic mechanism. The next step in elucidating this mechanism is to determine the mAChR necessary for KAR fEPSP depression. Pilocarpine at low concentrations is considered an M1 preferring agonist (Fisher et al., 1993). Additionally, M1 mAChRs are abundantly expressed in CA3

pyramidal neurons (Cortes et al., 1984; Cortés et al., 1986; Rodríguez-Puertas et al., 1997; Scarr et al., 2007). Thus, we sought to determine whether M1 mAChRs are

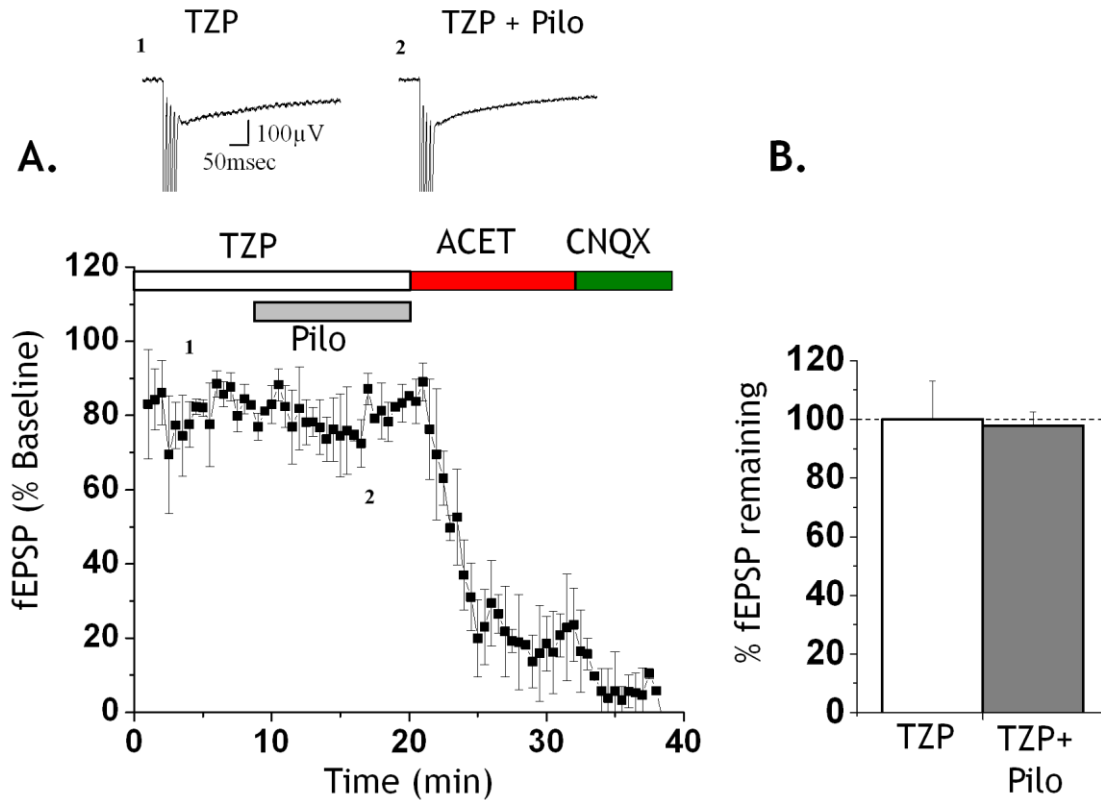


Figure 4.3 M1 mAChRs mediate the pilocarpine-induced depression of KAR fEPSPs

- A. Telenzepine (100 nM), an M1 mAChR antagonist, prevents pilocarpine-induced depression of KAR fEPSPs at mossy fiber – CA3 synapses.
- B. There is no significant difference in KAR fEPSP amplitude in the presence of telenzepine (100 nM) with or without pilocarpine (300 nM) co-application.

responsible for the pilocarpine – induced depression of KAR fEPSPs. To test this, we isolated the KAR fEPSP as previously described and preapplied an M1 mAChR

antagonist (telenzepine, 100 nM) for a minimum of 15 min before administering pilocarpine (300 nM). In the presence of telenzepine (100 nM), pilocarpine (300 nM) failed to depress the KAR fEPSP (Figure 4.3A). The presence of a KAR fEPSP was verified by complete inhibition of synaptic transmission by subsequent applications of ACET (1 μM) and CNQX (100 μM). A bar graph illustrates that neither application of telenzepine (100 nM) nor co-application of telenzepine (100 nM) and pilocarpine (300 nM) produced significant inhibition of the KAR fEPSP amplitude as compared to baseline KAR fEPSP amplitude prior to telenzepine (100 nM) application ($97.9 \pm 4.7\%$, $n = 7$) (Figure 4.3B).

4.3.4 PKC PHOSPHORYLATION IS NOT REQUIRED FOR MACHR – INDUCED DEPRESSION OF KAR fEPSPS

We discovered earlier that M1 mAChRs depresses KAR fEPSPs more than likely through a postsynaptic mechanism. One potential mechanism is by M1 mAChRs eliciting a G-protein mediated second messenger signaling cascade resulting in the phosphorylation of KARs. M1 mAChRs can couple to $G_{\alpha q}$ and are thought to initiate a second messenger cascade involving protein kinase C (PKC). To test whether PKC phosphorylation is necessary for M1 mAChR – induced depression of the KAR fEPSPs, we bath applied a PKC inhibitor (chelerythrine, 5 μM) at least 15 min. prior to and during pilocarpine (300 nM) application (Figure 4.4 A,B). In the presence of chelethryine (5 μM), pilocarpine still depressed the average KAR fEPSP amplitude to levels similar to that of pilocarpine application alone ($67.9 \pm 13.7\%$, $n = 6$). However, the depression in KAR fEPSPs was not statistically significant. Thus, PKC mediated phosphorylation of

KARs may not be fully responsible for M1 mAChR depression of KAR fEPSPs. Subsequent bath application of ACET (1 μ M) and CNQX (100 μ M) verified the presence of KAR fEPSPs.

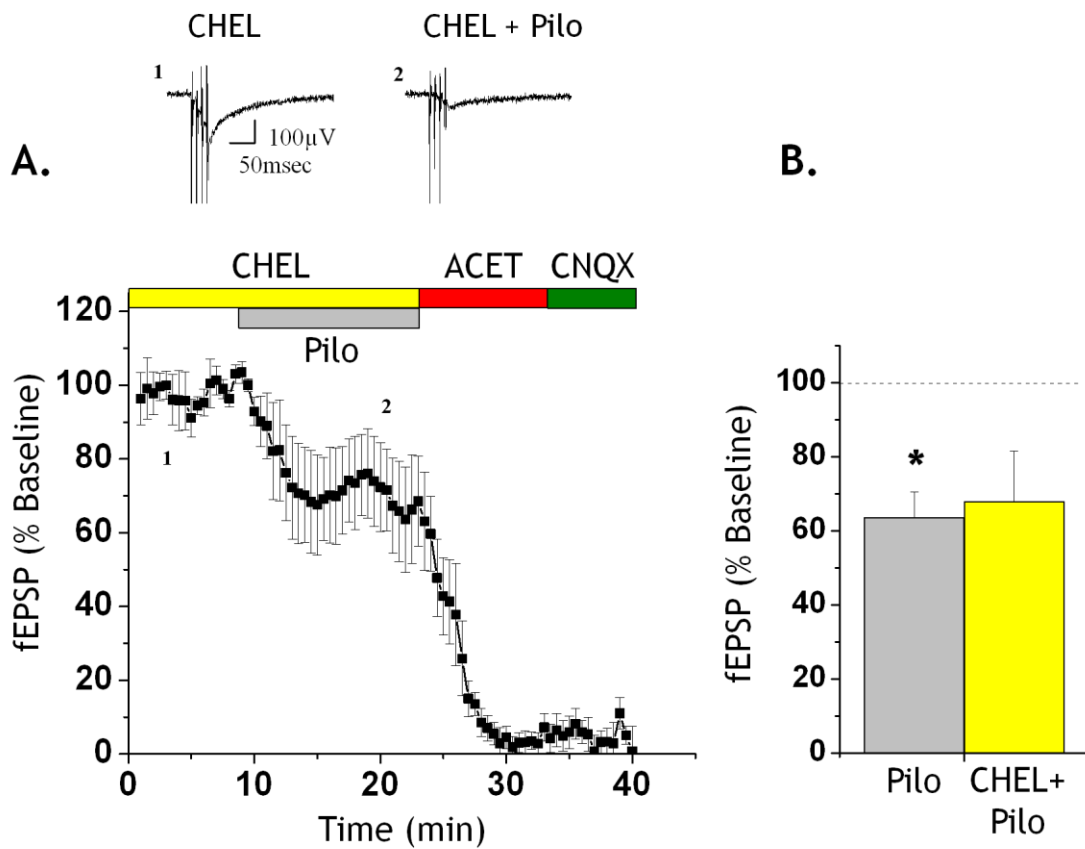


Figure 4.4 mAChRs depression of KARs is not mediated through PKC

- A. Chelerythrine (5 μ M), a PKC inhibitor, does not prevent pilocarpine-induced depression of KAR fEPSPs at mossy fiber – CA3 synapses.
- B. Pilocarpine (300 nM) depression of KAR fEPSPs is not significant in the presence of chelerythrine.

4.3.5 M1 mAChR DEPRESSION OF KAR fEPSP IS DEVELOPMENTALLY REGULATED

Kainate receptor expression and function is fundamentally different in the adolescent brain than the adult brain (Bahn et al., 1994). All data collected previously was using adolescent aged rats (P21 – P28). However, it is not known whether this regulation changes during further development. Thus, we examined whether this same phenomenon (M1 mAChR activation depresses KAR fEPSP) happens in an ‘adult’ aged brain. In this experiment, we used rats aged P65 – P80. The experimental set up was

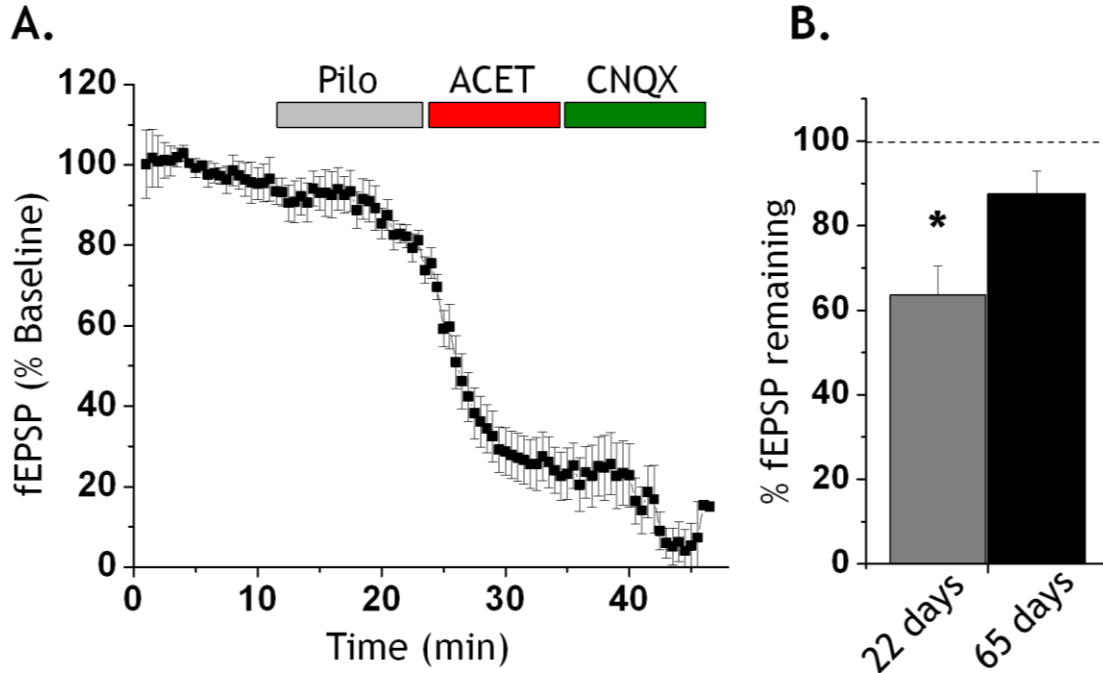


Figure 4.5 mAChR depression of KARs is developmentally regulated

- A. Pilocarpine (300 nM) fails to depress the kainate fEPSP in 65 days old rats.
- B. Pilocarpine (300 nM) significantly suppresses kainate fEPSP in 22 days old rats, but not 65 days old rats.

identical to previous experiments by isolating the KAR fEPSP with an antagonist cocktail. Then, we recorded a minimum of 20 min. of stable baseline (Figure 4.5A). Bath application of pilocarpine (300 nM) did not depress the KAR fEPSP. Subsequent bath application of ACET (1 μ M) and CNQX (100 μ M) confirmed the presence of KAR synaptic transmission. A bar graph illustrates that pilocarpine (300 nM) application significantly depressed the KAR fEPSPs in “adolescent” rat brains (22 days); however, pilocarpine (300 nM) failed to produce a significant depression of KAR fEPSPs in the “adult” rat brain (65 days) ($87.6 \pm 5.3\%$, $n = 5$).

