Biochemical Pharmacology 152 (2018) 201–210

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Constitutive activity of the metabotropic glutamate receptor 2 explored with a whole-cell label-free biosensor

Maarten L.J. Doornbos[a], Ilse Van der Linden[b], Liesbeth Vereyken[b], Gary Tresadern[b], Adriaan P. IJzerman[a], Hilde Lavreysen[b], Laura H. Heitman[a,*]

[a] Division of Drug Discovery and Safety, Leiden Academic Centre for Drug Research (LACDR), Leiden University, P.O. Box 9502, 2300RA Leiden, The Netherlands
[b] Janssen Research and Development, Turnhoutseweg 30, 2340 Beerse, Belgium

ARTICLE INFO

Keywords:
Biosensor
Impedance
Label-free
$mGlu_2$ receptor
Constitutive activity
Inverse agonism

ABSTRACT

Label-free cellular assays using a biosensor provide new opportunities for studying G protein-coupled receptor (GPCR) signaling. As opposed to conventional *in vitro* assays, integrated receptor-mediated cellular responses are determined in real-time rather than a single downstream signaling pathway. In this study, we examined the potential of a label-free whole cell impedance-based biosensor system (i.e. xCELLigence) to study the pharmacology of one GPCR in particular, the $mGlu_2$ receptor. This receptor is a target for the treatment of several psychiatric diseases such as schizophrenia and depression.

After optimization of assay conditions to prevent interference of endogenous glutamate in the culture medium, detailed pharmacological assessments were performed. Concentration-response curves showed a concentration-dependent increase in impedance for agonists and positive allosteric modulators, whereas receptor inhibition by an antagonist or negative allosteric modulator resulted in a concentration-dependent decrease in cellular impedance. Interestingly, constitutive receptor activity was observed that was decreased by LY341495, which therefore behaved as an inverse agonist here, a property that was heretofore unappreciated. This was confirmed by concentration-dependent modulation of LY341495 potency and efficacy by a allosteric modulators.

In summary, the use of the xCELLigence system to study $mGlu_2$ receptor pharmacology was validated. This is the first class C GPCR to be characterized extensively by such method, opening new avenues to study receptor pharmacology including inverse agonism and demonstrating its value for future drug discovery efforts of mGlu receptors as well as other GPCRs.

1. Introduction

Traditionally, drug discovery at G protein-coupled receptors (GPCRs) has been based on screening drug candidates using functional *in vitro* endpoint-based assays in which the ability of a compound to activate or inhibit a certain pathway is determined. Over the last decade on the other hand the use of label-free cellular assays has greatly increased [1,2]. These assays, typically using a biosensor, measure cellular responses upon ligand stimulation, which result in morphological changes of the whole cell [3]. Thereby some of the caveats of traditional endpoint assays are circumvented: no labels (e.g. radioactive, fluorescent) or reporter genes are required and measurements can be performed continuously [4]. Cell types used include recombinant cell lines, cell lines endogenously expressing the target of interest and even patient-derived cells [5]. Label-free assays used to study GPCR responses are mostly based on measurement of changes in cell morphology either optically, such as EPIC (Corning Inc.) or by changes in impedance, such as xCELLigence (ACEA Biosciences) [1,6]. These systems have proven valuable in studying GPCR pharmacology in different cell lines [7–9].

Being a presynaptic regulator of glutamate release, the $mGlu_2$ receptor has been of great interest in drug discovery over the last decade. This receptor, a class C GPCR, has emerged as therapeutic target for various psychiatric diseases amongst which schizophrenia, depression and anxiety, which are characterized by glutamatergic dysfunction [10,11]. A variety of glutamate-like agonists that can dampen glutamate hyperfunction was developed, of which LY2140023 of Eli Lilly, a prodrug of LY404039 showed improvement in positive and negative symptoms in schizophrenic patients, which could not be confirmed in later clinical studies [12,13]. Inhibition of the $mGlu_2$ receptor can be

* Corresponding author at: Division of Drug Discovery and Safety, Leiden Academic Centre for Drug Research, Leiden University, P.O. Box 9502, 2300RA Leiden, The Netherlands.
E-mail addresses: m.l.j.doornbos@lacdr.leidenuniv.nl (M.L.J. Doornbos), IVDLINDE@its.jnj.com (I. Van der Linden), LVEREYK1@its.jnj.com (L. Vereyken), gtresade@its.jnj.com (G. Tresadern), ijzerman@lacdr.leidenuniv.nl (A.P. IJzerman), HLAVREY1@its.jnj.com (H. Lavreysen), l.h.heitman@lacdr.leidenuniv.nl (L.H. Heitman).

https://doi.org/10.1016/j.bcp.2018.03.026
Received 24 January 2018; Accepted 27 March 2018
Available online 30 March 2018

beneficial for glutamate hypofunction in depression, and when considering the orthosteric binding site antagonist LY341495 has been the most studied for this purpose [14,15]. Since the orthosteric site is highly conserved among the mGlu receptor subtypes and medicinal chemistry of orthosteric ligands is constrained to hydrophilic glutamate derivatives, drug development efforts later shifted to allosteric modulators for the $mGlu_2$ receptor, which have been shown to bind in a less evolutionary conserved binding site within the transmembrane domain [16–18]. This allosteric binding site is more hydrophobic and allows more chemical diversity [19]. Therefore, subtype selectivity is obtained relatively easier and allosteric modulators are more prone to cross the blood brain barrier due their increased lipophilicity. Allosteric modulators enhance or inhibit the affinity and/or efficacy of the endogenous agonist glutamate and some positive allosteric modulators (PAMs) also show intrinsic efficacy, referred to as PAM agonism [20]. Reference PAMs in the field include JNJ-40068782 [21], BINA [22] and JNJ-46281222 [23]. Two PAMs entered clinical trials, AZD8529 of AstraZeneca and JNJ-40411813 (also known as ADX71149) from Janssen/Addex [24–29]. A number of $mGlu_2$ negative allosteric modulators (NAMs) have been characterized *in vivo*, including a recent series of NAMs from Janssen and RO4491533 and decoglurant from Roche, of which the latter also advanced into clinical trials [30–33]. Taken together, discovery efforts have resulted in a great diversity of selective and high affinity $mGlu_2$ ligands, but no compounds have successfully passed clinical evaluation.

To increase the understanding on ligand-induced $mGlu_2$ responses, we evaluated in this study the use of the label-free impedance-based xCELLigence system to study the pharmacology of the $mGlu_2$ receptor *in vitro*. A benefit of this biosensor is that experiments are performed under more relevant conditions than most traditional assays (i.e. living cells at 37 °C in culture medium). After optimization of pre-treatment conditions to reduce the concentration of endogenous glutamate in the assay medium, responses of an agonist, antagonist, PAM and NAM were studied. Furthermore, we evaluated constitutive receptor activity by using antagonist/inverse agonist and NAM pre-treatment experiments. Finally, the effects of a PAM or NAM on agonist efficacy were studied. Taken together, biosensor-based label-free systems are helpful in studying (novel) $mGlu_2$ pharmacology in a more physiologically relevant environment. Our studies may be equally relevant for other class C GPCRs.

2. Materials and methods

2.1. Materials

LY354740, RO4491533 and JNJ-46281222 were synthesized at Janssen Research and Development (Toledo, Spain). LY341495 was obtained from Tocris BioScience (Bristol, UK). Benzoylchloride, Dulbecco's modified Eagle's medium (DMEM), glutamate, glutamine, glutamate-d5 standards, glutamate-pyruvate transaminase (GPT), pertussis toxin (PTX) and sodium pyruvate were from Sigma–Aldrich (St. Louis, MO, USA). Penicillin, streptomycin, L-Proline and G418 were obtained from Duchefa Biochemie (Haarlem, The Netherlands). Fetal calf serum (FCS) was from Biowest (Nuaillé, France). PET E-plates 16 and 96 for the xCELLigence DP and SP system (ACEA Biosciences, San Diego, CA, USA) were obtained from Bioké (Leiden, the Netherlands). Chinese hamster ovary cells (CHO-K1, CCL-61) were from ATCC (Rockville, MD, USA). CHO-K1 cells stably expressing the $hmGlu_2$ receptor (CHO-K1_$hmGlu_2$) were from Janssen Research and Development (Beerse, Belgium). Acetonitrile ULC/MS-grade was purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid 98–100% and disodium tetraborate decahydrate were obtained from Merck (Darmstadt, Germany). High purity water was obtained with a Milli–Q Reference Ultrapure Water Purification System (Merck Millipore, Billerica, MA, USA). Other chemicals were of analytical grade and from standard commercial sources.

2.2. Cell culture

CHO-K1_hmGlu2 cells were grown in DMEM supplemented with 10% (v/v) FCS, 200 $IU \cdot mL^{-1}$ penicillin, 200 $\mu g \cdot mL^{-1}$ streptomycin, 30.5 $\mu g \cdot mL^{-1}$ L-proline and 400 $\mu g \cdot mL^{-1}$ G418 at 37 °C and 5% CO_2. CHO-K1 WT cells were cultured in the same medium without G418.

2.3. Analysis of glutamate concentration

2.3.1. Preparation of stock solutions and curves

As glutamate is present in the culture medium as an endogenous entity, a calibration curve had to be prepared in an artificial matrix, for which demineralised water was used. Therefore, a quality check was performed to validate quantification of the samples with a standard addition step (a known amount of glutamate is spiked to a sample). The calibration curve was prepared in a range of 0.010–50 μg/ml, starting from a stock solution of 100 μg/ml glutamate in water. Glutamate-d5 was used as a stable isotope labelled internal standard (STIL).

