A Real-Time Method for Measuring cAMP Production Modulated by Go/i-Coupled Metabotropic Glutamate Receptors

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mGluR-Modulated cAMP Production Measured by GloSensor Assay

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Nonstandard Abbreviations: GPCR, G protein-coupled receptor; mGlu, metabotropic glutamate; mGluR, mGlu receptor; PTX, pertussis toxin; CHO, Chinese hamster ovary; AC, adenylyl cyclase; RLU, relative light unit; PDE, phosphodiesterase; FRET, Förster resonance energy transfer. DCG-IV, (1R,2R)-3-[(1S)-1-amino-2-hydroxy-2-oxoethyl]cyclopropane-1,2-dicarboxylic acid; L-AP4, (2S)-2-amino-4-phosphonobutanoic acid; LY341495, 2-[(1S,2S)-2-carboxycyclopropyl]-3-(9H-xanthen-9-yl)-D-alanine; EGlu, (2S)-2-amino-2-ethylpentanedioic acid; CPPG, (RS)-α-Cyclopropyl-4-phosphonophenylglycine; IBMX, 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione.

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Abstract

Group II and group III metabotropic glutamate (mGlu) receptors are G protein-coupled receptors (GPCRs) that inhibit adenylyl cyclase via activation of Gαi/o. The purpose of this study was to design a universal method that overcomes previous challenges in consistently measuring group II and group III mGlu receptor activation in stably transfected systems. In CHO cells stably transfected with the GloSensor cAMP biosensor, we optimized conditions for simple and highly reproducible (<5% S.E.M.) measurements of cAMP in real time. The GloSensor cAMP biosensor is a recombinant firefly luciferase conjugated to a cAMP binding domain, where cAMP binding promotes a conformational shift within the GloSensor protein, inducing luciferase activity; cAMP levels are positively correlated with light output resulting from the luciferase-mediated breakdown of D-luciferin. Each group II and group III mGlu receptor was then stably transfected into the CHO-GloSensor cell line, and experimental conditions were optimized for each receptor. During assay optimization, we observed ion sensitivity of several receptors and inverse agonist activity of the antagonist, LY341495. While these phenomena have been previously reported, they remain poorly understood, emphasizing the GloSensor assay as an important tool with which to study group II and group III mGlu receptors. Our results highlight many advantages of using the GloSensor method for measuring activation of group II and group III mGlu receptors, and they further suggest that corresponding methods designed to measure activation of any Gαi0- or Gαs-coupled GPCR will be similarly advantageous.
Introduction

Group II metabotropic glutamate (mGlu) receptors, mGlu2 and mGlu3, and group III mGlu receptors, mGlu4, mGlu6, mGlu7, and mGlu8, inhibit cAMP production via Gαi/o activation. Despite this common signaling mechanism, it has been shown that each receptor has unique cognitive and neurotrophic properties (Lyon et al., 2008; Caraci et al., 2011). At least in part, these differences are due to the unique cellular expression profile of each mGlu receptor within the central nervous system and that the expression patterns of these receptors change during development (Catania et al., 1994). For example, high mGlu3 receptor expression is maintained in astrocytes through adulthood (Sun et al., 2013), while in the spinal cord mGlu3 receptor expression decreases during development (Berthele et al., 1999). Unfortunately, a thorough understanding of each member’s unique pharmacological, physiological, and pathophysiological role has been impeded by the lack of receptor-specific ligands. This deficiency emphasizes the need for receptor-selective ligands as tools for characterizing the individual signaling properties of each mGlu receptor.

Although no selective agonists exist, several nonselective mGlu receptor agonists have entered late-stage clinical trials (Patil et al., 2007; Adams et al., 2013), but none have successfully reached the market. These clinical setbacks may be due to the lack of subtype selectivity, as studies suggest that even within groups, individual mGlu receptors can play opposing physiological roles (Conn and Pin, 1997; Caraci et al., 2011). In turn, agents that preferentially target a single mGlu receptor may demonstrate enhanced clinical efficacy as compared to their nonselective counterparts. The discovery of receptor-specific ligands for each group II and group III mGlu receptor, however, has been challenging (Niswender et al., 2008). The lack of comparability between different experimental systems limits the quantification of ligand selectivity. These systems often rely on receptors from different species, cloned into different expression vectors, transfected into different cell types, and/or characterized using different assay techniques. A clear solution to this problem would be the development of a
single assay paradigm to measure activation of each stably transfected G\alpha_{i/o}-coupled mGlu receptor individually. To our knowledge, no such system is available, a technical insufficiency that has hindered the full pharmacological characterization of commonly used, supposedly selective, ligands.

Another advantage of a universal, robust assay for measuring group II and group III mGlu receptor activation would be to clarify discrepancies in the literature involving the activation mechanism of each receptor. Several reports have suggested that mGlu receptors are directly modulated by ions (Kubo et al., 1998; Kuang and Hampson, 2006). However, few overarching trends can be extracted due to contrasting, and even contradictory data. While one study proposed that mGlu receptors are a family of calcium sensing receptors (Kubo et al., 1998), another report shows that cations had no effect on ligand binding at mGlu3 receptors, but rather that anions were required (Kuang and Hampson, 2006). Except for these examples, ion sensitivity at group II and group III mGlu receptors has not been explored.