4.3.6 D1-LIKE DOPAMINE RECEPTORS DO NOT ALTER KAR fEPSP AT THE MOSSY FIBER – CA3 SYNAPSE

Dopamine receptors (DARs) are another GPCR with important modulatory affects in the brain (Ehringer and Hornykiewicz, 1960; Robinson and Berridge, 1993; Goto and Grace, 2007; Del Campo et al., 2011; Lodge and Grace, 2011). DARs are separated into two groups based upon the G protein in which they associate: D1 and D5 DARs (D1-like) couple to G_s proteins and stimulate adenylate cyclase activity, leading to PKA phosphorylation, while D2, D3, and D4 DARs (D2-like) couple to G_q proteins and inhibit adenylate cyclase activity. Specifically, the D1 DARs in the hippocampus are necessary for learning and memory (Huang and Kandel, 1995; Lemon and Manahan-Vaughan, 2006; Bethus et al., 2010; Clausen et al., 2011; da Silva et al., 2012). Previous studies have shown that D1-like DARs can mediate other ionotropic glutamate receptors,

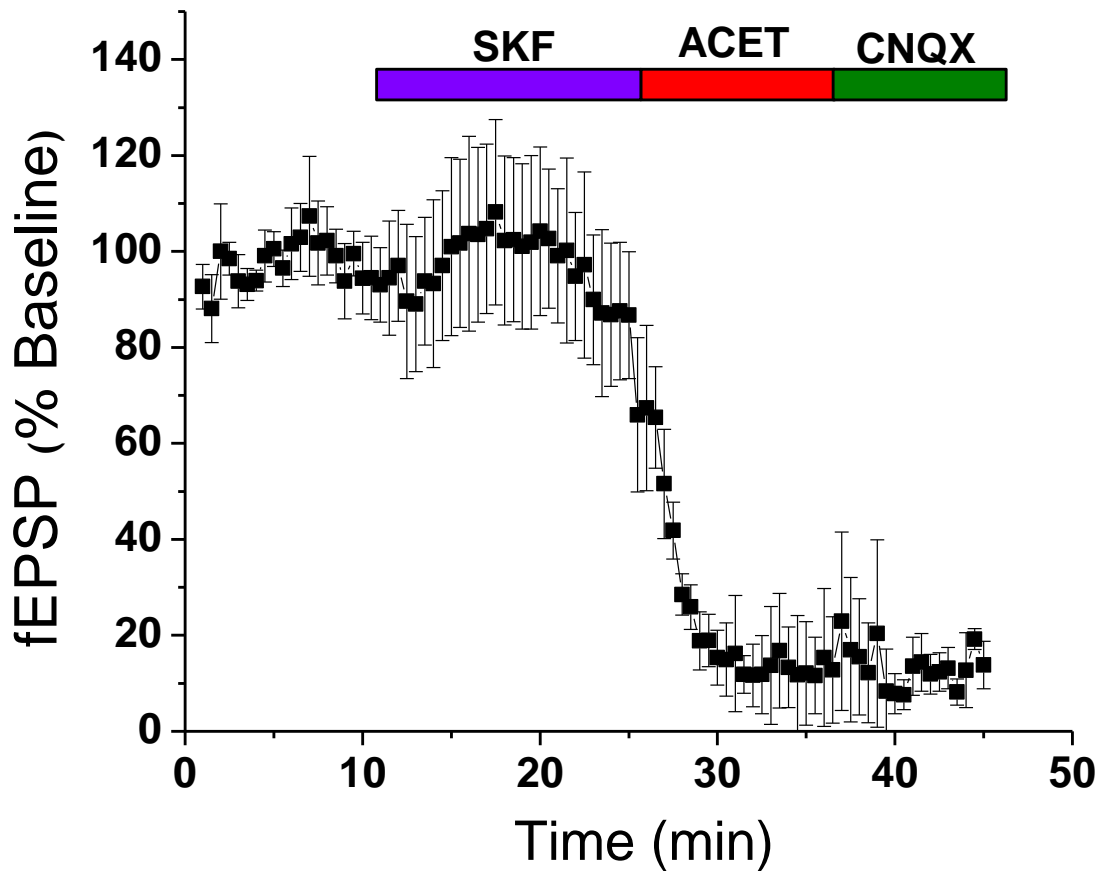


Figure 4.6 Activation of D1-like dopamine receptors did not alter KAR fEPSP

Activation of D1-like (D1 and D5) dopamine receptors (SKF38393, 50 μ M) did not alter KAR fEPSPs at the mossy fiber – CA3 synapse. Subsequent bath application of ACET (1 μ M) and then CNQX (100 μ M) verified the presence of KAR potentials and the lack of AMPAR potentials.

such as NMDAR and AMPAR, through either a classical PKA-mediated second messenger signaling pathway or a direct protein-protein interaction (Price et al., 1999; Lee et al., 2002b; Hallett et al., 2006; Paoletti et al., 2008). However, it is not known whether KAR activity is mediated by dopamine receptors. Recombinant data demonstrate

that KARs can be phosphorylated by PKA, yielding an increase in KAR current (Raymond et al., 1993; Wang et al., 1993; Kornreich et al., 2007). There is no data to demonstrate whether this can happen in the brain.

In this experiment, we sought to determine whether D1 dopamine receptors modulate KAR function in the brain. As previously described, we isolated KAR fEPSPs at the mossy fiber – CA3 synapse by stimulating the hilus of the dentate gyrus and recording in CA3 stratum lucidum in the presence of an antagonist cocktail to block AMPA, NMDA, GABA_A, and GABA_B synaptic potentials. After recording a stable baseline for a minimum of 20 min., we bath applied a selective D1-like DAR agonist (SKF38393, 50 μM) (Figure 4.6). Activation of D1-like DARs did not significantly alter the KAR fEPSP (101.9 ± 16.3 , $n = 4$). To verify the presence of a KAR fEPSP, we subsequently bath applied the selective KAR antagonist, ACET (1 μM). Furthermore, to ensure that we did not inadvertently measure AMPAR fEPSPs, we bath applied an AMPAR/KAR antagonist, CNQX (100 μM), which did not produce any further reduction of the measured KAR fEPSP. These data demonstrate a lack of D1 DAR modulation of KAR activity in the mossy fiber – CA3 synapse.

4.4 DISCUSSION

The major findings of our study are that M1 mAChRs, but not D1-like dopamine receptors, activation depresses KAR fEPSPs. This effect is likely through a postsynaptic mechanism, because the AMPAR fEPSP was unaffected by M1 mAChR activation by pilocarpine. M1 mAChRs are the major muscarinic receptor located in the hippocampus

(Cortes et al., 1984; Cortés et al., 1986; Rodríguez-Puertas et al., 1997; Scarr et al., 2007). It binds to the $G_{\alpha q}$ protein to signal PKC phosphorylation. Inhibition of PKC phosphorylation prevented the significant depression of KAR fEPSPs by pilocarpine. However, a depression in the KAR fEPSP did occur. This suggests that PKC phosphorylation has some affect on the magnitude of the depression. Interestingly, this depression of KAR fEPSPs by M1 mAChRs is developmentally regulated and goes away with age ('adult' P65 – P80).

4.4.1 M1 mAChRS DEPRESS KAR, BUT NOT AMPAR, AT MOSSY FIBER – CA3

SYNAPSES

This report is the first demonstration that M1 mAChRs can alter KARs activity in the brain. We demonstrated that pilocarpine, an M1 preferring agonist, depresses KAR fEPSPs at the mossy – fiber CA3 synapse. This depression was completely blocked by an M1 antagonist. In other studies, it was reported in a recombinant receptor that muscarinic activation by pilocarpine potentiated GluK2 containing heteromeric, but not homomeric, KARs, suggesting that potentiation of KARs by mACh receptors also requires the high-affinity KAR subunits (Benveniste et al., 2010). The difference in M1 mAChRs potentiating instead of inhibiting KAR activity, as in what we saw in our studies, could result from the experimental model being used. It is possible that the mACh receptors could increase channel conductance and receptor trafficking simultaneously. Recombinant experimental models are more sensitive to changes in channel conductance than trafficking. HEK-239T cell do not express synapse scaffolding proteins, and thus,

those experiments would not undergo changes in receptor trafficking that would resemble that in the actual brain slice. In the brain, it is possible that the amount of receptor trafficking away from the synapse could outperform the increase in channel conductance. Indeed, M1 mAChRs have been shown to alter NMDAR and AMPAR synaptic transmission primarily through trafficking of the receptors in or out of the synapse (Zheng et al., 1998; Dickinson et al., 2009; Nomura et al., 2012).

Although my research has yielded several interesting results, there are several questions that must be addressed. It was interesting to us that activation of M1 mAChRs can produce a significant depression of postsynaptic KAR fEPSPs but not AMPAR fEPSPs located in the same cell populations and synapses. Even more interesting is that both KARs and AMPARs can be phosphorylated by mAChRs and specifically M1 mAChRs. Thus, it seems interesting how there is a significant effect on KARs but not AMPARs. Further looking in the literature reveals that we are not the only laboratory to find such interesting divergence in the effect of muscarinic activation on glutamate receptors. Grishin et al. demonstrated that M1 mAChR activation depressed NMDAR currents on postsynaptic CA3 pyramidal neurons, but did not affect AMPAR currents at the same synapse (Grishin et al., 2005). Alternatively, other studies have shown that the same signaling cascade can elicit different effects on KARs and AMPARs due to the receptor association with the PDZ domain – interacting proteins PICK1 and GRIP (Hirbec et al., 2003). Dissociation of PICK1 with KARs caused a loss of KAR synaptic function, while the dissociation of PICK1 with AMPARs caused an increase in AMPAR synaptic function.

4.4.2 M1 mAChR DEPRESSION OF KAR ACTIVITY IS A FORM OF SHORT-TERM PLASTICITY

Another interesting result that deserves some explanation is that M1 mAChR depression of KAR fEPSP was a form of short-term plasticity. Short-term plasticity, as opposed to long-term plasticity, lasts for less than 1 hour after the initial stimulus. This form of plasticity is thought to be important for the dynamic regulation of synaptic function (Silva et al., 1996). Other studies have suggested that the determinate factor between a short-term versus long-term plasticity phenotype is the association with a second messenger system. Previous studies have reported that PICK1 plays an important role in the expression of long-term depression, and disruption of glutamate receptor interaction with PICK1 turns long-term plasticity into short-term plasticity (Xue et al., 2010). Thus, one mechanism that might explain the short-term effect is a dissociation of KARs with a PICK1 complex (Xue et al., 2010).

4.4.3 M1 mAChR DEPRESSION OF KAR ACTIVITY IS NOT MEDIATED BY PKC PHOSPHORYLATION

Another interesting result was that M1 mAChR depression of KAR fEPSPs at the mossy fiber – CA3 synapse was not prevented by application of the PKC inhibitor, chelethryine. M1 mAChRs couple to Gq proteins and are primarily thought to regulate other proteins, including glutamate receptors, through a PKC-dependent signaling cascade. We found this not to be true for M1 mAChR depression of KARs fEPSPs at the mossy fiber – CA3 synapse. As we previously discussed, Grishin et al demonstrated that

M1 mAChRs postsynaptically depressed NMDAR, but not AMPAR, current at CA3 pyramidal neurons (Grishin et al., 2005). This depression was mediated not through PKC phosphorylation but was dependent upon protein tyrosine phosphatases. Thus it is possible that our M1 mAChR depression of KARs located also on CA3 pyramidal neurons is mediated through protein tyrosine phosphatases.

An alternative explanation for the inability of chelethryine to inhibit the pilocarpine-induced depression of KAR fEPSPs at the mossy fiber – CA3 synapse is that chelethryine did not block PKC activity. Unlike the other drugs used in this chapter whose ligand binding sites are extracellular, chelethryine must maneuver inside the cell to inhibit PKC activity. We did not incorporate a positive control into our experiments to verify the inhibition of PKC activity by chelethryine.

4.4.4 M1 mAChR DEPRESSION OF KAR ACTIVITY IS DEVELOPMENTALLY REGULATED

We also discovered that M1 mAChR – induced depression of KAR fEPSPs was developmentally regulated. In rats around adolescent age (P21 – P28), the KAR fEPSP depression was robust; however, in rats around adult age (P65 – P80) we no longer saw a M1 mAChR depression of KAR fEPSP. Although we did not directly test the reason for this development ‘switch’, there are several possible mechanisms that we can speculate to be responsible. One such mechanism is the age-dependent change in mAChR expression. Previous studies have demonstrated that although mRNA expression of mAChRs does not change in the hippocampus, mAChR protein expression is age-dependently

downregulated in the hippocampus (Lee et al., 1994; Narang, 1995; Tice et al., 1996). Specifically, M1 mAChRs expression in the CA3 field of the hippocampus is significantly reduced in the aged rat brain compared to the young rat brain. A reduction in the expression of M1 mAChRs in the adult rat brain would occlude the M1 –dependent depression KAR fEPSPs.

