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A Putative Lysophosphatidylinositol Receptor GPR55 Modulates Hippocampal Synaptic Plasticity

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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 also 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.

Keywords

long-term potentiation; orphan G-protein coupled receptors; hippocampus; memory; anxiety; immobility; long-term depression

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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 (Scoville and Milner, 2000), declarative memory (Squire, 1992), and encoding spatial and contextual information (Burgess et al., 2002). The cellular mechanism of synaptic plasticity likely underlies these events. Synaptic plasticity includes enhancements in activity known as long-term potentiation (LTP) (Bliss and Collingridge, 1993; Bliss and Lomo, 1973) and decreases in activity known as long-term depression (LTD) (Dudek and Bear, 1992). LTP strengthens CA1 hippocampal synapses by increasing the number of postsynaptic glutamate receptors and enlarging the synapse (Malenka and Bear, 2004). On the other hand, LTD decreases the number of postsynaptic glutamate receptors (Malleret et al., 2010; Nicholls et al., 2008).

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) (Castillo et al., 2012; Gerdeman and Lovinger, 2003) and transient receptor potential vanilloid 1 (TRPV1) (Bennion et al., 2011; Chavez et al., 2010; Edwards, 2014; Gibson et al., 2008) . 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 (Hajos and Freund, 2002; Nemeth et al., 2008; Rouach and Nicoll, 2003). Similarly, a CB1/TRPV1-independent AEA-induced depression of excitatory transmission onto hippocampal stratum radiatum interneurons was demonstrated (Edwards et al., 2010). In addition, hippocampal AEA and 2-AG are present in high concentrations, but their effects are not fully explained by CB1 alone (Di Marzo et al., 2000). 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 (Gambi et al., 2005) with GPR55 being one potential candidate (Godlewski et al., 2009; Ryberg et al., 2007). In non-nervous tissues, GPR55 is characterized as a promoter of cancer cell proliferation (Andradas et al., 2011), a regulator of osteoclast number and function (Whyte et al., 2009), and a modulator of inflammatory and neuropathic pain (Staton et al., 2008). The role of GPR55 in the PNS/CNS, however, has only been demonstrated recently. In the CNS, GPR55 is involved in neuroprotection (Kallendrusch et al., 2013), hyperalgesia (Staton et al., 2008), motor coordination (Wu et al., 2013), pain perception (Deliu et al., 2015), and axon innervation/guidance (Cherif et al., 2015; Guy et al., 2015). GPR55 is widely distributed in the human brain (Sawzdargo et al., 1999) and in several regions of rodent brain (Coria et al., 2014; Serrano et al., 2012), including the hippocampus (Wu et al., 2013). Endogenously, GPR55 is activated by AEA (Yang et al., 2015), 2-AG, and LPI, the latter being a specific, natural ligand for GPR55 (Oka et al., 2007), which requires further investigation. GPR55 initiates a cascade which increases intracellular calcium (Sharir and Abood, 2010), including

in the dorsal root ganglion (Lauckner et al., 2008; Ryberg et al., 2007), likely through a RhoA-dependent mechanism (Henstridge et al., 2009). GPR55 also enhances neurotransmitter release in the hippocampal CA1 region (Sylantyev et al., 2013). GPR55 wild-type and knock-out mice were examined for potential effects on hippocampal plasticity, but no differences were noted (Wu et al., 2013). 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 P15–105. 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 (Bennion et al., 2011). 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; p15–26) 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) was used in the bath solution to reduce spiking. 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, average, 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 (Merrill et al., 2012). 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 4 °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.

