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Bidirectional variation in glutamate efflux in the medial prefrontal cortex induced by selective positive and negative allosteric mGluR5 modulators

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Abstract

Dysregulation of prefrontal cortical glutamatergic signalling *via* NMDA receptor hypofunction has been implicated in cognitive dysfunction and impaired inhibitory control in such neuropsychiatric disorders as schizophrenia, attention deficit hyperactivity disorder and drug addiction. Although

NMDA receptors functionally interact with metabotropic glutamate receptor 5 (mGluR5), the

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consequence of this interaction for glutamate release in the prefrontal cortex (PFC) is unknown. We therefore investigated the effects of positive and negative allosteric mGluR5 modulation on changes in extracellular glutamate efflux in the medial PFC (mPFC) induced by systemic administration of the non-competitive NMDA receptor antagonist dizocilpine (or MK801) in rats. Extracellular glutamate efflux was measured following systemic administration of the positive allosteric mGluR5 modulator [S-(4-Fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone] (ADX47273; 100 mg/kg, p.o.) and negative allosteric mGluR5 modulator [2-chloro-4-[[1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl]ethynyl]pyridine] (RO4917523; 0.3 mg/kg, p.o.), using a wireless glutamate biosensor in awake, freely-moving rats. The effect of MK801 (0.03-0.06 mg/kg, s.c.) on mPFC glutamate efflux was also investigated in addition to the effects of MK801 (0.03 mg/kg, s.c.) following ADX47273 (100 mg/kg, p.o.) pre-treatment. ADX47273 produced a sustained increase in glutamate efflux and increased the effect of NMDA receptor antagonism on glutamate efflux in the mPFC. By contrast, negative allosteric mGluR5 modulation with RO4917523 decreased glutamate efflux in the mPFC. These findings indicate that positive and negative allosteric mGluR5 modulators produce long lasting and opposing actions on extracellular glutamate efflux in the mPFC. Positive and negative allosteric modulators of mGluR5 may therefore be viable therapeutic agents to correct abnormalities in glutamatergic signalling present in a range of neuropsychiatric disorders.

Key words: Glutamate; mGluR5; NMDA receptors; prefrontal cortex; impulsivity

Abbreviations: mGluR5, metabotropic glutamate receptor 5; mGluR2/3, metabotropic glutamate receptor 2/3; GABA, Gamma-aminobutyric acid; PFC, prefrontal cortex; mPFC, medial prefrontal cortex; ADX47273, S-(4-Fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone; RO4917523, 2-chloro-4-[[1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl]ethynyl]pyridine; p.o., perioral; i.v., intravenous; s.c., subcutaneous; ADHD, attention-deficit hyperactivity disorder; NMDA, N-methyl-D-aspartate; PFA, paraformaldehyde; 5-CSRTT, 5-choice serial reaction time task;

Introduction

Dysregulation of prefrontal glutamatergic signalling is hypothesised to underlie impaired cognitive functioning in a number of neuropsychiatric disorders, including schizophrenia, attention-deficit hyperactivity disorder (ADHD) and drug addiction (Archer and Garcia, 2016; Deakin et al., 1989; Goff and Coyle, 2001; Kalivas and Volkow, 2011; Konradi and Heckers, 2003; Lindsley et al., 2006). In rodents and non-human primates, as well as in humans, N-methyl-D-aspartate (NMDA)

receptors contribute to cognitive functions dependent on the prefrontal cortex (PFC), including working memory (Aultman and Moghaddam, 2001; Barker and Warburton, 2015; Driesen et al., 2013; Karlsgodt et al., 2011; Wang and Arnsten, 2015) and executive attention (Castner and Williams, 2007; Graybeal et al., 2012; Malhotra et al., 1996).

We recently reported that positive allosteric mGluR5 modulation decreases impulsive responding in the 5-choice serial reaction time task (5-CSRTT) and attenuates the disruptive effects of NMDA receptor antagonism on this task (Isherwood et al., 2015). Although the neural mechanisms underlying these effects are unknown, optimal performance on this task depends on the PFC and glutamatergic signalling from this region to a variety of cortical and subcortical structures (Robbins, 2002). Supporting a putative locus within the PFC for these effects, NMDA receptor antagonists increase the activity of pyramidal neurons in this region (Jackson et al., 2004; Lecourtier et al., 2007; Suzuki et al., 2002) and locally increase glutamate release (Abekawa et al., 2003; Adams and Moghaddam, 1998; Ceglia et al., 2004; Lorrain et al., 2003; Moghaddam et al., 1997; Moghaddam and Adams, 1998). NMDA receptor antagonists also increase impulsivity in the 5-CSRTT (Amitai et al., 2007; Fletcher et al., 2011; Higgins et al., 2003b; Isherwood et al., 2015; Oliver et al., 2009; Paine et al., 2007; see Amitai and Markou, 2010 for review), which may result from increased synaptic availability of glutamate at non-NMDA receptors (Moghaddam et al., 1997; Moghaddam and Adams, 1998). Furthermore, suppressing glutamate release or pharmacologically blocking non-NMDA receptors reduces impulsivity and restores the cognitive impairment associated with NMDA receptor antagonism (Moghaddam et al., 1997; Moghaddam and Adams, 1998; Pozzi et al., 2011).

Previous studies have shown that activation of mGluR5 enhances NMDA receptor function (Campbell et al., 2004; Homayoun et al., 2004; Liu et al., 2008; Rosenbrock et al., 2010; Stefani and Moghaddam, 2010). Moreover positive allosteric modulation of mGluR5 reverses MK801-induced excessive activity and burst firing of neurons in the PFC of freely-moving rats (Lecourtier et al., 2007). However, it is unknown whether NMDA receptors interact with mGluR5 to regulate mechanisms affecting glutamate release in the PFC. Thus, although orthosteric mGluR5 agonists increase aspartate and glutamate release in the central nervous system (Musante et al., 2008; Park et al., 2004; Thomas et al., 2000), no *in-vivo* study to date has investigated whether mGluR5 PAMs affect the elevation in PFC glutamate release caused by NMDA receptor antagonists. Since positive allosteric mGluR5 modulation putatively activates GABA-ergic interneurons in the PFC (Chu and Hablitz, 1998; Lecourtier et al., 2007) which in turn act to inhibit glutamatergic pyramidal neurons in this region (Krystal et al., 2003), we hypothesised that mGluR5 PAMs may reverse the cognitive impairing effects of NMDA receptor antagonists by attenuating glutamate release in the PFC.