Another possible explanation is the expression level of KARs. KARs are necessary for the correct integration of mossy fiber – CA3 synapse (Tashiro et al., 2003; Lanore et al., 2012). Early in development KARs expression is high, peaking at P9 (Bahn et al., 1994). After P9 the expression of KARs steadily declines. In the transition from adolescent to adulthood, the ratio of GluK2 expression to GluK4 and GluK5 become higher (Bahn et al., 1994). Thus, it may be possible that the relative concentration of ACET-sensitive KAR responses decline with aging. However, the persistence of an ACET-sensitive KAR response in the adult mossy fiber – CA3 synapse verifies that the KARs necessary for mAChR regulation is present.

Lastly, another possible difference is the relative expression of proteins associated with translocation of KARs to the synapse, such as GRIP and PICK1. GRIP association has been shown to increase more than 100% from P7 to P11, while PICK1 remains unchanged (Xue et al., 2010). An age - induced increase in GRIP expression relative to PICK1 expression was demonstrated to cause a switch from a depressive plasticity to facilitation plasticity at the same synapse (Xue et al., 2010). If this trend continues through adulthood, we can contend that our M1 mAChR – induced depression of KAR fEPSPs would not persist with aging.

These data demonstrate that muscarinic cholinergic receptors can modulate KARs in the brain. Acetylcholine is released in the brain, specifically in the hippocampus, during different brain states, such as during exercise (Nakajima et al., 2003; Uchida et al., 2006) and just before a seizure (Hillert et al., 2014). Understanding what happens to the glutamate receptors, especially KARs, during these brain states can provide a better understanding for how the brain functions to improve cognition while exercising, and it will allow us to elucidate critical mechanism involved in the genesis of a seizure, which will lead to potential therapies.

4.4.5 KAINATE RECEPTOR ACTIVITY IN THE MOSSY FIBER – CA3 SYNAPSE IS NOT MEDIATED BY D1-LIKE DOPAMINE RECEPTORS

In contrast to our M1 mAChR data, we found that D1-like dopamine receptors did not alter KAR activity in the mossy fiber – CA3 synapse. D1-like dopamine receptors couple to Gs proteins to stimulate adenylate cyclase activity and PKA mediated phosphorylation pathways. Thus, these data suggest that KAR activity is more sensitive to M1 mAChR regulation than D1-like DAR mediated regulation. Indeed, there are few PKA phosphorylation sites on the C-terminus domain of KARs (GluK2, S856 and S868) (Rojas and Dingledine, 2013). These PKA phosphorylation at residues S856 and S868 have only been shown to potentiate kainate-evoked currents of recombinant receptor (Raymond et al., 1993; Wang et al., 1993). There is no evidence demonstrating that PKA phosphorylation can alter KAR activity in the brain.

CHAPTER 5

ALTERED DOPAMINERGIC REGULATION IN A CHRONIC MODEL OF TEMPORAL LOBE EPILEPSY

5.1 INTRODUCTION

Dopamine receptors in the hippocampus are important for modulating learning and memory (Packard and White, 1991; Gasbarri et al., 1996; Bernabeu et al., 1997; O'Carroll et al., 2006). The hippocampus receives dense dopaminergic innervation from the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) (Simon et al., 1979; Scatton et al., 1980; Swanson, 1982), where dopamine is synthesized through the rate-limiting enzyme, tyrosine hydroxylase (TH). Dopamine released from these neurons can bind to two main types of dopamine receptors: D1-like (D1 and D5) and D2-like (D2L/S, D3, and D4), which are categorized based on pharmacological agonists and second messenger coupling. Particularly D1-like receptors have been shown to be important in learning and memory consolidation (Frey et al., 1990, 1991; Huang and Kandel, 1995; Otmakhova and Lisman, 1996, 1998; Navakkode et al., 2007; Granado et al., 2008; Ortiz et al., 2010). Pharmacological blockage of D1-like receptors prevents early and late phase long-term potentiation, a cellular model of learning, and blocks long-term memory storage (Frey et al., 1990, 1991). Additionally, D1 knockouts and adult rats undergoing D1 receptor reduction in the CA1 region of the hippocampus exhibited

significant decline in spatial learning, fear learning, classical conditioning of the eyelid response, as well as the associated activity-dependent synaptic plasticity in the hippocampal CA1-CA3 synapse (Ortiz et al., 2010). These studies provided compelling evidence that a reduction in D1-like receptors in the hippocampus is correlated with deficits learning and memory.

In temporal lobe epilepsy, D1-like receptors are also implicated in seizure generation, as previous studies have shown that a D1-like receptor agonist can elicit seizures in and of itself (O'Sullivan et al., 2008). Furthermore, a D1-like antagonist can prevent seizures (al-Tajir et al., 1990a, 1990b; Barone et al., 1990; Turski et al., 1990; DeNinno et al., 1991; Starr, 1996). To date, it is unknown whether temporal lobe epilepsy alters D1 and D5 dopamine receptor protein expression in the hippocampus. The chronic epilepsy model we use is clinically relevant as rats experience chronic spontaneous seizures with comorbid cognitive decline (Rice et al., 1998). However, it is not known whether dopamine receptors in this model have been pathologically altered.

One potential mechanism by which D1-like dopamine receptors mediate learning and memory and temporal lobe epilepsy is by altering ionotropic glutamate receptor function. D1-like dopamine receptors can alter both AMPAR and NMDAR function through either a phosphorylation-signaling cascade or direct protein-protein heterodimerization (Price et al., 1999; Snyder et al., 2000; Lee et al., 2002b; Dunah et al., 2004; Gao et al., 2006; Hallett et al., 2006). Although we previously did not see an interaction between D1-like dopamine receptor activation and KAR receptor – mediated synaptic transmission at the mossy fiber – CA3 synapse (Figure 4.6), evidence exists for a potential interaction in epilepsy. Previous studies have demonstrated that a D1-like

dopamine receptor antagonist can reduce the severity of seizures initiated by kainate receptor agonists (Bourne et al., 2001). Before we can investigate whether D1-like dopamine receptors regulate KAR function in the epileptic hippocampus, we must first determine how D1-like dopamine receptors are altered in the epileptic hippocampus.

The goal of this study was to compare and contrast the dopaminergic system between the normal and chronically epileptic brain. We were particularly interested in evaluating differences known to regulate learning and memory. Thus, we evaluated the effects of chronic epilepsy on D1 and D5 dopamine protein expression, dopamine concentration, dopamine metabolites, and dopamine transporter protein expression in the hippocampus. Our study provides further detail for one mechanism that may explain the cognitive decline commonly experienced by TLE patients.

5.2 MATERIALS AND METHODS

5.2.1 ANIMALS

Adult male Sprague Dawley rats (40-45 days old; 200-250 gm) were purchased from Harlan and were housed two-three per cage. Rats were kept in a temperature-controlled facility with access to food and water ad libitum. All experimental procedures were approved by the University of South Carolina Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering and to reduce the number of animals used.

5.2.2 INDUCTION OF STATUS EPILEPTICUS (SE)

The pilocarpine model of TLE is widely used in rodents (Cavalheiro, 1990) and reproduces the human condition closely (Curia et al., 2008). Specifically, rodents treated with pilocarpine exhibit spontaneous generalized and partial seizures, behavioral abnormalities, as well as the pathologic and histological signs commonly observed in human patients with TLE (Goffin et al., 2007). The mechanism by which pilocarpine administration causes spontaneous recurrent seizures and epilepsy is fairly well understood (Hamilton et al., 1997) and the stereotypic set of behavior produced by this cholinergic agonist is well characterized (Goffin et al., 2007).

For detailed methods on the pilocarpine model of induced status epilepticus, please see section 2.4.2.

5.2.3. IMMUNOHISTOCHEMISTRY

For detailed immunohistochemistry methods, please see section 2.6.

5.2.4. CELL COUNTS

Sections containing dorsal hippocampus (approximately 3.14-3.6 mm caudal to bregma; Paxinos and Watson, 1998) were selected for manual cell counts of GAD-67-positive somata. An area was selected encompassing all regions (stratum oriens [SO], stratum pyramidale [SP], and striatum radiatum [SR]) of CA1 at 2X magnification, and all immunopositive cells were counted using a Nikon ECLIPSE 80i microscope equipped

with Neurolucida software (v.9, MicroBrightField, Inc., Williston, VT, USA) at 20X magnification. For all counts, no hemispheric differences were noted in either sham or epileptic tissue in any neuronal marker, cells or terminals. Therefore, we unilaterally counted and averaged across two sections for each animal.

5.2.5. IMMUNOBLOT ANALYSIS

Please see section 2.5 for detailed immunoblot analysis methods.

5.2.6. SPECIFICITY OF THE ANTISERA

The D1 dopamine receptor antibody used in this study (#D2944, Sigma, St. Louis, MO, USA) is a rat monoclonal (clone 1-1-F11 s.E6) which recognizes the C-terminal 97 amino acid of D1 dopamine receptor. The immunogen was affinity purified. The specificity of this antibody, which does not cross-react with fusion proteins derived from the same regions of D2, D3, D4, D5 receptors, has been well documented (Levey et al., 1993).

The polyclonal D5 receptor antiserum (#SC-1441, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was raised in goat and is a widely used antiserum to D5 receptors. Previous absorption studies demonstrate that it recognizes D5 dopamine receptors, but not other dopamine receptor homologues (D1, D2, D3, D4) (Bodei et al., 2009).

5.2.7. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Please see section 2.7 for a detailed description of the high performance liquid chromatography methods.

5.2.8. STATISTICAL ANALYSES

Data are presented as mean \pm standard error. Student's *t*-test for independent samples was used to compare the difference between means. Two sample sets were considered significantly different when $p < 0.05$. All statistical data were analyzed and graphical representations made in Origin version 7.5 software (OriginLab Corp, Northampton, MA).

5.3 RESULTS

5.3.1 D1 DOPAMINE RECEPTORS ARE LOCALIZED DISTINCTLY IN INTERNEURONS, AND LESS APPARENT IN PYRAMIDAL NEURONS

To determine how D1-like dopamine receptors are altered in the chronic epileptic CA1 region of the hippocampus, we first sought to determine the localization of both D1 and D5 dopamine receptors in the control 'naïve' brain. A previous study identifies D1 dopamine receptor localization primarily in non-parvalbumin GABAergic interneurons (Gangarossa et al., 2012). Indeed, our immunostaining for D1 dopamine receptor clearly revealed interneurons in all layers (stratum oriens, stratum pyramidale, and stratum

radiatum) of hippocampal area CA1 (Fig. 5.1, B-D). Less distinct labeling was seen in the pyramidal cell body layer and proximal dendrites. No staining was observed in the 'no primary' control (Fig. 5.1, A).

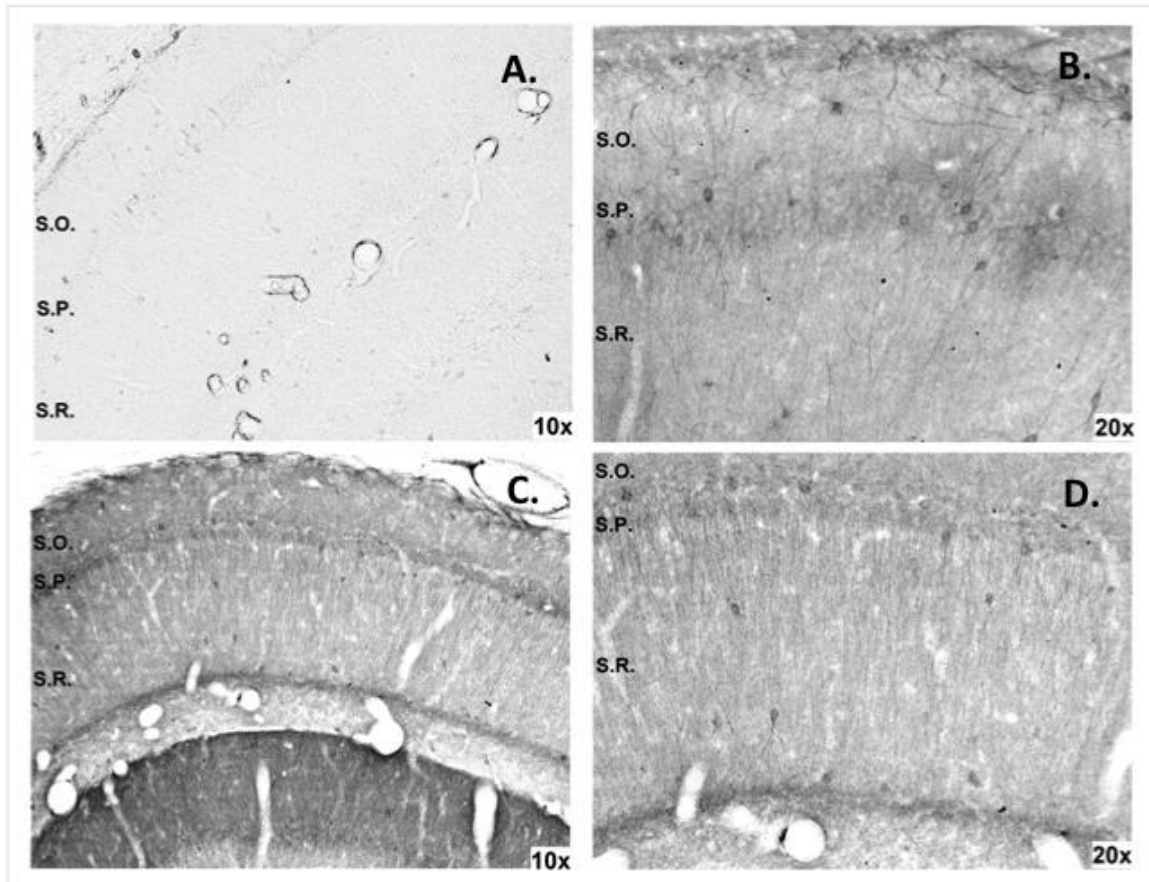


Figure 5.1 D1 dopamine receptors are located primarily in interneurons and less apparently in pyramidal neurons.