2.6. 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 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 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 ethanol (1mg into 100 μL EtOH + 100 μL DDH_2O) 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 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 experiments (Figure 4C). 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 (Bennion et al., 2011) and others via an eCB CB1-dependent mechanism (Chevaleyre and Castillo, 2004), 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 (Sylantyev et al., 2013), 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 (Edwards et al., 2012). 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 (Lauckner et al., 2008; Sharir and Abood, 2010), 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 (Wu et al., 2013). 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. (Sylantyev et al., 2013), 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 (Wu et al., 2013), 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 (Wu et al., 2013), 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 (Edwards et al., 2012), causing pyramidal cell disinhibition, which could in turn lead to enhanced pyramidal cell LTP (Bennion et al., 2011; Chevaleyre and Castillo, 2004). 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 (Sylantyev et al., 2013) 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 (Del Castillo and Katz, 1954), we saw an increase. However, in hippocampal slices and cultures, it was previously noted that presynaptic vesicle release machinery could 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 (Geppert et al., 1997). This phenomenon was seen while examining presynaptic transmitter release during hippocampal plasticity (Sun et al., 2007). 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 cannot say whether 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 (Hartley et al., 2005). 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 (Wu et al., 2013). However, ours is the first study to investigate potential differences in declarative memory thoroughly. 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 (Rahimi et al., 2015). However, GPR55$^{-/-}$ mice were recently shown to have decreased voluntary physical activity (Meadows et al., 2016). 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 (Wu et al., 2013). 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 (Marichal-Cancino et al., 2016). *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 (Anavi-Goffer et al., 2012; Pineiro and Falasca, 2012; Ruban et al., 2014). Identification of LPI forming enzymes in rat brain (Pik and Thompson, 1978) indicates that GPR55 agonists can be produced in brain tissue. Indeed LPI itself has been found in large quantities in the brain (Oka et al., 2009) and has been linked to inducing phosphorylation of ERK 1/2, in turn increasing intracellular calcium (Oka et al., 2007). *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 (Kallendrusch et al., 2013). In addition, functional LPI was reported to stimulate [35S]GTPγS binding in the rat hippocampus at 10μM (Rojo et al., 2012), 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 (Sylantyev et al., 2013). 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 (Henstridge et al., 2010). 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.

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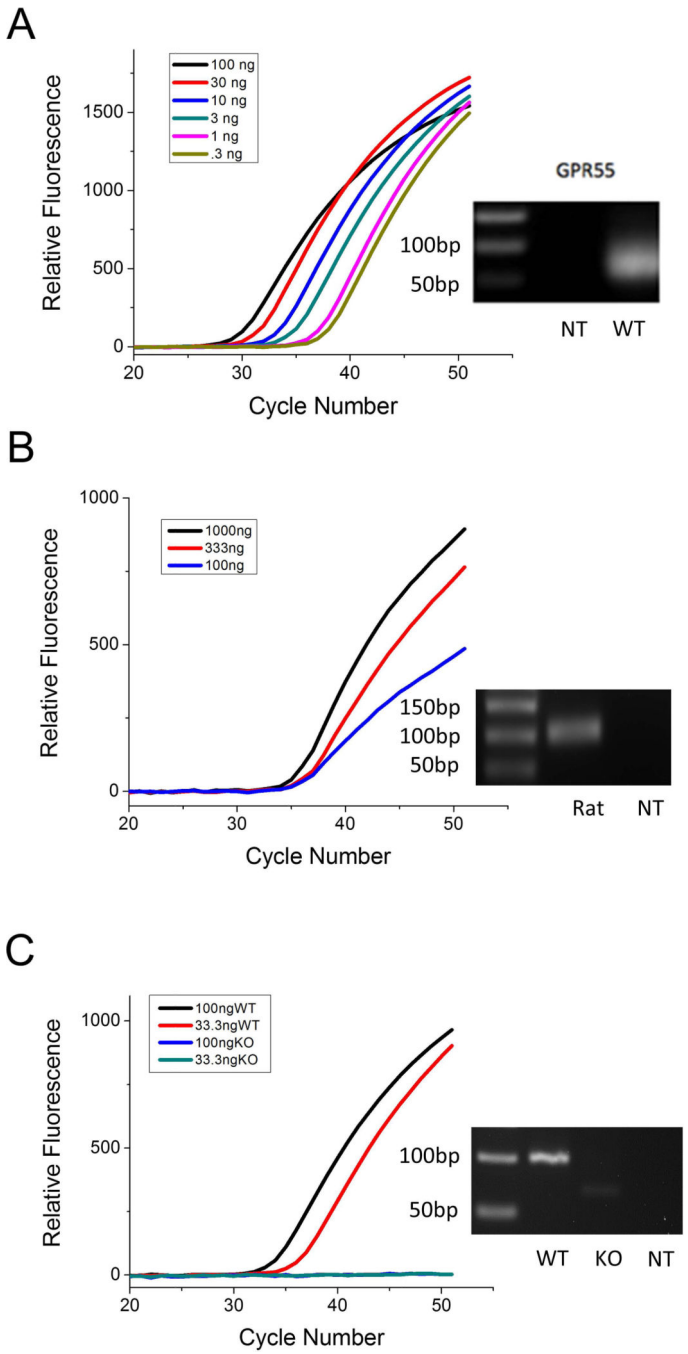

Figure 1.

