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Modulation of Synaptic Plasticity: Endocannabinoids and Novel G-protein Coupled Receptors Expression and Translational Effects in Interneurons

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ABSTRACT

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Learning and memory are important processes that occur in the brain. The brain is comprised of neurons that make connections with each other known as synapses. Synaptic plasticity is widely believed to be the physiologic mechanism by which learning and memory occur. Synapses can either be strengthened through a process known as long-term potentiation (LTP) or weakened through long-term depression (LTD). The area of the brain that is most studied for its role in learning and memory is the hippocampus, which has been shown to be involved in memory consolidation. The detection of endocannabinoids and their receptors has opened a whole new field of study in regards to synaptic plasticity. Cannabinoid receptor 1 (CB1) and transient receptor potential vanilloid 1 (TRPV1) are among the commonly studied endocannabinoid receptors found in the central nervous system. In the brain, these receptors' natural ligands, anandamide and 2-arachidonylglycerol (2-AG), are found in abundance. Yet not all forms of observed plasticity are accounted for by just these two receptors, so studies into other G-protein coupled receptors (GPCRs) continues. One GPCR, GPR55 is found in many regions of the brain, as well as lysophosphatidylinositol (LPI), its specific ligand. Here we have researched the role of GPR55 in modulating synaptic plasticity in the hippocampus. Using quantitative reverse transcription PCR and immunohistochemistry, we have found GPR55 to be expressed in the hippocampus with highest expression in pyramidal cells, the main excitatory neurons in the hippocampus. Using field and whole cell electrophysiology, we have investigated its effects on synaptic plasticity, discovering that activation of GPR55 by LPI significantly enhances LTP. In memory behavioral assays there are no significant differences between GPR55 KO mice and wild type littermates, indicating that it may not be involved in endogenous memory processes. However, our electrophysiology data makes GPR55 a potential target for treating memory disorders such as dementia.

We have also investigated GPR18 and GPR119 for their potential roles in synaptic plasticity. First, we confirmed their expression in the hippocampus and then investigated the effects of their agonists on plasticity. Another receptor, TRPV1 has been studied to alter plasticity. However, the study of how protein translation and RNA transcription involvement in TRPV1 plasticity in mammals has not been investigated. While translation and transcription are known to be important in many forms of LTP, it is unknown whether these processes are important for TRPV1-induced LTD. We are investigating their necessity via whole cell patching and using translation and transcription inhibitors Anisomycin and Actinomycin D, both previously used in slice electrophysiology.

Key words: synaptic plasticity, LTP, LTD, eCBs, GPR55, LPI, GPR18, GPR119, TRPV1

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CHAPTER 1: Introduction

Learning and memory are processes that are often taken for granted until these abilities are diminished or lost. Within the brain, the hippocampus has been identified as the structure primarily responsible for short-term declarative memory. When the hippocampus does not function properly, the ability to learn, the formation of new memories and spatial navigation are lost [92]. The hippocampus is organized into subfields called CA1, CA2, CA3 and CA4. The CA3 pyramidal cells extend axons, known as Schaffer collaterals, into the CA1 region. The organization of hippocampal circuitry has guided research in learning and memory; every circuit and every cell type plays an important role.

However, memories are not stored within cells—there isn't a cell for every memory or a new cell formed for each new experience. Rather, the physical, experience-dependent changes in the brain occur at the connections between neurons, called synapses. The ability of synapses to change in response to new stimuli or experience is known as synaptic plasticity.   In general, a synapse can experience plasticity as either a strengthening or weakening of the connection between neurons, known respectively as potentiation or depression. These changes can last a few minutes (i.e. short term potentiation or depression), or they can last an hour or more, called long-term potentiation (LTP) or long-term depression (LTD). Synaptic plasticity has been studied extensively since LTP was first described in 1973 [1]. LTP can also be separated into early-phase LTP, lasting a few hours, and late-phase LTP, extending for several hours and days. The more engaging an experience is, or the more thorough the repetition, the more synapses are activated, leading to the induction of plasticity at hippocampal synapses.

LTP and LTD are due to changes that occur in presynaptic and postsynaptic neurons in response to changes in activation. Plasticity affects the amount of postsynaptic depolarization that occurs in response to a given stimulus. LTP corresponds with an increase in neurotransmitter production and release presynaptically [2], and/or an increase in receptors in the postsynaptic membrane. In the case of excitatory neurotransmission, the excitatory neurotransmitter glutamate is released presynaptically to activate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are the main receptors responsible for depolarizing neurons postsynaptically [3]. When LTD occurs the result is a decrease in neurotransmitter release or decrease in receptors at the synapse, thus reducing neurotransmission, and post-synaptic depolarization, at that synapse

These differing forms of plasticity are induced by various receptors and signaling pathways. For example, some necessary mediators common to LTP include glutamate, N-methyl-D-aspartate (NMDA) receptors and intracellular calcium signaling. Glutamate released by the presynaptic neuron will activate AMPA and NMDA receptors on the post synaptic nerve depolarizing the nerve and allowing calcium to enter the cell and activate second messenger systems that will initiate insertion of AMPA receptors or retrograde signaling [4]. Some mediators of LTD that have been identified are Cannabinoid 1 receptor (CB1)[5], which is activated endogenously by anandamide, and Transient receptor potential vanilloid 1 (TRPV1)[6]. Alternatively, modulators, such as some types of G-protein coupled receptors (GPCRs), can enhance or reduce synaptic plasticity. This makes them potential targets for therapeutic agents because they do not completely disrupt or activate LTP or LTD.

GPCRs and endogenous lipid signaling molecules known as endocannabinoids (eCBs) are common mediators and modulators of synaptic plasticity [7]. Recently three orphan G-protein coupled receptors were characterized, and labeled GPR55, GPR18 and GPR119, which have great potential as modulators of synaptic plasticity as they are potential targets for eCBs. Studying their expression patterns, electrophysiology and behavioral effects can increase our understanding of eCBs and GPCRs involvement in learning and memory.

GPR55 is expressed in the human brain [36], and it binds several eCBs; anandamide, 2-arachidonylglycerol (2-AG), as well as binding to delta-9-tetrahydrocannabidiol (THC), the active component of marijuana [41]. In addition a specific endogenous ligand, lysophosphatidylinositol (LPI) [81], has been identified. Recent data also denotes GPR55 has a physiological effect in the hippocampus. LPI increases the frequency of miniature excitatory postsynaptic currents via release of presynaptic Ca^{2+} stores [45] suggesting that GPR55 activation leads to an increase in neurotransmitter release probability presynaptically. In addition to GPR55, there are also a couple other GPCRs with potential to modulate hippocampal synaptic activity.

GPR18 has been studied in microglia and dorsal root ganglia, but its expression in the brain and its role in neuronal function are not well understood. Research of GPR18 in dorsal root ganglion exhibited a block of heat-evoked firing of nociceptive neuron by N-palmitoyl glycine [72]. N-arachidonyl glycine, another GPR18 ligand, is also a potent activator of microglia [73]. Another GPCR, GPR119, binds anandamide and 2-AG [75, 76], two of the most abundant eCBs found in the brain. In rodents GPR119 is expressed in the pancreas, intestines and brain. There has been some research on GPR119 and its role with metabolism and effects in the

pancreas and intestines but it has not been investigated and very little is known regarding GPR119 in the CNS. GPR18 and GPR119 are candidate receptors that modulate eCB-mediated synaptic plasticity, because they bind several eCBs and are expressed in the central nervous system.

While TRPV1 is different from these GPCRs it has been studied as a mediator of interneuron LTD which has a disinhibiting effect in the hippocampus [17]. This disinhibition allows for enhanced LTP at the CA3 to CA1 connections, and understanding its mechanisms of action can also benefit our knowledge of how synaptic plasticity can occur and be effected by different receptors and molecular processes, such as translation and transcription.

Translation and transcription have been shown to be necessary for late-phase LTP in hippocampal neurons [65]. There is a significant decrease in LTP 30 minutes post stimulation when translation is inhibited by Anisomycin and there is a similar reduction in potentiation when transcription is blocked with Actinomycin D 90 minutes after initiation (see figure 1.1) [65]. Recently translation and transcription were investigated in TRPV-like receptor induced LTD in leeches, where transcription and translation were found to be necessary postsynaptically and translation is required presynaptically for the induction of LTD [63]. Examining whether these processes are necessary in mammal induction of TRPV1 dependent LTD may shed light on their involvement in eCB mediated or modulated synaptic plasticity.

As eCBs play a relatively new and less understood role in synaptic plasticity it is critical to understand these novel eCB receptors' functions in hippocampal plasticity and thus learning and memory. The hippocampus is highly organized and known to be involved in memory consolidation so it is a great region to continue researching how synaptic plasticity is mediated

or modulated by the various receptors and signaling molecules that have been identified. By investigating these novel eCB receptors and their role in hippocampal function we will attain better understanding of normal hippocampal function and divulge any potential treatment of neurodegenerative diseases.

Specific Aims

As the number of people suffering from neurodegenerative diseases increases the need for treatment options does as well. In the quest for better understanding the processes of learning and memory we sought to understand eCBs and their receptors as potential modulators of synaptic plasticity, with the hope of furthering research in treatments for neurodegenerative diseases. GPR55 was recently identified. We investigated its mRNA expression patterns using reverse transcription quantitative PCR (qRT-PCR) as well as protein expression and location using Immunohistochemistry (IHC). We also investigated physiological effects utilizing field and whole cell electrophysiology. We compared GPR55 KO and wild type (WT) mice littermates with and without the application of agonists and antagonists. Lastly we analyzed differences between the GPR55 KO and WT mice in four diverse behavioral tasks. Examining memory, spatial navigation and anxiety with novel object recognition, Morris water maze, radial arm maze and elevated plus maze.

We have also begun research into GPR18 and GPR119, first examining their expression in the hippocampus. Using qRT-PCR we have shown their expression in mouse hippocampus. With GPR18 we have also been able to show its protein expression using IHC. We are currently researching these receptors and their potential effects on physiology.

To further understand the mechanisms of TRPV1 and induction of LTD in interneurons in the hippocampus of mammals we are investigating the effects of translation and transcription inhibitors using whole cell electrophysiology in rat hippocampal slices.
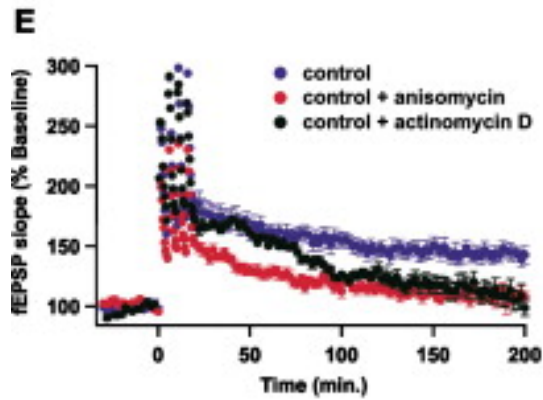
Figure 1.1: Translation and Transcription are Necessary for Late-phase LTP [65].

CHAPTER 2: A Putative Lysophosphatidylinositol Receptor GPR55 Modulates
Hippocampal Synaptic Plasticity

Abstract

GPR55, an orphan G-protein coupled receptor, is activated by lysophosphatidylinositol (LPI) and the endocannabinoid anandamide, as well as by other compounds including THC. Such signaling molecules are capable of modulating synaptic plasticity. LPI is a potent endogenous ligand of GPR55 and neither GPR55 nor LPIs' functions in the brain are well understood. While endocannabinoids are well known to modulate brain synaptic plasticity, the potential role LPI could have on brain plasticity has never been demonstrated. Therefore, we examined not only GPR55 expression, but the role its endogenous ligand could play in long-term potentiation, a common form of synaptic plasticity. Using quantitative RT-PCR, electrophysiology, and behavioral assays, we examined hippocampal GPR55 expression and function. qRT-PCR results indicate that GPR55 is expressed in hippocampi of both rats and mice. Immunohistochemistry and single cell PCR demonstrates GPR55 protein in pyramidal cells of CA1 and CA3 layers in the hippocampus. Application of the GPR55 endogenous agonist LPI to hippocampal slices of GPR55$^{+/+}$ mice significantly enhanced CA1 LTP. This effect was absent in GPR55$^{-/-}$ mice, and blocked by the GPR55 antagonist CID 16020046. We also examined paired-pulse ratios of GPR55$^{-/-}$ and GPR55$^{+/+}$ mice with or without LPI and noted significant enhancement in paired-pulse ratios by LPI in GPR55$^{+/+}$ mice. Behaviorally, GPR55$^{-/-}$ and GPR55$^{+/+}$ mice did not differ in memory tasks including novel object recognition, radial arm maze, or Morris water maze. However, performance on radial arm maze and elevated plus maze task suggests GPR55$^{-/-}$ mice have a higher frequency of immobile behavior. This is the first demonstration of LPI involvement in hippocampal synaptic plasticity.

Introduction

The hippocampus plays a vital role in learning and memory for humans and other

mammals and has interconnections with cortical association areas. It has been implicated in

many processes, including memory consolidation of recent events [8], declarative memory [9],

and encoding spatial and contextual information [10]. The cellular mechanism of synaptic

plasticity likely underlies these events. Synaptic plasticity includes enhancements in activity

known as long-term potentiation (LTP) [11, 1] and decreases in activity known as long-term

depression (LTD) [12]. LTP strengthens CA1 hippocampal synapses by increasing the number of

postsynaptic glutamate receptors and enlarging the synapse [4]. On the other hand, LTD

decreases the number of postsynaptic glutamate receptors [13,14]. While the role N-methyl-D-

aspartate (NMDA) receptors have in many forms of synaptic plasticity is well established, recent

studies indicate lipid-based signaling molecules such as endocannabinoids (eCBs) also are

involved in plasticity via cannabinoid receptor 1 (CB1)[15,16] and transient receptor potential

vanilloid 1 (TRPV1) [17, 18, 19, 6]. The classified eCB receptors, CB1 and CB2, are activated by

endogenous eCBs such as 2-arachidonlyglycerol (2-AG) and anandamide (AEA). However, lipids

such as eCBs mediating hippocampal plasticity via mechanisms independent of CB1/CB2/TRPV1

is clear [20, 21, 22]. Similarly, a CB1/TRPV1-independent AEA-induced depression of excitatory

transmission onto hippocampal stratum radiatum interneurons was demonstrated [23]. In

addition, hippocampal AEA and 2-AG are present in high concentrations, but their effects are

not fully explained by CB1 alone [24]. Therefore, additional uncharacterized receptors or lipid-

based signaling molecules are involved in modulating hippocampal synaptic plasticity. One such

potential lipid-signaling molecule is lysophosphatidylinositol (LPI), whose role is plasticity is unknown to date, but which activates the orphan G-protein coupled receptor GPR55.

Many have suggested the presence of a putative "CB3" receptor [25] with GPR55 being one potential candidate [26, 27]. In non-nervous tissues, GPR55 is characterized as a promoter of cancer cell proliferation [28], a regulator of osteoclast number and function [29], and a modulator of inflammatory and neuropathic pain [30]. The role of GPR55 in the PNS/CNS, however, has only been demonstrated recently. In the CNS, GPR55 is involved in neuroprotection [31], hyperalgesia [30], motor coordination [32], pain perception [33], and axon innervation/guidance [34, 35]. GPR55 is widely distributed in the human brain [36] and in several regions of rodent brain [37,38], including the hippocampus [32]. Endogenously, GPR55 is activated by AEA [39], 2-AG, and LPI, the latter being a specific, natural ligand for GPR55 [40], which requires further investigation. GPR55 initiates a cascade which increases intracellular calcium [41], including in the dorsal root ganglion [42, 27], likely through a RhoA-dependent mechanism [43]. GPR55 also enhances neurotransmitter release in the hippocampal CA1 region [44]. GPR55 wild-type and knock-out mice were examined for potential effects on hippocampal plasticity, but no differences were noted [32]. However, activating GPR55 with agonists such as LPI were never examined in that report. In addition, GPR55 is known to enhance internal calcium release so examining the potential role of LPI on synaptic plasticity when targeting the lysophosphatidylinositol receptor GPR55 would demonstrate a novel role for LPI in the brain.

Finally, the widespread expression of GPR55 and its ligand LPI makes discovering their function and mechanism of action within the hippocampus pressing research, as it may

modulate learning and memory systems in mammals. Therefore, we examined the significance of GPR55 on hippocampal memory at the molecular, physiological, and behavioral level. We hypothesized that GPR55 is a modulator of hippocampal plasticity. Here we present evidence, supporting GPR55 as a lysophosphatidylinositol receptor capable of modulating hippocampal plasticity.

Materials and Methods

The experiments were conducted in accordance with the Brigham Young University Institutional Animal Care and Use Committee standards and National Institute of Health guidelines to minimize pain and suffering of animals. Male Sprague-Dawley rats used for PCR were aged P20-27 (Charles River) and male GPR55 knockout and littermate wild type mice (Lexicon Pharmaceuticals via the Mutant Mouse Regional Resource Center through University of North Carolina) used for PCR, physiology and behavior were aged P14-105. Different cohorts of mice were used for each behavioral experiment to avoid confounding influences of prior exposures. Animals were housed in approved conditions with a 12-hour light-dark cycle.

Preparation of Brain Slices

All mice used for electrophysiology were deeply anesthetized with isoflurane using a rodent vapomatic chamber and decapitated, after which their brains were removed rapidly and placed in ice-cold, oxygenated artificial cerebrospinal fluid (ACSF) medium containing (in mM): NaCl, 119; $NaHCO_3$, 26; KCl, 2.5; NaH_2PO_4, 1.0; $CaCl_2$, 2.5; $MgSO_4$, 0.6; glucose, 11; saturated with 95% O_2, 5% CO_2 (pH 7.4). The posterior aspect of the brain was cut into 400 μm coronal

slices using a vibratome, and then transferred to a holding chamber containing oxygenated

ACSF at room temperature.

