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Metabotropic glutamate 2,3 receptor stimulation desensitizes agonist activation of G-protein signaling and alters transcription regulators in mesocorticolimbic brain regions

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Abstract

Metabotropic glutamate (mGlu) receptors are regulators of glutamate release and targets for development of therapies for hyperactive glutamatergic signaling. However, the effects of long-term stimulation of mGlu receptors on cellular signaling in the brain have not been described. This study investigated the effects of 2-day and 14-day osmotic mini-pump administration of the mGlu_{2,3} agonist LY379268 (3.0 mg/kg/day) to rats on receptor-mediated G protein activation and signaling in mesocorticolimbic regions in rat brain sections. A significant reduction in LY379268-stimulated [³⁵S]GTPγS binding was observed in the 14-day group in some cortical regions, prefrontal cortex, nucleus accumbens, and ventral pallidum. The 14-day LY379268 treatment group exhibited mGlu₂ mRNA levels significantly lower in hippocampus, nucleus accumbens, caudate and ventral pallidum. In both 2-day and 14-day treatment groups immunodetectable phosphorylated cAMP Response Element Binding protein (CREB) was significantly reduced across all brain regions. In the 2-day group, we observed significantly lower immunodetectable CREB protein across all brain regions, which was subsequently increased in the 14-day group but failed to achieve control values. Neither immunodetectable extracellular signal-regulated kinase (ERK) protein nor phosphorylated ERK from 2-day or 14-day treatment groups differed significantly from control across all brain regions. However, the ratio of phosphorylated ERK to total ERK protein was significantly greater in the 14-day treatment group compared with control. These results identify compensatory changes to mGlu_{2,3} signal transduction in rat brains after chronic systemic administration of agonist, which could be predictive of the mechanism of action in human pharmacotherapies.

Keywords

GTPγS binding; LY379268; signal transduction; desensitization; cyclic AMP response element binding protein (CREB); extracellular signal-regulated kinase (ERK)

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Declarations of interest: none

1. Introduction

Dysregulated glutamate transmission is implicated in various neurological disorders including substance use disorder, schizophrenia, autism and epilepsy (Celli et al 2019, Maksymetz et al 2017, Niswender et al 2005). The effects of glutamate are mediated by multiple receptors that belong to either the ionotropic ligand-gated ion channels (iGlu) or metabotropic (mGlu) G protein-coupled receptor superfamilies. The mGlu receptors fall within three sub-groups based on sequence homology, signal transduction mechanisms, and pharmacological specificity (Uys & LaLumiere 2008, Willard & Koochekpour 2013). Group I encompass mGlu1 and mGlu5, which functionally couple to G_q proteins to activate the phospholipase C/inositol triphosphate and diacylglycerol (PLC/IP₃/DAG) pathway (Uys & LaLumiere 2008). Group II includes the mGlu2 and mGlu3 subtypes, which are coupled to $G_{i/o}$ proteins that inhibit cAMP synthesis, inhibit calcium channels and activate potassium channels. Group II (mGlu2 and 3) and Group III mGlu (mGlu4, 6, 7, and 8) receptors are expressed either post-synaptically, or pre-synaptically where they modulate neurotransmitter release and neuronal excitability (Crawford et al 2013, Uys & LaLumiere 2008, Willard & Koochekpour 2013). Because mGlu2 and mGlu3 receptors play a critical role in the regulation of glutamate release, these receptor subtypes are potential therapeutic targets for the regulation of hyperactive glutamatergic synapses (Crawford et al 2013, Enz 2012, Johnson et al 2011).

In recent years, mGlu2 and mGlu3 receptor agonists have been under development for treatment of neuropsychological disorders including schizophrenia (Patil et al 2007, Wieroska et al 2016) and substance abuse disorder (Cross et al 2018, Moussawi & Kalivas 2010). One prominent lead, LY379268 ((-)-2-Oxa-4-aminobicyclohexane-4,6-dicarboxylic acid), is >80-fold selective as an agonist for the group II mGlu receptors, with no affinity for iGlu or non-glutamate receptors such as the dopaminergic receptors (Crawford et al 2013, Xi et al 2011). Based upon preclinical studies in animal models, LY379268 may be promising in treatments for schizophrenia, substance use disorder and other CNS disorders that have been intransigent to other pharmacotherapies (Imre 2007). Pretreatment with LY379268, both systemic and intra-NAc core, successfully inhibited cocaine-seeking behaviors after a cocaine priming injection in self-administering rats (Peters & Kalivas 2006). LY379268 also effectively attenuated cue-induced cocaine-seeking in rats in the late phase of the withdrawal process (Lu et al 2007). LY379268 has been shown to inhibit drug-seeking behavior and to attenuate cue-induced reinstatement in rats (Lu et al 2007, Peters & Kalivas 2006). LY379268 could be effective in preventing relapse caused by anxiety and increased sensitivity to stress from withdrawal. Aujla and colleagues (Aujla et al 2007) demonstrated that LY379268 exhibited anxiolytic effects in cocaine-dependent rats using defensive burying behavior as a measure for stress after a shock stimulus. More importantly, LY379268 has not shown signs of addictive properties or desensitization of therapeutic efficacy (Imre 2007). Secondary effects of LY379268 included temporary suppression of motor activity and inhibition of food-seeking behaviors (Imre 2007), both effects seen only at high doses.