Herein, we report the design and implementation of a single assay paradigm with which to compare the pharmacological properties of the group II and group III mGlu receptors. This assay utilizes the GloSensor cAMP Biosensor (Promega), a recombinant firefly luciferase conjugated to a cAMP binding domain (Binkowski et al., 2011). cAMP binding promotes a conformational shift within the GloSensor protein, which induces luciferase activity. cAMP levels, which are modulated by group II and group III mGlu receptor activation, are positively correlated with light output resulting from the luciferase-mediated breakdown of D-luciferin. We have generated mGlu2, mGlu3, mGlu4, mGlu6, and mGlu8 receptor-expressing CHO-GloSensor cell lines and characterized each receptors coupling to cAMP inhibition using a simple, homogeneous, robust, reproducible, and real-time assay paradigm.
Materials and Methods

Materials. cDNA encoding rat mGlu2, mGlu3, mGlu4, mGlu6, and mGlu8 receptors were cloned into the pIRES2-AcGFP1 vector, digested with EcoRI (New England Biolabs, Ipswich, MA) using the In-Fusion cloning method (Clontech, Mountain View, CA). PCR reactions to amplify mGlu receptor cDNAs (for primers, see Table 1) for insertion into the pIRES2-AcGFP1 vector were performed using the Phusion High-Fidelity DNA Polymerase Kit (New England Biolabs). The entire sequence of each mGlu receptor construct was confirmed by sequence analysis (Genewiz, South Plainfield, NJ). The pGloSensor-22F cAMP plasmid was purchased from the Promega Corporation (Madison, WI). DMEM, proline, and fetal bovine serum for cell cultures were purchased from Invitrogen (Carlsbad, CA). Receptor agonists glutamate, DCG-IV, and L-AP4, antagonists LY341495, EGlu, and CPPG, forskolin, IBMX, and pertussis toxin (PTX) were obtained from Tocris Bioscience (Ellisville, MO). D-Luciferin potassium salt was purchased from Gold Biotechnology (St. Louis, MO). [3H]-LY341495, with a specific activity of 40 Ci/mmol, was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Cell Cultures. Chinese hamster ovary-K1 (CHO) cells were transfected (Lipofectamine LTX, Invitrogen) with the pGloSensor-22F plasmid and selected using 200 µg/ml hygromycin (Invitrogen). CHO cells stably expressing the pGloSensor-22F construct (CHO-Glo) were then transfected with pIRES-AcGFP1 encoding mGlu2, mGlu3, mGlu4, mGlu6, or mGlu8 receptors and selected using 200 µg/ml G-418 (Research Products International, Mount Prospect, IL). Stable cell lines were maintained with 0.8 µg/ml hygromycin and 0.8 µg/ml G-418. All cells were cultured in 6% CO2 at 37°C in DMEM (high glucose) containing 10% fetal bovine serum, 300 µM proline, 2 mM glutamine and antibiotic-antimycotic (Invitrogen) and in the presence of G-418 and/or hygromycin. Cell lines were maintained in 6 cm polystyrene dishes. Cells for assay were plated on 96-well, white-walled, clear-bottom plates (Corning Lifesciences, Tewksbury, MA) and grown to confluence, without G-418 or hygromycin.
Buffers. All assays were performed in either Locke’s buffer or a modified Locke’s buffer to replace specific ions (for concentrations of buffer substituents, see Table 2). To substitute for sodium (Locke –Na+), NaCl, and NaHCO₃ were replaced with CholineCl and CholineHCO₃, respectively. To substitute for chloride (Locke –Cl⁻), NaCl, KCl, MgCl₂, and CaCl₂ were replaced with NaGluconate, KGluconate, MgGluconate₂, and CaGluconate₂, respectively. Potassium (Locke –K+), magnesium (Locke –Mg²⁺), or calcium (Locke –Ca²⁺) was replaced with NaCl. HEPES (Locke –HEPES) was replaced with Tris and adjusted to pH 7.4 with gluconic acid. All buffer components were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of mGlu-Glo Membranes. mGlu-Glo cell membranes were prepared by harvesting adherent cells grown to confluency on 10 cm polystyrene plates. Scraped cells were resuspended in 20 ml Tris buffer (20 mM Tris HCl, pH 7.4) and centrifuged at 1000 x g for 5 minutes at 4°C. The supernatant was discarded and the pelleted cells were frozen at -80°C overnight. The membrane pellets were resuspended in 20 ml of fresh Tris buffer, homogenized for 20 sec with a Brinkman polytron homogenizer, and centrifuged at 35,000 x g for 10 minutes at 4°C. After discarding the supernatant, this protocol was repeated once. The final pellet was resuspended in fresh Tris buffer and kept on ice until starting the binding assay. Protein concentration was determined using a Bradford protein assay.

Radioligand Binding. Membrane homogenates were incubated with the indicated concentrations of [³H]-LY341495 in Tris buffer, in the absence or presence of the indicated concentrations of glutamate, to measure total and nonspecific binding, respectively. After incubating for 1 hour on ice, samples were collected on S and S #30 filters by vacuum filtration. Radioactivity captured on the filters was measured by liquid scintillation counting (Beckman LS6500) and specific [³H]-LY341495 binding was calculated as the difference between total and nonspecific binding.

