

Pharmacological characterisation of *N*-[(2*S*)-5-(6-fluoro-3-pyridinyl)-2, 3-dihydro-1*H*-inden-2-yl]-2-propanesulfonamide: a novel, clinical AMPA receptor positive allosteric modulator

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Abbreviations: AMPA, α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; AMPAR, AMPA receptor; CNS, central nervous system; GluA, subunit of AMPA subtype ionotropic glutamate receptor; GRIA, gene encoding subunit of AMPA subtype ionotropic glutamate receptor ; LTD, long term depression; LTP, long term potentiation; MEST, maximal electroshock threshold test; NMDA, *N*-methyl-D-aspartic acid; NOR, novel object recognition test; sem, standard error of means; TARP, transmembrane AMPA receptor regulatory protein

Abstract

Background and Purpose

AMPA receptor positive allosteric modulators represent a potential therapeutic strategy to improve cognition in people with schizophrenia. These studies collectively constitute the pre-clinical pharmacology data package used to build confidence in the pharmacology of this molecule and enable a clinical trial application.

Experimental Approach

[*N*-[(2*S*)-5-(6-fluoro-3-pyridinyl)-2,3-dihydro-1*H*-inden-2-yl]-2-propanesulfonamide] (**UoS12258**) was profiled in a number of *in vitro* and *in vivo* studies to highlight its suitability as a novel therapeutic agent.

Key Results

We demonstrated that **UoS12258** is a selective, positive allosteric modulator of the AMPA receptor. At rat native hetero-oligomeric AMPARs **UoS12258** displayed a minimum effective concentration of approximately 10 nM *in vitro* and enhanced AMPAR-mediated synaptic transmission at an estimated free brain concentration of approximately 15 nM *in vivo*. **UoS12258** reversed a delay-induced deficit in novel object recognition (NOR) in rats

after both acute and sub-chronic dosing. Sub-chronic dosing reduced the minimum effective dose from 0.3 mg/kg to 0.03 mg/kg. **UoS12258** was also effective at improving performance in two other cognition models i.e. passive avoidance in scopolamine-impaired rats and water maze learning and retention in aged rats. In side-effect profiling studies, **UoS12258** did not produce significant changes in the maximal electroshock threshold (MEST) test at doses below 10 mg/kg.

Conclusion and Implications

We conclude that **UoS12258** is a potent and selective AMPAR modulator exhibiting cognition enhancing properties in several rat behavioural models superior to other molecules which have previously entered clinical evaluation.

1. Introduction

Many lines of evidence support the premise that enhancing AMPAR (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) function should be a viable route to treating a variety of disorders that are underpinned by a hypoglutamatergic state, such as schizophrenia (Goff 2001, Ozawa 1998, Sanacora 2008, Zarate 2008). The AMPA receptor itself is a family of tetrameric ionotropic receptors arising from four genes that encode four distinct subunits, named GluA1-4, or previously GluR1-4/A-D (Collingridge et al 2009). This diversity of subunits gives rise to heterogeneity of AMPAR composition in native brain states (Benyeto 2004), further complicated by both the presence of splice variants and post-translational editing (Seeburg 2001, Sommer 1990). Two main sub-populations arise from the editing of a Ca^{2+} -permeable glutamine residue in the re-entrant M2 loop of the GluA2 subunit to a Ca^{2+} -impermeable arginine, which renders the edited receptors permeable to just Na^+ and K^+ (Gouaux 2004). Other than this re-entrant M2 loop, each subunit also comprises three transmembrane domains (M1, 3, 4), an intra-cellular C-terminal domain, key for regulation of trafficking, as well as an extensive extracellular N-terminal domain containing both the glutamate-binding site domain (commonly referred to as the ligand-binding or agonist-binding domain) and an N-terminal domain (Traynelis 2010). Functionally active AMPARs can be formed from any combination of the GluA subunits, although different combinations of different subunits display different biophysical and kinetic properties as well as distinct regional distributions and trafficking potential (Herguedas 2016). Specifically for