Slice Electrophysiology

Following an interval of at least 1 hour, slices were transferred to a submerged

recording chamber and perfused with oxygenated ACSF of the same composition as that in the

holding chamber at a temperature between 28-32°C. Slices were continuously perfused with

ACSF at a flow rate of 2-3 ml/min. A bipolar stainless steel stimulating electrode was placed in

the stratum radiatum, at least 400-700 μm from the recording electrode to stimulate CA3

glutamatergic afferents of the Schaffer Collateral pathway at 6-50 μA for 100 μsec at 0.1Hz.

Recordings were performed in current clamp mode to measure excitatory postsynaptic

potentials using an Axopatch 200B or 700B amplifier. Data was filtered at 4 kHz, acquired with

an axon 1440A digitizer (Molecular Devices), and inputted onto a Dell personal computer with

pClamp10.4 Clampex software (Molecular Devices). Stimulation intensity was adjusted to elicit

an EPSP of 0.5 to 0.7 mV at the beginning of each experiment. Borosilicate glass patch pipettes

(2-3 MΩ) were filled with 1 M NaCl for field recording electrodes. EPSPs were evoked and

monitored for at least 60 minutes.

Field recordings (mice; P16-35) were analyzed as noted previously [17]. Briefly, the

EPSPs slopes was calculated using pClamp10.2 Clampfit software (Molecular Devices). Values

were normalized to control slope values 5-10 minutes immediately prior to theta burst. Theta

burst was used to mimic more natural hippocampal activation patterns. Two bursts were given

20 seconds apart. An increase in EPSP slope that persisted for longer than 60 min indicated that

LTP had been induced. EPSP normalized slope values 20-25 min post high frequency stimulus

were compared to baseline for significance (unpaired, two tailed t-test). Only one experiment was performed per slice, and the reported N is the number of slices not the number of animals. In general, 1-3 slices were used per animal. Microsoft Excel and Origin (North Hampton, MA) were used to organize, average, graph, and perform statistical analysis on the data.

Whole-cell recordings (mice; p14-26) were performed in voltage clamp at -65 mV using a Multiclamp 700B amplifier (Molecular Devices) and EPSCs were evoked by two pulses separated by 50msec. Borosilicate glass patch pipettes (2-6 MΩ) were filled with Cs^+-gluconate based internal solutions containing (in mM): NaCl, 2; $MgCl_2$, 5; HEPES, 20; ATP, 2; GTP, 0.3; QX 314 bromide, 1 and EGTA, 0.6. High divalent ACSF in mM: NaCl, 119; $NaHCO_3$, 26; KCl, 2.5; NaH_2PO_4, 1.0; $CaCl_2$, 2.5; $MgSO_4$, 1.3; glucose, 11; saturated with 95% O_2, 5% CO_2 (pH 7.4) AMPAR-mediated currents were measured while blocking $GABA_A$ receptors with picrotoxin (10μM). Stable baseline recordings of AMPAR-mediated currents were obtained at the frequency of 0.1 Hz. The cell input resistance was monitored throughout the experiment, and if changed by more than 10%, the cell was discarded. Interneurons were distinguished from pyramidal cells visually by their location in the radiatum and electrophysiologically by their higher input resistance. After 10 minutes of a consistent baseline, LPI (4 μM) was applied in the bath for 10 minutes and the recording continued for as long as the cell was stable. Peak glutamate responses were normalized to control values 5-10 minutes immediately prior to application of LPI. Microsoft Excel and Origin were used to organize, graph, and perform statistical analysis on the data.

For paired pulse ratio (PPR) determination, we first measured control conditions at different interpulse intervals ranging from 10 ms to 500 ms. Subsequently, in the same slice, we

then added LPI (4µM) to the perfused ACSF for 10 min and again examined PPRs in both

GPR55$^{+/+}$ and GPR55$^{-/-}$ mice and compared these to initial results.

All physiological experiments for both control and experimental variable took into

account differences in animal age, time of slice experiment since cutting and other

considerations to ensure unrelated variables did not contribute to differences seen in the

results.

Quantitative RT-PCR

All PCR reactions (rat/mouse; P20-35) for whole brain, hippocampus homogenate and

whole cell as well as primer and probe designs were prepared as described previously [46]. The

mRNA from whole brain and hippocampal homogenate was isolated using Trizol, while single

cells proceeded directly to reverse transcription. The iScript cDNA synthesis Kit (BioRad) was

used to convert mRNA to cDNA. The GPR55 cDNA from whole brain and hippocampal

homogenates was amplified in a dose dependent manner. After amplification, GPR55 cDNA

from rat whole brain and rat and mouse hippocampal homogenates was run out on a 4%

agarose gel illustrating the appropriate amplicon size (Figure 1 insets). The mouse GPR55 cDNA

band was then sequenced to confirm it was indeed the GPR55 receptor. The cDNA from cells

were pre-amplified in a multiplex reaction with 10-fold diluted primers, in a C1000

Thermocycler (BioRad). The preamplified samples were then run with the probe (Invitrogen) in

triplicate on a CFX96 qPCR machine (BioRad). For Rat GPR55 sequences we used two different

primer sets designed around the same probe to confirm GPR55 was the real target being

amplified. The first set was forward primer GTCGTCTTCGTGGTCTCCTT, reverse primer

GATGTTAGAGAAACACAGAGACAACTG, and probe TCCCAGTGCACCTGGGTTTGTTC with the

second set employing an alternate reverse sequence of CAAGATAAAGCCGTTCCTTACC. Mouse GPR55 forward primer sequence was CAGGGAAGTGGAGAGATACAAGTG, reverse primer GGGAAGGAGGAAGCCAAAG, and probe TTTCACAACATGTCGGATGTCACCTGG.

Immunohistochemistry

Mice used for immunohistochemistry were either GAD67-GFP knock-in, or GPR55$^{+/+}$ and GPR55$^{-/-}$ littermates. Brains were transcardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were dissected out, cryoprotected in 30% sucrose solution, frozen in OCT, sliced into 30 μm sections, and collected into 0.1 M PBS for a free-floating staining procedure. Slices were permeabilized with 0.2% Triton-X (Fisher Bioreagents) for 30 minutes, washed with 5% normal goat serum and 1% bovine serum albumin in 0.1 M PBS for 2 hours, and treated with primary antibody for anti-GPR55 (1:500; rabbit polyclonal; AB_2617111; Dr. Ken Mackie) in 5% normal goat serum and 1% bovine serum albumin in PBS overnight at 10 °C. Slices were then washed twice with 0.1 M PBS, followed by one wash of 0.2% Triton-X (Fisher Bioreagents) in 0.1 M PBS for 30 minutes, one wash of 5% normal goat serum and 1% bovine serum albumin in 0.1 M PBS for 2 hours, and a final wash of anti-rabbit secondary antibody (1:500, AlexaFluor 546, Invitrogen) in 5% normal goat serum and 1% bovine serum albumin in PBS for 2 hours at room temperature. Slices were washed three times with tris-buffered saline and mounted onto Superfrost Plus microscope slides (VWR). After drying overnight, slides were coverslipped with DAPI Fluoromount-G (Southern Biotech) and imaged on an Olympus FluoView FV1000 laser scanning confocal microscope including Z stack microscopy. Image capture was performed by sequential excitation of each fluorophore.

Novel Object Recognition

For the novel object recognition test, mice 1.5-2 months old were placed in a large, round, 45-centimeter diameter container with bedding material for 10 minutes each day for 4 days. Four different objects were used: a red funnel, a blue retainer case, a yellow rubber duck, and a fist-sized rock. Day 1 was acclimation, and no objects were introduced. Days 2 and 3, three random objects were introduced for the mice to explore. Day 4, one of the three objects was switched with a novel object, and the time each mouse spent with each object was recorded. Time spent with an object was defined as touching or nose pointing towards the object within approximately 2 cm. The objects were rotated so that not all mice had the same three objects on days 2 and 3. Likewise, the switched object was varied to account for any bias towards a certain object. Statistics were done using Excel two-tailed unequal variance t-test and graphed in Origin. The data points compared were the novel object recognition index (time spent with the novel object divided by total time spent with all objects) between GPR55$^{+/+}$ and GPR55$^{-/-}$ mice.

Radial Arm Maze

An eight arm radial maze (67 cm diameter) was constructed out of 1 cm thick plastic. Each arm was 8.9 cm wide, 22.9 cm long, and 17.8 cm tall with a 2.5 cm hole at each end. Various pictures served as visual cues at the end of each arm. Testing consisted of 6 trials per day, 5 days per week, for 7 weeks. Week 1 consisted of acclimation sessions where all 8 arms were baited with cheddar cheese. After the first week, the same 2 arms were baited with cheese. Mice were given 6 trials each day to learn the maze and between each trial the maze

was cleaned. Each trial was considered complete at consumption of both pieces of cheese or after 3 minutes.

During trials, video was captured and analyzed using ANY-maze software (Stoelting) to determine the distance traveled, time to completion and time spent immobile, which was designated as over 200 ms in one location. Two types of errors were analyzed: working memory errors and reference memory errors. Working memory errors occurred when an arm was entered more than once in a trial, and reference memory errors occurred when an arm not associated with food was entered.

Mice were 1.5-2 months old when trials were initiated. Mice were given food ab libitum until testing started; then food was restricted to 4 hours a day starting between 5 pm and 7 pm Monday-Thursday. Mice were weighed at the beginning and end of each week during the testing period to ensure healthy body weight maintenance. If mice lost more than 15% of their body weight, they were given extra time to feed. Mice had complete access to food Friday night through Sunday night. For statistics an average of each animal's time, errors, distance traveled, and time immobile for each week were compared using Excel two-tailed unequal variance t-test and graphed in Origin.

Morris Water Maze

Mice ranged in age from 1.5 to 2 months. The water maze consisted of a large, circular pool (1.67 m in diameter, .4 m in height). A clear platform (7.6 cm in diameter) was placed inside, and the tank was filled with water (22°C) until the top of the platform was submerged by 1 cm. A sufficient amount of powdered skim milk (Augason Farms) was added to ensure that the water was opaque in order to conceal the platform. Four sheets of paper with black and

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white geometric designs served as visual cues and were displayed in four quadrants around the pool. Swim paths were recorded by digital overhead camera (HeroHD) for each subject, and time to platform was recorded using a stopwatch. Pre-training acclimation sessions, consisting of free swim in the pool for 5 min without the platform, were performed the day before acquisition trials began. Initially, all mice swam around the perimeter of the pool and spent progressively less time there as the pre-training session continued. Mice were observed during the 5 min pre-training session before data collection to identify any phenotypic differences in their initial reactions to being placed in water. Two of the GPR55$^{-/-}$ and two of the GPR55$^{+/+}$ mice had more labored swimming, characterized by rapid, jerky movements, but improved over subsequent training sessions. No other health or motor deficits were observed. The training session consisted of 7 consecutive days where mice searched for the stationary platform four times from four different start points, designated North, South, East, and West, and the two groups were compared to see if GPR55 played a role in learning spatial navigation. Acquisition trials occurred over seven consecutive days of four trials per day. The position of the platform remained fixed for all trials for all mice days 1-7. Four points along the periphery of the pool served as trial start points designating North, South, East, and West, where the mice would be placed facing the wall of the pool. Each starting point was used once per session, with order determined arbitrarily by the tester. Once the platform had been located, the mouse was allowed to stay for 30 s before removal. If a subject failed to locate the platform in less than 120 s, it was manually guided to the platform. After the initial seven acquisition sessions, mice were subjected to a reversal test in which the platform was moved to the opposite side of the pool. All other task parameters remained the same. For statistics time to platform and time in

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quadrant were compared between genotypes using an ANOVA and two-tailed unequal variance t-test and graphed in Origin.

Elevated Plus Maze

The elevated plus maze task was conducted using the radial arm maze with 4 of the 8 arms blocked. Two additional sets of walls were made out of the same material to enclose 2 of the 4 arms being used for the elevated plus maze. The maze was placed on a small stool elevating it 80 cm.   Mice were 1.5-2 months old when the trials were conducted. Each mouse was placed in the center of the maze at the beginning of the trial and given 8 minutes to explore the maze. Videos of the trials were recorded and analyzed using ANY-maze software to determine distance traveled, time spent immobile, time spent in open arms, and time spent in closed arms. For statistics each animal's distance traveled, time immobile, time in open arms, and time in closed arms were compared. All statistics were analyzed using a two-tailed unequal variance t-test and graphed in Origin.

Materials and Solutions

All salts were purchased from Sigma-Aldrich, Mallinkrodt-Baker, or Fisher Scientific. Picrotoxin, CID16020046 and Lysophosphatidylinositol were purchased from Sigma-Aldrich. QX 314 bromide was purchased from Tocris. LPI was dissolved in diluted 200 proof ethanol (1mg /(100 μL EtOH + 100 μL DDH$_2$O)) and aliquots were stored in -20 freezer.

Results

GPR55 Expression in the Hippocampus

GPR55 is a lysophosphatidylinositol receptor that also binds lipid-based ligands such as anandamide, THC and cannabidiol, and therefore could mediate some novel forms of CB1/CB2/TRPV1-independent hippocampal synaptic plasticity. Therefore, we initially sought to confirm GPR55 receptor expression location in the rodent hippocampus. Using quantitative real-time PCR (qRT-PCR), we identified the expression of GPR55 in rat whole-brain as well as rat and mouse hippocampal homogenate (Figure 1). Mouse hippocampal cDNA was confirmed to be GPR55 by amplicon size (Figure 1 inset) and DNA sequencing. The expression of GPR55 mRNA in GPR55$^{+/+}$ mice and absence in GPR55$^{-/-}$ mice used in this study was also confirmed (Figure 1C). We next examined the cellular expression of GPR55 using single cell qRT-PCR. In CA3 and CA1 pyramidal cells, we noted its expression in 8 of 9 and 6 of 8 pyramidal cells respectively. In rat stratum radiatum interneurons, GPR55 was only noted in one of 30 cells. To confirm GPR55 mRNA was translated to protein, we used immunohistochemistry and a GPR55 antibody to examine its hippocampal expression (Figures 2 & 3). We noted GPR55 expression in stratum radiatum, stratum pyramidale, and stratum oriens, with highest expression in pyramidale (Figure 2M, N). Expression was not seen in GPR55$^{-/-}$ mice (Figure 2D-F, J-L). Immunohistochemistry data support GPR55 expression in pyramidal cells as well as in some interneurons of the pyramidal cell layer though it was relatively rare in stratum radiatum interneurons (Figure 3), similar to PCR data. To confirm GPR55 punctate labeling localization we performed a Z stack with confocal microscopy and note GPR55 and pyramidal cells overlap in the same focal plane. GPR55 punctate labeling is always localized near pyramidal cell nuclei, but

not in them. Therefore, GPR55 appears to be localized to the pyramidal cell cytosol, though we cannot exclude that some punctate could come from other sources such as inputs to the pyramidal cells, etc. Collectively, these data strongly suggest GPR55 expression in rodent hippocampus pyramidal cells.

Lysophosphatidylinositol Enhances Hippocampal CA1 LTP via GPR55

Next, we examined the potential GPR55 has in modifying hippocampal physiology as several reports indicate that other novel pathways, including eCBs can modulate hippocampal plasticity. Therefore, we examined the role of GPR55 activation by LPI, its more potent endogenous ligand, on CA1 LTP. This was done while recording fEPSPs from hippocampal CA1 stratum radiatum in mouse slices while using a theta-burst protocol to induce LTP. LPI was applied for at least 15 minutes prior to LTP induction protocols. We discovered that the endogenous GPR55 agonist LPI (2-4 µM) indeed caused a significant enhancement of LTP ($p < 0.05$; 199% LTP) when compared to EtOH vehicle control in GPR55$^{+/+}$ mice (176% LTP; Figure 4A). After LTP induction, if LPI was washed out no change was noted in fEPSP slopes and thus GPR55 activation via LPI is not likely involved in LTP maintenance. To confirm the LPI-induced enhancement of LTP was mediated directly by GPR55, we performed identical experiments on littermate GPR55$^{-/-}$ mice lacking the receptor. While GPR55$^{-/-}$ mice demonstrated extremely similar levels of control LTP ($p > 0.5$; 169%) when compared to littermate GPR55$^{+/+}$ mice, LPI did not induce enhanced LTP (155%; Figure 4B), which was significantly different from wild-type LTP in the presence of LPI ($p < 0.05$). In addition, GPR55 antagonist, CID16020046 (10µM) significantly ($p < 0.05$) blocked enhancement of LTP by LPI in GPR55$^{+/+}$ mice (153%; Figure 4C) to levels similar to control LTP. To confirm that CID16020046

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did not in and of itself alter LTP, additional experiments were completed with CID16020046 in the absence of LPI, and in this case LTP was not significantly different from control or LPI + CID16020046 (Figure 4C) experiments. In addition, to confirm CID16020046 did not alter basal synaptic transmission or that GPR55 was not already endogenously activated and blocking GPR55 could induce a depression in synaptic activity, we applied CID16020046 to the bath while recording baseline synaptic activity (Figure 4D). CID16020046 did not significantly alter basal synaptic activity. Finally, LTD induced by 3Hz stimulation for 5 minutes did not differ in wild-type mice in the presence or absence of LPI (data not shown), suggesting GRP55 is not involved in LTD.