GloSensor cAMP Assay, Data Analysis, and Statistics. All GloSensor assays were performed using live cells, in a 96-well format, on sterile, white-walled, clear-bottom plates.
Culture medium was aspirated and replaced with 100 µl Locke’s buffer containing 450 µg/ml D-Luciferin (Locke-Luc). To equilibrate the cells with substrate, plates were pre-incubated in the dark at room temperature for 1 hour. Bioluminescence was quantified using the EnVision Multilabel Plate Reader (Perkin-Elmer, Waltham, MA), using a one second integration time. Prior to drug addition, each plate was read five times, at two minute intervals, to establish basal bioluminescence levels from each well. The average of these five pre-readings was used to normalize each well’s response to account for differences in GloSensor expression and cell density. After the five pre-readings, a final concentration of 1 µM forskolin with the appropriate final concentration of agonist and/or antagonist was added in 50 µl of Locke-Luc buffer (150 µl final volume). Measurements were taken every two minutes for 18 minutes after drug addition. The bioluminescence measured at 16 minutes was used for “end-point” experiments.

For the real-time competition experiments (Fig. 5.), a second treatment (vehicle, agonist, or antagonist) was applied at 18 minutes in 50 µl of Locke-Luc buffer (200 µl final volume). To account for the volume increase, forskolin and the first treatment drug were also added to maintain initial concentrations.

In the experiments with PTX, 1 µg/ml of PTX was added to each well 24 hours prior to assay.

For “end-point” assays, statistical significance was assessed using Student’s T-test. Concentration-response curves were fit to data points by nonlinear regression using a 4-parameter logistic equation. All calculations were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA).
Results

The GloSensor Assay Measures Forskolin-Stimulated cAMP production in CHO-Glo cells. A new assay for measuring group II and group III mGlu receptor activity was designed to use the GloSensor cAMP biosensor (Promega). In this system, increased cAMP levels are directly proportional to increased luminescence, which results from the enzymatic breakdown of the substrate, $D$-luciferin. CHO cells were transfected with the GloSensor plasmid (Binkowski et al., 2011) and multiple clones were tested for maximal luminescence in response to 10 μM forskolin. The clone with the largest increase in luminescence in response to forskolin was selected, and a single cell line (CHO-Glo) was established. Untransfected CHO cells lacking the GloSensor construct did not produce a detectable signal, while treatment of CHO-Glo cells with the adenylyl cyclase (AC) activator forskolin caused a concentration-dependent increase in relative light units (RLU) over time (Fig. 1A). The concentration-dependency of cAMP production is shown using data measured 16 minutes after the addition of forskolin (Fig. 1B). The EC$_{50}$ for forskolin was calculated in this system to be 9.44 ± 0.60 μM, which is comparable to the reported forskolin EC$_{50}$ of 5-10 μM (Seamon et al., 1981). From these data, 1 μM forskolin was chosen as a concentration sufficient to stimulate cAMP production with minimal potential for substrate depletion and a reliable signal-to-noise ratio. Hence, this concentration was used throughout the study. The group II and group III mGlu receptor ligands used in this study were tested on CHO-Glo cells to ensure that these compounds would not affect cAMP levels in the absence of transfected mGlu receptors. Several group II and/or group III mGlu receptor agonists (glutamate, DCG-IV, and L-AP4) and antagonists (LY341945, EGlu, and CPPG) were assayed at saturating concentrations. Due to the lack of endogenous mGlu receptors, none of the drugs used in this study significantly altered cAMP production in CHO-Glo cells (Fig. 1C).

The GloSensor Assay Measures Glutamate-Mediated Decreases in cAMP Production in mGlu-Glo Cells. CHO-Glo cells were transfected with individual group II or III
mGlu receptor cDNAs, previously cloned into pIRES2-AcGFP1. Stable clones of CHO-Glo cells expressing individual mGlu receptor types (mGlu2-Glo, mGlu3-Glo, mGlu4-Glo, mGlu6-Glo, mGlu7-Glo, and mGlu8-Glo) were selected based on AcGFP fluorescence and maximal light output in response to forskolin treatment. In Locke buffer, all mGlu-Glo cell lines, except for mGlu3-Glo, showed significant decreases in forskolin-stimulated cAMP production in response to 100 µM glutamate (Fig. 2). Since several studies have demonstrated that mGlu receptor activation is sensitive to ions (Kubo et al., 1998; Kuang and Hampson, 2006), we examined whether a buffer constituent was precluding mGlu3-Glo cells from inhibiting forskolin-stimulated cAMP production in response to glutamate treatment. Each buffer constituent was individually replaced with a structurally distinct ion of like charge (Table 2). Sodium was replaced with choline (Locke –Na⁺). Potassium, magnesium, or calcium was replaced with sodium (Locke –K⁺, Locke –Mg²⁺, Locke –Ca²⁺). Chloride, replaced with gluconate, was reduced from 164.2 mM to 4.6 mM (Locke and Locke –Cl⁻, respectively). HEPES was replaced with Tris, and the pH was adjusted to 7.4 with gluconic acid (Locke –HEPES). The removal of K⁺, Mg²⁺, or Ca²⁺ had no significant effect upon cAMP production in any mGlu-Glo cell line. Locke –Na⁺ or Locke –HEPES showed non-specific, decreases in cAMP inhibition at all mGlu-Glo cells (Fig. 2). mGlu7-Glo cells did not respond to glutamate under any of these conditions.

