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Title Page

Effects of Positive Allosteric Modulators on Single Cell Oscillatory Ca^{2+} Signaling Initiated by the Type 5 Metabotropic Glutamate (mGlu5) Receptor

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Running Title Page

Running title: Allosteric modulation of mGlu5 receptor signaling

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Text Pages	29
Figures	11
Tables	1
References	39
Abstract	250 words
Introduction	719 words
Discussion	1387 words

ABBREVIATIONS: ADX47273, ***S***-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidinl-1-yl}-methanone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CDPPB, 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide; CPPHA, N-{4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide; DFB, 3,3'-difluorobenzaldazine; mGlu, metabotropic glutamate; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; 5MPEP, 5-methyl-2-(phenylethynyl)pyridine; M-5MPEP, 2-(2-(3-methoxyphenyl)ethynyl)-5-methylpyridine; NAM, negative allosteric modulator; NMDA, *N*-methyl-D-aspartate; PAM, positive allosteric modulator

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Abstract

Agonist stimulation of the type 5 metabotropic glutamate (mGlu5) receptor initiates robust oscillatory changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in single cells by rapid, repeated cycles of phosphorylation/dephosphorylation of the mGlu5 receptor, involving protein kinase C and as yet unspecified protein phosphatase activities. An emergent property of this type of Ca^{2+} oscillation-generating mechanism (termed "dynamic uncoupling") is that once a threshold concentration has been reached to initiate the Ca^{2+} oscillation its frequency is largely insensitive to further increases in orthosteric agonist concentration. Here, we report the effects of positive allosteric modulators (PAMs) on the patterns of single-cell Ca^{2+} signaling in recombinant and native mGlu5 receptor-expressing systems. In a Chinese hamster ovary cell-line (CHO-*lac*-mGlu5a), none of the mGlu5 receptor PAMs studied (3,3'-difluorobenzaldazine (DFB), N-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl}-2-hydroxy-benzamide (CPPHA), 3-cyano-N-(1,3-diphenyl-1H-prazol-5-yl)benzamide (CDPPB), S-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidinl-1-yl}-methanone (ADX47273)), stimulated a Ca^{2+} response when applied alone, but each PAM concentration-dependently increased the frequency, without affecting the amplitude, of Ca^{2+} oscillations induced by glutamate or quisqualate. Therefore, PAMs can cause graded increases (and NAMs graded decreases) in the Ca^{2+} oscillation frequency stimulated by orthosteric agonist. Initial data in rat cerebrocortical astrocytes demonstrated that similar effects of PAMs could be observed in a native cell background, although at high orthosteric agonist concentrations PAM addition could much more often be seen to drive rapid Ca^{2+} oscillations into peak-plateau responses. These data demonstrate that allosteric modulators can "tune" the Ca^{2+} oscillation frequency initiated by mGlu5 receptor activation and this might allow pharmacological modification of the downstream processes (e.g. transcriptional regulation) unachievable through orthosteric ligand interactions.

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Introduction

Glutamate, the major excitatory neurotransmitter in the central nervous system, acts on ionotropic glutamate receptors to elicit fast excitatory responses, and on metabotropic glutamate (mGlu) receptors to modulate and fine tune synaptic transmission (Conn and Pin, 1997). There are eight subtypes of the mammalian mGlu receptor, which can be divided into three sub-groups, based on sequence homologies, agonist and antagonist binding profiles, and preferred coupling to signal transduction pathways. The group I mGlu receptors, mGlu1 and mGlu5, both preferentially couple via $G_{q/11}$ proteins to stimulate phospholipase C activity, but are differentially localized and likely fulfill distinct physiological functions (Hermans and Challiss, 2001; Mannaioni et al., 2001; Ferraguti and Shigemoto, 2006; Kumar et al., 2008). Activation of these receptors also elicits highly distinct Ca^{2+} responses at a single cell level, with the mGlu1 receptor primarily eliciting a peak-plateau type of Ca^{2+} response, while mGlu5 receptor activation leads to oscillatory changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in both recombinant and native (e.g. astrocyte) cell backgrounds (Kawabata et al., 1996; Nakahara et al, 1997; Nash et al., 2001, 2002; Atkinson et al., 2006).

The robust oscillatory pattern of Ca^{2+} signaling initiated by the mGlu 5 receptor has been proposed to be a result of a “dynamic uncoupling” mechanism, involving rapid cycles of receptor phosphorylation and dephosphorylation (Kawabata et al., 1996), with Ser-839 being most recently implicated as the site of reversible covalent modification (Kim et al., 2005). Following agonist stimulation the mGlu5 receptor is rapidly phosphorylated by protein kinase C (PKC) turning-off productive receptor-G protein coupling (Kawabata et al., 1996; Uchino et al., 2004; Kim et al., 2005). A protein phosphatase, perhaps tightly associated with the receptor, efficiently dephosphorylates Ser-839 allowing reactivation of the receptor, and through the rapid turn-on/turn-off of receptor activity a Ca^{2+} oscillation is generated (Nash et al., 2001, 2002; Atkinson et al., 2006). This mechanism is probably similar to that reported for Ca^{2+} oscillations initiated by Ca^{2+} sensing receptor activation (Young et al., 2002), but clearly differs from the Ca^{2+}-induced Ca^{2+} release mechanism proposed to explain the majority of Ca^{2+} oscillatory behaviors elicited by

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(sub-maximal) agonist stimulation of a variety of G protein-coupled receptors (Berridge et al., 2000).

An intriguing property of mGlu5 receptor-stimulated Ca^{2+} oscillations is that once a concentration of agonist (*e.g.* glutamate, quisqualate, (***S***)-3,5-dihydroxyphenylglycine) has been reached to initiate a response, both the frequency and amplitude of the Ca^{2+} oscillation is essentially insensitive to further increases in agonist concentration, *i.e.* an "all-or-nothing" response (Nash et al., 2002). In contrast, altering the expression level of the mGlu5 receptor has marked effects of the frequency of the Ca^{2+} oscillation stimulated by agonist, and Ca^{2+} oscillation frequency can be reduced by addition of sub-maximally effective concentrations of the negative allosteric modulator (NAM) MPEP (1-100 nM) (Nash et al., 2002).

Positive allosteric modulators (PAMs) of the mGlu5 receptor are thought to be of potential clinical use in a variety of neurological and psychiatric disorders, including schizophrenia (Gasparini et al., 2002; Kew, 2004). The mGlu5 receptor is known to potentiate the function of *N*-methyl-D-aspartate (NMDA) receptors in various brain regions, and so PAMs, which bind to an allosteric site on the mGlu5 receptor and increase the response of the receptor to glutamate, may be able to counteract the NMDA receptor hypofunction proposed to be associated with this condition (Lindsley et al., 2006). Several PAMs acting at the mGlu5 receptor have been identified, including DFB (O'Brien et al., 2003), CPPHA (O'Brien et al., 2004), CDPPB (Kinney et al., 2005) and ADX47273 (Liu et al., 2008). Like MPEP, these PAMs bind to 7-transmembrane domains of the mGlu5 receptor to modulate the effects of orthosteric agonists (Pagano et al., 2000; Malherbe et al., 2003; Mühlemann et al., 2006). In addition an MPEP analog has been identified, 5-MPEP, which antagonizes the actions of both NAMs and PAMs at the mGlu5 receptor and thus acts as a neutral allosteric site ligand (Rodriguez et al., 2005)

Considering the mechanism utilized by the mGlu5 receptor to generate Ca^{2+} oscillations and the unusual emergent pharmacology, we have systematically investigated the effects of PAMs on orthosteric mGlu5 receptor agonist-stimulated responses at a single cell level. These new data

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add an important new dimension to previous investigations of the pharmacological properties of PAMs at the mGlu5 receptor, which to date have relied on signaling readouts that quantify cell population responses.

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Materials and Methods

Compounds. L-quisqualic acid, L-glutamic acid, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) and 3,3'-difluorobenzaldazine (DFB) were obtained from Tocris Cookson Ltd. (Bristol, UK). N-{4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide (CPPHA), 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), S-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidinl-1-yl}-methanone (ADX47273) and 5-methyl-2-(phenylethynyl)pyridine (5MPEP) were synthesized in-house by GlaxoSmithKline (Harlow, UK). 2-(2-(3-methoxyphenyl)ethynyl)-5-methylpyridine (M-5MPEP) was a kind gift from Dr. P.J. Conn (Vanderbilt Program in Drug Discovery, Nashville, TN).

Cell Culture. Chinese hamster ovary (CHO) cells expressing the human mGlu5a receptor under the control of a *lac*-repressor system (Hermans et al., 1998; Nash et al., 2002) were maintained in Dulbecco's minimum essential medium (DMEM) containing GlutaMAX-1 with sodium pyruvate, 4500 mg L^{-1} glucose, 10% fetal bovine serum (FBS), 44 μg mL^{-1} proline, 2.5 μg mL^{-1} amphotericin B, 10^5 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 300 μg mL^{-1} G418. Once confluent, flasks of CHO-*lac*-mGlu5a cells were washed twice with PBS (without Ca^{2+}/Mg^{2+}) and harvested with 0.25% (w/v) trypsin, 0.02% (w/v) EDTA. Cells were maintained at 37°C in a humidified 5% CO_2 : air atmosphere. For experiments, cells were seeded on to multiwell plates in medium with dialyzed FBS (substituted for FBS) and devoid of G418. mGlu5 receptor expression was induced by incubating CHO-*lac*-mGlu5a cells with 100 μM IPTG/10 mM butyrate for 24 h prior to experimentation.