- A. Representative normal CA1 demonstrating lack of immunostaining in the absence of primary antiserum.
- B. Representative D1 dopamine receptor labeled CA1 illustrating D1 labeling prominently in interneurons throughout the stratum oriens (S.O.), stratum pyramidale (S.P.), and stratum radiatum (S.R.). Faint labeling was observed diffused throughout the pyramidal cell body layer.

5.3.2. D5 DOPAMINE RECEPTORS ARE LOCALIZED PREDOMINANTLY IN PYRAMIDAL NEURONS

D5 dopamine receptor pattern of labeling was substantially different from D1 dopamine receptors. Previous studies report D5 labeling in pyramidal cell bodies and apical dendrites (Khan et al., 2000). Indeed, our D5 labeling was almost exclusive to pyramidal neurons (Fig. 5.2, B-D). Labeling extended from the pyramidal cell soma surface down the length of the dendrites into the stratum lacunocum-moleculare (Fig. 5.2, B-D). This resulting pattern of labeling suggests that D5 DARs may elicit its functional effects predominantly through excitatory inputs.

5.3.3. REMAINING INTERNEURONS CONTAIN MORE D1 DOPAMINE RECEPTORS

To investigate whether D1-like receptor protein expression and/or localization are altered in the chronically TLE CA1 region of the hippocampus, we performed western blots from microdissected CA1 and immunohistochemistry from sham-treated and epileptic animals. Immunoblot analysis revealed that D1 protein expression in the epileptic CA1 ($113.326 \pm 6.832\%$, $n = 8$) was not significantly different from sham-treated animals (Fig. 5.3, D). This result was particularly interesting based on previous reports that interneurons are selectively susceptible to neuron loss in epilepsy (Houser and Esclapez, 1996; Morin et al., 1998; André et al., 2001; Cossart et al., 2001; Dinocourt et al., 2003). Immunostaining with D1 receptor antibody revealed no change in labeling pattern between sham-treated and epileptic animals (Fig. 5.3, A-C). However, D1 DAR

labeled interneurons appeared more intensely stained in the epileptic condition than sham-treated (Fig. 5.3, A-C). These results suggest that the remaining interneurons are more tightly regulated by D1 dopamine receptors.

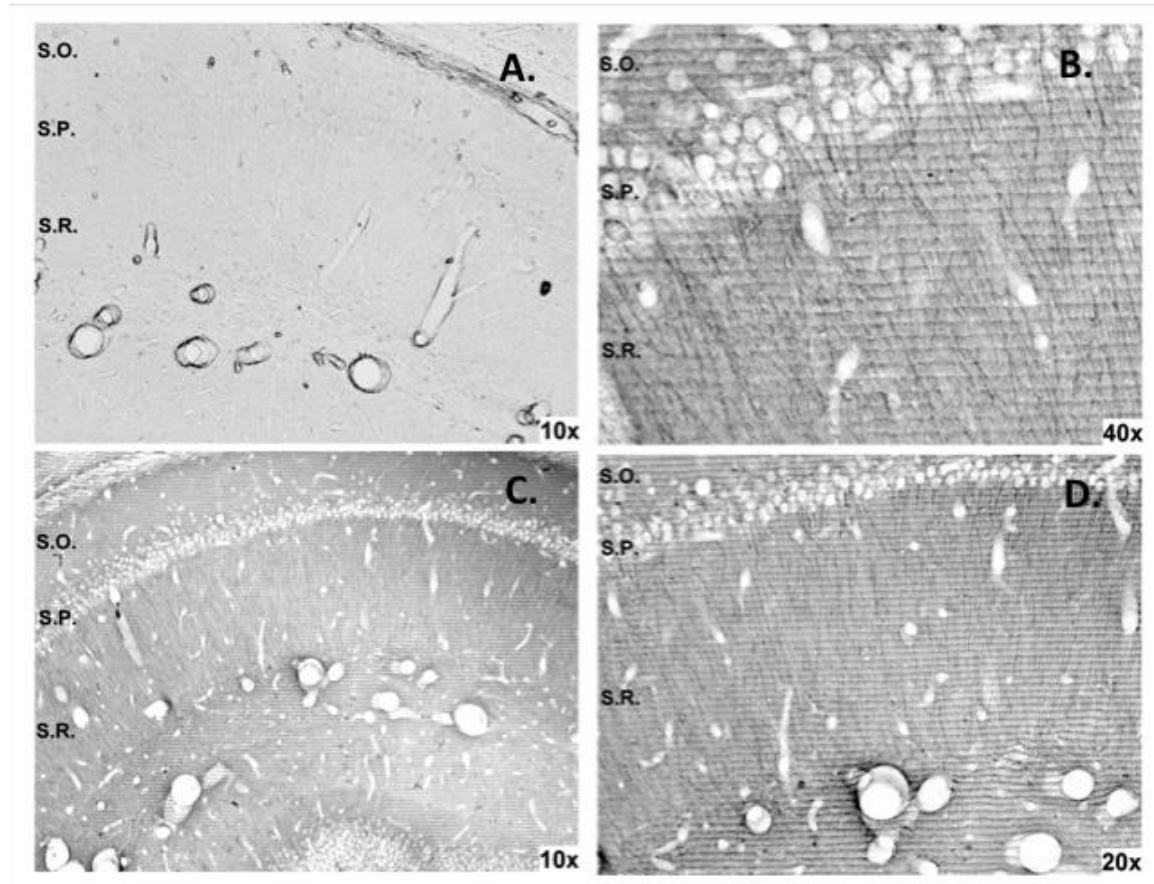


Figure 5.2 D5 dopamine receptors are located primarily on pyramidal neuron soma surface and dendrites.

- A. Representative CA1 from a naïve rat demonstrating lack of immunostaining in the absence of D5 primary antiserum.
- B. Representative D5 dopamine receptor labeled CA1 illustrating D5 labeling exclusively to pyramidal neuron soma surface and dendrites that extend throughout stratum radiatum (S.R.) and terminate in the stratum lacunosum molecular (SLM). Striatum oriens (S.O.), striatum pyramidale (S.P.)

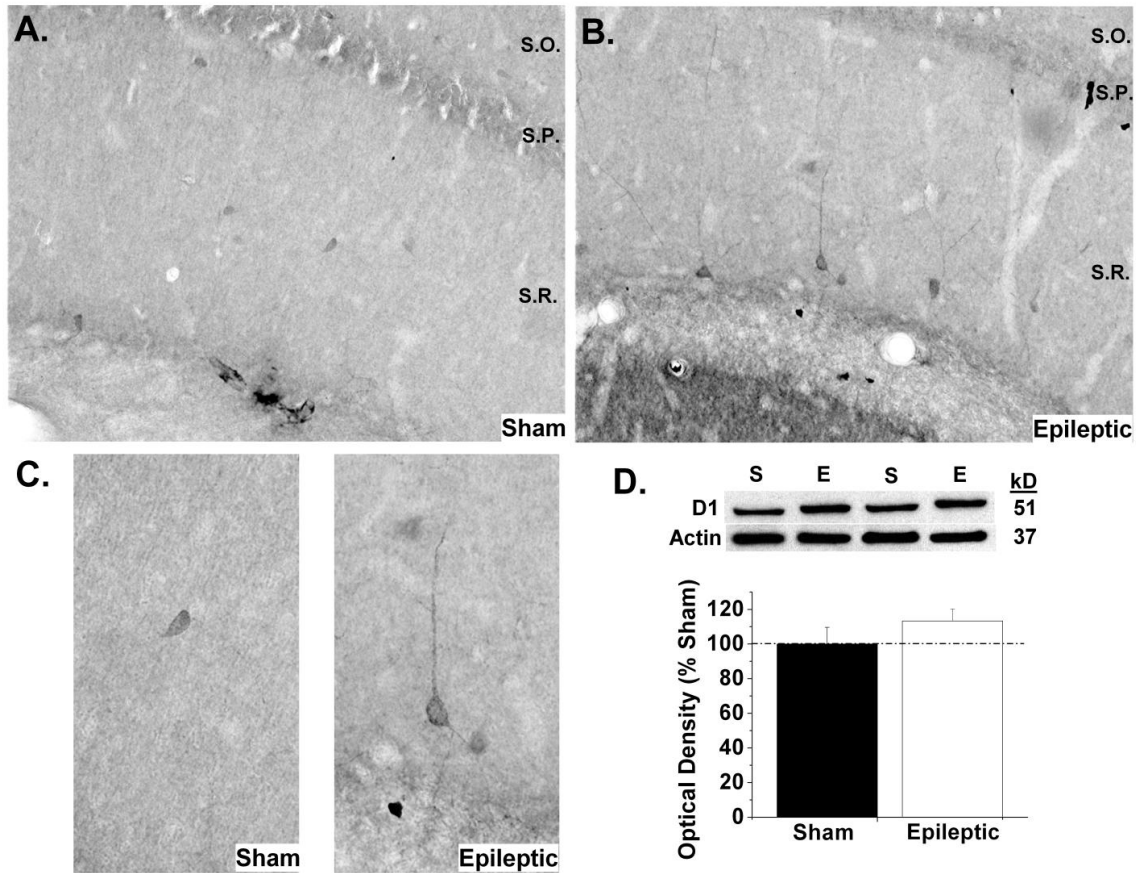


Figure 5.3 D1 dopamine receptor expression does not change in epilepsy CA1, but the intensity of interneuron labeling increases.

- Immunohistochemistry for D1 dopamine receptor labeling in area CA1 of sham-treated rats.
- Immunohistochemistry for D1 dopamine receptor labeling in area CA1 of epileptic rats reveal no difference in labeling pattern.
- However, closer analysis suggests that D1 dopamine receptor labeling in interneurons are more intense in the epileptic CA1 area than sham-treated.
- Western blot analysis demonstrated no significant change in total D1 dopamine protein expression between epileptic and sham-treated CA1.

5.3.4 DOPAMINE D5 RECEPTORS ARE REDUCED IN EPILEPSY

We then sought to determine whether the localization and/or protein expression of D5 dopamine receptors were altered in the chronically epileptic CA1 region of the hippocampus. Immunoblot analysis of D5 protein expression in microdissected CA1 revealed a 72% significant reduction in epilepsy ($27.701 \pm 4.574\%$, $n = 10$, $p < 0.0001$) compared to sham-treated animals (Fig. 5.4, E). This result was reproduced by an unbiased colleague. Immunostaining also confirmed the immunoblot analysis. D5 labeling in the epileptic condition was visibly reduced predominately in the pyramidal dendrites (Fig. 5.4, A-D). Instead of dense D5 dopamine receptor labeling extending down into the stratum lacunocum-molecularis as in the sham condition (Fig. 5.4, A,C), in the epileptic conditions D5 labeled staining was less dense and remained proximal to the pyramidal cell body layer (Fig. 5.4, B,D).

5.3.5 INTERNEURONS IN STRATUM ORIENS ARE SELECTIVELY LOSS IN EPILEPSY

To ensure that GABAergic interneurons are selectively lost in our chronic TLE model, we completed immunostaining for GAD67 and counted the positively labeled neurons. Results were normalized to sham-treated animals. As previously reported, we observed a significant reduction in GAD67 positively labeled interneurons, particularly in stratum oriens, in epilepsy ($27.19 \pm 0.809\%$, $n = 5$) when compared to sham. No significant difference was observed in the epileptic stratum pyramidale ($82.895 \pm 6.644\%$, $n = 5$) or stratum radiatum ($82.915 \pm 8.814\%$, $n = 5$) when compared to sham-treated animals (Fig. 5.4). This data confirms previous reports that interneurons are selectively lost in epilepsy

(Houser and Esclapez, 1996; Morin et al., 1998; André et al., 2001; Cossart et al., 2001; Dinocourt et al., 2003). Thus, knowing that D1 expression is located primarily in interneurons and interneurons are selective loss in epilepsy, demonstrating that total D1 expression does not change suggests that D1 expression in remaining interneurons may have increased.