LPI Does Not Alter Baseline Glutamate Responses

As CA1 LTP enhancement could be mediated by postsynaptic GPR55 as well as via disinhibition of pyramidal cells via GPR55-induced depression of GABAergic cell activity, which we have noted previously via an eCB TRPV1-dependent mechanism [17] and others via an eCB CB1-dependent mechanism [44] we examined whether disinhibition was involved. If disinhibition was potentially involved then LPI would depress Schaeffer Collateral (CA3) excitatory glutamatergic inputs to CA1 stratum radiatum interneurons as measured using whole-cell electrophysiology, which technique was done to isolate interneuron currents from pyramidal cells, the major cell type in the area. While performing whole-cell patch clamp recordings on stratum radiatum interneurons we applied the endogenous GPR55 agonist LPI (4µM) and looked for alterations in glutamatergic transmission. LPI caused no significant (p > 0.05) depression (Figure 5A), suggesting GPR55 did not alter neurotransmission at this synapse. Therefore, GPR55 does not act via disinhibition to enhance CA1 LTP, but more likely via

postsynaptic GPR55, which is supported by our PCR and IHC data illustrating GPR55 expression in CA1 pyramidal cells.

In addition, as temporary LPI-induced enhancement of presynaptic neurotransmission was noted at the CA3-CA1 synapse previously [45], we examined as another alternative whether LPI (4µM) could alter CA3-CA1 glutamatergic transmission by applying LPI to hippocampal slices while recording extracellular fEPSPs. Recording extracellular fEPSPs allows us to record mainly from CA1 pyramidal cells and to note any effect LPI may have at the CA3-CA1 pyramidal cell synapse. Using our system, which was somewhat different from the prior report, we did not note any significant ($p > 0.5$) change in evoked responses in the presence of LPI compared to baseline responses (Figure 5B). This suggests that in our recording system LPI-enhanced LTP is not likely via enhancing glutamate neurotransmission at the CA3-CA1 pyramidal cell synapse. While these data collectively suggests a postsynaptic site of LTP enhancement for GPR55, we decided to examine another approach to investigate a potential presynaptic mechanism of modulation, known as paired pulse ratios (PPRs). PPRs can be used as an indicator of presynaptic probability of transmitter release and were examined on GPR55$^{+/+}$ and GPR55$^{-/-}$ mice in the presence and absence of LPI (4µM; Figure 5C). We recorded fEPSPs in order to examine CA3-CA1 pyramidal cells connections. In the absence of LPI we noted that PPRs were not significantly different between GPR55$^{+/+}$ and GPR55$^{-/-}$ except at 500 ms ($p < 0.05$), though GPR55$^{+/+}$ tended to be higher. However, in the presence of LPI in GPR55$^{+/+}$, we noted significantly enhanced PPRs at several interpulse intervals ($p < 0.05$ comparing GPR55$^{+/+}$ and GPR55$^{-/-}$ with LPI), suggesting GPR55 may play a role on spontaneous release more than evoked release. This suggested LPI is in some way modifying transmitter

release, though not in a manner that was measurable by our evoked currents. Collectively, while LPI-enhanced CA1 LTP is not a disinhibition phenomenon and it appears likely that GPR55 enhancement is more a classic postsynaptic mechanism, we cannot rule out some presynaptic involvement as discussed below.

GPR55$^{-/-}$ and GPR55$^{+/+}$ Littermate Behavioral Memory Tasks

As GPR55 activation enhanced LTP in wild-type mice and as the hippocampus is involved in memory formation, particularly spatial memory, we wanted to examine the effect GPR55 might have on memory behaviorally to determine if GPR55 had an impact at the animal level. We employed three different behavioral memory assays: novel object recognition, Morris water maze, and radial arm maze, as well as the elevated plus maze to examine immobile behavior as it may relate to anxiety.

The novel object recognition assay was used to examine memory formation of new objects compared to familiar ones, based on the fact that mice will spend more time with a novel object. The results demonstrated near identical time spent with the new object by both GPR55$^{-/-}$ (43.2 ± 4.3%) and GPR55$^{+/+}$ (43.4 ± 4.8%; Figure 6A), suggesting no difference in object recognition memory.

Next, we used two assays that examine spatial memory: the Morris water maze and the radial arm maze. Regarding the Morris water maze, Both genotypes demonstrated decreased time to platform across training sessions (p < 0.05, Figure 6B), indicating that both groups were learning the location of the platform over time, however the genotypes were not significantly different from each other. Time in quadrant during Day 7 trials and the reversal test on Day 8 in which the platform was placed on the opposite side of the pool, showed no difference between

genotypes either (Figure 6C). This result indicates that there is no difference between the GPR55$^{-/-}$ and GPR55$^{+/+}$ mice in ability to replace the previously learned platform location.

Lastly, we employed the radial arm maze. Both wild-type and heterozygous GPR55 mice as well as GPR55$^{-/-}$ mice were slow to explore the maze during the first few acclimation trials. Once they started exploring, both GPR55$^{+/+}$ and GPR55$^{-/-}$ mice were able to learn the maze; however, wild-type mice exhibited significantly shorter trial times during weeks 2-4 ($p < 0.05$, Figure 7A). While this finding initially suggested that GPR55$^{-/-}$ mice exhibit decreased spatial memory compared to wild-type controls, to confirm this we also examined distance traveled, working memory errors and reference memory errors (Figure 7B-D). No significant difference was noted between GPR55$^{+/+}$ and GPR55$^{-/-}$ mice in distance traveled or memory errors suggesting performance in time was decreased for reasons unrelated to memory, but could be related more to immobility. GPR55$^{-/-}$ mice indeed navigated the maze more slowly due to significantly increased immobility in weeks 2-4 (Figure 7E). GPR55$^{-/-}$ mice were more likely to spend an extended period sitting after obtaining rewards before moving on. While 2 out of 12 GPR55$^{+/+}$ mice exhibited some degree of immobile behavior, in contrast 7 out of 8 GPR55$^{-/-}$ mice did. These results indicate that GPR55$^{-/-}$ mice do not differ from wild-type mice in spatial or novel object memory tasks but had higher immobility.

To more closely examine immobility and the possibility that it was due to increased anxiety in GPR55$^{-/-}$ mice, we conducted an elevated plus maze test with 2 open arms and 2 closed arms, where time spent in closed arms is more closely associated with anxiety. We looked at the distance traveled, time spent in open arms, time spent in closed arms and time spent immobile. GPR55$^{-/-}$ spent significantly more time immobile ($p = 0.05$, Figure 7F) and

showed some difference in their total distance traveled, where GPR55$^{-/-}$ mice tended to travel less distance (p = 0.09, Figure 7G). However, GPR55$^{+/+}$ and GPR55$^{-/-}$ mice spent similar amounts of time in open and closed arms (Figure 7H, I). This data suggests that the immobility of GPR55$^{-/-}$ is more due to general inactivity, and less likely related to anxiety.

Discussion

This study is the first to demonstrate that lysophosphatidylinositol can modify synaptic plasticity in the CNS and does so via GPR55. Specifically, our observations confirm that GPR55 is present in the hippocampus and indicate that GPR55 activation by LPI enhances CA1 LTP, the cellular mechanism associated with learning and memory. While GPR55 does not appear to be involved endogenously to a significant extent in memory behavioral tasks, GPR55 appears to play a role in decreased physical activity.

GPR55 Expression and Function

In a previous study, our lab identified an anandamide-mediated plasticity that was independent of CB1 and TRPV1 [47]. Therefore, we chose to examine GPR55 as a candidate for this effect based on its ability to bind eCBs such as anandamide and the fact that lysophosphatidylinositol has never been examined for its role in plasticity that we know of. This is relevant, as GPR55 is known to induce release of intracellular calcium [42, 41], a key signaling molecule in neuronal plasticity.

Our qRT-PCR experiments confirmed previous results detailing GPR55 expression in the brain and, specifically, in the hippocampus [32]. In addition, our data demonstrated GPR55 cellular localization in pyramidal cells for the first time by qPCR. Immunohistochemical assays

confirmed the protein expression of GPR55 in the hippocampus similar to Sylantyev et al. [45], who also reported hippocampal GPR55 expression in the CA1 region by IHC. Both their study and ours note punctate-type localization in the stratum radiatum and stratum pyramidale of GPR55$^{+/+}$ that is almost completely absent in GPR55$^{-/-}$ mice; however, we demonstrated a higher concentration of GPR55 in stratum pyramidale compared to stratum radiatum in contrast to this prior study. While the rationale for this difference is unknown, clearly both our PCR and immunohistochemical data, combined with that of others, strongly support GPR55 mRNA and protein expression in the hippocampus.

Our results also suggest hippocampal LPI is functionally relevant as the endogenous ligand of GPR55. LPI enhanced LTP significantly in GPR55$^{+/+}$ but not GPR55$^{-/-}$ mice, confirming LTP enhancement was mediated by LPI specifically targeting GPR55. While another study examined LTP in GPR55$^{-/-}$ and GPR55$^{+/+}$ mice [32], they did not apply LPI or GPR55 agonist, nor examine the ability of LPI to alter plasticity and therefore this is the first demonstration of such that we are aware of. Interestingly, as LTP in the absence of LPI in GPR55$^{-/-}$ mice was no different compared to wild-type littermate mice in this or other studies [32], therefore GPR55 is not likely involved in typical endogenous LTP, at least in *ex vivo* brain slices. One hypothesis of the potential mechanism for this LTP enhancement is that GPR55 might be the CB1/TRPV1-independent eCB mediator of CA1 stratum radiatum interneuron LTD [47], causing pyramidal cell disinhibition, which could in turn lead to enhanced pyramidal cell LTP [17, 44]. However, whole-cell patch clamp recordings of mouse CA1 interneurons showed no depression in response to LPI, and thus, LTP enhancement is not likely a disinhibition phenomenon. Alternatively, GPR55-mediated increased transmitter release [45] could result in some of the

LTP we noted. However, LPI did not alter glutamate neurotransmission in our study, and as standard CA1 pyramidal cell LTP is usually postsynaptic, this seems less likely. That being said, the ability of GPR55 to increase PPR suggests GPR55 does have a presynaptic role. Unexpectedly, while we initially thought to see a decrease in PPR to accompany an increase in neurotransmitter release, as PPR assesses release probability [48], we saw an increase. However, in hippocampal slices and cultures, it was previously noted that presynaptic vesicle release machinery can be modulated to increase efficiency of vesicle fusion without necessarily increasing vesicle fusion probability. Indeed others have also noted increased PPR while still detecting increased transmitter release [49]. This phenomenon was seen while examining presynaptic transmitter release during hippocampal plasticity [50]. It is possible that GPR55 modulates a mechanism that normally limits synaptic release to single quantum vesicles, allowing more than one vesicle to be released. In this way GPR55 activation could affect spontaneous release without effecting evoked release.

Collectively, while we can't say whether or not the GPR55-mediated presynaptic effect has any role in enhancing LTP, and still the most likely explanation is that postsynaptic GPR55 increases intracellular Ca^{2+} to enhance LTP, there are several other possibilities. Indeed, activation of either presynaptic GPR55 directly or alternatively postsynaptic GPR55 initiating a retrograde signal that acts presynaptically to either enhance plasticity or enhance short-term plasticity during LTP induction leading to larger LTP are also possibilities. This question is the subject of ongoing studies.

Spatial Memory in GPR55$^{+/+}$ and GPR55$^{-/-}$ Mice

The hippocampus, particularly the CA1 region, is widely believed to process spatial

memory [51]. This fact, in conjunction with GPR55 activation enhancing LTP, suggests GPR55

may be involved in memory processing in some manner. Previously, GPR55$^{+/+}$ and GPR55$^{-/-}$ mice

were examined behaviorally to determine major phenotypes through experiments such as

elevated plus maze, open field, and motor skills, with differences noted only in motor

coordination [32]. However, ours is the first study to thoroughly investigate potential

differences in declarative memory. Interestingly, while no significant differences in memory

impairment/enhancement were identified by novel object recognition, Morris water maze, or

radial arm maze, a surprising increase in immobility time in GPR55$^{-/-}$ compared to GPR55$^{+/+}$

mice in the radial arm maze and elevated plus maze suggests GPR55$^{-/-}$ mice had either

decreased physical movement or increased anxiety. A recent report demonstrated GPR55

antagonists increased anxiety-like behaviors, which is comparable to our GPR55$^{-/-}$ mice [52].

However, GPR55$^{-/-}$ mice were recently shown to have decreased voluntary physical activity [53].

Based on our data, the latter appears to be the more likely candidate for their immobility, as

the elevated plus maze test results indicated no difference in fear or anxiety. In either case,

immobility is not likely mediated by general motor issues that slow the mice as distance

traveled and speed (m/s) while mobile were not different in the radial arm task in our data, nor

in behavioral exams performed by others [32]. The fact that memory performance was similar

in all behavioral assays between GPR55$^{-/-}$ and GPR55$^{+/+}$ mice confirms our finding that no

difference was noted in control LTP conditions in the absence of LPI between the two

genotypes. Therefore, while it appears that GPR55 might not be involved in normal declarative

memory formation, it could potentially play a role in enhancing memory performance when exogenously activated. For example, applying GPR55 agonists before a behavioral learning task may improve performance. Indeed, a new report demonstrates GPR55 agonist when injected into striatum enhanced procedural memory using a T-maze [54]. *In vivo* injections of GPR55 agonists during learning of memory assays would be one way to examine a role for GPR55 activation in enhancing memory in future studies.

GPR55 as a Lysophosphatidylinositol Receptor

While GPR55 is proposed as a putative endocannabinoid receptor, LPI appears to be the endogenous ligand most specific for GPR55 as it is arguably the most potent endogenous activator of GPR55 identified thus far that does not also activate CB1 [55, 56, 57]. Identification of LPI forming enzymes in rat brain [58] indicates that GPR55 agonists can be produced in brain tissue. Indeed LPI itself has been found in large quantities in the brain [59] and has been linked to inducing phosphorylation of ERK 1/2, in turn increasing intracellular calcium [60]. *In vivo*, LPI showed neuroprotective properties in a model of transient global ischemia, specifically in CA1 pyramidal cells where we note its expression, even when given 30 min after the ischemic insult [31]. In addition, functional LPI was reported to stimulate [35S] GTPγS binding in the rat hippocampus at 10μM [61], confirming that it activates hippocampal GPCRs. While specific GPCR targets of LPI could be variable, GPR55 is one of them. Also the finding of enhanced glutamate release from CA3 to CA1 hippocampal cells in response to LPI suggests a viable function for GPR55 in the hippocampus [45]. These prior reports, combined with the current data, suggest GPR55 may play an important role in hippocampal function. Furthermore, the downstream effects and signaling mechanisms of GPR55 reported previously vary with both

ligand binding and tissue type, which may allow this receptor to mediate various processes within the body [62]. Overall, while the question of *in vivo* GPR55 activation remains yet to be fully answered; GPR55 appears to be emerging as an interesting novel lysophospholipid-type receptor, making understanding its function in the hippocampus and its role in memory a worthwhile pursuit.

Conclusions and Future Directions

Collectively, exogenous activation of GPR55 enhances plasticity – and thus potentially, memory – in the hippocampus which is responsible for the tasks of learning and encoding memory. Our intriguing findings may have relevance to assisting those with accelerated memory loss observed in neurodegenerative disease, via exogenous activation of GPR55. Further investigation into the role of GPR55 in hippocampal function may assist us in better understanding mechanisms of memory formation, thereby opening new channels of research in therapy development for devastating disorders like dementia.