Rat cerebrocortical astrocyte preparation. Wistar rats (1-2 days of age) were decapitated and the cortices removed. During the dissection, cortices were placed into ice-cold Earle's balanced salt solution (EBSS, Invitrogen), supplemented with 3.2 mM $MgSO_4$, 0.3% (w/v) BSA (fraction V) and 16.7 mM glucose. Tissue was cut up into small pieces and incubated at 37°C for 15 min in 10 mL EBSS solution containing 0.025% (w/v) (bovine pancreatic) trypsin with gentle agitation. After 15 min, 10 mL modified EBSS solution (containing 50 μM $MgSO_4$, DNase I

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(type IV, 150 Kunitz Units), and 0.02% (w/v) trypsin inhibitor) was added and the suspension left to settle for 5 min. The supernatant was subsequently decanted, and 2.5 mL EBSS solution containing 320 μM $MgSO_4$, DNase I (800 Kunitz Units), and 0.12% (w/v) trypsin inhibitor was added. Tissue was slowly triturated using a glass fire-polished Pasteur pipette and 2.5 mL EBSS solution (supplemented with 0.4% (w/v) BSA and 250 μM $MgSO_4$) added. The cell suspension was centrifuged (1000 r.p.m.; 8 min), and pellet re-suspended in Dulbecco's minimum essential medium (DMEM) containing GlutaMAX-1 with sodium pyruvate, 4500 mg L^{-1} glucose, 15% heat-inactivated FBS, 2.5 μg mL^{-1} fungizone and 0.1 μg mL^{-1} gentamycin. Cells were plated into poly-D-lysine-coated cell culture flasks and incubated at 37°C in a 5% CO_2, humidified air atmosphere for 7 days, with medium being replaced after 4 days. At 7 DIV, medium was replaced again and flasks were transferred to a shaking incubator overnight (37°C; 320 r.p.m.). On the following day (8 DIV), cells were washed twice with PBS (without Ca^{2+}/Mg^{2+}) and harvested with 0.25% (w/v) trypsin, 0.02% (w/v) EDTA. Cells were subsequently seeded onto pre-coated poly-D-lysine tissue culture plates for experiments. After 24 h (DIV 9), medium was replaced with Dulbecco's minimum essential medium (DMEM) containing GlutaMAX-1 with sodium pyruvate, 4500 mg L^{-1} glucose, 2.5 μg mL^{-1} fungizone and 0.1 μg mL^{-1} gentamycin and G-5 supplement. Cells were used for experiments at DIV 11-13.

Single-Cell Intracellular Ca^{2+} Concentration ($[Ca^{2+}]_i$) Assay. CHO-*lac*-mGlu5a cells were seeded onto 22 mm borosilicate coverslips and grown to approx. 80% confluency. Cells were loaded with Fura-2 AM (2 μM) in KHB containing 1 mg mL^{-1} bovine serum albumin for 60-90 min at room temperature. Coverslips of astrocyte (DIV 11-13) were incubated with Fura-2 AM (2 μM) similarly to the CHO-*lac* cells except that the loading period was 40 min at room temperature. Coverslips were then transferred to the stage of a Nikon Diaphot inverted epifluorescence microscope, with an oil immersion objective (x40) and a SpectraMASTER II module (PerkinElmer Life Sciences). Cells were excited at wavelengths of 340 and 380 nm using a SpectraMASTER II monochromator and emission was recorded at wavelengths above 520 nm. The ratio of fluorescence intensities at these wavelengths is given as an index of $[Ca^{2+}]_i$. All experiments were performed at 37°C; drug additions were made via a perfusion line.

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Cell Population $[Ca^{2+}]_i$ Assay. CHO-*lac*-mGlu5a cells were seeded onto 96-well black-walled cell culture plates (Corning Costar) and induced on the following day with IPTG (100 μM) and sodium butyrate (10 mM) for 24 h prior to experimentation. Cells were loaded in Tyrode's solution containing the Ca^{2+} sensitive fluorescent dye, calcium-3 (Calcium 3 assay kit; Molecular Devices, Sunnyvale, CA), 1.5 mM $CaCl_2$ and 2.5 mM probenecid, for 1 h. Allosteric modulators were pre-incubated for 30 min prior to the addition of the agonist on the FLIPR. Changes in fluorescence intensity are recorded as an index of $[Ca^{2+}]_i$.

Data Analysis. Concentration-response relationships were analyzed by non-linear regression using GraphPad Prism 5.0 software (San Diego, CA). For statistical tests, where only two datasets were being compared an unpaired Student's *t*-test (two-tailed) was used, where $P<0.05$ was deemed statistically significant. Where greater than two datasets were compared, one- or two-way analysis of variance (ANOVA) tests were used with $P<0.05$ being accepted as significantly different. ANOVA tests were followed by the Bonferroni's *post-hoc* test. All statistical analyses were performed using GraphPad Prism 5.0 software.

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Results

Effects of Positive Allosteric Modulators (PAMs) on Ca^{2+} Oscillation Frequency. All of the mGlu5 receptor PAMs studied, DFB, CPPHA, CDPPB or ADX47273, caused significant (2-3 fold) increases in the frequency (but not the amplitude) of Ca^{2+} oscillations initiated by either glutamate or quisqualate in CHO-*lac*-mGlu5 cells (Table 1). Representative single cell traces are shown for the effects of DFB (100 μM), CPPHA (3 μM), CDPPB (10 μM) and ADX47273 (10 μM) on glutamate- and quisqualate-stimulated Ca^{2+} oscillations (Fig. 1). To further explore how PAMs affect the Ca^{2+} oscillation frequency initiated by an orthosteric agonist, we stimulated CHO-*lac*-mGlu5a cells with either glutamate (100 μM) or quisqualate (10 μM) and then co-added increasing concentrations of CDPPB or ADX47273 (0.01-10 μM). Analysis of these data revealed that the PAMs caused concentration-dependent increases in orthosteric agonist-stimulated Ca^{2+} oscillation frequency (pEC_{50} (M) values: for CDPPB, 6.46 ± 0.26 (+glutamate; Fig. 2B) and 6.95 ± 0.27 (+quisqualate; Fig. 2D); for ADX47273, 6.33 ± 0.13 (+ glutamate; Fig. 2F) and 6.66 ± 0.19 (+ quisqualate; Fig. 2H)).

Effects of PAMs on the Threshold for Glutamate-Evoked Ca^{2+} Oscillations. Following mGlu5 receptor induction (IPTG/butyrate addition for 24 h), Ca^{2+} oscillations were observed in the vast majority of Fura-2-loaded CHO-*lac*-mGlu5a cells challenged with either L-glutamate or quisqualate. In the presence of 1 μM glutamate only a small number of cells (<15%) responded, however, increasing the glutamate concentration to 3 μM initiated baseline Ca^{2+} oscillations in most cells (Fig. 3A, C) and the amplitude and frequency of the Ca^{2+} oscillation was not significantly altered by further increases in the concentration of glutamate (3-100 μM). Similar concentration-independent effects were observed for quisqualate, where 0.1 μM quisqualate was sufficient to initiate Ca^{2+} oscillations in the majority of CHO-*lac*-mGlu5a cells and further increases in quisqualate concentration (0.3-10 μM) did not significantly alter either the amplitude or frequency of the agonist-stimulated Ca^{2+} oscillation (data not shown). We have also evaluated the effects of DFB (30 μM) on the concentration of glutamate (threshold) required to evoke Ca^{2+} oscillations (Fig. 3B). The presence of DFB left-shifted the threshold for the stimulation of a Ca^{2+}

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oscillation by the orthosteric agonist (glutamate or quisqualate), as well as increasing the maximal oscillation frequency achieved (Fig. 3B, C). It should also be noted that in the presence of the PAM it is possible to discern a more graded increase in Ca^{2+} oscillation frequency compared to the steep, "all-or-nothing" glutamate concentration-response curve seen in the absence of an allosteric ligand (see Fig. 3; Nash et al., 2002).

Effects of PAMs on Single-Cell Ca^{2+} Responses. Although this study focuses on Ca^{2+} oscillatory responses, other patterns of change in $[Ca^{2+}]_i$ can be observed in individual CHO-*lac*-mGlu5a cells following agonist addition. Previously, we have undertaken analyses of Ca^{2+} signaling patterns in CHO-*lac*-mGlu5a (and CHO-*lac*-mGlu1a) cells (Atkinson et al., 2006) and in that study classified responses into 4 categories (non-responders (NR); single peak (SP), oscillatory (OS), peak-and-plateau (PP)). Here, we undertook similar analyses to assess how PAMs alter the occurrence of the different categories of Ca^{2+} response. Representative data are shown for ADX47273 effects on glutamate- and quisqualate-stimulated Ca^{2+} signaling patterns (Fig. 4). In the absence of the PAM, orthosteric agonists stimulate a Ca^{2+} oscillatory response in the majority (>70%) of cells, with essentially no cells showing peak-and-plateau responses. Following co-addition of orthosteric agonist plus ADX47273, the number of cells that either fail to respond (NR) or respond with only a single peak (SP) diminish towards zero, while the number of cells driven into a peak-and-plateau (PP) response increases somewhat (to approx. 10-20% of all cells analyzed; Fig. 4). Importantly, in both the absence and presence of the PAM, Ca^{2+} oscillations are the predominant response seen following orthosteric agonist addition to CHO-*lac*-mGlu5a cells.