The purpose of the present study was to identify pharmacodynamic changes associated with long-term stimulation of mGlu2,3 receptors such as might occur under therapeutic regimens

with agonists. We utilized a rat model of mGlu2,3 receptor stimulation by continuous systemic delivery of LY379268 via osmotic mini-pumps compared with sham control rats in order to avoid the stress of daily multiple injections. We assessed brain signal transduction after 2-day and 14-day minipump administration, taking into consideration that the first 24 hrs. include latency to onset of release, distribution of drug to tissues, and achievement of steady-state levels. The data identified cellular signaling changes associated with long-term agonist exposure on parameters of G protein activation, mGlu2 receptor mRNA, and phosphorylation of cyclic AMP response element binding protein (CREB) and extracellular signal-regulated kinase (ERK) in mesocorticolimbic brain regions of the rat.

2. Methods

2.1 Reagents

LY341495, DCG-IV and LY379268 were from Tocris Bioscience (Minneapolis, MN, USA). 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was from Research Biochemicals International (Natick, MA, USA). [35 S]GTP γ S was purchased from Perkin Elmer (Waltham, MA, USA).

2.2 Animals

Male Sprague Dawley rats (300–350 g) (Harlan Industries, Indianapolis, IN, USA) were acclimated for seven days in a reverse 12-hr light/dark cycle, with food and water *ad libitum*. Animals were pair-housed before mini-pump implant surgery and were single-housed after the surgery. Animal procedure protocols were approved by the Wake Forest School of Medicine Institutional Animal Care and Use Committee and conformed to the principles in the NIH Guide for the Care and Use of Laboratory Animals.

Rats were randomly assigned to one of three treatment groups: control (n=8), 2-day LY379268 (n=8), or 14-day LY379268 (n=8). To implant the mini-pumps, anesthesia was induced with 5% inhaled isoflurane and maintained with 1–2.5% isoflurane. For 2-day and 14-day treatment groups, a 3.0 cm mini-pump (ALZET® Osmotic Pumps, Cupertino, CA, USA) was inserted subcutaneously over either shoulder. After a 12-hr latency, each pump (208 μ l fill volume) released 1.0 μ l/hr., providing a 7-day dosage of 3.0 mg/kg/day LY379268. This dose was chosen to represent the range of doses used in previous studies showing efficacy of LY379268 in reducing cocaine and methamphetamine self-administration in (Baptista et al 2004, Crawford et al 2013, Hao et al 2010, Karkhanis et al 2016). Rats in the 14-day treatment group underwent the implantation procedure twice consecutively to give 14 days of continuous drug exposure. The drug-naïve, sham control group underwent procedures identical to the 14-day group minus the drug pump. All rats received an injection of 3.0 mg/kg ketoprofen after surgery for analgesia. The rats were sacrificed by guillotine, and the brains were quick-frozen in isopentane at -30°C and stored at -80°C . The frozen brains were sliced in coronal sections at the indicated thickness for the determinations described below, using a Leica cryostat microtome (Leica Biosystems Inc., Buffalo Grove, IL, USA) at -22°C . Tissue sections for all the experiments performed in this study, including [35 S]GTP γ S autoradiography, mRNA analysis of mGlu2,3 receptors, and staining for pCREB and pERK, were from the same animals. Sections for [35 S]GTP γ S

autoradiography and mRNA analysis were cut at 20 μm thickness, while sections for pCREB and pERK analysis were cut at 30 μm for optimal post-staining and immunofluorescence results. Alternating sections from the same brain were used for each assay described below. Coordinates for the five levels used (relative to bregma): Level 1, +2.70 mm to +0.70 mm; Level 2, +0.20 mm to -1.40 mm; Level 3, -2.12 mm to -4.30 mm; Level 4, -4.80 mm to -6.30 mm; Level 5, -8.72 mm to -10.04 mm.

2.3 [^{35}S]GTP γS binding analyses

Agonist-stimulated [^{35}S]GTP γS binding was performed in membranes from rat frontal cortex in order to determine optimal concentrations of agonist to assay mGlu2,3-activated G proteins, and to confirm the pharmacological specificity of this assay, using a modification of previously described methods (Breivogel et al 1999, Sim-Selley et al 2000a, Sim-Selley et al 2000b). Membranes prepared from frontal cortex of untreated rats were suspended in assay buffer (50 mM Tris-HCl, 3 mM MgCl_2 , 0.2 mM EGTA, 100 mM NaCl, pH 7.7) with the indicated concentrations of mGlu2,3 agonists (LY379268 and DCG-IV), in the presence or absence of antagonist (LY341495), 30 μM GDP, 0.05 nM [^{35}S]GTP γS , 4 mU/ml adenosine deaminase, 5–15 μg protein and assay buffer in a final volume of 1 ml. Basal binding was determined in the presence of GDP and absence of drug, and nonspecific binding was assessed in the presence of 10 μM unlabeled GTP γS . Assays were incubated at 30° for 2 hr. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters followed by three washes with 3 ml cold 50 mM Tris-HCl buffer pH 7.7. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [^{35}S] after overnight extraction of the filters in 4 ml ScintiSafe Econo scintillation fluid. Data are reported as mean $\pm$ standard error of three separate experiments each performed in triplicate. Percent stimulation is defined as (net stimulated binding / basal binding) $\times$ 100%.