In the present study we investigated this hypothesis using wireless glutamate biosensor technology in awake, freely-moving rats to assess the effects of a selective mGluR5 PAM and a selective mGluR5 NAM on the extracellular concentration of glutamate in the mPFC. Changes in extracellular glutamate concentration were measured following the systemic administration of the NMDA receptor antagonist MK801, the positive allosteric mGluR5 modulator [S-(4-Fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone] (ADX47273; Isherwood et al., 2015; Liu et al., 2008) and the negative allosteric mGluR5 modulator [2-chloro-4-[[1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl]ethynyl]pyridine] (RO4917523; Isherwood et al., 2015; Lindemann et al., 2015). Subsequently, we investigated whether ADX47273 modulates glutamate efflux induced by systemic MK801 administration.

Materials and Methods

Subjects

Male Wistar Han rats (RRID:RGD_2308816), weighing 250 – 280 g on arrival (Charles River, Germany), were initially housed in groups of four under a 12 hour light/dark cycle, at ca. 22°C, with food and water available *ad libitum* and environmental enrichment consistent of a plexiglas tube and gnawing sticks. At least one week's acclimatisation was given before surgery, during which time all rats were habituated with daily handling. Following surgery to implant the glutamate biosensor, all rats were individually housed for the remainder of the study. The experiments were conducted between the hours of 07:00 and 15:00 with at least a week washout period between each experiment. All experimental procedures were authorised by the Local Animal Care and Use Committee (VVH 12-029 & 15-002) in accordance with local animal care guidelines, AAALAC regulations and the USDA Animal Welfare Act. The study was not preregistered.

Drugs and drug bioavailability

The tool compounds, [S-(4-Fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone] (ADX47273) and [2-chloro-4-[[1-(4-fluorophenyl)-2,5-dimethyl-1H-imidazol-4-yl]ethynyl]pyridine] (RO4917523), were synthesised and supplied by Boehringer Ingelheim Pharma GmbH & Co. KG (Germany). Both compounds were dissolved in 10 % Tween 80 (0.1 % v/v) and 90 % natrosol (0.5 % v/v) and administered orally (p.o.) at 2 ml/kg. (+)-MK801 hydrogen maleate was purchased from Sigma-Aldrich (Germany), dissolved in saline (0.9 %), and administered subcutaneously (s.c.) at 1 ml/kg after adjustment to pH 7.4. The doses of ADX47273, RO4917523 and MK801 were selected based on previously published behavioural data (Isherwood et al., 2015). Plasma levels of MK801, ADX47273 and RO4917523 were measured to determine drug bioavailability and to ensure suitable drug exposures were attained in Wistar Han rats

(compared with Lister-Hooded rats used previously, Isherwood et al., 2015). Blood samples were taken 15 min after MK801 (0.03 mg/kg, s.c.), 1 hour 30 min after ADX47273 (100 mg/kg, p.o.) and 2 hours after RO4917523 (0.1 mg/kg, p.o.) administration *via* the sublingual vein. Rats were lightly anaesthetised under 5 % isoflurane (with oxygen) until all reflex responses were absent. The tongue was gently extended and, using a sharp needle, the sublingual vein was punctured. Whole blood was collected (enough to provide 100 µl plasma) in potassium EDTA pre-conditioned vials (Kabe Labortechnik GmbH, Germany), to avoid blood coagulation, and stored on ice until preparation of the plasma by centrifugation. The blood samples were centrifuged no more than 30 min after blood sampling at 10000 rpm for 10 min at 4 °C. The plasma supernatant was subsequently collected and frozen at -20 °C until analysis. Drug levels were measured using mass spectroscopy.

Surgery

For glutamate sensor guide cannulae implantation, rats were anaesthetised with ‘MMF’ (1 ml/kg, i.m; medetomidine hydrochloride (150 µg/kg; Komtur Apotheke, Germany), midazolam (2 mg/kg; HEXAL AG, Germany) and fentanyl (5 µg/kg; Janssen, Germany), dissolved in saline (0.9 %), with co-administration of the analgesic meloxicam (0.5 mg/kg, 0.1 ml s.c., Boehringer Ingelheim, Germany). MMF was chosen for sensor guide cannulae implantation as (i) anesthesia is fully reversible, (ii) well tolerated by rats with low incidence of adverse events or surgery-related complication, (iii) the oro-nasal inhalation mask required for isoflurane anesthesia interferes sterically with the head caps required to protect the guide cannulae and sensor system. Rats were secured into a stereotaxic frame (David Kopf Instruments, USA) fitted with atraumatic ear bars. The incisor bar was adjusted to ensure a flat skull position. Local anaesthetic (bupivacain, 0.5 %; JenaPharm®, Germany) was applied to the surgical wound for 2-3 min before exposing the skull. Using standard stereotaxic techniques, glutamate sensor guide cannulae (BAS Rat Cannula, Pinnacle Technology Inc, USA) were implanted unilaterally directly above the left mPFC and secured to the skull with bone screws (Pinnacle Technology Inc, USA) and dental cement (PermaCem Automix; DMG, Germany). The following coordinates were used: AP +2.7 or 3.2 mm; ML -0.5 mm; DV -1.2 mm, relative to dura and bregma (Paxinos and Watson, 1997). Upon completion of surgery, MMF anaesthesia was antagonised with 3 ml/kg, s.c, 750 µg/kg atipamezole hydrochloride (Zoetis, Germany) and 0.2 mg/kg flumazenil (Hameln, Germany), dissolved in 0.9% saline. Rats also received 0.1 ml meloxicam analgesic for two days post-surgery and a prophylactic antibiotic (marbofloxacin, 1 %, Germany) administered 0.2 ml/kg, s.c. for one week. Rats recovered for at least one week before the experiments commenced.

Glutamate biosensor

Extracellular glutamate efflux in the mPFC was measured using a wireless glutamate biosensor system (Pinnacle Technology Inc, USA). Each sensor was constructed with a platinum-iridium electrode coated with glutamate oxidase, which catalyses the breakdown of glutamate to α -ketoglutarate and hydrogen-peroxide (H_2O_2). The sensor was additionally coated with ascorbate oxidase to catalyse the breakdown of ascorbic acid, thereby reducing interference. The oxidation current (nA) of H_2O_2 provided an index of the extracellular concentration of glutamate and was signalled wirelessly *via* Bluetooth[®] to a control computer.