In low chloride buffer (Locke –Cl⁻), glutamate significantly inhibited forskolin-stimulated cAMP production in mGlu3-Glo cells (Fig. 2, mGlu3-Glo). In contrast, the removal of chloride decreased glutamate efficacy at mGlu4-Glo cells (Fig. 2, mGlu4-Glo), a result consistent with a previous report (Kuang and Hampson, 2006). Chloride removal showed no significant effect at any other mGlu-Glo cell line. Because this finding allowed reliable measurements of mGlu3 receptor activation, all subsequent mGlu3 receptor GloSensor assays were performed in Locke’s buffer containing 4.6 mM chloride (Locke –Cl⁻) while all mGlu2, mGlu4, mGlu6 and mGlu8 receptor GloSensor assays were performed using Locke’s buffer (Table 2). Nearly all reports of mGlu receptor modulation of cAMP production used end-point assays in the presence of a buffer containing no chloride.
of IBMX to inhibit phosphodiesterases (PDE). In this study, experiments were performed to compare the efficacy of glutamate in the absence or presence of IBMX at all mGlu-Glo cell lines. As expected, in the presence of IBMX, total luminescence was increased (Supplemental Fig. 1A), although no difference in glutamate efficacy was observed at any mGlu-Glo cell line (Supplemental Fig. 1B).

**Radioligand Binding Studies Using Membranes From mGlu-Glo Cell Lines.**

Radioligand binding studies using [3H]-LY341495 were conducted both to estimate the levels of receptor expression in all mGlu-Glo cell lines and to determine whether mGlu7-Glo cells were unresponsive due to insufficient receptor expression. To approximate levels of receptor expression, K_d concentrations of [3H]-LY341495 determined for human mGlu receptors (Wright et al., 2000) were used with membranes (50 μg of protein) from each mGlu-Glo cell line. Only membranes from mGlu2-, mGlu3-, mGlu7-, and mGlu8-Glo cells demonstrated reliable specific binding. To our knowledge, the K_d values of [3H]-LY341495 at rat mGlu receptors have not been reported; therefore B_max values were calculated using reported K_d values for human mGlu receptors (Wright et al., 2001). Calculated B_max values ranged from 0.26 to 1.5 pmol/mg (Supplemental Table 1).

**The GloSensor Assay Provides a Real-Time Measurement of Concentration-Dependent Agonist-Stimulated, Gα_i/o-Activation in mGlu-Glo Cell Lines.** When assayed in Locke-Luc at mGlu2-, mGlu4-, mGlu6- and mGlu8-Glo and Locke –Cl at mGlu3-Glo cells, all cell lines showed glutamate-mediated, concentration-dependent decreases in forskolin-stimulated cAMP production, in real-time (Fig. 3). By 8 minutes after drug addition, glutamate mediated decreases in cAMP production were proportional at all subsequent time points. Data from the 16 minute time point showed the best signal-to-noise ratio and were used for all concentration-response curves (Fig. 4).

To ensure that effects on cAMP levels were entirely mediated by mGlu receptor activation of Gα_i/o proteins, the ability of glutamate to inhibit cAMP production was measured in
the presence of pertussis toxin (PTX). PTX is a bacterial exotoxin which inactivates Gαi/o proteins via ADP-ribosylation of Gαi/o subunits. As previously demonstrated, 100 μM glutamate was sufficient to significantly reduce cAMP levels at all mGlu-Glo cell lines (Fig. 2). However, in cells pretreated overnight with 1 μg/ml PTX, glutamate failed to inhibit cAMP production (Fig. 4). PTX treatment had no effect on forskolin-stimulated cAMP production in CHO-Glo cells (data not shown). These results indicate that the inhibition of cAMP production in mGlu-Glo cell lines is receptor-mediated and entirely modulated by Gαi/o proteins.

To further characterize these cell lines, the potencies of two agonists were determined at each receptor. All cell lines were assayed with the endogenous mGlu receptor agonist, glutamate. Additionally, mGlu2- and mGlu3-Glo cell lines were assayed with the group II mGlu receptor-selective agonist DCG-IV; mGlu4-, mGlu6-, and mGlu8-Glo cell lines were assayed with the group III mGlu receptor-selective agonist L-AP4 (Fig. 4). Both DCG-IV and L-AP4 were more potent than glutamate at their respective receptors (Conn and Pin, 1997). As expected, DCG-IV and L-AP4 were inactive up to 100 μM at Group III and Group II mGlu receptors, respectively. EC50 values calculated for glutamate, DCG-IV, and L-AP4 are summarized in Table 3.

