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The Role of RGS14 in Learning, Memory, and Synaptic Plasticity

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The Role of RGS14 in Learning, Memory, and Synaptic Plasticity

By

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B.S., University of West Georgia, 2003

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An abstract of a dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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2012

Abstract

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By Sarah Emerson Lee

The hippocampus is crucial for converting new experiences into long-term memories following initial learning. Learning and memory are closely linked to synaptic plasticity, which involves altering the strength of connections between of neurons especially within the dentate gyrus (DG)-CA3-CA1 trisynaptic circuit of the hippocampus. Conspicuously absent from this circuit is the intervening CA2 whose existence as a distinct region has been subject to debate. The CA2 only recently been implicated in learning and memory. CA2 neurons have a striking lack of synaptic long-term potentiation (LTP). RGS14 is differentially expressed during postnatal development and is highly enriched in CA2 pyramidal neurons. RGS14 is critically important for suppressing synaptic plasticity in these cells and hippocampal learning and memory. RGS14 is an unusual scaffolding protein that integrates G protein and MAP kinase signaling pathways making it well positioned to suppress plasticity in CA2 neurons. Supporting this idea, we find that deletion of exons 2-7 of the RGS14 gene yields mice that lack RGS14 (RGS14-KO) that also express robust LTP following high frequency stimulation of Schaffer collateral synapses, but with no impact on synaptic plasticity in CA1 neurons. When tested behaviorally, RGS14-KO mice exhibited marked enhancement in the acquisition of spatial learning and of object recognition memory compared with their wild type littermates, but showed no differences in their performance on tests of non-hippocampal-dependent behaviors. These results demonstrate that RGS14 is a key regulator of signaling pathways linking synaptic plasticity in CA2 pyramidal neurons to hippocampal-based learning and memory, and RGS14 may serve as a memory filter that could be a pharmacological target to provide general cognitive enhancement in patients with neurodegenerative disorders.

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Abbreviations

AD	Alzheimer's Disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CA	Corrnus ammonis
CAMKII	Calcium/calmodulin dependent kinase II
cAMP	cyclic adenosine monophosphate
CNG	Cyclic-nucleotide gated ion channel
CREB	cAMP response element binding
DAG	Diaclyglycerol
DG	Dentate Gyrus
EC	Entorhinal Cortex
EPAC	Exchange protein activated by cAMP
ERK	Extracellular signal related kinase
FGF	Fibroblast growth factor
GAP	GTPase accelerating Protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G protein coupled receptor
GPR	G protein regulatory/GoLoco
GTP	Guaninosine-5'- triphosphate
HPC	Hippocampus
$InsP_3$	inositol1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
KO	Knock-out
LII	Layer II of the Entorhinal cortex
LIII	Layer III of the Entorhinal cortex
LTD	Long-term depression

LTP	Long-term potentiation
MAP kinase	Mitogen activated protein kinase
NMDA	N-Methyl-D-Aspartate
p38	P38 MAP kinase
PCL	Pyramidal cell layer
PDGF	Platelet-derived growth factor
PKA	Protein kinase A
PLC	Phospholipase C
PTX	Pertussis toxin
Rap	Ras related protein
RBD	Ras/Rap binding domain
RGS	Regulator of G protein Signaling
SC	Schaffer colaterals
SLM	Stratum-lacunosum moleculare
SO	Stratum oriens
SR	Stratum radiatum
WT	Wild-type

Chapter 1: Introduction

1.1 Learning and Memory

Learning and memory are essential to the human experience. Learning is the acquisition of new knowledge or skills while memory is the usage of knowledge or skills. Generally, learning takes place over time with repetition and can often be a laborious task. Memory is recalling stored experiences or learning built over a lifetime. Memory forms the essence of the human spirit and allows individuals to benefit from past experiences, guided by these experiences and not simply by present perceptions. Therefore, storing knowledge is a critical element of memory; the ability to recall and reflect on life experiences is a profound and powerful element of being human. The loss of this ability is the most devastating feature of Alzheimer's disease and other neurodegenerative disorders.

1.2 The role of the hippocampus in memory.

Memory can be divided into groups based on the type of information learned. Declarative memory is learning facts- the who, what, when, and where of an event. Declarative memory can be further separated into semantic or episodic memory. Semantic memory is factual knowledge, meanings, and understanding concepts. Episodic memory is the life story of a person or an autobiographical recording which includes all the details of events and may be recalled as a whole episode similar to a scene in a play. The second type of memory is procedural memory- learning how to do something. It is skill learning like riding a bicycle or playing an instrument. Procedural memories can be automatic and performed without conscious awareness

of past events such as specific lessons or experiences in which the skill was learned. Declarative and procedural memories are encoded in different regions of the brain. Procedural memory encoding occurs in the cerebellum, putamen, caudate nucleus and the motor cortex (Figure 1.1) (Budson and Price, 2005). Declarative memories are encoded in the CA regions and dentate gyrus of the hippocampus, entorhinal cortex, and perirhinal cortex(Budson and Price, 2005). The importance of the medial temporal lobe in the formation of new declarative memories was revealed by William Scoville and Brenda Milner through studies with patient H.M (Scoville and Milner, 1957). Following bilateral medial temporal lobe resection, patient H.M. experienced anterograde amnesia in that he was unable to form new declarative memories; however, he could form new procedural memories and recall memories from childhood. He experienced retrograde amnesia for events that occurred within a few years prior to the surgery, indicating that once memories are consolidated, they are then stored long term outside the medial temporal lobe in the neocortex (Scoville and Milner, 1957). Patient H.M. provided direct evidence for the role of the hippocampus in acquisition of declarative memories.

In addition to the role of the hippocampus in declarative memory, the hippocampus is involved in spatial memory. In freely moving animals, the position of the animal within an environment activates a specific group or collection of neurons known as place cells (Nakazawa et al., 2004). Place cells are found in pyramidal cells of the hippocampus proper and in granule cells of the dentate gyrus(van Strien et al., 2009). These neurons tend to be excitatory although inhibitory interneurons may be activated as well. Because place cells are activated based on spatial

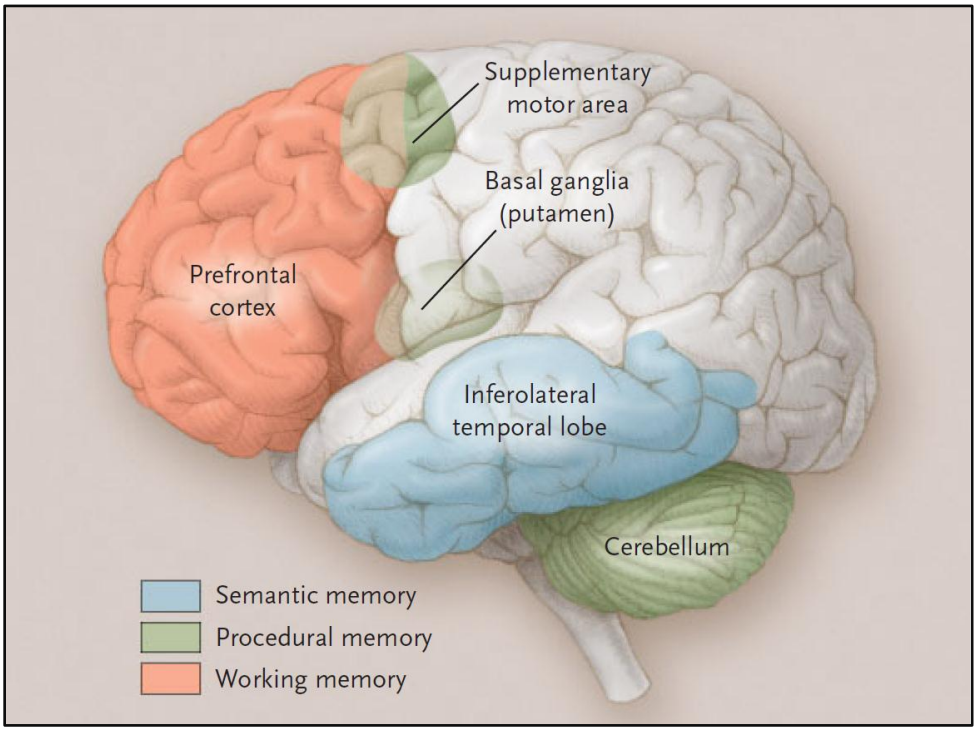


Figure 1.1. Semantic, Procedural, and Working Memories.

The inferolateral temporal lobes are important in the naming and categorization tasks by which semantic memory is typically assessed. However, in the broadest sense, semantic memory may reside in multiple and diverse cortical areas that are related to various types of knowledge. The basal ganglia, cerebellum, and supplementary motor area are critical for procedural memory. The prefrontal cortex is active in virtually all working memory tasks. Other cortical and subcortical brain regions will also be active, depending on the type and complexity of the working memory task.positioning, the hippocampus has been proposed to form a type cognitive map or a neuronal pattern of activation specific to different positions and environments (Morris et al., 1982; O'Keefe and Conway, 1978). The distinct functions of the hippocampus in memory are likely due to its cellular architecture. Figure from (Budson and Price, 2005).

1.3 Neuroanatomy and Circuitry of the Hippocampus

The hippocampus has a unique three-dimensional gross anatomy. Named for its similarity in appearance to the sea horse, *Hippocampus leria*, the hippocampus proper can be divided into three segments of the *cornu ammonis:* CA3, CA2, and CA1(Andersen, 2007). The hippocampal formation also includes the dentate gyrus, subiculum, presubiculum, parasubiculum, and the entorhinal cortex. Within the brain, the hippocampus receives highly processed sensory input from a range of different neocortical regions indicating that the hippocampus is a site for integration of information from different sensory points of origin (Andersen, 2007). Transmission in the hippocampus forms a primarily unidirectional pathway. Projections originating from layer III of the entorhinal cortex follow the perforant and alvear pathways to synapse with neurons in the CA1 and subiculum (Andersen, 2007). Neurons in layer II of the entorhinal cortex (EC) project to dendrites in the dentate gyrus and CA3. Projections from the EC to the dentate gyrus are not reciprocal in that the dentate gyrus does not send projections back to the EC making the pathway unidirectional. The dentate gyrus sends projections from granule cells. These axonal projections are known as mossy fibers because of swellings or varicosities that give them a mossy appearance. Mossy fibers synapse with pyramidal neurons in the CA3 region, yet again no projections return from the CA3 to dentate gyrus granule cells. CA3 pyramidal neurons send axonal projections to the CA1 via Schaffer collaterals. CA1 does not send projections to CA3 rather to the subiculum. This series of unidirectional pathways from the EC to dentate gyrus to

CA3 to CA1 forms the basis of the classical trisynaptic circuit of the hippocampus (Figure 1.2) (Andersen, 2007).

Excluded from the trisynaptic circuit is the CA2 region. The CA2 was long believed to be little more than a transition region between the cells of the CA1 and CA3. CA2 neurons have properties that are reminiscent of both CA1 and CA3 regions. Like CA1, the CA2 receives neither mossy fiber input from dentate gyrus granule cells nor has thorny excrescences characteristic of CA3 pyramidal neurons (Lorente de No, 1934). Yet the large size of the pyramidal neurons in CA2 is closer to that of CA3 than of CA1. The CA2 is the only region of the hippocampus to receive input from the supramammillary nucleus of the hypothalamus indicating its unique place in hippocampal function (Magloczky et al., 1994; Soussi et al., 2010). CA2 pyramidal cells exhibit more branching in the Stratum-lacunosum moleculare (SLM) than CA1 pyramidal cells (Andersen, 2007). Compared to CA1 pyramidal cells, CA2 pyramidal cells exhibit lower input resistance and higher membrane capacitance (Jones and McHugh, 2011). Typically, synaptic input on proximal dendrites strongly excites neurons. In the CA2, pyramidal neurons receive input from distinct sources. Schaffer collateral projections from the CA3 region synapse

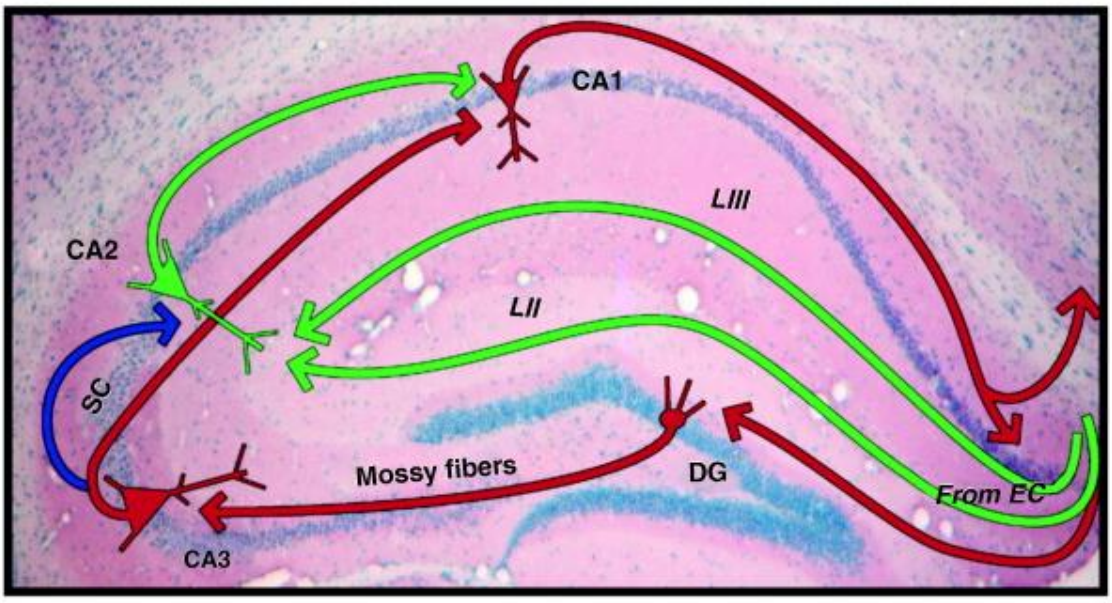


Figure 1.2 Hippocampal Circuitry. Diagram of HPC circuitry. Red arrows indicate trisynaptic circuit. Input from the entorhinal cortex (EC) synapses on neurons in the dentate gyrus (DG). Mossy fiber projections from DG synapse on CA3 pyramidal neurons. Schaffer Collateral projections from CA3 synapse on CA1 neurons to complete the trisynaptic circuit. Blue arrow indicates CA3 Schaffer collaterals also synapse on CA2 pyramidal neurons. Green arrows indicate a separate circuit in which EC inputs synapse on CA2 dendrites.

on proximal CA2 dendrites, and projections originating in the entorhinal cortex synapse on distal dendrites (Sekino and Shirao, 2007). CA2 pyramidal cells receive weaker input from CA3 and stronger input from the EC (Chevaleyre and Siegelbaum, 2010b). Projections from CA2 pyramidal cells synapse with CA1 neurons (Jones and McHugh, 2011; Sekino and Shirao, 2007).

Additionally the CA2 exhibits a different pattern of gene expression. Gene expression profiles can be used to distinguish each of the hippocampal subfields (Lein et al., 2005b). A discrete set of genes including vasopressin 1b receptors, adenosine A1 receptors, fibroblast growth factor 2 (FGF2), and the regulator of G protein signaling 14 (RGS14) are found in the CA2 further indicating that its role in neurophysiology is more than a simple transition region between CA1 and CA3 (Bland et al., 2007; Lee et al., 2010; Ochiishi et al., 1999; Young et al., 2006) .

1.4 The Hippocampus in Human Disease

Alzheimer's disease and temporal lobe epilepsy are two of the most common neurological disorders. Damage to the hippocampus is a critical indicator of the appearance of these two diseases. Alzheimer's disease (AD) is the most common cause of dementia. According to the Alzheimer's Association, there are currently 5.4 million Americans living with AD. Once diagnosed, people with AD live an average of four to eight years. The cost to the economy in 2011 was $183 billion an increase of $11 billion over the previous year. The most distinguishing feature of AD is memory loss. Early symptoms include an inability

to recall life events, current affairs, and the names and faces of friends as well as a poor sense of direction and failing to remember familiar routes. As the disease progresses memory loss becomes more profound. Word recall becomes difficult and eventually problems with word comprehension become apparent. The progressive nature of impairments in AD is reflective of the nature of degeneration of different brain regions including frontal, temporal, and parietal lobes. Accompanying cognitive decline are psychological symptoms such as depression, changes in personality, hallucinations and delusions, as well as behavioral changes including aggression, agitation, and night wandering. Behavioral disturbances tend to increase as the disease progresses. Treatments aim to enhance cholinergic function or block glutamate-mediated synaptic signaling(Brody et al., 2005). While these treatment methods improve cognitive performance, neither can slow nor alter the natural progression of AD.

Pathological features of AD include accumulation of extracellular β-amyloid plaques and intracellular neurofibrillary tangles. Amyloid plaques are comprised of a splice variant of the amyloid precursor protein (APP) surrounded by activated microglia that secrete inflammatory mediators (O'Brien and Wong, 2011). Intracellular neurofibrillary tangles made of tau, a microtubule associated protein, that is hyperphosphorylated at atypical amino acid residues (Avila et al., 2004). Both plaques and neurofibrillary tangles appear in areas with high degrees of neuronal degeneration. Degeneration in the hippocampus seems to follow the flow of anatomical synapses. Decreased hippocampal volume is preceded by degeneration of the entorhinal cortex pointing to the distinct

connections and circuitry in the hippocampus (Andersen, 2007; Brody et al., 2005).

The hippocampus is especially vulnerable to oxygen deprivation from ischemic/hypoxic events. Likely due to its role in memory formation, the hippocampus requires and receives significant blood supply. Blood arrives from the anterior choroidal artery and branches of posterior cerebral artery. Blockage of blood flow by an embolic event due to cerebrovascular disease or other cause leads to ischemic insult that can cause damage to the hippocampal formation. The CA1 region is particularly sensitive with ischemic events causing massive neuronal cell death, but the CA2 region is largely spared (Andersen, 2007).

Epilepsy is the propensity to have seizures resulting from increased neuronal activity and excitability, and according the Centers for Disease Control and Prevention affects about 2 million Americans. Seizures can originate in different brain regions; however, seizures that arise in the temporal lobe are associated with hippocampal sclerosis, which is the loss of pyramidal neurons, proliferation of glia, and dispersion of granule cells (Andersen, 2007). Temporal lobe resection can be used as a last resort when seizures do not respond to pharmacological treatment (Andersen, 2007). Neuronal cell loss occurs following prolonged seizures. Pyramidal cells of the CA1 are susceptible to damage; however, pyramidal cells of the CA2 and dentate granule cells are more resistant (Corsellis and Bruton, 1983; Sloviter, 1987; Sloviter, 1991). Cell loss leads to structural changes and changes in neuronal connectivity. Alterations in neurotransmission, decreased inhibitory signaling through GABAergic

interneurons, increased excitatory signaling of glutamatergic neurons, and changes in neuronal properties can lead to epilepsy (Andersen, 2007). Therefore, maintaining a balance between excitability and inhibition is critical for normal hippocampal function.

Psychological disorders such as schizophrenia produce hippocampal pathologies including a reduction in hippocampal volume. Reductions in hippocampal volume are primarily due to decreases in neuron size rather than number with the exception of area CA2 (Benes et al., 1998). The CA2 of schizophrenic and bipolar disorder patients suffer a decrease in the density of inhibitory interneurons (Benes et al., 1998). The sparing of CA2 during ischemia/epilepsy and its unique pathology in psychological disorders points to a differential role for the CA2 in hippocampal function.

1.5 Cellular and Molecular Mechanisms of Memory hippocampal-based memory

Memory is thought to be encoded within the brain as biochemical and physical changes at synapses leading to alterations in neurotransmission, a process known as synaptic plasticity. One such form of synaptic plasticity is the long lasting increase in the strength of excitatory glutamatergic synaptic transmission, long-term potentiation (LTP,) that can be induced with high frequency afferent stimulation. LTP has been best characterized within the well-defined dentate gyrus (DG)-CA3-CA1 trisynaptic circuit of the hippocampus

(Figure 1.2), with the overwhelming majority of studies performed in the CA1 region (Nakazawa et al., 2003; Neves et al., 2008; Rolls and Kesner, 2006).

The DG-CA3-CA1 pathway (Figure 1.2, red) consists of 1) input from the entorhinal cortex (EC), forming synapses on granule neurons in the DG; 2) axons originating from the DG that project to area CA3, forming large mossy fiber synapses on dendrites of CA3 pyramidal neurons; and 3) CA3 axons, also known as the Schaffer collateral (SC) fibers, connecting to the dendrites of CA1 pyramidal neurons in an area known as the Stratum Radiatum. Plasticity at synapses is dependent on their activation and may be manifest as short or long-term changes in synaptic strength. Short bursts of stimulation that mimic brain activity of an animal exploring a new environment induce LTP.

Considerable work has been done to dissect the molecular signaling pathways that underlie LTP. N-Methyl-D-aspartate and AMPA glutamate receptors are known to be important mediators of synaptic plasticity, and calcium influx through AMPA and NMDA receptors allows for the reorganization of the actin cytoskeleton to alter dendritic spine size (Sheng and Kim, 2002). Downstream of NMDA receptors, the calcium/calmodulin dependent protein kinase II (CAMKII) pathway is a critical mediator of actin cytoskeleton remodeling and is responsible for dendritic spine dynamics (Kennedy et al., 2005). The actin cytoskeleton is rearranged during learning and LTP, which facilitate increases in the spine size and the number of cell-surface AMPA receptors (Chen et al., 2007; Sheng and Kim, 2002; Tada and Sheng, 2006). Long-term depression, LTD, also induces changes in dendritic spines, however, LTD induces a decrease in

spine size and decreases the expression of cell surface AMPA receptors (Collingridge et al., 2004). Several signaling mediators expressed in dendritic spines control AMPA receptor expression.

Increasing the number of AMPA receptors on the cell surface boosts synaptic transmission, which is concomitantly accomplished by raising current passage through AMPA receptors through CAMKII, Protein Kinase A, or Protein Kinase C induced phosphorylation of certain serine residues on AMPA receptors. The monomeric GTPase, Ras, in concert with CAMKII influence insertion of AMPA receptors in the post-synaptic density(Kennedy et al., 2005). Ras, best known for its role as an oncogene that controls cell growth and survival, activates downstream effectors of the mitogen activated kinase pathway (MAP kinase) to regulate gene transcription. Activation of the MAP kinase pathway is required for certain types of learning and induction of LTP (Kennedy et al., 2005; Sweatt, 2004). Blocking NMDA receptor signaling and MAP kinase pathways prevents LTP and blocks learning (English and Sweatt, 1997; Sweatt, 2004).

Genetic and pharmacological alterations to the function of NMDA receptors change synaptic signaling properties as well as learning and memory in rodents (Morris et al., 1986; Nakazawa et al., 2003). Studies using transgenic and knock-out animal models have given insight into the cellular and molecular mechanisms of learning and memory. While most genetic alterations result in deficits in cognitive function, a significant number have been shown to enhance learning and memory (Lee and Silva, 2009). Changes in synaptic strength are

believed to underlie learning and memory storage. Therefore, it is not surprising that a large percentage of these mutations exhibit enhancements in LTP.