For [^{35}S]GTP γS autoradiography, frozen rat brain coronal sections were thawed onto glass slides as described previously (Sim et al 1995, Sim et al 1997). Briefly, brain sections were washed with TME buffer (50 mM Tris-HCl, pH 7.4; 3 mM MgCl_2 ; 0.2 mM EGTA; 100 mM NaCl) for 10 min at 25°C prior to incubation with TME buffer containing 2 mM GDP for 15 min at 25°C. Sections were then incubated for 2 hr. at 25°C in TME buffer containing 2 mM GDP, 100 nM DPCPX (an A_1 adenosine receptor antagonist), and 0.04 nM [^{35}S]GTP γS in the presence or absence of 1 μM LY379268. The sections were washed twice with 50 mM Tris-HCl, pH 7.4 at 4°C, rinsed once with deionized water, and were exposed to phosphor-imaging screens overnight. Screen images were digitally captured, and quantitative densitometric analysis was performed on regions of interest using NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA). Regions of interest were defined by user-defined settings in NIH ImageJ software that selected areas of highest optical density. Optical densities were quantitated by comparison with [^{14}C] brain paste standards and values corrected to nCi/g [^{35}S]. [^{35}S]GTP γS binding data were expressed as percent of net agonist-stimulated binding in sections from control rats.

2.4 RNA isolation and quantitation

RNA was isolated from frozen brain sections thawed on ice, and tissue from appropriate brain areas was isolated with punches. Total RNA was isolated using the RNeasy isolation kit (Qiagen, Valencia, CA, USA). High-purity RNA was quantitated on the NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and was converted to single-stranded cDNA using a high-capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA). Oligonucleotide primers for quantitative real-time polymerase chain reaction (qPCR) were designed using the NCBI Primer-BLAST program, and were synthesized by Integrated DNA technologies (Coralville, IA, USA): Rat mGlu2 Forward: 5'-GTGACAAGTCCCGTTACGATTA-3'; Reverse: 5'-CTCAATGCCTGTCTCACCATAG-3'; Rat mGlu3 Forward: 5'-GGAGTCATTGGTGGTTCATACA-3'; Reverse: 5'-ACGGTCCTGGCAAAGTAATC-3'; Rat β -actin Forward: 5'-ACAGGATGCAGAAGGAGATTAC-3'; Reverse: 5'-ACAGTGAGGCCAGGATAGA-3'. qPCR was performed with Fast SYBR Green Master Mix (Invitrogen) in a 96-well format using an ABI 7500 Fast real-time PCR System (Applied Biosystems, Foster City, CA, USA). Samples containing no cDNA template and no reverse transcriptase were run as controls for contamination and amplification of genomic DNA, respectively. For each gene, all treatment groups were determined concurrently in triplicate on the same 96-well plate and were normalized to rat β -actin using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001).

2.5 Immunofluorescence staining for total and phosphorylated CREB and ERK1/2 proteins

Frozen rat brain coronal sections were placed flat onto frozen phosphate-buffered saline/4% paraformaldehyde/30% sucrose solution in 24-well plates, which thawed simultaneously and were stored at 4°C. The brain slices were washed with Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.4; 137 mM NaCl) to remove the fixative, transferred to 24-well plates using a camel hair brush (0), and washed twice with TBS. The sections were blocked with immunohisto-chemistry (IHC) buffer comprised of (20 mM Tris-HCl, 137 mM NaCl, 0.1% NP40) : Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) (1:1) at 4°C for 24 hr., followed by primary antibodies (1:250 dilution in IHC buffer) at 4°C for 24 hr. Primary antibodies which recognize α , β , and δ CREB isoforms (CREB-1 (sc-377154), and phospho-CREB-1 (sc-101663)) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies detecting ERK (L34F12) and phospho-ERK (Y204) were from Cell Signaling (Danvers, MA, USA). The brain sections were washed 4 times with TBS containing 0.1% Tween-20 before incubation with the secondary antibodies conjugated to 680 nm or 800 nm IRDye® fluorophores (LI-COR Biosciences®, Lincoln, NE, USA) at 1:1500 dilution in IHC blocking buffer, at room temperature for 2 hr. After washing the brain sections 3 times, the TBS-Tween20 was slowly aspirated while the brain slice was carefully adjusted to be flat on at the bottom of the well using a camel hair brush, avoiding wrinkles or bubbles. The brain slices were dried for 4 days in the dark at room temperature before imaging using LI-COR Odyssey® CLx Infrared Imaging System (LI-COR Biosciences®, scanning settings: 84 μ m resolution, 2.5 mm offset, and highest quality). Densitometric data were analyzed using the Image Studio™ Odyssey software version 1.0.11. Each brain region of interest was hand-drawn on each brain section for

a more accurate measurement following the parameters of *The Rat Brain in Stereotaxic Coordinates. Second edition* (Paxinos & Watson 1986). Background signal was subtracted from the total signal of the channel on the brain slice and divided by the area, giving a normalized intensity signal in arbitrary units for the specific region. The right and left hemisphere intensities were averaged to obtain one measurement per region per rat. To determine the fraction of the phosphorylated protein, a ratio was determined by dividing the intensities of the phosphorylated protein signal by the total protein signal. The mean and SEM values were obtained and expressed as a percent of the mean of control rats as 100%.