Glutamate sensor calibration and implantation

Following recovery from surgery, and approximately 24 hours before the start of the experiment, each sensor was calibrated *in vitro* to confirm the sensitivity of the recording system to glutamate. Once calibrated, the sensors were implanted. Experimentation and glutamate recordings started approximately 12 hours later. The procedure of calibration involved immersing the sensor in a 20 ml solution of a magnesium- and calcium-free phosphate-buffered saline (PBS; Gibco[®] by life technologies, Germany), diluted 1:10, and adjusted on a magnetic stirrer to pH 7.4 and 37 °C. The sensor was equilibrated for one hour until the rate of decline in the oxidation current was less than 0.2 nA/5 minutes. At this point, the current was recorded for an additional 10 minutes as a baseline period. A fresh calibration stock solution of 5 mM glutamate was made prior to calibration (L-glutamic acid, Roth, Germany). Forty-microliter aliquots of the glutamate stock solution were added three times, every 5 mins, into the PBS solution. In addition, 50 μl of L-(+)-ascorbic acid (100 mM) was added to the PBS solution. The final concentration of ascorbic acid of 250 μM was in excess of the estimated concentration within the brain (40-60 μM ; (Tsai et al., 1996). Sensors which responded to ascorbic acid (> 1 nA increase in current) were discarded.

Following calibration, rats were lightly anaesthetized with isoflurane (5 % in oxygen). The dummy cannulae were removed and the sensors were implanted and attached to a potentiostat powered by a 3V lithium battery (Energizer[®], Germany). Once awake, rats were returned to the animal holding room until the following day when the experiment began.

On the day of the experiment, all rats were habituated to the testing room for at least one hour with the recording experiment taking place in the rats' home cage. Recordings commenced 10 min before drug administration (baseline) and continued for 2-3 hours after drug administration. A largely within-subjects design was used since preliminary experiments indicated a high individual variability in glutamate efflux evoked by NMDA receptor antagonism. Drug doses and pre-treatment times were chosen on the basis of previously published behavioural and pharmacokinetic data (Isherwood et al., 2015). To ensure that the bioavailability of compounds did not differ between rat strains (i.e. between Lister-hooded rats used in previous behavioural studies (Isherwood

et al., 2015) and Wistar Han rats used in the present studies), plasma levels of MK801, ADX47273 and RO4917523 were measured in a separate cohort of Wistar Han rats (Table 1).

Experimental Outline

Experiment 1 was conducted in a single group of rats, using a between-subjects design (three experimental groups with the random allocation of rats into groups using the random number generator function on Windows Excel; sensor placements shown in Fig.1A). Experiments 2 and 3 were conducted in a separate group of rats, using a within-subjects design (each rat received each treatment in each experiment (no blinding of experimenter); sensor placements shown in Fig.1B). A timeline diagram of experimental procedure, including number of animals per single experiment, excluded animals and reason for exclusion is given in supplementary figure 1.

By adopting a within-subjects design in experiment 2 & 3, the data variability and thus the number of animals required for statistical significance could be reduced. The animals' suffering during experiments was minimized using a wireless glutamate biosensor technology which allows performing experiments in awake, freely-moving rats. Adequate anaesthesia during surgery and pain medication, as outlined above, together with close monitoring of the animals after the surgery ensured reducing the animals' pain or discomfort during experiments.

Experiment 1: Effect of NMDA receptor antagonism on mPFC glutamate efflux

A single group of rats (n = 4-6 per treatment group) received saline (s.c.) followed 1 hour later by either saline (n = 2; individual data shown), 0.03 mg/kg (n = 4) or 0.06 mg/kg (n = 6) MK801 (s.c.). 'Time' and 'drug' (vehicle, MK801) served as within-subject factors, whereas 'dose' (0, 0.03, 0.06 mg/kg) served as a between-subjects factor. A vehicle-vehicle group was used as an additional control to assess the stability of the glutamate-related signal following repeated injections.

Experiment 2: Effects of positive and negative mGluR5 allosteric modulation on mPFC glutamate efflux

The effects of ADX47273 and RO4917523 on extracellular glutamate levels in the mPFC were evaluated. 'Time' and 'drug' served as within-subject factors. Rats were administered vehicle (10 % Tween 80 (0.1 % v/v) and 90 % natrosol (0.5 % v/v); p.o) followed 3 hours later by ADX47273 (100 mg/kg, p.o.; n = 10) or RO4917523 (0.3 mg/kg, p.o.; n = 8).

Experiment 3: Interactive effects of NMDA receptor antagonism and positive allosteric mGluR5 modulation on mPFC glutamate efflux

The interactive effects of MK801 (0.03 mg/kg) on mPFC extracellular glutamate efflux and ADX47273 pre-treatment were assessed. 'Time' and 'drug' served as within-subject factors. Rats were administered saline (s.c.) *or* vehicle (10 % Tween 80 (0.1 % v/v) and 90 % natrosol (0.5 % v/v); p.o.) followed in turn by MK801 (s.c.) *or* ADX47273 (p.o.) followed by MK801 (s.c.) in a counter-balanced fashion (n = 10). ADX47273 was administered before MK801 to ensure appropriate drug exposures were achieved. ADX47273 has a $T_{\max}$ of 1.5 hours, whereas MK801 has a $T_{\max}$ of 15 minutes. Thus, the time points at which glutamate efflux was measured, maximal exposures of ADX47273 and MK801 were attained. As an additional control, rats were subsequently administered ADX47273 (p.o.) followed by saline (s.c.). We did not assess the effects of RO4917523 on MK801-induced glutamate efflux since RO4917523 failed to modulate the disruptive effects of MK801 on behavioural performance on the 5-CSRTT (Isherwood et al., 2015).

Histology

At the end of the experiments, rats were anaesthetised with a lethal dose of sodium pentobarbital (0.16 mg/kg, 2 ml/kg; Merial, Germany) and perfused transcardially at a rate of 10 ml/min with PBS (Sigma-Aldrich, Germany) followed by 4 % paraformaldehyde (PFA, Alfa Aesar[®], Germany). The brains were carefully removed, post-fixed in 4 % PFA at room temperature for 24 hours and subsequently transferred to 30 % sucrose for cryo-protection for 24 hours. The brains were then removed and frozen at -20 °C until sectioned. Coronal sections, cut at 50 µm (LEICA cm3050, Biosystems, Germany) were thawed onto Superfrost Ultra Plus[®] microscope slides (Thermo Scientific, Germany) and frozen at -20 °C until placement assessment. Brain sections were defrosted, dried and subsequently incubated in 4 % PFA for 5 min, before being allowed to dry overnight before analysis of probe placement.