Acknowledgements
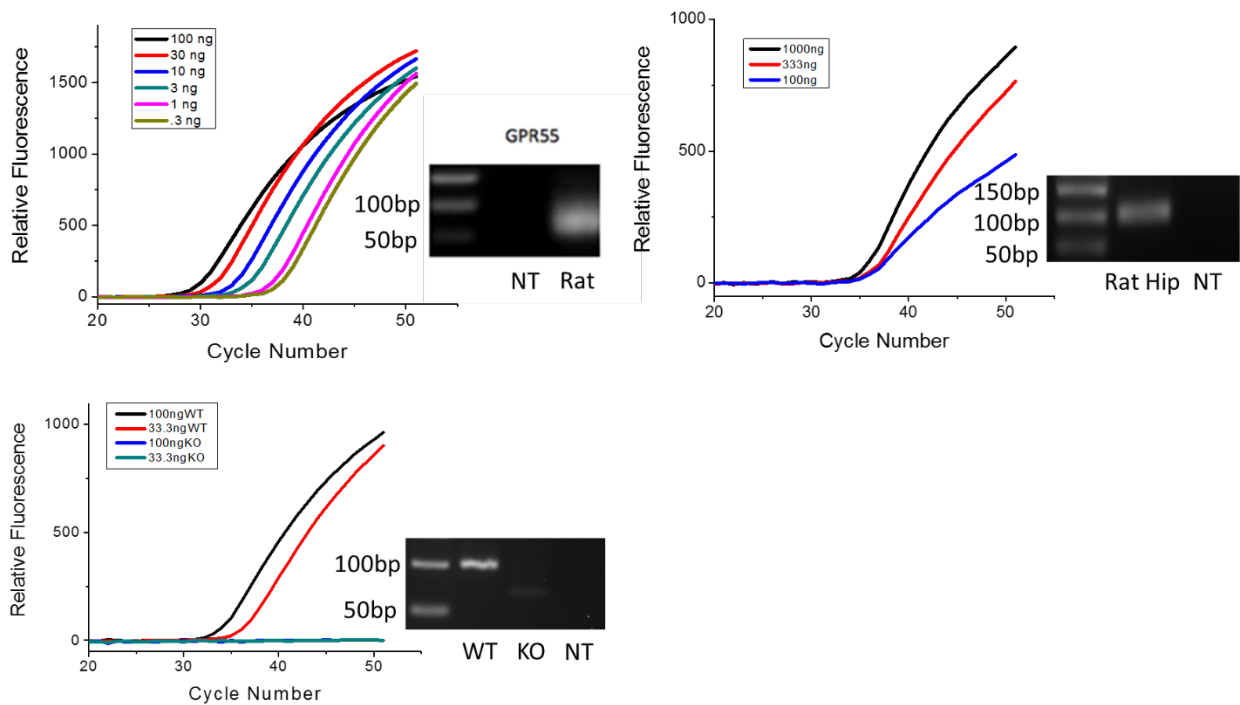
Figure 2.1: GPR55 Expression in Rodent Hippocampus. A) GPR55 mRNA is expressed in rat whole brain, as noted in concentration dependent fashion (black 100 ng, red 30 ng, blue 10 ng, green 3 ng, pink 1 ng, and tan 0.3 ng) using a GPR55 FAM-TAMRA fluorescent probe. Inset: 4% agarose gel to confirm amplicon size (left-right: ladder; no template control; second GPR55 rat primer set PCR product anticipated was 76 bp). B) Rat hippocampus also demonstrate GPR55 mRNA in dose dependent fashion (black 1000 ng, red 333 ng, blue 100 ng). Inset: 4% agarose gel to confirm amplicon size (left-right: ladder; first GPR55 rat primer set; no template control; first GPR55 rat primer set 140 bp). C) GPR55$^{+/+}$ and GPR55$^{-/-}$ mouse hippocampus template with relative fluorescence of GPR55 FAM-TAMRA probe, shows GPR55 mRNA in GPR55$^{+/+}$ mouse hippocampus that is absent in GPR55$^{-/-}$ hippocampus (black wild-type (WT) 100 ng, red WT 33 ng, blue knock-out (KO) 100 ng, green KO 33 ng). Inset: 4% agarose gel to confirm amplicon size (left-right: ladder; WT 100 ng cDNA with anticipated amplicon size of 100 bp; KO 100 ng cDNA; and no template; NT). The cDNA from this band was isolated from the gel, sequenced, and blasted to confirm GPR55 mRNA sequence identity.
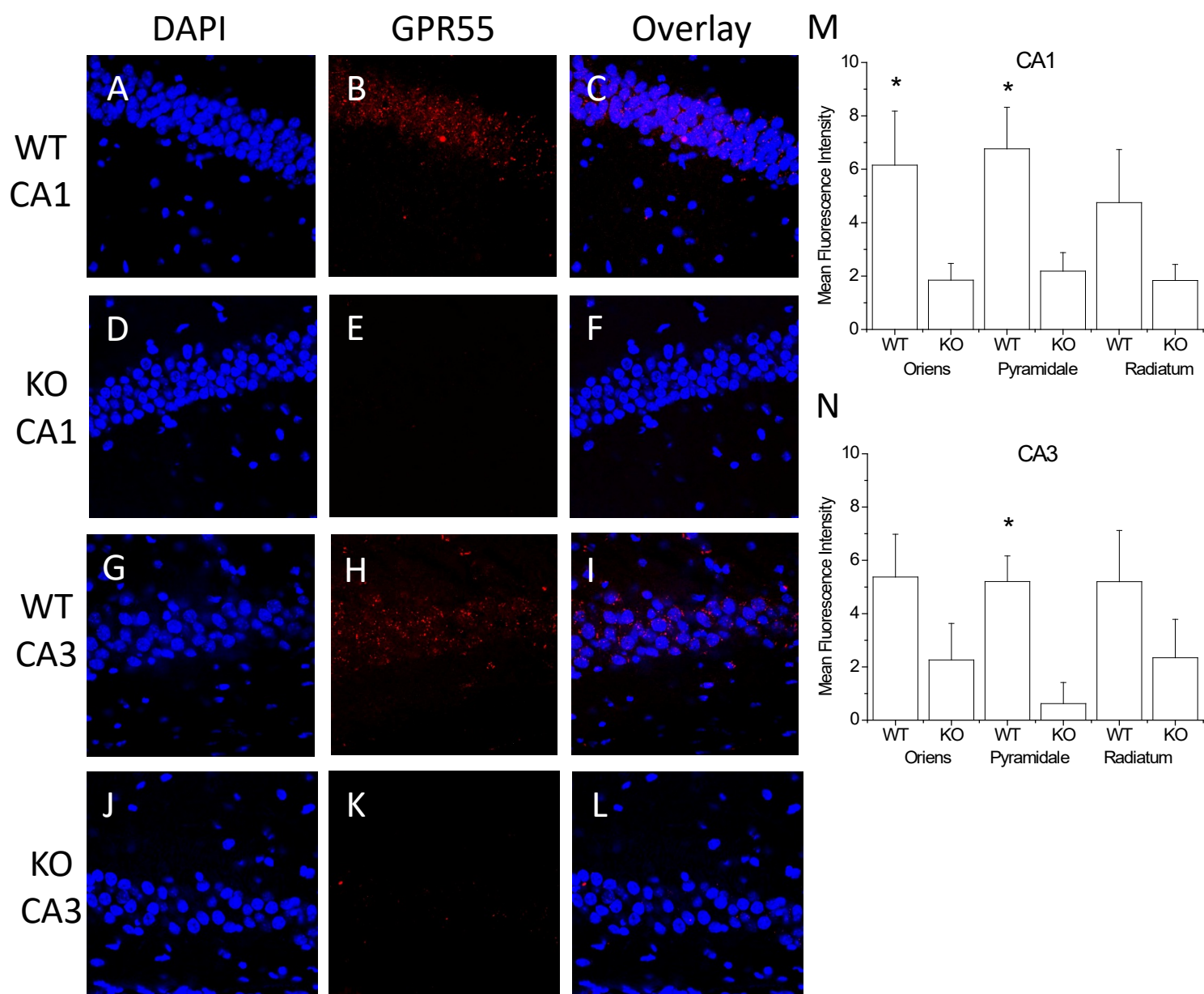
Figure 2.2: Immunohistochemistry Demonstrates GPR55 Protein in CA1 and CA3 Pyramidal Cells. Immunohistochemistry illustrates DAPI staining in wild-type CA1 and CA3, respectively to identify nuclei (A & G) and GPR55 antibody staining (B & H). GPR55 punctate presence is noted in the CA1 and CA3 pyramidal cells as indicated in the overlay images (C & I, scale bar 50 μ). In GPR55$^{-/-}$ mice, while DAPI staining of nuclei is present in CA1 and CA3 (D & J), GPR55 staining is absent (E & K). The overlays are also shown illustrating absence of GPR55 in the CA1 and CA3 pyramidal cells (F & L, scale bar 50 μm). Mean fluorescence intensity of CA1 (M) and CA3 (N) in GPR55$^{+/+}$ and GPR55$^{-/-}$ mice. Labeling is significantly greater in CA3 and CA1 stratum pyramidale and significant or approaching significance ($p < 0.1$) in stratum oriens and stratum radiatum (n=5 per genotype in CA1 and n=4 in CA3).