PAMs at the mGlu5 Receptor are Allosteric Modulators Devoid of Intrinsic Activity. None of the PAMs studied here stimulated a Ca^{2+} response *per se*, and required the presence of orthosteric agonist to exert their positive modulator effect (Fig. 5). It should be noted that the medium over the CHO-*lac*-mGlu5a cells was rapidly exchanging throughout the time-course (perfusion rate 5 mL min^{-1}); if the perfusion rate was slowed or stopped then a Ca^{2+} oscillatory response was quickly initiated (within a few seconds) in the presence of PAM only (data not

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shown). It is likely that CHO-*lac*-mGlu5a cells release glutamate into the medium and in static incubation systems the accumulation of glutamate is sufficient to synergize with the PAM to produce a Ca^{2+} response.

Neutral Allosteric Modulator Effects on Orthosteric/Allosteric Interactions to Regulate $[Ca^{2+}]_i$. At a sufficiently high concentration (100 nM), the NAM, MPEP is able to completely abolish orthosteric agonist-stimulated Ca^{2+} oscillations in CHO-*lac*-mGlu5a cells (Fig. 6A; Nash et al., 2002), whereas the neutral allosteric modulator 5MPEP (10 μM; Rodriguez et al., 2005) was without effect (Fig. 6B). The effects of increasing concentrations of MPEP (here shown for CHO-*lac*-mGlu5a cells using FLIPR technology to assess population Ca^{2+} responses) on the glutamate concentration-response curve illustrate the non-competitive nature of the interaction, with increasing MPEP concentrations causing a progressive suppression of the maximal response with no significant effect on the glutamate EC_{50} value (Fig. 6C). In contrast, 5MPEP was without effect on the glutamate concentration-response curve (Fig. 6D).

To explore further the mechanism of action of the PAMs, we investigated whether their effects on orthosteric agonist-stimulated Ca^{2+} oscillatory responses are sensitive to the neutral allosteric antagonist 5MPEP. A representative trace illustrating the experimental design is shown in Fig. 7A. Addition of 5MPEP (10 μM), after sequential additions of glutamate (100 μM) and glutamate-plus-ADX47273 (10 μM), caused a complete ablation of the PAM-mediated frequency increase in the glutamate-stimulated Ca^{2+} oscillation. Fig. 7 also shows that 5MPEP could completely reverse the positive modulator effect of ADX47273, DFB or CDPPB (Fig. 7B-D). In contrast, the positive modulator effect of CPPHA on glutamate-stimulated Ca^{2+} oscillatory responses in CHO-*lac*-mGlu5a cells was unaffected by 5MPEP (Fig. 8).

A comparison of the effects of a positive (ADX47273), negative (MPEP) and neutral (5MPEP) allosteric modulator on orthosteric agonist-stimulated Ca^{2+} oscillation frequency in CHO-*lac*-mGlu5a cells is shown in Fig. 9. In addition, we have found that the previously reported mGlu5 receptor allosteric partial inverse agonist, M-5MPEP (Rodriguez et al., 2005) also causes a

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concentration-dependent decrease in the glutamate-evoked Ca^{2+} oscillations. Although this compound exhibited a lower potency with respect to inhibiting the glutamate-stimulated Ca^{2+} response, at a sufficiently high concentration (10 μM), M-5MPEP displayed a negative efficacy approaching that of MPEP (Fig. 9).

Allosteric Modulator Effects on Glutamate-Stimulated Ca^{2+} responses in Astrocytes. Addition of glutamate (100 μM) to G5-differentiated rat cerebrocortical astrocytes initiated Ca^{2+} oscillations that were typically of a higher frequency (≥2 oscillations per min) than observed in the CHO-*lac*-mGlu5a cells and occurred on a raised baseline (Fig. 10A). Addition of increasing concentrations of MPEP (0.01-1 μM) initially reduced oscillation frequency and then completely suppressed orthosteric agonist-evoked oscillations (Fig. 10A, B). The potency of the MPEP-evoked suppression was pharmacologically indistinguishable to that seen previously in CHO-*lac*-mGlu5a cells (pIC_{50} (M) ≈8; Fig. 10B). Similarly, the glutamate-stimulated Ca^{2+} oscillation was completely unaffected by the neutral allosteric modulator 5MPEP (Fig. 10C).

Similar to their effects in CHO-*lac*-mGlu5a cells, the PAMs investigated here possessed no intrinsic agonist activity in cerebrocortical astrocytes (data for CDPPB shown; Fig. 11A), but were able to reduce the threshold for orthosteric agonist-evoked Ca^{2+} responses in astrocytes. Thus, when perfused alone glutamate (0.3 μM) did not evoke a Ca^{2+} response in the vast majority of astrocytes, however the presence of CDPPB (10 μM) resulted in Ca^{2+} oscillations being observed in the majority of cells (Fig. 11C, D).

PAMs increased the Ca^{2+} oscillation frequency stimulated by sub-maximal concentrations of orthosteric agonist (Fig. 11G-J), whereas at maximally effective concentrations of glutamate, which already caused rapid Ca^{2+} oscillations, addition of the PAM most often caused glutamate-mediated Ca^{2+} oscillations to transition into a sustained peak-plateau Ca^{2+} responses (Fig. 11I-L). These data suggest that above a certain oscillation frequency (2-3 min^{-1}) PAMs can drive orthosteric agonist-stimulated Ca^{2+} oscillatory responses into peak-plateau responses.

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Discussion

In the present study we have compared and contrasted the actions of orthosteric and allosteric ligands at the mGlu5 receptor using an assay readout that allows single cell responses to be evaluated. In contrast to previous studies conducted in cell populations, these new data indicate that positive allosteric modulators (PAMs) can uniquely affect mGlu5 receptor-mediated signal transduction in ways not achievable by orthosteric agonists alone.

Stimulation of the mGlu5 receptor has been shown to elicit robust intracellular Ca^{2+} oscillations in astrocytes and neurons (Nakahara et al., 1997; Flint et al., 1999; D'Ascenzo et al., 2007) as well as recombinant model systems, such as the CHO-*lac*-mGlu5 cell-line used here (Nash et al., 2002; Atkinson et al., 2006). Considerable evidence has accrued to support the idea that this oscillatory pattern of Ca^{2+} signaling is brought about by a process termed 'dynamic uncoupling', which involves repetitive cycles of phosphorylation and dephosphorylation of a key residue (Ser-839) in the proximal *C*-terminal domain of the mGlu5 receptor, which respectively uncouple and restore signal transduction from mGlu5 receptor to G protein (Kawabata et al., 1996; Nash et al., 2001, 2002; Uchino et al., 2004; Kim et al., 2005). An interesting emergent pharmacological property of Ca^{2+} oscillations generated by the dynamic uncoupling mechanism is that once an orthosteric agonist concentration sufficient to initiate receptor-driven Ca^{2+} signaling has been reached then the frequency and amplitude of the oscillatory signal changes little over a broad agonist concentration range. This "hard-wiring" of the mGlu5 receptor signaling output at a single cell level might be of physiological importance as it should allow an invariant signal to be maintained over a wide range of glutamate concentrations.

Here we report that four previously described mGlu5 receptor-selective PAMs (DFB, CPPHA, CDPPB and ADX47273) all significantly increase the frequency (not the amplitude) of glutamate- or quisqualate-stimulated Ca^{2+} oscillations in single CHO-*lac*-mGlu5 cells compared

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to stimulation with orthosteric agonist alone. None of the PAMs elicited a Ca^{2+} response when applied to the cells in the absence of orthosteric agonist, confirming initial reports for each of the compounds that they are true allosteric modulators possessing no intrinsic agonist activity (O'Brien et al., 2003, 2004; Kinney et al., 2005; Le Poul et al., 2005). At maximally effective concentrations in CHO-*lac*-mGlu5 cells the PAMs caused 2-3 fold increases in the Ca^{2+} oscillation frequency stimulated by a maximally effective orthosteric agonist concentration. In rat cerebrocortical astrocytes the Ca^{2+} oscillation frequencies observed in response to orthosteric agonist concentration were generally higher and PAM addition could either increase Ca^{2+} oscillation frequency further or drive the cell into a peak-plateau response (see Fig. 11), suggesting that above a certain frequency (~3 Ca^{2+} oscillations min^{-1}) dynamic uncoupling transitions to a different Ca^{2+} signature in this cell background.