The GloSensor Assay Measures Real-Time Reversibility of Agonists and Antagonists in mGlu-Glo Cell Lines. To ensure that decreases in cAMP mediated by agonists were due to receptor-selective, orthosteric interactions, competition assays were performed to determine if the competitive antagonist LY341495 would reverse glutamate-mediated inhibition of cAMP production. Vehicle, glutamate (100 μM), or LY341495 (1 μM) was added to mGlu-Glo cells and light output was measured every two minutes for 18 min. A second drug treatment (vehicle, 1 mM glutamate, or 10 μM LY341495) was applied 18 minutes after the first addition, and light output was measured every two minutes for 18 min. (Fig. 5). Ten-fold higher concentrations of drug were used during the second treatment to ensure full competition with the prior treatment. mGlu3-Glo cells were assayed using Locke –Cl−. During the first addition
time = 0 to 18 min), glutamate prevented cAMP production in all mGlu-Glo cell lines, but not in
CHO-Glo cells. Notably, during the first addition, treatment with the antagonist LY341495
showed inverse efficacy, increasing cAMP production above vehicle treatment in mGlu4,
mGlu6, and mGlu8-Glo cell lines, suggesting its action as an inverse agonist. After the second
addition (time = 18 to 36 min), glutamate inhibited cAMP production in all vehicle-treated and
LY341495-treated mGlu-Glo cells, but not in CHO-Glo cells. Additionally during the second
addition, glutamate-treated mGlu-Glo cells challenged with LY341495 not only reversed
 glutamate-mediated inhibition of cAMP production, but also showed inverse agonism at all cell
lines (Fig. 5). Interestingly, inverse agonism was present in mGlu2- and mGlu3-Glo cell lines
only when LY341495 was added to agonist treated cells, while mGlu4-, mGlu6-, and mGlu8-Glo
cell lines showed inverse agonism in both the presence and absence of agonist. After the
second treatment, non-specific, transient decreases in RLU signal, likely due to the volume
change in the wells, were observed at all cell lines, including the control CHO-Glo cell line.
Decreases in luminescence were also observed after 30 minutes, possibly due to PDE activity,
desensitization of adenylyl cyclase, or cellular exhaustion.

The GloSensor Assay Accurately Approximates Antagonist Affinity. Our data show
that the GloSensor assay effectively measures the concentration-dependency of agonist-
mediated cAMP inhibition. To further examine the behavior of antagonists, LY341495 was used
at increasing concentrations to shift the concentration-response curve of glutamate at mGlu3
receptors (Fig. 6). As expected for competitive antagonists, a rightward shift in the glutamate
concentration-response curve was observed with increasing concentrations of LY341495. Each
curve was normalized to its own maximal RLU in the absence of glutamate, and glutamate EC50
values were determined at each concentration of LY341495. Based on these calculations, a
Schild plot was constructed, yielding the $K_b$ of LY341495 at rat mGlu3 receptors to be 1.8 nM.
This estimate is consistent with the $K_b$ value of 0.75 nM, determined empirically for LY341495 at
transfected human mGlu3 receptors (Johnson et al., 1999).
Discussion

Group II and group III mGlu receptors have been implicated as therapeutic targets for treating a variety of cognitive and neurodegenerative diseases, including schizophrenia, stress and anxiety disorders, Parkinson’s disease, and Alzheimer’s disease (Calabresi et al., 1999; Niswender and Conn, 2010; Caraci et al., 2011). Although the group II and group III mGlu receptors share a common signaling mechanism, selective activation of just one receptor subtype can have unique physiological consequences (Corti et al., 2007). Unfortunately, a thorough understanding of these differences has been hindered as few subtype-selective ligands have been identified. A universal, robust, and reproducible method to evaluate and compare the individual pharmacological properties of each receptor subtype would provide an essential tool for the discovery of subgroup-selective drugs. While a variety of techniques for measuring mGlu receptor-induced $G_{\alpha_i/o}$ activity have been successful, few groups have measured group II and group III mGlu receptor activation with the same method, in the same system. This may be due to the challenge of engineering a method to reliably measure $G_{\alpha_i/o}$-coupled mGlu receptor activation. Groups have resorted to measuring non-canonical $G_{\beta/\gamma}$ activation (Malherbe et al., 2001; Niswender et al., 2008), using complicated FRET assays that require artificial receptor constructs (Yanagawa et al., 2011), engineering chimeric receptors (Wroblewska et al., 1997), or employing cell-free radioligand binding assays (Schweitzer et al., 2000). Herein, we report a novel, comprehensive method which reliably measures group II and III mGlu receptor-mediated inhibition of AC. A schematic of the GloSensor method is presented in Fig. 7.

As others have reported, our group also faced difficulties measuring mGlu receptor-mediated $G_{\alpha_i/o}$ activation (Niswender et al., 2008). While establishing the GloSensor assay to measure mGlu2, mGlu4, mGlu6, and mGlu8 receptor activation was surprisingly straightforward, we encountered challenges establishing an assay for mGlu7 and mGlu3 receptors. Surprisingly, mGlu7-Glo cells showed significant specific binding of the radioligand
[³H]-LY341495, while no measurable specific binding was observed at mGlu4-Glo or mGlu6-Glo cell lines. Previous reports indicate that [³H]-LY341495 is not suitable for binding studies using human mGlu4 receptors, likely due to a high K_d value (Wright et al., 2000), and this may be true for rat mGlu4 receptors. Similarly, although the mGlu6-Glo cell line is functional, differences between the K_d values of [³H]-LY341495 at human vs. rat mGlu6 receptors could explain the lack of specific binding observed in this study. Finally, the calculated B_max values indicate that mGlu2-, mGlu3-, mGlu7-, and mGlu8-Glo cell lines have a lower receptor density than previously reported for human mGlu receptor-expressing cell lines (Wright et al., 2000) and group II mGlu receptors in rat brain tissue (Wright et al., 2001).