2.3.2. Sample preparation

Glutamate was quantified after derivatization with benzoylchloride. 20 μL medium was transferred to a sample tube, subsequently 20 μL of the calibration curve in water was added followed by 20 μL of STIL Glu-d5 and 20 μL of NaBorate solution (100 mM, pH 8). For derivatization, 40 μL benzoylchloride (3% v/v in acetonitrile) was transferred to the sample tubes, after which samples were incubated for 15 min at 50 °C and subsequently cooled to room temperature for 10 min. Finally, 200 μL 0.1% formic acid was added. Samples were vortexed and centrifuged for 3 min at 6000*g*, after which the supernatant was analyzed.

2.3.3. Liquid chromatography – Mass spectrometry

Chromatographic separation was performed with an Acquity UPLC system (Waters, Millford, Boston, USA). Injections of 2 μL were made on an Acquity UPLC BEH C18 2.1 × 50 mm (1.7 μm particles) column held at 55 °C at a flow rate of 0.400 ml/min. Chromatographic elution started isocratically at 2% solvent B (acetonitrile) and 98% solvent A (0.1% formic acid) for 0.2 min. A subsequent linear gradient to 30% B in 1.8 min, followed by a wash step (0.2 min at 98% solvent B) and 0.8 min equilibration time at 2%B resulted in a total run time of 3 min/injection.

Instrument parameters were optimized by injecting a glutamate standard solution (2 ng/ml in 0.1% formic acid/acetonitrile, v/v; 80/20) at a flow rate of 10 μL/min. The following source parameters were used: source temperature 150 °C, desolvation temperature 450 °C, capillary 2.9 kV, sampling cone 30 V, source offset 50 V, cone gas 150 L/h, desolvation gas 900 L/h, collision gas flow 0.18 ml/min and nebulizer gas 6 ml/min. Glutamate was analyzed in the positive ion mode with electrospray ionization and detected by multiple reaction monitoring (MRM) (collision energy 20 eV and dwell time 75 ms) on a triple quad Xevo TQ-s system (Waters, Manchester, UK), monitoring the channels of m/z 252.1 - > 105.1 (quantifier) and m/z 252.1- > 130.1 (qualifier), respectively. The MRM transition monitored for the STIL Glu-d5 is m/z 257.1 - > 105.1. During method optimization, special consideration was given to the chromatographic separation of glutamate and glutamine. The incubation medium has an excessive amount of glutamine present and it is known that glutamine, with a MW 1 Da lower than glutamate, gives crosstalk in the MRM channel of glutamate. Therefore, glutamine was also monitored, at m/z 251.1 - > 105.1 as a qualifier. Quantitative values were obtained by relating chromatographic peak areas to those derived from separately run calibration standards as described above. Calibration curves were plotted using a non-weighted log-log linear regression.

2.4. Label-free whole-cell assays

Label-free whole-cell assays were performed using the xCELLigence

real-time cell analyzer (RTCA) system [3,34] as previously described [7].

The system measures whole-cell responses based on changes in electrical impedance generated by adherence of cells to gold-coated electrodes at the bottom of microelectronic E-plates. Relative changes in impedance (Z) are recorded continuously and displayed in the dimensionless parameter Cell Index (CI), which is defined as $(Z_i - Z_0)\ \Omega / 15\ \Omega$. Z_i is the impedance at a given time and Z_0 is the baseline impedance measured at the start of the experiment in the absence of cells.

Z_0 was measured after adding 45 μL, or in the case of pre-incubation experiments 40 μL, culture medium per well in 16 or 96 well E-plates. 50 μL of cell suspension was added to the plate containing 40,000 cells per well. After resting at room temperature for 30 min, E-plates were mounted in the recording station within a humidified 37 °C, 5% CO_2 incubator. Subsequently, impedance was measured overnight every 15 min. To remove endogenous glutamate, refreshment of culture medium by culture medium or serum-free culture medium with GPT (3 U/ml) and additional pyruvate (3 mM) was performed 3 h before stimulation unless stated otherwise. For evaluation of constitutive receptor activity, pre-incubation with antagonist LY341495 or NAM RO4491533 was performed 1 h before stimulation. After 19 h, cells were stimulated with increasing concentrations of ligand (i.e. agonist, antagonist, PAM or NAM). Final well volumes after addition of compounds were 100 μL in all cases. DMSO concentrations were constant between wells and were ≤0.1%. For inhibition of $G\alpha_i$ signaling, cells were seeded in culture medium containing PTX (300 ng/ml) when indicated. PTX concentrations were kept constant also after medium refreshment.

2.5. Data analysis

RTCA software 2.0 (ACEA Biosciences) was used to obtain the experimental data. All analyses were performed using Prism 7.00 (GraphPad software, San Diego, CA, USA). Ligand responses were normalized to Δ cell index (ΔCI) after subtracting baseline (vehicle control) to correct for ligand-independent effects. Maximum or minimum peak responses were considered as the highest or lowest level of ΔCI within 60 min after compound stimulation and were used for bar graphs and concentration-response curves. pEC_{50} and pIC_{50} values were obtained using non-linear regression curve fitting into a sigmoidal dose-response curve with variable slope. Data shown are the mean ± SEM of at least three individual experiments performed in duplicate. Statistical analyses were performed as indicated. If p-values were below 0.05, observed differences were considered statistically significant.

3. Results and discussion

3.1. Serum-free assay medium with GPT resulted in lowest levels of endogenous glutamate

One of the benefits of the xCELLigence biosensor is that experiments can be performed under more relevant conditions than most traditional assays (i.e. living cells at 37 °C in culture medium). However, endogenous glutamate is often found in culture medium and is also continuously produced by cells [35]. As high levels of glutamate will interfere with functional responses by other $mGlu_2$ receptor agonists, we first determined the concentration of endogenous glutamate in culture medium using HPLC analysis, which was found to be 78 ± 2.0 μM (Table 1). Since the potency of glutamate to activate the $mGlu_2$ receptor is around 10 μM, this concentration is capable to activate the $mGlu_2$ receptor to a high level [23,36]. Hence, we aimed to reduce the concentration of endogenous glutamate in the culture medium. For this purpose, glutamate-pyruvate transaminase (GPT; 3 U/ml) was added in the presence of additional pyruvate (3 mM), a procedure commonly used in the field of mGlu receptors, e.g. [37,38]. This treatment led to an almost 4-fold lower concentration of glutamate in the culture medium: 23 ± 0.09 μM. At this concentration, glutamate is still able to activate the receptor over 50% and will thus significantly interfere with the functional readout. Next, we switched to serum-free medium, which we determined to contain only 0.22 ± 0.005 μM endogenous glutamate, indicating that the fetal calf serum (FCS) present in the culture medium was the main source of glutamate.

Importantly, when seeded in serum-free medium, cells did not attach to the bottom of the wells, did not proliferate and therefore did not show any remarkable level of impedance (data not shown). This clearly indicated that cells required FCS for growth and proliferation. Therefore, serum-free medium was added to the wells by medium refreshment three hours before stimulation only. Of note, during these first three hours of the serum-free medium being on the cells, the concentration of glutamate increased to 13 ± 0.47 μM at the time of stimulation (Table 1). Therefore, GPT and pyruvate were added to this serum-free assay medium, resulting in a stable concentration of endogenous glutamate during the signaling read-out of 1 h (i.e. from 1.6 ± 0.09 μM at the moment of stimulation to 2.3 ± 0.62 μM two hours after stimulation, Table 1). This residual level of endogenous glutamate only slightly activates the receptor to a maximum level of approximately 10%, leaving a large experimental window for further (exogenous) agonist-induced receptor signaling studies.

3.2. Endogenous glutamate reduced agonist yet enhanced antagonist responses

The $mGlu_2$ receptor-mediated signaling was monitored on the xCELLigence RTCA system. In the field of class C GPCRs, this technique was never characterized extensively, yet it was used as a tool in two studies: Scandroglio et al. [39] presented a small proof of concept study on the $mGlu_1$ receptor and recently we reported on the $mGlu_2$ receptor using the xCELLigence as a tool to assess the effect of residence time on functional efficacy of $mGlu_2$ PAMs [40]. Here, CHO-K1_$hmGlu_2$ cells stably expressing the $hmGlu_2$ receptor were used. 40,000 cells/well were seeded in culture medium 19 h prior to stimulation (Fig. 1A). The cells adhered well to the bottom of the wells on the gold-coated electrodes, which is essential for sufficient measurements of impedance [41]. Therefore no additional well coatings had to be used. Overnight proliferation generally led to cell indices (CI) between 3 and 5 (Fig. 1B for two representative traces), dependent on cell passage number and confluency.

As discussed above, the concentration of endogenous glutamate varied between different pre-treatment conditions (Table 1). To assess how this affected the response of an $mGlu_2$ receptor agonist or antagonist, responses of a single 1 μM concentration of LY354740 and LY341495 were obtained, respectively. This concentration was chosen such that it is well above the respective potencies of the two ligands. The traces of the agonist LY354740 at 1 μM were always “positive”, inducing an increase in Cell Index and thus impedance (Fig. 1C,D). The responses after different pre-treatment conditions varied greatly. When using normal culture medium containing a high concentration of endogenous glutamate at the time of stimulation (100 ± 3.0 μM), a small LY354740 response was observed, with peaks at an average maximum ΔCI level of 0.098 ± 0.012 (Fig. 1D). In the presence of GPT and pyruvate the glutamate concentration was reduced to 28 ± 1.1 μM at stimulation, resulting in an increased LY354740 response with maximum peaks of 0.24 ± 0.05 ΔCI. This response reached a similar maximum level as when culture medium was replaced by serum-free medium, although the LY354740 response was more prompt in that case (Fig. 1C). The highest and significantly increased LY354740 response was obtained using medium refreshment with serum-free medium containing GPT and pyruvate, which contained 1.6 ± 0.09 μM endogenous glutamate at the time of stimulation, leading to significantly increased maximum peaks of 0.47 ± 0.04 ΔCI. Even in the presence of a high concentration of endogenous glutamate, LY354740 was still able to induce responses (Fig. 1C, D), which exemplified the

Table 1
Glutamate concentrations (μM) in culture medium or serum-free medium with and without addition of GPT (3 U/ml) and pyruvate (3 mM). Medium was added to the cells at the time of medium refreshment (−3 hrs).