Overexpression of the NR2B subtype of the NMDA receptor in the adult forebrain results in enhanced learning and memory in a variety of tasks and increases in LTP (Tang et al., 1999). Mice with alterations in other genes related to NMDA receptor signaling show enhancements in learning and memory as well. Conditional KO of cyclin-dependent kinase 5 (Cdk5) in mouse forebrain reduced degradation of NR2B subunits and may increase NMDA receptor activity (Hawasli et al., 2007). Loss of the nociception receptor, ORL1, show normal responses to pain but enhanced learning and memory in the water maze, passive avoidance task, and LTP(Manabe et al., 1998). Activation of ORL1 inhibits N-type Ca^{2+} channels and promotes channel internalization (Altier et al., 2006). Loss of ORL1 may enhance N-type Ca^{2+} conductance to increase intracellular Ca^{2+} levels. Changes in Ca^{2+} homeostasis can alter learning and LTP. Extrusion of Ca^{2+} is a critical mechanism for maintaining homeostatic balance. The Na^{+}/Ca^{2+} exchanger (NCX) pumps removes Ca^{2+} from neurons at a high rate. Mice lacking NCX2 exhibit increased performance in hippocampal-dependent learning tasks and an enhancement in LTP (Jeon et al., 2003). Other gene knockouts that lead to memory improvements include proto-oncogene, Cbl-b, transcriptional regulator, GCN2, monoamine oxidase A, and others (Lee and Silva, 2009)

One mechanism for enhancement of learning may be due to increases in intracellular calcium ions following activation of NMDA receptors; G protein

coupled receptors, and Growth Factor receptors activate a host of downstream signaling molecules and effectors. Activation of these downstream pathways can influence learning, memory, and LTP. Increased intracellular Ca^{2+} activates Ras, which stimulates insertion of AMPA type glutamate receptors in the dendritic spine membrane and leads to increases in spine size (Zhu et al., 2002). Additionally, transgenic expression of a constitutively active form of H-Ras produces mice with enhancements in learning, memory and LTP (Kushner et al., 2005). Rap is another monomeric GTPase and a close relative of Ras; however Rap GTPases that are activated by cAMP have opposing actions on dendritic spine dynamics than Ras. Calcium-calmodulin activates adenylyl cyclase, which stimulates production of cAMP. The cAMP second messenger activates exchange protein directly activated by cAMP (EPAC), a RapGEF that directly activates Rap GTPases. Raf kinase is then activated by Rap and activates components of the MAP kinase pathway. Rap signaling results in the removal of synaptic AMPA receptors and is associated with decreased spine size, long-term depression, depotentiation and deficits in hippocampal dependent memory performance (Figure 1.2) (Fu et al., 2007; Ryu et al., 2008; Tada and Sheng, 2006; Zhu et al., 2005). Signaling interplay between ion channels, G protein coupled receptors and growth factor receptors is becoming more apparent and integrators of these signaling pathways may have important roles in learning and memory. Regulation of LTP and synaptic plasticity has been extensively studied in the DG-CA3-CA1 trisynaptic circuit; however, area CA2 is usually not mentioned in the literature, likely due to its earlier

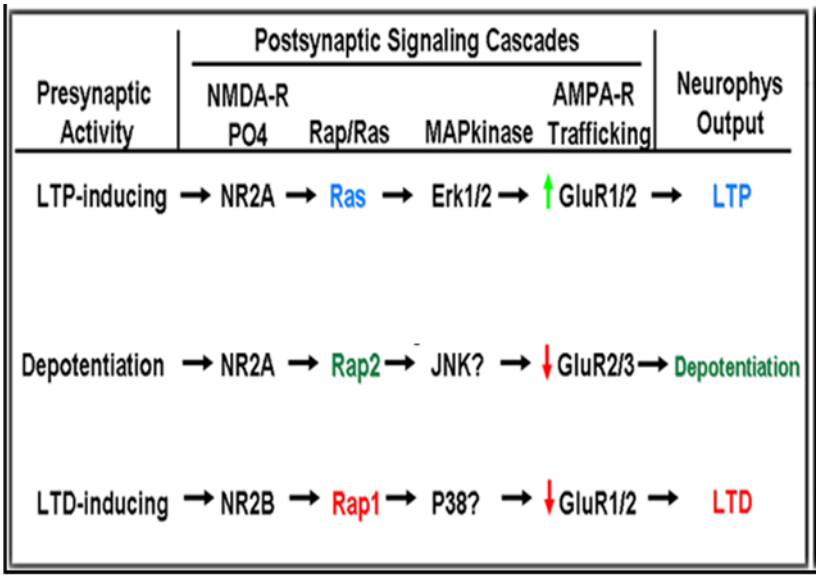


Figure 1.2 Postsynaptic signaling in dendritic spines. Activation of NMDA receptors stimulates downstream activation of signaling pathways. NMDA Top: LTP inducing activity induces NR2A subunit activation triggering Ras activation followed by ERK1/2 phosphorylation, which increases surface expression of AMPA receptor GluR1/2 subunits that lead to increases in neurotransmission (LTP). Center: Stimuli leading to depotentiation result in RAP2 activation, JNK phosphorylation, and decreased surface expression of GluR2/3 subunits. Lower: LTD inducing stimuli activate NR2B NMDA receptor subunits that activate Rap1, which leads to phosphorylation of p38 and decreased surface expression of GluR1/2 subunits

controversial status as a transition zone between CA1 and CA3 rather than as a separate area.

The CA2 was clearly defined decades earlier as an area consisting of unique neurons that are similar both in size to the large CA3 neurons and also in that they receive no mossy fiber synaptic input from the DG. Instead, CA2 neurons receive their main input from the CA3 Schaffer collaterals (Figure 1.3, black) (Lorente de No, 1934). Most commonly, LTP is studied in CA1 neurons that receive the CA3-derived Schaffer collaterals where the potentiation of neurotransmission is robust. Unlike synapses on their CA1 neighbors, though, the Schaffer collateral synapses on CA2 pyramidal neurons do not typically exhibit LTP in response to high frequency stimulation (Simons et al., 2009; Zhao et al., 2007). This lack of LTP in CA2 pyramidal neurons is attributed to increased calcium buffering capacity and increased calcium extrusion (Simons et al., 2009)

In addition to the Schaffer collateral input from CA3, CA2 neurons also receive distinct synaptic input from layer II (LII) and III (LIII) neurons of the EC (Figure 1.3, green). Neurons of the CA1, CA2 and CA3 region are large and pyramidal in shape and have dendrites that extend from the apex of the cell body (apical dendrites) that are either proximal (close to the cell body) or distal (extend far away) (Figure 1.3). Unlike the CA3-Schaffer collateral input to CA2 neurons that synapse on the proximal dendrites and show no LTP, the CA2 input from the EC synapse on distal dendrites in a region known as the

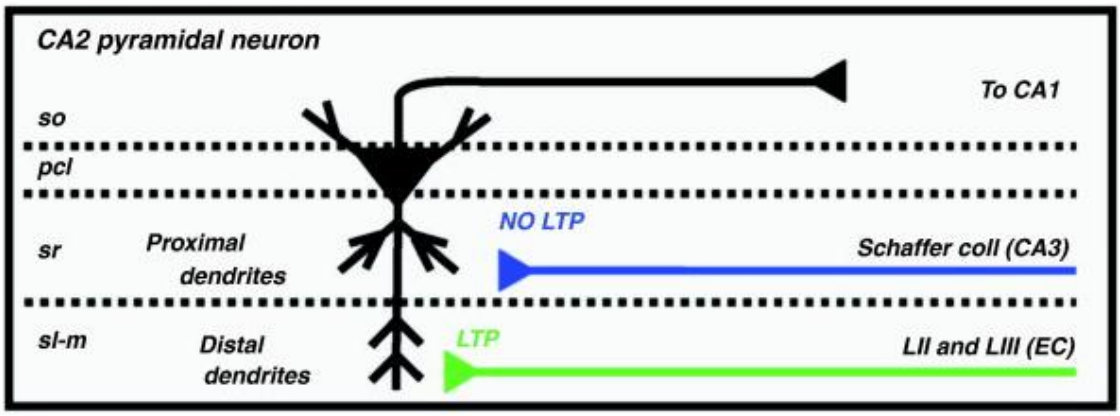


Figure 1.3 Distinct synaptic inputs on CA2 pyramidal neurons. Hippocampus cell layers shown at left. Stratum Oriens (so), Pyramidal Cell Layer (pcl), Stratum Radiatum (sr), Stratum-lacunosum moleculare (sl-m). CA3 Schaffer Coll. (blue) synapse on proximal dendrites of CA2 neurons within the sr. High frequency stimulation of Schaffer Coll. generates no LTP in CA2 proximal dendrites. Projections from layer II and III of the EC (green) synapse on distal dendrites within the sl-m. Stimulation of EC inputs generates LTP in CA2 distal dendrites.

Stratum Lacunosum Moleculare. Recently, Chevaleyre and Siegelbaum found that both LII and LIII EC pathways are capable of expressing robust NMDA receptor-dependent LTP in CA2 neurons in contrast to the synapses from CA3 that fail to express LTP (Chevaleyre and Siegelbaum, 2010a). Typically proximal dendrites exert the strongest excitatory effect on neurons. CA2 pyramidal neurons appear to have a reversal of this property in that distal dendrites actually are strongly excited by input from the EC (Chevaleyre and Siegelbaum, 2010b). These findings suggest that regional differences or compartmentalization of molecular signaling machinery within CA2 neurons may provide distinct synaptic outputs in response to activity from CA3 or from the different layers of the EC.

Dendritic spines may act to create synaptic microenvironments with distinct protein expression profiles, calcium handling properties, or other synaptic properties. CA2 proximal dendritic spines activate following stimulation of Schaffer collaterals, but these synapses are resistant to induction of LTP (Simons et al., 2009). These synapses exhibit high levels of calcium buffering capacity and increased calcium extrusion (Simons et al., 2009). The CA2 region expresses a subset of signaling proteins, some of which have been shown to have roles in learning and memory. Arginine vasopressin1b receptors are concentrated in the CA2. Mice lacking the vasopressin1b receptor exhibit deficits in temporal learning, social memory, and decreased aggression(DeVito et al., 2009). Blocking vasopressin1b receptors decreases aggression in hamsters, decreases anxiety, and has anti-depressive effects in animal models (Blanchard

et al., 2005; Griebel et al., 2002). Adenosine 1A receptors are concentrated in the CA2 and have been shown to mediate the cognitive enhancing properties of caffeine (Simons et al., 2012). Adenosine and vasopressin cell surface receptors that are members of the G protein coupled receptor family.

1.6 G Protein Coupled Receptors

G protein coupled receptors (GPCR) make up a large cell surface receptor superfamily. Nearly 800 genes in the human genome encode for GPCRs, and they are responsible for transmitting nearly 80% of signal transduction across cell membranes (Kroeze et al., 2003). Many different types of ligands including single photons, ions, odorants, amino acids, fatty acids, neurotransmitters, peptides, as well as proteolytic enzymes that cleave off receptor fragments to generate an activating ligand and adhesion molecules activate GPCRs (Neves et al., 2002). Embedded in the plasma membrane and consisting of seven-transmembrane domains, connected by extracellular and intracellular loop, GPCRs all share a common architecture. Given their cell surface localization and important roles in cell physiology, GPCRs make excellent pharmacological targets; indeed, more than one third of all current therapeutics are directed at them (Millar and Newton, 2010). GPCRs are classified into distinct families based on sequence homology. With 672 family members more than half of which are odorant receptors, the largest GPCR family is Class A, the Rhodopsin-like family. The other families include Class B, the secretin family with 15

members, Class C, the metabotropic Glutamate family with 22 members, and others that include the adhesion family with 33 members, and the Frizzled/Taste receptors with 36 members (Millar and Newton, 2010).

1.7 G Protein Coupled Receptor Signal Transduction

G protein coupled receptors bind to extracellular ligands. Activation of the receptor following ligand binding is transmitted across the cell membrane where the intracellular loops of GPCRs bind to heterotrimeric G proteins. The heterotrimeric G protein complex includes three subunits: one α subunit, one β subunit, and one γ subunit. The β and γ subunits exist as an obligatory heterodimer, Gβγ, whereas the Gα subunit binds to and is activated by guanine triphosphate (GTP). Established models of signaling propose that GPCRs activate heterotrimeric G proteins (Gαβγ) by serving as guanine nucleotide exchange factors (GEFs) to trigger GDP release from the Gα subunit and promote GTP binding which is followed by Gβγ dissociation. Activated Gα-GTP and free Gβγ are signaling molecules that interact with downstream effectors and signaling pathways to regulate cell and organ physiology(Oldham and Hamm, 2008). Signaling is terminated by hydrolysis of GTP to GDP through the intrinsic GTPase activity of the Gα subunit.

Heterotrimeric G proteins are classified into four families: Gs, Gi/o, Gq, and G12/13, based on amino acid sequence homology and functional similarity of the Gα subunit. The Gαs family members are stimulatory. Ligand binding to

GPCRs coupled to Gαs triggers exchange of GDP for GTP and dissociation of the Gαs from Gβγ. The Gαs subunit activates adenylyl cyclase to produce the second messenger cAMP. cAMP activates Protein Kinase A (PKA), which directly phosphorylates downstream signaling proteins or activates cAMP response element binding (CREB) to modulate gene transcription. Additionally, cAMP activates EPACs (Exchange Protein Activated by cAMP) and the CNG (Cyclic-Nucleotide Gated Ion Channel). CNG activation leads to Ca2+ influx. cAMP activates Rap1A (Ras-Related Protein-1A) and Rap1B (Ras-Related Protein Rap1B) through the PKA-independent and EPAC-dependent pathway. The Gαi/o family members of G proteins are inhibitory in nature having effects that oppose those of Gαs. Activation of GPCRs coupled to Gαi/o results in inhibition of adenylyl cyclase activity thereby preventing the formation of the second messenger, cAMP. Activity of Gαi/o is pertussis toxin sensitive, which is a defining feature of Gαi/o family members. Members of the Gαq/11 family couple to a subclass of receptors and ubiquitously activate the beta isoforms of phospholipase C (PLCβ). PLCβ isoforms catalyze the formation of 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) second messengers. $InsP_3$ binds to receptors to mobilize Ca^{2+} release from intracellular stores. Gαq/11 family members also directly bind and activate p63RhoGEF which activates RhoA GTPases to regulate the actin cytoskeleton. Gα12/13 family members activate a different Rho-GEF, p115RhoGEF, to also regulate cytoskeleton dynamics (Kristiansen, 2004). Because of the diverse nature of G proteins in cell physiology, tight regulation of G protein signaling is a critical component for

maintaining homeostasis. Signal regulation and termination may be accomplished by inactivating the GPCR or the G protein. G protein-coupled receptor kinases phosphorylate the receptor, which then interacts with arrestin causing inactivation by uncoupling of the receptor from its G protein (Ferguson, 2001). Arrestin binding causes G protein uncoupling and may facilitate receptor internalization via clathrin-coated vesicles. Once internalized the receptor may be targeted to lysosomes for degradation or recycled to the plasma membrane (Ferguson, 2001). Regulators of G protein signaling are responsible for inactivation of G protein signaling.

1.8 The Regulators of G Protein Signaling

The Regulators of G protein Signaling (RGS) family of proteins are important components of G protein coupled-receptor (GPCR) signaling pathways (De Vries et al., 2000; Hollinger and Hepler, 2002b; Ross and Wilkie, 2000a; Willars, 2006). RGS proteins recognize both receptors and active Gα-GTP to selectively accelerate Gα GTPase activity and limit the duration of G protein signaling (Neitzel and Hepler, 2006). This large family of nearly 40 distinct signaling proteins is classified into eight subfamilies according to sequence identities and shared functions as GTPase accelerating proteins (GAPs) (De Vries et al., 2000; Hollinger and Hepler, 2002b; Ross and Wilkie, 2000a; Willars, 2006). RGS protein structures range from simple with a single domain to highly complex with multiple binding domains (De Vries et al., 2000; Hollinger and

Hepler, 2002b; Ross and Wilkie, 2000a; Willars, 2006). The protein architecture of complex family members suggests that many RGS proteins serve as multifunctional integrators of G protein signaling pathways. In addition to acting as GAPs towards activated Gα subunits, certain complex RGS proteins exhibit other functions on Gα subunits and other binding partners and signaling pathways (Hollinger and Hepler, 2002b; Willars, 2006).

1.9 Structure and interactions of RGS14

RGS14 is a structurally complex RGS protein. RGS14 is a 61 kDa protein classified within the D/R12 subfamily of RGS proteins. RGS14's closest relatives are RGS12 and RGS10 though RGS10 is much smaller and shares only a single RGS domain in common with RGS14. Besides the conserved RGS domain, RGS14 contains a second Gα binding domain (GPR/GoLoco domain) and two Ras/Rap-binding domains (RBDs) (Figure 1), suggesting that RGS14 serves signaling functions in addition to or distinct from the canonical GAP functions of the RGS protein (Snow et al., 1997b; Traver et al., 2000b). In particular, the presence of distinct binding sites on both RGS14 and RGS12 for Gα in either its active GTP-bound or inactive GDP-bound form indicates that RGS14 and its closest relative are unique among RGS proteins.

RGS14 is a protein enriched in brain but also is expressed in the spleen, thymus, and lymphocytes of rodents (Cho et al., 2000b; Snow et al., 1997b; Traver et al., 2000b). Of these tissues, RGS14 is most abundant in adult brain,

specifically within terminally differentiated neurons. The precise molecular mechanism of RGS14 function in the brain is currently unknown, but RGS14 can act as a scaffold to bind G proteins and components of the MAP kinase signaling pathway that are important for synaptic plasticity, learning, and memory (Shu et al., 2010). RGS14 was first discovered as a Rap1/2 binding partner (Traver et al., 2000b), and each of the identified RGS14 binding partners Gαi/o, H-Ras, Rap2, and Raf kinases have been shown to control various aspects of synaptic plasticity within hippocampal neurons (Fu et al., 2007; Kennedy et al., 2005; Kushner et al., 2005; Pineda et al., 2004; Ryu et al., 2008; Zhu et al., 2005). Following an initial report that one of the isolated purified RBDs of RGS14 can interact with H-Ras *in vitro* (Kiel et al., 2005), we and others discovered that RGS14 binds both activated H-Ras and Raf-1 in cells (Shu et al., 2010; Willard et al., 2009) to inhibit ERK-mediated MAP kinase signaling by platelet-derived growth factor (PDGF) (Shu et al., 2010).

Activated H-Ras recruits RGS14 to the plasma membrane in the absence of exogenous Gαi, allowing RGS14 to regulate PDGF-induced signaling (Shu et al., 2010). Co-expressed wild-type Gαi1 reverses RGS14's capacity to inhibit PDGF-induced ERK phosphorylation. In this case, RGS14 binds Gαi1 instead of Raf-1, indicating that RGS14 may act as a molecular switch between binding/regulating Gαi1 and binding/regulating Raf-1 and subsequent Raf-1-induced ERK phosphorylation (Shu et al., 2010).

Although RGS14 regulates PDGF-induced ERK phosphorylation in an H-Ras- and Gαi1-dependent manner, how this occurs remains unknown (Shu et al.,

2010). Various groups have reported unconventional roles for G proteins and interactions of G proteins with receptors that are not GPCRs (Marty and Ye, 2010). Relevant to RGS14, recent studies have examined the role of Gαi in directly regulating PDGF receptor/ERK-mediated MAP kinase signaling. Pertussis toxin (PTX) treatment of cells prevents Gαi/o-coupling to receptors, which subsequently blocks c-Src activation and ERK phosphorylation by PDGF, indicating a possible role for Gαi in PDGF receptor regulation of c-Src signaling (Conway et al., 1999). The PDGFβ receptor is also shown to induce phosphorylation of Gαi upon stimulation, which enhances ERK phosphorylation (Alderton et al., 2001). A key element to the involvement of Gαi in this process is the potential role of a GPCR. Germane to this point was the discovery that the PDGFβ receptor interacts with the EDG1 receptor, a Gαi-linked GPCR (Alderton et al., 2001). Co-expression of both PDGFβ receptor and EDG1 stimulates an increase in both Gαi phosphorylation and subsequent ERK activation following PDGF treatment (Alderton et al., 2001). How or even if RGS14 participates in PDGFβ/EDG1 receptor signaling is not known, but these studies highlight potential mechanisms for how RGS14 may switch from binding Gαi to binding activated H-Ras and regulating MAP kinase signaling. Stimulation of a GPCR may induce Gαi activation, subsequently influencing RGS14 localization and its preference for binding partners (*e.g.* Gαi vs. Raf-1). Formation of a GPCR:Gαi:RGS14 complex may also sequester RGS14 from binding its MAP kinase signaling partners. Additional studies will be necessary to distinguish between these possibilities, though studies indicate that RGS14 can functionally

interact with GPCR:Gαi pairs (Vellano et al., 2011). Taken together, these findings suggest that RGS14 may engage in both conventional and unconventional G protein signaling mechanisms in order to integrate G protein and MAP kinase signaling pathways underlying learning, memory, and synaptic transmission.

1.10 Objective of this dissertation.

Going into these studies, very little was known about RGS14 in the brain. While protein binding partners and some signaling functions of RGS14 had been described by our lab and others, nothing was known about RGS14's role in cell and organ physiology. Due to the limited expression of RGS14 in brain and white blood cells, I decided to examine the expression pattern of RGS14 protein and mRNA in the mouse brain during postnatal development through adulthood. Understanding the differential expression of RGS14 during developmental periods will provide us with essential clues about the functional role of RGS14 in brain physiology. My second objective was to follow-up on these findings to explore RGS14 functions in those brain regions where it is most highly expressed. I found that RGS14 was expressed almost exclusively in the CA2 region of the hippocampus. Because of the central role of the hippocampus in learning and memory, I examined the functional role of RGS14 in synaptic transmission, learning, and memory using a mouse model that lacked the RGS14 gene. My studies characterized the functional role RGS14 *in vivo*. By having a

better understanding of the signaling and function of this important signaling protein, we hope to better understand the pathways that regulate synaptic signaling and lead to learning and memory, and to perhaps lead to better treatments for neurological disorders in the future.

Chapter 2: Differential Expression of RGS14 during postnatal mouse brain development.

2.1 Introduction

A large number of GPCRs expressed in the brain mediate critical components of brain physiology. GPCRs in the brain play roles in addiction, fear, pleasure, reward, memory, and synaptic plasticity. Members of the regulators of G protein signaling family play important roles in cell and organism physiology. because they are responsible for modulating the signaling of G protein coupled receptors (GPCRs) by increasing the intrinsic rate of GTP hydrolysis of the Gα subunit. When GTP is hydrolyzed into GDP, Gα protein signaling is terminated. RGS proteins may indirectly limit Gβγ signaling by increasing the number of inactive Gα-GDP subunits available for interaction with Gβγ thereby facilitating formation of the heterotrimer.

RGS proteins vary in size and complexity. Some RGS proteins serve only as GAPs for Gα proteins. Other RGS proteins interact with multiple binding partners and act as scaffolds to integrate different signaling pathways. RGS proteins are classified by sequence homology within the RGS domain. Messenger RNA expression patterns for many different RGS family members in the brain have been examined revealing distinct patterns of expression in brain regions. Variations in RGS expression in concert with GPCR and Gβγ specificity indicate that RGS proteins participate in regulation of specific neurotransmitter systems. One RGS protein, RGS14, interacts with activated Gαi/o-GTP family members. In addition to the canonical RGS domain, RGS14 has tandem RBD domains that bind to active monomeric GTPases, H-Ras and Rap2A. An

additional functional domain, a G protein regulatory domain or GPR domain, binds tightly and specifically to inactive Gαi1/3 preventing activation of the Gα subunit and reformation of the Gαβγ heterotrimer. The capacity to bind to both active GαiGTP and inactive GαiGDP is an identifying characteristic of RGS14 and RGS12.