GPR55 expression in rodent hippocampus. A) GPR55 mRNA is expressed in rat whole brain, as noted in dose 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). 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.

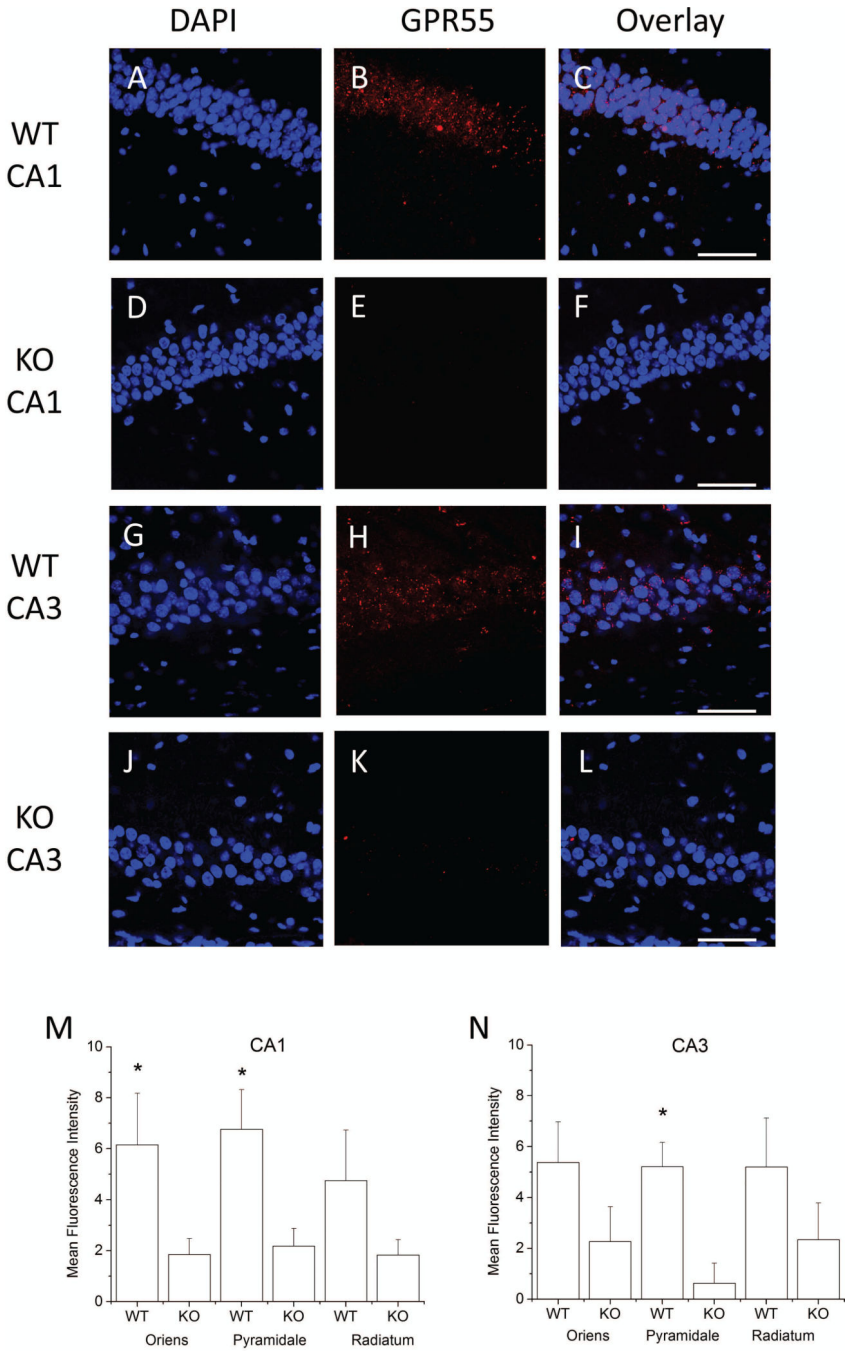

Figure 2.