CA1 GAD67 with GPR55
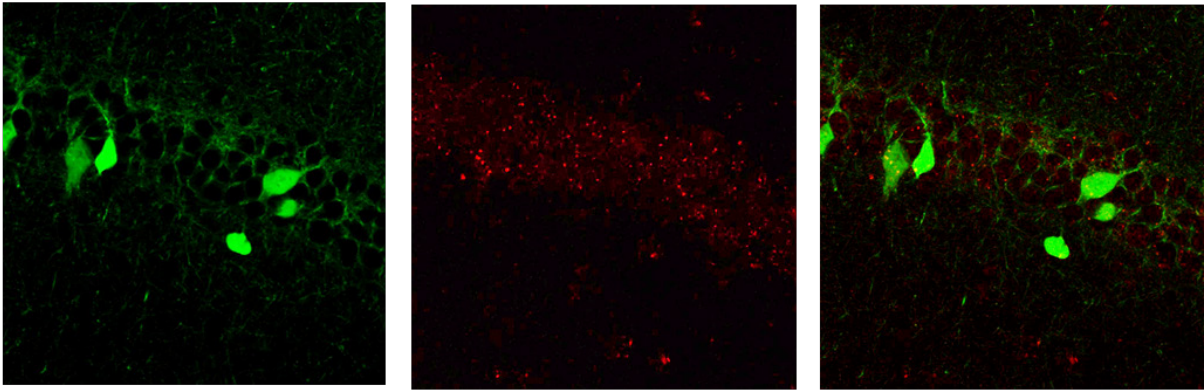
CA3 GAD67 with GPR55
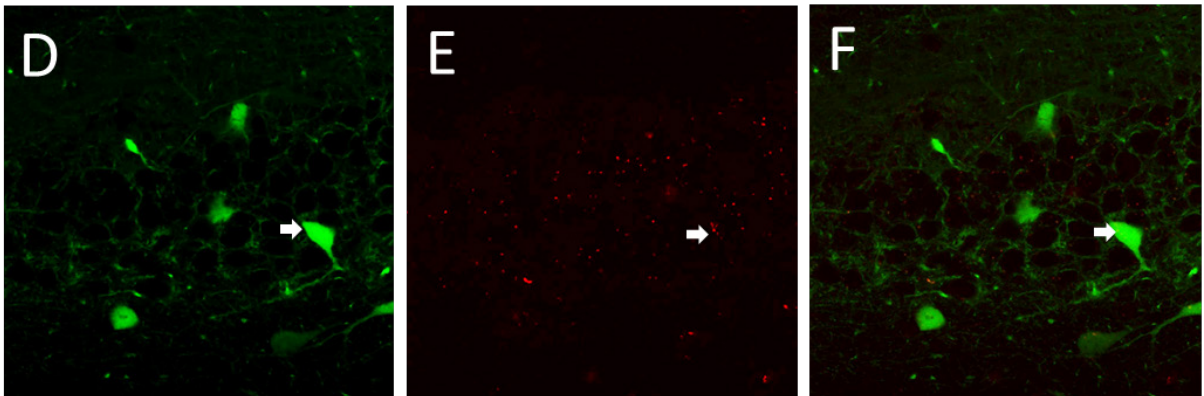
Secondary only control
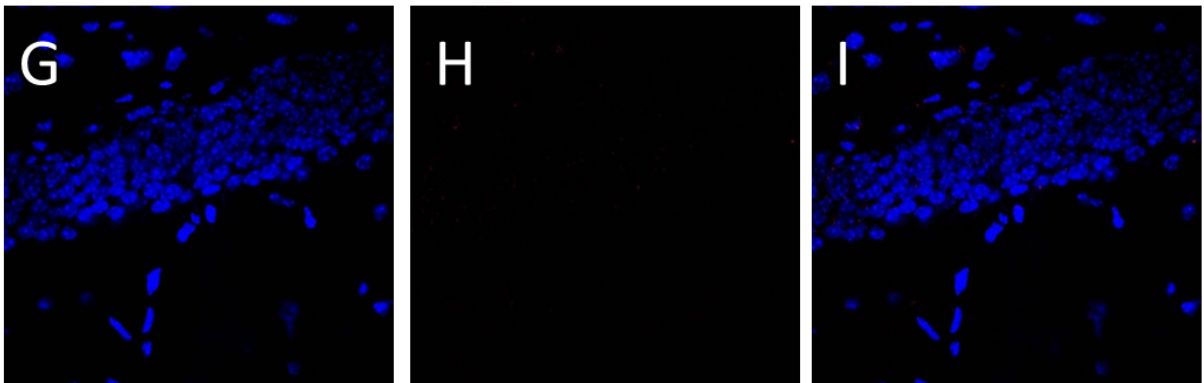
Figure 2.3: Immunohistochemistry Demonstrate Some Expression of GPR55 in Hippocampal Interneurons. GFP in GAD67-GFP knock-in mice illustrate interneurons in CA1 (A) and CA3 (D) stratum pyramidale. GPR55 staining in CA1 and CA3 region demonstrate GPR55 expression within some interneurons (B & E). C & F) Colocalization demonstrated in overlay (scale bar 50 μm).   G) DAPI staining to show nuclei in secondary control. Note that secondary has minimal binding without GPR55 primary antibody (H & overlay I).
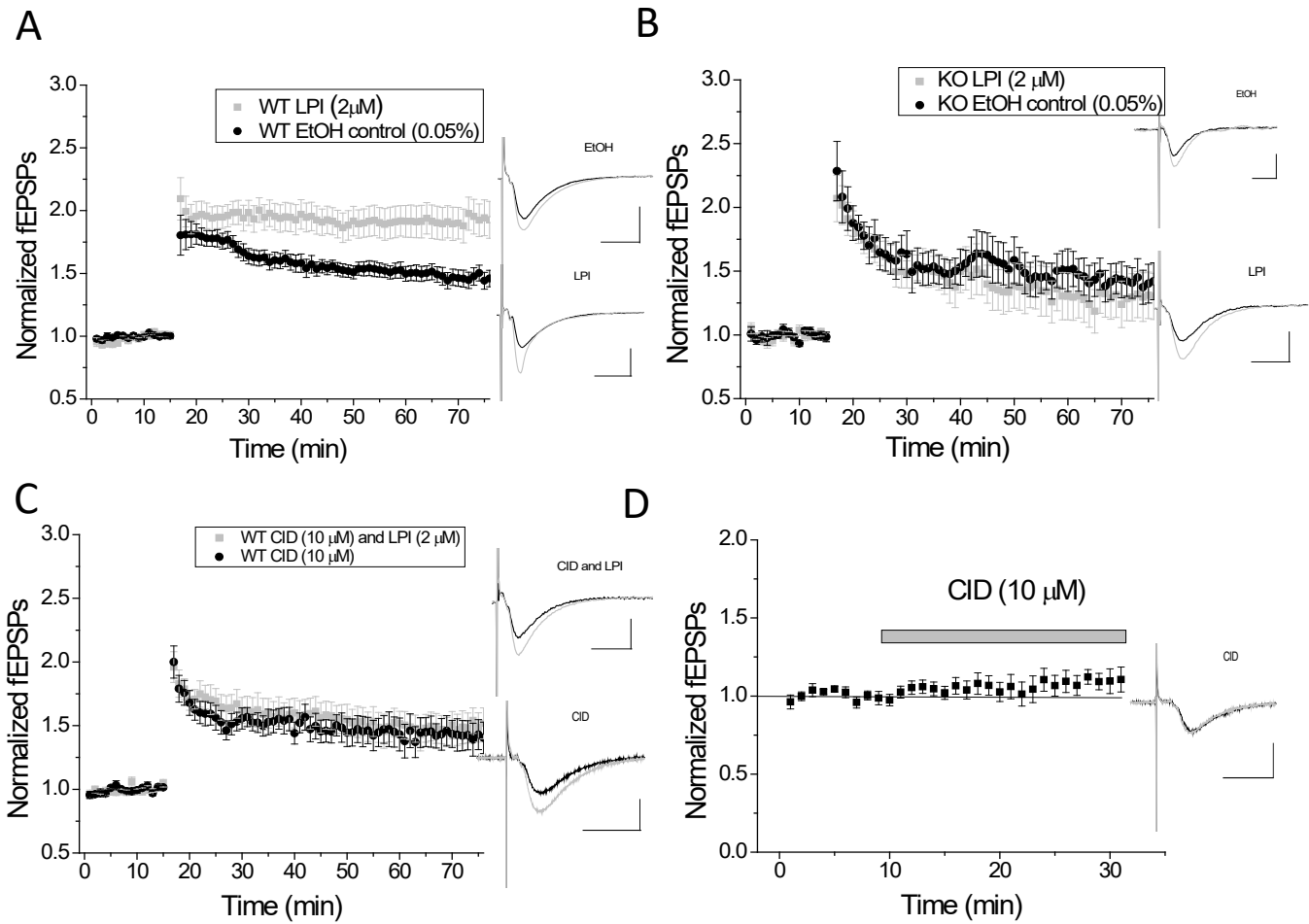
Figure 2.4: GPR55 Agonist Lysophosphatidylinositol (LPI, 2 μM) Enhances LTP via GPR55. A) LPI significantly enhanced LTP in GPR55^{+/+} ($p < 0.05$, grey, n=23) mice compared to ethanol vehicle controls (0.05%; black, n=21). B) In GPR55^{-/-} mice, LPI (grey; n=12) did not alter LTP compared to ethanol vehicle controls (0.05%; black, n=9). LPI significantly ($p < 0.05$) enhanced LTP in GPR55^{+/+} (199%) compared to GPR55^{-/-} mice (155%). C) LPI-enhanced LTP is blocked by GPR55 antagonist CID 16020046 (10 μM; 153%) in GPR55^{+/+} mouse hippocampal slices ($p < 0.05$, grey, n=10) compared to controls in A. CID16020046 by itself did not alter LTP (black, n=7), as LTP in its presence alone was not significantly ($p > 0.5$) different from control, or LPI + CID16020046. Error bars indicate SEM. Insets: representative EPSP traces, scale bar: 100 pA, 10 ms. D) CID16020046 (10 μM) when applied to the bath also did not alter basal synaptic transmission ($p > 0.5$; n = 5), suggesting GPR55 is not basally activated and that CID16020046 does not alter transmission by itself.
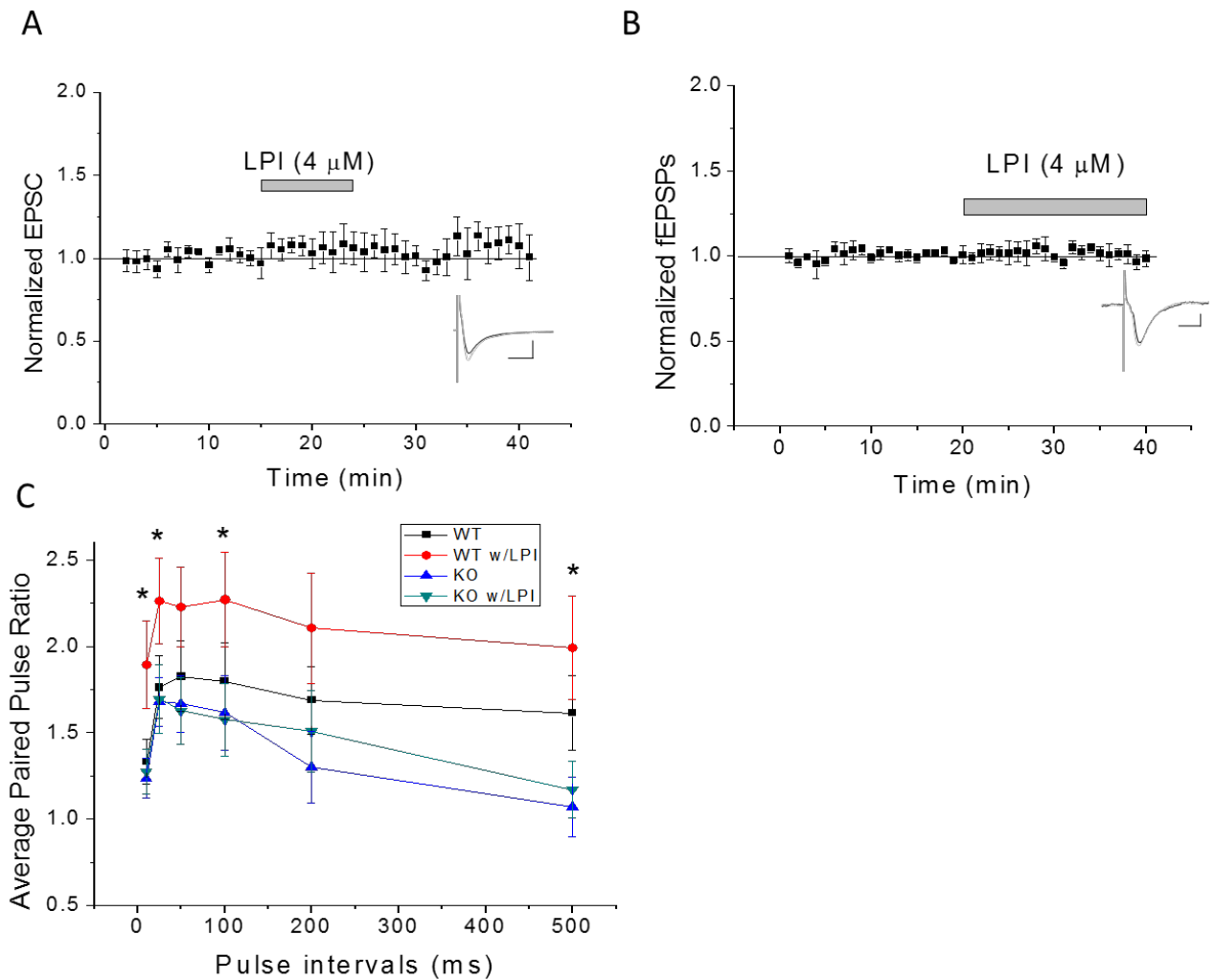
Figure 2.5: LPI-mediated Effects on Glutamate Neurotransmission. A) LPI (4 µM) does not depress the glutamate response of EPSCs in CA1 interneurons (n=7). Error bars indicate SEM. B) LPI (4 µM) also does not alter glutamate neurotransmission of CA1 field EPSPs (n=6). C) LPI increased paired pulse ratios (PPRs) as measured using fEPSPs in GPR55^{+/+} hippocampal slices (red) compared to control PPRs (black), which was absent in GPR55^{-/-} hippocampal slices (green, * p < 0.05 comparing GPR55^{+/+} to GPR55^{-/-} with LPI; # p < 0.05 comparing GPR55^{+/+} to GPR55^{-/-} without LPI; GPR55^{+/+} n=14, GPR55^{-/-} n=11. LPI also significantly (p < 0.05) increased PPR at 10ms and 20ms in GPR55^{+/+} mice when compared to in its absence, which is not demarcated in the figure).
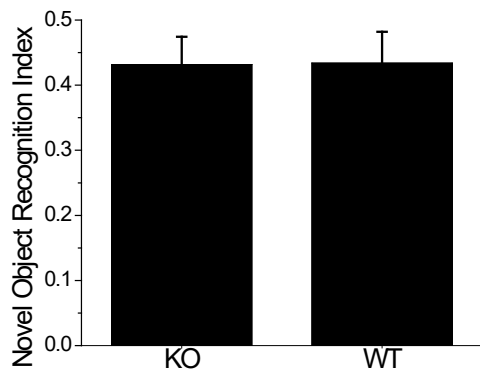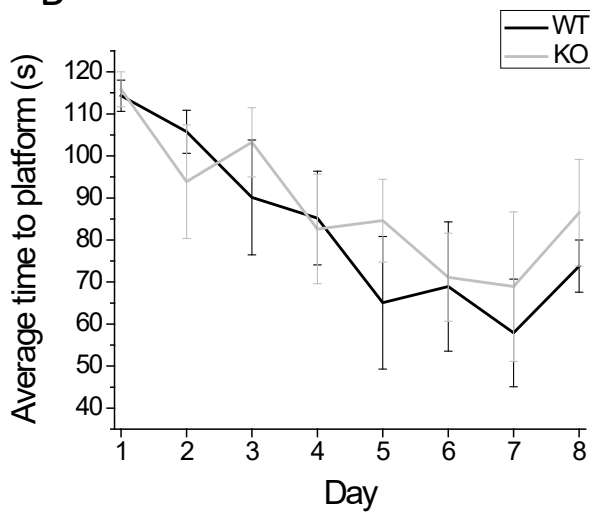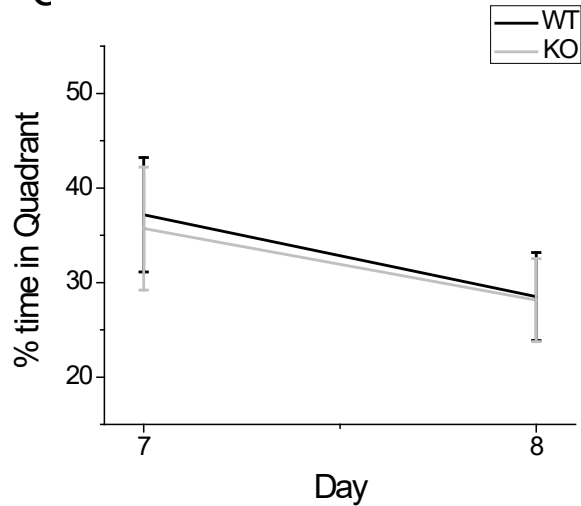
Figure 2.6: GPR55+/+ and GPR55-/- Mice Do Not Differ in Novel Object Recognition Task or Morris Water Maze. A) GPR55$^{+/+}$ mice (n=9) and GPR55$^{-/-}$ mice (n=14) spent a similar percentage of time with the novel object. Reference index indicates the time spent with the novel object divided by total time spent with all objects. B) GPR55$^{-/-}$ (n=7; grey) and GPR55 $^{+/+}$ mice (n=7; black) did not differ in time to platform in the water maze task. Both learned to find the platform at the same rate over the 7-day period as well as on day 8 reversal, when the platform was moved to the opposite quadrant ($p > 0.05$). C) Examining time in quadrant on day 7 and reversal day 8 also showed no difference between GPR55$^{+/+}$ and GPR55$^{-/-}$ mice ($p > 0.05$). Error bars indicate SEM.
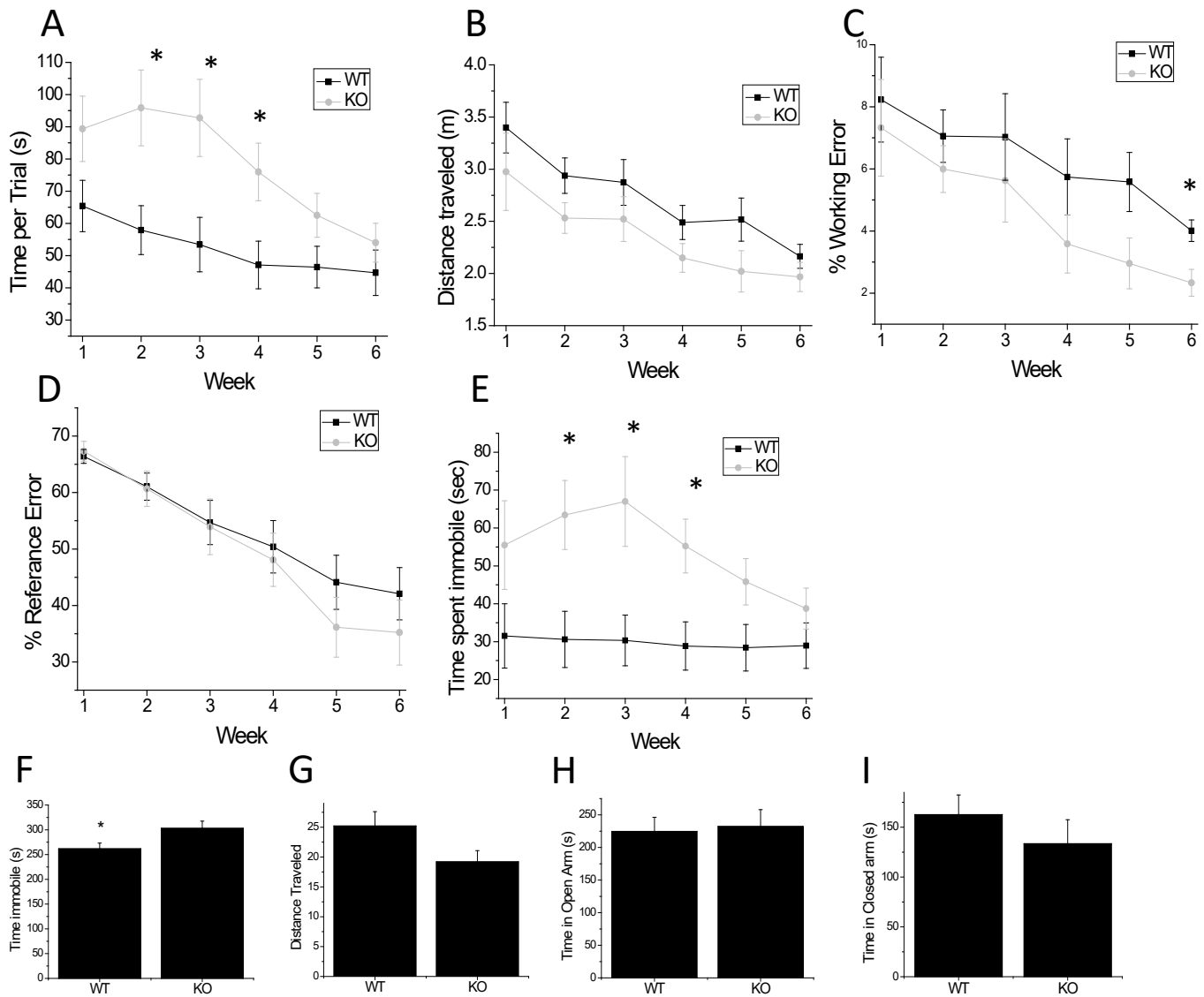
Figure 2.7: GPR55+/+ and GPR55-/- Mice Perform Similarly in the Radial Arm Maze Task, but Exhibit Increased Immobility. A) GPR55$^{-/-}$ mice (grey, n=8) take longer to complete trials weeks 2-4 (* p < 0.05) compared to GPR55$^{+/+}$ mice (black, n=12). B) GPR55$^{-/-}$ and GPR55$^{+/+}$ mice travel similar distances during trials. C) GPR55$^{-/-}$ and GPR55$^{+/+}$ mice made similar working memory errors except for on week 6 where GPR55$^{-/-}$ made fewer errors (* p < 0.05). E) GPR55$^{+/+}$ and GPR55$^{-/-}$ also perform similar in reference memory errors. E) However, GPR55$^{-/-}$ mice spend more time immobile weeks 2-4 (C, * p < 0.05) thereby increasing total time per trial. F) Analysis of elevated maze data demonstrate that GPR55$^{-/-}$ mice spend signficantly more time immobile (p = 0.05) compared to GPR55$^{+/+}$ mice. G) GPR55$^{-/-}$ tend to travel shorter distances in limited time (p < 0.1). H) GPR55$^{-/-}$ and GPR55$^{+/+}$ mice spend similar time in open arms as well as closed arms (I). These data indicate that immobile behavior may not be due to increased anxiety but increased immobility in GPR55$^{-/-}$.

CHAPTER 3: Expression and Physiology of GPR18 and GPR119 in Rodent Hippocampus

Abstract

When investigating learning and memory there are many different approaches to examine function and mechanisms. Synaptic plasticity is widely accepted as the molecular mechanism for learning and memory. However, not all mechanisms of plasticity are fully understood. Our research has been in the novel field of endocannabinoids (eCBs) and G-protein coupled receptors (GPCRs) and how they may be involved in modulating synaptic plasticity. We have investigated the GPCR GPR55 extensively and found that it can enhance long-term potentiation (LTP), a form of synaptic plasticity that strengthens synapses and thus could enhance learning and memory. We have continued investigating other similar GPCRs, GPR18 and GPR119 that have similar ligands to GPR55 and Cannabinoid Receptor 1 (CB1). Here we have used quantitative reverse transcription PCR (qRT-PCR) to confirm that GPR18 and GPR119 are expressed in the hippocampus of rodents. We have also demonstrated the protein expression of GPR18 using immunohistochemistry, with its presence seen mainly in pyramidal cells of the hippocampus. Initial electrophysiology experiments show, using O-1602 (5µM) an agonist for GPR18, had no effect on baseline and no significant difference in hippocampal CA1 LTP. Using GPR119 agonist PSN375963 (20 µM) on WT hippocampal slices no significant change in baseline and LTP experiments were observed either. While these agonists did not affect baseline or LTP in hippocampal slices these GPCRs may have effects in other regions of the brain or antagonists may exhibit a change in physiology.

Introduction

   The brain is an intricate compilation of neurons connecting and communicating with each other. This communication can occur through many different signaling molecules, each molecule can bind different protein receptors that will then respond accordingly. Depending on the region of the brain and the cell type these neurotransmitters can induce different responses. Glutamate is the most widely used excitatory neurotransmitter which is released from presynaptic neurons to activate postsynaptic AMPA and NMDA receptors that depolarize cells and induce synaptic change. These synaptic changes can be either strengthening, called long-term potentiation (LTP), or weakening, called long-term depression (LTD), to increase or decrease in activity, respectively. The ability that neurons have to change these synapses is called synaptic plasticity and is the molecular mechanism of learning and memory.

   While AMPA and NMDA receptors play a vital part in synaptic plasticity, other molecules are also involved in this process. A group of endogenous lipid signaling molecules known as endocannabinoids (eCBs), the most common being anandamide and 2-arachidonylglycerol (2-AG), have been identified as having roles in synaptic plasticity. The most understood mechanisms of these eCBs is via Cannabinoid receptor 1 (CB1) as well as the CB2 receptor. Another receptor known to regulate plasticity is transient receptor vanilloid 1 (TRPV1), but not all of the effects of eCBs are explained by these receptors. There have been reports of non-CB1/TRPV1 mediated plasticity [24]. G-protein coupled receptors (GPCRs) are great candidates for investigating as mediators or modulators of plasticity because they are involved in a variety of cellular processes. GPCRs are known to be involved in the release of intracellular calcium, which is an important signaling cascade for cells to insert more AMPA receptors which will

increase signaling, or LTP [4]. One GPCR we examined previously was GPR55, which is expressed in the hippocampus, the region involved in memory consolidation, and its activation enhanced hippocampal LTP [82]. Like GPR55, GPR18 and GPR119 also bind eCBs such as anandamide and THC [69, 70].

As there appears to be more than one CB1/TRPV1-independent eCB pathway, which are often described as uncharacterized "CB3" receptors, GPR18 and GPR119 are other potential receptors to examine. GPR18 has been studied in microglia and dorsal root ganglia, but its expression in the brain and its role in neuronal function are not as of yet explained. THC, from marijuana, along with the endogenous cannabinoids: N-palmitoyl glycine (PalGly) and N-arachidonoyl glycine (NAGly) are full agonists for GPR18 [71]. Initial research of GPR18 in dorsal root ganglion showed a block of heat-evoked firing of nociceptive neuron by PalGly, an endogenous lipid. PalGly induced transient calcium influx via extracellular calcium in these neurons, and cellular stimulation induced the production of PalGly [72]. NAGly is a potent microglia activator, via GPR18 which is expressed abundantly in primary microglia [73]. Microglial migration was decreased after GPR18 knockdown with siRNA [74]. While these data were acquired using cultured BV-2 microglia and not *in vivo/ex vivo*, this data is suggestive of a potential role for GPR18 in the brain, as well as implications in neurodegenerative diseases which can be induced by over activated microglia. As microglia can regulate neurotransmitter reuptake GPR18 has possible implications as a synaptic plasticity modulator. NAGly has also been shown to regulate levels of anandamide in rat and bovine brain tissue [88], this could be another mechanism by which GPR18 may affect synaptic plasticity. Investigating where GPR18

is expressed and physiological effects will help elucidate potential involvement in synaptic plasticity, neuroprotection, learning and memory.