Time of:	Time from stimulation (hrs)	Culture medium		Serum-free medium	
		− GPT	+ GPT	− GPT	+ GPT
Medium refreshment	−3	78 ± 2.0	23 ± 0.09	0.22 ± 0.005	0.098 ± 0.004
	−2.5	83 ± 1.6	23 ± 0.58	3.7 ± 0.20	1.2 ± 0.09
Pre-treatment	−1	88 ± 1.3	25 ± 0.90	9.8 ± 0.09	1.2 ± 0.10
Stimulation	0	100 ± 3.0	28 ± 1.1	13 ± 0.47	1.6 ± 0.09
	2	100 ± 9.4	33 ± 1.9	17 ± 2.1	2.3 ± 0.62

Data are shown as mean ± SEM of at least three individual experiments performed in duplicate.

sensitivity of the xCELLigence system, registering very small ligand-induced responses.

In contrast to the positive LY354740 responses, 1 μM antagonist LY341495 induced opposite negative responses in Cell Index (Fig. 1E). As for the agonist LY354740, the peak level (i.e. minimum peak in case of LY341495) was dependent on the medium refreshment conditions, although in reversed order. When using normal culture medium the peak of LY341495 was the largest, −1.62 ± 0.06 ΔCI (Fig. 1F). Treatment of culture medium with GPT already reduced the peak slightly, but when medium was refreshed by serum-free medium peaks were significantly reduced to minimum levels of −0.92 ± 0.08 ΔCI. Moreover, addition of GPT did not further increase the minimum peak of LY341495 (−0.98 ± 0.06 ΔCI, Fig. 1F), indicating a relatively large response of the compound on its own. Interestingly, in serum-free medium with GPT, the trace returned to baseline more readily after the peak (Fig. 1E), indicating that the difference in glutamate levels still slightly altered the shape of the trace. Importantly, no compound-induced responses were observed for both LY354740 and LY341495 at 1 μM in parental wild-type CHO-K1 cells (Fig. 1C, E), confirming that all responses were $mGlu_2$ receptor-mediated. This was in line with our previous observation that PAM JNJ-46281222 did not induce significant responses on parental CHO-K1 cells [42].

Taken together, the lowest level of endogenous glutamate was reached by medium refreshment with serum-free medium supplemented with GPT, which was used for all further experiments. Of note, the presence of the glutamate degrading enzyme GPT excluded further experimentation with exogenous glutamate, since such experiments would not result in meaningful parameters for potency and/or efficacy. The responses of LY354740 and LY341495 were affected by the different endogenous glutamate concentrations from the culture medium. LY354740 peaks increased upon reduction of endogenous glutamate levels. On the contrary, LY341495 peaks were reduced when endogenous glutamate levels were lowered. Both observations indicate competition of the two molecules for the orthosteric glutamate binding site, which has been shown in radioligand binding experiments with [^{3}H]LY354740 and [^{3}H]LY341495 as the radioligands [43,44].

3.3. Agonist LY354740 and antagonist LY341495 induced concentration-dependent responses at low levels of endogenous glutamate

Once the assay conditions were optimized, the potency of agonist LY354740 and antagonist LY341495 were determined. For LY354740 the impedance increased concentration-dependently with a “positive” peak (i.e. positive ΔCI values) around 10 min, then gradually decreasing with a second ‘shoulder’ peak around 20 min (Fig. 2A). A concentration-response curve obtained from the maximum peak levels within the first 60 min after stimulation revealed a pEC_{50} value of 6.70 ± 0.24 (Table 2; Fig. 2B). This potency was lower than observed in other assay types such as Ca^{2+} mobilization (pEC_{50} 7.4; [45]) and similar to the potency obtained in electrophysiology experiments on hippocampal slices (pEC50 6.9; [46]).

Stimulation with increasing concentrations of the antagonist LY341495 resulted in concentration-dependent negative peaks with a minimum around 15 min (Fig. 2A). For low concentrations it took around 30 min to return to baseline, while at 0.1 and 1 μM LY341495 the trace returned to baseline after 120 or 180 min, respectively (data not shown). A pIC_{50} value of 8.15 ± 0.09 was obtained from the minimum peak levels (Fig. 2B; Table 2), which was in agreement with potencies reported before using cAMP, Ca^{2+}-mobilization and [^{35}S]GTPγS assays [14,31]. The concentration-response curve of LY354740 had a pseudo-Hill coefficient of around unity (n_H 0.92) compared to the curve of LY341495 that was much steeper (n_H −1.81). As mentioned before, LY341495 showed negative responses in Cell Index, indicating that it exhibits an opposite pharmacological effect compared to the agonist LY354740, resulting in an opposite impedance measured. Similar observations have been seen using other label-free systems (i.e. the CellKey and EPIC system), in which agonists induced an increase in response, while inverse agonists caused a decrease in response [1,47]. However, these studies do not present a mechanistic understanding as they were performed on two non-disclosed GPCRs. Given that in these studies inverse agonists induced negative responses, LY341495 may also be an inverse agonist at the $mGlu_2$ receptor, especially since the concentration of endogenous glutamate was reduced to a level only slightly able to activate the receptor.

Finally, the selective $G\alpha_{i/o}$ inhibitor PTX was used to evaluate whether the effects induced by the agonist LY354740 and antagonist LY341495 were mediated via $G\alpha_{i/o}$ protein-dependent signaling. Responses of LY354740 and LY341495 were almost completely lost in the presence of PTX, which indicated that the opposite compound-induced cellular responses were both resulting from signaling via $G\alpha_{i/o}$ (Fig. 2C,D). The residual responses in the presence of PTX most likely represent incomplete inhibition of the $G\alpha_{i/o}$-mediated signaling.

3.4. Agonist efficacy was increased by PAM and decreased by NAM treatment

Before measuring the effect of allosteric modulators on agonist efficacy, responses of a PAM and a NAM in the absence of exogenous agonist were determined. Both allosteric modulators showed responses on their own without added agonist, which were likely caused by the low level of endogenous glutamate (1.6 ± 0.09 μM) still present in the serum-free culture medium with GPT (Table 1). Stimulation with the PAM JNJ-46281222 resulted in positive dose-dependent responses, leading to a pEC_{50} of 7.17 ± 0.24 for this compound (Table 2; Fig. 3A, B). The potency of the PAM was lower than in a [^{35}S]GTPγS assay (pEC_{50} of 7.71; [23]). This may be caused by the concentration of endogenous glutamate that is lower than the typical EC_{20} glutamate concentrations used in standard [^{35}S]GTPγS assays used to determine the potency of allosteric modulators or by the differences in assay types [48,49]. Treatment with the NAM RO4491533 induced negative peaks in Cell Index yielding a pIC_{50} value of 7.34 ± 0.19, which was lower than observed before in a [^{35}S]GTPγS assay (pIC_{50} 8.7; [48]). In this case the difference may be due to the high EC_{80} glutamate concentration used in the [^{35}S]GTPγS assay or, again, due to the differences in