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).

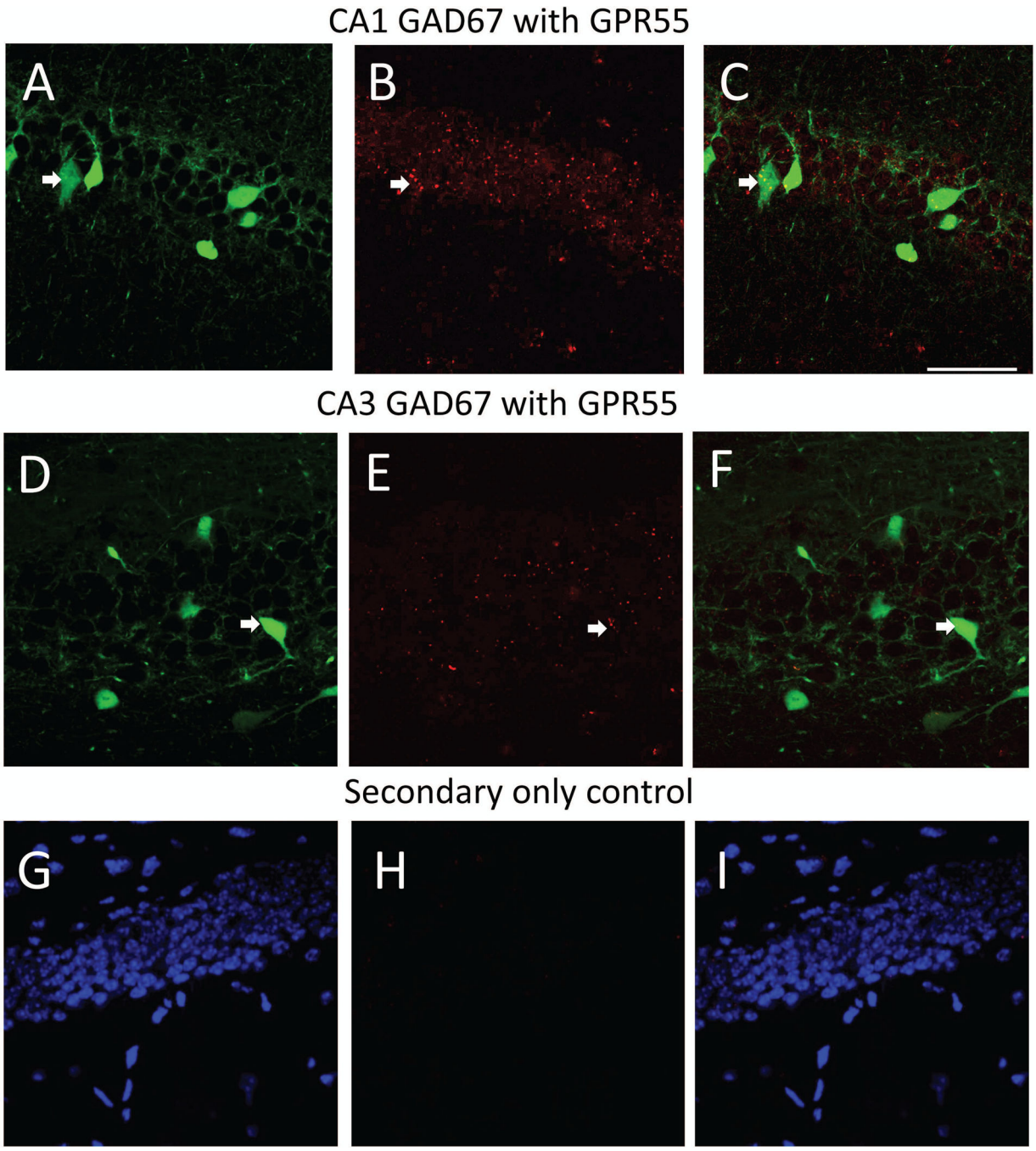

Figure 3.