GPR119 also binds the endogenous cannabinoids anandamide and 2-AG, as well as oleoylethanolamide (OEA), palmitoylethanolamide (PEA) and lysophosphatidylcholine (LPC) [75, 76], sharing some of the same ligands as GPR55 and GPR18 [70]. Because these endogenous ligands are expressed in the brain it suggests potential physiological roles for these GPCRs. In rodents GPR119 is expressed in the pancreas, intestines and the brain. OEA reduces food intake and weight gain by activation of GPR119 and is specific binding to GPR119 [77]. GPR119 also has implications as a regulator of insulin and cellular glucose levels [78, 79, 80]. GPR119 has also been studied in melanocytes, it was found to stimulate dendricity and pigmentation when activated by LPC [86]. GPR119 has been studied for its associations in nutrition but little is known regarding it affects in the central nervous system. GPR119 has possible implications in therapy of neurodegenerative diseases [87]. LPC, an endogenous ligand for GPR119, has been shown to potentiate the BDNF pathway in cerebellar granule neurons [89] as well as inhibit the SNARE complex disassembly [90]. LPC derived from neurons and astrocytes was shown to activate microglia in ischemic stroke penumbra [91]. These studies did not investigate the specific receptor mediating the effects of LPC but GPR119 is a possible mechanism. With its expression in the brain and ability to bind eCBs, GPR119 has great potential as a candidate receptor that modulates synaptic plasticity.

Material and Methods

The experiments conducted in this research closely resemble those carried out in previous research conducted in Dr. Edwards's lab [17]. GPR55 knockout (University of North

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Carolina via the Mutant Mouse Regional Resource Center) and wild type mice P16-32 were

used in these studies. Animals were housed in approved conditions with a 12 h light-dark cycle.

The experiments were conducted in accordance with the Brigham Young University Institutional

Animal Care and Use Committee standards and National Institute of Health guidelines to

minimize pain and suffering of animals.

PCR

A series of quantitative real-time polymerase chain reaction (qRT-PCR), gel

electrophoresis, immunohistochemistry (IHC), and electrophysiology experiments were

conducted. The presence of GPR18 and GPR119 in the hippocampus was demonstrated

through qRT-PCR by amplifying and sequencing a portion of the mRNA coding for each protein

from mouse hippocampal and VTA tissue extracts. For GPR18 in mice,

TTCCAGGCTCGAGTCATCAG was used as the forward primer sequence,

TGTTGCTAAGTGACCGTAAACTG for the reverse, and TGTACCGCAATTACCTTCGCAGTGTTCG for

the fluorescently tagged probe, producing an amplicon of 100 base pairs (bp). For GPR119 in

mice, TGTTTCACCCAAGGTTTGTG was used as the forward primer sequence,

AGAGGCAATCTTGAGCATGTC for the reverse, and TGGCTTCTTCCCAGCTGTGCTCCTCT for the

fluorescently tagged probe, also producing an amplicon of 100bp. For GPR119 in rat,

CGTTTCTCATCACTAGCATTGTG was used as the forward primer sequence,

AGGAGCCAGAGGTATTTTCC for the reverse, and CTGCCACAAATGCTGCCGCTACAA for the

fluorescently tagged probe, producing an amplicon of 100bp. All primers and probes were

purchased from Thermo Fisher Scientific's Invitrogen. Sso Fast EvaGreen Supermix and Sso

Advanced Universal Probes Supermixmaster mixes purchased from Bio-Rad were used for melt

curve and probe experiments, respectively.

IHC

GAD67-GFP knock-in mice were used for IHC experiments in order to visualize GABA-

ergic neurons. The brains extracted from these mice were transcardially perfused with 0.1 M

phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1 M PBS (pH 7.4). They were

cryoprotected in 30% sucrose solution, frozen in OCT, sliced into 30 µm sections, and placed in

0.1 M PBS for a free-floating staining procedure. These slices were permeablized with 0.2%

Triton-X (Fisher Bioreagents) for 30 minutes, washed with 1% bovine serum albumin in 0.1 M

PBS for 2 hours, and treated with primary antibody for anti-GPR18 (1:500, Novus) in 5% normal

goat serum and 1% bovine serum albumin in PBS overnight at 10 °C. Slices were then washed

twice with 0.1 M PBS, followed by one wash of 0.2% Triton-X (Fisher Bioreagents) in 0.1 M PBS

for 30 minutes, one wash of 1% bovine serum albumin and 5% normal goat serum in 0.1 M PBS

for 2 hours, and a final wash of anti-rabbit secondary antibody (1:500, AlexaFluor 647,

Invitrogen) secondary antibody in 5% normal goat serum, 1% bovine serum albumin in PBS for

2 hours at room temperature. Slices were washed three times with tris-buffered saline and

mounted onto Superfrost Plus microscope slides (VWR). After drying overnight, slides were

coverslipped with DAPI Fluoromount-G (Southern Biotech) and imaged on an Olympus

FluoView FV1000 laser scanning confocal microscope. Image capture was performed by

sequential excitation of each fluorophore. An attempt to image GPR119 by a similar process

was made but has so far been unsuccessful.

Gel Electrophoresis and Sequencing

2% agarose gel was made with 45ml double distilled water, 5mL 10X TAE buffer, 1g powdered agarose, and 3 μl of Ethidium Bromide. Once the gel was ready, the electrophoretic gel box was filled with enough 1X TAE buffer to cover the surface of the gel. 10μl of 50bp-increment DNA ladder was loaded into a well, and 6μl of corresponding PCR product was loaded in a neighboring well. The gels were run at 150V for approximately 45 minutes, the gels were removed, and the bands were imaged under a UV camera. The bands corresponding to GPR18 and GPR119 in mouse were excised from the gels, and the DNA was isolated, using QIAquick Gel Extraction Kit, and sequenced at the BYU Life Science Sequencing Center. Their results were compared with the sequence of the intended amplicons.

Slice Electrophysiology

All animals were deeply anesthetized with isoflurane using a vapomatic chamber and decapitated, following which their brains were removed rapidly and placed in ice-cold, oxygenated medium containing (in mM): NaCl, 119; $NaHCO_3$, 26; KCl, 2.5; NaH_2PO_4, 1.0; $CaCl_2$, 2.5; $MgSO_4$, 0.6; glucose, 11; saturated with 95% O_2, 5% CO_2 (pH 7.4). The posterior aspect of the brain was glued with cyanoacrylate adhesive to the cutting stage of a vibratome after which 400 μm coronal slices were cut and then transferred to a holding chamber containing oxygenated artificial cerebrospinal fluid (ACSF) at room temperature. This cutting technique maintains the integrity of the hippocampal circuitry. Following an interval of at least 1 h, slices were transferred to a submerged recording chamber and perfused with oxygenated ACSF medium of the same composition as that in the holding chamber at a temperature between 28-32°C. Slices were continuously perfused with ACSF at a flow rate of 2-3 ml/min. A bipolar

stainless steel stimulating electrode was placed in the stratum radiatum, at least 400-700 μm

from the recording electrode to stimulate CA3 glutamatergic afferents of the Schaffer Collateral

pathway at 6-50 μA for 100 μsec at 0.1Hz sampling rate. Recordings were performed in current

clamp mode to measure excitatory postsynaptic potentials using an Axopatch 200B amplifier.

Data was filtered at 4 kHz and acquired with an axon 1440A digitizer (Molecular Devices) and

inputted onto a Dell personal computer (OptiPlex 745) with pClamp10.2 clampex software

(Molecular Devices). Stimulation intensity was adjusted to elicit an EPSP of 0.5 mV at the

beginning of each experiment. Borosilicate glass patch pipettes (2-3 MΩ) were filled with 1 M

NaCl for field recording electrodes. EPSPs were evoked and monitored for at least 60 minutes.

To measure how baseline and LTP in hippocampal neurons are influenced by these

receptors, electrophysiology experiments were conducted using electrodes to measure

neuronal responses to electrical stimulation. O-1602 (5μM, Tocris), an agonist specific to

GPR55 and GPR18, was used in GPR55 KO mouse hippocampal slices to observe effects of

GPR18 on baseline and LTP. PSN375963 (20 μM, Cayman) an agonist for GPR119 was used in

WT mice to observe effects of GPR119 on baseline and LTP. Specifically, baseline pyramidal cell

activity was measured at the Schaffer collateral pathway in field electrophysiology recordings,

slices were exposed to electrical stimulation at 10 second intervals. After establishing a stable

15-20 min baseline response, O-1602 in GPR55 KO mice or PSN375963 in WT mice was applied

in the bath for 20 minutes and baseline was recorded to observe any changes in slope of

glutamate responses. In another experiment, theta frequency stimulation patterns, which

mimic brainwave activity during learning events, were used to induce LTP with or without

application of O-1602 or PSN375963 after 20 min of drug application and/or 15 min of stable

baseline were established. Results were analyzed and compared using Microsoft excel and

Origin, and additional experiments must be conducted to establish statistical significance.

Results

GPR18 and GPR119 are viable candidates for involvement in synaptic plasticity as they

both bind eCBs, so we first set out to establish their expression in the hippocampus. mRNA

from hippocampal homogenate was isolated and converted into cDNA. PCR was then run on

the sample to detect GPR18 mRNA is expression in mouse hippocampus (Figure 3.1A). To

confirm that GPR18 mRNA was being amplified in our PCR results the amplicon was run out on

a gel to confirm amplicon size of 100 base pairs (figure 3.1B) and then the amplicon was

extracted and sequenced, confirming GPR18 cDNA was amplified. To confirm GPR18 protein

expression, IHC was performed on GAD67-GFP (a line that labels interneurons) mouse

hippocampal slices, where expression is predominately found in a majority of the pyramidal

cells of the CA3 region and a couple interneurons (figure 3.2). Field electrophysiology

experiments with the GPR55 and GPR18 agonist O-1602 in GPR55 KO mice show no significant

difference in LTP compared to GPR55 KO control experiments, there also does not appear to be

an effect on baseline response (Figure 3.3). While these electrophysiology experiments did not

exhibit a significant difference in LTP, there is a difference in the initial PTP (p = 0.05), this may

be an effect of O-1602 or due to the differences in ages, O-1602 experiments were on mice 30-

100 days old, with an average of 60, vs control ages 14-40 days, averaging 30, further

experiments could resolve this issue.

To investigate GPR119 we tested hippocampal cDNA from rat and mouse, and GPR119 is

expressed in both (figure 3.4). We examined the amplicon size from the mouse sample by

running it on a gel (100 bp, figure 3.4) and then excising and sequencing the sample to confirm that GPR119 cDNA was being amplified. We then investigated GPR119s possible effects on physiology using the GPR119 agonist PSN375963 (20 µM) and examining baseline glutamate responses and LTP in hippocampal slices. PSN375963 does not show a significant change in baseline glutamate response and there is also no significant difference between control and PSN375963 LTP (Figure 3.5). Initial electrophysiology experiments may not have exhibited a change with GPR119 agonist, but further study into antagonists may show an effect if GPR119 is already being activated endogenously.

Discussion

GPR18 and GPR119 are candidates for an uncharacterized "CB3" receptors as they are capable of binding common eCBs such as 2-AG and anandamide as well as the cannabinoid THC [71, 75, 76]. Previous research of GPR18 in cultured BV-2 microglia has shown that GPR18 is expressed abundantly [73] and knockdown of GPR18 decreased microglial migration [74], this is suggestive of potential research for treatment of neurodegenerative diseases where microglia are over activated. GPR18 is also found in the dorsal root ganglion and has possible uses for pain relief in the PNS [72]. Our research has shown GPR18 expression in pyramidal cells indicating possible functions for GPR18 in the hippocampus, and potentially in learning and memory. With neurodegenerative diseases being so devastating to individuals and their families, finding practical treatments is indispensable. Further research into GPR18 in the hippocampus using a specific agonist and antagonist will assist in understanding if it could be targeted for treatments and what potential side effects may be encountered.

While GPR119 has positive effects on health via its role in metabolism [79, 80], it's difficult to draw comparisons between this and synaptic plasticity. GPR119 is also expressed in melanocytes, which are closely related to neurons as they both originate from the ectodermal layer during development, and was found to mediate dendricity and pigmentation when activated by LPC in these cells [86]. This regulation in melanocytes may give some indication of GPR119s role in the CNS. In a study done on Alzheimer's disease, GPR119s' agonist LPC was indicated as having potential as therapy of neurodegenerative diseases [87], in addition LPC derived from neurons and astrocytes was shown to activate microglia in ischemic stroke penumbra [91] which may indicate a role for GPR119 in neuroprotection. LPC potentiates the BDNF pathway in cerebellar granule neurons [89] and inhibits SNARE complex disassembly [90] these affects by GPR119s agonist point toward a role in synaptic plasticity. Whether these effects are via GPR119 was not part of the studies but with GPR119s expression in the brain and specifically in the hippocampus it is indeed a promising candidate. Future directions that would enlighten where and how these receptors may be involved in plasticity would be to record in interneurons of the hippocampus, looking at the effects of their antagonists, and examining other areas of the brain such as the VTA. These previous studies in combination with our current data argue for the continued research of these GPCRs, GPR18 and GPR119, and their potential roles in synaptic plasticity.
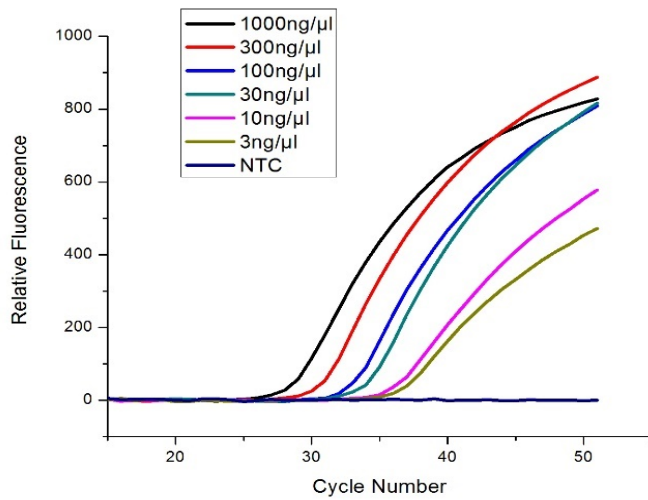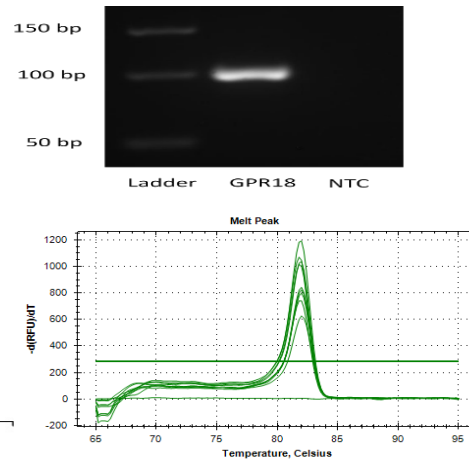
Figure 3.1: GPR18 is Expressed in Mouse Hippocampus. A) GPR18 qRT-PCR of mouse hippocampal cDNA (black-1000 ng/µl, red-300 ng/µl, blue- 100 ng/µl, green 30 ng/µl, pink- 10 ng/µl, tan-3 ng/µl). B) Top 4% agarose gel to confirm amplicon size (left-right: ladder; WT 1000 ng cDNA 100 bp; and no template; NT). Bottom PCR melt curve from PCR run in A, showing a single peak, indicating one product was amplified.
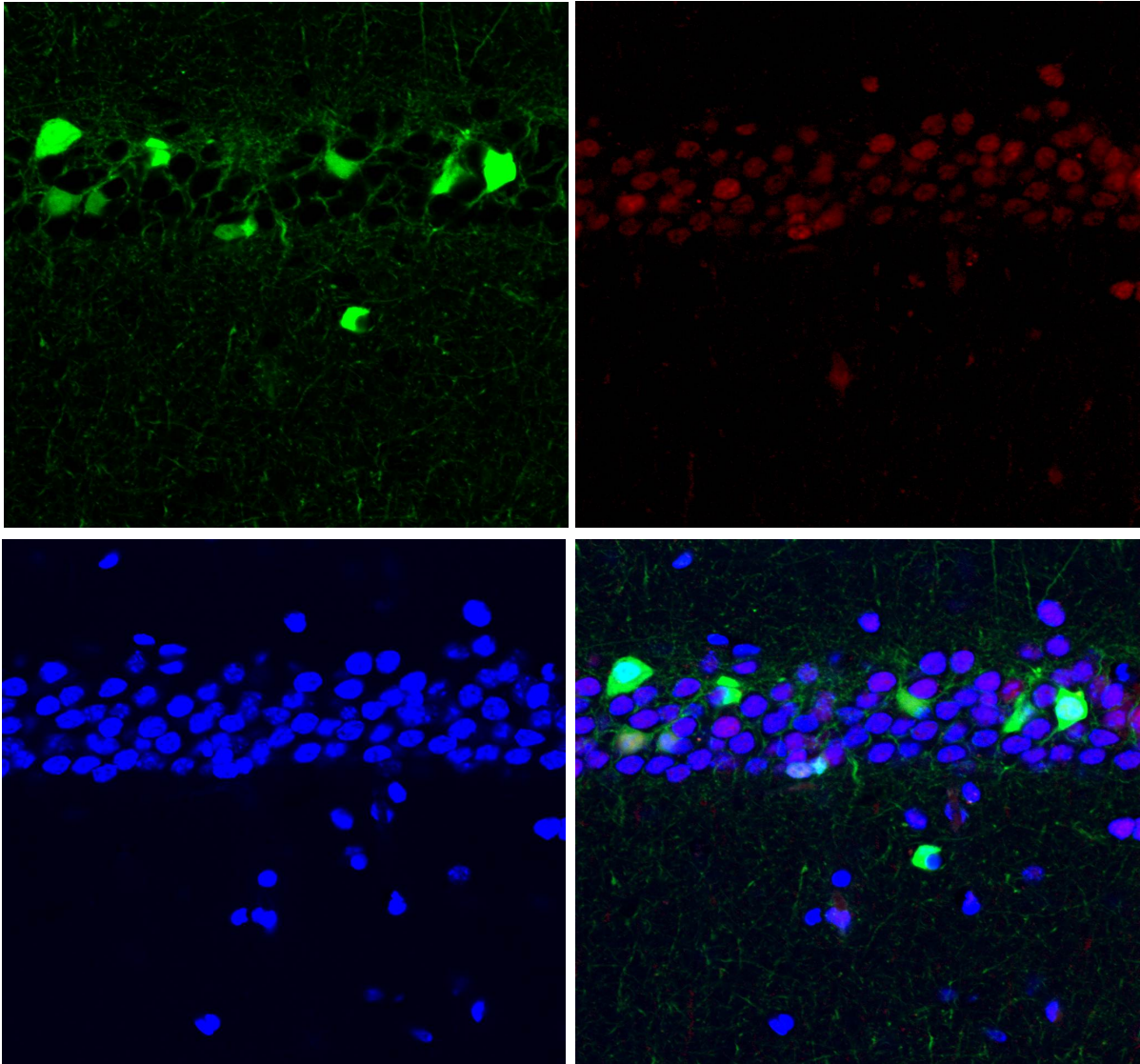
Figure 3.2: IHC Imaging of CA3 of Hippocampus. Top-left: GAD67-GFP positive (GABA-ergic) neurons of the CA3 region in the hippocampus in green; top-right: GPR18 positive neurons in red; bottom-left: DAPI stain for cell nuclei in blue; and bottom-right: overlay of all three, showing GPR18 in a majority of the pyramidal cells as well as a couple GABA-ergic neurons.
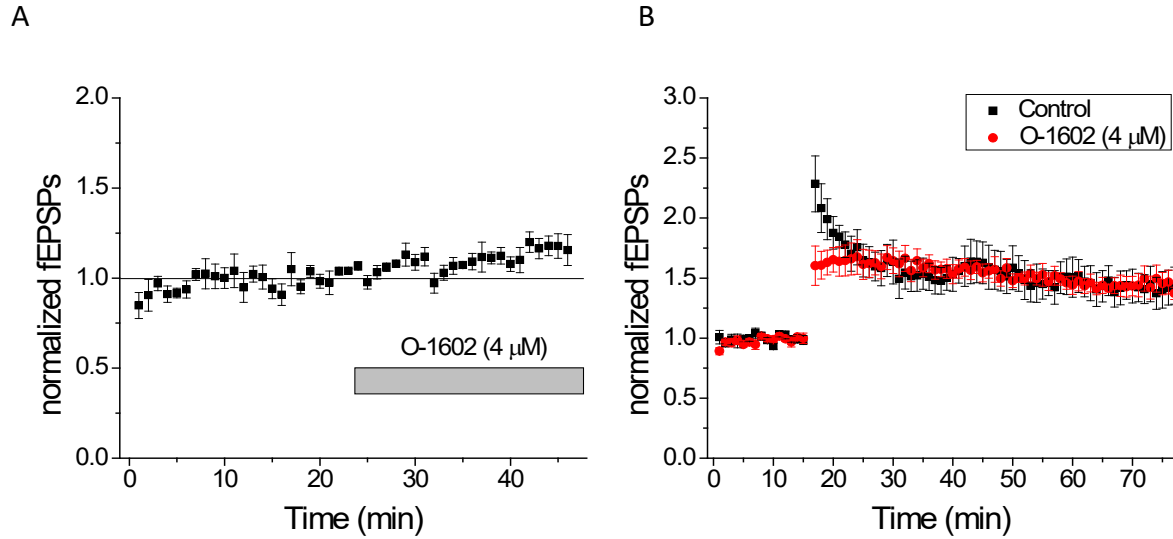
Figure 3.3: Effects of O-1602, a Potent GPR55 and GPR18 Agonist, in GPR55 KO Mice. A) O-1602 shows no significant effect on baseline (n=5). B) GPR55 KO control (black, n=9) LTP shows no difference in LTP compared to O-1602 (red, n=6), but PTP is significantly different (minutes 1-5 post theta burst, p = 0.05).
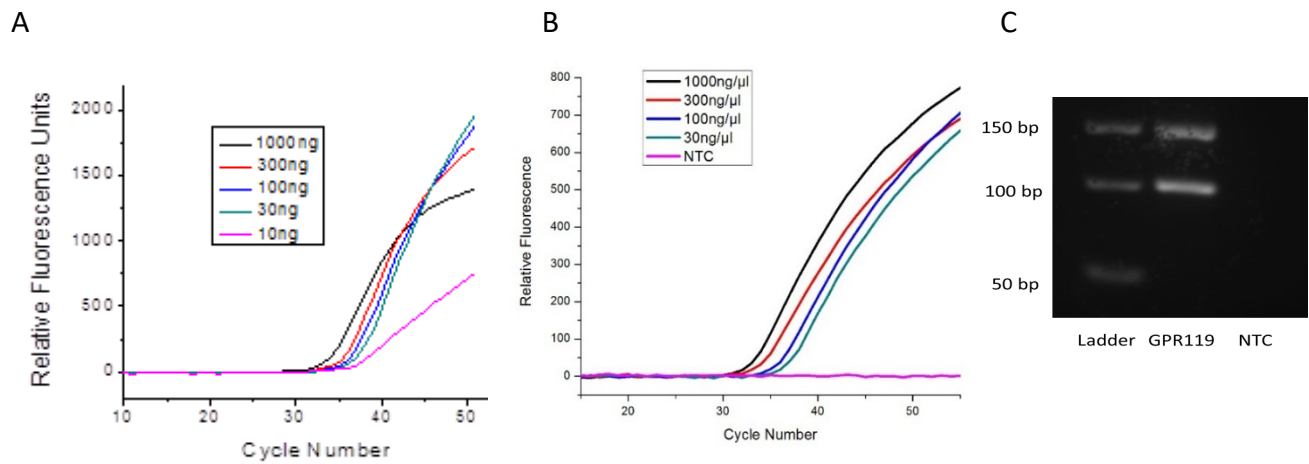