Data acquisition and statistical analysis

Data were acquired using Serenia[®] acquisition software (Pinnacle Technology Inc, USA) and analysed using Spike2 (Version 8; Cambridge Electronic Design Limited, UK). Glutamate levels were recorded every second for 2-3 hours following drug injection. Data were averaged into 10 min time bins. Raw data (nA) were converted to glutamate concentration (nM) based on the calibration curve generated for each sensor during the calibration procedure. The relative change in glutamate concentration (nM) is reported (i.e. raw data minus baseline). Sample sizes were predetermined based on previous studies and verified statistically by a non-clinical biostatistician using *Proc Power* in SAS Version 9.2 (SAS Institute Inc., Cary, North Carolina, USA). Primary parameter for sample size calculation was drug-induced 'change in glutamate release' vs. vehicle treatment

(power of 80 % to determine drug-induced change in glutamate release of at least 50 % vs control group with t-test, assuming heteroscedastic variance of $\alpha = 5\%$).

All statistical analyses were conducted using SPSS for Windows (version 21) and GraphPad Prism 7.02. Statistical significance was set at $p < 0.05$. Three animals that lost head caps in the course of the experiment were excluded from further analysis as outlined in detail in the results. No test of outliers was carried out. In all studies, data were analysed by repeated measures ANOVA. In the study involving MK801 alone, treatment and time served as within- and between-subject factors, respectively (mixed design ANOVA). In all other studies, treatment and time served as within-subject factors. When significant main effects and interactions were found, further analysis using Bonferroni *post hoc* tests were performed. No additional test of normality was performed. For data represented in the form of a histogram, positive and negative changes in glutamate are represented additively, with the baseline set as zero.

Results

Drug bioavailability

Plasma levels of MK801, ADX47273 and RO4917523 are shown in Table 1. MK801 levels 15 minutes after administration were comparable to previously published levels in Lister hooded rats (Isherwood et al., 2015), specifically 7.64 nM in Wistar Han rats compared to 6.74 nM in Lister-hooded rats. Plasma levels of ADX47273 and RO4917523 were also broadly similar to those previously reported (Isherwood et al., 2015). However, ADX47273 achieved moderately lower plasma exposures in Wistar Han rats compared with Lister-hooded rats, whereas RO4917523 achieved moderately higher exposures in this rat strain. However, it should be noted that blood samples were collected 15 min before those taken from the Lister-hooded rats. Nevertheless, plasma exposures in Wistar Han rats were sufficient to evoke a pharmacological response, based on functional *in vitro* data reported previously (Isherwood et al., 2015) and the present study.

Histology

Reconstructed active zones of the glutamate biosensors (1 mm) are shown in Fig.1A-B. Two rats were excluded from experiment 1 (MK801 dose-response) due to misaligned placements. Sensors were predominately located in the prelimbic and infralimbic sub-regions of the mPFC.

Experiment 1: Effect of NMDA receptor antagonism on mPFC glutamate efflux

Glutamate-related signals were stable approximately one hour after the start of the experiment, at which point a 10-min baseline period was recorded. The effects of a single injection of saline (s.c.) on glutamate efflux was then monitored for 1 hour. Consistent with a previous report (Moghaddam et al., 1997), the injection procedure produced an immediate but modest increase in glutamate

efflux (Fig.2A). One hour later, a new baseline was recorded, after which saline, 0.03 or 0.06 mg/kg MK801 (s.c.) was administered. Unfortunately, for technical reasons, two saline control animals were excluded from further analysis. Specifically, during the experiment the lithium batteries leaked inside the head caps, shorting the internal electrical circuit on the potentiationstat and preventing the glutamate signal being recorded. MK801 produced a significant, approximate 2-fold increase in glutamate efflux (main effect of drug, $F_{1,8} = 6.9$, $p < 0.05$) compared with the saline control (Fig.2A); an effect that did not depend on the dose of MK801 administered (main effect of dose; drug x dose interaction, *NS*). The increase in glutamate concentration following 0.03 and 0.06 mg/kg MK801 was apparent in the first 10-min time bin and peaked after 30-min at 322 ± 92 nM following the 0.03 mg/kg dose, and at 345 ± 45 nM, 40-min after the 0.06 mg/kg dose (Fig.2A). We also observed an apparent increase in glutamate efflux following the vehicle injection. Within two hours of MK801 administration, the extracellular concentration of glutamate in the mPFC returned to baseline. Despite modest differences in glutamate response between 0.03 and 0.06 mg/kg MK801, there was no overall difference in response, as revealed by the area under the curve (AUC) for the vehicle and MK801 groups (AUC represents one hour post vehicle/MK801 administration (i.e. time bin 0 to bin 8)) (Fig.2B).

Experiment 2: Effect of allosteric mGluR5 modulation on mPFC glutamate efflux

The effect the positive allosteric mGluR5 modulator, ADX47273, on glutamate efflux in the mPFC is shown in Fig.3. One rat lost his head cap and was removed from this experiment. Glutamate-related signals were stable after approximately 1-hour, whereupon a 10-min baseline was recorded. All rats then received vehicle (p.o.) followed by a further 3-hours of recording. The administration of vehicle evoked an immediate, but relatively small increase in glutamate efflux (~ 325 - 400 nM), which was maintained for approximately 2-hours. All rats were then administered ADX47273 (p.o.) following an additional 10-min re-baseline and glutamate efflux recorded for a further 3-hours. ADX47273 produced a significant but delayed increase in glutamate efflux within the mPFC compared with the vehicle control group; (drug x time interaction: $F_{18,162} = 3.4$, $p < 0.001$; main effect of drug, *NS*) (Fig.3A). *Post hoc* tests revealed that ADX47273 significantly increased glutamate efflux relative to the vehicle group 140-mins after its systemic administration. At this time, glutamate efflux was approximately 3-fold higher than the vehicle group (vehicle, 271 ± 112 nM vs. ADX47273, 902 ± 233 nM, $p < 0.01$). AUC data for the control and ADX47273 groups, collapsed over 3-hours (i.e. from time bin 0), are shown in Fig.3B.