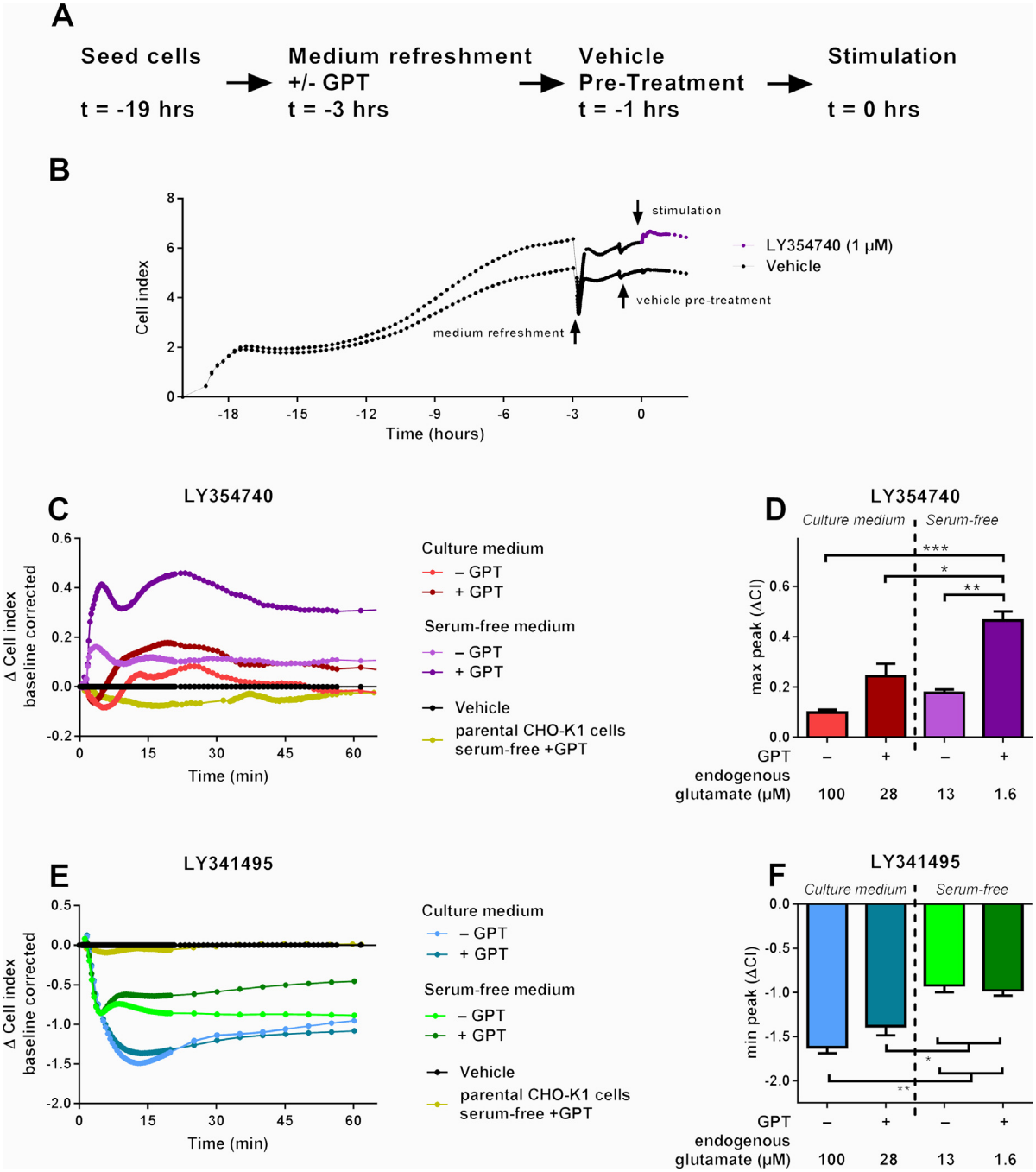

Fig. 1. Agonist LY354740 and antagonist LY341495-induced responses in the presence of different concentrations of endogenous glutamate. A) Set-up of experiments. B) Typical growth curve of CHO-K1 cells stably expressing the $mGlu_2$ receptor (40,000 cells/well). 3 h prior to stimulation culture medium was replaced by serum-free medium containing GPT (3 U/ml) and additional pyruvate (3 mM). Cells were stimulated with LY354740 (1 µM). Serum-free medium with 0.025% DMSO was used as vehicle control. C) Responses induced by 1 µM LY354740 after different pre-treatment conditions and the response of 1 µM LY354740 on CHO-K1 WT cells three hours after replacement of culture medium by serum-free medium containing GPT. D) Maximum peak values induced by 1 µM LY354740 in the presence of different concentrations of endogenous glutamate as presented in Table 1. E) Responses induced by 1 µM LY341495 after different pre-treatment conditions and the response of 1 µM LY341495 on CHO-K1 WT cells three hours after replacement of culture medium by serum-free medium containing GPT. F) Maximum peak values induced by 1 µM LY341495 in the presence of different concentrations of endogenous glutamate as presented in Table 1. All xCELLigence traces are from representative experiments performed in duplicate. Bar graphs represent mean ± SEM of at least three individual experiments performed in duplicate. Statistical analyses were performed using one-way ANOVA with Tukey's post-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

assay type. The concentration-response curve of JNJ-46281222 was shallow with a pseudo-Hill coefficient of 0.59 compared to RO4491533's which was around unity (n_H −1.02). Of note, this is the first observation that Cell Indices go up in the presence of a PAM and down when using a NAM, like JNJ-46281222 and RO4491533, respectively. In that sense, the effects of allosteric modulators for the $mGlu_2$ receptor resemble orthosteric compound-induced responses as described above.

To study the effects of a PAM on agonist efficacy, cellular responses to an LY354740 concentration equivalent to its EC_{20} response (30 nM) were determined in the absence and presence of 1 µM JNJ-46281222. This resulted in a significant 3-fold increase in the LY354740 peak

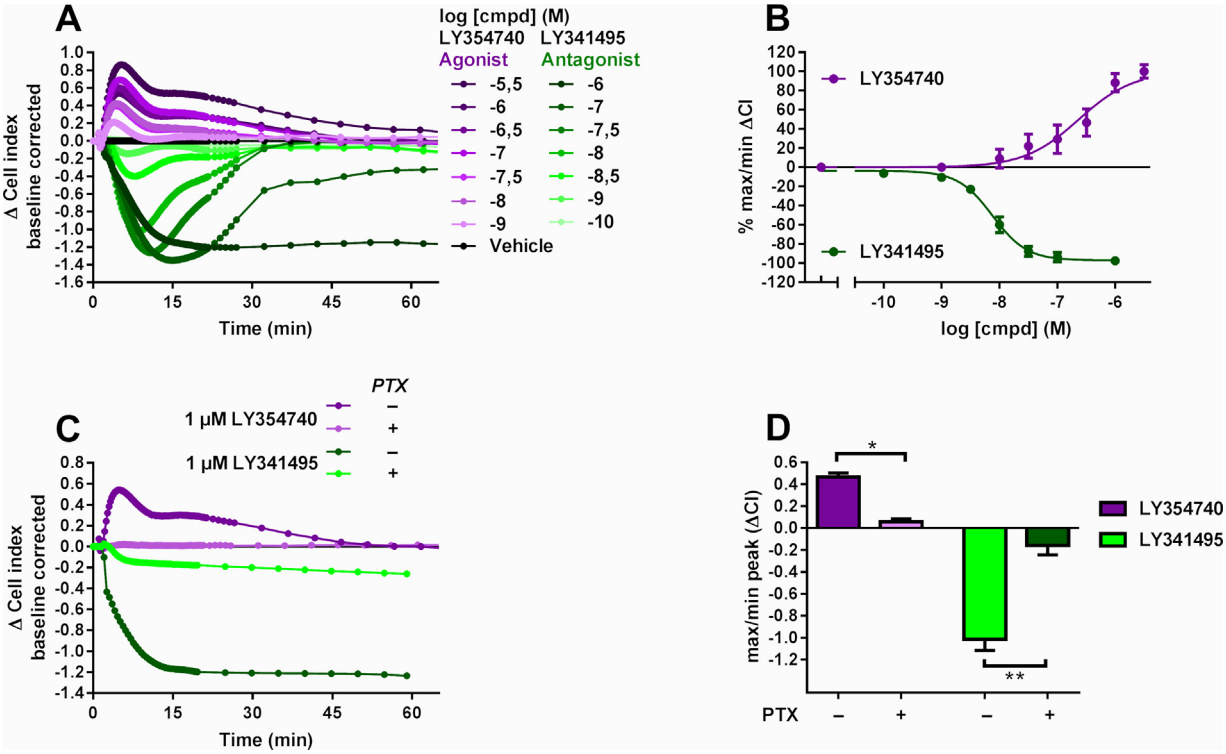

Fig. 2. Agonist LY354740 and antagonist LY341495-induced concentration-effect responses at low concentration of endogenous glutamate (1.6 ± 0.09 μM). A) Cells were stimulated by increasing concentrations of agonist LY354740 or antagonist LY341495. B) Concentration-response curves were obtained from maximum (LY354740) or minimum (LY341495) ΔCI values and normalized from vehicle-induced ΔCI (0%) to maximum or minimum ΔCI (100% or -100% respectively). C) Responses of 1 μM LY354740 and 1 μM LY341495 in the absence or presence of PTX (300 ng/ml). D) Comparison of maximum peak values induced by 1 μM LY354740 and minimum peak values induced by 1 μM LY341495 in the absence or presence of PTX (300 ng/ml). All xCELLigence traces are from representative experiments performed in duplicate. Concentration-response curves (panel B) represent mean ± SEM of at least three individual experiments performed in duplicate. Bar graphs represent mean ± SEM of at least three individual experiments performed in duplicate. Statistical analysis was performed using a two-tailed unpaired Student's *t*-test. $^{*}p < 0.01$, $^{**}p < 0.001$.

Table 2
Potency (pEC_{50} or pIC_{50}) of ligands at the $mGlu_2$ receptor in CHO-K1_hmGlu_2 cells after refreshment of medium by serum-free medium containing GPT (3 U/ml) and pyruvate (3 mM).

Compound		pEC_{50}	pIC_{50}
LY354740	Agonist	6.70 ± 0.24	–
LY341495	Antagonist	–	8.15 ± 0.09
JNJ-46281222	PAM	7.17 ± 0.24	–
RO4491533	NAM	–	7.34 ± 0.19

pEC_{50}/IC_{50} data are shown as mean ± SEM from at least three individual experiments performed in duplicate.

response, confirming that JNJ-46281222 behaves as a PAM for this agonist (Fig. 3C). The effect of 1 μM of the NAM RO4491533 was determined using an LY354740 concentration equivalent to its EC_{80} concentration (300 nM). This yielded a significant, more than twofold reduction in the response of LY354740 (Fig. 3D), which is a typical NAM effect. These experiments showed that xCELLigence is able to assess the effects of allosteric modulators on agonist efficacy, which is in line with work reported by Chen et al. [50] on allosteric modulation of the M_4 muscarinic acetylcholine receptor.