Figure 3.4: GPR119 is Expressed in Rodent Hippocampus. A) qRT-PCR of rat hippocampus (black-1000 ng/μl, red-300 ng/μl, blue- 100 ng/μl, green 30 ng/μl, pink-10 ng/μl). B) qRT-PCR of mouse hippocampus (black-1000 ng/μl, red-300 ng/μl, blue- 100 ng/μl, green 30 ng/μl, pink-NTC). C) 4% agarose gel to confirm amplicon size (left-right: ladder; WT 1000 ng cDNA 100 bp; and no template; NT), 100 bp band was excised, sequenced and blasted to confirm GPR119 sequence for amplicon.

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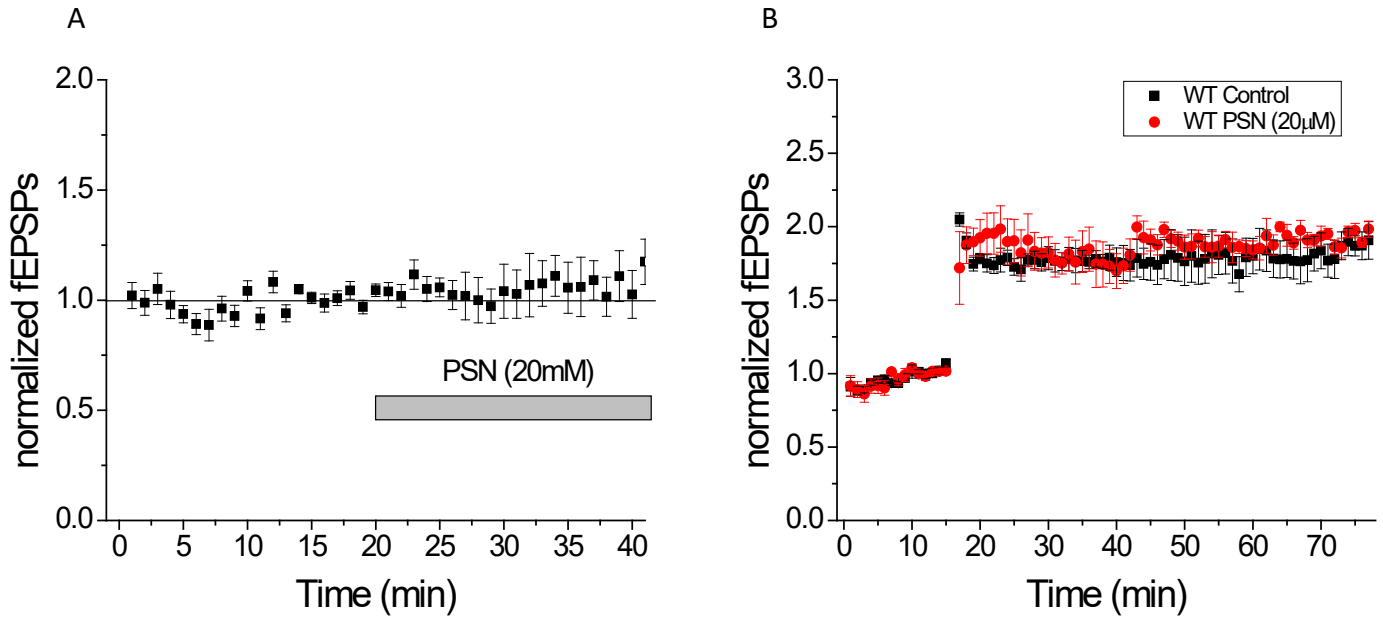
Figure 3.5: GPR119 Activation in WT Mice Hippocampus Do Not Show an Increase in Baseline Glutamate Responses or Change of LTP. A) Application of GPR119 agonist to hippocampal slices while recording CA1 field excitatory response exhibit no significant increase in baseline (n=5, p = 0.4). B) GPR119 agonist PSN375963 (20 µM, red, n=5) does not significantly enhance CA1 LTP compared to control (black, n=4, p = 0.8).

CHAPTER 4: Translational Effects of TRPV1-dependent Long-term Depression in
Hippocampal Interneurons

Abstract

Transient receptor potential vanilloid 1 (TRPV1) is recognized most commonly for its role in signaling heat, pain and the spicy sensation from chili peppers. In the PNS, it is studied for its effects on pain and inflammation, but it is also found in the CNS where its role has only recently been described. One role for TRPV1 is its involvement in mediating long-term depression (LTD) of CA1 hippocampal interneurons, causing a disinhibition of hippocampal pyramidal cells. To further investigate the necessary cellular processes for the TRPV1-dependent induction of LTD in interneurons we examined the effect of translation and transcription inhibitors, Anisomycin and Actinomycin D, respectively, had on LTD. Our data thus far do not show a significant difference in LTD when translation is blocked. Although there is not a significant difference in LTD there is a change in the ratio of short term depression to long term in the presence of Anisomycin, with an increase in the number of short term depression experiments and a decrease in the number exhibiting LTD.

Introduction

Learning and memory are an intriguing process that occurs in the brain. Exactly how and where these events occur are not entirely understood, but what we have discovered thus far is that the best molecular process that ensues in the brain to account for learning and memory is synaptic plasticity. The brain is comprised of neurons that make hundreds of connections with each other, these connections are called synapses, and plasticity is changes at these connections. Plasticity has been studied extensively since it was first described in 1973

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[1], and has been found to have many different mechanisms. Synapses can either be strengthened, called long-term potentiation, or weakened, called long-term depression. The area of the brain that is most studied for its role in learning and memory is the hippocampus, and is known to be involved in memory consolidation and spatial navigation [92]. This region of the brain is highly organized which has allowed for research in this area to thrive, much of what we know about synaptic plasticity has come from studying this area of the brain. The hippocampus is organized into subfields called CA1, CA2, CA3 and CA4; and the CA3 pyramidal cells extend axons, known as Schaffer collaterals, into the CA1 region. Despite the many forms of plasticity that have been unraveled the long-term cellular mechanism involved are not fully understood.

These differing forms of plasticity can be induced or modulated by several receptors and signaling pathways. One such receptor is transient receptor potential vanilloid 1 (TRPV1)[6], known for binding capsaicin the spicy ingredient in chili peppers. TRPV1, as well as other receptors are often activated by endogenous lipid signaling molecules known as endocannabinoids (eCBs) and have been shown to be mediators or modulators of synaptic plasticity [7]. TRPV1 is expressed in many different places throughout the body and because of TRPV1s involvement in pain [64] it is being investigated as a potential target for pain killers. Studying their expression, electrophysiology and mechanisms in the CNS can increase our understanding of eCBs and their receptors involvement in learning and memory, and how they may potentially be targeted to help with memory/learning disorders or even pain.

When attempting to find a target for treating different disorders an understanding of the proteins function and mechanism is crucial. TRPV1-mediated interneuron LTD was recently

described to occur at several synapses [4, 18] including CA3 pyramidal cell to CA1 radiatum interneuron synapses, TRPV1 is expressed presynaptically on CA3 pyramidal cells and when activated induces LTD at excitatory synapses onto stratum radiatum interneuron [6]. Because inhibitory interneurons depress the activation of the CA1 pyramidal cells they innervated, LTD of interneurons releases pyramidal cells from some of their inhibition, thereby disinhibiting them. This disinhibition of pyramidal cells allows them to exhibit greater LTP [17], which is usually associated behaviorally with better memory formation, and thus could be of importance to neurodegenerative disorders of memory systems. Therefore, understanding its mechanisms of action can also benefit our knowledge of how synaptic plasticity can occur and be effected by different receptors and molecular processes, such as translation and transcription.

Translation and transcription have been shown to be necessary for CA 1 late phase LTP in hippocampal Schaffer collateral synapses [65]. When translation is inhibited by Anisomycin within 30 minutes post high frequency stimulation (HFS) to induce LTP, there is a significant decrease in potentiation and when transcription is blocked with Actinomycin D there is a significant difference compared to control LTP experiments 90 minutes post HFS [65]. By three hours the neuron responses returned to baseline when either process was blocked, this effect was first described in 1984 when Anisomycin was injected intraventricularly in rats, LTP decayed 3-4 hours after tetanus [66]. The effects of translation has been studied in several different forms of plasticity from oxytocin promoted LTP [67] to ß-adrenergic and muscarinic conversion of short-term to long-term potentiation [68]. NMDA receptors have been linked to translation via eILF4E activation of NMDA receptors, resulting in activation of PKA and ERK dependent MNK1 activation and increased eILF4E phosphorylation, which leads to enhanced

translation [83,86], in mouse hippocampus [84]. mGluRs have also been shown to induce a form of LTP that is controlled by translation and Arc signaling in the hippocampus [85]. These studies display the role of translation and transcription in several forms of plasticity.

In addition to the study of protein translation in induction and maintenance of LTP this process has been implicated in several forms of LTD. A form of mGluR-dependent LTD was shown to be mediated by rapid translation of Arc/Arg 3.1 [87]. Protein synthesis was also shown to be necessary presynaptically for CB1 induced LTD onto GABA-ergic cells [88]. Recently translation and transcription have been described as important processes in the induction of TRPV-like mediated LTD in leeches, where postsynaptic transcription and translation and presynaptic translation are required for the induction of LTD [63]. However, we still do not understand the role of translation and transcription in mammalian TRPV1-dependent plasticity. Defining the roles of transcription and translation in TRPV1-mediated LTD in mammalian systems will help to understand how protein production may be regulated and involved in this form of plasticity or duration of plasticity in addition to other processes such as phosphorylation, dephosphorylation or endocytosis. Translation and transcription are important processes throughout the body and here we can see that they are also vital to synaptic plasticity and thus are necessary in learning and memory, particularly when studying long-term memory.

Materials and Methods

The experiments were conducted in accordance with the Brigham Young University Institutional Animal Care and Use Committee standards and National Institute of Health

guidelines to minimize pain and suffering of animals. Sprague-Dawley rats used were aged P14-27 (Charles River). Animals were housed in approved conditions with a 12-hour light-dark cycle.

All mice used for electrophysiology were deeply anesthetized with isoflurane using a rodent vapomatic chamber and decapitated, after which their brains were removed rapidly and placed in ice-cold, oxygenated artificial cerebrospinal fluid (ACSF) medium containing (in mM): NaCl, 119; $NaHCO_3$, 26; KCl, 2.5; NaH_2PO_4, 1.0; $CaCl_2$, 2.5; $MgSO_4$, 0.6; glucose, 11; saturated with 95% O_2, 5% CO_2 (pH 7.4). The posterior aspect of the brain was cut into 400 μm coronal slices using a vibratome, and then transferred to a holding chamber containing oxygenated ACSF at room temperature.

Following an interval of at least 1 hour, slices were transferred to a submerged recording chamber and perfused with oxygenated ACSF of the same composition as that in the holding chamber at a temperature between 28-32°C. Slices were continuously perfused with ACSF at a flow rate of 2-3 ml/min. A bipolar stainless steel stimulating electrode was placed in the stratum radiatum, at least 400-700 μm from the recording electrode to stimulate CA3 glutamatergic afferents of the Schaffer Collateral pathway at 6-50 μA for 100 μsec at 0.1Hz. Recordings were performed in current clamp mode to measure excitatory postsynaptic potentials using an Axopatch 200B or 700B amplifier. Data was filtered at 4 kHz, acquired with an axon 1440A digitizer (Molecular Devices), and inputted onto a Dell personal computer with pClamp10.4 Clampex software (Molecular Devices). Stimulation intensity was adjusted to elicit an EPSP of 0.5 to 0.7 mV at the beginning of each experiment. Borosilicate glass patch pipettes (2-3 MΩ) were filled with 1 M NaCl for field recording electrodes. EPSPs were evoked and monitored for at least 60 minutes.