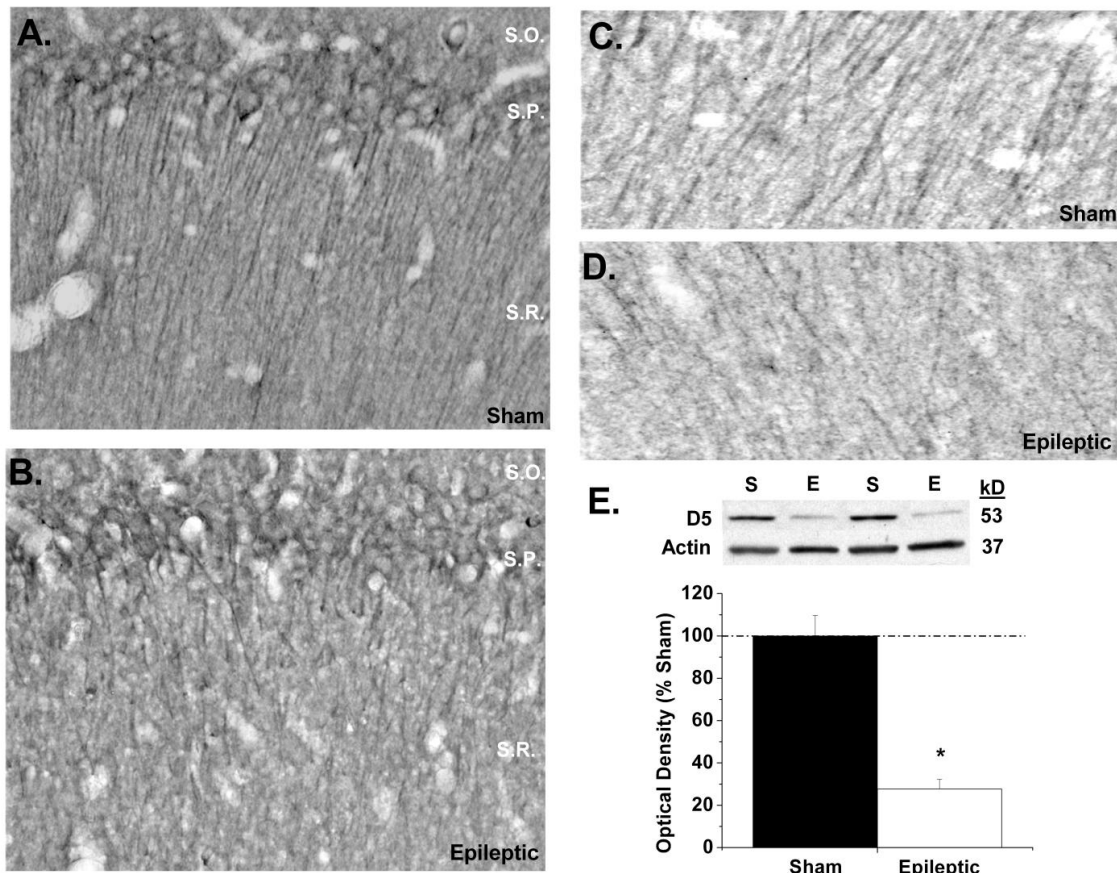


Figure 5.4 D5 dopamine receptor expression is reduced in the epileptic CA1

- A. Immunohistochemistry for D5 dopamine receptor labeling in area CA1 of sham-treated rats
- B. Immunohistochemistry for D5 dopamine receptor labeling in area CA1 of epileptic rats revealed a substantial decrease in immunostaining in the pyramidal neuron soma and dendrites.
- C. Higher magnification of D5 dopamine receptors labeled dendrites in striatum radiatum in sham-treated rats.

- D. Higher magnification of D5 dopamine receptors labeled dendrites in striatum radiatum in epileptic rats.
- E. Western blot analysis confirmed immunohistochemistry results, revealing a significantly reduction in D5 expression in microdissected CA1 area.

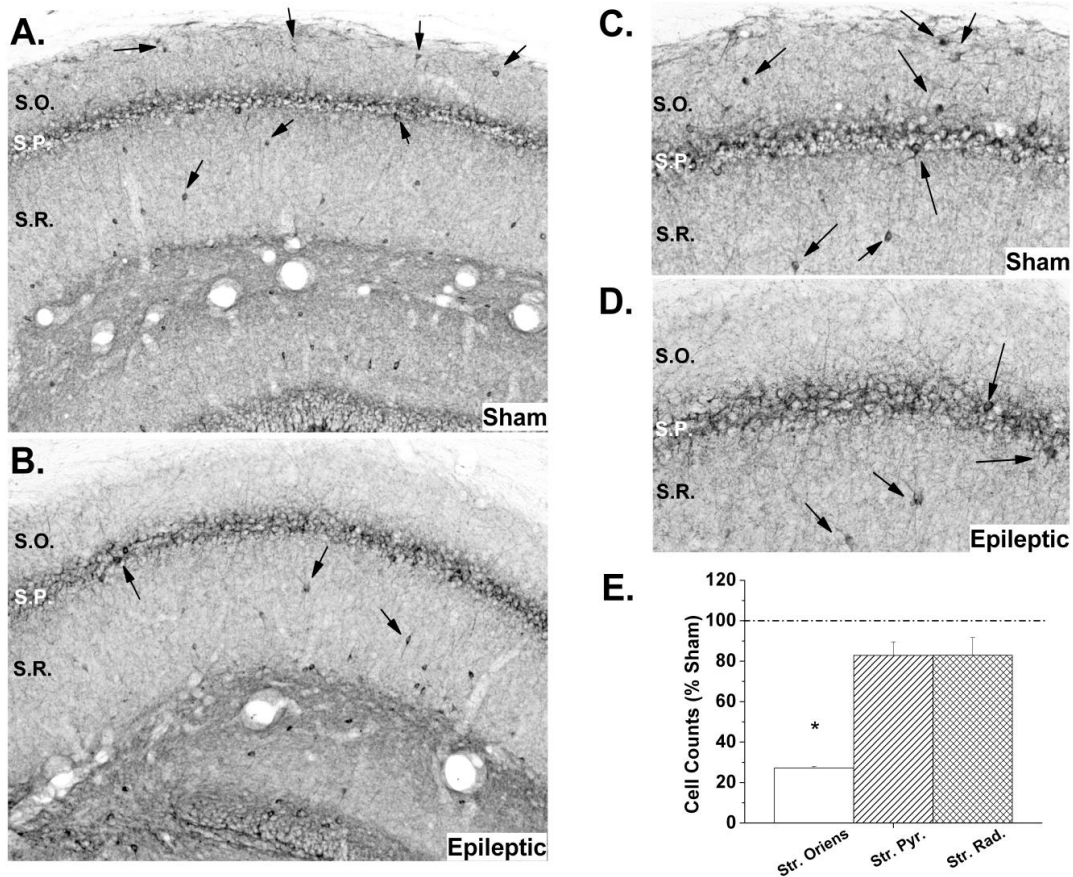


Figure 5.5 Epilepsy reduces the number of GABAergic interneurons in CA1.

- A. Immunohistochemistry for glutamate decarboxylase (GAD)-67 labeling in area CA1 of sham-treated rats.
- B. Immunohistochemistry for glutamate decarboxylase (GAD)-67 labeling in area CA1 of epileptic rats revealed seizure-related decreases in inhibitory neurons in stratum oriens (S.O.).
- C. Higher magnification of GABAergic labeling CA1 interneurons in sham treated rats.
- D. Higher magnification of GABAergic labeling CA1 interneurons in epileptic rats.
- E. Epilepsy was associated with a significant decrease in the number of GAD-67 immunoreactive cells in CA1 limited to S.O.

5.3.6 LOCALIZATION OF D1 DOPAMINE RECEPTORS IS UNCHANGED IN THE EPILEPTIC DENTATE GYRUS

D1 dopamine receptors are expressed more abundantly in the dentate gyrus than any other region in the hippocampus (Gangarossa et al., 2012), where they impact neuron excitability and learning and memory. Indeed, D1-like dopamine receptors activate during a “novel” or “rewarding” signal in freely moving animals, and consequentially increases DG excitability (Hamilton et al., 2010). Furthermore, inhibition of D1-like dopamine receptors inhibits LTP in the DG (Yanagihashi and Ishikawa, 1992; Kusuki et al., 1997; Swanson-Park et al., 1999). Thus, we investigated whether D1 dopamine receptor expression is altered in the epileptic dentate gyrus. In agreement with previous studies, D1 dopamine receptors demonstrated diffused labeling throughout the molecular layer (MOL) and hilus (H) of the dentate gyrus (Figure 5.6A). We also observed abundant D1 dopamine receptor labeling in the stratum lucidum. Notably, D1 dopamine receptor labeling was absent from granule cell layer (GCL). This pattern of labeling suggests that D1 dopamine receptors are expressed on dentate granule cell dendrites and axons (mossy fibers), but absent from dentate granule cell soma.

D1 dopamine receptor expression in the epileptic dentate gyrus demonstrated no observable difference in D1 dopamine receptor labeling pattern or strength in labeling signaling (Figure 5.6B). Thus, these results illustrate that D1 dopamine receptor expression is unchanged in the epileptic dentate gyrus.

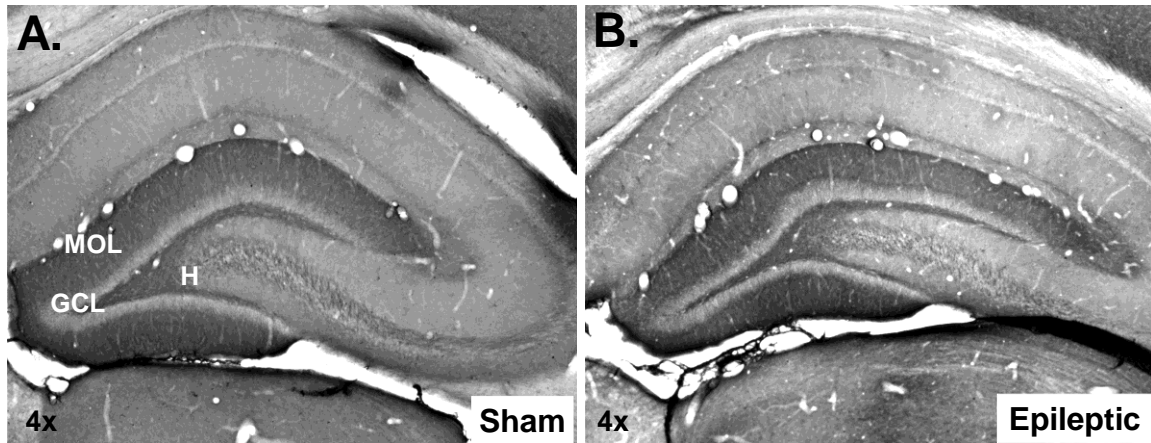


Figure 5.6 D1 dopamine receptor expression in the dentate gyrus does not change in epilepsy.

- A. Immunohistochemistry for D1 dopamine receptor labeling in the dentate gyrus of sham-treated rats. D1 dopamine receptors are localized throughout the molecular layer (MOL) and hilus (H) of the dentate gyrus but absent from the granule cell layer (GCL).
- B. Immunohistochemistry for D1 dopamine receptor labeling in the dentate gyrus of epileptic rats revealed a similar labeling pattern to sham-treated controls.

5.3.7 LOCALIZATION OF D1 DOPAMINE RECEPTORS IS UNCHANGED IN THE EPILEPTIC CA3 REGION

In the CA3 region, previous studies have demonstrated sparse localization of D1 dopamine receptors on GABAergic interneurons (Gangarossa et al., 2012). In this study, we also found that D1 dopamine receptors are scattered on interneurons (Figure 5.7A, B). However, in addition to D1 labeled interneurons, we also saw prominent labeling in the stratum lucidum, resembling mossy fiber axon labeling.

Mossy fiber sprouting and hyperexcitability is a hallmark symptom of chronic epilepsy (Tauck and Nadler, 1985; Sutula et al., 1988, 1989; Houser et al., 1990; Babb et al., 1991; Cronin et al., 1992). Thus, we investigated whether D1 dopamine receptor expression is altered in the epileptic CA3 region compared to sham-treated controls (Figure 5.7C, D). Our immunostaining demonstrated no observable differences in D1 dopamine receptor labeling in the epileptic CA3 region (Figure 5.7C) when compared to sham-treated control (Figure 5.7D).