In CHO-*lac*-mGlu5 cells CDPPB and ADX47273 caused concentration-dependent changes in the Ca^{2+} oscillatory frequency stimulated by orthosteric agonist. The EC_{50} values obtained for PAM effects (L-Glu + CDPPB = 0.35 μM; L-Glu + ADX47273 = 0.47 μM; Fig. 5) were intermediate between affinity estimates determined in radioligand binding assays (e.g K_i =4.3 μM for ADX47273 displacing [^{3}H]-MPEP binding; Lui et al., 2008) and functional (potentiation of Ca^{2+} release) assays (e.g. EC_{50} = 0.045 μM for CDPPB potentiation of the response to an EC_{20} concentration of L-Glu; Chen et al., 2007). Further, PAMs "sensitize" the mGlu5 receptor to changes in orthosteric agonist, reducing the threshold concentration of L-glutamate needed to elicit a regenerative Ca^{2+} oscillatory response. This was demonstrated here for DFB in CHO-*lac*-mGlu5 cells (Fig. 3) and for CDPPB in rat cerebrocortical astrocytes (Fig. 11). A striking feature of this "sensitization" is that in the presence of a PAM Ca^{2+} oscillations are observed in astrocytes in the presence of extracellular L-glutamate concentrations (0.3 μM; see Fig. 11B) approaching those considered to be below that required to exert excitatory actions in the CNS (see Herman and Jahr, 2007). These data suggest that mGlu5 receptor PAMs might trigger

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sustained Ca^{2+}-dependent signaling at low ambient concentrations of the endogenous transmitter, as well as when extracellular glutamate is elevated by normal exocytotic release.

Therefore, mGlu5 receptor PAMs increase (and NAMs decrease; Nash et al., 2002), the Ca^{2+} oscillation frequency stimulated by an orthosteric agonist. This pharmacological alteration in the "hard-wired" single cell Ca^{2+} oscillation frequency recapitulates the effects of increasing or decreasing mGlu5 receptor expression levels (through altering induction conditions in the CHO-*lac*-mGlu5 cell-line) (Nash et al. 2002).

The binding sites for each of the mGlu5 PAMs have previously been characterized by other groups using site-directed mutagenesis and radioligand binding approaches. Thus, DFB and CDPPB competitively displaced binding of [^{3}H]-MPEP (or a similarly radiolabeled MPEP analog) to the mGlu5 receptor and, in the case of CDPPB, this displacement was shown to be unaltered in the presence of glutamate (O'Brien et al., 2003; Kinney et al., 2005). In addition, mutation of Ala-809 in TM7 of the mGlu5 receptor leads to loss of MPEP and CDPPB binding (Chen et al., 2007). ADX47273 has also been reported to bind to the MPEP site (Le Poul et al., 2005; Liu et al., 2008). In contrast, CPPHA did not displace [^{3}H]-MPEP binding to the mGlu5 receptor, and while mutation of alanine-809 had no effect on its PAM activity, mutation of phenylalanine-585 in TM1 of the mGlu5 receptor led to loss of the positive modulatory effect of this compound (Zhao et al., 2007; Chen et al., 2008). Taken together, these previous data indicate that DFB, CDPPB, and ADX47273 exert their PAM effects by binding to the same or and overlapping site to the NAM, MPEP, but CPPHA binds to a distinct allosteric site (Conn et al., 2009). Here, we used the mGlu5 receptor neutral allosteric modulator, 5MPEP, to investigate this at a single cell level. 5MPEP binds to the MPEP site and has been shown to block both the effects of the NAM, MPEP, and the PAMs, DFB and CDPPB (Rodriguez et al., 2005). Our initial experiments confirmed that 5MPEP had no effect on glutamate-stimulated Ca^{2+} responses in both single cell and cell population assays. Our work clearly demonstrated that the increase in

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orthosteric agonist-induced Ca^{2+} oscillation frequency caused by DFB, CDPPB and ADX47273 was completely inhibited in the presence of 5MPEP, such that the frequency of Ca^{2+} oscillations was reduced to that stimulated by the orthosteric agonist alone. These data provide confirmatory evidence at a single cell level that DFB, CDPPB and ADX47273 all exert their modulatory activities via the MPEP/5MPEP binding site on the mGlu5 receptor. In contrast, the modulatory activity of CPPHA on orthosteric agonist stimulated Ca^{2+} oscillation frequency was completely unaffected by the presence of 5MPEP indicating that this PAM interacts with a distinct allosteric binding site on the mGlu5 receptor. Despite this difference in the locus of interaction of CPPHA, the activity of this PAM with respect to the modulation of glutamate- and quisqualate-stimulated Ca^{2+} responses was indistinguishable from that of the other PAMs at a single cell level. This contrasts with previous reports that allosteric potentiation at distinct sites of the mGlu5 receptor can result in differential effects downstream in rat cortical astrocytes (Zhang et al., 2005).

This present work has clearly demonstrated that the frequency of Ca^{2+} oscillations initiated by glutamate, or another mGlu5 receptor orthosteric agonist, can be concentration-dependently altered by the addition of either PAMs or NAMs in a recombinant mGlu5a receptor-expressing cell-line and rat cortical astrocytes. These findings have required the study of Ca^{2+} signaling behaviors using single cell assays and suggest that at this level PAMs and NAMs primarily mediate frequency rather than the amplitude modulation of the Ca^{2+} signal. Alteration of the Ca^{2+} oscillation frequency can have a number of (patho)physiological consequences, as changes in the frequency of Ca^{2+} oscillations have previously been shown to influence the activation of specific Ca^{2+}-dependent enzymes (De Koninck and Schulman., 1998), the synthesis and release of various gliotransmitters (Agulhon et al., 2008) and growth factors (Jean et al., 2008), and gene transcriptional activation patterns (Dolmetsch et al., 1998; Tomida et al., 2003). Therefore, the ability of mGlu5 receptor PAMs to alter the frequency of Ca^{2+} oscillations in single cells has the potential to alter fundamentally the cell's interpretation of the signal initiated at the cell surface. This will require a re-evaluation of how allosteric modulators are to be utilized to manipulate

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mGlu5 receptor signaling in a variety of CNS disorders including schizophrenia (Conn et al., 2009).

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Figure Legends

Fig. 1. Effects of the positive allosteric modulators (PAMs), DFB, CPPHA, CDPPB and ADX47273, on the frequency of Ca^{2+} oscillations in CHO-*lac*-mGlu5a cells. Representative traces showing the response of single CHO-*lac*-mGlu5a cells to perfusion with L-glutamate (100 μM; panels **A, C, E** and **G**) or quisqualate (30 μM; panels **B, D, F** and **H**) for 5 min followed immediately by the same concentration of agonist plus DFB (100 μM; panels **A** and **B**), CPPHA (3 μM; panels **C** and **D)**, CDPPB (10 μM; panels **E** and **F**) or ADX47273 (10 μM; panels **G** and **H**) perfused for a further 5 min. Data are representative of at least 50 individual cells recorded over at least 3 separate days.

Fig. 2. Concentration-dependent effects of CDPPB and ADX47273 on the frequency of Ca^{2+} oscillations stimulated by L-glutamate or quisqualate in CHO-*lac*-mGlu5a cells. Representative traces showing the effects of increasing concentrations of CDPPB (0.01, 0.1, 1, 10 μM; panels **A** and **C**) or ADX47273 (0.01, 0.1, 1, 10 μM; panels **E** and **G**) on Ca^{2+} oscillations elicited by glutamate (100 μM; panels **A** and **E**) or quisqualate (30 μM; panels **C** and **G**). Concentration-response curves showing the mean number of oscillations per min when cells were stimulated with glutamate (100 μM; panels **B** and **F**) or quisqualate (30 μM; panels **D** and **H**) plus increasing concentrations of CDPPB (panels **B** and **D**) or ADX47273 (panels **F** and **H**). Data are shown as means ± S.E.M. from 25 individual cells recorded over 4 separate days. Mean pEC_{50} (M) values for facilitation of glutamate and quisqualate responses were 6.46 ± 0.26 and 6.95 ± 0.27 for CDPPB, and 6.32 ± 0.26 and 6.71 ± 0.39 for ADX47273, respectively.

Fig. 3. Effects of DFB on the threshold for glutamate-evoked on Ca^{2+} oscillations in CHO-*lac*-mGlu5a cells. (**A**) Representative trace showing the effect of stimulating cells with increasing concentrations of glutamate (each concentration applied for 3 min). (**B**) A representative trace showing responses to increasing glutamate concentrations in the presence of DFB (30 μM). **(C)** Mean data showing the changes in oscillation frequency that occur when cells were stimulated

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with increasing concentrations of glutamate in the absence or presence of DFB (30 μM). Data are shown as means ± S.E.M. for at least 25 individual cells over at least 3 experiments.

Fig. 4. Effects of the positive allosteric modulator, ADX47273, on orthosteric agonist-stimulated Ca^{2+} responses in CHO-*lac*-mGlu5a cells. Data show the % of the total number of cells analyzed that gave no (**NR**), a single, transient peak (**SP**), an oscillatory (**OS**), or a peak-plateau (**PP**) response when stimulated with L-glutamate (100 μM; panel **A**) or quisqualate (30 μM; panel **B**) in the absence and presence of ADX47273 (10 μM). Data are shown as means ± S.E.M. from at least 50 individual cells recorded over 4 separate days. Criteria for classification of cell responses into NR, SP, OS and PP sub-groups were identical to those defined by Atkinson et al. (2006).