2.6 Data Analyses

The data were analyzed using Excel and GraphPad Prism 7 and the graphs were prepared using GraphPad Prism 7. Outlier values were assessed using the GraphPad ROUT method. A one-way analysis of variance (ANOVA) followed by a Tukey's or Fisher's LSD post-hoc test was used to determine differences between treatment groups. Statistical significance was assigned to a p value <0.05.

3. Results

3.1 mGlu2,3 receptor-stimulated G protein activation in rat brain membranes and brain sections.

In order to determine optimal concentrations of mGlu2,3 agonists to assay agonist activation of G proteins coupled to mGlu2,3 receptors, we performed concentration-effect experiments of [³⁵S]GTPγS binding in rat cortical membranes. Fig. 1A shows stimulation of [³⁵S]GTPγS binding by two mGlu2,3 agonists, LY379268 and DCG-IV. Both agonists activated G proteins with the same efficacy, but LY379268 was an order of magnitude more potent than DCG-IV, with an EC₅₀ value of 0.019 ± 0.012 μM for LY379268 compared to 0.16 ± 0.17 μM for DCG-IV. To confirm that activation of G proteins by LY379268 was mediated by mGlu2,3 receptors, concentration-effect curves for LY379268 were generated in the presence or absence of 100 nM of the specific mGlu2,3 antagonist LY341495. Fig. 1B shows that the effects of LY341495 were consistent with those of a competitive antagonist at mGlu2,3 receptors, shifting the LY379268 curve to the right with a K_e value of 2.6 ± 0.7 nM.

Fig. 2 shows the brain regional distribution of mGlu2,3-activated G proteins from LY379268-stimulated [³⁵S]GTPγS binding in five levels of frozen brain sections from untreated rats. Significant stimulation of [³⁵S]GTPγS binding by LY379268 was observed in numerous regions; quantitation of agonist-stimulated binding was performed in the following:

- Level 1: nucleus accumbens (L1), insular cortex (L1), cingulate cortex (L1), prefrontal cortex;
- Level 2: nucleus accumbens (L2), insular cortex (L2), cingulate cortex (L2), caudate-putamen, frontal cortex, ventral pallidum;
- Level 3: hippocampus (L3), cortex (L3), amygdala;

Level 4: hippocampus (L4), cortex (L4);

Level 5: cerebellum, parabrachial nucleus.

3.2 The effects of prolonged mGlu2,3 receptor stimulation *in vivo* on G protein activation in mesocorticolimbic brain regions.

To examine the effect of 14-day treatment with LY379268 on G protein activation by mGlu2,3 receptors, rats were implanted with osmotic mini-pumps delivering LY379268 for 2 days or 14 days as indicated. Brain sections were assayed for mGluR2,3-stimulated [³⁵S]GTPγS binding using 1 μM LY379268 as agonist. Table 1 shows net agonist-stimulated [³⁵S]GTPγS binding from the autoradiograms in rat brain sections from each treatment group, with brain regions selected as described above. These data show no significant effect of the 2-day treatment with LY379268 on agonist-stimulated binding in any region selected. However, the 14-day treatment resulted in significant decrease in agonist stimulated binding in four regions: nucleus accumbens (NAc), insular cortex (AI2), frontal cortex (FC), and ventral pallidum (VP), all regions in Level 2. In Fig. 3, the data in Table 1 are normalized as per cent of net agonist-stimulated binding in each region of control rat brains. These data confirm the results from Table 1: no significant effects on agonist-stimulated [³⁵S]GTPγS binding in any brain regions from rat after two days LY379268 treatment, and significant reduction in net agonist-stimulated binding in four regions, including NAc, AI2, FC, and VP, after LY379268 treatment for 14 days. These data are consistent with desensitization of the mGlu2,3 response in these regions after continued exposure to the mGlu2,3 agonist LY379268.

3.3 Effect of LY379268 treatment on mGlu2 and mGlu3 gene expression in mesocorticolimbic brain regions

Of the 19 regions that we quantified for mGlu2,3-activated G proteins by autoradiography, we chose five selected regions for mGlu2 and mGlu3 mRNA analyses, based on relatively high levels of agonist-stimulated activity, as well as containing sufficient tissue for reliable mRNA analysis. Desensitization of G protein-coupled receptor function can be accompanied by an internalization and degradation of receptors (Pavlos & Friedman 2017) which might require new protein synthesis to reinstate receptors. Fig. 4 shows that both 2-day and 14-day LY379268 treatment groups exhibited a significant increase of mGlu2 mRNA levels in the VP. However, the 2-day treatment group exhibited a small but significant increase in mGlu2 mRNA in the FC, with a small decrease in hippocampus (hipp) and NAc which was also reduced in the 14-day group. In contrast, LY379268 had no significant effect on mGlu3 mRNA levels except for a small but significant increase in the FC in the 2-day treatment group.