While a functional mGlu7-Glo cell line remains elusive, our data suggest that the reason measuring mGlu3 receptor signaling via Gα_i/o has proved difficult is because chloride prevents measurements of agonist-mediated mGlu3 receptor activation. This finding is consistent with other studies that have suggested that mGlu receptors are directly modulated by ions. For example, one study proposed that mGlu receptors are a family of calcium sensing receptors, where CaCl_2 was shown to activate mGlu3 receptors (Kubo et al., 1998). While Ca^{2+} did not affect any mGlu receptor activation in our system, Cl^- removal from the assay media greatly improved mGlu3 receptor-mediated responses to agonists. In agreement with our findings, a separate study showed that, while cations had no effect, small anions affected ligand binding at mGlu3 receptors (Kuang and Hampson, 2006). This report complements our data showing that agonist activation of the mGlu3 receptor is affected specifically by chloride. While reducing chloride concentration allows for reproducible measurements of mGlu3 receptor activation, further work is required to describe the mechanism of mGlu3 receptor chloride sensitivity.

Data obtained using saturating concentrations of the antagonist LY341495 revealed an increase in cAMP production, relative to vehicle treatment (Fig. 5). At mGlu2- and mGlu3-Glo cell lines, this phenomenon was only observed after agonist treatment. This finding supports other reports of Gα_i/o-coupled GPCR activation supersensitizing AC and it suggests that group II
mGlu receptor activation may supersensitize AC as well (Watts, 2002; Watts and Neve, 2005). At the group III mGlu receptors, LY341495 mediated increases in cAMP production in both the absence and presence of agonist. These observations suggest that, in mGlu4-, mGlu6-, and mGlu8-Glo cell lines, LY341495 was either competing with an unidentified agonist that had also supersensitized AC, or LY341495 was acting as an inverse agonist by inhibiting constitutive receptor activity. While these data are consistent with other reports of antagonist-mediated increases in cAMP production at many of the Goi/o-coupled mGlu receptors in transfected cells (Suzuki et al., 2007), it is not clear whether this phenomenon is present in native systems. Further investigation is required to determine the mechanism and physiological relevance of this inverse agonism.

The GloSensor cAMP biosensor provides many advantages over other methods for measuring mGlu receptor activation: (1) every assay to date that has measured mGlu receptor-mediated Goi/o activity has been an end-point assay, requiring IBMX to inhibit phosphodiesterases (PDEs). In contrast, the GloSensor method measures cAMP production, in real-time. This novelty allows us to stimulate and inhibit receptor activity, on the same cells, in the same assay, demonstrating the competitive and reversible nature of orthosteric ligands acting at the mGlu receptors. Furthermore, this real-time system has the capability to expose kinetic differences (i.e. efficiency of coupling to G proteins, ligand on-off rates, deactivation profiles, effects on PDE’s, etc.) between any Goi/o coupled receptors. (2) The GloSensor method allows direct, functional measurements of the classical signaling pathway modulated by group II and group III mGlu receptors. While competition binding assays allow for measurements of ligand affinity, they do not address any functional properties associated with the ligand, namely whether a ligand is an agonist, partial agonist, antagonist, or inverse-agonist. Furthermore, FRET-based methods, while useful, do not employ wild-type receptors. Other methods measure non-canonical Gβγ-mediated potassium flux as an alternative indicator for receptor activation. (3) In contrast to many electrophysiological and biophysical methods, the
GloSensor method does not require expensive equipment, as all measurements can be performed with any luminescence detector. The substrate is inexpensive, and we have performed experiments using several luciferin salts, all of which produced similar results. Set up and assay times are particularly short with the GloSensor method, making it an efficient assay paradigm that can facilitate rapid and reliable measurements of ligand activity in a high-throughput screening format. While a homogeneous assay system for measuring $\gamma_i/o$ activation mediated by the group II and III mGlu receptors will address many discrepancies in the literature, the emergence of GPCR-mediated G-protein-independent signaling suggests that using just one assay system to investigate drug-receptor interactions will provide incomplete information; rather it is necessary to integrate data from several distinct functional assays, ligand affinity experiments, and structural studies to provide a comprehensive ligand-receptor profile (Shoichet and Kobilka, 2012).

In summary, we developed a new method to measure $\gamma_i/o$-coupled mGlu receptor activity in stably transfected cells. This assay has already helped to clarify a discrepancy in the literature, showing that chloride, but not calcium, affects mGlu3 receptor signaling. Together with LY341495-mediated inverse agonism, these results demonstrate that the GloSensor method is a useful tool for investigating properties of mGlu receptor signaling. The GloSensor method provides a cost-effective, efficient, real-time approach for measuring the canonical signaling mechanism of group II and group III mGlu receptors, and it should prove equally applicable to any $\gamma_i/o$- or $\gamma_s$-coupled GPCR.
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**Authorship Contributions**

*Participated in research design:* DiRaddo, Miller, Wroblewska, Wolfe, Wroblewski

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*Contributed new reagents or analytic tools:* DiRaddo, Miller, Liotta, Wroblewski