Fig. 5. The positive allosteric modulators, DFB, CPPHA, CDPPB and ADX47273 possess no intrinsic agonist activity in the absence of orthosteric stimulation. Maximal concentrations of DFB (100 μM; panel **A**), CPPHA (3 μM; panel **B**), CDPPB (10 μM; panel **C**) and ADX47273 (10 μM; panel **D**) were perfused on to CHO-*lac*-mGlu5a cells for 5 min, followed by simultaneous perfusion of quisqualate (30 μM) plus each respective modulator. Traces are representative from at least 20 individual cells recorded over 3 separate days.

Fig. 6. Comparison of effects of MPEP and 5MPEP on Ca^{2+} responses in single and populations of CHO-*lac*-mGlu5a cells. Representative traces showing the effects of MPEP (100 nM; panel **A**), or 5MPEP (30 μM; panel **B**) on Ca^{2+} oscillations elicited by glutamate (100 μM) in CHO-*lac*-mGlu5a cells. Traces shown are representative of at least 50 cells recorded over 3 separate days. FLIPR cell population responses for glutamate-stimulated Ca^{2+} concentration-response curves performed in the absence or presence of 10, 30 or 100 nM MPEP (panel **C**) or 0.1, 1 or 10 μM 5MPEP (panel **D**). MPEP and 5MPEP were added 30 min before challenge with glutamate at the concentrations indicated. Data are shown as means ± S.E.M. for 3 separate experiments performed in duplicate.

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Fig. 7. 5MPEP abolishes the positive modulatory effects of ADX47273, DFB, or CDPPB on orthosteric agonist-stimulated Ca^{2+} oscillation frequency in CHO-*lac*-mGlu5a cells. Cells were perfused with glutamate (100 μM) for 5 min, followed by glutamate (100 μM) plus PAM for 5 min, and then glutamate (100 μM), PAM and 5MPEP (30 μM)(see panel **A**). Perfusion periods with glutamate ± PAM ± 5MPEP followed on from each other without any washout between additions. A representative trace showing the effects of 5MPEP on the Ca^{2+} oscillation frequency elicited by glutamate (100 μM) plus ADX47273 (10 μM) is shown (panel **A**). Mean data for each PAM are also shown: ADX47273 (10 μM; panel **B**), DFB (100 μM; panel **C**), and CDPPB (10 μM; panel **D**). Histograms show means ± S.E.M. for 20 individual cells recorded over 4 separate days, with statistically significant differences ($^{***}P < 0.001$) determined by one-way ANOVA.

Fig. 8. 5MPEP does not block the positive modulatory effect of CPPHA on orthosteric agonist-stimulated Ca^{2+} oscillation frequency in CHO-*lac*-mGlu5a cells. Cells were perfused with glutamate (100 μM) for 5 min, followed by glutamate (100 μM) plus CPPHA (3 μM) for 5 min, and then glutamate (100 μM), CPPHA (3 μM) and 5MPEP (30 μM)(see panel **A**). Perfusion periods with glutamate ± CPPHA ± 5MPEP followed on from each other without any washout between additions. A representative trace showing the effects of 5MPEP on the Ca^{2+} oscillation frequency elicited by glutamate plus CPPHA is shown (panel **A**), while panel **B** presents mean data. Data are shown as means ± S.E.M. for 35 individual cells recorded over 7 separate days, with statistically significant differences ($^{***}P < 0.001$) determined by one-way ANOVA.

Fig. 9. Allosteric modulator site pharmacology at the mGlu5 receptor. Concentration-dependent effects of ADX47273, MPEP, 5MPEP or M-5MPEP on glutamate (100 μM) evoked Ca^{2+} oscillations in CHO-*lac*-mGlu5a cells are summarized (pEC_{50}/IC_{50} (M) values: ADX47273, 6.33 ± 0.13; MPEP, 7.69 ± 0.14, M-5MPEP, 6.26 ± 0.21). Data are shown are means ± S.E.M. for at least 25 cells recorded over at least 3 separate days. Note that ordinate values shown are normalized to the oscillation frequency evoked by stimulation with glutamate (100 μM) alone.

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Fig. 10. Modulatory effects of MPEP and 5MPEP on L-glutamate-stimulated Ca^{2+} oscillations in rat cerebrocortical astrocytes. Representative trace (panel **A**) showing the pattern of Ca^{2+} oscillations evoked by glutamate (100 μM) and its attenuation by co-addition of increasing concentrations of MPEP (0.01-0.3 μM;). Summary data are shown (panel **B**) comparing the effects of MPEP on glutamate-stimulated Ca^{2+} oscillation frequency in astrocytes and CHO-*lac*-mGlu5a cells. Data are shown as means ± S.E.M. for at least 25 individual cells over at least 3 separate experiments. The lack of effect of 5MPEP (10 μM) on glutamate-stimulated Ca^{2+} oscillations in astrocytes is also illustrated by a representative trace (panel **C**).

Fig. 11. Effects of the mGlu5 receptor PAM, CDPPB, on glutamate-stimulated Ca^{2+} responses in rat cerebrocortical astrocytes. Under the experimental conditions used here (rapidly perfused (5 mL min^{-1}) cells on coverslips) addition of CDPPB (10 μM) alone did not evoke a Ca^{2+} response, in contrast to addition of glutamate (100 μM) (panel **A**). Representative traces are also shown illustrating the effect of CDPPB (10 μM) on Ca^{2+} responses evoked by a range of glutamate concentrations (**C**, 0.3 μM; **E**, 1 μM; **G**, 3 μM; **I**, 10 μM; **K**, 100 μM). Mean data are shown as the % of the total number of cells analyzed that gave no (**NR**), a single, transient peak (**SP**), an oscillatory (**OS**), or a peak-plateau (**PP**) response for each condition. These latter data are determined from analysis of least 50 individual astrocytes over at least 3 separate experiments. Note that CDPPB reduced the threshold for glutamate-stimulated Ca^{2+} oscillations (panel **C**), and while this PAM increased oscillation frequency at low orthosteric agonist concentrations (panels **E**/**F** and **G**/**H**), it transformed the response from oscillatory to peak-plateau at high agonist concentrations (panels **I**/**J** and **K**/**L**).

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TABLE 1 Effects of positive allosteric modulators (PAMs) on the frequency of the Ca^{2+} oscillatory response stimulated by either L-glutamate or quisqualate in CHO-*lac*-mGlu5a cells.

CHO-*lac*-mGlu5a cells were stimulated with either L-glutamate (100 μM) or quisqualate (30 μM) for 300 sec and then by the same concentration of orthosteric agonist plus the indicated concentration of DFB, CPPHA, CDPPB or ADX47273. At least 20 cells were analyzed from each coverslip and each experiment was repeated on at least 3 separate days to give the summary data shown (mean ± SEM). Data are shown for the number of oscillations in a 5 min period. Data were analyzed using two-way ANOVA. Statistically significant increases in oscillation frequency in the presence versus the absence of PAM are indicated as $^{***}P < 0.001$.

		+glutamate	**+quisqualate**
DFB (100 μM)	-	3.1 ± 0.3	4.3 ± 0.5
	+	11.1 ± 1.4***	10.9 ± 0.9***
CPPHA (3 μM)	-	5.1 ± 0.7	5.1 ± 0.5
	+	8.4 ± 1.1***	10.1 ± 0.8***
CDPPB (10 μM)	-	5.2 ± 0.5	4.2 ± 0.7
	+	12.7 ± 0.9***	14.8 ± 1.1***
ADX47273 (10 μM)	-	5.0 ± 0.4	5.7 ± 0.5
	+	13.4 ± 0.7***	14.4 ± 0.8***