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).

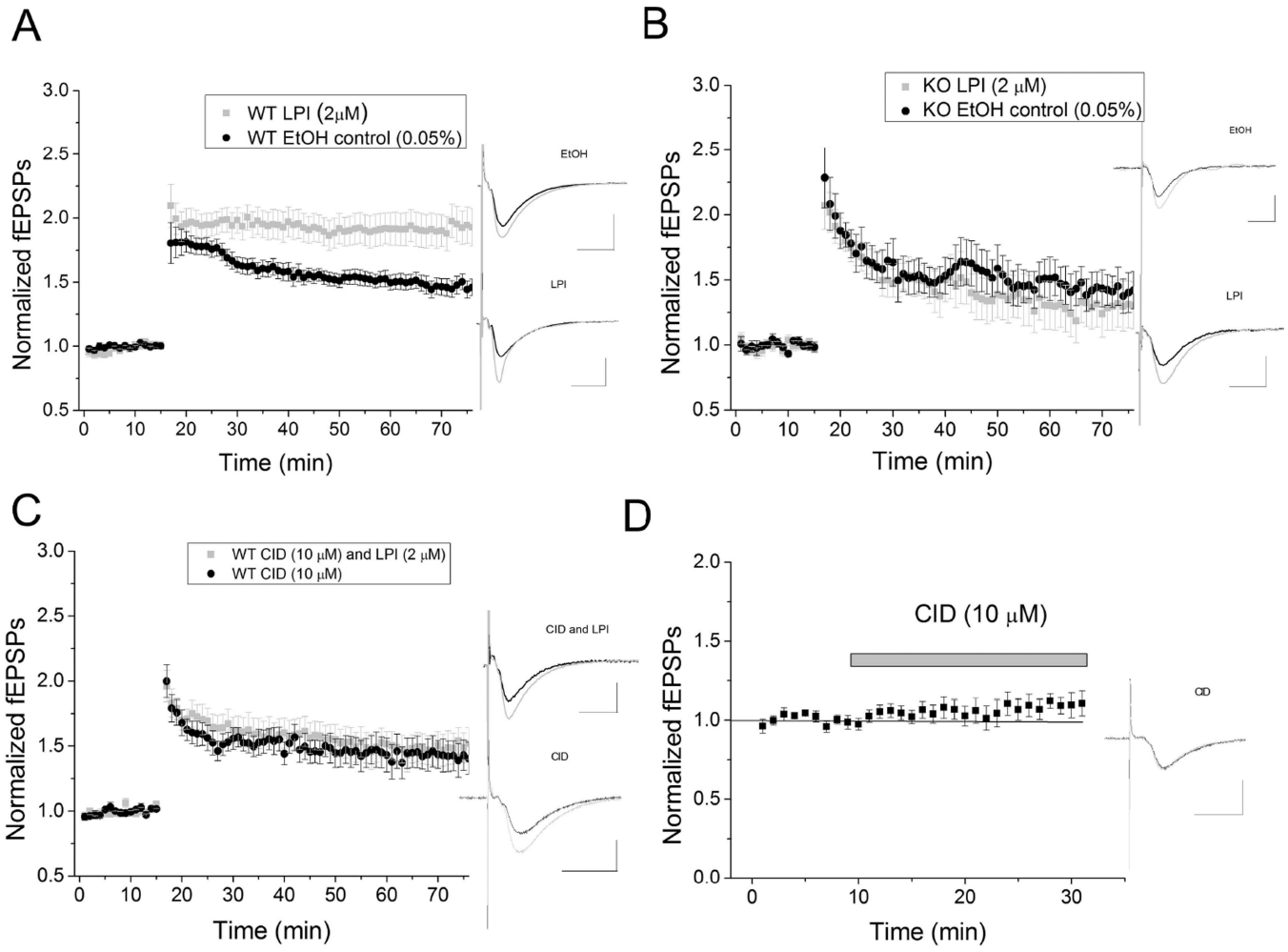

Figure 4.

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.