Due to lost head caps, two rats were excluded from the RO4917523 study. One additional rat was excluded from this group due to an unstable baseline, possibly caused by sensor damage during implantation. Glutamate-related signals were stable after 1-hour. Following a 10-min baseline, rats were then administered vehicle (p.o.) followed by 3-hours of recording. Again,

vehicle administration caused an immediate but small increase in glutamate efflux (~ 230 nM). This effect peaked after 20-min and returned to baseline levels within 70-min (Fig.4A). For the remainder of the experiment, glutamate efflux declined, eventually reaching levels approximately 300 nM below baseline. RO4917523 significantly decreased glutamate efflux (drug x time interaction: $F_{18,126} = 4.3$, $p < 0.001$; main effect of drug, *NS*), reaching statistical significance 60-min after its administration compared with the vehicle group. A maximal decrease in glutamate efflux of approximately observed after 80-min (vehicle, 129 ± 99 nM decrease vs. RO4917523, 475 ± 152 nM decrease, $p < 0.05$). A gradual increase in glutamate efflux was then observed, reaching levels that were largely indifferent to that measured at baseline suggesting a return of glutamatergic tone in the mPFC. AUC data for the control and RO4917523 groups, collapsed over 3-hours (i.e. from time bin 0), are shown in Fig.4B

Experiment 3: Combined effects of NMDA receptor antagonism and positive allosteric mGluR5 modulation on mPFC glutamate efflux

One rat was excluded from this study due to a lost head cap. Fig.5 shows the effect of pre-treatment with ADX47273 on glutamate efflux evoked by MK801; vehicle data are shown in Supplementary Fig.2. Within 1-hour of habituation, glutamate-related signals were stable and recording commenced. After a 10-min baseline, rats received either vehicle (p.o.) or ADX47273 (100 mg/kg, p.o.), which evoked an immediate, but relatively small, increase in glutamate efflux. Eighty minutes after ADX47273/vehicle treatment, rats received MK801 (0.03 mg/kg, s.c.) or vehicle (s.c.) with glutamate efflux recorded for an additional two hours (Fig.5A; Supplementary Fig.2). Overall, ANOVA revealed a significant drug x time interaction ($F_{24,216} = 3.5$, $p < 0.001$) and consistent with previous experiments glutamate levels declined steadily in the vehicle control group. Glutamate efflux increased maximally in ADX47273-treated and MK801-treated rats by 476 ± 119 nM and 257 ± 88 nM, respectively (Fig.5A). However, when combined, ADX7273 and MK801 increased glutamate efflux by 817 ± 176 nM. AUC data for the various groups, over 2-hours (i.e. from time bin 8), are shown in Fig.5B.

Discussion

We investigated the effects of allosteric mGluR5 modulation on prefrontal glutamate efflux and specifically whether the cognitive enhancing effects of positive mGluR5 modulation is reflected by a reversal of increased glutamate efflux induced by NMDA receptor antagonism. In this context numerous studies have demonstrated that NMDA receptor antagonists increase glutamate efflux and disrupt cognitive functions dependent on the PFC (Agnoli and Carli, 2012; Campbell et al., 2004;

Ceglia et al., 2004; Fletcher et al., 2011; Homayoun et al., 2004; Lopez-Gil et al., 2007; Moghaddam et al., 1997; Moghaddam and Adams, 1998; Stefani and Moghaddam, 2010).

In contrast, positive allosteric mGluR5 modulators are hypothesised to offset the disinhibiting effects of NMDA receptor antagonists by augmenting glutamatergic neurotransmission at postsynaptic NMDA receptors and/or by activating GABA-ergic interneurons (Alagarsamy et al., 2002; Kerner et al., 1997; Lecourtier et al., 2007; Muly et al., 2003; Romano et al., 1995; Shigemoto et al., 1993). *In vivo*, positive allosteric mGluR5 modulation blocks the effects of MK801 on neuronal firing in the orbitofrontal cortex and mPFC (Homayoun and Moghaddam, 2008; Lecourtier et al., 2007) whereas negative mGluR5 modulation has the opposite effect (Homayoun and Moghaddam, 2006). Therefore, we hypothesised that positive allosteric mGluR5 modulation by ADX47273 would decrease glutamate release by enhancing GABA-ergic inhibition of glutamatergic pyramidal neurons in the mPFC. Our findings, however, indicate that the positive allosteric mGluR5 modulator ADX47273 increases glutamate efflux in the mPFC when administered alone or when combined with the NMDA receptor antagonist MK801. Moreover, glutamate efflux in the mPFC was decreased by the negative allosteric mGluR5 modulator RO4917523. These findings indicate that cognitive enhancing effects of positive allosteric mGluR5 modulators in tasks that depend on NMDA receptor function may not be due to an attenuation of glutamate release in the PFC.

We previously reported that MK801 increases impulsivity in the 5-CSRTT (Isherwood et al., 2015); an effect hypothesised to originate from increased glutamatergic transmission in the mPFC, specifically within the infralimbic cortex (Carli et al., 2004; Ceglia et al., 2004; Fletcher et al., 2011; Higgins et al., 2003b; Moghaddam et al., 1997; Moghaddam and Adams, 1998; Murphy et al., 2005; Pozzi et al., 2011). Indeed, ketamine and other NMDA receptor antagonists increase glutamatergic neuronal activity (Homayoun et al., 2005; Jackson et al., 2004; Jodo et al., 2003; Suzuki et al., 2002) and consequently extracellular glutamate levels in the mPFC (Adams and Moghaddam, 1998; Ceglia et al., 2004; Lopez-Gil et al., 2007; Moghaddam et al., 1997). Increased availability of glutamate at non-NMDA receptors is thought to mediate cognitive impairment and impaired response inhibitory control produced by NMDA receptor antagonists (Moghaddam et al., 1997; Pozzi et al., 2011). Indeed, non-NMDA receptor antagonists and drugs that suppress glutamate release in the mPFC show efficacy in alleviating behavioural deficits evoked by NMDA receptor antagonism (Bubser et al., 1995; Moghaddam et al., 1997; Moghaddam and Adams, 1998; Pozzi et al., 2011). For example, mGluR2/3 agonists block NMDA receptor antagonist-induced glutamate efflux, primarily by decreasing neuronal firing in the mPFC (Homayoun et al., 2005; Moghaddam and Adams, 1998; Pozzi et al., 2011), whilst also attenuating impulsivity in the 5-CSRTT, locomotion, stereotypy, and deficits in working memory (Homayoun et al., 2005; Moghaddam and Adams, 1998; Pozzi et al., 2011).