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Figure 1

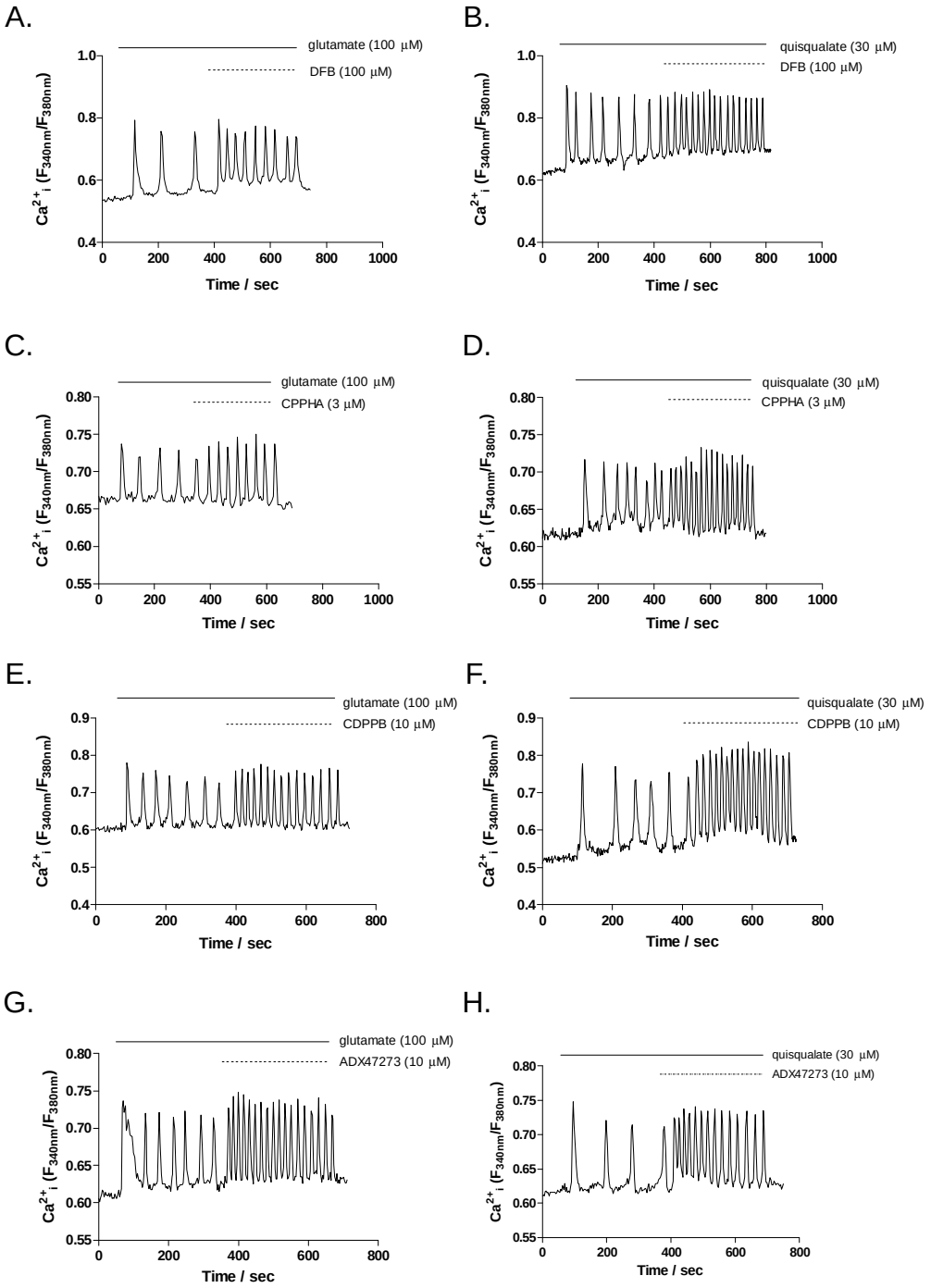

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Figure 2

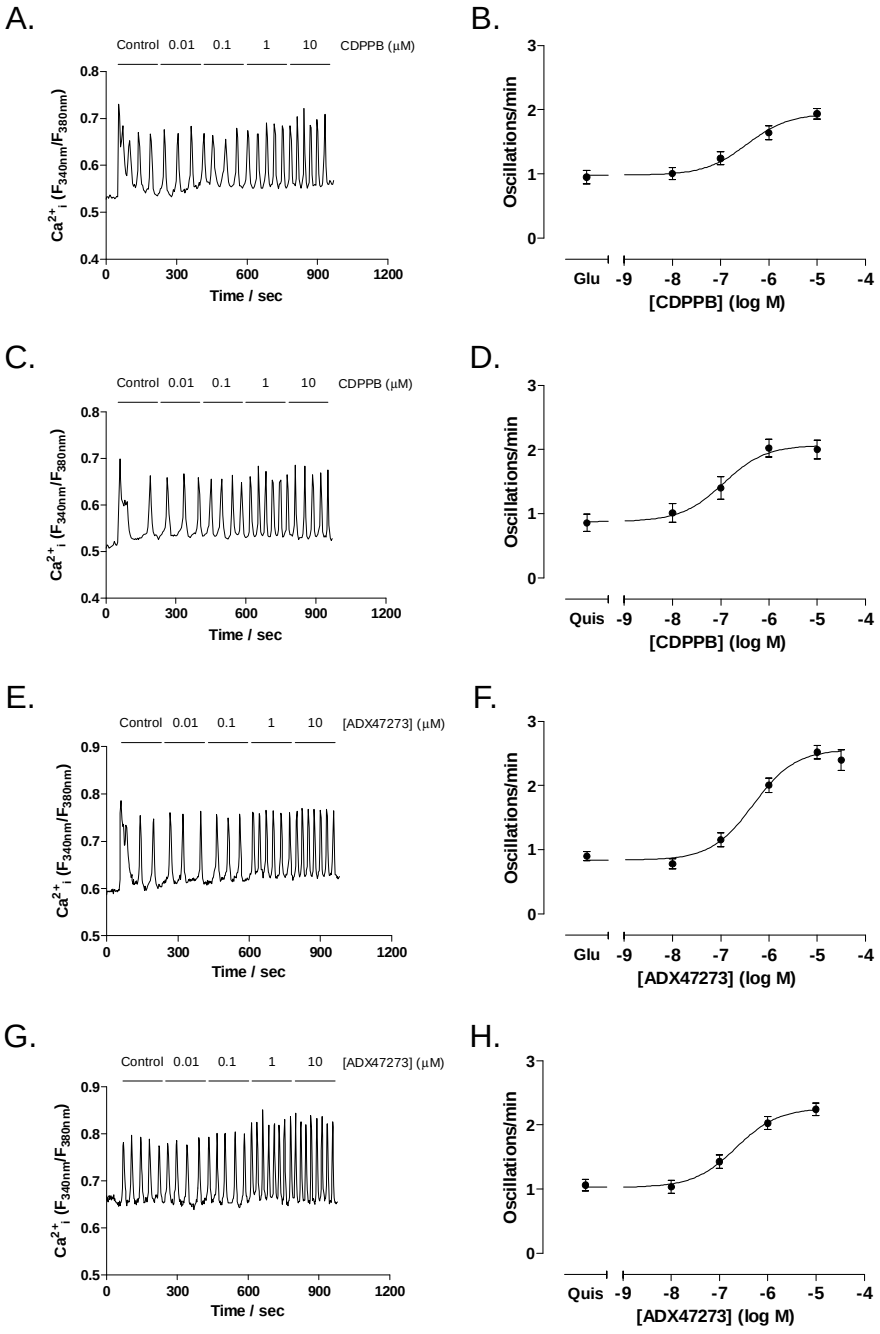

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Figure 3

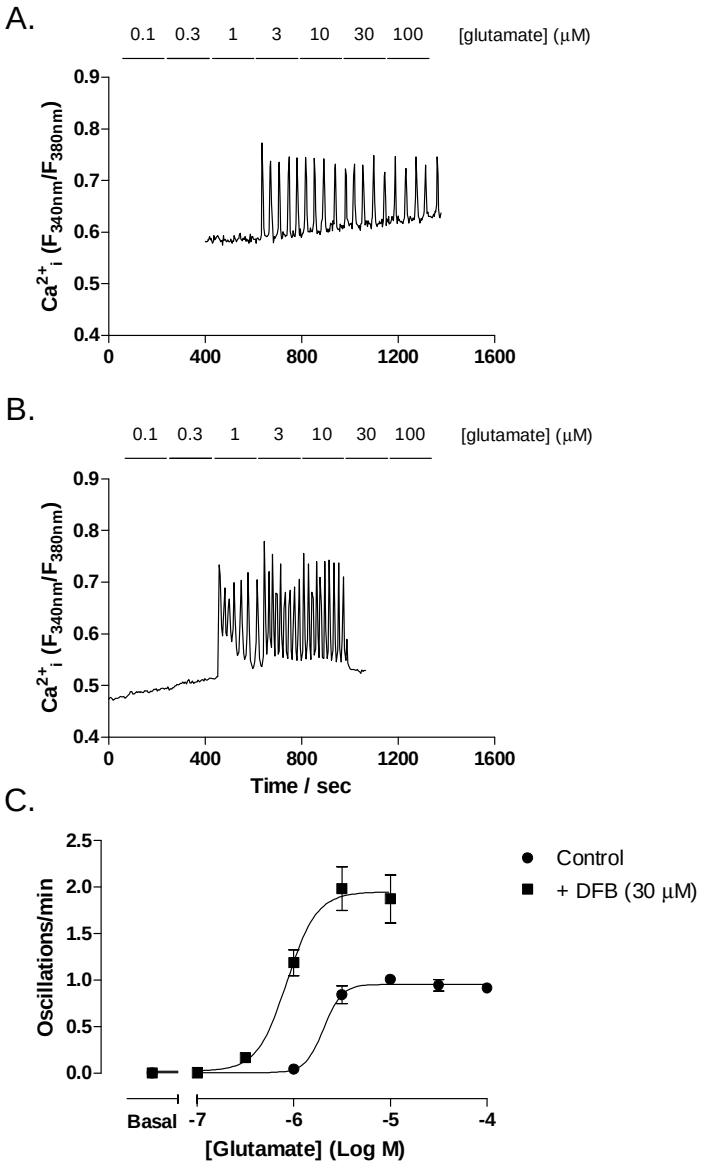

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Figure 4