RGS14 mRNA has been found in the hippocampus and in lymphocytes (Cho et al., 2000a; Grafstein-Dunn et al., 2001; Traver et al., 2000a). Examination of RGS14 protein revealed that its expression in tissues is limited but is detected at high levels in rat brain and lymphocytes (Hollinger et al., 2001). Even though RGS14's functional role in these tissues is unclear, RGS14 can act as a scaffold binding G proteins and components of the MAP kinase signaling pathways that are known for their roles in synaptic plasticity and learning (Kennedy et al., 2005). Gαi/o, H-Ras, Rap-2, and Raf kinases mediate different aspects of synaptic plasticity within hippocampal neurons. In HeLa cellsRGS14 binds to H-Ras and Raf-1 to inhibit ERK-mediated MAP kinase signaling by PDGF (Shu et al., 2010; Willard et al., 2009). RGS14 is a cytosolic protein, but inactive Gαi and active H-Ras recruit it to the plasma membrane. The presence of RGA14, Gαi, and H-Ras in the same cells prevents RGS14 from inhibiting PDGF-induced ERK phosphorylation because RGS14 preferentially binds to Gαi instead of Raf-1 (Shu et al., 2010). Therefore, RGS14 may be link between Raf-ERK pathways and Gαi signaling pathways.

Because of the highly restricted nature of RGS14 mRNA expression in brain, we decided to characterize the expression of endogenous RGS14 protein

from early postnatal development to adulthood. The postnatal period is a highly dynamic time of synapse development and remodeling, neuronal expansion in the cerebellum, olfactory bulb, and hippocampus, growth of glial cell populations, and protein expression changes. The NR2B subunit of the NMDA receptor is gradually replaced with the NR2A subunit as synapses mature. Expression of adenylyl cyclase 1 and certain G proteins begins in the second postnatal week. Examining changes in the expression of RGS14 may reveal clues about the role of RGS14 in brain physiology. These experiments were performed using a new mouse monoclonal antibody, which we first characterized for sensitivity and specificity.

2.2 Materials and Methods

2.2.1 Plasmids and Cell lysates

RGS14-Flag, RGS2-HA, RGS4-HA, RGS16-HA, RGS10-Flag, all in pcDNA 3.1 plasmid vectors, were transiently transfected into HEK293 cells using Lipofectamine 2000 reagent (Invitrogen). RGS14-Flag truncation mutants were described by (Shu et al., 2007) Cells were cultured in DMEM supplemented with L-glutamine, 10% FBS, and 1% Pennicillin/streptomycin and grown in a humidified incubator 5% CO_2 at 37^{o}C. To prepare cell lysates, media was aspirated and cells were rinsed in PBS. Cells were scraped from the culture dish and centrifuged at low speed.

2.2.2 Antibodies

Anti-RGS14 mouse monoclonal was obtained from Neuromabs. and used at 1:1000 in blocking buffer (5% fat free milk in TBS-T) at 4^{o}C overnight. Anti-HA mouse monoclonal was obtained from (SigmaAldrich) and used 1:1000 in blocking buffer for 1 hr. at room temperature. Anti-GFP antibody was obtained from Invitrogen and diluted 1:1000 in blocking buffer for 1hr at room temperature. Anti-RGS10 goat antibody was a generous gift from Dr. Malu Tansey and was used at 1:200 in blocking buffer at 4^{o}C overnight. Anti-Flag-HRP antibody was obtained from (SigmaAldrich) and used at 1:25,000 in TBS-T for 1 hour at room temperature. All membranes were then washed 3 HRP conjugated secondary antibodies were all diluted in TBS-T and incubated for 1 hour at room temperature. were used as follows: goat anti-mouse 1:5000, goat anti-rabbit 1:25,000, rabbit anti-goat 1:3000.

2.2.3 Preparation of mouse brain lysates

Mice were deeply anesthetized using isoflurane, brain tissue harvested, and immediately flash frozen in liquid nitrogen then stored at -80^{o}C. To prepare lysates, brain tissue was weighed and resuspended in 25 mM Tris: 7.5 pH, 150 mM NaCl, 2 mM dithiothreitol, 1 µM phenylmethylsulfonyl fluoride. Tissue was homogenized using a glass dounce, and nuclei and cell debris cleared by low speed centrifugation.

2.2.4 Western Blot Analysis

Lysates were subject to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in Tris-buffered saline with 5% milk, 0.5% Tween 20, 0.02% sodium azide for 1 h at room temperature, and then incubated overnight with a specific anti-RGS14 mouse monoclonal antibody (1:2000). This antibody (N133/21) was generated in a collaborative effort with the NIH-sponsored Neuromab facility using purified full-length RGS14 as antigen as described (www.Neuromab.org). Membranes were washed with Tris-buffered saline + 0.1% Tween 20 then probed with horseradish peroxidase-conjugated goat anti-mouse secondary antibody in Tris-buffered saline + 0.1% Tween 20. The protein bands were visualized using chemiluminescence and exposure to X-ray film.

2.2.5 Immunohistochemistry:

Mice were deeply anesthetized; brains were fixed by perfusion with a 4% paraformaldehyde in PBS solution. Tissue was harvested and post-fixed with the same solution. Whole brains were embedded in paraffin, and 8 µm sections cut and mounted onto glass slides. Paraffin was removed from sections through incubation with xylenes, acetone and a series of graded alcohols. Antigen retrieval was accomplished by microwaving at high power in 1mM citrate monohydrate pH 6.0. Endogenous peroxidase activity was quenched with a 3% H_2O_2 in methanol incubation for 5 min at 40^{o}C. Sections were blocked with 5% normal goat serum and goat anti-mouse Fab fragment (1:250) (Jackson

ImmunoResearch) diluted in Tris-Saline Brij, pH7.5. Slides were incubated in primary anti-RGS14 monoclonal antibody (1:2000) (Neuromabs) overnight at 4^{o}C in a humidified chamber. Sections were washed and incubated in biotinylated goat anti-mouse secondary antibody (1:200) (Vector Labs BA-9200) followed by avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Labs) and developed with 3,3'-diaminobenzidine (DAB substrate kit, Vector Labs SK-4100). Nuclei were counterstained with hematoxylin (blue). For pre-adsorption of antibody, pure thyoredoxin-his-tagged RGS14 was incubated with anti-RGS14 antibody at a protein ratio of 10 to 1 for overnight at 4^{o}C.

2.2.6 Electron Microscopy (EM)

Mice were transcardially perfused with Ringer's solution followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer (0.1 M; pH 7.4). Tissue was cut, labeled using preembedding immunoperoxidase staining with RGS14 monoclonal antibody and prepared for EM as described (Mitrano et al., 2008). Sections were examined on the Zeiss EM-10C electron microscope. Electron micrographs were taken and saved with a CCD camera (DualView 300W, Gatan, Pleasanton, CA) controlled by Digital Micrograph software (version 3.10.1, Gatan).

2.3 Results

2.3.1 RGS14 Monoclonal Antibody Characterization

In collaboration with Neuromab (UC Davis), we generated a mouse monoclonal antibody to rat RGS14. To determine the epitope recognized by this antibody, we used Flag-tagged truncation mutants of RGS14. Each mutant expressed one functional domain of RGS14 as well as some linker region. The RGS domain was contained in the first mutant, which included the N-terminus to 220 amino acids. The second mutant contained amino acids 220 to 490, which included the RBD domains. The final mutant contained amino acids 440 to the carboxy-terminus and included the GPR motif. The truncation mutants were transfected into HeLa cells and were detected by immunoblotting with a Flag antibody. The RGS14 monoclonal antibody did not detect the mutant containing the first 220 amino acids and the RGS domain but strongly detected the mutant that included amino acids 220 to 490, the RBD domains (Figure 2.1). Additionally, the third mutant was detected at a low level. Since the second and third truncation mutants overlap by 40 amino acids, the RGS14 antibody appears to be specific for the region between the second RBD domain and the GPR domain.

To test the sensitivity of the RGS14 monoclonal antibody, a dilution curve with purified RGS14 protein was performed (Figure 2.2). The RGS-14 monoclonal antibody can detect pure, thioredoxin tagged RSG14 at

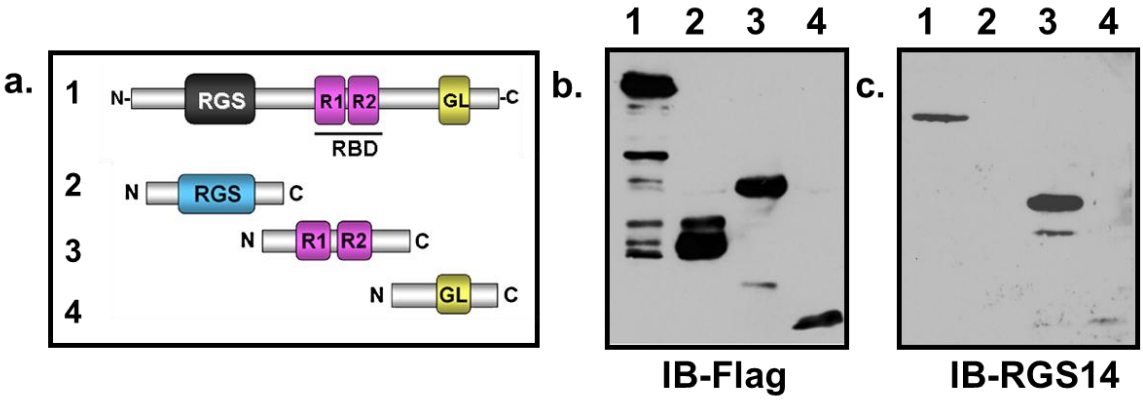


Figure 2.1 anti-RGS14 antibody epitope. (a) Flag-tagged RGS14 functional domains and truncation mutants 1. Full-length RGS14, 2. Amino acids 1-220, including the RGS domain, 3. Amino acids 220-490, including the RBD domains, 4. Amino acids 440-to carboxy terminus, including the GoLoco domain. (b) Immunoblot analysis of HeLa cells with transiently transfected constructs using anti-Flag antibody to show each RGS14 construct is expressed. (c) Immunoblot analysis with the anti-RGS14 monoclonal antibody shows the antibody recognizes Full-length RGS14, the RBD construct, and a faintly recognizes the GoLoco construct.

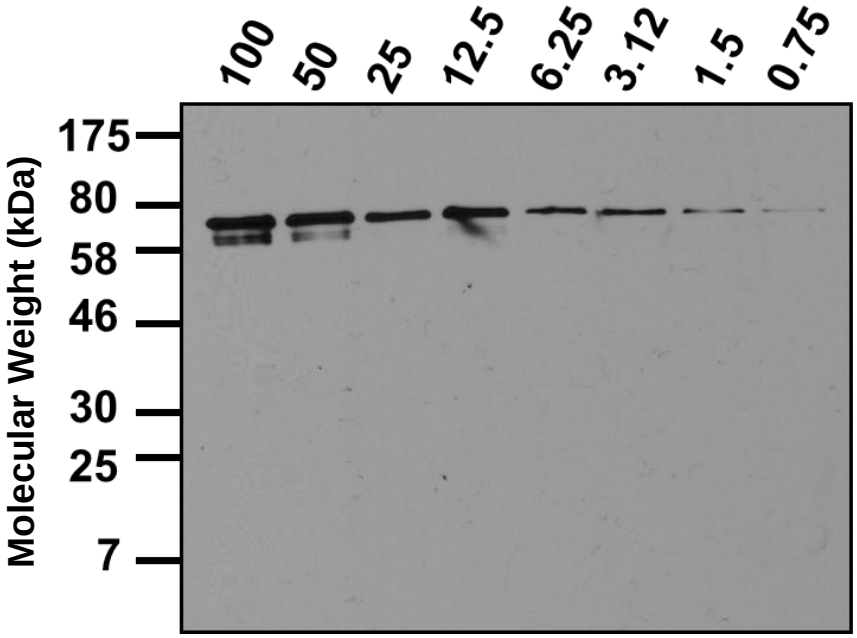


Figure 2.2 Dilution curve using RGS14-trx. Serial dilutions of pure RGS14 tagged with thioredoxin. Concentrations are indicated 100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng, 1.5 ng, and 0.75 ng. Immunoblot analysis using the RGS14 monoclonal antibody.

concentrations as low as 0.75 ng. Serial dilutions of the pure protein show that the monoclonal is highly sensitive to very low quantities of RGS14.

2.3.2 RGS14 antibody is specific for RGS14 protein.

The RGS14 monoclonal antibody is specific for RGS14 and does not detect other RGS proteins (Figure 2.3). HEK293 Cells were transiently transfected with RGS2, RGS4, RGS16, RGS10, RGS12, RGS14 or empty plasmid vector. Each RGS protein was detected with the antibody for its respective tag; however while the RGS14 monoclonal antibody strongly detected recombinant and endogenous RGS14 from mouse brain lysates, it did not detect any of the other RGS proteins.

2.3.3 RGS14 antibody shows faint staining in RGS14-KO mice

The RGS14 monoclonal antibody shows residual staining in RGS14-KO mice (Figure 2.4). DAB staining appears in the CA2 region of the hippocampus and is greatly reduced compared to staining intensity in WT mouse brain. WT mouse brain shows light staining in cortical regions including the olfactory cortex. There appears to be no RGS14-KO staining outside the CA2 region of the hippocampus. Western blot analysis of WT and RGS14-KO mouse brain lysates shows the loss of full-length RGS14; however, there appears to be a faint smaller

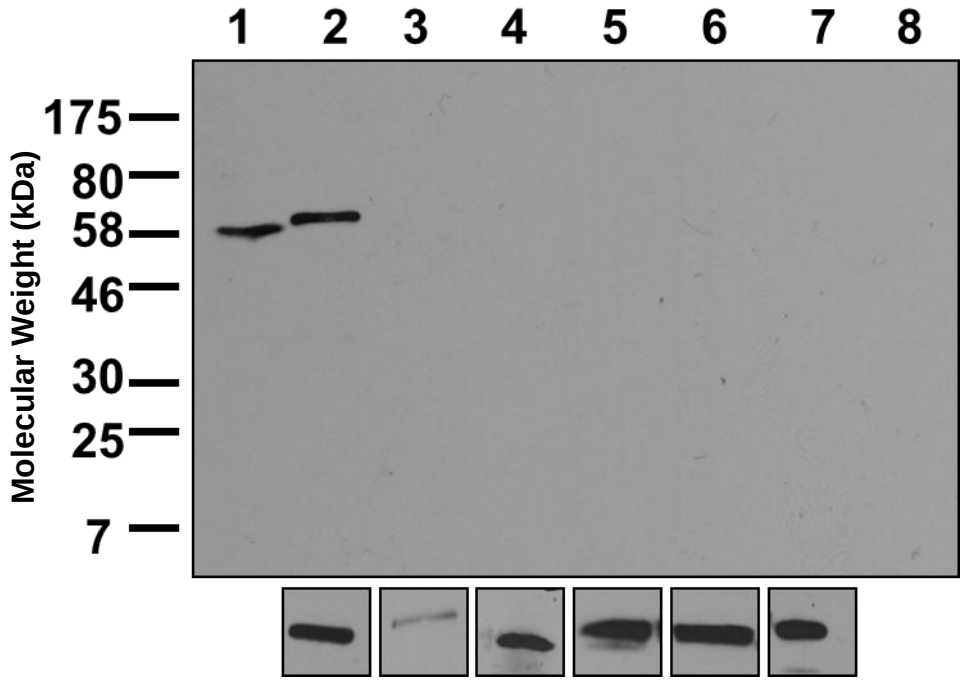


Figure 2.3 RGS14 monoclonal antibody is specific for RGS14. Lane 1 Mouse Brain Lysates, Lane 2 Flag-Rat RGS14, Lane 3 GFP-RGS12 TS, Lane 4 RGS10, Lane 5 RGS2-HA, Lane 6 RGS4-HA, Lane 7 RGS16-HA, Lane 8 pcDNA 3.1 The Upper panel shows immunoblot analysis using the RGS14-monoclonal antibody. The lower panel shows immunoblot analysis using antibodies specific for each protein or tag. Lane 2 anti-Flag, Lane 3 anti-GFP, Lane 4 anti-RGS10, Lanes 5, 6, and 7 anti-HA.

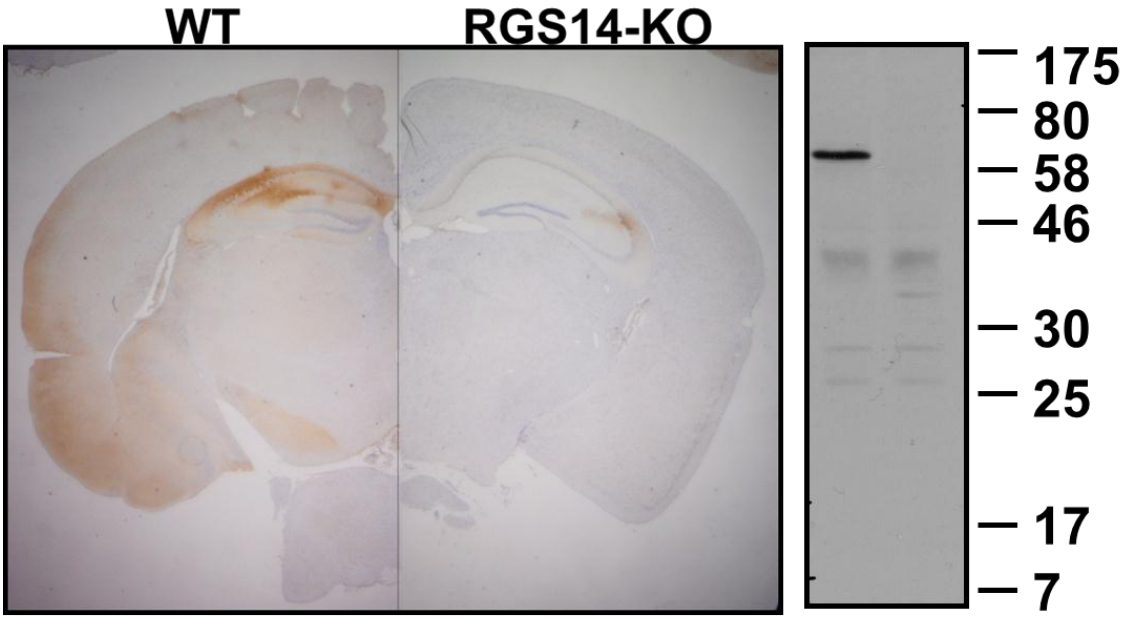


Figure 2.4 Expression of RGS14 in WT and RGS14-KO mouse brain (Left panel). Fixed paraffin-embedded mouse brain sections stained with anti-RGS14 monoclonal antibody (Brown; DAB staining with specific anti-RGS14 monoclonal antibody) and counterstained with hematoxylin (Blue). (Right panel) Protein immunoblot for RGS14 protein using a specific anti-RGS14 monoclonal antibody. Lane 1 WT RGS14 (+/+) brain lysates, Lane 2 RGS14-KO brain lysates.

band of approximately 27 KDa that does not correspond to any band in the WT brain lysates.

2.3.4 Temporal Expression of RGS14 during postnatal brain development

Western blot analysis detected changes in the temporal expression of RGS14 protein during postnatal development (Figure 2.5). RGS14 protein was not detectible in brain lysates from newborn mice at postnatal day 0 (P0) or P3 mice. A faint band at 60 kDa appeared at P5 and increased in intensity from P7, P10, P14, to P21. Expression in adult mice aged 4, 8, and 12 months revealed unchanged levels of the expression of RGS14 protein.

Quantitative RT-PCR experiments were performed to probe for mRNA expression of RGS14 during postnatal development. Expression of mRNA may be transient and is not always reflected in detectible levels of immunoreactivity in immunoblot analysis. However, the pattern of RGS14 mRNA expression was similar to the expression found in protein levels (Data not shown). No RGS14 message was detected in newborn mice.

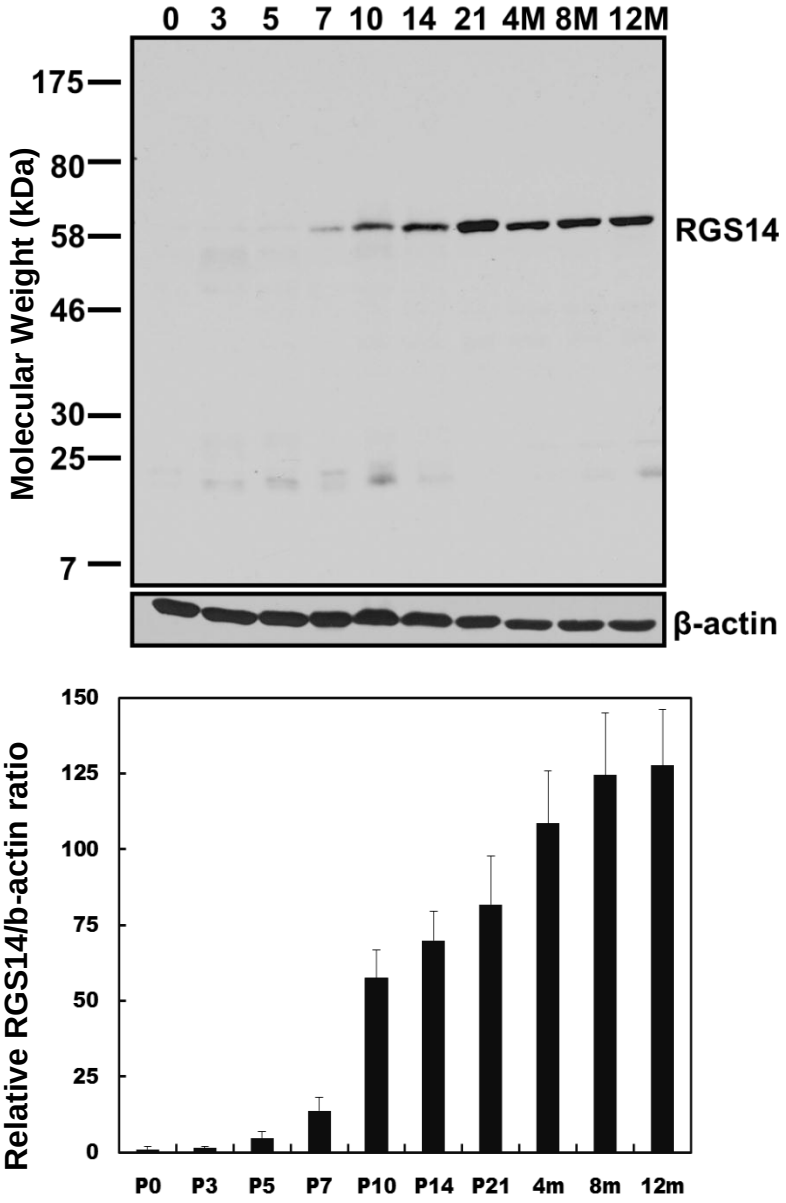


Figure 2.5 Temporal expression of RGS14 in mouse brain. Mouse brain lysates from pups at 0, 3, 5, 7, 10, 14, and 21 days of age, adult mouse brain lysates at 4, 8, and 12 months of age. Immunoblot with anti-RGS14 monoclonal antibody. Center panel shows immunoblot with anti-β-actin as a loading control. (n=3-5 for each age). Lower panel shows relative RGS14/ β-actin densitometry ratios.