3.5. LY341495 pre-treatment revealed constitutive receptor activity

The maximum peak height of the agonist LY354740 was much lower compared to the negative peaks of the antagonist LY341495 (Fig. 2A, C). In order to study the underlying mechanism, cells were pretreated with 1 μM LY341495 1 h prior to stimulation (Fig. 4A). The response of subsequent stimulation by 1 μM LY354740 was increased significantly from an average max peak of 0.47 ± 0.04 ΔCI up to a level of 1.21 ± 0.16 (Fig. 4B, C). This was opposite to the expected loss of agonist response by the pre-treatment with a high concentration of a competitive antagonist that is still present at the time of stimulation with agonist. The effect of LY341495 pre-treatment on the LY354740 peak was concentration-dependent: pre-treatment with 10 nM LY341495 slightly increased LY354740 traces, whereas 100 nM and 1 μM LY341495 were able to further and significantly increase the peak height of 1 μM LY354740 stimulation (Fig. 4C). All traces reached baseline after 30–45 min. In contrast to the other traces, the trace of 1 μM LY354740 after pre-treatment with 1 μM LY341495 increased slightly after that point reaching a plateau around 0.4 ΔCI. Treatment with the selective $G\alpha_{i/o}$ inhibitor PTX revealed that the LY354740 response after pre-treatment with LY341495 was lost in the presence of PTX, indicating that this is also a mostly, if not fully, $G\alpha_{i/o}$-mediated response (Fig. 4B, C). In contrast to LY341495, pre-treatment with 1 μM of the NAM RO4491533 resulted in a typical reduced agonist response of LY354740 (Fig. 4D, E).

Together, these results show that LY341495 increased the window of agonist response by almost 3-fold. This is a larger increase than can be explained by the level of endogenous glutamate (1.6 μM at stimulation), which might enable approximately 10% receptor activation. Blockade of glutamate binding may thus increase the agonist-induced response by a similar level. Therefore, we believe that the increased agonist response after pre-treatment with LY341495 indicates that the receptor is constitutively active and that LY341495 is behaving as an inverse agonist. This was confirmed by the fact that, in contrast to pre-treatment with LY341495, pre-treatment by the NAM RO4491533 resulted in a decreased agonist response (Fig. 4D, E). Such a decreased agonist response is similar to the expected effect of pre-treatment with a

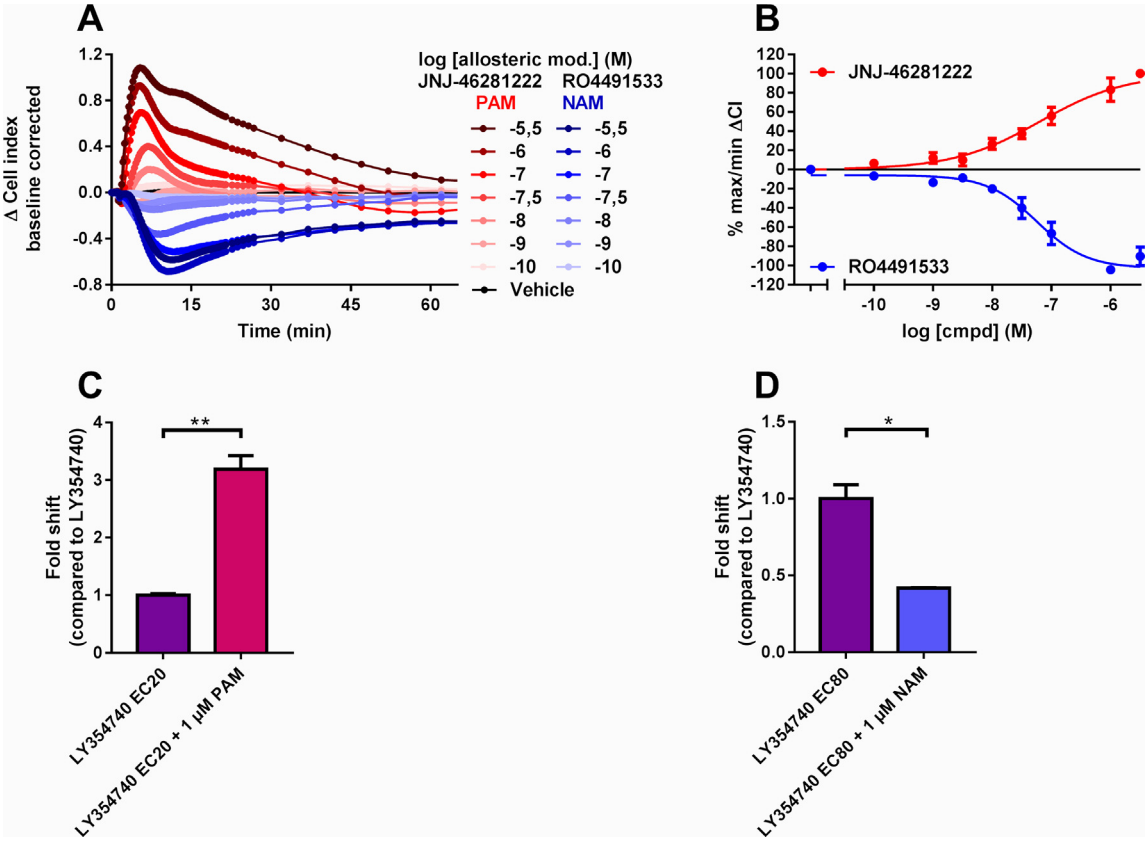

Fig. 3. Positive allosteric modulator (PAM) JNJ-46281222 and negative allosteric modulator (NAM) RO4491533-induced responses at low concentration (1.6 ± 0.09 μM) of endogenous glutamate. A) Cells were stimulated by increasing concentrations of JNJ-46281222 or RO4491533. B) Concentration-response curves were obtained from maximum (JNJ-46281222) or minimum (RO4491533) ΔCI values and normalized from vehicle-induced ΔCI values (0%) to maximum (100%) or minimum (−100%) ΔCI values. C) Responses induced by an EC_{20} LY354740 concentration in the absence and presence of 1 μM JNJ-46281222. D) Responses induced by an EC_{80} LY354740 concentration in the absence and presence of 1 μM RO4491533. xCELLigence traces are from representative experiments performed in duplicate. Bar graphs represent mean ± SEM of at least three experiments performed in duplicate, where data was normalized to the response of PAM or NAM on itself (1.0). Statistical analysis was performed using a two-tailed unpaired Student's *t*-test. $^{*}p < 0.01$, $^{**}p < 0.001$.

neutral antagonist.

To further assess the inverse agonism of LY341495, concentration-response curves were obtained after pre-treatment with increasing concentrations of the PAM JNJ-46281222 (Fig. 4F, G). The curves shifted from a pIC_{50} of 8.15 ± 0.09 without PAM pre-treatment to 7.89 ± 0.13 in the presence of 10 nM PAM, to 7.55 ± 0.13 in the presence of 100 nM PAM. Such a modulatory shift in potency in the presence of a PAM is typical for an inverse agonist and is not expected for a neutral antagonist [51,52]. Next to the shift in potency, increasing concentrations of PAM pre-treatment resulted in a larger window of LY341495 response. On the contrary, pre-treatment with NAM RO4491533 did not result in significant alteration in LY341495 potency (Fig. 4H), a pIC_{50} values of 8.22 ± 0.14 in the presence of 100 nM NAM and 8.35 ± 0.12 in the presence of 1 μM NAM were found. The efficacy window of LY341495 was reduced dose-dependently up to more than 60% at 1 μM NAM, showing that the NAM is not only able to strongly reduce agonist efficacy without changing potency [31], but similarly affects the inverse agonist activity of LY341495.

A shift in LY341495 potency induced by the PAM could be the reason of increased potency of endogenous glutamate. However, the large shift in efficacy cannot be explained by presence of endogenous agonist, nor can the large reduction of efficacy after NAM pre-treatment. Together, inverse agonism by LY341495 was further confirmed by these PAM and NAM pre-treatment experiments in this system that measures phenotypic morphological responses of the whole cell, in this case via the $G\alpha_{i/o}$ signaling pathway. On a separate note, receptor desensitization induced by endogenous glutamate could be of concern here. However, the $mGlu_2$ receptor is known to be resistant to agonist-induced desensitization which was also seen in our hands in repeated stimulation experiments (data not shown) [53,54]. Doumazane et al. (2013) [55] found a higher FRET signal after application of 1 μM LY341495, similar to the effect of LY341495 pre-treatment presented here. These authors reasoned that this effect was mediated by either constitutive activity or presence of endogenous glutamate, which was not elucidated any further. Inverse agonism by LY341495 has been reported before. For example, DiRaddo et al. (2014) [56] found that LY341495 was behaving as inverse agonist after pre-treatment with agonist, when using the Glo-sensor cAMP assay with CHO-K1 cells stably expressing the $mGlu_2$ receptor. Moreover, inverse agonism by LY341495 at the $mGlu_2$ receptor was also seen in studies investigating receptor cross-signaling in $mGlu_2$-$5HT_{2A}$ heterocomplexes [57,58]. Generally, LY341495 is considered a neutral antagonist for the $mGlu_2$ receptor [14,15] as most *in vitro* assays did not yield inverse agonism, which is likely due to differences in cell lines or assay systems used.