*Performed data analysis:* DiRaddo, Hathaway, Wroblewski

*Wrote or contributed to the writing of the manuscript:* DiRaddo, Miller, Hathaway, Wroblewska, Wolfe, Liotta, Wroblewski
References


Footnotes

a) N/A

b) N/A

c) Jarda T. Wroblewski, Ph.D.
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**Figure Legends**

**Fig. 1.** Forskolin-mediated cAMP production in stably transfected CHO cells. (A) CHO cells, stably transfected with the GloSensor cAMP biosensor (CHO-Glo), show concentration-dependent increases in RLUs over time when treated with increasing concentrations of the adenyl cyclase activator forskolin. The reading from each well was normalized to its own basal luminescence, determined before forskolin addition. (B) The forskolin concentration-response curve was generated from data obtained 16 min after forskolin treatment. (C) Group II and group III mGlu receptor agonists and antagonists do not affect forskolin-mediated cAMP production in CHO-Glo cells (lacking a transfected receptor). Data are means ± S.E.M. of three individual experiments performed in triplicate.

**Fig. 2.** Ion sensitivity of glutamate-mediated group II and group III mGlu receptor activation. CHO-Glo or mGlu-Glo cells were treated with vehicle (white bars) or 100 µM glutamate (gray bars) in a series of modified Locke's buffers (Table 2). In each buffer, data was normalized as a percent of vehicle treatment. Glutamate-mediated decreases in cAMP production were statistically significant (p<0.01) at all mGlu-Glo cell lines except mGlu3-Glo. Glutamate activation of mGlu3 receptors was significant only when Locke–Cl⁻ was used as the assay buffer. Statistical differences in the data were assessed by a Student's t-test. Data are mean ± S.E.M. of three individual experiments performed in triplicate. ***, p<0.001.

**Fig. 3.** mGlu receptor-mediated inhibition of cAMP production in stably transfected CHO cells. mGlu-Glo cell lines were treated with 1 µM forskolin and increasing concentrations of glutamate. Concentration-dependent decreases in RLUs were observed at all cell lines. Data from each treatment was normalized to its own basal luminescence. Data are mean ± S.E.M. of three individual experiments performed in triplicate.

**Fig. 4.** mGlu receptor agonists decrease cAMP production in mGlu-Glo cell lines in a PTX-sensitive, concentration-dependent manner. mGlu-Glo cells were pretreated with (or
without) PTX before each assay. Vehicle (white bars) or 100 μM glutamate (gray bars), +/- PTX, were normalized to their respective vehicle-treated values. Vehicle or increasing concentrations of mGlu receptor agonists, glutamate, or either DCG-IV or L-AP4. Statistical significance was assessed by a Student’s t-test. Data for concentration-response curves were normalized to vehicle-treated values. Data are mean ± S.E.M. of three individual experiments performed in triplicate. ***, p<0.001.

Fig. 5. Real-time reversibility of agonists and antagonists effects on cAMP production. CHO-Glo or mGlu-Glo cells were pre-equilibrated with the substrate in Locke’s buffer (Locke 4.6 at mGlu3-Glo) and basal luminescence was measured as before. Vehicle (black line), glutamate (100 μM, green, dashed line), or LY341495 (1 μM, red, dashed line) was added first. A second treatment of vehicle (black line), glutamate (1 mM, green, solid line), or LY341495 (10 μM, red, solid line) was added at 18 minutes. For the sake of clarity, S.E.M. is not shown, but averaged 8% through the experiment. Data represent the means of three individual experiments performed in triplicate.

Fig. 6. The GloSensor assay measures antagonist activity. mGlu3-Glo cells were pre-equilibrated with substrate in Locke –Cl-. Increasing concentrations of glutamate were added, without or with several concentrations of LY341495 (0, white circles; 10^-8, black circles; 10^-7, black squares; 10^-6, black triangles). The Kᵦ value were calculated using GraphPad Prism software. Data are mean ± S.E.M. of three individual experiments performed in triplicate.

Fig. 7. Schematic representation of the GloSensor assay. Forskolin stimulates cAMP production via adenylyl cyclase (AC). cAMP binding to the GloSensor construct causes a conformational shift that increases luciferase activity, whereby the D-luciferin substrate is oxidized to produce oxyluciferin and light. Agonist stimulation of group II or group III mGlu receptors activates heterotrimeric Gᵦᵢ proteins, where the liberated α-subunit then inhibits AC activation. Agonist activity is measured as decreased light output, relative to vehicle treatment.
Tables

Table 1
Primers used to insert individual mGlu receptors into EcoRI-cut pIRES2-AcGFP1

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td>mGlu2F</td>
<td>Forward</td>
<td>GACTCAGATCTCGAGGGGCCATGGAATCAGCTGCTTGGGTTTCTG</td>
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<tr>
<td>mGlu2R</td>
<td>Reverse</td>
<td>AATTCGAAGCTTGAGTCACAGTGAGGTGGTGGAGTC</td>
</tr>
<tr>
<td>mGlu3F</td>
<td>Forward</td>
<td>GACTCAGATCTCGAGGATTCTGAGATGTTGACAAAGACTACAA</td>
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<tr>
<td>mGlu3R</td>
<td>Reverse</td>
<td>AATTCGAAGCTTGAGTCACAGTGAGGTGGTGGAGTC</td>
</tr>
<tr>
<td>mGlu4F</td>
<td>Forward</td>
<td>CGTCAGATCCGCTAGTGCCAAATGTCGGGAGG</td>
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<tr>
<td>mGlu4R</td>
<td>Reverse</td>
<td>GTCCCGTAGCGCTAGGATCTGAGGATGGGATG</td>
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Table 2