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The paradoxical effect of NMDA receptor antagonists to increase glutamate release within the mPFC is hypothesised to result from the inhibition of GABA-ergic interneurons and thus the disinhibition of glutamatergic pyramidal neurons (Homayoun and Moghaddam, 2007; Krystal et al., 2003; Moghaddam et al., 1997). However, although local infusion of 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), a competitive NMDA receptor antagonist, increases mPFC glutamate release (Ceglia et al., 2004; Pozzi et al., 2011), MK801 and other non-competitive NMDA receptor antagonists have no effect on glutamate release (Lopez-Gil et al., 2007; Lorrain et al., 2003; Suzuki et al., 2002) or glutamatergic neuronal firing in the mPFC (Jodo et al., 2005; Suzuki et al., 2002). Thus, the population of NMDA receptors responsible for the effects of MK801 on glutamate release appear to be located outside the PFC, possibly on GABA-ergic neurons tonically involved in inhibiting glutamatergic input to the PFC (Lopez-Gil et al., 2007).

In the present study ADX47273 increased glutamate efflux in the mPFC when administered alone. This effect is compatible with a previous report describing the excitatory effects of the positive allosteric mGluR5 modulator CDPBB on neuronal firing in the mPFC (Lecourtier et al., 2007). Since ADX47273 increased rather than decreased glutamate efflux our results suggest that excitation of glutamatergic neurons by mGluR5 PAM was sufficient to overcome the assumed enhanced inhibition of these neurons by mGluR5-induced depolarization of GABA-ergic neurons, as depicted in Fig.6. Moreover, the temporal profile and sustained increase in glutamate efflux evoked by ADX47273 we report mirrors the bioavailability of this compound in plasma after oral dosing (Isherwood et al., 2015). Of particular importance, our findings support the conclusion that allosteric modulators do not lead to a rapid desensitization of the target receptor (Urwyler, 2011), unlike orthosteric modulators (Gereau and Heinemann, 1998).

Negative allosteric mGluR5 modulation by RO4917523 produced a significant reduction in glutamate efflux in the mPFC, a finding consistent with the effects of the mGluR5 antagonist MPEP, which decreases burst activity and firing of mPFC neurons (Homayoun and Moghaddam, 2006). Again, these findings suggest a post-synaptic site of action of RO4917523 on glutamatergic pyramidal neurons. However, the initial and rapid effect of RO4917523 on glutamate efflux was surprising given the known pharmacokinetic properties of RO4917523 (T_{max} 2 hours) (Isherwood et al., 2015); changes in glutamate efflux occurred *before* optimal plasma exposures were attained (i.e. within 30 min). In the pharmacokinetic study described, a dose of 5.8 mg/kg (p.o.) was used. An additional pharmacokinetic study was therefore performed using a lower dose of RO4917523. Importantly, the C_{max} and T_{max} of RO4917523 (0.1 mg/kg, p.o.) were determined to be 96 nM and 25 min, respectively whilst *in vitro*, the IC_{50} of RO4917523 was ~ 4.5 nM (Isherwood et al., 2015). Since the brain/plasma ratio of RO491723 is ~ 2 to 3 and the predicted free-fraction is 3% we can assume that following 0.1 mg/kg RO4917523 administration, an active brain exposure of ~ 6 nM would be attained. This is well within the range of the *in vitro* IC_{50} . In the present study a dose of

0.3 mg/kg was used, suggesting with certainty that appropriate brain exposures were attained within 25 min of administration to evoke a pharmacological effect at mGluR5. Moreover, our analysis also revealed that within two hours of administration (0.1 mg/kg), the predicted exposure of this compound in the brain would fall below the *in vitro* IC₅₀. This would explain the return of glutamatergic tone in the mPFC at this time.

It is important to note that some variability in glutamate release was observed in experiments 1 and 2 following the administration of the vehicle. Thus, in experiment 1, the vehicle injection produced an increase in glutamate efflux of approximately 200 nM whereas in experiment 2 the vehicle injection increased glutamate release by almost 400 nM. The reason for this variation is unclear but may be due to different vehicle solutions for experiment 1 (saline) and experiment 2 (10% Tween 80), differences in the dynamic in-vivo performance of the sets of biosensors used for each experiment, and/or that the biosensors were implanted in a slightly different anterior-posterior location. Thus, whereas probe placements in experiment 1 ranged between 3.2 and 3.7 mm along the anterior-posterior axis, placements in experiment 2 ranged between 2.2 and 3.2 mm. It is therefore possible that the observed variability in glutamate release resulted from differential effects of injection stress on glutamatergic neurons located in posterior and anterior regions of the mPFC.

The main findings of this study indicate that positive allosteric mGluR5 modulation and NMDA receptor antagonism lead the same outcome of increasing glutamate release in the PFC. It is unlikely therefore that the reported ability of mGluR5 PAMs to reverse cognitive impairments induced by NMDA receptor antagonism is related to a correction of glutamate release in this region. Indeed, results from several studies indicate that mGluR5 PAMs (e.g. ADX47273 and CDPBB) ameliorate a range of deficits caused by MK801 and PCP, including set-shifting (LaCrosse et al., 2015; Stefani and Moghaddam, 2010), novel object recognition (Horio et al., 2013) and impulsivity on the 5-choice task (Isherwood et al., 2015). Although the neural substrates underlying the cognitive enhancing effects of mGluR5 PAMs are presently unknown these may be mediated in part by effects within the mesolimbic dopamine system. Thus, in addition to stimulating glutamatergic transmission, NMDA receptor blockade increases dopamine efflux in the nucleus accumbens (Adams and Moghaddam, 1998, 2001; Mathé et al., 1999; Moghaddam and Adams, 1998), possibly by stimulating glutamatergic outflow from the PFC to subsequently enhance activity in the mesolimbic dopamine system (Mathé et al., 1999; Murase et al., 1993). Moreover, systemic ADX47273 decreases dopamine efflux in the nucleus accumbens (Liu et al., 2008). However, although this may account for the ability of ADX47273 to reduce MK801-induced impulsivity in the 5-choice task (Isherwood et al., 2015), for which performance depends on dopaminergic mechanisms in the nucleus accumbens (Robbins, 2002), there is at least one report showing that CDPBB does not alter basal or MK801-induced dopamine release in the nucleus accumbens (Lecourtier et al., 2007). Thus, it remains an open question whether the pro-cognitive effects of

ADX47272 are mediated by an attenuation of MK801-induced release of dopamine and other neurotransmitters (e.g. within in the nucleus accumbens). Fig. 6 proposes a hypothesised mechanism for the modulation of glutamate release within the mPFC by NMDA receptor antagonism and mGluR5 modulation.

