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In vitro characterisation of the novel positive allosteric modulators of the $mGlu_5$ receptor, LSN2463359 and LSN2814617, and their effects on sleep architecture and operant responding in the rat

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ABSTRACT

The demonstrated functional interaction of metabotropic glutamate 5 ($mGlu_5$) receptors with N-methyl-D-aspartate (NMDA) receptors has prompted speculation that their activation may offer a potential treatment for aspects of schizophrenia. Development of selective $mGlu_5$ agonists has been difficult, but several different positive allosteric modulator (PAM) molecules have now been identified. This study describes two novel $mGlu_5$ PAMs, LSN2463359 (N-(1-methylethyl)-5-(pyridin-4-ylethynyl)pyridine-2-carboxamide) and LSN2814617 [(7S)-3-tert-butyl-7-[3-(4-fluorophenyl)-1,2,4-oxadiazol-5-yl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-A]pyridine], which are useful tools for this field of research. Both compounds are potent and selective potentiators of human and rat $mGlu_5$ receptors *in vitro*, displaying curve shift ratios of two to three fold in the concentration–response relationship to glutamate or the glutamate receptor agonist, DHPG, with no detectable intrinsic agonist properties. Both compounds displaced the $mGlu_5$ receptor antagonist radioligand, [^{3}H]MPEP *in vitro* and, following oral administration reached brain concentrations sufficient to occupy hippocampal $mGlu_5$ receptors as measured *in vivo* by dose-dependent displacement from the hippocampus of intravenously administered MPEPy. *In vivo* EEG studies demonstrated that these $mGlu_5$ PAMs have marked wake-promoting properties but little in the way of rebound hypersomnolence. In contrast, the previously described $mGlu_5$ PAMs CDPPB and ADX47273 showed relatively poor evidence of *in vivo* target engagement in either receptor occupancy assays or EEG disturbance. Wake-promoting doses of LSN2463359 and LSN2814617 attenuated deficits in performance induced by the competitive NMDA receptor antagonist SDZ 220,581 in two tests of operant behaviour: the variable interval 30 s task and the DMTP task. These effects were lost if the dose of either compound extended into the range which disrupted performance in the baseline DMTP task. However, the improvements in response accuracy induced by the $mGlu_5$ potentiators in SDZ 220,581-treated rats were not delay-dependent and, therefore, perhaps more likely reflected optimization of general arousal than specific beneficial effects on discrete cognitive processes. The systematic profiling of LSN2463359 and LSN2814617 alongside other previously described molecules will help determine more precisely how $mGlu_5$ potentiator pharmacology might provide therapeutic benefit.

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Abbreviations: ADX47273, [S-(4-fluorophenyl)-{3-[3-(4-fluorophenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone]; ANCOVA, analysis of covariance; ANOVA, analysis of variance; CDPPB, 3-Cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; CHPG, (RS)-2-chloro-5-hydro-xyphenylglycine; CT, circadian time; DMTP, delayed-matching-to-position; EEG, electroencephalogram; mGlu, metabotropic glutamate receptor; EMG, electromyogram; LMA, locomotor activity; LSN2463359, N-(1-methylethyl)-5-(pyridin-4-ylethynyl)pyridine-2-carboxamide; LSN2814617, (7S)-3-tert-butyl-7-[3-(4-fluorophenyl)-1,2,4-oxadiazol-5-yl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-A]pyridine; MPEPy, 3-Methoxy-5-(pyridin-2-ylethynyl)pyridine; NMDA, N-methyl-D-aspartate; PAM, positive allosteric modulator; RFU, relative fluorescent unit; SDZ 220,581, ((S)-α-amino-2′-chloro-5-(phosphonomethyl)[1,1′-biphenyl]-3-propanoic acid); VI, variable interval.

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1. Introduction

The neurotransmitter glutamate can mediate signal transduction events via ionotropic and metabotropic receptors, and as such represents one of the predominant means of excitatory signalling in the brain. Ionotropic and metabotropic glutamate (mGlu) receptors subserve different types of signal transduction events in neurons and glia, both from the perspective of temporal dynamics of the signal generated and the molecular pharmacological interactions of the signal. By convention, mGlu receptors for glutamate have been classified into three main groups (Groups I, II and III) according to their sequence homology, pharmacology and coupling to second messenger signalling pathways. Group I receptors comprise two subtypes, $mGlu_1$ and $mGlu_5$ receptors, both of which couple via G_q to activation of phospholipase C and hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to generation of the second messengers diacyl glycerol and inositol triphosphate (Schoepp et al., 1999). In addition to this classical G-protein mediated intracellular signalling mechanism, G-protein independent signalling pathways have also recently been described for group I mGlu receptors (Emery et al., 2010).