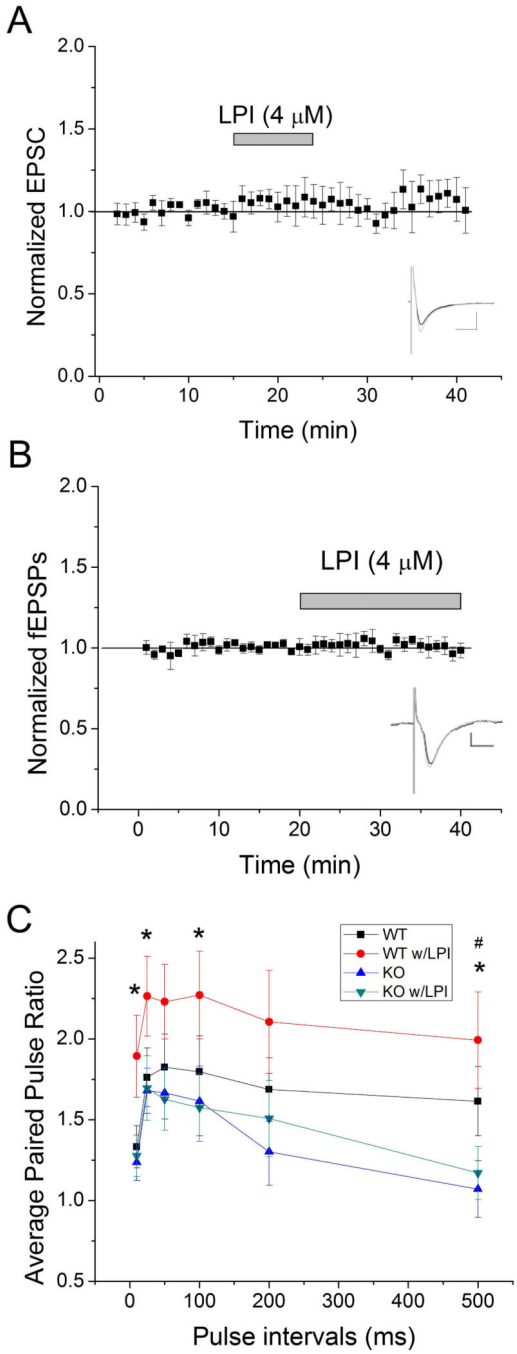

Figure 5.

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).

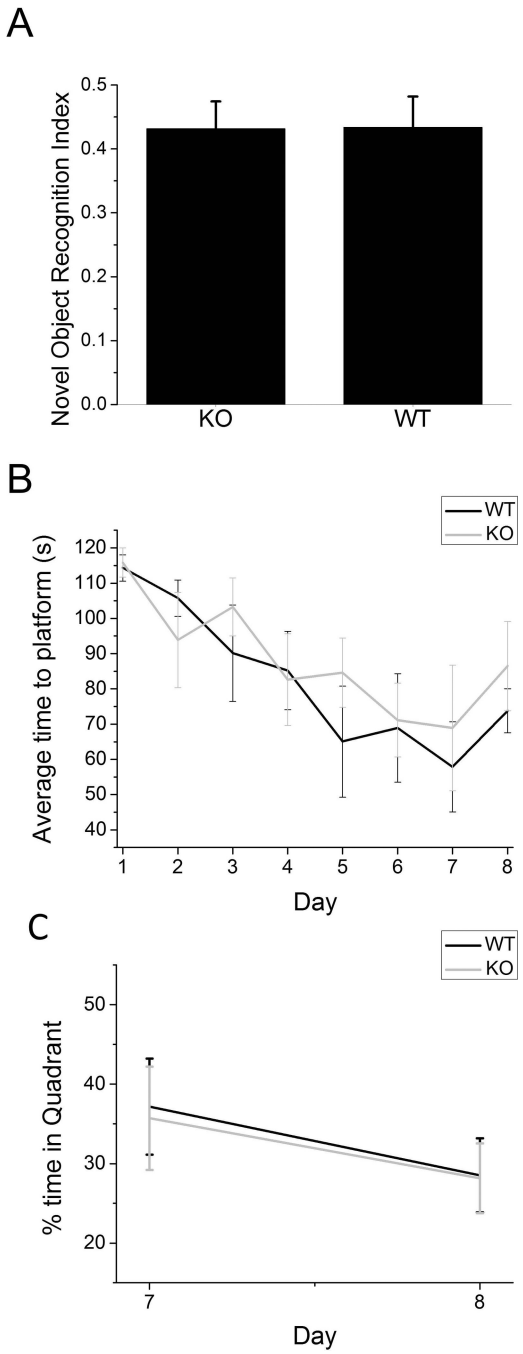

Figure 6.