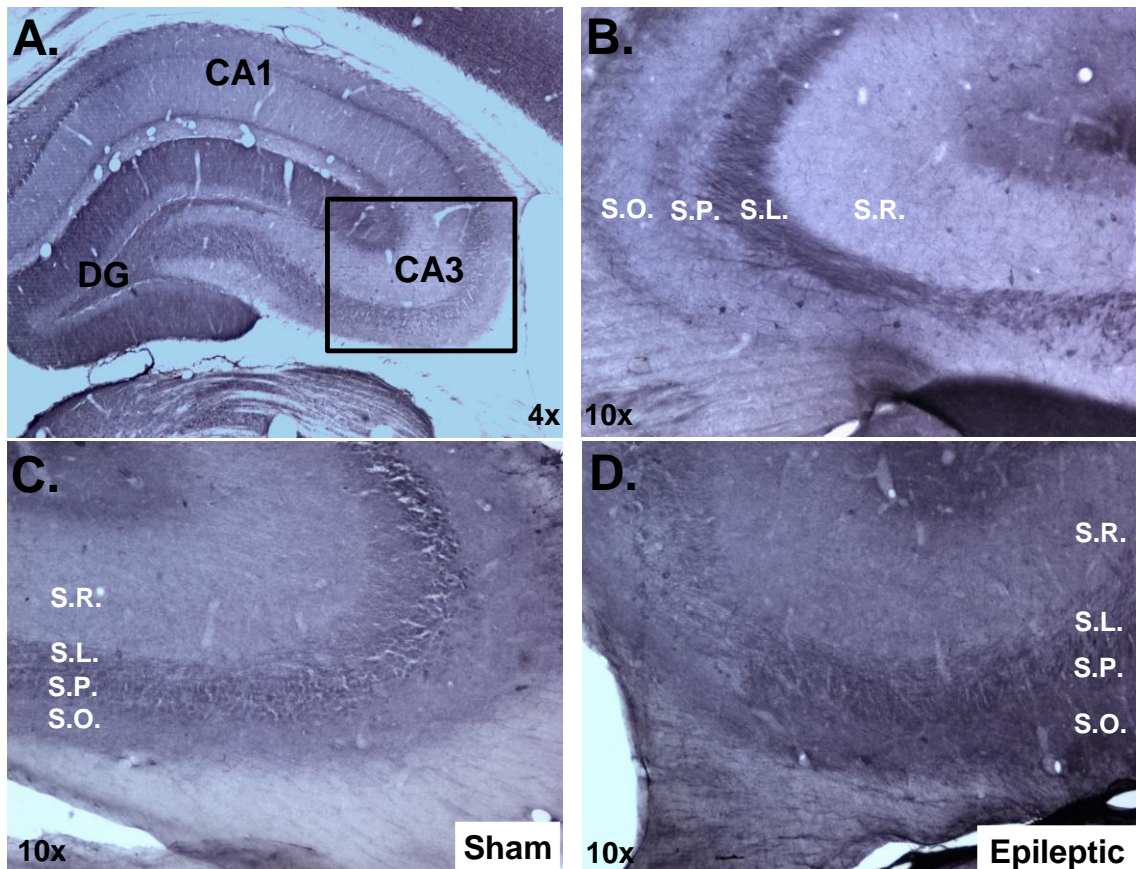


Figure 5.7 D1 dopamine receptor expression does not change in the epileptic CA3 region.

- A. Representative normal hippocampus illustrating the CA1, CA3, and dentate gyrus (DG) regions. The black box indicates the CA3 region.
- B. Magnification of the CA3 region from a naïve rat. D1 dopamine receptor immunoblotting prominently labeled mossy fiber axons in the stratum lucidum (S.L.). D1 dopamine receptors were also expressed on sparse interneurons throughout the stratum oriens (S.O.), stratum radiatum (S.R.), stratum pyramidale (S.P.), and stratum lucidum (S.L.).
- C. Immunohistochemistry for D1 dopamine receptor labeling in the CA3 region of sham-treated rats.
- D. Immunohistochemistry for D1 dopamine receptor labeling in the CA3 region of chronically epileptic rats demonstrates similar labeling pattern and intensity to the sham-treated rats.

5.3.8 TOTAL DOPAMINE AND DOPAMINE METABOLITE LEVELS REMAIN UNCHANGED IN TLE HIPPOCAMPUS

We next sought to better understand how changes in the D1-like receptor expression and localization might alter overall dopaminergic function. Our leading hypothesis was overall D1-like receptor expression in the TLE hippocampal was suppressed due to homeostatic compensation for upregulated dopamine. Thus, we used HPLC to measure dopamine, dopamine metabolites, and norepinephrine concentration in whole TLE hippocampus and sham-treated controls. Several experimental controls were applied to ensure we identified the correct peak as dopamine. In addition to running standard controls at various concentrations, sample from a sham-treated hippocampus was also spiked with DA standard. The solemn differentiating peak was considered dopamine (Fig. 5.6, C).

Total dopamine (DA), dopamine metabolites (DOPAC), and norepinephrine (NE) in whole hippocampus were unaffected by TLE (Fig. 5.6, A-B,D). Monoamine levels

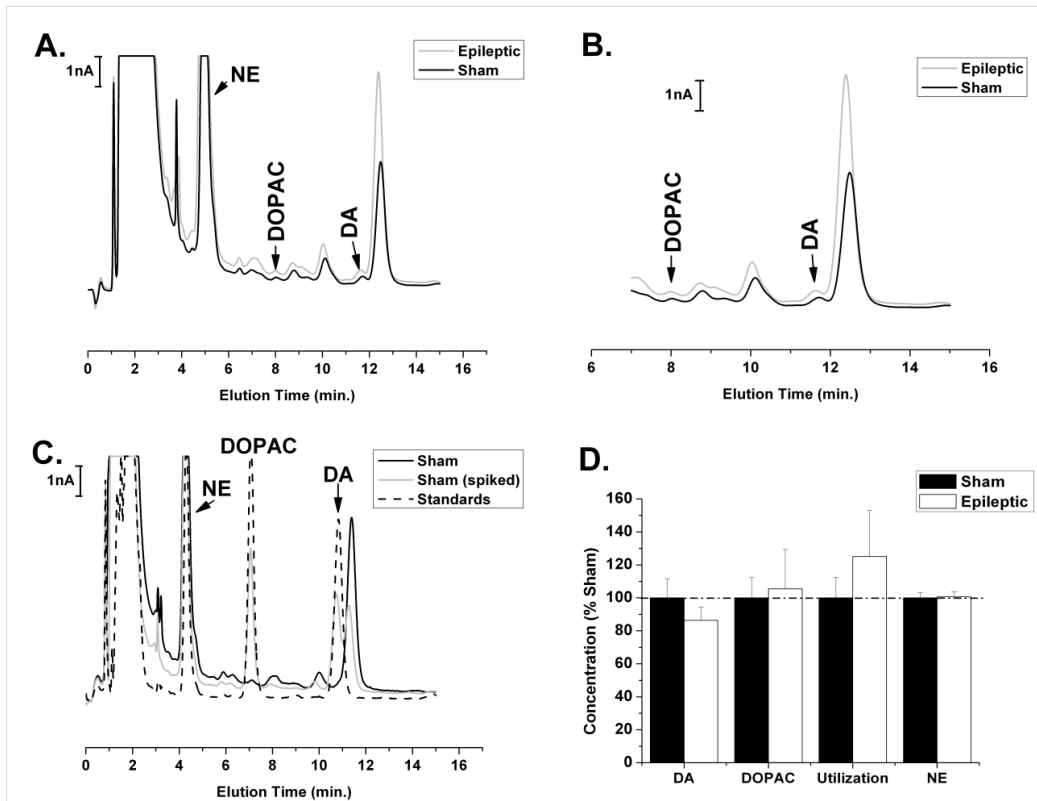


Figure 5.8 Total dopamine and dopamine metabolite levels remain unchanged in the TLE hippocampus.

- A. High performance liquid chromatography (HPLC) chromatograms for sham-treated (black line) superimposed with epileptic (gray line) illustrated no change in total hippocampus concentration of dopamine (DA), dopamine metabolite (3,4-dihydroxyphenylacetic acid (DOPAC)), or norepinephrine (NE, downstream synthesized catecholamine).
- B. Chromatographs of a combination standard (100 nM of NE, DOPAC, and DA; black dash line), sham-treated sample (black solid line), and sham-treated sample “spiked” with 100 nM combination standard (gray line) superimposed to demonstrate correct identification of each monoamine.
- C. Quantification of DA, DOPAC, dopamine utilization (DOPAC/DA, a measure of released dopamine), and NE revealed no significant differences in monoamine concentrations between the epileptic and sham-treated hippocampus.

were reported as a percentage of Sham-treated controls. Total levels of DA in epileptic animals were $86.376 \pm 8.114\%$ of that in sham-treated animals ($n = 4$ sham-treated, $n = 7$ epileptic) (Fig. 5.6, A-B,D). Total DOPAC levels in epileptic animals were $105.466 \pm 23.817\%$ of that in sham-treated animals. Total NE levels in epileptic animals were $100.609 \pm 3.115\%$ of that in sham-treated animals. Dopamine utilization was calculated by dividing total DOPAC by DA concentration as a measure of released dopamine. No difference was observed in the epileptic hippocampus ($125.133 \pm 27.885\%$) from sham-treated animals. Thus, the amount of dopamine produced is not different between the sham and epileptic hippocampus. These results suggest that changes in dopamine receptor expression are more significant.

5.3.9. REDUCED DOPAMINE CLEARANCE IN THE EPILEPTIC HIPPOCAMPAL CA1

REGION

After observing that dopamine concentration did not change in epilepsy, we then evaluated whether dopamine clearance is altered in the chronically epileptic hippocampus. Western blots for COMT in microdissected CA1 from epileptic and sham-treated animals displayed two bands (35kD and 25kD) (Fig. 7, B). Immunoblot analysis revealed no significant difference at either protein size (35kD, $109.921 \pm 10.073\%$; 25kD, $98.658 \pm 12.286\%$; $n = 11$) when compared to sham-treated animals (Fig. 7, B).

Immunoblots for DAT also displayed a band at two protein sizes (80kD and 55kD) (Fig. 7, A). Epilepsy demonstrated a significant reduction in DAT protein expression in the 80kD protein size ($50.588 \pm 10.867\%$, $n = 11$, $p = 0.03$) but not the

55kD protein size ($84.534 \pm 8.287\%$, $n = 11$), when compared to sham-treated animals (Fig. 7, A). Thus, we observed reduced dopamine clearance in the epileptic hippocampal CA1 region.

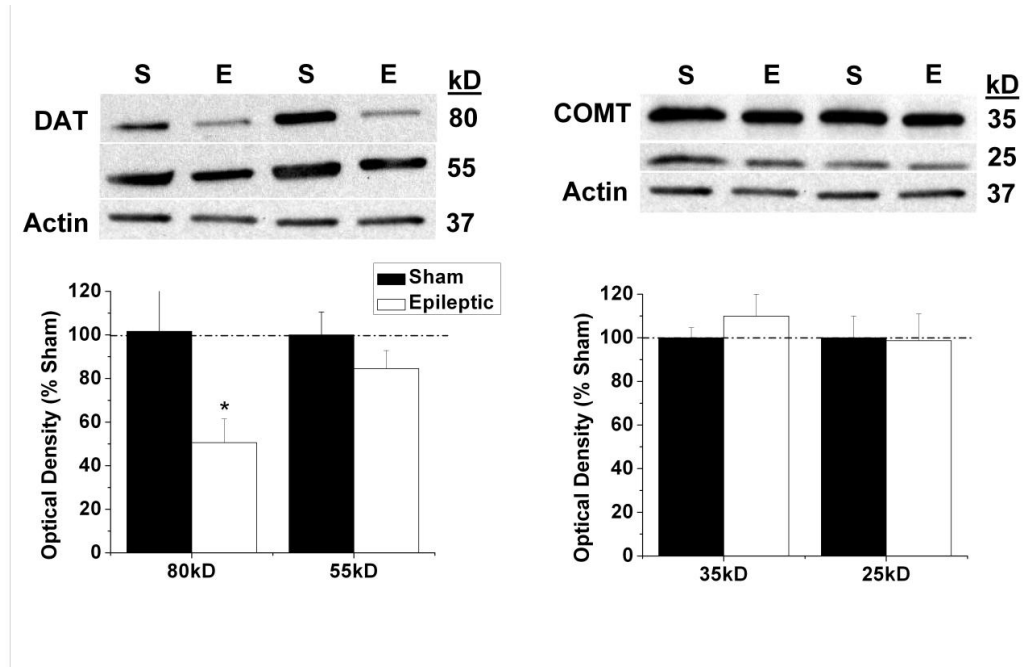


Figure 5.9 Reduced dopamine clearance in the epileptic hippocampal CA1 region.

- A. Antigen for DAT recognized bands at 80 kilodaltons (kD) and 55 kD. Western blot analysis revealed a significant reduction in dopamine transporter (DAT) protein expression in the epileptic CA1 area only at 80 kD when compared to sham-treated rats.
- B. Antigen for COMT recognized bands at 35 kD and 25 kD. Western blot analysis revealed no difference in catechol-O-methyl transferase (COMT) protein expression in the epileptic CA1 area when compared to sham-treated rats. Immunoblot analyses were corrected for actin.

5.3.10 NO CHANGE IN DOPAMINERGIC FIBER EXPRESSION IN THE EPILEPTIC HIPPOCAMPAL CA1 REGION

To determine whether dopaminergic fibers expression and/or localization were altered in the epileptic condition, we performed immunoblots with microdissected hippocampal CA1 and immunohistochemistry with epileptic and sham-treated animals. Immunoblot analysis revealed no significant change in epileptic tyrosine hydroxide (TH) expression ($97.047 \pm 15.919\%$, $n = 8$) from sham-treated animals (Fig. 8, C). This result was confirmed by immunostaining. TH labeled catecholamine fibers that dispersed randomly throughout all CA1 layers (Fig. 7, A-B). Thus, no change was observed in the amount of dopaminergic fibers.