2.3.5 Regional RGS14 expression in the developing mouse brain.

We next examined expression of RGS14 by immunohistochemistry to determine protein expression patterns during postnatal mouse brain development. At P0, no RGS14 staining is apparent in any brain region including cortex and hippocampus. By P7, a very faint DAB signal can be seen in CA2 of the hippocampus, but no other region appears positive for RGS14 expression. However, by two weeks of age expression of RGS14 appears abundant and widespread. RGS14 protein staining is apparent in the visual and olfactory cortex. The hippocampus shows expression of RGS14 at high levels in the CA2 and in the dentate gyrus. Additionally staining is apparent in the cerebellum in cells morphologically consistent with purkinje neurons, however we cannot rule out the possibility that these cells are Bergmann glia.

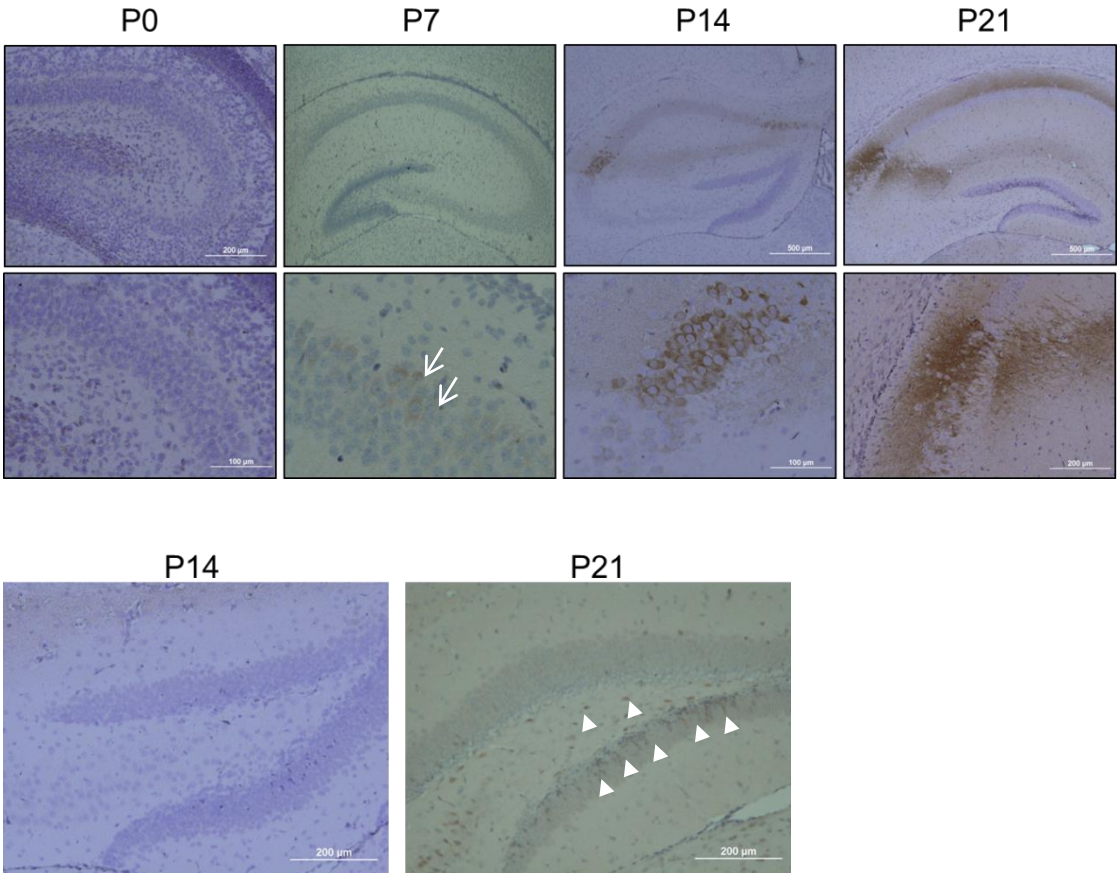


Figure 2.6 Expression of RGS14 in the postnatal hippocampus. Immunohistochemical staining of RGS14 protein in mouse brain using RGS14 monoclonal antibody DAB immunoperoxidase staining with hematoxylin blue counterstain. RGS14 immunoreactivity is not evident in newborn mice, Postnatal day 0 (P0), by P7 light DAB (Brown) immunoreactivity is apparent, white arrows, in the CA2 of the hippocampus. By P14, DAB staining intensity has increased and at P21 staining has reached peak levels. Bottom panel shows expression of RGS14 (white arrow heads) in the Dentate Gyrus at P14 and P21.

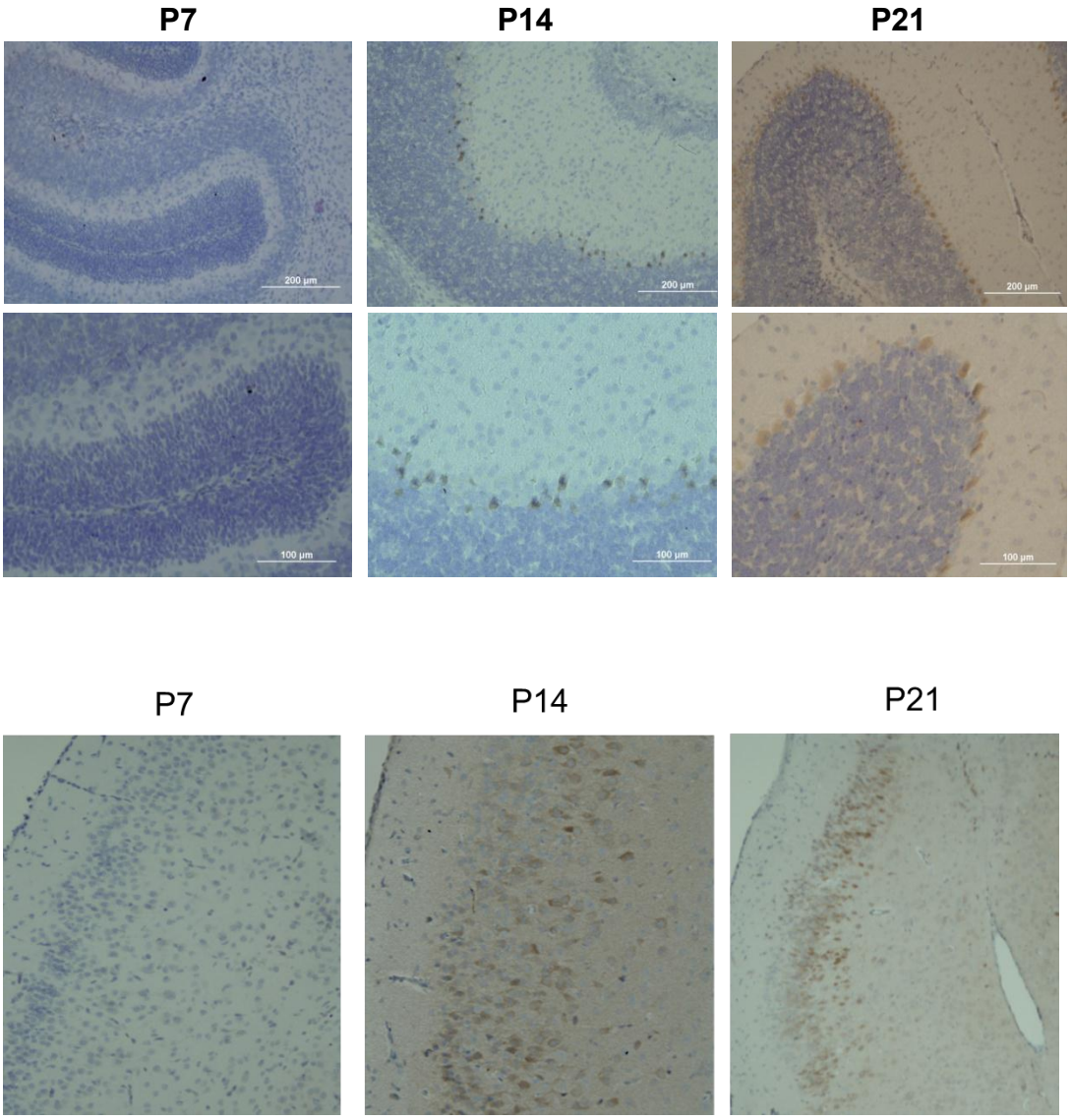


Figure 2.7 Expression of RGS14 in the Cerebellum Top and center panels show DAB staining of RGS14 in the cerebellum at P14 and P21 in cells that resemble Purkinje neurons. Expression of RGS14 in the Olfactory Cortex Bottom panels show DAB staining of RGS14 in the olfactory cortex of mouse brain. Brown staining of RGS14 is apparent at P14 and P21.

2.3.6 RGS14 Protein Expression in Adult Mouse Brain

RGS14 protein is expressed most prominently in brain and exclusively within neurons (Hollinger et al., 2001). In order to understand the role of RGS14 in brain function, we determined its protein distribution pattern in mouse brain sections using a specific anti-RGS14 monoclonal antibody. We observed that RGS14 exhibits a striking expression pattern with strong immunohistochemical staining that is mostly limited to the CA2 sub-region of the hippocampus (Figure 2.9 a and 2.9 b); light staining is also seen in the olfactory cortex. This protein staining pattern very closely matches the mRNA distribution pattern of RGS14 in mouse brain (http://mouse.brain-map.org; Figure 2.10). The staining is completely eliminated by pre-adsorption of antibody with purified RGS14 protein (Figure 2.9 c). Within the hippocampus, strong immunoreactivity is evident in soma and neurites of CA2 pyramidal neurons that appear to project through the stratum lacunosum-moleculare and stratum radiatum to the fasciola cinerea (FC) (Figure 2.9 b and 2.9 d). Within the FC, strong staining is evident in pyramidal neurons in soma and neurites (Figure 2.9 f). With this finding, RGS14 joins a discrete fraternity of signaling proteins that share this highly restricted expression pattern in both CA2 and FC neurons (Lein et al., 2005b), suggesting a possible functional link between these proteins and these two poorly understood hippocampal regions. Staining is also observed in soma and neurites of sporadic, unidentified neurons within the CA1 pyramidal cell layer, which we speculate to be either inhibitory interneurons or a distinct subset of pyramidal neurons (Figure 2.9 e). At the electron microscopic (EM) level, RGS14 labeling

in CA2 hippocampal neurons is predominantly found in dendritic shafts (Figure 2.9 g), as well as necks or heads of dendritic spines (Figure 2.9 h,i), including an apparent enrichment at some post-synaptic densities (PSD) (Figure 2.9 i). This conspicuous distribution pattern of RGS14 within the hippocampus highlights the divergent molecular nature and anatomical circuitry of neurons in the CA2, and suggests that CA2 functions may be distinct from those of the neighboring CA1 and CA3 regions. The distribution pattern of RGS14 within the hippocampus (Table 2.1) highlights the divergent molecular nature and anatomical circuitry of neurons in the CA2 and suggests that CA2 functions may be distinct from those of the neighboring CA1 and CA3 regions.

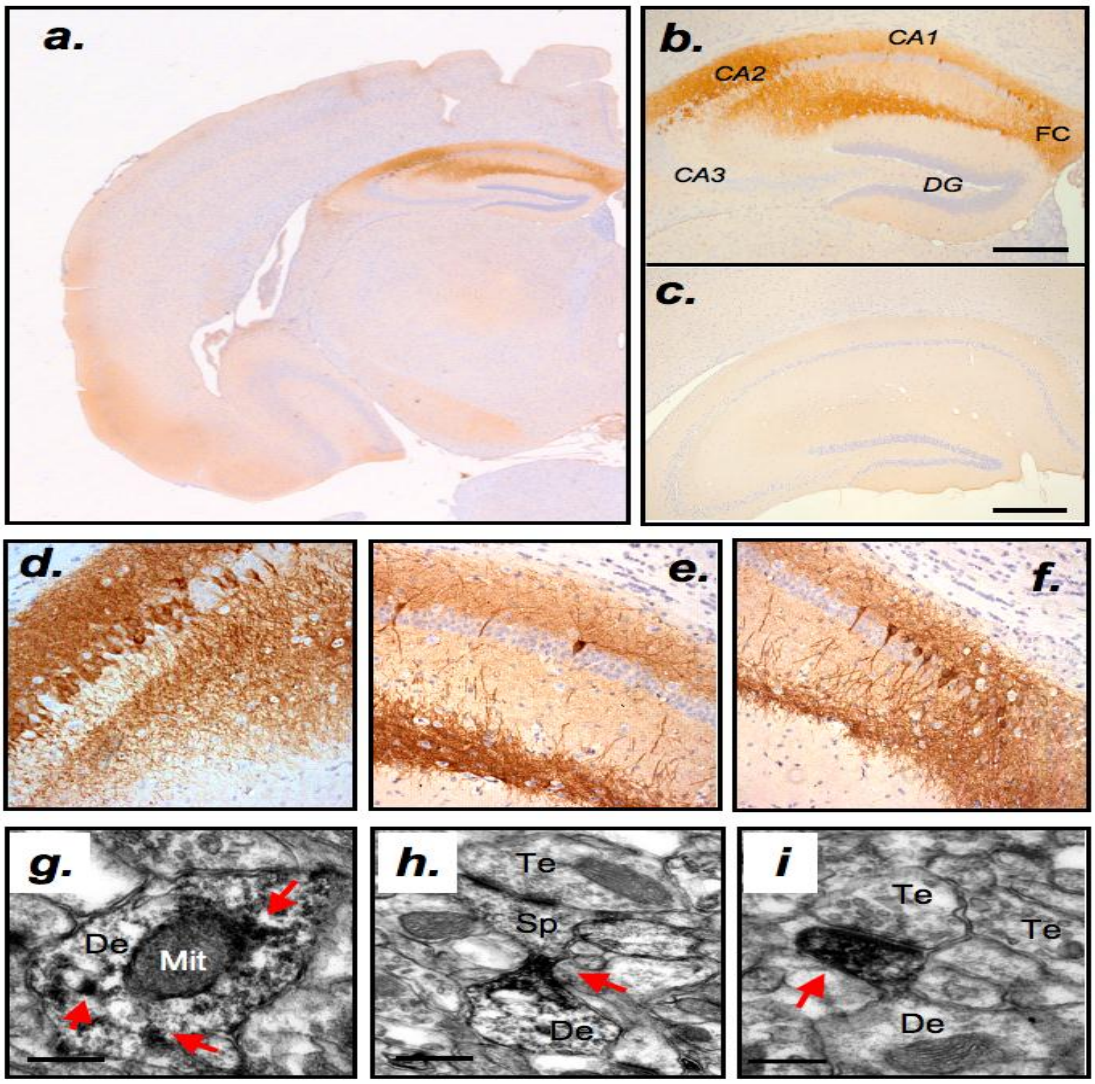


Figure 2.8: RGS14 is enriched in hippocampal CA2 neurons. (a,b) Fixed paraffin-embedded mouse brain sections stained with anti-RGS14 monoclonal antibody (Brown; DAB staining with specific anti-RGS14 monoclonal antibody) and counterstained with hematoxylin (Blue). (c) Elimination of RGS14 staining by pre-adsorption of antibody with purified recombinant RGS14 protein; scale bar represents 400 µm. (d) RGS14 expression in CA2 neurons, (e) in a discrete subset of CA1 neurons, and (f) in neurons within the fasciola cinerea (FC).

Electron micrographs of RGS14-immunoreactive (red arrows) (g) dendritic shaft (de), (h) spine (sp) neck, and (i) spine head in the stratum oriens region of mouse CA2 hippocampus; scale bar represents 0.2 µm (DAB staining with RGS14 antibody). Other recognized structures are mitochondria (mit).

	P0	P7	P14	P21	4M
Hippocampus CA1	-	-	+	+	+
Hippocampus CA2	-	+	++	+++	++++
Hippocampus CA3	-	-	-	-	-
Hippocampus DG	-	-	-	+	-
Fasciola cinerea	-	-	+	++	++++
Visual Cortex	-	-	+	+	-
Olfactory Cortex	-	+	++	++	+
Cerebellum	-	-	+	+	-

Table 2.1 Summary of relative expression of RGS14 protein in brain different brain regions each week from Postnatal day 0 to 4 months or age. Highest expression was observed on P21 in the CA2 of the hippocampus. +++ Very high concentration of DAB staining, ++ moderate DAB staining, + light DAB staining, - no DAB staining.

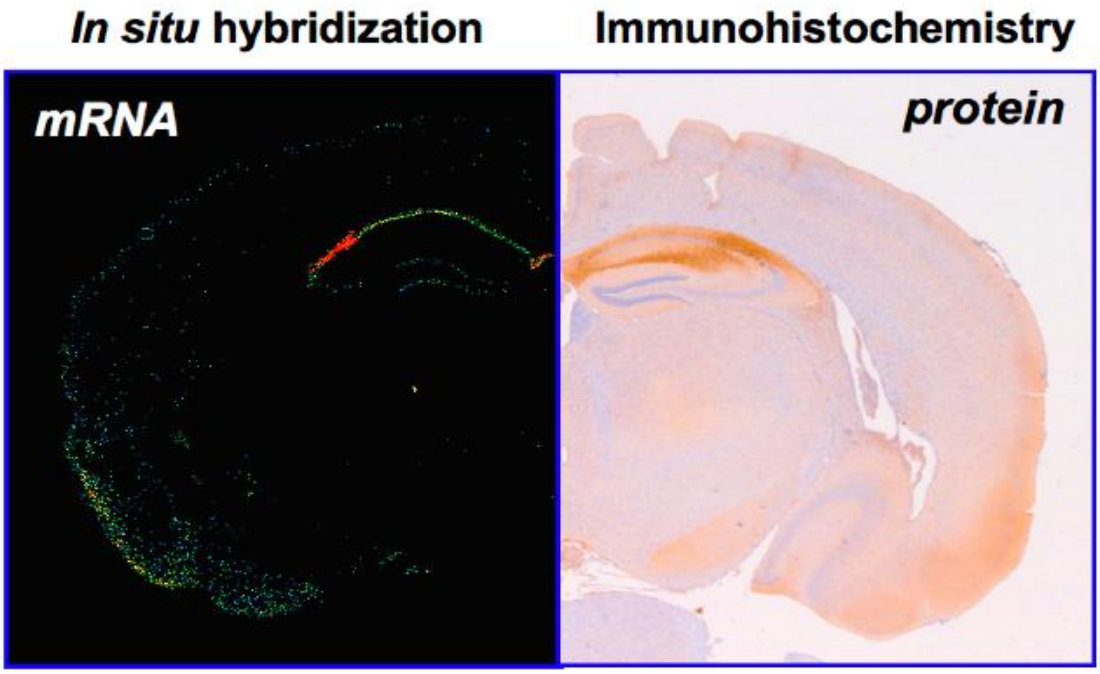


Figure 2.10: RGS14 mRNA and protein expression and distribution in mouse brain. (a) *In situ hybridization* showing expression of RGS14 mRNA in mouse brain. Allen Mouse Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Available from: http://mouse.brain-map.org. (b) Mouse brain sections stained with anti-RGS14 monoclonal antibody (Brown; DAB staining) and counterstained with hematoxylin (Blue).

2.4 Discussion

Previous studies show that RGS14 is found at high levels in rodent brain with discrete expression in other tissues such as thymus and spleen (Hollinger et al., 2001). To further characterize the expression of native RGS14, a new mouse monoclonal antibody was generated and characterized to determine protein expression of endogenous RGS14. The RGS14 monoclonal antibody is specific for RGS14, and does not recognize other RGS proteins including RGS14's closest relatives RGS10 and RGS12. This new RGS14 monoclonal antibody is sensitive in that it can detect concentrations as low as 0.75 ng of purified RGS14 protein.

Within rodent brain, RGS14 is expressed at high levels in neurons of the hippocampal CA2 subregion and appears to be enriched in dendrites and spines, with much less protein present in cell bodies and synaptic terminals (Figure 2.9) (Lee et al., 2010). These findings suggest that RGS14 is trafficked within its host neurons to regulate postsynaptic signaling events.

Pyramidal cells of the CA subfields are primarily formed before birth during embryonic days 10 to 18 in the mouse and begin to express some region specific markers by day 15.5. Formation of the granule cells in the dentate gyrus also begins around embryonic day 10, but granule cell development persists into the postnatal period and may continue into adulthood. The postnatal period is highly dynamic in terms of dendritic spine formation and elimination. Since the neonatal period in rodents is analogous to the third trimester of human gestation and is a

period of critical importance for brain development, we wanted to examine the expression of RGS14 during this period. Previous studies detected RGS14 mRNA during early embryonic development but did not examine the postnatal period (Martin-McCaffrey et al., 2004). We found that RGS14 protein was not detectable in newly born mouse brain, but, of note, very low level expression of RGS14 mRNA was detected by qRT-PCR (Paul Evans, Hepler lab, data not shown). RGS14 mRNA increased in quantity up through adulthood, and RGS14 protein was clearly detectable by 7 days following parturition. It is likely that little or no RGS14 protein is expressed during the first week of life based on the level of detection of mRNA though we cannot rule out that the protein is present in amounts below detectable levels for immunoblot analysis. Expression of RGS14 mRNA and protein levels mirror one another as they increase during postnatal development. This period is a highly plastic period in which synapses are being formed and pruned. Increasing expression levels of RGS14 suggest that perhaps it plays a role in neurons as the synapses mature. RGS14 protein first appears in the CA2, which is not surprising given its high level expression there in adult mouse brain. In P21 mouse brain, RGS14 protein can be detected in the cerebellum in cells that appear to be Purkinje neurons. Purkinje cells are inhibitory neurons that are the main output of the cerebellum and are characterized by their propensity to undergo long-term depression of neurotransmission. LTD acts as the functional opposite of LTP. LTD in the cerebellum is proposed to be an underlying mechanism of motor learning, but others suggest it may be a protective mechanism to reduce calcium-mediated

excitotoxicity (Llinas et al., 1997). Expression of RGS14 here suggests that it may be involved in dampening or reducing synaptic activity during the postnatal period. Given the synaptic stability of the CA2 and the loss of this stability with the concurrent loss of RGS14, it would be interesting to test for differences in cerebellar LTD in RGS14–KO mice. RGS14-KO mice had normal locomotor activity but were not tested for motor memory using associative eyelid conditioning or adaptation of the vestibulo-ocular reflex. Juvenile RGS14-KO mice may exhibit some differences in cerebellar function.

Chapter 3: RGS14 is a natural suppressor of Learning, Memory, and Synaptic Plasticity.

3.1 Introduction

Many aspects of brain plasticity, including those associated with learning and memory, are thought to be mediated by long-term potentiation (LTP) of excitatory glutamatergic synaptic transmission (Neves et al., 2008). Plasticity at synapses of the DG-CA3-CA1 circuit within the hippocampus, in particular, plays a key role in acquisition and consolidation of certain forms of learning and memory (Nakazawa et al., 2003; Rolls and Kesner, 2006). Absent from this canonical DG-CA3-CA1 circuit is the CA2 region, which differs from these regions by its distinct anatomy, pattern of gene expression, and electrophysiological characteristics (Ishizuka et al., 1995; Lein et al., 2005b; Simons et al., 2009; Woodhams et al., 1993; Zhao et al., 2007). CA2 pyramidal neurons also are uniquely resistant to injury in several animal models of epilepsy and hypoxia but exhibit a unique pathology in brains from schizophrenic patients (Benes et al., 1998; Kirino, 1982; Sadowski et al., 1999). Unexplained, however, is the fact that protocols typically effective at inducing LTP in hippocampal CA1/CA3 subfields are largely ineffective in CA2 neurons at Schaffer collateral synapses (Zhao et al., 2007).