In conclusion, we have validated the use of the impedance-based biosensor xCELLigence system to study $mGlu_2$ pharmacology. High levels of endogenous glutamate reduced the agonist activation window and therefore efforts were made to reduce these. We characterized $mGlu_2$ signaling with an agonist, antagonist and both positive and negative allosteric modulators; the latter two modulated the response of both orthosteric agonist and antagonist. Remarkable and opposite effects on impedance were observed for compounds with opposite pharmacological effects. Both the agonist and PAM increased impedance whereas the antagonist and NAM decreased this read-out. Importantly,

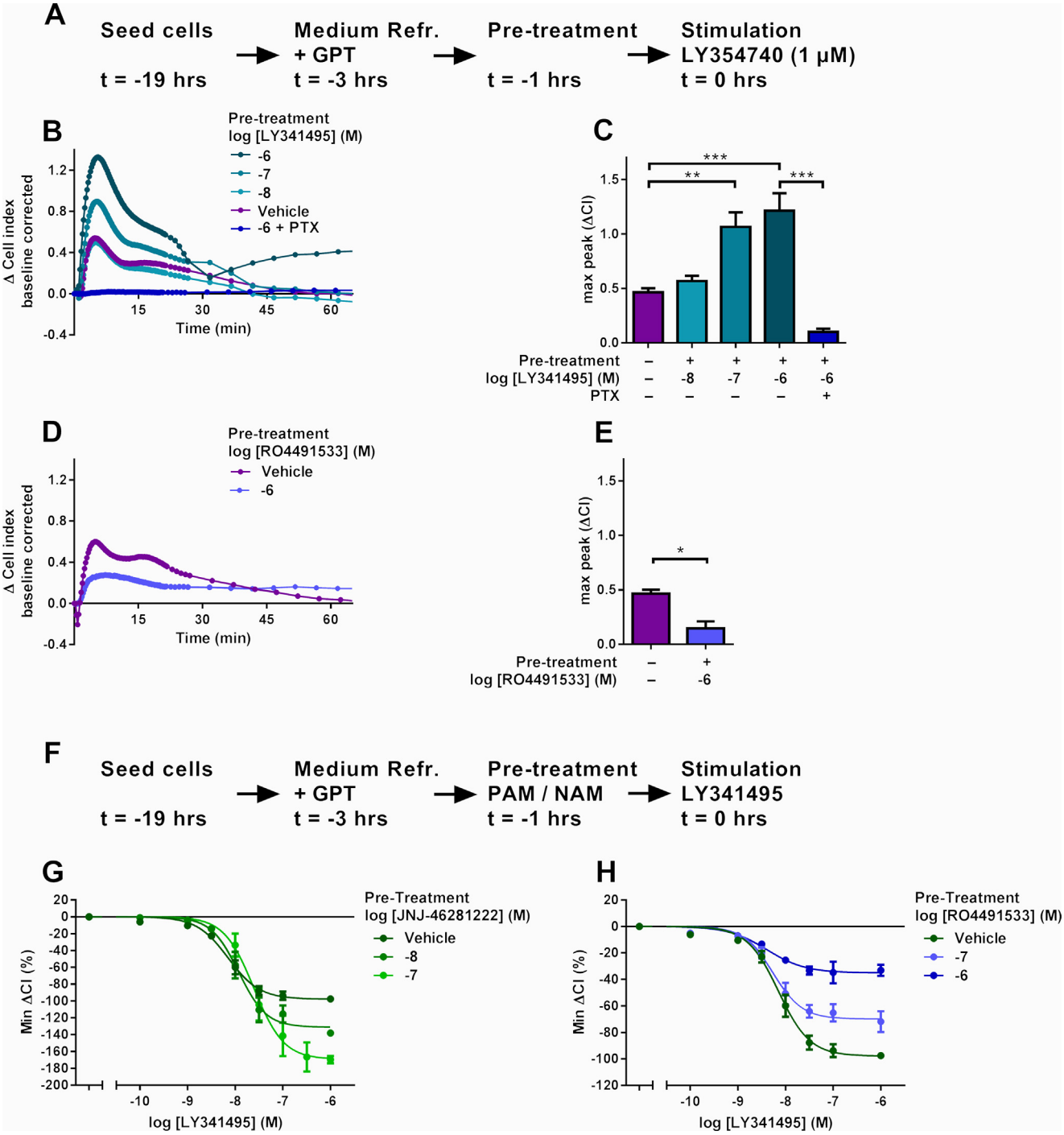

Fig. 4. Evaluation of LY341495 inverse agonism. A) Set-up of experiments. B) Cells were stimulated with 1 µM LY354740 after pre-treatment with increasing concentrations of LY341495. Pre-treatment with 1 µM LY341495 was also performed in the presence of PTX (300 ng/ml). C) Comparison of maximum peak values induced by 1 µM LY354740 after pre-treatment with increasing concentrations of LY341495. Pre-treatment with 1 µM LY341495 was also performed in the presence of PTX (300 ng/ml). D) Cells were stimulated with 1 µM LY354740 after pre-treatment with 1 µM NAM RO4491533 or vehicle. E) Comparison of maximum peak values induced by 1 µM LY354740 after pre-treatment with 1 µM RO4491533 or vehicle. F) Set-up of experiments. G, H) Concentration-response curves were obtained from minimum ΔCI values and normalized from vehicle-induced ΔCI values (0%) to minimum (−100%) ΔCI values after vehicle pre-treatment. xCELLigence traces are from representative experiments performed in duplicate. Bar graphs represent mean ± SEM of at least three individual experiments performed in duplicate. Concentration-response curves represent mean ± SEM of at least three individual experiments performed in duplicate. Statistical analysis was performed using a one-way ANOVA with Dunnett's (C) post-test or using a two-tailed unpaired Student's *t*-test (E). $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$.

we show that constitutive $mGlu_2$ receptor activity could be inhibited by the antagonist LY341495 which therefore behaved as an inverse agonist in the system used. The present study provides an extended ensemble of the properties of $mGlu_2$ ligands in comparison to conventional *in vitro* assays. Together the results show that label-free biosensors, such as the xCELLigence, are valuable tools in drug discovery and pharmacological profiling at the $mGlu_2$ receptor as well as other GPCRs.

Conflict of interest

None.

List of author contributions

Participated in research design: Doornbos, Van der Linden, Vereyken, Tresadern, IJzerman, Lavreysen, Heitman.

Conducted experiments: Doornbos, Vereyken.

Performed data analysis: Doornbos, Vereyken.

Wrote or contributed to writing of the manuscript: Doornbos, Van der Linden, Vereyken, Tresadern, IJzerman, Lavreysen, Heitman.

Acknowledgements

The authors thank Lieve Heylen for technical assistance.

This project was financially supported by Vlaams Agentschap Innoveren & Ondernemen project number: 120491.

References

[1] C.W. Scott, M.F. Peters, Label-free whole-cell assays: expanding the scope of GPCR screening, Drug Discov. Today 15 (2010) 704–716, http://dx.doi.org/10.1016/j.drudis.2010.06.008.

[2] Y. Fang, Combining label-free cell phenotypic profiling with computational approaches for novel drug discovery, Expert Opin. Drug Discov. 10 (2015) 331–343, http://dx.doi.org/10.1517/17460441.2015.1020788.

[3] N. Yu, J.M. Atienza, J. Bernard, S. Blanc, J. Zhu, X. Wang, X. Xu, Y. Abassi, Real-time monitoring of morphological changes in living cells by electronic cell sensors arrays: an approach to study G protein-coupled receptors, Anal. Chem. 78 (2006) 35–43.

[4] R. Halai, M.A. Cooper, Using label-free screening technology to improve efficiency in drug discovery, Expert Opin. Drug Discov. 7 (2012) 123–131, http://dx.doi.org/10.1517/17460441.2012.651121.

[5] J.M. Hillger, W.-L. Lieuw, L.H. Heitman, A.P. IJzerman, Label-free technology and patient cells: from early drug development to precision medicine, Drug Discov. Today. (2017), http://dx.doi.org/10.1016/j.drudis.2017.07.015.

[6] K. Lundstrom, Cell-impedance-based label-free technology for the identification of new drugs, Expert Opin. Drug Discov. 12 (2017) 335–343, http://dx.doi.org/10.1080/17460441.2017.1297419.

[7] J.M. Hillger, J. Schoop, D.I. Boomsma, P. Eline Slagboom, A.P. IJzerman, L.H. Heitman, Whole-cell biosensor for label-free detection of GPCR-mediated drug responses in personal cell lines, Biosens. Bioelectron. 74 (2015) 233–242, http://dx.doi.org/10.1016/j.bios.2015.06.031.

[8] I. Nederpelt, R.D. Vergroesen, A.P. IJzerman, L.H. Heitman, Persistent GnRH receptor activation in pituitary αT3-1 cells analyzed with a label-free technology, Biosens. Bioelectron. 79 (2016) 721–727, http://dx.doi.org/10.1016/j.bios.2015.12.066.

[9] M. Grundmann, I.G. Tikhonova, B.D. Hudson, G. Milligan, T. Kenakin, M. Grundmann, I.G. Tikhonova, B.D. Hudson, N.J. Smith, K. Mohr, T. Ulven, Article A molecular mechanism for sequential activation of a G protein-coupled receptor article A molecular mechanism for sequential activation of a G protein-coupled receptor, Cell Chem. Biol. 23 (2016) 392–403, http://dx.doi.org/10.1016/j.chembiol.2016.02.014.

[10] G.J. Marek, Metabotropic glutamate2/3 (mGlu2/3) receptors, schizophrenia and cognition, Eur. J. Pharmacol. 639 (2010) 81–90, http://dx.doi.org/10.1016/j.ejphar.2010.02.058.

[11] C.M. Niswender, P.J. Conn, Metabotropic glutamate receptors: physiology, pharmacology, and disease, Annu. Rev. Pharmacol. Toxicol. 50 (2010) 295–322, http://dx.doi.org/10.1146/annurev.pharmtox.011008.145533.

[12] S.T. Patil, L. Zhang, F. Martenyi, S.L. Lowe, K.A. Jackson, B.V. Andreev, A.S. Avedisova, L.M. Bardenstein, I.Y. Gurovich, M.A. Morozova, S.N. Mosolov, N.G. Neznanov, A.M. Reznik, A.B. Smulevich, V.A. Tochilov, B.G. Johnson, J.A. Monn, D.D. Schoepp, Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial, Nat. Med. 13 (2007) 1102–1107, http://dx.doi.org/10.1038/nm1632.

[13] D.H. Adams, B.J. Kinon, S. Baygani, B.A. Millen, I. Velona, S. Kollack-Walker, D.P. Walling, A long-term, phase 2, multicenter, randomized, open-label, comparative safety study of pomaglumetad methionil (LY2140023 monohydrate) versus atypical antipsychotic standard of care in patients with schizophrenia, BMC Psychiatry. 13 (2013) 143, http://dx.doi.org/10.1186/1471-244X-13-143.