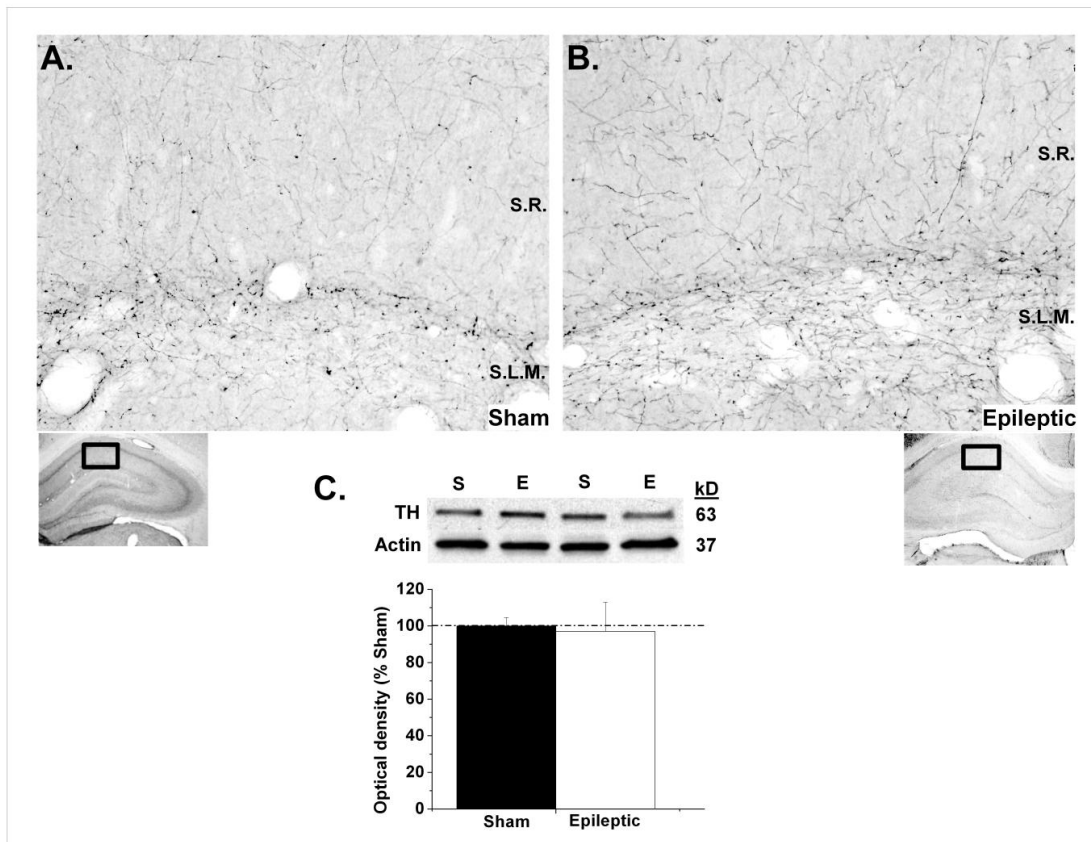


Figure 5.10 No change in dopaminergic fiber expression in the epileptic hippocampal CA1 region.

- A. Immunohistochemistry for tyrosine hydroxylase (TH) labeling in area CA1 of sham-treated rats.
- B. Immunohistochemistry for tyrosine hydroxylase (TH) labeling in area CA1 of epileptic rats revealed no difference in dopaminergic fiber innervation.
- C. Western blots confirmed immunostaining results, demonstrating no difference in TH protein expression when comparing epileptic to sham-treated CA1. TH protein expression was normalized to actin.

5.4 DISCUSSION

The principal finding of this study is that the dopaminergic system is altered in a chronic model of temporal lobe epilepsy. In our study we have identified differential patterns of labeling between D1 and D5 receptors. D1 DARs were located primarily on interneurons, diffused throughout the molecular layer of the dentate gyrus, and on mossy fiber axons, while D5 receptors were located predominately in pyramidal neurons dendrites. D5 DAR protein expression was significantly reduced in proximal dendrites of the epileptic hippocampal CA1 area. No change in protein expression was seen in D1 DARs; however we did observe a possible increase in interneuron D1 DAR expression in the epileptic CA1. These results were accompanied by a reduction in dopamine clearance, but no change in overall dopamine content.

We identify that D5 dopamine receptors are significantly reduced in the epileptic hippocampus. This deficit in D1-like receptor may result in the cognitive decline associated with epilepsy. To date the role of D5 dopamine receptors are not well characterized. However, previous studies have elucidated a role for D5 dopamine

receptors in learning and memory. D5 dopamine receptors are particularly important in regulating plasticity of the medial perforant path onto dentate granule cells (Yang and Dani, 2014) Furthermore, a rat model of ADHD that demonstrates impaired learning also has significantly lower D5 dopamine receptor expression than healthy controls (Medin et al., 2013).

Previous studies have identified D1 DARs primarily on interneurons (Mansour et al., 1990, 1991; Fremeau et al., 1991; O’Sullivan et al., 2008; Gangarossa et al., 2012). However, other studies suggest that D1 DARs are predominately in pyramidal neurons (Bergson et al., 1995). We identify D1 DARs to be predominately located on interneurons, with diffuse labeling throughout the stratum pyramidal cell body layer and dendrites. In our hands, we identified no change in D1 DAR expression. This was of particular interest to us because we identified D1 DARs to be primarily located on interneurons. It has been widely demonstrated in previous literature and also replicated in this study that interneurons, particularly in the stratum oriens, are susceptible to cell death in epilepsy (Dinocourt et al., 2003). Thus, no change in D1 DAR expression may indicate that the remaining interneurons have upregulated D1 DAR expression.

There is not a consensus on how the dopaminergic system is altered in epilepsy. Some studies indicated that dopamine concentration increases (Cavalheiro et al., 1994), while others indicate no change dopamine content but an increase in dopamine metabolites (Freitas et al., 2004). We identified no significant change in dopamine concentration. We went to great lengths to identify the correct peak for each monoamine measured. Not only were various concentration of each monoamine standard used to complete the standard curves for measuring accurate concentration and retention times,

but a hippocampus sample was “spiked” with a combination of each monoamine standard. The spiked sample, original hippocampus sample, and the monoamine standard were all superimposed onto each other, and the peaks in common were identified as the respective monoamine.

We demonstrated a significant reduction of dopamine clearance in the epileptic hippocampal CA1 region. This reduction in dopamine clearance suggests that even though dopamine concentration is unchanged in the epileptic hippocampus, the dopamine may be allowed to diffuse further away from the synapse. This would allow dopamine to bind to receptors it would not normally interact with, in effect, increasing its sphere of influence. Immunoblots for both COMT and DAT produced two bands. COMT immunoblots produced bands at 35kD and 25kD. The 35kD band represents the phosphorylated form of COMT, which is transported to the cell surface where it can be more active. The 25kD COMT band represents the non-phosphorylated form of COMT, which is left in the cytoplasm and is considered the less active form. DAT immunoblots demonstrated bands at 80kD and 55kD. The 80kD band represents the glycosylated, mature form of DAT, which is packaged and transported to the cell membrane. The 55kD band is thought to be the non-glycosylated, immature form of DAT, which is left in the endoplasmic reticulum.

Based on our findings, we suggest that dopamine D1-like receptor expression is differentially regulated in the epileptic hippocampus. A decrease in D5 dopamine receptors located in CA1 hippocampus pyramidal neurons and reduction in dopamine clearance may speak to the deficits in learning and memory seen in patients with temporal lobe epilepsy.

CHAPTER 6

GENERAL DISCUSSION AND SIGNIFICANCE

6.1 FINDINGS OF THE STUDY

1. ACET selectively inhibits the GluK4 and GluK5 subunits in GluK2/K4 and GluK2/K5 heteromeric receptor. Kynurenate selectively inhibits the GluK2 subunits. We used both drugs to determine that glutamate binding to either the “high-affinity” subunits (GluK4 or GluK5) or the GluK2 subunit is sufficient to activate the heteromeric receptor. However, desensitization occurred only when three or more subunits are bound to glutamate. Furthermore, we demonstrated that perfusing ACET onto brain slices inhibits kainate receptor synaptic transmission at the mossy fiber – CA3 synapse. See Figure 6.1 for an illustration of these conclusions.

2. Activation of M1 mAChRs, but not D1 dopamine receptors, depressed KAR synaptic transmission at the mossy fiber – CA3 synapse. This effect is mediated through co-localized M1 and KARs on the postsynaptic CA3 neuron. However, PKC phosphorylation does not explain this depression. Furthermore, M1 mAChR depression of KAR synaptic transmission goes away in adulthood. See Figure 6.2 for an illustration of these conclusions.

3. Dopamine D5 receptor is significantly reduced in the epileptic CA3 region of the hippocampus, while D1 dopamine receptor expression in remaining interneurons is upregulated. Additionally, dopamine transporter and COMT were significantly reduced although tyrosine hydroxylase and dopamine content were unchanged. Thus, total dopamine in the epileptic hippocampus is unchanged but dopamine clearance is reduced. This suggests that more dopamine is floating in the extrasynaptic space. See figure 6.3 for an illustration of these conclusions.

6.2 MUSCARINIC MODULATION OF KAINATE RECEPTORS: IMPLICATIONS FOR EXERCISE AND EPILEPSY

Exercise alters the cholinergic system in healthy and diseased brains. Particularly, in the hippocampus, exercise has been shown to double acetylcholine release during moderate intensity exercise (Nakajima et al., 2003; Uchida et al., 2006), while inhibiting acetylcholine degradation by inhibiting acetylcholinesterase (Tsakiris et al., 2006). Thus, more acetylcholine is released and remains in the extrasynaptic space in the exercising brain. This increase in acetylcholine content would increase binding and activation of acetylcholine receptors, including abundantly expressed M1 muscarinic acetylcholine receptors (Cortes et al., 1984; Cortés et al., 1986; Rodríguez-Puertas et al., 1997; Scarr et al., 2007). In the CA3 region, we proved that M1 mAChR activation depressed KAR-mediated synaptic transmission, where they are co-localized on CA3 pyramidal neurons (Chapter 4). Thus, an exercise-induced increase in acetylcholine in the hippocampus would increase binding to M1 mAChRs in principal neurons, including in the CA3. This

Closed

Open, Non-desensitizing

Open, desensitized

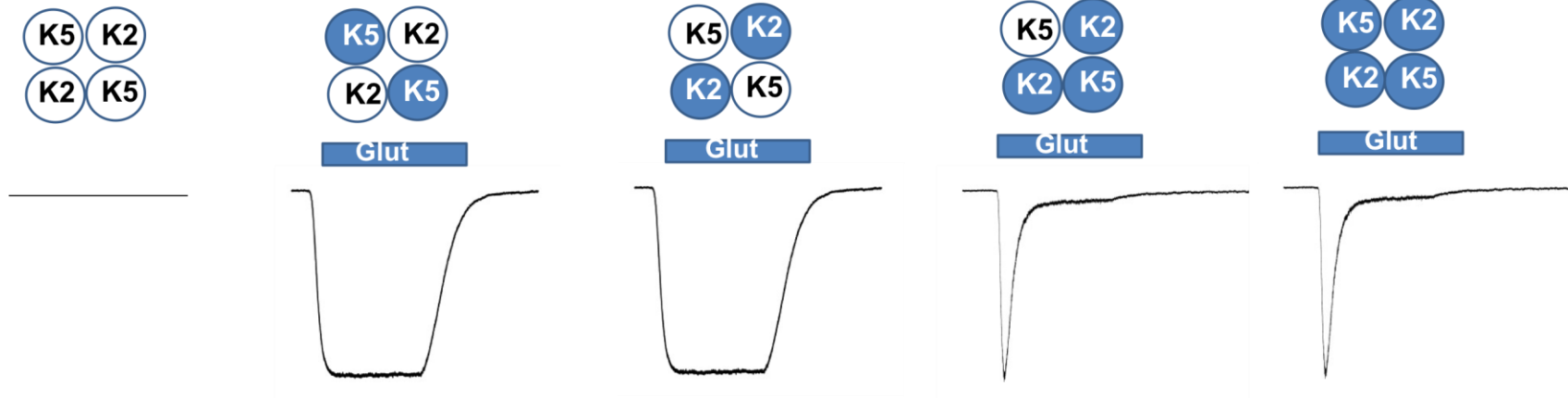


Figure 6.1 Illustration of the receptor occupancy model for KAR activation and desensitization. When no glutamate is present, the kainate receptor is closed and no current is produced. If either GluK2 or the GLuK5 subunits in the GluK2/K5 heteromeric tetramer are bound to glutamate, the receptor opens to a non-desensitizing current. However, when three or more subunits in the tetramer are bound to glutamate, the kainate receptor will desensitize.