Cellular signaling underlying LTP and synaptic plasticity includes key mechanistic roles for calcium, Ras/MAP kinases and G protein signaling pathways (Kennedy et al., 2005). The regulators of G protein signaling (RGS) proteins are a structurally diverse family of greater than 30 member proteins enriched in brain that typically limit neurotransmitter receptor- and G protein-linked signaling in the CNS and elsewhere by serving as GTPase activating

proteins (GAPs) (Hollinger and Hepler, 2002a; Ross and Wilkie, 2000b). One particular family member, RGS14, is uniquely situated to regulate signaling pathways involved with synaptic plasticity. Originally identified as a binding partner for Rap GTPases (Snow et al., 1997a; Traver et al., 2000a), RGS14 has binding domains for multiple signaling proteins including active Gαi/o-GTP (RGS domain), Ras/Rap GTPases (tandem RBD domains) and for inactive Gαi1-GDP and Gαi3-GDP (a single GPR motif) (Cho et al., 2000a; Hollinger et al., 2001; Kimple et al., 2001; Snow et al., 1997a; Traver et al., 2000a; Traver et al., 2004). Most recently, we have shown that RGS14 can act as a scaffold to assemble Gαi, H-Ras, and Raf kinases, in turn, to integrate G protein and ERK/MAPK signaling pathways and inhibit growth factor receptor signaling (Shu et al., 2010). Here we have tested the idea that RGS14 serves to regulate synaptic plasticity in the brain, perhaps with a resulting role in learning and memory.

3.2 Materials and Methods

3.2.1 Generation, genotyping, and RT-PCR of RGS14-KO mutant mice

Mice lacking RGS14 (RGS14tm1-lex) were generated by Lexicon Genetics through the NIH-sponsored Mutant Mouse Regional Resource Center: http://www.informatics.jax.org/searches/accession_report.cgi?id=MGI:3528963. Embryos were implanted into C57/BL6 females, and founder mice crossed with C57/BL6 to establish the novel knock-out line (RGS14-KO). Genotypes were determined by PCR of genomic DNA from tail biopsies. Wild type (WT) forward

primer was 5’ cagcgcatcgccttctatc 3’. Primer for the targeting vector was 5’ gcagcgcatcgccttctatc 3’ with a shared reverse primer (5’ agactggcagaagaattcagg 3’). PCR reactions were performed with Platinum Taq (Invitrogen) under the following amplification conditions: 94^{o} C for 3 min, and 30 cycles of 94^{o}C for 30 sec, 64^{o}C for 30 sec, 72^{o}C for 45 sec and completing with 72^{o}C for 1.5 min. For reverse transcription-PCR (RT-PCR), mRNA from brain tissue of WT and RGS14-KO animals was isolated using Invitrogen PureLink Kit with forward (#1: 5’ caaatccccgctgtaccaagagtg 3’ #3: 5’ acttgggtgtccccaacgggc 3’), and reverse (#2: 5’ ggaagccgtgccgtcaggtagata 3’ #4: 5’ gaacatatctggccggggctgg 3’) primers.

3.2.3 Hippocampal Slice Preparation

Hippocampal slices were prepared from RGS14-KO and WT mice (CA2: postnatal day 14 – 18, CA1: 8-12 weeks) as described (Zhao et al., 2007). Animals were decapitated, and the brains rapidly removed. Coronal brain slices (340-400 μm thick) containing the hippocampus were cut using a vibrating blade microtome in aerated, ice-cold artificial cerebral spinal fluid (ACSF). Freshly cut slices were allowed to recover for at least 1 h in ACSF at room temperature and were then transferred to a recording chamber in which they were bathed continuously with room temperature ACSF.

3.2.4 Whole-cell patch recordings

Recordings were made from individual CA2 neurons due to the difficulty in localizing CA2 dendrites, which makes assessment of field potentials unreliable. Recordings of CA2 neurons were made with patch pipettes (4–6 MΩ) only when

the CA2 could be visually distinguished from neighboring regions CA1 and CA3. Cluster type stimulating electrodes (FHC, Bowdoinham, ME) were placed in the Stratum Radiatum approximately 150 µm from the patched neuron to stimulate the Schaffer collateral axons and measure excitatory postsynaptic currents (EPSCs)(English and Sweatt, 1997). Neuronal excitability was examined by recording the response of each neuron to depolarizing current steps in current clamp mode (0.1 - 0.6 µA), measurement of input resistance, and by recording action potentials in response to 1s of 100 Hz (LTP-inducing) synaptic stimulation. Action potential threshold was identified using previously published mathematical methods(Henze et al., 2000). Electrically-evoked synaptic responses were recorded at -70 and +40mV in ACSF containing 1mM gabazine for measurement of NMDA and AMPA type synaptic currents(Myme et al., 2003).

3.2.5 Field potential recordings

Extracellular field potential recordings in the stratum radiatum area of CA1 were recorded in response to Schafer collateral inputs. Population excitatory postsynaptic potential (EPSP) output was measured in response to varying input currents to determine baseline synaptic transmission. For induction of LTP, WT and RGS14-KO hippocampal slices were prepared and maintained as described above. Slices were then subjected to stimulation (2 X 1 sec, 100 Hz, 20 sec intervals) to induce LTP and post-synaptic neurotransmission was monitored every 15 sec for 180 min. Data presented are pooled mean +/- standard deviation (SD).

3.2.6 Novel-object recognition

The object recognition apparatus consisted of an open box (44×44×8 cm) made of white PVC placed in a sound-isolated testing room. Four objects (approximately 7 cm height and 6 cm diameter) made of a combination of plastic, metal, and rubber were employed in this task. The weight of the objects ensured that they could not be displaced by mice. Training and testing sessions were recorded with a video camera mounted over the training arena and analyzed using LimeLight video-tracking software (Coulbourn, Whitehall, PA). Novel-object recognition tests were carried out as described (Heldt et al., 2007). Time spent exploring and number of contacts for each object were expressed as percentages of total time or number of contacts.

3.2.7 Morris water maze

Adult RGS14-KO and WT littermates age 2-6 months were used. The water maze consisted of a circular swim arena (diameter of 116 cm, height of 75 cm) surrounded by extra-maze visual cues that remained in the same position for the duration of training. Water at 22^{o}C filled the maze to cover the platform by 1cm and was made opaque with non-toxic, white tempera paint. The escape platform was a circular, non-skid surface (area 127 cm^{2}) placed in the NW quadrant of the maze. Acquisition training consisted of five test days with four daily trials. Mice entered the maze facing the wall and began each trial at a different entry point in a semi-random order. Trials lasted 60 seconds or until the animal mounted the platform with a 15 minute inter-trial interval. A probe trial

was conducted on day six wherein the platform was removed, and the animal swam for 60 seconds, and the time spent in the target quadrant (NW) versus the adjacent and opposite quadrants was recorded. A video camera mounted above the swim arena and linked to TopScan software recorded swim distance, swim speed, time to platform and was used for tracking and analysis. Statistics were ANOVA and *post hoc* Dunnett's test unless otherwise stated.

3.2.8 Locomotor Activity

Baseline motor activity was measured by examining the total ambulatory distance (in cm) during the 20-min open field test session. Activity for each mouse was measured using individual activity chambers constructed from clear polycarbonate and equipped with four 24-beam infrared arrays across the base of each chamber wall (MED Associates, Model, OFA-MS). Activities measured include distance traveled, ambulatory counts, stereotypy counts, vertical counts, jumping activity, average velocity of movement for ambulatory episodes. Data was collected via computer and was analyzed with the MED Associates' Activity Monitor Data Analysis software.

3.2.9 Startle Response

Each system consisted of a nonrestrictive Plexiglas cylinder, 5.5 cm in diameter and 13 cm long, mounted on a Plexiglas platform which was located in a ventilated, sound-attenuated chamber. Cylinder movements were sampled each millisecond (ms) by a piezoelectric accelerometer mounted under each platform. Startle amplitude was defined as the peak accelerometer voltage that

occurred during the first 100 ms after the onset of the startle stimulus. The output sensitivity of all response systems was calibrated to be nearly identical (SR-LAB Startle Calibration System). Startle, prepulse and background stimuli were presented through a high-frequency speaker located 15 cm above the startle chambers. Stimuli intensities were measured by a sound level meter (Radio Shack, #33-2055) directed inside of the cylinder. Stimuli presentation and data acquisition were controlled by an IBM PC-compatible computer using SR-Lab software. Acclimation: For three consecutive days, each mouse was placed in the cylinder for approximately 15 min during which time no stimuli were presented. The purpose of this acclimation procedure was to familiarize the mice to handling and the startle apparatus. Startle: On the fourth day, mice were placed in the cylinder and after 5 min were given 10 startle stimuli at each of four different startle stimulus intensities (90, 100, 110, 120 dB) with an interstimulus interval (ISI) of 30 s. All startle stimuli were presented in a pseudorandom sequence with the constraint that each stimulus intensity occur only once in each consecutive four-trial block. Mean startle amplitudes were calculated for each mouse by computing the average startle response at each of four different startle stimulus intensities (90, 100, 110, 120 dB). PPI test sessions: Each PPI test session consisted of five different trial types. Startle stimuli (115 dB, 50 ms) were presented alone or were preceded by noise prepulses (20 ms) of 2, 4, 8, 10, or 12 dB above a 63dB white noise background (i.e. 65, 67, 71, 73, or 75 dB) with a fixed interval (100 ms) between onsets of the prepulse and startle stimuli. The session began with a 5-min acclimation period followed by the five different trial

types presented in random order nine times for a total of 45 trials. Intertrial intervals ranged from 20 to 40 s. Mean startle amplitudes for the startle-alone trials and each of the 5 prepulse+startle trials were calculated for each mouse by averaging the startle amplitude of each trial type. Each mean prepulse+startle amplitude score was converted to a percent PPI. The percent PPI was obtained as follows; Percent PPI = 100 x (mean startle-alone amplitude - mean prepulse+startle amplitude)/(mean startle-alone amplitude).

3.2.10 Elevated-Plus Maze Test

During each 5-min test session, the animal is placed at the far end of an enclosed arm, and the timer started. Each closed arm entry and open arm entry, total arm entries and time in open arms were recorded. An entry (or exit) is defined as the animal having all four paws inside or outside an arm. To record time spent in open arms, a stopwatch was started with each open arm entry and was paused when the mouse left the open arm. At the end the session, the total number of entries (open+closed), the percent of open arm entries (open/total) and percent time in open arms (open arms time in seconds/300s) were tabulated.

3.3 Results

3.3.1 Generation of an RGS14 Knock-out Mouse Model

To determine RGS14 functions in brain and hippocampal physiology, we characterized a novel line of knockout mice lacking full-length RGS14 (RGS14-KO) generated by inserting a LacZ/Neo cassette that deletes exons 2-7 of the RGS14 gene (http://www.informatics.jax.org) (Figure 3.1). As shown in Figure 3.2, PCR analysis of genomic DNA shows loss of the wild type (WT) gene in RGS14-KO mice and the presence of the targeting vector (Figure 3.2a, top). RT-PCR analysis of mRNA shows loss of RGS14 message (Figure 3.2a, bottom). Immunoblot analysis of mouse brain lysates shows RGS14 as a single band of expected molecular weight (60 kDa) in WT brain, and no corresponding band in RGS14-KO brain lysates (Figure 3.2b), indicating complete loss of full-length protein. RGS14-KO mice appear healthy with no obvious differences in their growth, fertility or any apparent physiological phenotype compared to their wild type and heterozygous littermates.

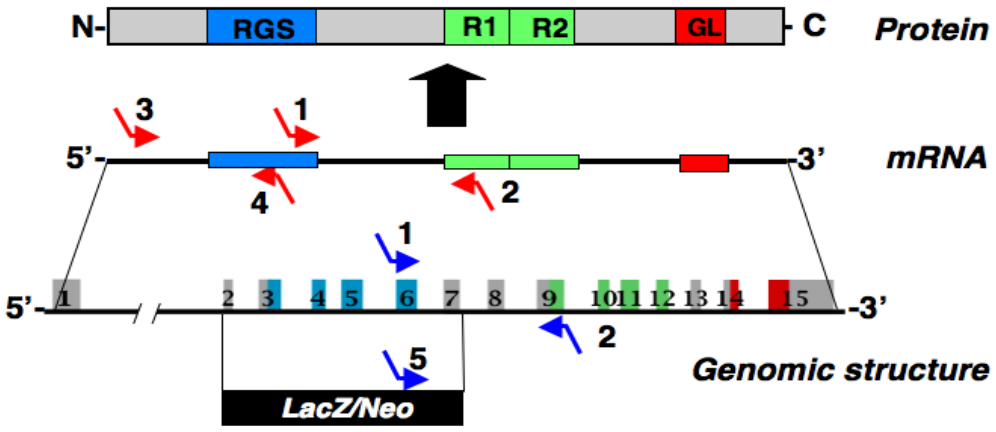


Figure 3.1 Deletion of the RGS14 gene and protein in mice. Schematic of RGS14 gene and mRNA including protein domain structures. Red arrows indicate the location of primers used in RT-PCR for mRNA. Center panel shows the structure of the genomic DNA intron and exon arrangement indicating targeting vector location and insertion site for the lacZ/neo cassette replacing exons 2 through 7. Blue arrows indicate location of primers used for PCR genotyping. Oligo pairs used for PCR reaction are shown in above as blue arrows 1, 2, and 5. Oligo pairs used on cDNA derived from mRNA are shown above as red arrows 1, 2, 3, and 4.

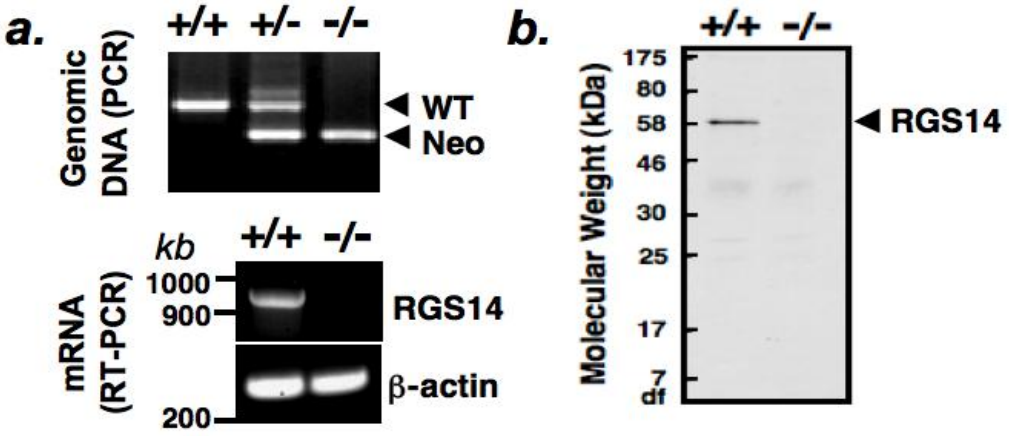


Figure 3.2 Loss of full-length RGS14 gene, mRNA, and Protein (a; top) PCR genotyping. Multiplex PCR reaction shows single larger band for wild type (WT) genomic DNA, two bands for heterozygous RGS14 (+/-) genomic DNA, and a single lower band for knockout RGS14 (+/-) (RGS14-KO) genomic DNA indicating loss of RGS14 gene and insertion of lacZ/neo cassette. (A; bottom) RT-PCR analysis of RGS14 mRNA. No mRNA product seen for any of the RT-PCR primers used in RGS14-KO. (b) Protein immunoblot for RGS14 protein using a specific anti-RGS14 monoclonal antibody. Lane 1 WT RGS14 (+/+) brain lysates, Lane 2 RGS14-KO brain lysates.

3.3.2 Determining the function of RGS14 in synaptic activity.

Because RGS14 is expressed primarily in CA2 hippocampal neurons, we tested the effects of loss of RGS14 on synaptic transmission in CA2 compared with CA1 neurons (Figure 3.3a,b). In rodents, high frequency synaptic stimulation (HFS) in the stratum-radiatum results in a sustained marked enhancement of postsynaptic responses – i.e. long term potentiation (LTP) – in CA1 and CA3 neurons (Bliss and Collingridge, 1993). However, CA2 neurons differ from their neighboring neurons by their curious lack of plasticity following synaptic stimulation to Schaffer collateral synapses (Zhao et al., 2007). We discovered that the loss of RGS14 confers to CA2 neurons a robust capacity for this type of plasticity (Figure 3.3a), whereas CA2 neurons from wild type animals exhibited little LTP following HFS as expected. On the other hand, both the RGS14-KO and WT mice displayed normal LTP in CA1 neurons assessed in field potentials (Figure 3.3b). The basal synaptic transmission in CA2 and CA1 neurons was also normal in both WT and KO animal. Hence, RGS14 may act as a natural brake to limit synaptic plasticity within the CA2 following stimulation.

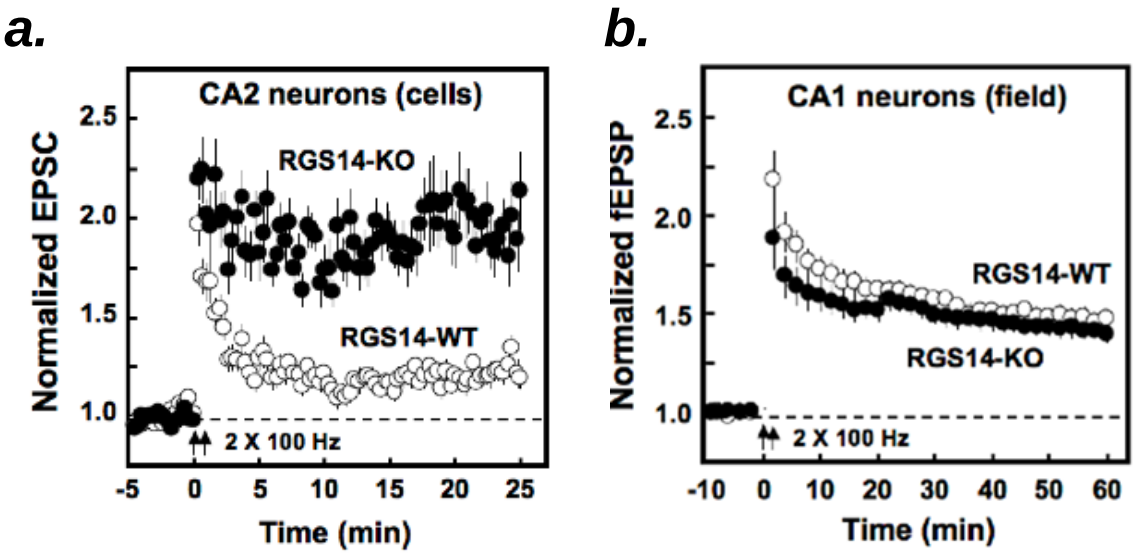


Figure 3.3 Loss of RGS14 allows for induction of nascent LTP in CA2 neurons but is unchanged in CA1 following high frequency stimulation (a) For induction of LTP, CA2 neurons were stimulated (2 X 1 sec, 100 Hz, 20 sec intervals) and excitatory postsynaptic currents (EPSCs) measured WT (N= 5 mice, 22 neurons), RGS14-KO (N= 6 mice, 24 neurons). Plotted are means ± SEMs (b) For induction of LTP in CA1, hippocampal slices from WT and RGS14-KO mice were stimulated (2 X 1 sec, 100 Hz, 20 sec intervals) and post-synaptic neurotransmission was monitored every 15 sec for 180 min. Data are means ± SEMs.

3.3.3 LTP in CA2 is blocked by MEK inhibition

We next investigated possible underlying molecular mechanisms to explain how losing RGS14 enhances synaptic plasticity in CA2 neurons. RGS14 binds Rap2, Gαi1/3-GDP, H-Ras, and Raf kinases to inhibit growth factor-directed MAP kinase signaling (Shu et al., 2010; Shu et al., 2007; Traver et al., 2000a). Therefore, we tested whether treatment of CA2 neurons with a specific inhibitor of ERK/MAP kinase signaling affected the increased capacity for LTP caused by the loss of RGS14. We find that the MEK inhibitor U0126 completely prevented this robust LTP induction in CA2 neurons (Figure 3.4a), thus implicating RGS14 as a suppressor of MAP kinase signaling in this process. The MEK inhibitor also blocked LTP in CA1 neurons (Figure 3.4b), as expected, suggesting a key role for MEK/ERK signaling in the regulation of LTP in both CA2 and CA1 neurons (English and Sweatt, 1997). This effect of the MEK inhibitor on LTP in CA2 neurons does not rule out a possible additional role for G protein signaling and regulation of potassium channel function. Consistent with evidence that MAPK or G protein signaling can influence neuronal excitability (Cohen-Matsliah et al., 2007; Lei et al., 2003; Mark and Herlitze, 2000; Yuan et al., 2002), we also observed that loss of RGS14 results in a modest increase in input resistance in CA2 neurons (Figure 3.5 a, b), which could be due to loss of regulation of either RGS14 binding partner H-Ras or G□i. This concomitant increase in neuronal excitability, however, is not sufficient to substantially impact the action potential response to high frequency stimulation (Figure 3.5c). Taken together, these findings support our hypothesis that RGS14 serves

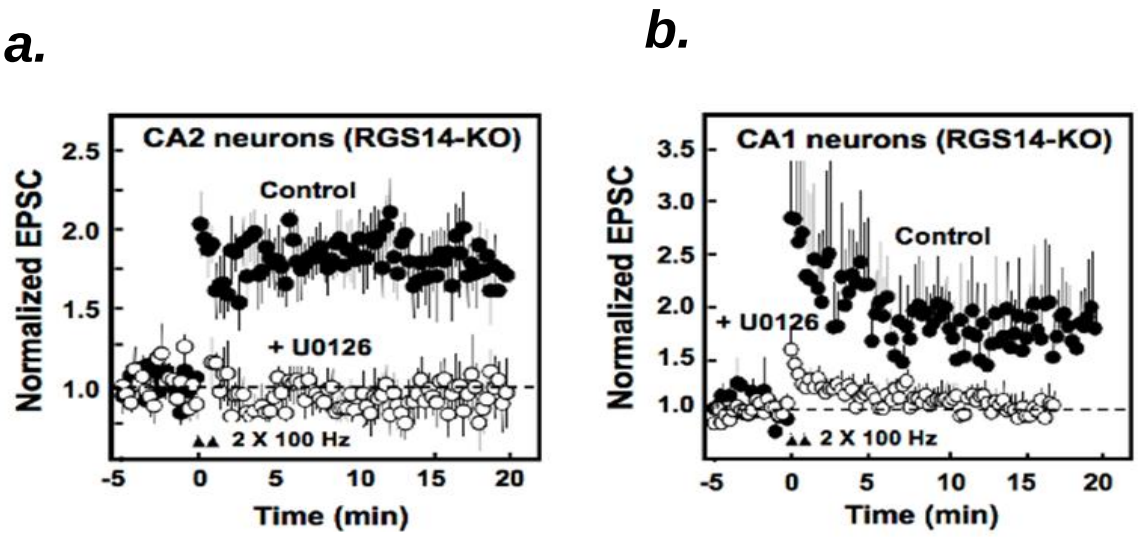


Figure 3.4 LTP in CA2 neurons following high frequency stimulation that is blocked by a specific MEK inhibitor For inhibition of LTP in CA2 neurons by treatment with a MEK inhibitor, experiments on individual CA2 neurons from RGS14-KO mice were performed as in (3.5a) except that 500 nM U0126 was included in the bath solution (N = 7 with U0126 and N = 9 without). Plotted are means ± SEMs. (b) For inhibition of LTP in CA1 neurons by treatment with a MEK inhibitor, experiments on individual CA1 neurons from RGS14-KO mice were performed as in (a) except that 500 nM U0126 was included in the bath solution (N = 7 with U0126 and N = 4 without). Plotted are means ± SEMs.