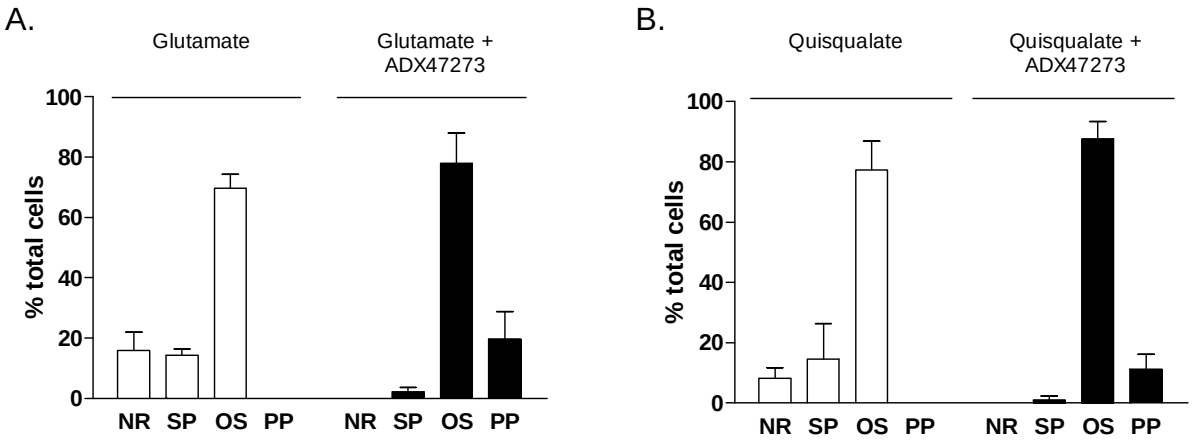

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Figure 5

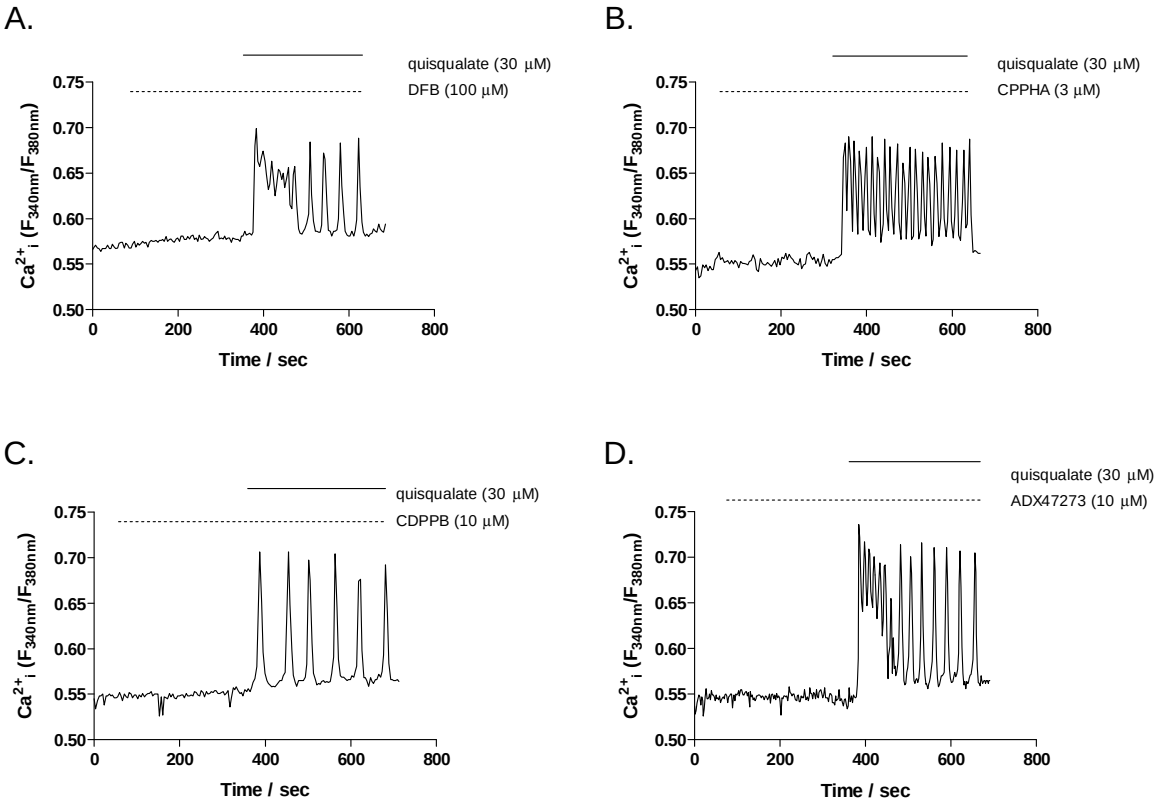

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Figure 6

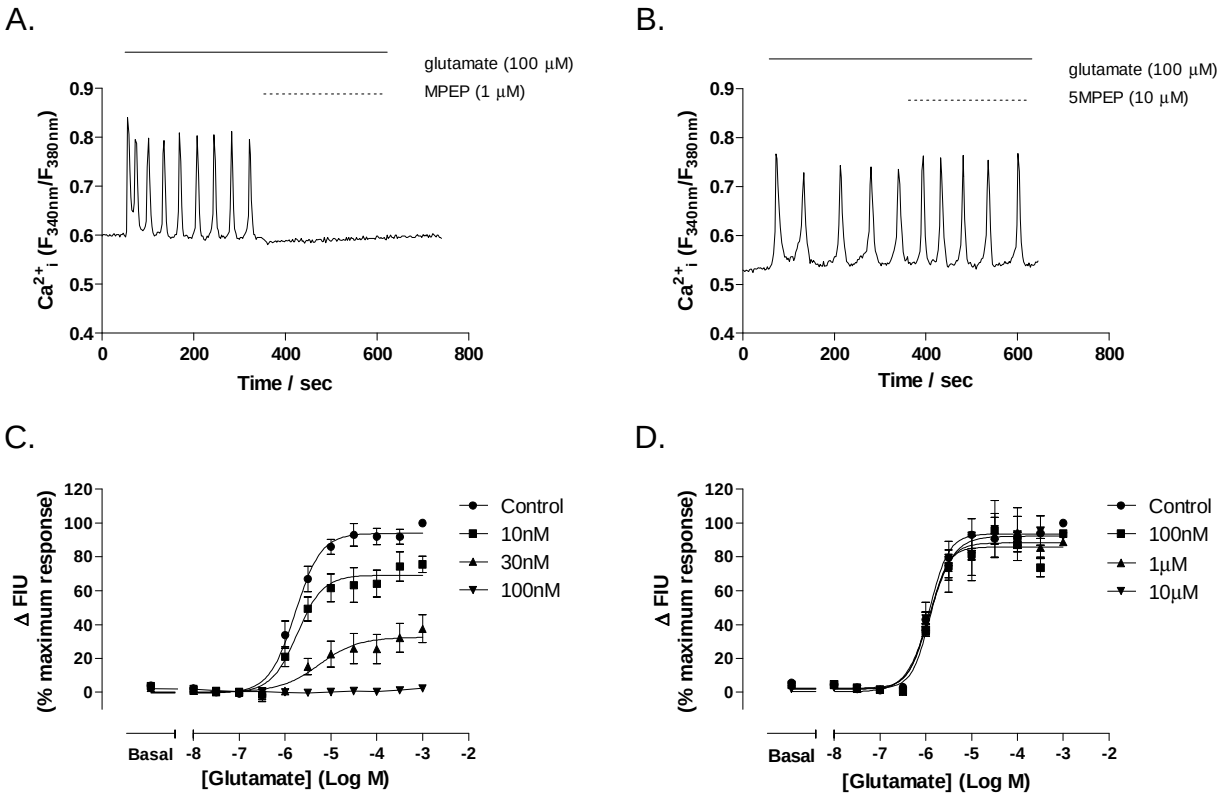

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Figure 7

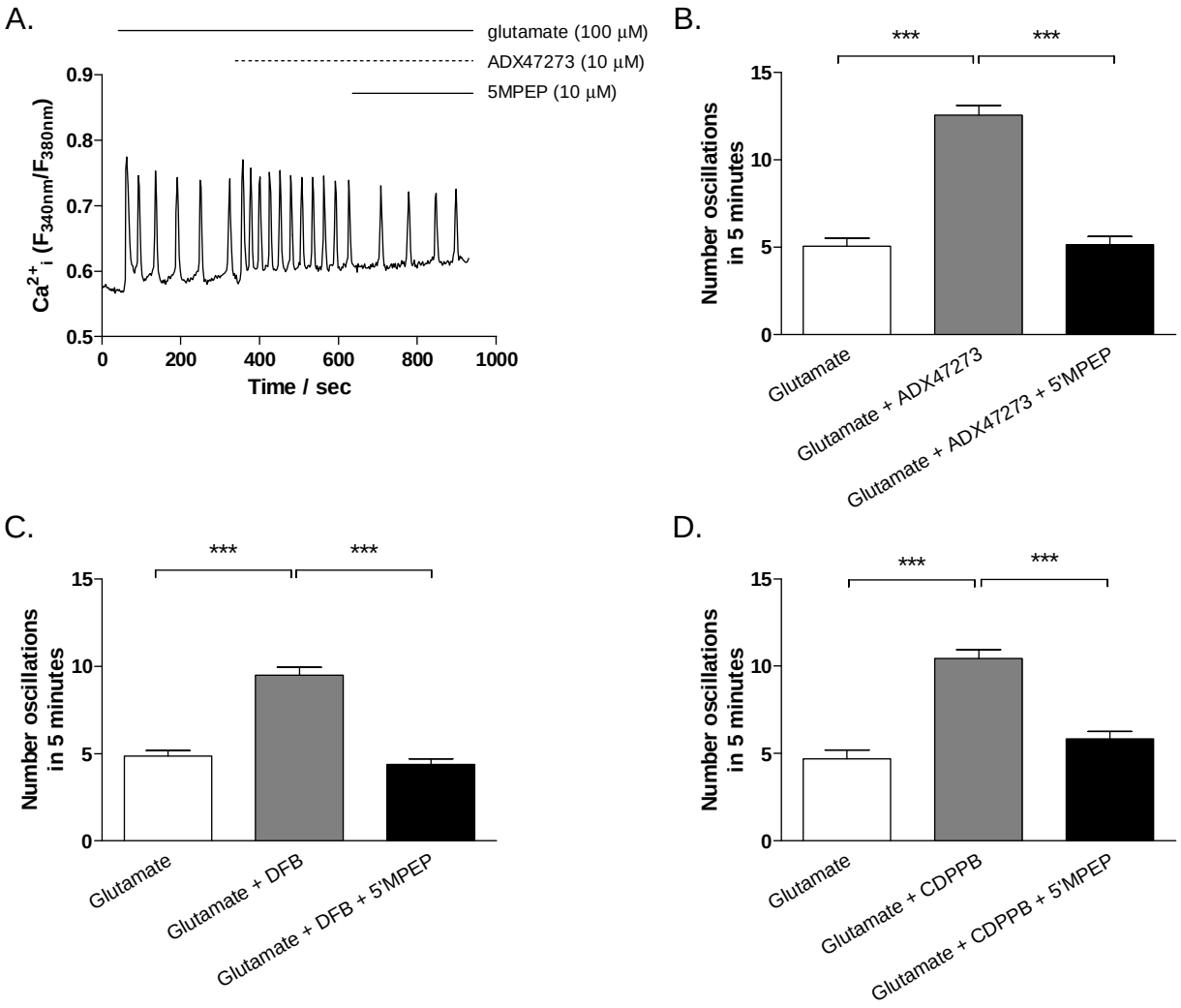