In conclusion, our findings demonstrate that positive allosteric mGluR5 modulation by systemic ADX47272 leads to a sustained elevation in extracellular glutamate concentration in the mPFC. Rather than reverse the effects of the MK801 on glutamate efflux in this region, as expected, ADX47272 produced an additive increase in glutamate availability that contrasted with the effects of the negative allosteric mGluR5 modulator RO4917523. Therefore, the cognitive enhancing effects of mGluR5 PAMs in tasks dependent on NMDA receptor function appear to be dissociable from alterations in PFC glutamate release. These findings imply an additional mechanism responsible for the cognitive enhancing effects of mGluR5 PAMs separate from the reversal of the NMDA receptor antagonist-induced disinhibition of glutamatergic neurons in the PFC (Homayoun and Moghaddam, 2007; Krystal et al., 2003; Moghaddam et al., 1997). Defining the neural loci underlying the pro-cognitive effects of mGluR5 PAMs may provide new opportunities to ameliorate cognitive dysfunction present in a range of neuropsychiatric disorders including schizophrenia.

Conflict of Interest

SNI and AP are employees of Boehringer Ingelheim Pharma GmbH & Co. KG. TWR consults for Cambridge Cognition, Lundbeck, Teva, Shire Pharmaceuticals, Otsuka, and has research grants from Lundbeck and GSK, royalties from Cambridge Cognition, and editorial honoraria from Springer and Elsevier. JWD has received research grants from Boehringer Ingelheim and editorial honoraria from Wiley and Sage.

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Table 1. Mean plasma levels following the administration of 0.03 mg/kg MK801 (15 minutes post-administration), 100 mg/kg ADX472723 (1 hour and 30 minutes post-administration) and 0.1 mg/kg RO4917523 (2 hours post-administration) in Wistar Han rats. For comparison, mean plasma levels of 0.03 mg/kg MK801 (10 minutes post-administration) in Lister-hooded rats are also shown.

	Mean plasma concentration (nM); Wistar Han	Mean plasma concentration (nM); Lister-hooded
MK801		
(0.03 mg/kg; s.c.)	7.64 ± 0.1	6.74 ± 0.1
ADX47273		
(100 mg/kg; p.o.)	1740.0 ± 176.2	2550 ± 210.0 (Isherwood et al., 2015)
RO4917523		
(0.1 mg/kg; p.o.)	72.4 ± 4.77	25 ± 0* (Isherwood et al., 2015)

Values represent mean ± SEM (n = 3 per group). * n = 1 (two samples below detectable range)

Figure legends

Fig.1. Schematic diagrams of coronal rat brain sections depicting reconstructed biosensor placements within the mPFC. Black lines represent the glutamate-sensitive region of each sensor. Coordinates are mm forward of bregma. **(A)** Sensor placements for MK801 dose-response experiment (n = 14 rats; two rats were excluded due to probe placements outside of the mPFC (shown in red)). **(B)** Sensor placements for mGluR5 modulation experiments (n = 11 rats). Drawings adapted from Paxinos and Watson (1998).

Fig.2. Effect of NMDA receptor antagonism, by 0.03 or 0.06 mg/kg MK801 (s.c.), on extracellular glutamate efflux in the mPFC. **(A)** All rats received vehicle (n = 12 rats; s.c.; time bin zero), followed by vehicle (grey) (n = 2 rats; individual data shown) or MK801 (0.03 mg/kg (n = 4 rats) or 0.06 mg/kg (n = 6 rats) one hour later. Data points represent mean ± 1SEM. Repeated measures ANOVA (mixed design) (main effect of drug, * $p < 0.05$ vehicle versus MK801 (up to 1 hour post injection)). **(B)** AUC presented, as a histogram, for vehicle and MK801 treatment, one-hour post-injection. **(C)** Summary of experimental timeline (BL = baseline).

Fig.3. Effect of the positive allosteric mGluR5 modulator, ADX47273, on extracellular glutamate efflux in the mPFC (n = 10 rats). **(A)** All rats received vehicle (p.o.; time bin zero). Three hours

later, all rats received ADX47273 (100 mg/kg, p.o.; time bin zero). Data points represent mean $\pm$ 1SEM. Repeated measures ANOVA, Bonferroni *post hoc*, * $p < 0.05$, ** $p < 0.01$, ADX47273 versus vehicle treatment. (B) AUC presented, as a histogram, for vehicle and ADX47273 conditions. (C) Summary of experimental timeline (BL = baseline).

Fig.4. Effect of the negative allosteric mGluR5 modulator, RO4917523, on extracellular glutamate efflux in the mPFC (n = 8 rats). (A) All rats received vehicle (p.o.; time bin zero). Three hours later, rats received RO4917523 (0.3 mg/kg, p.o.; time bin zero). Data points represent mean $\pm$ 1SEM. Repeated measures ANOVA, Bonferroni *post hoc*, * $p < 0.05$, ** $p < 0.01$, RO4917523 versus vehicle condition. (B) AUC presented, as a histogram, for vehicle and RO4917523 conditions. (C) Summary of experimental timeline (BL = baseline).

Fig.5. Effect of pre-treatment with ADX47273 on glutamate efflux in the mPFC evoked by the NMDA receptor antagonist MK801 (n = 10 rats). (A) Rats received vehicle (p.o.) or ADX47273 (100 mg/kg, p.o.; time bin zero), followed by vehicle (s.c.) or MK801 (0.03, s.c.) 80 min later. Data points represent mean $\pm$ 1SEM. Repeated measures ANOVA. (B) AUC presented as a histogram, for vehicle or ADX47273 or MK801 or ADX47273 and MK801 treatment (two hours post MK801/vehicle injection). (C) Summary of experimental timeline (BL = baseline).