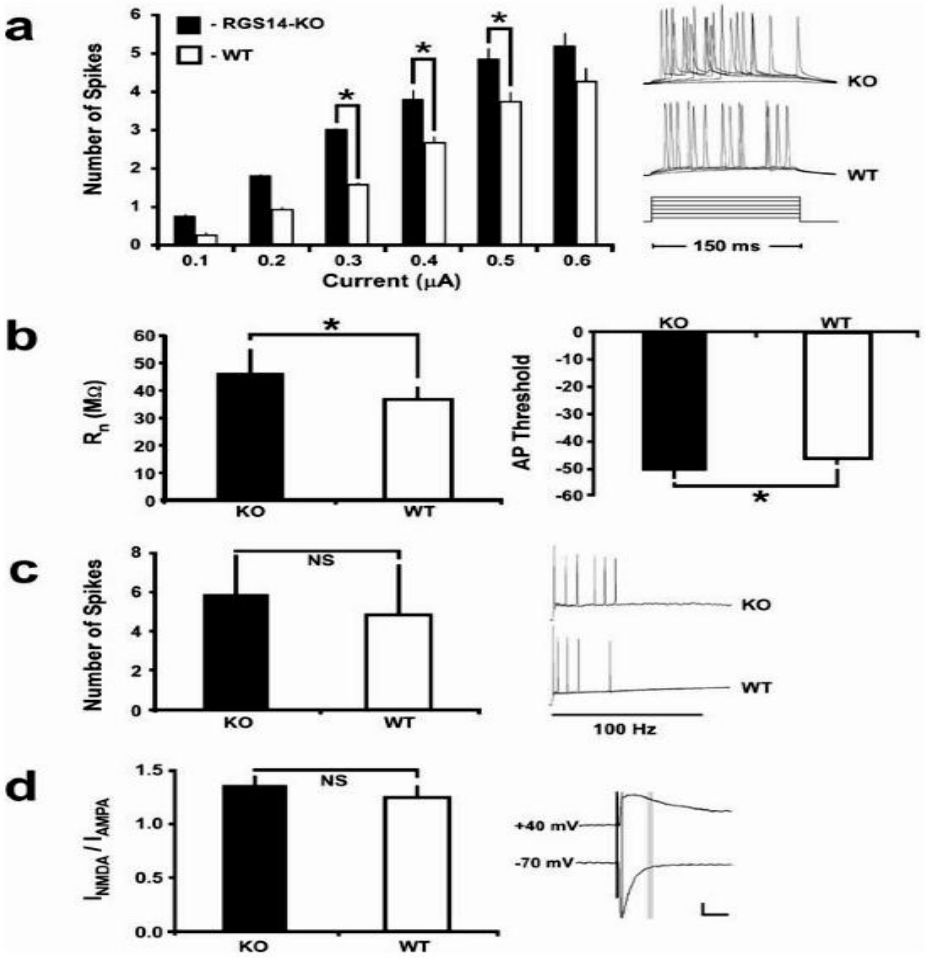


Figure 3.5: CA2 neurons from RGS14-KO mice are more excitable than those from WT mice, but their synaptic properties are similar. (a) Average number of 2 action potentials (spikes) evoked with 150 ms current injections in KO and WT CA2 neurons. Examples of single neuron responses and current injection waveforms are shown to the right. (b) Average input resistances (Rn) (left) and action potential thresholds (right) differ between KO and WT CA2 neurons. (c) Average number of spikes evoked during 1s of 100Hz synaptic stimulation does not differ between WT and KO. Examples of representative single neuron

responses are shown to the right. Delivery of the 100Hz stimulus is indicated with a bar. (d) Ratios of average synaptic NMDA receptor and AMPA receptor currents did not differ between neurons from WT and KO mice. Measurements of NMDA receptor and AMPA receptor-dependent components were measured at 10 and 1ms respectively and are indicated by the light gray bar and dark gray bar, indicated in the examples on the right, which were recorded from a single CA2 neuron from an RGS14-KO. Both measurements were acquired at +40mV. Scale bars are 50pA and 50ms. In all panels (*) indicates differences are statistically significant $p<0.05$. NS, differences not significant. n=12 neurons from 5 RGS14-KO mice and 11 neurons from 4 WT mice.

as a previously unknown natural suppressor of signaling pathways important for LTP in CA2 neurons.

3.3.4 RGS14-KO mice exhibit enhanced learning and memory

Because RGS14-KO mice exhibited markedly enhanced synaptic plasticity in CA2 neurons, we hypothesized that hippocampal-based spatial learning and memory in RGS14-KO mice might also be enhanced (Figure 3.3). To test this idea, we first measured declarative memory using a novel object recognition task (Ennaceur and Delacour, 1988). Mice were allowed to explore two identical objects. Four and 24 hours later one object was replaced with a novel object to test for signs of object recognition. Although both groups of mice showed similar baseline exploratory behavior by spending an equivalent amount of time with the identical objects, after four hours RGS14-KO mice spent significantly more time than WT mice exploring (Figure 3.6a) and made more contacts with the novel object (Figure 3.6b) in the test trials, indicating enhanced recognition memory. Of note, our findings here are inconsistent with a recent report that shows over-expression of RGS14 in the visual cortex enhances novel-object recognition (Lopez-Aranda et al., 2009). Because those studies involve over-expression of recombinant RGS14 in a brain region where it is not naturally expressed, we believe our findings more accurately reflect the physiological role for RGS14 in the context of novel-object recognition.

In a second test of hippocampal function, we subjected mice to the Morris water maze (MWM) to test for spatial learning. In this task, rodents navigate a swim arena using visual cues to locate a submerged escape platform (Vorhees, 2006). In repeated trials of the MWM, paired WT and RGS14-KO littermates showed a similar latency to escape on day one, and exhibited typical learning behavior improving each day, reaching a plateau by day five. Surprisingly though, RGS14-KO animals exhibited a significantly enhanced initial learning rate that was sustained each day (Figure 3.6c). In probe trials after six days of testing, the platform was removed to determine whether the animals would perseverate in their search for the platform in the previous location. WT and RGS14-KO mice spent more and similar amounts of time in the trained quadrant (Figure 3.6d), indicating that both groups learned the platform location.

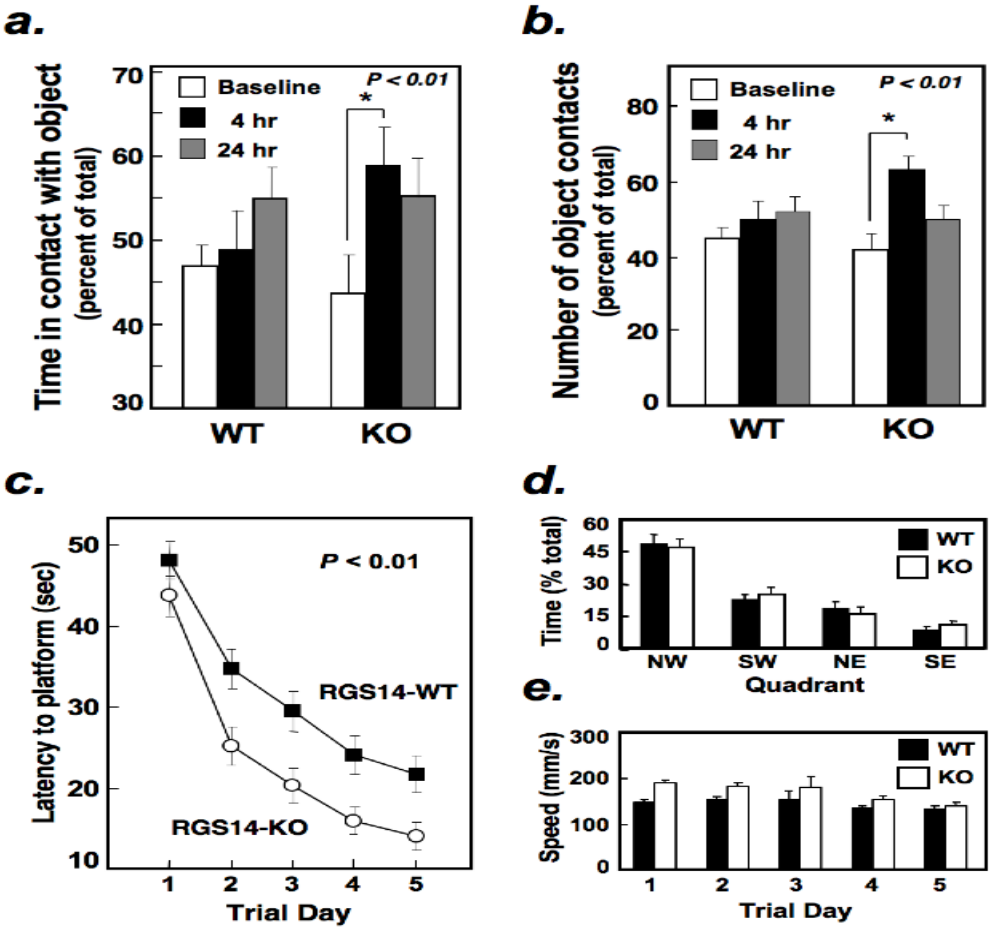


Figure 3.6: Loss of RGS14 enhances hippocampal-based spatial learning and object working memory. (a-b) Novel object recognition task: (a) percentage of total time spent exploring and percentage of total contacts made on two objects for five min during training, and memory for object at 4 and 24 h after training (paired t-test, *P<0.01; N=34, WT; N=20, RGS14-KO). (c) Morris water maze task: latency for WT and RGS14-KO mice to reach a hidden platform in acquisition trials over five days (two factor, repeated measures ANOVA; *P<0.01). (d) Probe trial on day six for WT and RGS14-KO mice; time spent in

each quadrant with escape platform removed. (e) Average swim speed over five days of acquisition training (c-e: N=17, WT; N=16, RGS14-KO).

3.3.5 RGS14-KO mouse performance in non-hippocampus dependent tasks

Differences in behavior were limited to those tasks linked to the hippocampus, as both groups swam at similar speeds (Figure 3.6e), and we observed no significant differences in ambulatory behavior (Figure 3.7), baseline startle response, sensory-motor gating (Figure 3.8), or anxiety in the elevated plus maze (Figure 3.9). However, there was a trend toward increased anxiety in the elevated plus maze for the RGS14-KO mice. These altered behaviors coupled with the observed enhancement of CA2-specific plasticity of postsynaptic transmission in RGS14-KO animals indicate that the presence of RGS14 limits hippocampal-based learning and memory without altering other behaviors.

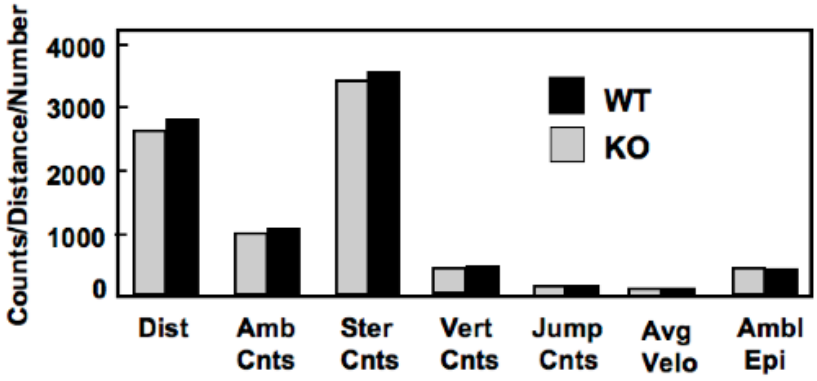


Figure 3.7 Locomotor Activity: a) Open-field locomotor activity. Baseline motor activities were measured by examining the total ambulatory distance (in cm) during the 20-min open field test session equipped with four 24-beam infrared arrays across the base of each chamber wall. Activity data was collected via computer and analyzed with the MED Associates' Activity Monitor Data Analysis software. Motor activities measured include distance travelled (Dist), ambulatory counts (Amb cnts), stereotypy counts (Ser cnts), vertical counts (Vert. Cnts), jumping activity (Jump cnts), average velocity of movement (avg velo), and ambulatory episodes (Amb epi). Wild-type (N = 34) and RGS14-KO (N = 20).

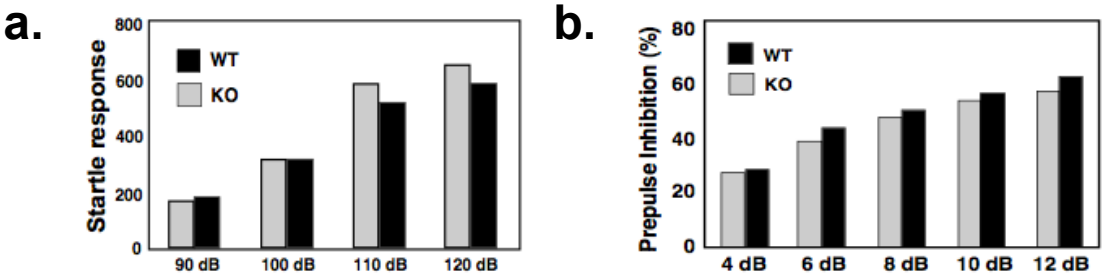
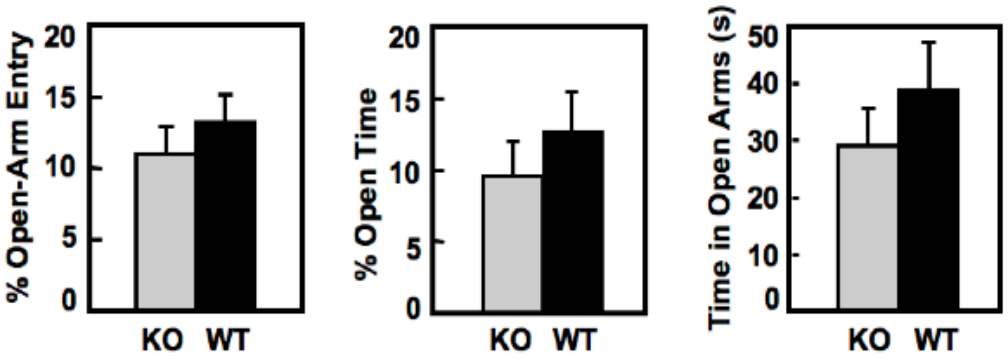


Figure 3.8 RGS14-KO and WT mice exhibit similar responses to startle and sensory motor gating (a) Startle response. Ten startle stimuli at each of four different startle stimulus intensities (90, 100, 110, 120 dB) with an interstimulus interval (ISI) of 30s. All startle stimuli were presented in a pseudorandom sequence with the constraint that each stimulus intensity occur only once in each consecutive four-trial block. Mean startle amplitudes were calculated for each mouse by computing the average startle response at each of four different startle stimulus intensities. Wild-type (n=34) and RGS14-KO (n=20). (b) Pre-pulse inhibition of startle. Startle stimuli (115 dB, 50 ms) were presented alone or were preceded by noise prepulses (20 ms) of 2, 4, 8, 10, or 12 dB above a 63dB white noise background (i.e. 65, 67, 71, 73, or 75 dB) with a fixed interval (100 ms) between onsets of the prepulse and startle stimuli. Five different trial types were presented in random order nine times for a total of 45 trials. Intertrial intervals ranged from 20 to 40 s. Mean startle amplitudes for the startle-alone trials and each of the 5 prepulse+startle trials were calculated for each mouse by averaging the startle amplitude of each trial type. Each mean prepulse+startle amplitude score was converted to a percent PPI obtained as follows: Percent PPI = 100 x



(mean startle-alone amplitude - mean prepulse+startle amplitude)/(mean startle-alone amplitude). Wild-type (N=34) RGS14-KO (N=20).

Figure 3.9 RGS14-KO and WT mice show no significant differences in anxiety behaviors. Elevated-plus maze for anxiety-related behavior. Mice were placed in an elevated-plus maze and closed arm entries, open arm entries, total arm entries and time in open arms were recorded. The total number of entries (open + closed); the percent of open arm entries (open/total) and percent time in open arms (open arms time in seconds/30). Wild-type (N=34) RGS14-KO (N=20).

3.4 Discussion

Very little is known about the role of the CA2 sub-region in hippocampal function and behavior. CA2 synapses are unusually stable and resistant to plasticity (LTP) following typical modes of stimulation (Zhao et al., 2007). No previous studies implicate a role for the CA2 in spatial learning or memory except that it may be involved in social recognition memory and memory for temporal order (DeVito et al., 2009). Yet, we show here that the loss of RGS14, a single CA2-enriched gene, abrogates this synaptic stability resulting in both robust LTP in CA2 neurons and an enhancement of spatial learning and object recognition memory. Only a few signaling proteins that could contribute to its unusually weak LTP have been reported to be localized in CA2 neurons (Lein et al., 2005b; Simons et al., 2009), and possible roles for those proteins (if any) in synaptic plasticity have not been defined. By contrast, many signaling proteins and pathways have been implicated in the positive regulation of LTP, synaptic plasticity, and learning and memory in other, non-CA2 hippocampal regions (Malenka and Bear, 2004; Neves et al., 2008). Among them are the identified RGS14 binding partners Gαi1, active H-Ras and Rap2, and associated MAP kinase signaling pathways (Kennedy et al., 2005).

At present, the exact molecular mechanism by which RGS14 regulates LTP in CA2 neurons remains uncertain. Our findings here show that loss of RGS14 results in nascent LTP that is prevented by inhibiting MEK (Figure 3.4b), indicating that RGS14 may be acting as a natural suppressor of ERK1/2 signaling pathways underlying synaptic plasticity. Consistent with this idea, we

have shown that RGS14 integrates G protein and H-Ras/Raf signaling to inhibit growth factor stimulated ERK1/2 signaling (Shu et al., 2010). We found that RGS14 is localized at dendritic spines (Lee et al., 2010) and may be enriched in Triton-X 100-insoluble post-synaptic densities (Hollinger et al., 2001), i.e. well-established focal points for synaptic plasticity-related signaling. RGS14 binding partners H-Ras and Rap2 modulate the activity of various MAP kinase pathways in hippocampal neurons (Zhu et al., 2005) and have been implicated in different aspects of LTP and synaptic plasticity in the hippocampus (Fu et al., 2007; Kushner et al., 2005; Manabe et al., 2000; Pineda et al., 2004; Ryu et al., 2008; Tada and Sheng, 2006; Zhu et al., 2002; Zhu et al., 2005). However, RGS14 also binds Gi/o family members at both the GPR domain and the RGS domain, and our findings do not rule out a possible role for RGS14 regulation of Gi/o protein signaling in this process. The G protein-regulated second messengers cAMP and calcium each play crucial roles in learning, memory and synaptic plasticity by regulating gene expression and modulating postsynaptic signaling events (Bliss and Collingridge, 1993; Bourtchuladze et al., 1994). Thus, loss of RGS14 and with it, its capacity to limit Gαi/o signaling, may alter postsynaptic cAMP and/or calcium levels to enhance LTP and learning. Further studies are necessary to determine the role for each of these binding partners and their linked signaling pathways in RGS14-mediated suppression of synaptic plasticity in CA2 neurons.

In summary, RGS14 is a natural suppressor of signaling pathways critically involved with regulating synaptic plasticity, and its host CA2 neurons

may represent a newly recognized module that functions either distinct from or in concerted action with the canonical trisynaptic circuit to mediate hippocampal-based learning and memory. Given the very discrete protein expression pattern of RGS14 and the fact that RGS14-KO mice exhibit no obvious deleterious phenotypes, inhibition of RGS14 function may serve as an attractive therapeutic target for future cognitive enhancers (Blazer and Neubig, 2008; Lee and Silva, 2009).

Chapter 4: Seizure susceptibility and extinction of learning in RGS14-KO Mice

4.1 Introduction

The regulator of G protein signaling, RGS14, plays an important role in brain function. RGS14 is a natural suppressor of synaptic activity in the CA2 of the hippocampus and is involved in modulating memory formation and consolidation in hippocampus-dependent memory tasks. Loss of full-length RGS14 leads to enhancement of LTP in the CA2 region of the hippocampus. The CA2 is unusually stable, in that high-frequency stimulation to the Schaffer collateral synapses does not induce LTP, whereas the same stimulation generates robust LTP in the CA1. This apparent synaptic stability may be beneficial for the region because CA2 pyramidal neurons are largely spared following ischemic insult as well as during status epilepticus. The CA1 pyramidal neurons suffer tremendous cell loss during such events. The sparing of the CA2 may be due to its increased calcium buffering capacity and to the presence of RGS14 (Simons et al., 2009). We decided to test mice missing RGS14 to determine if there are any differences in the manifestation of status epilepticus following pilocarpine induced seizures. We also examined increases in activation of the MAP kinase pathway by measuring levels of phospho-ERK1/2 following status epilepticus.

RGS14-KO mice exhibit enhanced acquisition learning in the Morris water maze. Since animal models showing enhancements is cognition may also exhibit impairments in extinction or reversal learning. Conditional inhibition of calcineurin, a calcium-dependent phosphatase, in mice leads to increases in LTP, improved performance in the Morris water maze, and impaired extinction

(Baumgartel et al., 2008; Malleret et al., 2001). Additionally, overexpression of type-1 adenylyl cyclase in mouse forebrain enhances LTP and object memory but impairs extinction (Wang et al., 2004). To determine if RGS14-KO mice also exhibit differences in reversal or extinction, we tested RGS14-KO mice for extinction and reversal learning in the Morris water maze.

RGS14 protein appears to suppress LTP and learning in mice, and loss of full length RGS14 enhances LTP in the CA2 region of the hippocampus and improves performance in hippocampus-dependent memory tasks. RGS14 acts as a molecular scaffold that binds effectors of G protein coupled receptor signaling and upstream mediators of the MAP kinase pathway. To determine if RGS14 is indeed a suppressor of synaptic activity, we generated a lentivirus expression vector to overexpress EGPF-RGS14 in rat brain.

4.2 Materials and Methods

4.2.1 Pilocarpine-induced seizures

The systemic administration of pilocarpine for induction of generalized clonic seizures in rodents is widely employed to identify potential anticonvulsants. Mice received 1 of 2 intraperitoneal test injections of pilocarpine (300 or 400mg/kg in 0.9% NaCl). Immediately following injection, mice were placed individually in acrylic observation chambers cage (450 mm ×350 mm×300 mm) for a 1hr observation period. Latencies (in seconds) to forelimb clonic, rearing, and tonic-clonic seizures were recorded. Tonic-clonic seizures generally begin with running, followed by the loss of righting ability, then a short tonic phase (flexion or extension of fore and hind limbs) progressing to tonus of all four limbs leading to the death of the animal.

4.2.2 Immunohistochemistry

Mice were perfused with PBS followed by fixative, 4% paraformaldehyde in PBS and tissue post-fixed with the same solution for 18 hrs. Whole brains were frozen, cut into 20 µm sections, stained free-floating and mounted onto glass slides. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol incubation for 5 min. Sections were blocked with 5% normal goat serum diluted in 0.3% TritonX-PBS-BSA, pH7.5, and incubated in primary anti-phosphoERK1/2 antibody (Cell Signaling #9102) overnight at 4^oC. Sections were then washed, and incubated in biotinylated goat anti-rabbit secondary antibody (Vector Labs BA-1000) followed by avidin-biotin-peroxidase complex

(Vectastain Elite ABC kit) and developed with 3,3'-diaminobenzidine (DAB substrate kit, Vector Labs SK-4100). Nuclei were counterstained with hematoxylin.