Field recordings (rats; P14-24) were analyzed as noted previously [17]. Briefly, the EPSPs slopes was calculated using pClamp10.2 Clampfit software (Molecular Devices). Values were normalized to control slope values 5-10 minutes immediately prior to theta burst. Theta burst was used to mimic more natural hippocampal activation patterns. Two bursts were given 20 seconds apart. An increase in EPSP slope that persisted for longer than 60 min indicated that LTP had been induced and glutamate response was recorded for 90 minutes post conditioning. EPSP normalized slope values 20-25 min post high frequency stimulus were compared to baseline for significance (unpaired, two tailed t-test). Only one experiment was performed per slice, and the reported N is the number of slices not the number of animals. In general, 4-6 slices were used per animal. Microsoft Excel and Origin (North Hampton, MA) were used to organize, average, graph, and perform statistical analysis on the data.

Whole-cell recordings (rats; p14-24) were performed in voltage clamp at -65 mV using a Multiclamp 700B amplifier (Molecular Devices) and EPSCs were evoked by two pulses separated by 50msec. Borosilicate glass patch pipettes (2-6 MΩ) were filled with Cs^+-gluconate based internal solutions containing (in mM): NaCl, 2; $MgCl_2$, 5; HEPES, 20; ATP, 2; GTP, 0.3; QX 314 bromide, 1 and EGTA, 0.6. High divalent ACSF in mM: NaCl, 119; $NaHCO_3$, 26; KCl, 2.5; NaH_2PO_4, 1.0; $CaCl_2$, 2.5; $MgSO_4$, 1.3; glucose, 11; saturated with 95% O_2, 5% CO_2 (pH 7.4) AMPAR-mediated currents were measured while blocking $GABA_A$ receptors with picrotoxin (10μM). The cell input resistance was monitored throughout the experiment, and if changed by more than 10%, the cell was discarded. Interneurons were distinguished from pyramidal cells visually by their location in the radiatum and electrophysiologically by their higher input resistance. Peak glutamate responses were normalized to control values 5-10 minutes

immediately prior to conditioning. Microsoft Excel and Origin were used to organize, graph, and perform statistical analysis on the data. Stable baseline recordings of AMPAR-mediated currents were obtained at the frequency of 0.1 Hz. After 15 minutes of a consistent baseline, 2 X 100 Hz stimulus at 150% voltage 20 seconds apart was delivered to the slice and then baseline was recorded post conditioning for as long as the cell was stable.

Anisomycin and Actinomycin D were dissolved into DMSO and stored in 20 mM stock aliquots of 100 μL that was suspended in 100 ml ACSF to make 20μM Anisomycin or Actinomycin D final concentration. For all electrophysiology experiments with drug slices were stored in ACSF with Anisomycin or Actinomycin D for an hour before experiments were conducted.

Results

To investigate the role of translation in TRPV1-induced LTD we bathed slices in ACSF with 20 μM Anisomycin for at least one hour before initiating whole cell plasticity experiments to inhibit protein translation. Among the 11 control experiments included there were 7 cells that exhibited LTD, 3 cells with STD and 1 cell that did not exhibit plasticity or a change in glutamate response post conditioning. There are different subtypes of interneurons within the hippocampus so this range in responses is not uncommon. When examining experiments where slices were bathed with Anisomycin there was a slight decrease in the number of LTD experiments with only 5 cells out of 11 demonstrating LTD, 4 cells that did STD and 2 cells that exhibited no plasticity. While LTD was not significantly affected by blocking translation, there was a trend towards a higher frequency of short term depression (STD, figure 4.1, p = 0.06). To investigate if Anisomycin is active in our preparation we conducted positive control field

experiments using Anisomycin at the same concentrations and examined their effects on LTP. Field LTP experiments using Anisomycin did yield the expected decrease in LTP (figure 4.2, $p = 0.05$), reassuring our findings in whole cell experiments, that Anisomycin is blocking translation in our slices. Positive control Actinomycin D experiments, to block transcription, also yielded a decrease in LTP but not to the significance or in the time frame anticipated (figure 4.2, $p = 0.1$). Examination of the drug dilution and application needs to be done before further experiments into whole cell patching is conducted.

Discussion

Translation has been demonstrated as being vital in other forms of LTD in mammals, both in mGluR [87] and CB1 [88] induced LTD, which indicates its potential role in TRPV1 induced LTD in mammals. While reports in leeches indicated a necessity for translation in TRPV-like induced LTD [63], it is difficult to determine if this is the same case in mammalian systems. In current study there are clear occurrences of LTD when translation is inhibited but there is a decrease in the frequency of LTD and among the experiments that did exhibit LTD the amount of depression was slightly less than control LTD experiments. In interneurons LTD occurs about 50% of the time, STD 35% and no change 15% [6], these ratios are similar to what we analyzed in control and Anisomycin experiments. While we may not have seen a significant change in LTD, this may be due to the shortness of whole cell experiments, perhaps the effects of blocking translation would be more apparent in mammals at later time points than we were able to procure using whole cell physiology.

While translation has been shown to be vital for many mechanisms of plasticity, transcription has also been shown to play a pivotal role in synaptic plasticity. The effects of

inhibiting transcription are often seen after longer time periods, in the late-phase of LTP the differences were not noted until around 90 minutes post conditioning [65]. Although, in the study done in leeches the effects of blocking transcription were noted almost immediately [63], this is not likely in mammals considering our data thus far from blocking translation. While there is still a chance that transcription is necessary for TRPV1-induced LTD this data would be challenging to obtain with the difficulty of acquiring lengthy whole cell experiments. But without a clear answer to the importance of translation in TRPV1-induced LTD, the argument for transcription being necessary or unnecessary seems imprudent. As Actinomycin D also did not significantly block LTP in field control experiments we can't be certain that data on whole cell physiology would accurately represent the effects of transcription inhibition on TRPV1-induced LTD. Examination of another transcription inhibitor may be necessary.

In summary, translation is likely to be involved in TRPV1-induced LTD in mammals while this effect was not significant in our experiments there is a trend in the data and a more significant differences may be found with more experiments or in longer lasting experiments. This would be in accordance with other research on synaptic plasticity and translation in mammals. As for transcription needing even longer to exhibit a difference when blocked examining its role in TRPV1-induced LTD via whole cell patching may prove unsuccessful. Future directions would be to investigate inducing LTD via TRPV1 agonists while inhibiting translation, this may result in a more significant difference, although the effects may still take a longer period of time in mammals than was reported in leeches [63].
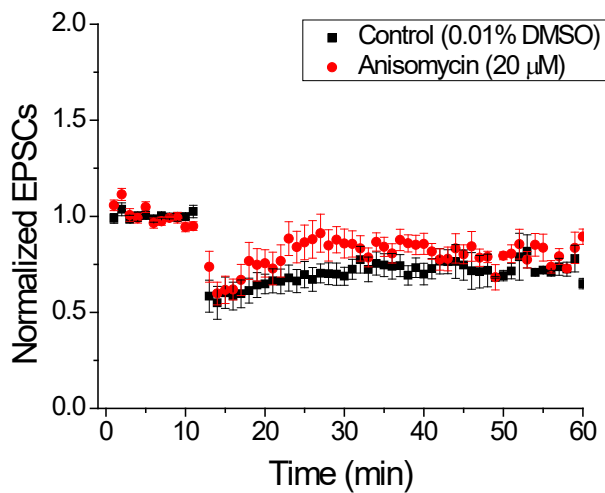
Figure 4.1: Translation Inhibition by Anisomycin Effects on Hippocampal Interneuron TRPV1-induced LTD. Slices bathed in Anisomycin (red, n=11) for at least an hour before and during experiments are similar to control (black, n=11) LTD experiments with a trend towards more short-term depression (p = 0.08, 12-16 min post 100 Hz, p = 0.06, 25-30 min post 100 Hz).
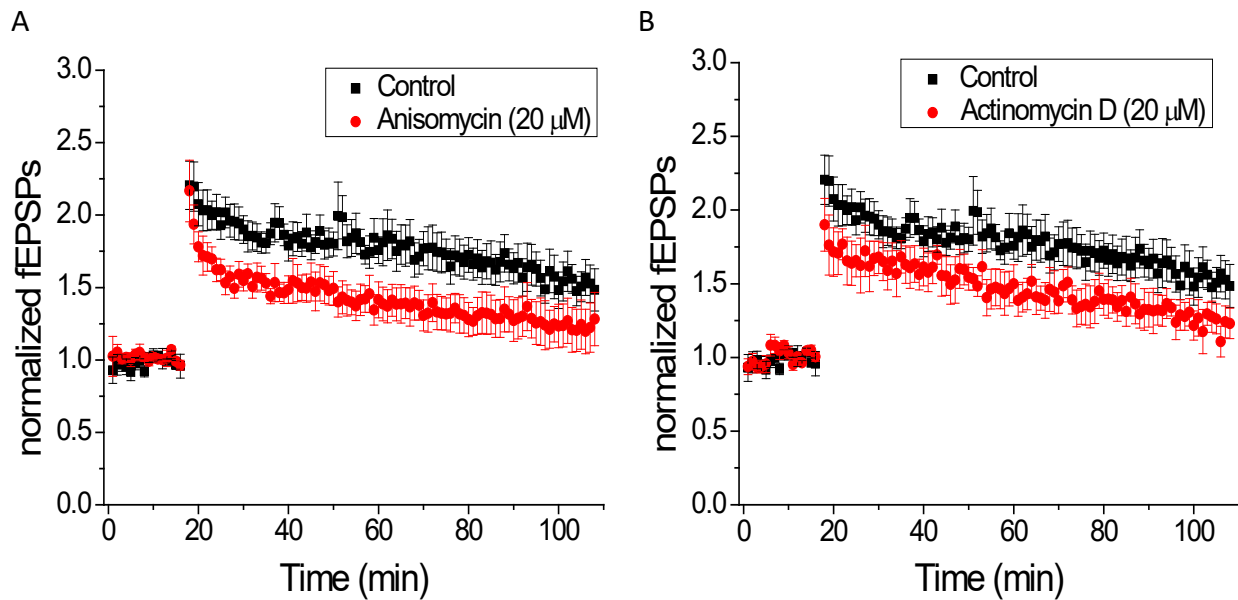
Figure 4.2: Translation and Transcription Inhibition, via Anisomycin and Actinomycin D Respectively, on Hippocampal Field LTP. A) Anisomycin (red, n=6) when applied for an hour before and during LTP experiments significantly decreases LTP (p = 0.05, 25-30 min post theta burst) compared to control (black, n=5) LTP experiments. B) Actinomycin D (red, n=5), also applied one hour before and during experiments, did not significantly decrease LTP (p = 0.1, 25-30 and 85-90 min post theta burst) compared to control (black, n=5) LTP experiments.

CHAPTER 5: Conclusion

The purpose of this research was to uncover the potential roles G-protein coupled

receptors, GPR55, GPR18, and GPR119 in hippocampal synaptic plasticity. We have described

the expression of GPR55, GPR18 and GPR119 in the hippocampus. In regard to GPR55, we have

done an in depth study of its physiological effects in the hippocampus and investigated its

importance in behavioral assays. We found that GPR55 when activated with LPI was able to

enhance LTP and also increased paired pulse ratios indicating a potential presynaptic

mechanism. In behavior GPR55 KO mice overall did not differ in memory and spatial navigation

tasks, but exhibited an increase in immobile behavior. Therefore, GPR55 could potentially

enhance memory making it a potential target for treatment of neurodegenerative diseases.

GPR18 and GPR119 are expressed in the hippocampus. We see further studies are

needed into their physiological effects. For GPR18 a more specific agonist and investigation of

antagonists, to determine if GPR18 is endogenously activated, should be used in WT mice to

examine if there is an effect on synaptic plasticity. More physiology experiments are needed

with GPR119 agonist and antagonist on baseline and LTP to distinguish whether it has an effect

on plasticity, even though our data thus far does not show an enhancement of baseline or LTP.

We also examined another eCB-like receptor TRPV1 and its requirement of translation

and transcription to induce LTD. Translation and transcription are essential in late-phase LTP,

also in TRPV-like induced LTD in leeches but this necessity for early LTD in mammal

hippocampal interneurons seems less likely, but more experiments may tease out a significant

difference. LTD was still found to occur when translation is blocked with Anisomycin, blocking

transcription with Actinomycin D may show better results, although this is less likely since translation needs to follow transcription for the effects to emerge. The necessity of translation and transcription may be more apparent in later phases of TRPV1-induced LTD, examining this via whole cell patching is challenging as maintaining cell viability for more than an hour is difficult.

The study of synaptic plasticity has been exciting, the brain is still an unknown organ in the body and is fascinating to unfold. Our research on GPR55, especially, has yielded interesting findings that could lead to future studies and treatments of neurodegenerative diseases. So far our research of GPR18 and GPR119 have not yielded such exciting results but they still have great potential and need further investigation. As for the study of translation and transcription in TRPV1-induced LTD, this study is difficult using whole cell techniques. As there is likely an important role for translation and transcription, the effect of blocking them are more probable to take place at later time periods in mammals which is difficult to study with whole cell patching. While there are many mechanisms by which synaptic plasticity can occur, these studies have shed light on some unknowns and also opened doors to other questions about the processes by which plasticity can be effected.

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EDUCATION
- PhD Physiology and Developmental Biology, Brigham Young University (July 2012-Aug 2017)
  - GPA: 3.8
  - Graduate course work: Molecular Biology of the Cell, Cell and Molecular Physiology, Developmental Genetics and Genomics, Molecular Evolution and Developmental Biology, Biophysics, and Pharmacology
- Bachelor of Science, Physiology and Developmental Biology, Brigham Young University
  - GPA: 3.72
  - Undergraduate course work: Advanced Physiology, Developmental Biology, Advanced Molecular Biology, Cell biology, Genetics, Advanced Neuroscience, and Biochemistry 1, 2

RESEARCH EXPERIENCE
- Graduate Research Experience with Dr. Jeff Edwards, Neuroscience, electrophysiology and molecular biology (May 2013- present)
- Graduate Research Experience with Dr. Juan Arroyo, cell and molecular biology research (January 2013-April 2013)
- Graduate Research Experience with Dr. Michael Stark, developmental biology research (July 2012-December 2012)
- Undergraduate Research Experience with Dr. David Kooyman, cell and molecular biology research (January 2010-December 2010)

TEACHING EXPERIENCE
- Teacher's Assistant for Developmental Biology (August 2012-December 2012, August 2013-December 2014, August 2015-April 2016, and January 2017-current)
- Physiology Lab Instructor (January 2013-June 2013, April 2015-June 2015, August 2016-December 2016)
- Physiology Lecturer (teaching practicum, January 2015-April 2015)
- Yoga teacher (August 2016-current), Spin instructor (January 2017-current)
- Teacher's Assistant for Molecular Biology (January 2011-April 2011)

PUBLICATIONS/ABSTRACTS

Hurst, K., Badgley, C., Ellsworth, T., Bell, S., Friend, L., Prince, B., Welch, J., Cowan, Z., Williamson, R., Lyon, C., Anderson, B., Poole, B., Christensen, M., McNeil, M., Call, J., Edwards, J. (2017). A Putative Lysophosphatidylinositol Receptor GPR55 Modulates Hippocampal Synaptic Plasticity. Hippocampus, doi:10.1002/hipo22747

K. Hurst, C. Badgley, S. Bell, D. Haynie and J.G. Edwards. 2016. The Putative Cannabinoid Receptor GPR55: Expression, Modulation of Hippocampal Plasticity and Behavior. Society for Neuroscience International Conference.

K. Hurst, C. Badgley, S. Bell, D. Haynie and J.G. Edwards. 2015. The Putative Cannabinoid Receptor GPR55: Expression, Modulation of Hippocampal Plasticity and Behavior. Society for Neuroscience International Conference.

K. Hurst, C. Badgley, S. Bell, D. Haynie and J.G. Edwards. 2014. The Putative Cannabinoid Receptor GPR55: Expression, Modulation of Hippocampal Plasticity and Behavior. Society for Neuroscience International Conference.

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Hurst K, Badgley C, Bell S, Edwards JG. 2013. The Putative Cannabinoid Receptor GPR55 Modulates Synaptic Plasticity in the Hippocampus. Intermountain SfN Chapter Meeting, Page 4.

CONFERENCE PRESENTATIONS
- Society for Neuroscience conference in San Diego, CA. (Nov. 2016)
- Neuroscience conference in Snowbird, UT. (Oct 2016)
- Society for Neuroscience conference in Chicago, IL (Oct. 2015)
- Neuroscience conference in Snowbird, UT. (Oct 2014)
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- Neuroscience conference in Snowbird, UT. (Oct 2013)
- Society for Neuroscience conference in San Diego, CA. (Nov. 2013)

SCHOLARSHIPS and AWARDS
- Research Presentation Award Fall 2016 (BYU Graduate Student Society)
- Research Presentation Award Fall 2015 (BYU Graduate Student Society)
- Academic scholarship 2012-current (Brigham Young University)
- Academic scholarship 2010-2011 (Brigham Young University)
- Academic scholarship 2005-2007 (Brigham Young University-Idaho)

LEADERSHIP and SERVICE
- BYU Women in Science Club (April 2011-August 2011)- Promoted women in scientific field, participate in club activities, forums and service projects
- Brain Awareness Week volunteer (April 2011)- Visited high schools in Provo/Orem area and taught about brain awareness
- Volunteer Representative for the Church of Jesus Christ of Latter-day Saints (January 2008-June 2009)- Taught religious principles, conducted daily and weekly planning sessions, taught other volunteers necessary skills and teaching techniques.

PROFESSIONAL AFFILIATIONS
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