Fig.6. Illustrative diagram showing putative mechanisms underlying the effects of NMDA receptor antagonism and allosteric mGluR5 modulation on glutamate efflux in the mPFC. (A) NMDA receptor antagonism inhibits GABA-ergic neurons leading to a disinhibition of glutamatergic neurons and increased glutamate efflux. (B) Positive allosteric mGluR5 modulation activates GABA-ergic neurons and glutamatergic neurons *via* the activation of pre- and post-synaptic mGluR5 resulting in increased glutamate efflux. (C) NMDA receptor antagonism and positive allosteric mGluR5 modulation leads to a reversal of (i) NMDA receptor antagonist-induced disinhibition; (ii) post-synaptic mGluR5 activation of glutamatergic neurons; (iii) pre-synaptic mGluR5 autoreceptor activation, leading to increased glutamate efflux. (D) Negative allosteric mGluR5 modulation inhibits GABA-ergic neurons and glutamatergic neurons leading to decreased glutamate efflux. This is a hypothesised proposal since we did not assess GABA-ergic mechanisms.

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Figure 1.

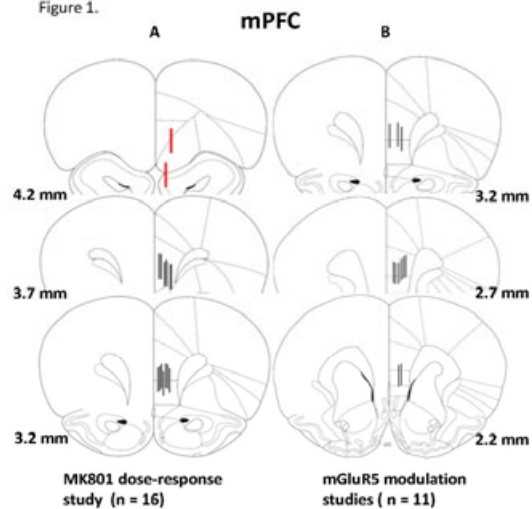


Figure 2.

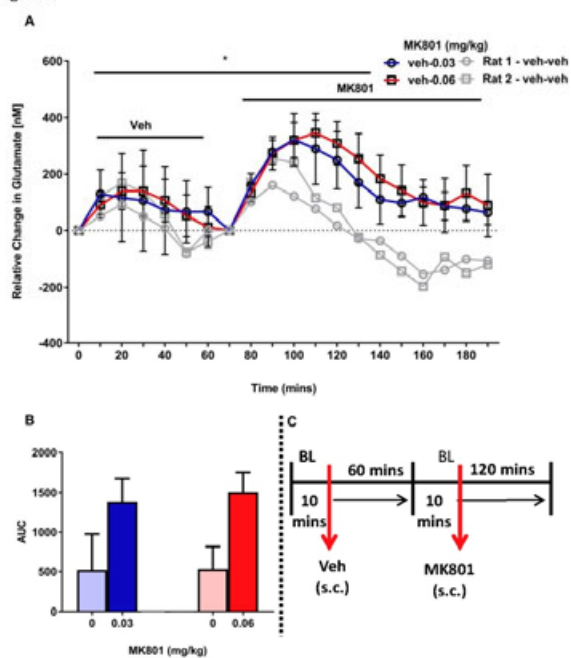


Figure 3.

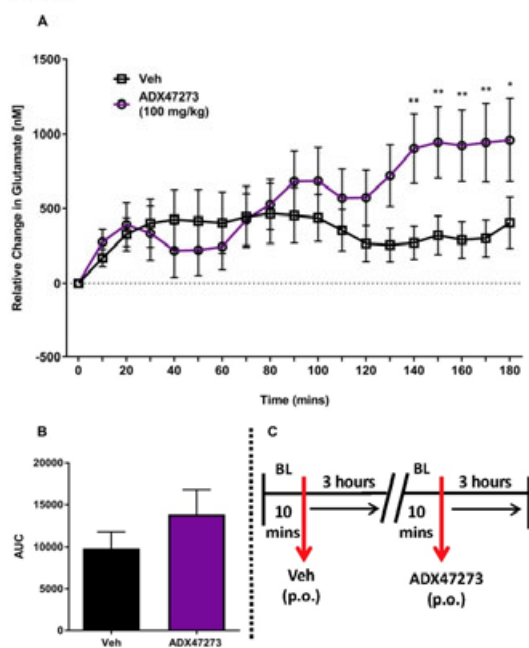


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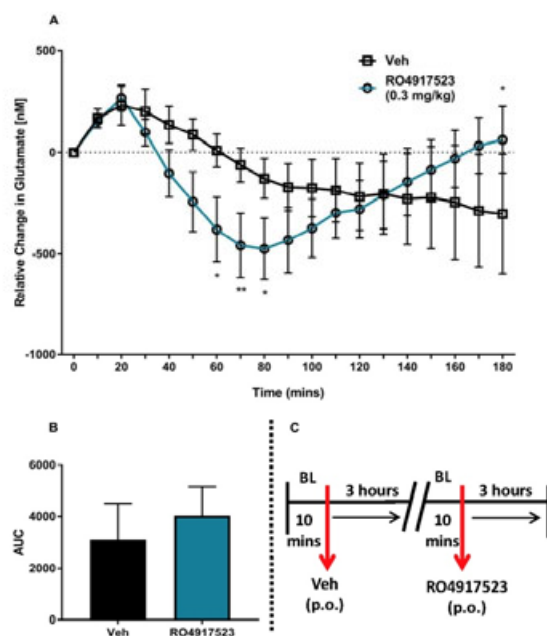


Figure 5.

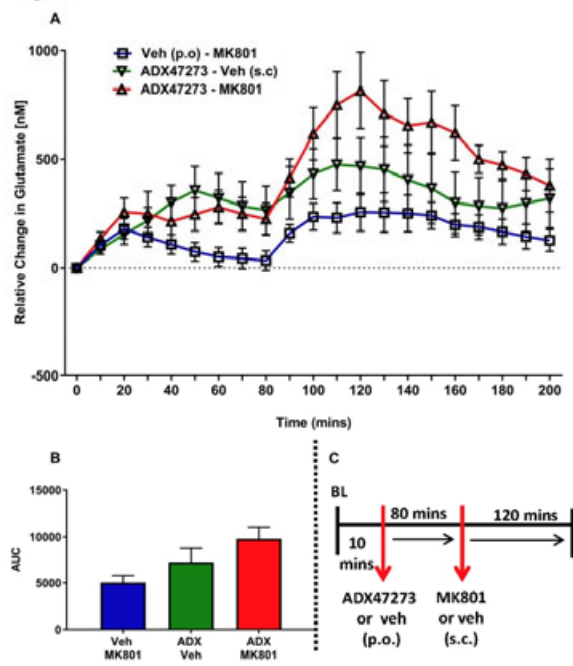


Figure 6.