4.2.3 Morris water maze

Adult RGS14-KO and WT littermates age 2-6 months were used. The water maze consisted of a circular swim arena (diameter of 116 cm, height of 75 cm) surrounded by extra-maze visual cues that remained in the same position for the duration of training. The maze was filled with water at 22^{o} C to cover the platform by 1cm, and the water was made opaque with non-toxic, white tempera paint. For reversal learning, the escape platform was a circular, non-skid surface (area 127 cm^2) placed in the SE quadrant of the maze. Reversal training consisted of five test days with four daily trials. Mice entered the maze facing the wall and began each trial at a different entry point in a semi-random order. Trials lasted 60 seconds or until the animal mounted the platform with a 15 minute inter-trial interval. Extinction trials were conducted wherein the platform was removed, and the animal swam for 60 seconds; the time spent in the target quadrant (NW) versus the adjacent and opposite quadrants was recorded. A video camera mounted above the swim arena and linked to TopScan software recorded swim distance, swim speed, and time to platform and was used for tracking and analysis. Statistics were ANOVA and *post hoc* Dunnett's test unless otherwise stated.

4.2.4 HeLa cells expressing EGFP-RGS14 Lentivirus. Cells were cultured in DMEM supplemented with L-glutamine, 10% FBS, and 1% Penicillin/streptomycin and grown in a humidified incubator 5% CO_2 at 37^{o}C. To prepare cell lysates, media was aspirated, and cells were rinsed in PBS with protease inhibitor cocktail (Roche). Cells were scraped from the culture dish and centrifuged at low speed. The supernatant was discarded and sample buffer added to the cell pellet. The cell pellet was sonicated with pulsation for 1 minute on ice. Samples were boiled in a dry bath for 5 minutes and centrifuged at 14 x g for 2 minutes. Samples were stored at -20^{o}C. Samples were subjected to SDS-PAGE transferred to nitrocellulose membranes for immunoblot analysis. RGS14 monoclonal antibody (Neruomabs) was used to detect RGS14 in samples 48 hours after infection. EGFP-RGS14 was detected using an Olympus fluorescence microscope.

4.3 Results

4.3.1 RGS14-KO Mice are resistant to Pilocarpine induced seizures

The hippocampus is known for its role in epilepsy. Hippocampal sclerosis, which is hardening or damage to the hippocampus as the result of traumatic brain injury, can often lead to the occurrence of seizures. Since RGS14 appears to be a negative regulator of synaptic activity, we tested RGS14-KO mice in the pilocarpine induced seizure model. Pilocarpine is a nonselective muscarinic acetylcholine receptor agonist and high dose, systemic treatment causes status epilepticus in rodents. The pilocarpine model triggers intense grand mal seizures initially. Rodents that survive the initial seizures will periodically experience recurrence of status epilepticus for the rest of their lives. WT and RGS14-KO mice were injected with 300mg/kg pilocarpine, and the time to clonic, rearing, and tonic seizures was measured. RGS14-KO mice appear resistant to pilocarpine induced seizures taking significantly longer to reach tonic seizures (Figure 4.1). This result appears to contradict our findings about enhanced LTP in the CA2 region of the hippocampus. However, the greatest area of damage during status epilepticus is the CA1 region of the hippocampus while the CA2 region is spared. RGS14-KO mice exhibited no differences in synaptic activity in CA1 excitatory field potentials. Additionally, the high dose pilocarpine induced seizures may not be representative of the actual events that occur during kindling. Perhaps a better study would be to examine the spontaneous seizures that occur following the initial pilocarpine treatment. Activation of the MAP kinase pathway occurs

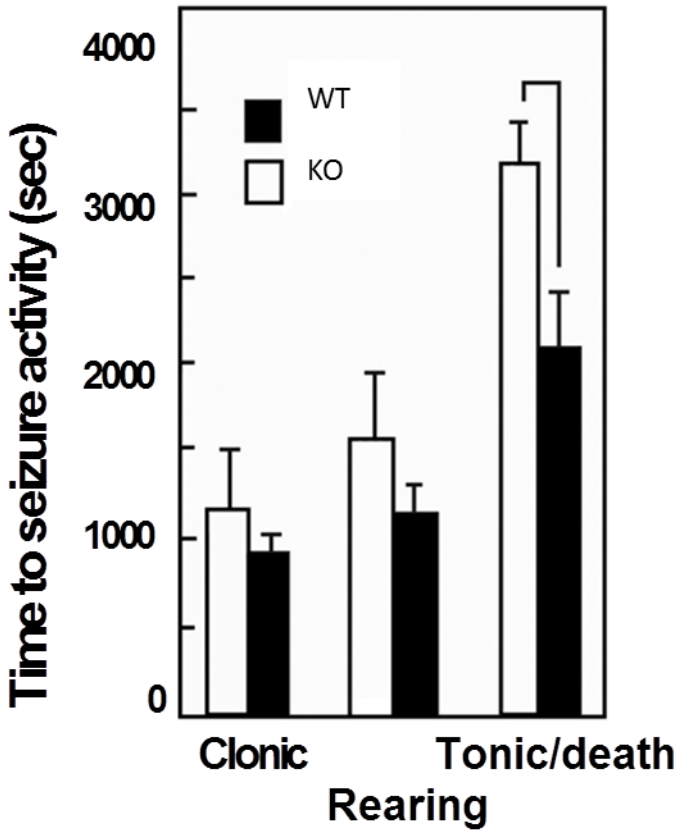


Figure 4.1 RGS14-KO mice are resistant to pilocarpine induced seizures. The systemic administration of pilocarpine for induction of generalized clonic seizures in rodents is widely employed to identify potential anticonvulsants. Latencies (in seconds) to forelimb clonic, rearing, and tonic-clonic seizures were recorded. Tonic-clonic seizures generally begin with running, followed by the loss of righting ability, then a short tonic phase (flexion or extension of fore and hind limbs) progressing to tonus of all four limbs leading to the death of the animal. RGS14-KO mice had significantly longer latencies to tonic phase seizures than their WT littermates.

following pilocarpine induced seizures. Mice were administered methyl scopolamine to reduce the peripheral muscarinic actions of pilocarpine. Mice were injected with 300mg/kg pilocarpine and were sacrificed 15 minutes following status epilepticus. Mice were transcardially perfused with saline followed by 4% paraformaldehyde fixative. Tissues were immunoperoxidase stained for phospho-ERK1/2. Mice that were subject to pilocarpine induced status epilepticus showed an increase in phospho-ERK1/2; however, the staining was not in pyramidal neurons as was expected and there were no differences between WT and RGS14-KO mice (Figure 4.2). Increases in phospho-ERK1/2 occur rapidly following pilocarpine administration, in order to more accurately measure activated phospho-ERK1/2 mice may need to be sacrificed at 15 minutes following pilocarpine administration rather than following the onset of status epilepticus.

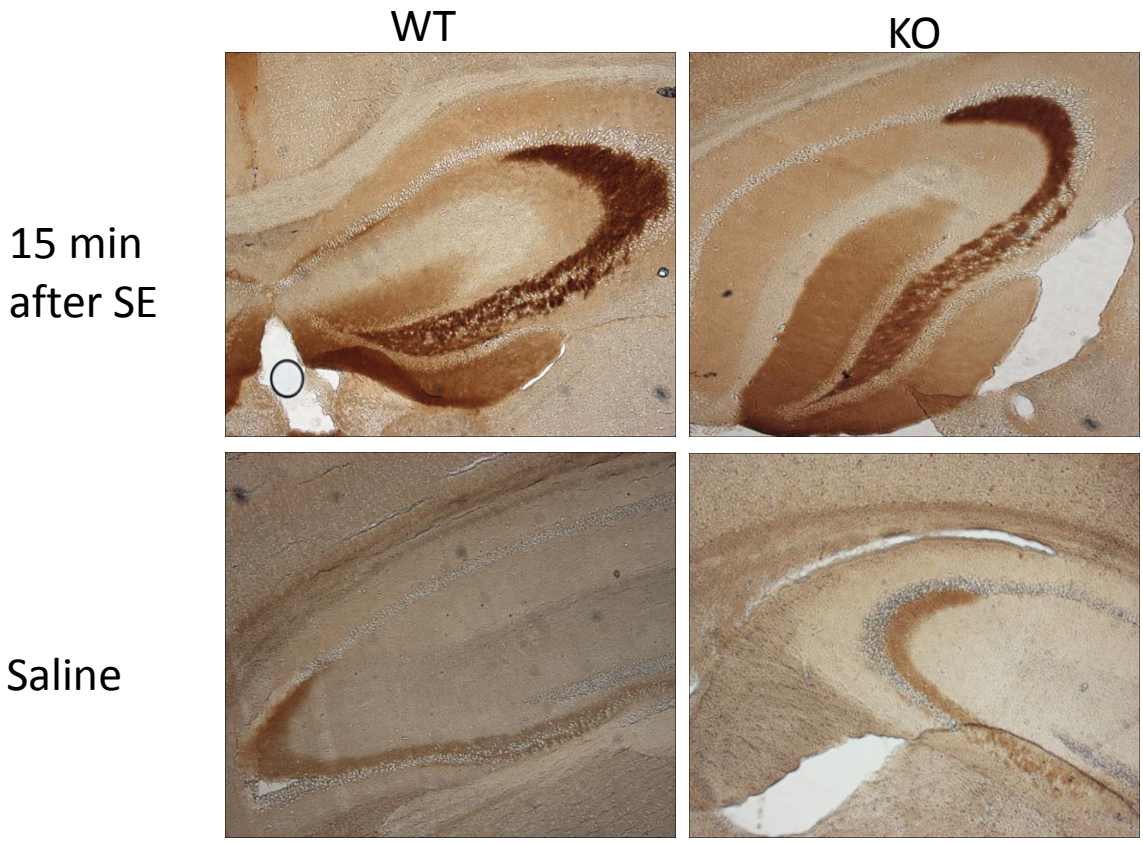


Figure 4.2 Phosphorylation of ERK1/2 following pilocarpine induced status epilepticus. WT and RGS14-KO mice were injected i.p. with 270 mg/kg pilocarpine. Tissue was collected 15 minutes following seizure onset. and was stained using DAB immunoperoxidase for phospho-ERK1/2. Phospho-ERK1/2 was increased over saline, but staining was not evident in pyramidal cell bodies. There was no difference in phospho-ERK1/2 between WT and RGS14-KO mice.

4.3.2 Reversal and Extinction in the Morris water maze

To further examine the role of RGS14 in learning and memory, we tested WT and RGS14-KO mice for reversal and extinction in the Morris water maze. Reversal involves moving the escape platform to the opposite quadrant from the original platform location. Reversal is dependent on the hippocampus and reveals whether the test subject can extinguish the original learning and learn the location of a new target. RGS14-KO mice showed no significant differences in reversal tasks (Figure 4.3). The extinction task measures whether mice have the ability to extinguish prior learning. Some mouse models with enhanced acquisition learning have difficulty extinguishing previous learning. However RGS14-KO mice show no deficits in reversal or extinction in the water maze.

4.3.4 Lentivirus expression of RGS14

Discrete point mutations disrupt interactions with each of RGS14's binding partners. Single point mutations in the sequence of RGS14 could potentially be used to dissect which of the binding partners is most important for the function of RGS14 in LTP and learning. We generated twolentivirus vectors to infect hippocampal neurons. Initial tests show (Figure 4.5) that EGFP-RGS14 is expressed at a high level in HeLa cells 36 hours after infection. The second vector contains RGS14 followes by an IRES-EGFP. Immunoblot analysis shows a 60 kDa band in infected HeLa cells. EGFP expression does not appear at high levels compared to the EGFP-RGS14 vector.

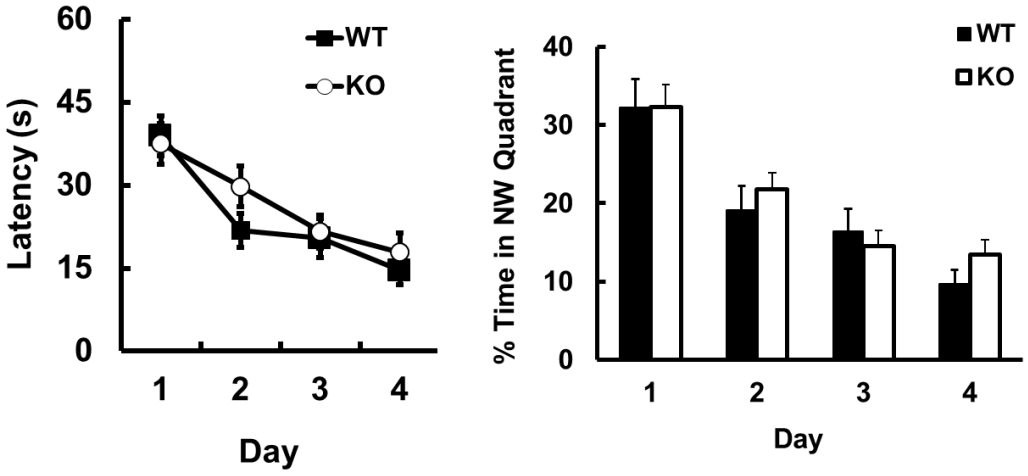


Figure 4.3 Reversal learning in Morris water maze. For reversal tasks, the escape platform was moved to the SE quadrant, opposite the original platform location. Four trials over the course of four testing days revealed no significant differences in reversal learning between WT and RGS14-KO mice.

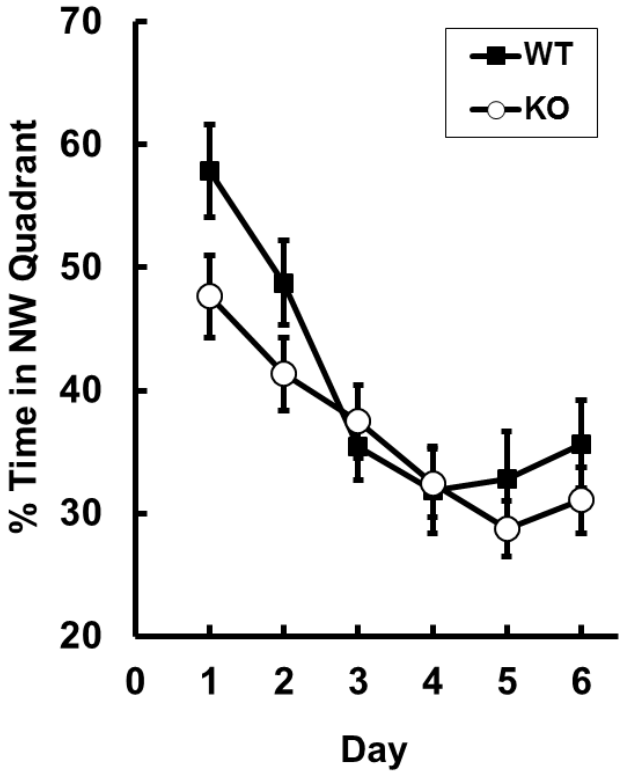


Figure 4.4 Extinction in the Morris water maze. RGS14-KO and WT littermates were tested for time to extinguish prior learning of the platform location. Training trials were a series of 60 second trials over six training days. Percent time spent in the NW quadrant greater than 40% indicates that the previous training has not been extinguished. Less than 25% is correlated with extinction of the former platform location. RGS14-KO and WT littermates showed

no difference in extinction.

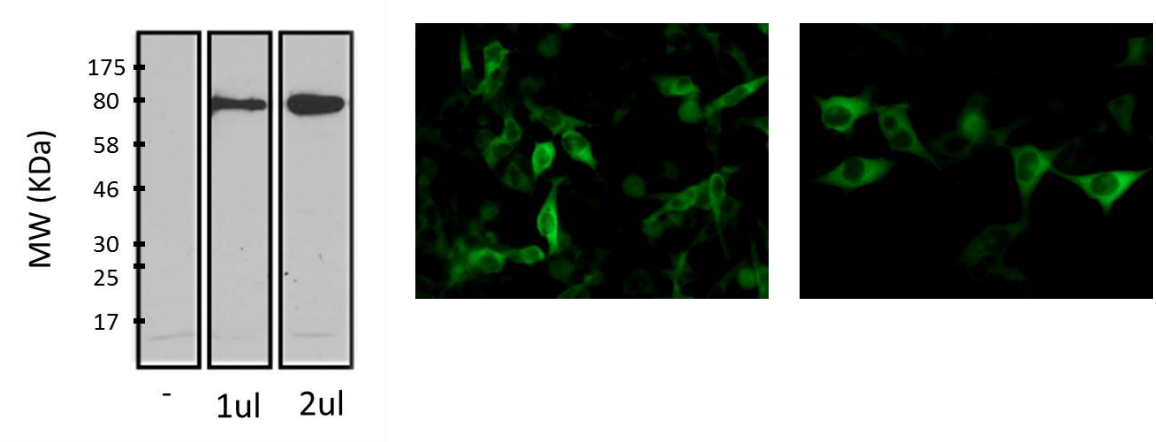


Figure 4.5 EGFP-RGS14 Lentivirus 48 hours following infection of HeLa cells with EGFP-RGS14 lentivirus. Each 1 ul contains 2 x 10^9 MOI. Left panel shows immunoblot analysis for RGS14. An 80 kDa band corresponds to RGS14 with the EGFP tag. Uninfected HeLa cell lysates show no detectable RGS14. Center and right panels show HeLa cells expressing EGFP-RGS14.

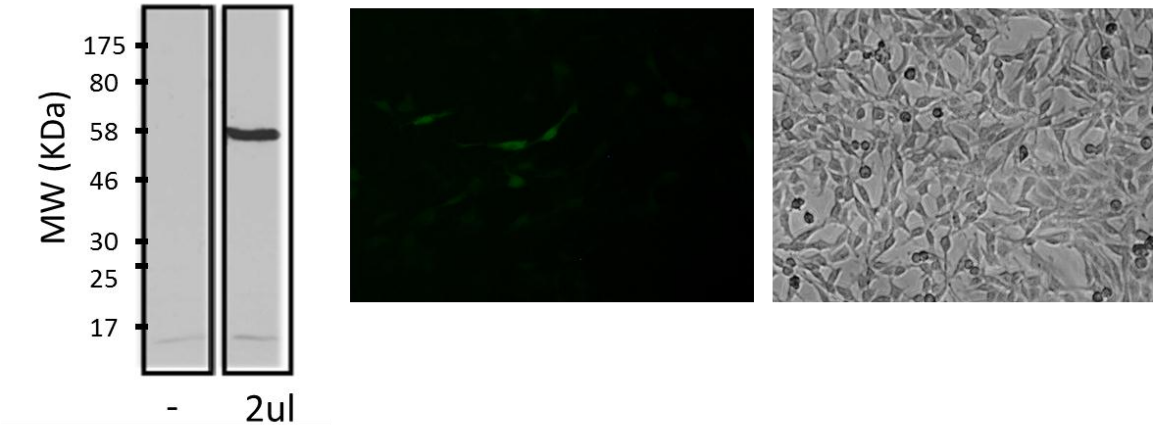


Figure 4.6 RGS14 IRES-EGFP Lentivirus 48 hours following infection of HeLa cells with EGFP-RGS14 lentivirus. Each 1 ul contains 1 x 10^8 MOI. Right panel shows immunoblot analysis for RGS14. A 60 kDa band corresponds to untagged RGS14. Uninfected HeLa cell lysates show no detectable RGS14. Center and panel shows HeLa cells expressing EGFP. Right panel shows the field of view. RGS14-IRES-EGFP vector expresses a much lower level of EFGP.

Even though the virus has a lower titer, 1 x 10^8 versus 2 x 10^9 for the EGFP-RGS14 virus. The IRES-EGFP acts as a marker for infected neurons but does not contain a large-EGFP tag attached to RGS14 that may interfere with binding partner interactions. These viruses will be injected into rodent brain to overexpress RGS14. We will measure changes in synaptic properties in EGFP expressing neurons. RGS14 lentiviruses could be used in behavior experiments.

4.4 Discussion

4.4.1 Pilocarpine induced seizure resistance in RGS14-KO mice

Loss of RGS14 alters synaptic activity and memory performance. RGS14-KO mice exhibit normal LTP of neurotransmission in the classical trisynaptic circuit. However, these animals also exhibit enhanced LTP in the CA2 region of the hippocampus, which is best characterized for its inability to undergo LTP. Seizures occur due to uncontrollable synaptic firing. We hypothesized that RGS14-KO mice would exhibit differences in susceptibility to seizures. Indeed this is the case; however, RGS14-KO mice appear to be resistant to pilocarpine induced seizures taking a longer time to reach clonic, rearing, and tonic, late stage seizure endpoints. Resistance to pilocarpine induced seizures was an unexpected result. It is possible that this result is due to alterations in G protein signaling. Pilocarpine acts as a nonselective muscarinic receptor agonist, but the M_1 muscarinic receptor has been shown to be primarily responsible for seizure activity following pilocarpine administration (Hamilton et al., 1997). Without

RGS14 in the brain, signaling through the inhibitory Gαi proteins may be prolonged following activation of M_2/M_4 muscarinic receptor subtypes. While blockade of inhibitory M_2 muscarinic receptors by subtype specific antagonists, gallamine and methoctramine, is sufficient to induce seizures in the absence of pilocarpine, likely by altering the balance of signaling between Gαi and Gαq coupled muscarinic receptors. Signaling through Gαq coupled muscarinic receptors in RGS14-KO mice should be unchanged with rapid signal termination by other RGS proteins leading to the apparent resistance to pilocarpine induced seizures.

We observed no differences in localization or amount of phospho-ERK1/2 between WT or RGS14-KO mice following pilocarpine induced status epilepticus. Tissues were collected 15 minutes following the onset of SE. Activation of the MAP kinase pathway occurs rapidly following pilocarpine administration; therefore, tissues may need to be collected at an earlier time point to observe any difference.

4.4.2 Extinction and Reversal in Morris water maze

Extinction and reversal learning can be impaired when a genetically modified animal exhibits enhanced acquisition learning. We tested RGS14-KO mice and their WT littermates and found no significant differences in extinction or reversal in the Morris water maze. Future studies could examine differences in contextual versus cued fear responses as well as extinction of fear learning.

Other memory tests, particularly those for social memory and learning, could provide interesting insight and further characterize the role of RGS14 in behavior. Studies using RGS14-KO mice have provided insight into the function of RGS14 as a natural suppressor of learning and synaptic activity. To further test this hypothesis and dissect the roles of the three functional domains, we needed to restore RGS14 or over-express RGS14 in the brain. We decided to use viral expression systems to accomplish this task.

4.4.3 Lentivirus vector expression of RGS14

Lentiviruses have the capacity to infect and produce proteins in non-dividing cells such as neurons. Since RGS14 is predominantly found in neurons of the CA2, we generated a lentivirus to express EGFP-RGS14. This virus has the potential to be used in electrophysiology or in behavioral studies. Preliminary studies show that the virus can infect HeLa cells and robustly and stably express EGFP-RGS14. Future studies will use this virus to examine synaptic activity of neurons that express RGS14. The EGFP acts as a marker for RGS14 expression to selectively examine LTP in neurons that overexpress RGS14. Discrete point mutations in the functional domains of RGS14 disrupt interaction of RGS14 with its binding partners. Each mutation is selective such that disruption with one binding partner in its functional domain does not affect interaction with the other binding partners. Use of these mutants could be

powerful tools to provide insight into the mechanism of RGS14 effects on learning, memory, and synaptic activity.