3.4 Effects of prolonged mGlu2,3 receptor stimulation on cellular signaling kinases in mesocorticolimbic brain regions

One of the effector pathways for mGlu2,3 receptor signaling is Gi-mediated inhibition of adenylyl cyclase, leading to reduced protein kinase A (PKA) activity. Phosphorylation of CREB Ser-133 by PKA allows association with CREB binding protein and promotes gene transcription (Gonzalez & Montminy 1989). Table 2 shows the relative fluorescence units

of CREB and phosphoCREB for individual brain regions. Fig. 5A shows that compared with control (17.42 ± 0.553), phosphorylated CREB was significantly reduced in the 2-day (11.91 ± 0.528), and 14-day (10.54 ± 0.40) LY379268 treatment groups. Fig. 5B shows that compared with control (5.62 ± 0.140), CREB protein density was significantly reduced (3.87 ± 0.147) in the 2-day treatment group. However, the 14-day CREB protein density (5.015 ± 0.211) was increased relative to the 2-day treatment group but still lower than control. Fig. 5C shows that compared with control (3.24 ± 0.067), the ratio of phosphorylated to total CREB was increased (3.88 ± 0.242) in the 2-day LY379268 treatment group. It should be noted that the two regions that contributed primarily to this effect were the ventral tegmental area (VTA) and the substantia nigra (SN). The ratio of phosphorylated to total CREB was significantly decreased (2.15 ± 0.091) in the 14-day treatment group compared with control or 2-day groups.

Activation of the Tyr-204/187 and Thr-202/185 of ERK1 and ERK2, respectively, occurs upon phosphorylation by MAPK-ERK Kinase (MEK) (Naor et al 2000). MEK can be phosphorylated by PKA, which reduces the MEK activity, thereby reducing ERK phosphorylation. Reduced PKA activity would be expected to increase phosphorylation of ERK1/2, allowing dimerization and exposure of the nuclear localization signal, and translocation of the ERK dimer into the nucleus (Lidke et al 2010). Table 2 shows the relative fluorescence units of ERK and phosphoERK for individual brain regions. In contrast to the changes in CREB, there was not a significant difference in phosphoERK (Fig. 6A) or the ERK protein (Fig. 6B) immunodensities between control, 2-day or 14-day treatment groups. Although the small increase in the ratio of phosphorylated to total ERK in the 2-day LY379268 treatment group (9.17 ± 0.424) was not statistically different from control (7.60 ± 0.334), a significant increase in the ratio of phosphorylated to total ERK was observed in the 14-day LY379268 treatment group (9.98 ± 0.590) compared with control (Fig. 6C). These data indicate that 14-day agonist exposure of mGlu2,3 receptors led to continued increase in the fraction of ERK in the phosphorylated state.

4. Discussion

The present study was designed to examine effects of long-term mGlu2,3 agonist exposure on three parameters of receptor signaling in mesocorticolimbic brain regions of the rat: mGlu2,3 receptor activation of G proteins, regulation of mGlu2,3 receptor mRNA levels, and signaling through kinases that govern transcription. One of the strengths of this design is that all three of these parameters were studied from frozen sections from brains obtained from the same treated animals.

The finding that 14-day, but not 2-day, treatment with LY379268 decreased agonist-stimulated [^{35}S]GTP γ S binding to G proteins is consistent with agonist-induced desensitization. The fact that this desensitization effect varied between different brain regions is consistent with 14-day agonist-induced desensitization measured previously with mu opioid (Sim-Selley et al 2000b), CB₁ cannabinoid (Breivogel et al 1999) and 5HT_{1A} serotonergic receptors (Sim-Selley et al 2000b). It is interesting to note that the regions that exhibited chronic agonist-induced desensitization in this study are not the same regions exhibiting similar effects in other G protein-coupled receptor studies, nor do they correspond

to regions with either highest or lowest levels of mGluR2,3-stimulated [^{35}S]GTP γ S binding (Table 1). The factors that regulate these chronic effects in different brain regions clearly vary with different receptors.

In the present study, we observed that phosphorylated CREB was reduced after both 2-day and 14-day LY379268 treatments. These data are consistent with an mGlu2,3 receptor- and Gi/o-mediated reduction in cAMP production, and subsequent reduction in PKA-mediated CREB phosphorylation. We also observed that stimulation of mGlu2,3 receptors with LY379268 decreased immuno-detectable CREB protein, which could indicate homeostatic adjustments are occurring over the 14-day treatment at the level of cellular regulation of CREB synthesis and/or degradation rates to counteract the chronic agonist-induced desensitization of receptor activated G proteins. Through immunostaining experiments, it has been shown that phosphorylated CREB co-localizes with the active form of SUMO-1. This co-localization allows SUMO to inactivate CREB and recruit transcriptional regulators in a nuclear compartment for stabilization or degradation (Navascués et al 2007).