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Figure 8

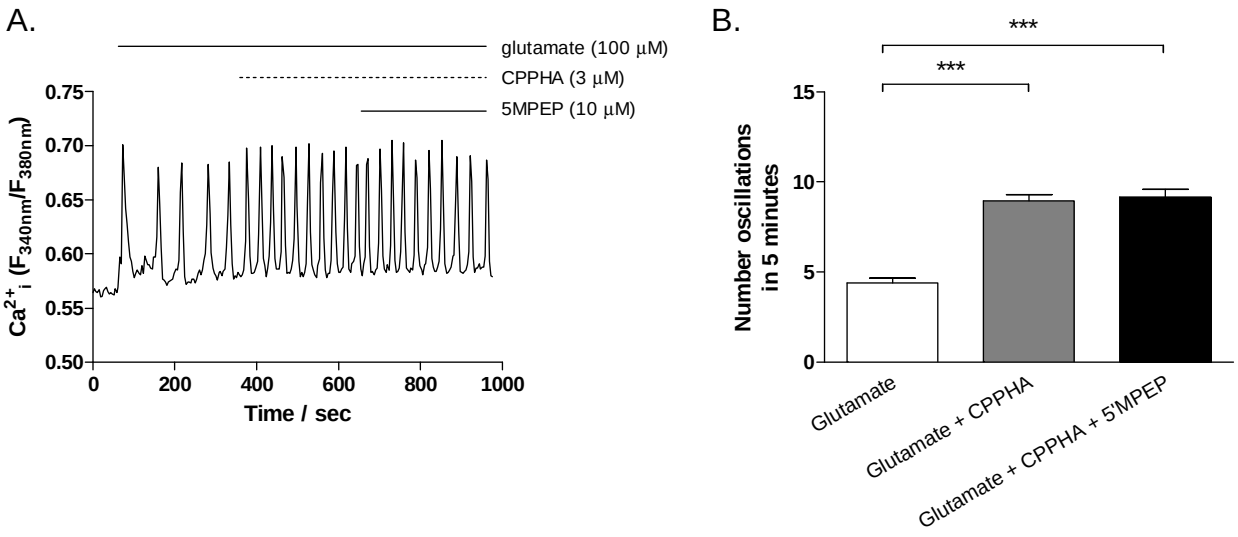

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Figure 9

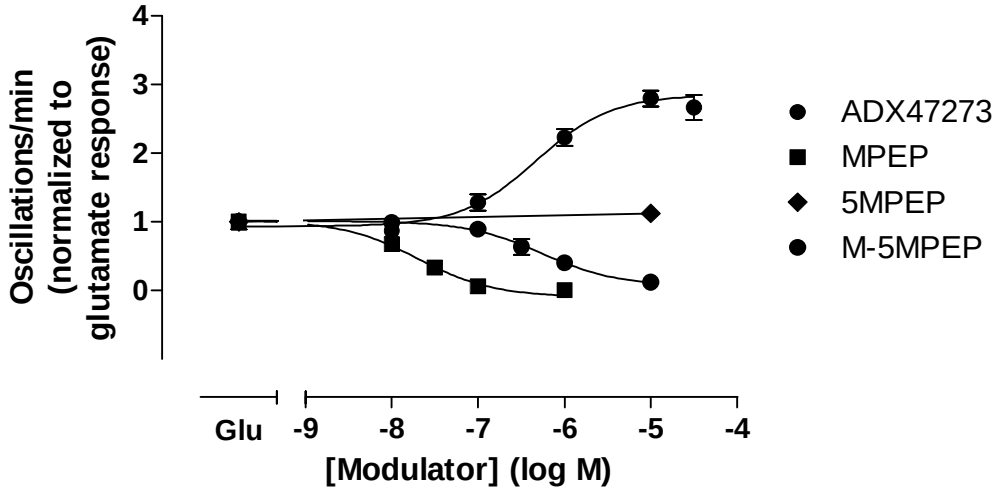

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Figure 10

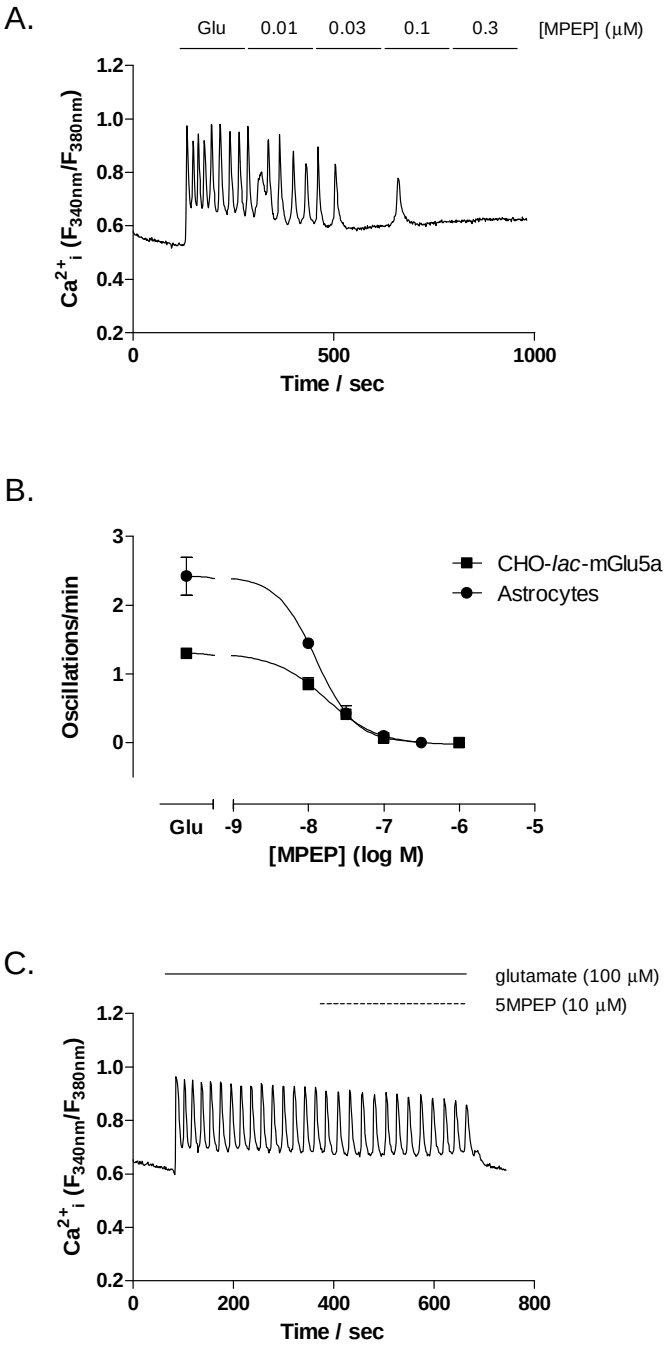

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Figure 11