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.

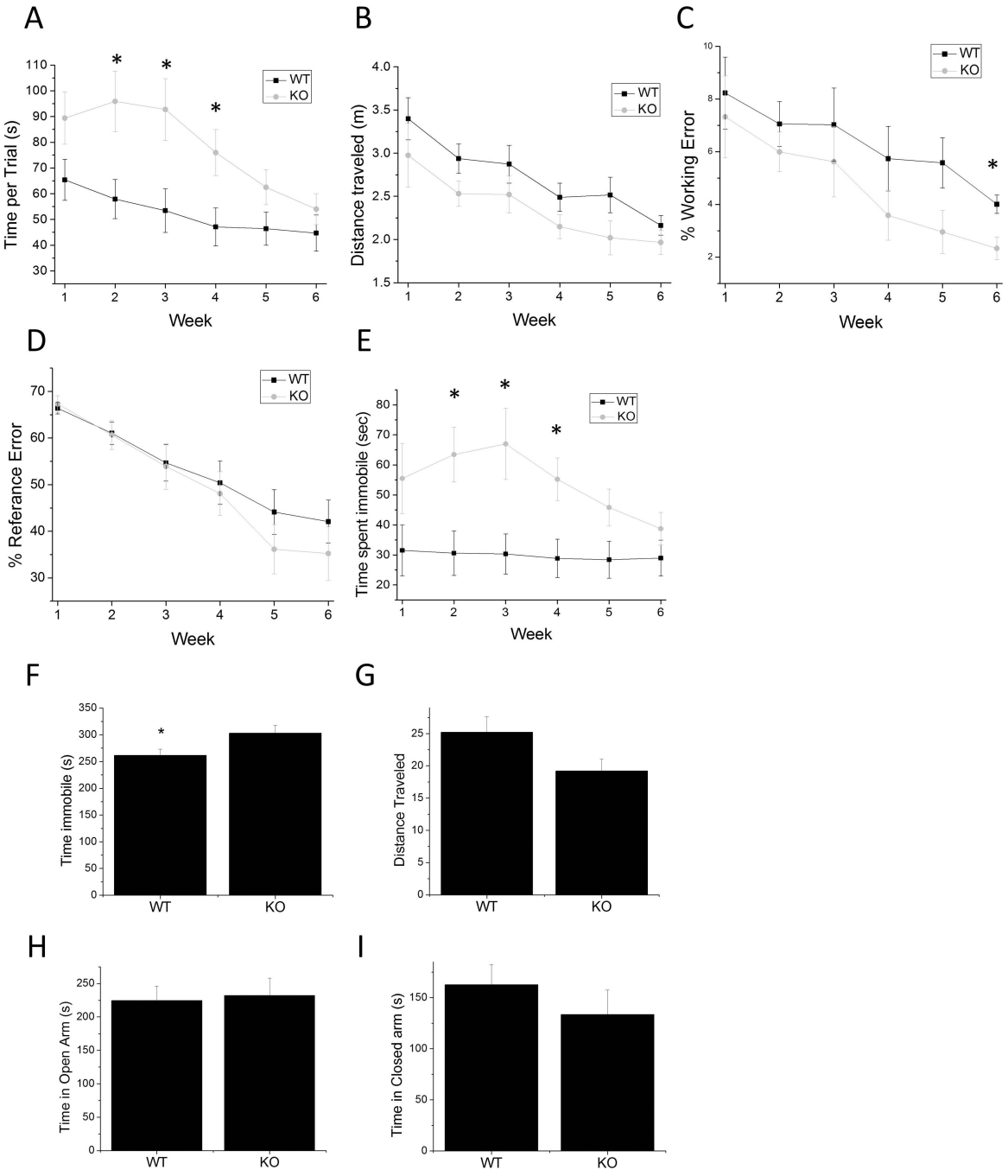

Figure 7.

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$^{-/-}$.