Our study identified chronic agonist-induced desensitization of mGluR2,3 receptor-activated G proteins in some brain regions, yet continued diminished CREB phosphorylation and protein levels after 14-day mGlu2,3 receptor stimulation. Larson et al. (2011) demonstrated that increases in CREB expression increased reinforcement of cocaine self-administration and motivation to seek cocaine, whereas decreased CREB expression facilitated the extinction of cocaine-seeking and reduced reinforcement effects. Not only is CREB a key regulator of reinforcement independent of the learning association process, CREB function is also important in withdrawal and craving (Larson et al., 2011). These findings, in addition to our data described herein, can explain at least one cellular mechanism for LY379268's inhibition of drug-seeking and attenuation of craving (Lu et al 2007, Peters & Kalivas 2006).

We observed significant differences in the ratio of phosphorylated to total ERK protein levels in rats after 14-day exposure to LY379268. It is possible that the dose was below that necessary to produce significant changes in ERK after 2-day exposure. Alternatively, the regulation of this kinase is complex, and can be initiated with different time courses by mGlu2,3 receptor-mediated release of the Gi $\beta\gamma$ dimer, or by a process regulated by β -arrestins (Pouyssegur et al 2002). Phosphatase-mediated dephosphorylation also regulates ERK1/2 signaling (Pouyssegur et al 2002). mGlu2,3-mediated ERK phosphorylation plays a role in brain function in multiple cell types that would be present in the brain sections. For example, Chia-Ho and colleagues observed that LY379268 has cytoprotective effects through the activation of ERK in cultured astrocytes (Chia-Ho et al 2014, Ciccarelli et al 2007); and Wang and colleagues observed LY379268-mediated ERK phosphorylation associated with AMPA receptor cell surface localization in cultured prefrontal cortex neurons (Wang et al 2013). In *in vitro* studies such as these, the time necessary to increase ERK phosphorylation could range from minutes to hours, and ERK protein levels may not be altered within this timeframe. Irrespective of the responding cell type or cellular mechanisms, our data indicating differences between the G protein desensitization and the ERK phosphorylation upon chronic exposure provides an intriguing consideration for "biased agonism" in pharmacotherapeutic design. The distinction between desensitization (uncoupling of receptor from the G protein signal) versus internalization of the receptor-G

protein complex is one source for biased agonist effects. Biased agonism can also arise through signaling endosomal retention intracellularly versus lysosomal degradation.

Our observation of desensitization of mGluR2 following chronic exposure could be related to an increase in mGluR2 gene expression as a compensatory change. However, it is interesting to note that in the experimental treatments with LY379268, the mGlu2 mRNA transcript levels were reduced in a number of regions, concurrently with a reduction in phosphoCREB densities associated with 2-day and 14-day treatment. Based on Qiagen GeneBlobe analysis of transcription factors, the mGlu2 promoter region possesses a cAMP response element that CREB binds, but not the c-fos serum response element (SRE) that ERK binds (<https://www.genecards.org/cgi-bin/carddisp.pl?gene=GRM2>). Thus, the decreased activity of CREB evidenced by phosphoCREB suggests that activation of transcription of mGluR2 gene could be attenuated.

It is tempting to attribute development of tolerance to effects of long-term exposure in intact animals to the neurochemical changes in cellular signaling parameters that we report herein. In preclinical models of LY379268 for neuropsychological diseases, tolerance developed to the primary untoward effect of motor suppression, but not to promising therapeutic responses (Imre 2007). For example, behavioral tolerance has been reported for the mGlu2 agonist-mediated disruption of REM sleep in rats after three days of daily oral administration (Ahnaou et al 2015). Tolerance occurred to the untoward motor impairment (rotorod performance) observed after four days of high dose daily oral administration LY379268; however, tolerance failed to occur to the therapeutic response to ameliorate phencyclidine-evoked ambulation excitation after low dose daily oral administration (Cartmell et al 2000). This observation reinforces the goal of separating beneficial versus untoward effects of drugs by understanding the cellular signaling pathway leading to each, and taking advantage of desensitized versus prolonged signaling responses in drug design and development.

5. Conclusion

In sum, our findings provide insight to modifications to signal transduction that occur in brain areas receiving long-term stimulation of mGlu2,3 receptors. The neurochemical consequences of chronic systemic administration may be important when mGlu2 and mGlu3 agonists are considered for pharmacotherapeutic treatment of diseases of glutamatergic dysregulation.

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Data Availability Statement:

Original data files will be made available upon request.

Abbreviations:

AI	insular cortex
Amyg	amygdala
Cereb	cerebellum
Cing	cingulate cortex
Cort	cortex
CPu	Caudate Putamen
CREB	cAMP response element binding protein
ERK	extracellular signal-regulated kinase
FC	Frontal Cortex
Hipp	Hippocampus
Hypo	hypothalamus
iGlu	Ionotropic glutamate receptors
mGlu	metabotropic glutamate receptors
NAc	nucleus accumbens
PAG	periaqueductal grey
PBN	parabrachial nucleus
PFC	prefrontal cortex
VP	ventral pallidum
VTa	Ventral Tegmental Area

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Figure 1. Effect of mGlu2,3 agonists and antagonists on [35 S]GTP γ S binding in rat cortical membranes. A. LY379268 and DCG-IV.