Chapter 5: Conclusion

5.1 Roles for RGS14 during postnatal development.

Neurons that make up the rodent brain are largely formed before birth; however, the postnatal period is a time of great neuronal expansion in the cerebellum, olfactory bulb, and hippocampus (Dumas, 2005). The postnatal period is highly dynamic in that synapses are forming, being pruned rapidly, and glial cell populations are rapidly expanding (Dumas, 2005; Watson et al., 2006). Proper development is crucial to brain function and abnormalities that disrupt development may lead to mental health disorders such as bipolar disorder or schizophrenia (Benes et al., 1991; Weinberger and Lipska, 1995). Protein expression patterns as well as synaptic properties change throughout development, and hippocampus-dependent memory tasks are largely impaired at ages prior to P21(Dumas, 2005). Indicating that maturation of signaling systems is important for hippocampal function are not complete immediately after birth. Certain components of G protein signaling pathways begin to be expressed after the first postnatal week including adenylyl cyclase 1, Gαs (Dumas, 2005), and, as my work shows here RGS14 as well. Changes in the protein expression profile in juvenile pups correspond to impairments in hippocampus dependent behavior even though electrical properties of synapses appear to mature sooner by P15 indicating that the LTP machinery is present but that development of downstream signaling mediators is not complete. RGS14 expression begins around P7 and increases until P21, the point at which rodents no longer display impairments in hippocampal dependent memory tasks. The peak of RGS14 function may be in

mature synapses rather than developing ones as mediator signaling pathways perhaps suppressing synaptic activity and learning.

5.2 RGS14 is important for the acquisition of hippocampal-based spatial learning and object memory.

The highly restricted brain distribution pattern and its temporal expression during postnatal development for RGS14 suggests it serves a key role in hippocampal function. The hippocampus is a well-defined center for spatial learning and memory. In tests of wild-type mice and their littermates lacking the RGS14 gene/protein, we found that the presence of RGS14 within CA2 neurons suppresses hippocampal-based spatial learning and object memory (Lee et al., 2010). RGS14 knockout (RGS14-KO) mice learn more quickly to navigate a water-maze and locate a submerged escape platform, indicating that loss of RGS14 significantly enhances the rate of acquisition of spatial learning. Additional tests of hippocampal memory with these mice show that loss of RGS14 improves novel-object memory without altering other behaviors not directly associated with the hippocampus, such as open field locomotor activity, startle response, and anxiety (Lee et al., 2010). Taken together, these findings suggest that RGS14 naturally inhibits certain forms of hippocampal-based learning and memory.

5.3 RGS14 is a natural suppressor of synaptic plasticity in CA2 neurons

Memory is thought to be encoded within the brain as biochemical and physical changes at synapses lead to alterations in neurotransmission, a process known as synaptic plasticity. One such form of synaptic plasticity is the long lasting increase in the strength of excitatory glutamatergic synaptic transmission (long-term potentiation; LTP) that can be induced with high frequency afferent stimulation. LTP has been best characterized within the well-defined dentate gyrus (DG)-CA3-CA1 trisynaptic circuit of the hippocampus (Nakazawa et al., 2003; Neves et al., 2008; Rolls and Kesner, 2006) (Figure 1.2 Red), with the overwhelming majority of studies performed in the CA1 region. Mice lacking RGS14 show no differences in LTP in CA1, a somewhat surprising result given the well-established link between LTP in the CA1 region and learning and memory (Lee et al., 2010). On the other hand, the result is less surprising considering that RGS14 is most highly expressed in the adjacent CA2 region, an anatomically and biophysically distinct region with contributions to hippocampal function that are largely unknown.

The trisynaptic circuit consists of input from the entorhinal cortex forming synapses on granule neurons in the DG. Axons originating from the DG that project to area CA3 form mossy fiber synapses on dendrites of CA3 pyramidal neurons; and CA3 axons, via the Schaffer collateral fibers, connect to the dendrites of CA1 pyramidal neurons in the Stratum Radiatum. Neurons of area CA2 consist of unique neurons that are similar both in size to the large CA3 neurons and in that they receive no mossy fiber synaptic input from the DG.

Instead, CA2 neurons receive their main input from the CA3 Schaffer collaterals (Figure 1.2 green). Most commonly, LTP is studied in CA1 neurons that receive the CA3-derived Schaffer collaterals where the potentiation of neurotransmission is robust. Unlike synapses on their CA1 neighbors, though, the Schaffer collateral synapses on CA2 pyramidal neurons do not typically exhibit LTP in response to high frequency stimulation (Simons et al., 2009; Zhao et al., 2007). This lack of LTP in CA2 pyramidal neurons is attributed to increased calcium buffering capacity and increased calcium extrusion (Simons et al., 2009). We found that even with the very active calcium handling in CA2 neurons loss of RGS14 apparently permits Schaffer collateral synapses in CA2 to now exhibit robust LTP, strongly suggesting that RGS14 is a natural suppressor of LTP in most CA2 synapses (Lee et al., 2010). The presence of RGS14 may inhibit signaling pathways that lead to the induction of LTP in CA2 neurons. These findings provide a direct link between RGS14 expression and hippocampal learning, memory, and synaptic plasticity that may depend on region-specific protein expression.

In addition to the Schaffer collateral input from CA3, CA2 neurons also receive distinct synaptic input from layer II (LII) and III (LIII) neurons of the EC (Figure 1.2A, green). Neurons of the CA1, CA2 and CA3 region are large and pyramidal in shape and have dendrites that extend from the apex of the cell body (apical dendrites) that are either proximal (close to the cell body) or distal (extend far away) (Figure 1.3). Unlike the CA3-Schaffer collateral input to CA2 neurons that synapse on the proximal dendrites and show no LTP, the CA2 input

originating in the EC form synapses on distal dendrites in a region known as the Stratum Lacunosum Moleculare. Recently, Chevaleyre and Siegelbaum found that both LII and LIII EC pathways are capable of expressing robust NMDA receptor-dependent LTP in CA2 neurons, in contrast to the synapses from CA3 that fail to express LTP (Chevaleyre and Siegelbaum, 2010a). These findings suggest that regional differences or compartmentalization of molecular signaling machinery within CA2 neurons may provide distinct synaptic outputs in response to activity from CA3 or from the different layers of the EC. Consistent with this idea, we have found that RGS14 protein is differentially localized to a subset of CA2 dendritic spines and spine necks (Lee et al., 2010). However, we saw no obvious exclusion of RGS14 expression from the Stratum Lacunosum Moleculare, suggesting the involvement of other modulators at these more distal synapses. Thus, our data showing enhancement of both memory and Schaffer collateral LTP in mice lacking RGS14 strongly suggest that CA2's role in learning and memory is likely dependent on RGS14-containing dendritic spines of CA2 synapses in general and not necessarily restricted to the trisynaptic DG-CA3-CA1 pathway. Dendritic spines act to limit the synaptic microenvironments with distinct protein expression profiles, calcium handling properties, or other synaptic properties. Of note, we found that a subset of RGS14 protein appears to localize to the PSD of CA2 dendritic spines (Hollinger et al., 2001; Lee et al., 2010), indicating that RGS14 is well positioned to modulate signaling events important for synaptic plasticity. We will next discuss how RGS14 may act as a multifunctional integrator of signaling pathways important for synaptic plasticity.

5.4 Possible mechanistic roles for RGS14 and its binding partners in synaptic plasticity

RGS14 appears to localize to post-synaptic densities of dendritic spines, well-established focal points for synaptic plasticity (Hollinger et al., 2001; Lee et al., 2010). Furthermore, as outlined above, RGS14 binds specific G proteins and key components of the MAP kinase pathway that are important for synaptic plasticity. Based on these findings, we postulate that RGS14 serves as a regulatory brake or filter to reduce LTP and synaptic plasticity initiated by presynaptic input. CA2 neurons express a unique profile of signaling genes and proteins, RGS14 among them that may contribute to the region's unusual regulation of LTP (Figure 5.1) (Lein et al., 2005a; Lein et al., 2004). How RGS14 may interact with these or other signaling proteins to integrate G protein and MAP kinase signaling pathways and modulate synaptic plasticity is unclear. Here we will entertain various possibilities based on what is currently known about CA2 synaptic transmission.

The G protein-regulated second messengers calcium and cAMP each play crucial roles in learning, memory, and synaptic plasticity by regulating gene expression and modulating postsynaptic signaling events. Calcium in particular is important in mediating MAP kinase signaling in neurons (Kennedy et al., 2005; Sheng and Hoogenraad, 2007; Sweatt, 2004). Within CA2 neurons, there exist both calcium-dependent and calcium-independent mechanisms that regulate LTP. RGS14 may therefore regulate calcium signaling directly or indirectly by one or more mechanisms.

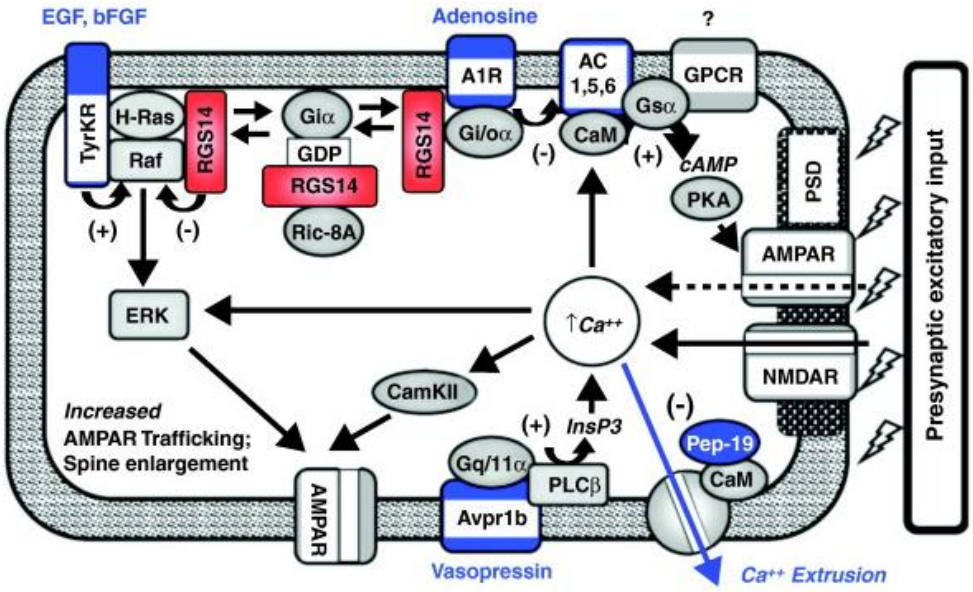


Figure 5.1 Cartoon model of a dendritic spine from CA2 neurons that express RGS14, and speculative roles for RGS14 in the negative regulation of CA2 synaptic plasticity. Shown are distinct properties and signaling proteins that are uniquely or highly expressed in CA2 neurons (blue), additional signaling proteins that are involved in synaptic plasticity (grey), and proposed roles for RGS14 (red).

A particularly robust calcium extrusion system normally suppresses LTP in proximal dendrites of CA2 neurons, yet stimulation of Gq/11-linked vasopressin (Avp1b) receptors potentiates postsynaptic transmission that normally requires calcium and calmodulin kinase II (CamKII) (Simons et al., 2009; Zhao et al., 2007; Zhao, 2010). Potentiation of postsynaptic transmission in CA2 neurons is also induced by calcium *in*dependent-mechanisms requiring cAMP/PKA following inhibition of Gi/o-linked adenosine A1 receptors (Dudek, 2010). Loss of RGS14 and the capacity of its RGS domain to limit Gαi/o signaling may alter postsynaptic cAMP levels to enhance LTP and learning. Since GPR motifs compete with Gβγ for Gαi binding (Ghosh et al., 2003), loss of RGS14 may allow activated Gβγ to bind free Gαi to form an inactive complex, thus terminating any Gβγ-mediated effects on calcium channels.

Alternatively, RGS14 actions on synaptic plasticity in CA2 neurons may be linked to its capacity to bind H-Ras, Rap2 and Raf kinase to regulate MAP kinase signaling. Both H-Ras and Rap2 are implicated in different forms of synaptic plasticity in the hippocampus. Expression of active Rap2A in hippocampal neurons of mice results in spatial learning deficits that are coupled with increases in JNKinase activity, decreases in synaptic AMPA receptor activity and dendritic branch complexity, and an enhancement of long-term depression (LTD) of neurotransmission (Fu et al., 2007; Ryu et al., 2008; Zhu et al., 2005). By contrast, H-Ras enhances AMPA receptor-directed synaptic transmission and induces up-regulation of AMPA receptors on spine surfaces (Zhu et al., 2002), while other studies show that Ras regulates LTP in hippocampal neurons by

facilitating NMDA receptor phosphorylation (Manabe et al., 2000). Furthermore, transgenic mice that express active H-Ras exhibit enhanced spatial learning and LTP (Kushner et al., 2005), a phenotype similar to the RGS14-KO mice (Lee et al., 2010). These findings with Ras, when considered alongside our observation that the nascent LTP caused by the loss of RGS14 is blocked by MEK inhibitors, suggests that RGS14 modulates synaptic plasticity through the inhibition of Ras/Raf/MAP kinase signaling pathways in hippocampal neurons (Figure 3.4) (Lee et al., 2010). Of note, CA2 neurons express high amounts of the EGF receptor (EGFR) and basic-FGF receptor, FGF1R, both tyrosine kinase receptors that activate H-Ras and ERK signaling (Lein et al., 2005a; Lein et al., 2004). However, H-Ras-mediated ERK signaling is also directly activated by G proteins, calcium, and/or cAMP regulated pathways (Kennedy et al., 2005), so RGS14 may serve as a brake on ERK signaling downstream of any of these pathways

Despite this wealth of information, much remains unknown about negative regulation of synaptic plasticity and the role of RGS14 in this process. Although evidence suggests a role for H-Ras/MEK/ERK signaling, we cannot rule out the possibility that RGS14 suppresses LTP in CA2 neurons through modulation of conventional or unconventional Gαi/o signaling, Rap2 signaling, or some combination of these and H-Ras/MEK/ERK signaling. Binding of RGS14 to these proteins may either inhibit and/or redirect their signaling to alter regulation of synaptic plasticity. Studies are ongoing to determine which of these pathways underlie RGS14-mediated suppression of synaptic plasticity in CA2 neurons.

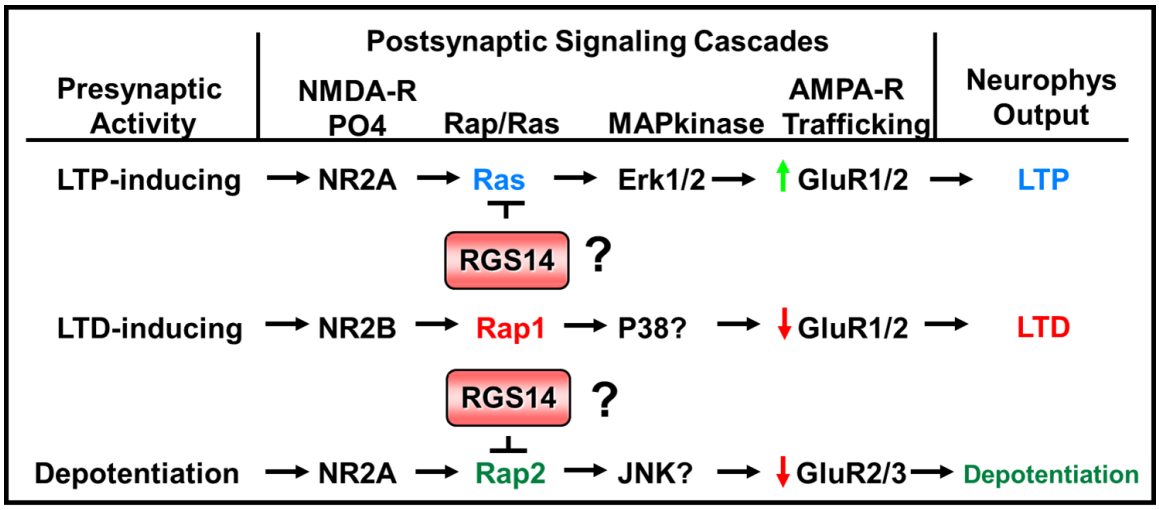


Figure 5.2 Proposed role for RGS14 in modulating Ras/Rap2 signaling. RGS14 may act by modulating the signaling activitiy of its binding partners. RGS14 may negatively regulate Ras activity leading to decreases in LTP in cells that express RGS14. RGS14 also binds Rap2 and may modulate its signaling.

Compelling evidence now indicates that RGS14 is a multifunctional scaffold that integrates G protein and MAP kinase signaling pathways important for synaptic plasticity in CA2 hippocampal neurons. While much is known about RGS14 binding partners and how they interact, more studies are needed to examine how these proteins and RGS14 may work together to suppress hippocampal synaptic plasticity in CA2 neurons. RGS14 can be added to a growing list of genes/proteins that have been linked to enhanced cognition (Lee and Silva, 2009). The challenge going forward will be to determine how RGS14 fits into these key pathways to suppress LTP and how this process is regulated. Besides these signaling proteins involved with enhanced cognition, other GPR proteins that share similarities with RGS14 are also important for brain function. The *m*ammalian *p*artner of *ins*cutable (mPins, aka LGN) and AGS3 both contain GPR domains that bind Gαi/o-GDP to stabilize their association with membranes, are regulated by Ric-8A, and are enriched in brain. AGS3 is localized within neurons throughout most of the CNS, including the hippocampus (Blumer et al., 2002), and AGS3 in the prefrontal cortex and nucleus accumbens is reported to be important for cocaine-seeking and ethanol-seeking relapse behavior, respectively (Bowers et al., 2008; Bowers et al., 2004). mPins/LGN is enriched in synaptic membranes of CA1 hippocampal neurons, where it associates with PSD-95 and MAGUK scaffolding proteins in a Gαi1-dependent manner to influence their trafficking, NMDA receptor surface expression, and dendritic remodeling (Sans et al., 2005). RGS14 and its binding partners in CA2 neurons likely serve roles mechanistically similar to, though functionally distinct from

those of mPins/LGN and AGS3 in brain physiology. Together, these proteins and RGS14 represent a novel class of G protein binding partners important for brain physiology/disease that could serve as future therapeutic targets for a range of CNS pathologies.

5.5 Future Studies

Discrete mutations in the functional domains of RGS14 disrupt binding and interaction of RGS14 with its binding partners. These mutants could be used to determine the possible signaling pathways that are modulated by RGS14. A lentivirus expressing EGFP-RGS14 and injected into rodent brain is being used to determine if the overexpression of full length RGS14 further impairs LTP in Schaffer collateral CA2 neurons. Point mutants could be injected into rodent brain to determine which functional domain is responsible for the function of RGS14. The mutants disrupt binding within each domain without altering interactions of the other binding partners. A Double point mutation, R92A,N93A, in the RGS domain prevents the interaction of RGS14 with Gαi/o-GTP. Loss of Gαi/o-GTP binding would prolong inhibitory signaling through the Gαi/o-GTP until intrinsic GTPase activity or another RGS protein terminates signaling. Interaction with Ras and Raf occurs via the RBD domains, and alteration of arginine at position 333, R333L, prevents RGS-Ras binding. I think it is likely that the RBD is the region responsible for the suppression of LTP in WT mice. RGS14-KO mice are missing this domain and may have prolonged Ras/Raf/MAP kinase

signaling following stimulation even though there is no change in basal levels of phosphorylated ERK1/2, which are downstream of MEK. Additionally CA2 LTP in RGS14-KO is blocked by inhibiting MEK signaling. The final mutant, Q515A,R516F, in the GL/GPR domain loses its ability to bind to inactive Gαi1/3. Losing this interaction may indirectly shorten the duration of Gβγ signaling because the GPR domain sequesters Gαi1/3 and prevents the reassociation of the heterotrimer. If Gα-GDP is free, it will likely reform the heterotrimeric complex.

Another important future experiment is to examine if RGS14 is found in postsynaptic densities. Preliminary evidence from detergent insensitive protein fractions and immuneperoxidase EM indicates that RGS14 is at the PSD (Hollinger et al., 2001; Lee et al., 2010). Immunogold labeling EM and purification protocols to purify PSDs using protein samples from mouse brain could conclusively pinpoint if RGS14 is indeed found at the PSD.

RGS14 appears to reduce seizure susceptibility in the pilocarpine model of status epilepticus. Following up these finding is an important future direction. Pilocarpine acts as a non-selective muscarinic receptor agonist. Effects observed in RGS14-KO mice could be related to receptor activation and signaling rather than alterations in synaptic activity. Perhaps a better model would be to observe spontaneous seizures following initial pilocarpine administration or to use the kindling method to induce seizures.

RGS14-KO mice perform better in tests of hippocampal-dependent memory and exhibit an increase, albeit not a significant one, in anxiety in the elevated plus maze. Although RGS14-KO mice did not exhibit deficits in extinction using the water maze, testing fear learning in RGS14-KO mice is an important follow-up study. RGS14-KO may exhibit a differential response following conditioned or cued fear learning. Tests of the olfactory system are also important since RGS14 is expressed in the olfactory cortex and in the olfactory bulb.

5.6 Pharmacological Relevance

Cognitive impairments are associated with a large number of neurological disorders including Alzheimer's and Parkinson's disease, Schizophrenia, depression, mental retardation, and learning disabilities. These disorders stem from a large number of deficiencies and genetic alterations even within the same type of disorder. The diverse etiology and mechanisms responsible for deficits make creating targeted and specific therapeutics to treat each disorder difficult, costly, and may limit the drug's profitability. Therapeutics that enhance cognition in a more general way are an attractive alternative to targeted therapeutics. RGS14 may be a good candidate for improving overall cognition without adverse side effects. Mice missing the full-length RGS14 gene and protein exhibit enhanced cognition with no apparent adverse phenotypes. One caveat, however, is that the functional domain responsible for the action of RGS14 is

unknown. Further studies are needed to determine the best approach for blocking RGS14 function.

5.7 Summary

In summary, RGS14 is a natural suppressor of signaling pathways critically involved with regulating synaptic plasticity, and its host CA2 neurons may represent a newly recognized module that functions either distinct from, or in concerted action with, the canonical trisynaptic circuit to mediate hippocampal-based learning and memory. Given the very discrete protein expression pattern of RGS14 and the fact that RGS14-KO mice exhibit no obvious deleterious phenotypes, inhibition of RGS14 function may serve as an attractive therapeutic target for future cognitive enhancers (Blazer and Neubig, 2008; Lee and Silva, 2009). Evidence presented here points to the function of RGS14 in the brain as a “Homer Simpson” gene. The presence of RGS14 in mice reduces the capacity for LTP in the CA2 and limits performance in learning and memory tasks. The question that remains, is does RGS14 act as a true inhibitor of learning, memory and LTP? One possibility is that the CA2 acts as a filter to screen input received by the brain and determine which information is important enough to consolidate and store. RGS14-KO mice do not have deficits in extinction or forgetting, but they may more efficiently store or simply store more incoming sensory input. Storing more information may not be an advantage in that we are inundated daily with information that is not important. How our brain decides what information

should be consolidated is not known. Future studies could examine fear learning in RGS14-KO mice since memories associated with fearful events tend to be vivid and stored long term.

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