Components of buffers used to examine specific ion's effects on mGlu receptor activation

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<tr>
<th>Component (mM)</th>
<th>Locke</th>
<th>Locke -Na⁺</th>
<th>Locke -Cl⁻</th>
<th>Locke -K⁺</th>
<th>Locke -Ca²⁺</th>
<th>Locke -Mg²⁺</th>
<th>Locke -HEPES</th>
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<td>NaCl</td>
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<tr>
<td>KCl</td>
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<td>-</td>
<td>-</td>
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<tr>
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<td>CholineHCO₃</td>
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<td>NaGluconate</td>
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<td>KGlucnate</td>
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<td>Tris</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
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Table 3

EC₅₀ (µM) values calculated from the GloSensor assay are comparable to reported values. EC₅₀ values calculated from data shown in Fig. 4. using the 3-parameter logistic equation. Errors are reported as confidence interval (C.I.).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Glutamate EC₅₀</th>
<th>95% C.I.</th>
<th>Reported</th>
<th>DCG-IV EC₅₀</th>
<th>95% C.I.</th>
<th>Reported</th>
<th>L-AP4 EC₅₀</th>
<th>95% C.I.</th>
<th>Reported</th>
</tr>
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<tbody>
<tr>
<td>mGlu2 Receptor</td>
<td>2.9</td>
<td>1.8 to 4.7</td>
<td>4-20ᵃ</td>
<td>0.91</td>
<td>0.50 to 1.7</td>
<td>0.3ᵃ</td>
<td>&gt;100</td>
<td>-</td>
<td>&gt;100</td>
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<tr>
<td>mGlu3 Receptor</td>
<td>3.2</td>
<td>2.4 to 4.3</td>
<td>4-5ᵃ</td>
<td>0.87</td>
<td>0.79 to 0.96</td>
<td>0.2ᵃ</td>
<td>&gt;100</td>
<td>-</td>
<td>&gt;100</td>
</tr>
<tr>
<td>mGlu4 Receptor</td>
<td>3.9</td>
<td>2.2 to 6.9</td>
<td>3-20ᵃ</td>
<td>&gt;100</td>
<td>-</td>
<td>&gt;100</td>
<td>0.26</td>
<td>0.16 to 0.42</td>
<td>0.4-1.2ᵃ</td>
</tr>
<tr>
<td>mGlu6 Receptor</td>
<td>1.2</td>
<td>0.67 to 2.1</td>
<td>16ᵃ</td>
<td>&gt;100</td>
<td>-</td>
<td>&gt;100</td>
<td>0.28</td>
<td>0.19 to 0.42</td>
<td>0.9ᵃ</td>
</tr>
<tr>
<td>mGlu8 Receptor</td>
<td>0.62</td>
<td>0.35 to 1.1</td>
<td>0.02ᵃ</td>
<td>&gt;100</td>
<td>-</td>
<td>&gt;100</td>
<td>0.19</td>
<td>0.06 to 0.56</td>
<td>0.4ᵃ</td>
</tr>
</tbody>
</table>

Fig. 1
Fig. 4
Fig. 5
mGlu3-Glo

Fig. 6
Fig. 7
Supplemental Fig. 1. Effects of IBMX on glutamate-mediated inhibition of cAMP production in mGlu-Glo cell lines. (A) The addition of IBMX increases relative luminescence when added to the GloSensor assay in the absence (white bars) or in the presence of 100 µM glutamate (gray bars). (B) Percent inhibition of cAMP production by glutamate remains unchanged in the absence or presence of IBMX. Data are mean ± S.E.M. of three individual experiments performed in triplicate.
**Supplemental Table 1**

Radioligand binding of \[^{3}H\]-LY341495 to mGlu-Glo cell membranes. (n=3)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>B(_{\text{max}}) (pmol/mg)</th>
<th>S.D. (pmol/mg)</th>
<th>(K_{d})(^a)</th>
<th>L-Glutamate (mM)</th>
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<tbody>
<tr>
<td>mGlu2 Receptor</td>
<td>0.260</td>
<td>0.080</td>
<td>1.7</td>
<td>1</td>
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<tr>
<td>mGlu3 Receptor</td>
<td>1.516</td>
<td>0.335</td>
<td>0.7</td>
<td>0.3</td>
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<tr>
<td>mGlu4 Receptor</td>
<td>0.103</td>
<td>0.029</td>
<td>32</td>
<td>1</td>
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<tr>
<td>mGlu6 Receptor</td>
<td>0.048</td>
<td>0.021</td>
<td>32</td>
<td>1</td>
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<tr>
<td>mGlu7 Receptor</td>
<td>0.540</td>
<td>0.032</td>
<td>72</td>
<td>30</td>
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<tr>
<td>mGlu8 Receptor</td>
<td>1.434</td>
<td>0.470</td>
<td>14</td>
<td>1</td>
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