Membranes were prepared from rat frontal cortex and assayed with agonists for [35 S]GTP γ S binding in solution as described in 2.3. **B. agonist LY379268 in the presence or absence of antagonist LY341495.** Membranes from rat frontal cortex were incubated with LY379268 in the presence or absence of 100 nM LY341495. Results are expressed as percent stimulation of [35 S]GTP γ S binding above basal and represent mean $\pm$ SEM of three separate experiments.

Figure 2. Autoradiography of LY379268-stimulated [35 S]GTP γ S binding in rat brain. Frozen brain sections at five levels were incubated with 1 μ M LY379268 and images were visualized on phosphor-imaging screens as described in 2.3. The figure is a typical autoradiogram from an untreated rat.

Figure 3. Effect of 2-day and 14-day treatment of rats with LY379268 on LY379268-stimulated [35 S]GTP γ S binding in brain sections.

Rats implanted with mini-pumps releasing LY379268 in 2-day or 14-days protocols were sacrificed and [35 S]GTP γ S binding and autoradiography were quantitated as described on 2.3. Results of agonist-stimulated [35 S]GTP γ S binding are expressed as a percent of net binding in control animals as 100%. Data were analyzed by one-way ANOVA and Fisher's LSD post-hoc test, with significant differences from control (* $p < 0.05$) in the NAc ($p=0.04$), AI2 ($p=0.001$), FC ($p=0.001$), and VP ($p=0.03$) ($N=6$ rats per group).

Figure 4. The effect of LY379268 treatment on relative mRNA levels for mGlu2 and mGlu3 receptors.

Relative mRNA levels were determined as described in 2.4, and analyzed for each region by ANOVA followed by Tukey's test. The mRNA for the mGlu2 receptor was significantly different in 2-day treatment compared with control in frontal cortex (FC) ($p=0.0214$), nucleus accumbens (NAc) ($p=0.0005$), ventral pallidum (VP) ($p<0.0001$), and hippocampus (Hipp) ($p=0.0022$). The mRNA in the 14-day treatment was significantly different compared with control in the caudate putamen (CPu) ($p=0.0029$), NAc ($p=0.0001$), VP ($p=0.0003$), and Hipp ($p=0.0003$). The mRNA for the mGlu3 was significantly different in the 2-day treatment group compared with control in the FC ($p=0.0145$). (N=6 rats per group).

Figure 5. The effect of LY379268 treatment on phosphorylated and total CREB immunofluorescence density and phosphorylation ratio.

Brain sections were alternate slices from animals depicted in Figs. 3 and 4, and were immunostained and analyzed as described in 2.5. **A. Phosphorylated CREB immunodensity.** A one-way ANOVA with a Tukey's post-hoc test showed the 2-day and 14-day treatment groups significantly differed from the control group ($F(2, 33) = 53.86$, $p < 0.0001$); **B. Total CREB immunodensity.** A one-way ANOVA and a Tukey's test showed a significant difference ($F(2, 33) = 27.32$) between the 2-day treatment group and the control group ($p < 0.0001$) and the 14-day treatment group ($p < 0.0001$). The 14-day treatment group also differed from the control treatment group ($p < 0.05$). **C. Phospho-CREB to total CREB ratio.** A one-way ANOVA and a Tukey's test showed a significant difference ($F(2, 33) = 32.00$) between the control group and the 2-day treatment group ($p < 0.05$) and the 14-day group ($p < 0.0001$). The 2-day treatment differed significantly from the 14-day treatment group ($p < 0.0001$) (*= $p < 0.05$, **= $p < 0.0001$) (N=6 rats per group). A.U.: Arbitrary Units

Figure 6. The effect of LY379268 treatment on phosphorylated and total ERK levels and phosphorylation ratio.

Brain sections were alternate slices from animals depicted in Figs.3 and 4, and methods were described in 2.5.

A. Phosphorylated ERK immunodensity. A one-way ANOVA showed no significant difference between treatment groups ($F(2, 33) = 3.272, p=0.051$).

B. Total ERK immunodensity. A one-way ANOVA showed no significant differences ($F(2, 33) = 2.708, p=0.082$).

C. Phospho-ERK to total ERK ratio. A one-way ANOVA and a Tukey's test showed a significant difference ($F(2, 33) = 6.910, p=0.0031$) between the control group and the 14-day treatment group (*= $p<0.05$). (N=6 rats per group). A.U.: Arbitrary Units

Table 1.