[14] A. Kingston, P. Ornstein, R. Wright, B. Johnson, N. Mayne, J. Burnett, R. Belagaje, S. Wu, D. Schoepp, LY341495 is a nanomolar potent and selective antagonist of group II metabotropic glutamate receptors, Neuropharmacology 37 (1998) 1–12, http://dx.doi.org/10.1016/S0028-3908(97)00191-3.

[15] J.M. Witkin, S. Mitchell, K. Wafford, G. Carter, G. Gilmour, J. Li, B. Eastwood, C. Overshiner, X. Li, L. Rorick-Kehn, K. Rasmussen, W. Anderson, A. Nikolayev, V. Tolstikov, M.-S. Kuo, J. Catlow, R. Li, S. Smith, C. Mitch, P. Ornstein, S. Swanson, J. Monn, Comparative effects of LY3020371, a potent and selective mGlu2/3 receptor antagonist, and ketamine, a non-competitive NMDA receptor antagonist, in rodents: evidence supporting use for the treatment of depression, J. Pharmacol. Exp. Ther. (2017), http://dx.doi.org/10.1124/jpet.116.238121.

[16] P.J. Conn, C.W. Lindsley, J. Meiler, C.M. Niswender, Opportunities and challenges in the discovery of allosteric modulators of GPCRs for treating CNS disorders, Nat. Rev. Drug Discov. 13 (2014) 692–708, http://dx.doi.org/10.1038/nrd4308.

[17] H. Wu, C. Wang, K.J. Gregory, G.W. Han, H.P. Cho, Y. Xia, C.M. Niswender, V. Katritch, J. Meiler, V. Cherezov, P.J. Conn, R.C. Stevens, Structure of a class C GPCR metabotropic glutamate receptor 1 bound to an allosteric modulator, Science 344 (2014) 58–64, http://dx.doi.org/10.1126/science.1249489.

[18] A.S. Doré, K. Okrasa, J.C. Patel, M. Serrano-Vega, K. Bennett, R.M. Cooke, J.C. Errey, A. Jazayeri, S. Khan, B. Tehan, M. Weir, G.R. Wiggin, F.H. Marshall, Structure of class C GPCR metabotropic glutamate receptor 5 transmembrane domain, Nature. 511 (2014) 557–562, http://dx.doi.org/10.1038/nature13396.

[19] K.J. Gregory, E.N. Dong, J. Meiler, P.J. Conn, Allosteric modulation of metabotropic glutamate receptors: structural insights and therapeutic potential, Neuropharmacology 60 (2011) 66–81, http://dx.doi.org/10.1016/j.neuropharm.2010.07.007.

[20] A. Christopoulos, J.-P. Changeux, W.A. Catterall, D. Fabbro, T.P. Burris, J.A. Cidlowski, R.W. Olsen, J.A. Peters, R.R. Neubig, J.-P. Pin, P.M. Sexton, T.P. Kenakin, F.J. Ehlert, M. Spedding, C.J. Langmead, International union of basic and clinical pharmacology. XC. Multisite pharmacology: recommendations for the nomenclature of receptor allosterism and allosteric ligands, Pharmacol. Rev. 66 (2014) 918–947, http://dx.doi.org/10.1124/pr.114.008862.

[21] H. Lavreysen, X. Langlois, A. Ahnaou, W. Drinkenburg, P. te Riele, I. Biesmans, I. Van der Linden, L. Peeters, A. Megens, C. Wintmolders, J.M. Cid, A.A. Trabanco, J.I. Andrés, F.M. Dautzenberg, R. Lütjens, G. Macdonald, J.R. Atack, Pharmacological characterization of JNJ-40068782, a new potent, selective, and systemically active positive allosteric modulator of the mGlu2 receptor and its radioligand [3H]JNJ-40068782, J. Pharmacol. Exp. Ther. 346 (2013) 514–527, http://dx.doi.org/10.1124/jpet.113.204990.

[22] R. Galici, N.G. Echemendia, A.L. Rodriguez, P.J. Conn, A selective allosteric potentiator of metabotropic glutamate (mGlu) 2 receptors has effects similar to an orthosteric mGlu2/3 receptor agonist in mouse models predictive of antipsychotic activity, J. Pharmacol. Exp. Ther. 315 (2005) 1181–1187, http://dx.doi.org/10.1124/jpet.105.091074.

[23] M.L.J. Doornbos, L. Pérez-Benito, G. Tresadern, T. Mulder-Krieger, I. Biesmans, A.A. Trabanco, J.M. Cid, H. Lavreysen, A.P. IJzerman, L.H. Heitman, Molecular mechanism of positive allosteric modulation of the metabotropic glutamate receptor 2 by JNJ-46281222, Br. J. Pharmacol. 173 (2016) 588–600, http://dx.doi.org/10.1111/bph.13390.

[24] J.M. Cid, G. Tresadern, G. Duvey, R. Lütjens, T. Finn, J. Rocher, S. Poli, J.A. Vega, A.I. de Lucas, E. Matesanz, M.L. Linares, J.I. Andrés, J. Alcazar, J.M. Alonso, G.J. Macdonald, D. Oehlrich, H. Lavreysen, A. Ahnaou, W. Drinkenburg, C. Mackie, S. Pype, D. Gallacher, A.A. Trabanco, Discovery of 1-butyl-3-chloro-4-(4-phenyl-1-piperidinyl)-(1H)-pyridone (JNJ-40411813): a novel positive allosteric modulator of the metabotropic glutamate 2 receptor, J. Med. Chem. 57 (2014) 6495–6512, http://dx.doi.org/10.1021/jm500496m.

[25] H. Lavreysen, A. Ahnaou, W. Drinkenburg, X. Langlois, C. Mackie, S. Pype, R. Lütjens, E. Le Poul, A.A. Trabanco, J.M.C. Nuñez, Pharmacological and pharmacokinetic properties of JNJ-40411813, a positive allosteric modulator of the mGlu2 receptor, Pharmacol. Res. Perspect. 3 (2015) e00096, http://dx.doi.org/10.1002/prp2.96.

[26] H. Lavreysen, X. Langlois, L. Ver Donck, J.M. Cid Nuñez, S. Pype, R. Lütjens, A. Megens, Preclinical evaluation of the antipsychotic potential of the mGlu2-positive allosteric modulator JNJ-40411813, Pharmacol. Res. Perspect. 3 (2015) e00097, http://dx.doi.org/10.1002/prp2.97.

[27] H. Salih, I. Anghelescu, I. Kezic, V. Sinha, E. Hoeben, L. Van Nueten, H. De Smedt, P. De Boer, Pharmacokinetic and pharmacodynamic characterisation of JNJ-40411813, a positive allosteric modulator of mGluR2, in two randomised, double-blind phase-I studies, J. Psychopharmacol. 29 (2015) 414–425, http://dx.doi.org/10.1177/0269881115573403.

[28] J.M. Kent, E. Daly, I. Kezic, R. Lane, P. Lim, H. De Smedt, P. De Boer, L. Van Nueten, W.C. Drevets, M. Ceusters, Efficacy and safety of an adjunctive mGlu2 receptor positive allosteric modulator to a SSRI/SNRI in anxious depression, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 67 (2016) 66–73, http://dx.doi.org/10.1016/j.pnpbp.2016.01.009.

[29] D. Cook, D. Brown, R. Alexander, R. March, P. Morgan, G. Satterthwaite, M.N. Pangalos, Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework, Nat. Rev. Drug Discov. 13 (2014) 419–431, http://dx.doi.org/10.1038/nrd4309.

[30] C. Goeldner, T.M. Ballard, F. Knoflach, J. Wichmann, S. Gatti, D. Umbricht, Cognitive impairment in major depression and the mGlu2 receptor as a therapeutic target, Neuropharmacology 64 (2013) 337–346, http://dx.doi.org/10.1016/j.neuropharm.2012.08.001.

[31] B. Campo, M. Kalinichev, N. Lambeng, M. El Yacoubi, I. Royer-Urios, M. Schneider, C. Legrand, D. Parron, F. Girard, A. Bessif, S. Poli, J.-M. Vaugeois, E. Le Poul, S. Celanire, Characterization of an mGluR2/3 negative allosteric modulator in rodent models of depression, J. Neurogenet. 25 (2011) 152–166, http://dx.doi.org/10.3109/01677063.2011.627485.

[32] M. Van Gool, S.A. Alonso De Diego, O. Delgado, A.A. Trabanco, F. Jourdan, G.J. Macdonald, M. Somers, L. Ver, Donck, 1,3,5-Trisubstituted Pyrazoles as potent negative allosteric modulators of the mGlu2/3 receptors, ChemMedChem 12 (2017) 905–912, http://dx.doi.org/10.1002/cmdc.201700101.