A notable feature of $mGlu_5$ receptors is their ability to positively modulate ionotropic glutamate transmission via the N-methyl-D-aspartate (NMDA) receptor complex. This was first demonstrated by Doherty et al. (1997) using the $mGlu_5$ selective receptor agonist, (RS)-2-chloro-5-hydroxy-phenylglycine (CHPG). In this study, NMDA-induced depolarisation was enhanced in CHO cells expressing $mGlu_5$ receptors following CHPG administration, but not in those expressing $mGlu_1$ receptors. Since this initial report, there have been numerous other examples described of $mGlu_5$-NMDA receptor interactions, both *in vitro* and *in vivo* (Awad et al., 2000; Attucci et al., 2001; Mannaioni et al., 2001; Pisani et al., 2001; Kinney et al., 2003, 2005, 2006; Lecourtier et al., 2007). Such results have significantly influenced thinking on the potential therapeutic utility of novel ligands with affinity for the $mGlu_5$ receptor (Bird and Lawrence, 2009; Cleva and Olive, 2011). Amongst the indications proposed, the ability of $mGlu_5$ receptor activation to positively modulate NMDA-induced synaptic currents has suggested the possibility that $mGlu_5$ receptor agonists might have antipsychotic-like effects (Mannaioni et al., 2001). Indeed, from the opposite perspective, the behavioural response to NMDA receptor antagonists can be enhanced by antagonists of $mGlu_5$ receptors (Campbell et al., 2004) whilst, similar to treatment with PCP, mice in which the $mGlu_5$ receptor gene has been deleted show deficits in prepulse inhibition of the auditory startle response (Kinney et al., 2003), a response often diminished in schizophrenic patients (Swerdlow et al., 2008).

Development of clinically viable, selective $mGlu_5$ receptor orthosteric agonists has been unsuccessful to date, for a number of reasons (Conn et al., 2009). In the search for molecules that will selectively enhance $mGlu_5$ activity, positive allosteric modulators (PAMs) offer practical and theoretical advantages over orthosteric agonists, not least being the possibility of overcoming the narrow structural requirements (and hence lack of specificity) associated with interaction with orthosteric binding sites on metabotropic glutamate receptors (Conn and Pin, 1997). Theoretically, PAMs also offer the possibility of amplifying ongoing, physiological tonic and phasic glutamatergic signalling since their effects are most apparent in the presence of released transmitter. In contrast, orthosteric agonists will amplify neurotransmission independent of glutamate release (May et al., 2004). Such increased physiological specificity should provide benefit in terms of precision of the ultimate preclinical and clinical behavioural responses, hopefully affording more potential efficacy-related observations and less adverse effects.

In the present report, we describe some of the *in vitro* and *in vivo* pharmacological properties of the novel $mGlu_5$ positive allosteric modulators, LSN2463359 (N-(1-methylethyl)-5-(pyridin-4-ylethynyl)pyridine-2-carboxamide) and the structurally unrelated molecule LSN2814617 [(7S)-3-tert-butyl-7-[3-(4-fluorophenyl)-1,2,4-oxadiazol-5-yl]-5,6,7,8-tetrahydro[1,2,4]triazolo[4,3-A]pyridine]. These molecules represent important new tools for investigation of the role of $mGlu_5$ receptors in normal and disease states, and offer *in vitro* and *in vivo* properties that surpass many of the presently available $mGlu_5$ PAMs.

2. Methods

2.1. In vitro assays

2.1.1. Primary cortical cultures

Pregnant Sprague-Dawley rats (Harlan, Bicester, Oxon) were killed by cervical dislocation following exposure to a rising concentration of CO_2. Cortices of 18-day old foetuses were dissected, trypsinized, triturated and isolated cells were plated into black-walled, transparent bottomed, poly-D-lysine coated 96-well FLIPR plates (Corning or Becton–Dickenson) at a density of 6.6×10^5 cells/ml, in a volume of 0.1 ml of neurobasal medium containing B27 supplement (GIBCO BRL, Gathersburg, MD). Cultures were maintained for 7–10 days in a humidified atmosphere containing 95% air and 5% CO_2, at 37 °C.