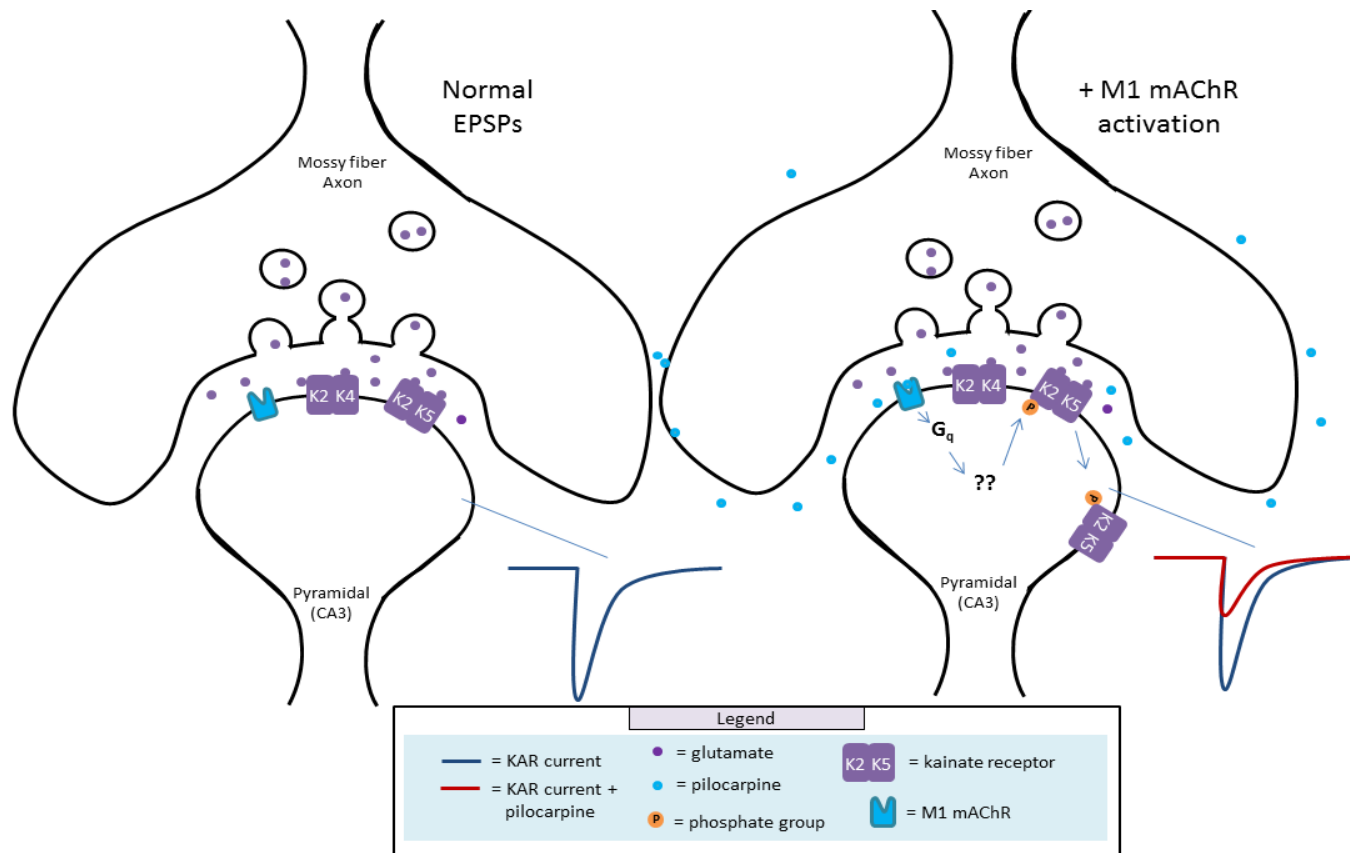


Figure 6.2 Illustration of M1 mAChR depression of kainite receptor EPSPs. During normal synaptic transmission, depolarization of mossy fibers causes release of glutamate from mossy fiber terminals. This released glutamate binds to GluK2/K4 and/or GluK2/K5 receptors located postsynaptically on CA3 pyramidal neurons and produces a KAR EPSP. Bath application of pilocarpine activates M1 mAChRs located on CA3 pyramidal neurons, which elicits a signaling cascade that either phosphorylates or dephosphorylates GluK2/K4 and GluK2/K5 receptors. The end result is KARs trafficking away from the synapse and smaller KAR fEPSP amplitudes.

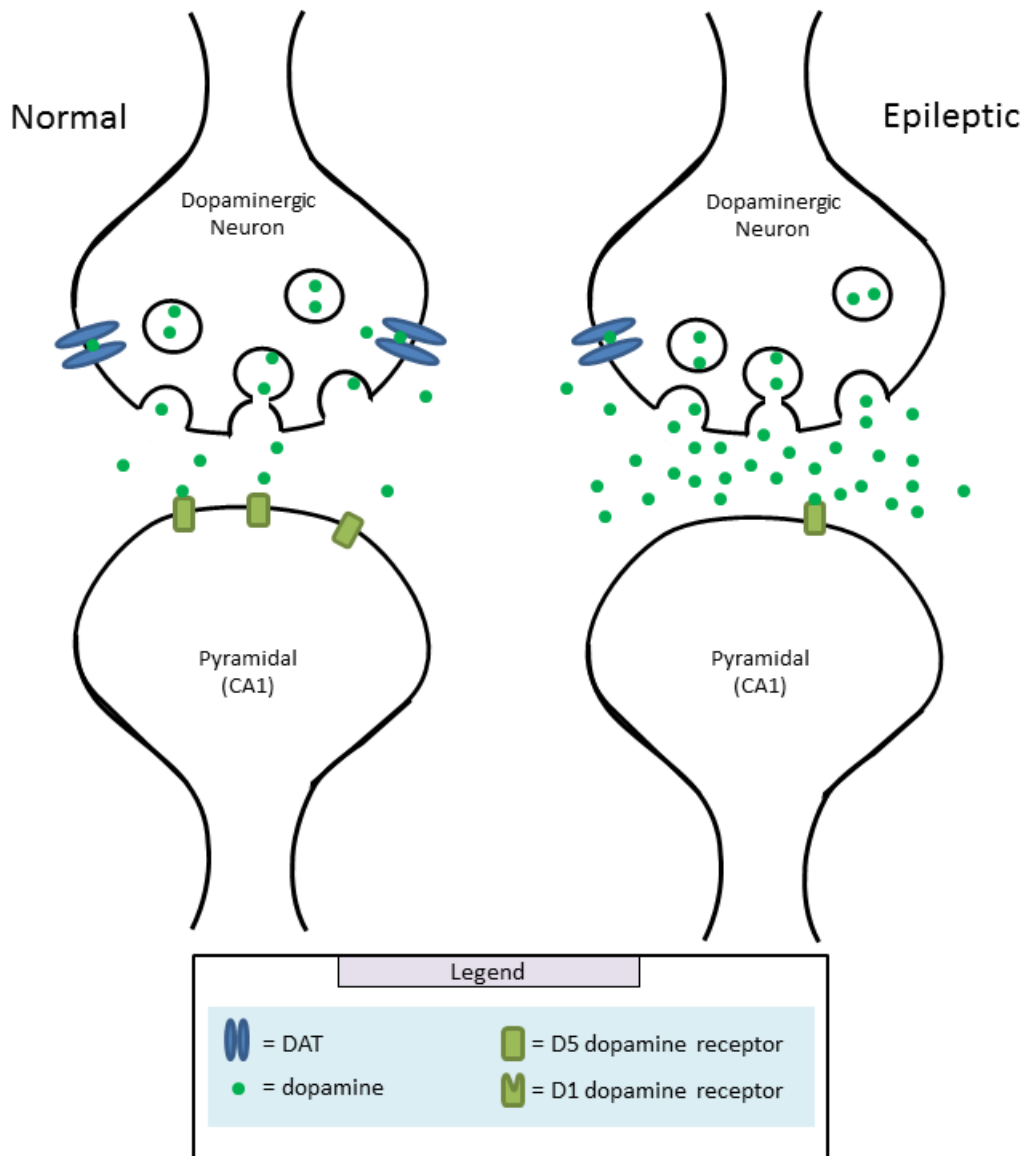


Figure 6.3 Illustration of dopaminergic system alteration in the epileptic CA1 compared to the normal brain. We discovered that D5 dopamine receptors and dopamine clearance is reduced in the epileptic hippocampus, while dopamine concentration and the amount of dopaminergic fiber is unchanged. Thus, we reasoned that released dopamine remains in the extrasynaptic space longer in the epileptic brain compared to the normal brain.

increased M1 mAChR activation would subsequently depress KAR synaptic transmission.

Overactivation of KARs have been implicated in epilepsy (Ben-Ari and Cossart, 2000; Vincent and Mulle, 2009; Contractor et al., 2011; Lerma and Marques, 2013). First, due to their slow deactivation kinetics, KARs facilitate temporal summation of postsynaptic excitatory potentials and epileptiform bursting (Castillo et al., 1997; Vignes and Collingridge, 1997; Frerking et al., 1998; Kidd and Isaac, 2008). Secondly, postsynaptic KARs elicits a metabotropic function that reduces the slow afterhyperpolarization, causing an increase in spike firing and neuronal excitability (Melyan et al., 2002, 2004). Furthermore, a hallmark symptom of chronic epilepsy in both human and animal models is mossy fiber sprouting, where mossy fibers in the CA3 make aberrant synaptic connections onto dentate granule cell neurons (Buckmaster et al., 2002). This sprouting creates an excitatory feed forward circuit that is susceptible to epileptiform bursting. KARs also located on these sprouted mossy fibers and can respond to glutamate (Epsztein et al., 2005). Lastly, KARs are also located presynaptically on principal and interneurons where they facilitate glutamate release and inhibit GABA release (Contractor et al., 2011; Carta et al., 2014). Overactivation of presynaptic KARs would tilt the excitatory/inhibitory brain balance to being more excitatory. Thus, an exercised-induced suppression of KARs activity on CA3 pyramidal neurons would be beneficial for patients with epilepsy. Indeed, humans and animals with a chronic model of temporal lobe epilepsy have demonstrated reduced seizure frequency and susceptibility to developing seizures when engaged in exercise training (Westerberg et al., 1984; Arida et al., 1999, 2004, 2009a, 2013; de Lima et al., 2011; Gomes da Silva et al., 2011; Nyberg

et al., 2013). Acutely, seizures susceptibility is higher during rest than during or immediately after bouts of physical activity, which has been shown to silence epileptiform discharges (Camilo et al., 2009; Vancini et al., 2010; de Lima et al., 2011).

6.3 ALTERATIONS IN THE DOPAMINERGIC SYSTEM IN TEMPORAL LOBE EPILEPSY: IMPLICATIONS FOR EXERCISE

Forty years ago, epilepsy was thought to be a disease of dopaminergic hypoactivity versus schizophrenia (Lamprecht, 1977; Starr, 1996), which is thought to be a disease of dopaminergic hyperactivity. This theory was conceived as a result of studies demonstrating that application of a general dopamine receptor agonist reduces seizure susceptibility (Lamprecht, 1977; Lal, 1988), whereas blockage of dopamine receptors induced seizures (Laird et al., 1984; Jann et al., 1993). We now know that there are two subtypes of dopamine receptors: D1-like and D2-like. These dopamine receptors have opposite effects on seizure susceptibility. D1-like dopamine receptor activation precipitates seizures (Starr et al., 1987; al-Tajir et al., 1990a, 1990b), while D2-like dopamine receptors reduce seizure susceptibility (al-Tajir et al., 1990a; Al-Tajir and Starr, 1991).

In the chronically epileptic brain, we found that D5 dopamine receptor expression is reduced in CA1, while total D1 dopamine receptor expression is unchanged but likely upregulated in remaining interneurons in CA1. Additionally, we discovered that total dopamine concentration remains unchanged, while dopamine clearance is reduced in the whole hippocampus. Taken together, these results suggest that more dopamine is

remaining in the extrasynaptic space. An increase in extrasynaptic dopamine would allow more dopamine binding to both D1-like and D2-like dopamine receptors. In theory, these anatomical changes of increased extrasynaptic dopamine and reduced D1-like dopamine receptor expression are beneficial for the epileptic condition.

Interestingly, these changes mimic exercise's effects on the dopaminergic system. Briefly, exercise also increased dopamine concentration in the brain while reducing D1 DA receptor density (de Castro and Duncan, 1985; MacRae et al., 1987; Greenwood et al., 2011). Exercise also decreases dopamine turnover (a ratio of dopamine metabolites to dopamine concentration) in Parkinson patients (Aguiar et al., 2015), suggesting that a higher percentage of released dopamine remains in the extrasynaptic space. Also, exercise has been shown to increase the sensitivity of dopamine receptors to dopamine (Zigmond et al., 2012). As stated in the previous section, exercise is beneficial for people with epilepsy and can be used as an alternative therapy in conjunction with pharmaceutical therapies to help patients manage their seizures.

6.4 FUTURE DIRECTIONS

1. We used ACET and kynurenate to discover that binding to either the GluK2 or GluK5 subunit is sufficient to activate the receptors, and that binding or both are required for desensitization. However, we did not investigate the subunit contribution to deactivation, which is fundamentally different mechanism than desensitization. Also, deactivation has significant physiological relevance. Therefore, it would be important to

use the pharmaceutical tools, ACET and kynurenate, to delineate the function roles of GluK2 and GluK5 to deactivation.

2. We found that M1 mAChRs depresses postsynaptic KAR-mediated synaptic transmission at the mossy fiber – CA3 synapse. Epilepsy has been shown to modulate both the cholinergic system and kainate receptor expression. Thus, it would be important to determine whether a chronic model of epilepsy alters this M1 mAChR-mediated depression of KARs.

3. The dopaminergic system is altered in a chronic model of temporal lobe epilepsy. Exercise is a potent therapy demonstrated to both reduce seizure frequency and alter the dopaminergic system. Thus, it would be important to evaluate the anatomical and functional differences between the dopaminergic system in a chronically epileptic rat and a chronically epileptic rat that is allowed to undergo physical activity.

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