[33] Homepage: https://clinicaltrials.gov/show/NCT01457677. ARTDeCo Study: A study of RO4995819 in patients with major depressive disorder and inadequate

response to ongoing antidepressant treatment. (Accessed December 5, 2017), (n.d.).
[34] B. Xi, N. Yu, X. Wang, X. Xu, Y.A. Abassi, The application of cell-based label-free technology in drug discovery, Biotechnol. J. 3 (2008) 484–495, http://dx.doi.org/10.1002/biot.200800020.
[35] J. Wahrheit, A. Nicolae, E. Heinzle, Investigation of glutamine metabolism in CHO cells by dynamic metabolic flux analysis, BMC Proc. 7 (2013) P44, http://dx.doi.org/10.1186/1753-6561-7-S6-P44.
[36] J.P. Pin, C. De Colle, A.S. Bessis, F. Acher, New perspectives for the development of selective metabotropic glutamate receptor ligands, Eur. J. Pharmacol. 375 (1999) 277–294.
[37] H. Lavreysen, E. Le Poul, P. Van Gompel, L. Dillen, J.E. Leysen, A.S.J. Lesage, Supersensitivity of human metabotropic glutamate 1a receptor signaling in L929sA cells, Mol. Pharmacol. 61 (2002) 1244–1254.
[38] K. Sengmany, J. Singh, G.D. Stewart, P.J. Conn, A. Christopoulos, K.J. Gregory, Biased allosteric agonism and modulation of metabotropic glutamate receptor 5: implications for optimizing preclinical neuroscience drug discovery, Neuropharmacology 115 (2017) 60–72, http://dx.doi.org/10.1016/j.neuropharm.2016.07.001.
[39] P. Scandroglio, R. Brusa, G. Lozza, I. Mancini, R. Petrò, A. Reggiani, M. Beltramo, Evaluation of cannabinoid receptor 2 and metabotropic glutamate receptor 1 functional responses using a cell impedance-based technology, J. Biomol. Screen 15 (2010) 1238–1247, http://dx.doi.org/10.1177/1087057110375615.
[40] M.L.J. Doornbos, J.M. Cid, J. Haubrich, A. Nunes, J.W. van de Sande, S.C. Vermond, T. Mulder-Krieger, A.A. Trabanco, A. Ahnaou, W.H. Drinkenburg, H. Lavreysen, L.H. Heitman, A.P. IJzerman, G. Tresadern, Discovery and kinetic profiling of 7-Aryl-1,2,4-triazolo[4,3-*a*]pyridines: positive allosteric modulators of the metabotropic glutamate receptor 2, J. Med. Chem. 60 (2017) 6704–6720, http://dx.doi.org/10.1021/acs.jmedchem.7b00669.
[41] S. Lieb, S. Michaelis, N. Plank, G. Bernhardt, A. Buschauer, J. Wegener, Label-free analysis of GPCR-stimulation: the critical impact of cell adhesion, Pharmacol. Res. 108 (2016) 65–74, http://dx.doi.org/10.1016/j.phrs.2016.04.026.
[42] M.L.J.J. Doornbos, J.M. Cid, J. Haubrich, A. Nunes, J.W. van de Sande, S.C. Vermond, T. Mulder-Krieger, A.A. Trabanco, A. Ahnaou, W.H. Drinkenburg, H. Lavreysen, L.H. Heitman, A.P. IJzerman, G. Tresadern, Discovery and kinetic profiling of 7-Aryl-1,2,4-triazolo[4,3- a]pyridines: positive allosteric modulators of the metabotropic glutamate receptor 2, J. Med. Chem. 60 (2017) 6704–6720, http://dx.doi.org/10.1021/acs.jmedchem.7b00669.
[43] B.G. Johnson, R.a Wright, M.B. Arnold, W.J. Wheeler, P.L. Ornstein, D.D. Schoepp, [3H]-LY341495 as a novel antagonist radioligand for group II metabotropic glutamate (mGlu) receptors: characterization of binding to membranes of mGlu receptor subtype expressing cells, Neuropharmacology 38 (1999) 1519–1529 10530814.
[44] H. Schaffhauser, J.G. Richards, J. Cartmell, S. Chaboz, J.A. Kemp, A. Klingelschmidt, J. Messer, H. Stadler, T. Woltering, V. Mutel, In vitro binding characteristics of a new selective group II metabotropic glutamate receptor radioligand, [3H]LY354740, in rat brain, Mol. Pharmacol. 53 (1998) 228–233, http://dx.doi.org/10.1124/mol.53.2.228.
[45] J.A. Monn, L. Prieto, L. Taboada, C. Pedregal, J. Hao, M.R. Reinhard, S.S. Henry, P.J. Goldsmith, C.D. Beadle, L. Walton, T. Man, H. Rudyk, B. Clark, D. Tupper, S.R. Baker, C. Lamas, C. Montero, A. Marcos, J. Blanco, M. Bures, D.K. Clawson, S. Atwell, F. Lu, J. Wang, M. Russell, B.A. Heinz, X. Wang, J.H. Carter, C. Xiang, J.T. Catlow, S. Swanson, H. Sanger, L.M. Broad, M.P. Johnson, K.L. Knopp, R.M.a Simmons, B.G. Johnson, D.B. Shaw, D.L. McKinzie, Synthesis and pharmacological characterization of C4-disubstituted analogs of 1 S,2 S,5 R,6 S -2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate: identification of a potent, selective metabotropic glutamate receptor agonist and determination of agonist-bou, J. Med. Chem. 58 (2015) 1776–1794, http://dx.doi.org/10.1021/jm501612y.
[46] J. Kilbride, L. Huang, M.J. Rowan, R. Anwyl, Presynaptic inhibitory action of the group II metabotropic glutamate receptor agonists, LY354740 and DCG-IV, Eur. J. Pharmacol. 356 (1998) 149–157, http://dx.doi.org/10.1016/S0014-2999(98)00526-3.
[47] P.H. Lee, A. Gao, C. van Staden, J. Ly, J. Salon, A. Xu, Y. Fang, R. Verkleeren, Evaluation of dynamic mass redistribution technology for pharmacological studies of recombinant and endogenously expressed g protein-coupled receptors, Assay Drug Dev. Technol. 6 (2008) 83–94, http://dx.doi.org/10.1089/adt.2007.126.
[48] L. Pérez-Benito, M.L.J. Doornbos, A. Cordomí, L. Peeters, H. Lavreysen, L. Pardo, G. Tresadern, Molecular switches of allosteric modulation of the metabotropic glutamate 2 receptor, Structure 25 (2017) 1–10, http://dx.doi.org/10.1016/j.str.2017.05.021.
[49] T.P. Kenakin, A Pharmacology Primer – fourth edition, Academic Press, San Diego, 2014.
[50] A.N.Y. Chen, D.T. Malone, K. Pabreja, P.M. Sexton, A. Christopoulos, M. Canals, Detection and quantification of allosteric modulation of endogenous M4 muscarinic acetylcholine receptor using impedance-based label-free technology in a neuronal cell line, J. Biomol. Screen 20 (2015) 646–654, http://dx.doi.org/10.1177/1087057114563025.
[51] J.R. Lane, L.T. May, R.G. Parton, P.M. Sexton, A. Christopoulos, A kinetic view of GPCR allostery and biased agonism, Nat. Chem. Biol. 13 (2017) 929–937, http://dx.doi.org/10.1038/nchembio.2431.
[52] M. Canals, J.R. Lane, A. Wen, P.J. Scammells, P.M. Sexton, A. Christopoulos, A monod-wyman-changeux mechanism can explain G protein-coupled receptor (GPCR) allosteric modulation, J. Biol. Chem. 287 (2012) 650–659, http://dx.doi.org/10.1074/jbc.M111.314278.
[53] L. Iacovelli, G. Molinaro, G. Battaglia, M. Motolese, L. Di Menna, M. Alfiero, J. Blahos, F. Matrisciano, M. Corsi, C. Corti, V. Bruno, A. De Blasi, F. Nicoletti, Regulation of group II metabotropic glutamate receptors by G protein-coupled receptor kinases: mGlu2 receptors are resistant to homologous desensitization, Mol. Pharmacol. 75 (2009) 991–1003, http://dx.doi.org/10.1124/mol.108.052316.
[54] S.M. Lennon, G. Rivero, A. Matharu, P.a Howson, D.E. Jane, P.J. Roberts, E. Kelly, Metabotropic glutamate receptor mGlu2 is resistant to homologous agonist-induced desensitization but undergoes protein kinase C-mediated heterologous desensitization, Eur. J. Pharmacol. 649 (2010) 29–37, http://dx.doi.org/10.1016/j.ejphar.2010.08.038.
[55] E. Doumazane, P. Scholler, L. Fabre, J.M. Zwier, E. Trinquet, J.-P. Pin, P. Rondard, Illuminating the activation mechanisms and allosteric properties of metabotropic glutamate receptors, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) E1416–E1425, http://dx.doi.org/10.1073/pnas.1215615110.
[56] J.O. DiRaddo, E.J. Miller, H.a Hathaway, E. Grajkowska, B. Wroblewska, B.B. Wolfe, D.C. Liotta, J.T. Wroblewski, A real-time method for measuring cAMP production modulated by Gαi/o-coupled metabotropic glutamate receptors, J. Pharmacol. Exp. Ther. 349 (2014) 373–382, http://dx.doi.org/10.1124/jpet.113.211532.
[57] M. Fribourg, J.L. Moreno, T. Holloway, D. Provasi, L. Baki, R. Mahajan, G. Park, S.K. Adney, C. Hatcher, J.M. Eltit, J.D. Ruta, L. Albizu, Z. Li, A. Umali, J. Shim, A. Fabiato, A.D. MacKerell, V. Brezina, S.C. Sealfon, M. Filizola, J. González-Maeso, D.E. Logothetis, Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs, Cell 147 (2011) 1011–1023, http://dx.doi.org/10.1016/j.cell.2011.09.055.
[58] L. Baki, M. Fribourg, J. Younkin, J.M. Eltit, J.L. Moreno, G. Park, Z. Vysotskaya, A. Narahari, S.C. Sealfon, J. Gonzalez-Maeso, D.E. Logothetis, Cross-signaling in metabotropic glutamate 2 and serotonin 2A receptor heteromers in mammalian cells, Pflügers Arch. – Eur. J. Physiol. 468 (2016) 775–793, http://dx.doi.org/10.1007/s00424-015-1780-7.