2.1.2. Recombinant cell lines

AV-12 cell lines stably expressing recombinant human and rat $mGlu_{5a}$ receptors were cultured in DMEM with high glucose and pyridoxine hydrochloride supplemented with 5% heat inactivated, dialysed fetal bovine serum, 1 mM sodium pyruvate, 10 mM (hu) or 1 mM (rat) HEPES, 0.75 mg/mL geneticin, 0.25 mg/mL hygromycin B and 1 mM L-glutamine. Confluent cultures were passaged biweekly and maintained in a 37 °C humidified incubator with 95% air and 5% CO_2. Cells were harvested 24 (human) or 48 (rat) hours prior to assay using 0.25% trypsin–EDTA and plated at a density of 65,000 (human) or 50,000 (rat) cells per well in poly D lysine-coated (human) or tissue culture treated (rat) 96-well FLIPR plates in complete growth medium.

AV-12 cell lines stably expressing human mGlu1, mGlu2/Gα15, mGlu3/Gα15, mGlu4/Gα15, $mGlu_5$, mGlu7/Gα15, mGlu8/Gα15 and $GABA_B$ receptors with rat glutamate transporter EAAT1 were maintained by culturing in DMEM with high glucose and pyridoxine hydrochloride (without L-glutamine and sodium pyruvate) supplemented with 5% heat inactivated, dialysed fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES and varied concentrations of L-glutamine and selection agents (geneticin, hygromycin B, zeocin, and blasticidin), depending on the cell line. Confluent cultures were passaged biweekly. Cells were harvested 24 h prior to assay and plated in a variable concentration of glutamine and at a variable density of cells per well, depending on cell line, in poly-D-lysine coated 96-well FLIPR plates.

2.1.3. FLIPR-based assays

Receptor-mediated changes in intracellular calcium concentration were determined using a calcium-sensitive fluorescent dye, Fluo3AM (Invitrogen) and a fluorometric imaging plate reader (FLIPR – Molecular Devices Corporation, Sunnyvale, CA, USA). The Hank's Balanced Salt Solution (HBSS) assay buffer, supplied by Invitrogen (Gibco 14025-050), was supplemented with 10 mM (rat $mGlu_5$) or 20 mM (all other cell lines) HEPES and adjusted to pH 7.2. At the start of the assay, growth media was removed from the 96-well cell plates by inversion and gentle tapping and media was replaced with assay buffer containing 8–10 μM Fluo-3AM/0.05% pluoronic F-127. Cell plates were stored in the dark at room temperature for 75–120 min, depending on the cell line, to allow dye loading into cells. Subsequently, the dye solution was removed and replaced with assay buffer at which stage cell plates were transferred to the FLIPR for assay. 1 μM TTX was included in the assay buffer when using rat cortical neurons.

Intracellular calcium levels were monitored before and after the addition of compounds, with data collection ranging from 1 image every second to 1 image every 3 s. A single-addition FLIPR assay generating a 10-point concentration response curve for the agonist glutamate or DHPG was conducted prior to each experiment. The results were analysed (GraphPad Prism v4) to calculate the concentration of agonist needed to induce EC_{90} (antagonist assay) and EC_{10-25} (potentiator assay) responses. Responses were measured as the maximal peak height in Relative Fluorescent Units (RFUs), and the assay window was defined as the maximal response obtained by agonist-stimulated wells. All RFU values were corrected for basal fluorescence in the absence of agonist. Antagonist effects were quantified by calculating the percent inhibition of the EC_{90} glutamate response caused by the compound. Agonist effects were quantified as percent stimulation induced by compound alone relative to the maximal glutamate or DHPG response. Potentiator effects were quantified as percent increase in agonist EC_{10-25} response relative to the maximal agonist response. All data were calculated as relative IC_{50} or EC_{50} values using a four-parameter logistic curve fitting program (ActivityBase v6.1.2.15. or Excel Fit 4 v4.2.1).