Effect of LY379268 treatment (2 days and 14 days) on net mGluR2,3 agonist-stimulated [^{35}S]GTP γ S binding in rat brain regions

Region	Rat treatment		
	Control	LY 2 days	LY 14 days
Cingulate cortex L1	99.8 $\pm$ 6.9	105 $\pm$ 7.0	104 $\pm$ 9.6
Nucleus accumbens L1	78.2 $\pm$ 6.8	75.6 $\pm$ 8.1	74.5 $\pm$ 7.4
Insular cortex L1	106 $\pm$ 5.5	102 $\pm$ 7.6	109 $\pm$ 5.9
Prefrontal cortex L1	101 $\pm$ 5.9	92.8 $\pm$ 7.1	104 $\pm$ 5.7
Cingulate cortex L2	73.3 $\pm$ 5.7	67.4 $\pm$ 4.9	68.3 $\pm$ 3.9
Nucleus accumbens L2	75.1 $\pm$ 5.4	68.8 $\pm$ 5.8	62.8 $\pm$ 4.6*
Insular cortex L2	101 $\pm$ 6.4	88.9 $\pm$ 6.5	63.5 $\pm$ 5.4*
Cortex L2	106 $\pm$ 3.8	94.4 $\pm$ 4.5	78.7 $\pm$ 4.4*
Caudate putamen L2	75.3 $\pm$ 4.2	67.1 $\pm$ 5.6	67.4 $\pm$ 4.2
Ventral pallidum L2	69.8 $\pm$ 4.3	63.5 $\pm$ 4.8	58.6 $\pm$ 4.1*
Hippocampus L3	47.1 $\pm$ 3.5	48.0 $\pm$ 3.8	47.6 $\pm$ 2.7
Amygdala L3	65.2 $\pm$ 16.9	70.8 $\pm$ 13	68.8 $\pm$ 7.9
Cortex L3	107 $\pm$ 3.4	115 $\pm$ 5.0	117 $\pm$ 3.1
Hypothalamus L3	77.5 $\pm$ 8.8	86.1 $\pm$ 8.0	75.9 $\pm$ 5.0
Hippocampus L4	42.2 $\pm$ 3.5	39.6 $\pm$ 3.7	45.9 $\pm$ 4.0
Cortex L4	92.2 $\pm$ 5.0	83.7 $\pm$ 3.6	86.2 $\pm$ 4.3
Periaqueductal grey L4	36.8 $\pm$ 4.6	38.0 $\pm$ 6.6	42.8 $\pm$ 6.5
Cerebellum L5	55.5 $\pm$ 3.5	51.1 $\pm$ 4.7	53.5 $\pm$ 4.0
Parabrachial nucleus L5	52.9 $\pm$ 6.2	54.1 $\pm$ 12	50.4 $\pm$ 9.1

Rats were treated with LY379268 (3 mg/kg/day) for two or fourteen days, or with vehicle. Brain sections from these groups were assayed for mGluR2,3-stimulated [^{35}S]GTP γ S binding autoradiography, and autoradiograms were quantified as described in Methods. Data are presented as mean values $\pm$ SEM of net agonist stimulated [^{35}S]GTP γ S binding (nCi/g tissue). Data were analyzed by one-way ANOVA and Fisher's LSD post-hoc test, with significant differences from control (* $p < 0.05$) in the nucleus accumbens L2 ($p = 0.04$), insular cortex L2 ($p = 0.001$), cortex L2 ($p = 0.001$), and ventral pallidum L2 ($p = 0.03$) ($N = 6-8$ rats per group).

Table 2.

Relative fluorescence units of CREB, phosphoCREB, ERK and phosphoERK for individual brain regions.

Region	CREB	pCREB	ERK	pERK
Prefrontal cortex L1	5.43 ± 0.45	16.7 ± 2.9	5.94 ± 0.76	41.1 ± 7.6
Nucleus accumbens L1	5.67 ± 0.57	17.8 ± 2.7	7.02 ± 0.83	50.1 ± 9.2
Cortex L2	5.34 ± 0.65	17.1 ± 2.7	5.05 ± 0.71	43.3 ± 8.9
Ventral pallidum L2	6.08 ± 1.36	18.9 ± 2.5	8.06 ± 1.36	47.9 ± 9.2
Insular cortex L2	4.51 ± 0.57	14.1 ± 2.3	5.77 ± 0.95	43.6 ± 10.0
Caudate putamen L2	5.93 ± 0.83	16.4 ± 2.0	5.99 ± 0.74	38.3 ± 7.1
Cingulate cortex L2	5.87 ± 0.62	19.3 ± 2.7	5.85 ± 0.74	47.7 ± 8.1
Amygdala L3	5.24 ± 0.67	16.7 ± 3.0	6.62 ± 0.79	47.3 ± 6.6
Hippocampus L3	5.31 ± 0.79	14.6 ± 2.6	5.60 ± 1.00	36.7 ± 7.6
Hippocampus L4	6.21 ± 0.92	17.5 ± 2.8	8.21 ± 0.96	71.8 ± 13.3
Substantia Nigra L4	5.67 ± 0.80	19.9 ± 4.1	7.11 ± 1.15	38.9 ± 7.8
Ventral Tegmental Area L4	6.16 ± 0.64	20.2 ± 3.4	6.91 ± 1.10	42.1 ± 7.4

Data are presented as mean values ± SEM of relative fluorescence units.