distribution, GluA1, GluA2 and GluA3 subunits are found at high levels in the hippocampus, basal ganglia, amygdala, lateral septum and cerebellum in rat and macaque, whereas GluA4 is most highly expressed in the cerebellum (Boutler 1990, Keinanen, 1990, Beneyto 2004). Glutamate is the major excitatory neurotransmitter, and AMPA receptors, are responsible for mediating the majority of fast, excitatory synaptic transmission, and as such, offer a great opportunity for modulation for therapeutic benefit. Repetitive AMPAR stimulation leads to activation of another group of ionotropic glutamate receptors, NMDA receptors, by voltage-mediated release of magnesium ion block. This enables greater influx of calcium ions into the post synaptic neuron, and onward synaptic transmission and processes required for memory deposition and learning, which are further strengthened by increased trafficking of AMPA receptors to the membrane (Lynch 2002).

1.1 Glutamate receptors and schizophrenia

The considerable body of data linking glutamate receptors to schizophrenia comprises both empirical observations and genetic associations. Studies in schizophrenic patients have identified various supporting lines of data, including reduced glutamate concentrations in cerebro-spinal fluid (Kim 1980), reduced hippocampal glutamate from *post mortem* studies (Harrison 2000), reduced glutamate neurotransmission, reduced carboxypeptidase II (Tsai 2004) and increased *N*-acetyl-aspartyl glutamate (endogenous iGluR antagonist) (Jessen 2013). Additionally, evidence from immunocytochemical studies on different brain regions of patients as well as analysis of mRNA transcript level changes in hippocampal regions of schizophrenia brains indicates reductions in AMPAR levels in prefrontal & temporal cortical and thalamic regions that are associated with the performance of cognitive tasks (Akbarian 1995, Wright 2012). Wider evidence comes from the NMDA receptor, for which it is clear that administration of an NMDAR blocker, such as phencyclidine (PCP) or ketamine, is able to both amplify the symptoms of schizophrenia in schizophrenic patients (Lahti 1995), and also induce a 'schizophrenia-like' state including psychosis and disrupted cognition in healthy volunteers (Lahti 2001). These data are more compelling for NMDAR blocking agents than for other agents, such as dopamine-releasers such as the amphetamines (Le Pen 2003).

1.2 AMPAR positive modulators

Given the evidence associating reduced AMPA receptor-mediated neurotransmission with various disease states, it is not surprising that considerable effort has been expended in trying to identify ways to increase glutamatergic transmission. Direct activation approaches suffer from the loss of spatial and temporal control of activation of endogenous glutamate binding and also will run a significant safety and tolerability risk of generating undesirably high excitatory signalling levels. To overcome both of these liabilities, positive allosteric modulators have been targeted over the recent decades, such that the glutamate signalling will be enhanced if, and only if, both glutamate and positive modulator are bound at the receptor. A range of molecules has been reported, from various groups, which have been studied in different pre-clinical species as well as clinically. These molecules have generated considerable *in vitro* data to support their mechanisms of action, and in particular, have been shown to be selective for the AMPAR subtype of glutamate receptor over the other iGluR and mGluR receptor subtypes (unlike the majority of orthosteric-site binding ligands). In addition to effects directly at the receptor, these molecules have demonstrated potentiation in native tissue and hippocampal slice preparations, particularly generating facilitation of polysynaptic responses (Arai 1996, Lynch 2002), long-term potentiation, increased BDNF levels and activity in a wide range of animal models of learning and memory (Woolley 2009). Specifically for models of cognition, molecules have demonstrated improved performance in olfactory discrimination, radial arm maze (Staubli et al 1994), conditioned fear (Rogan et al 1997), water maze performance (Zivkovic et al 1995; Quirk and Nisenbaum 2001), delayed non match to sample (Hampson et al 1998), novel object recognition (Lebrun et al 2000), passive avoidance (Lebrun 2000, Quirk and Nisenbaum 2001) in rodents and have also demonstrated encouraging results in non-human primates (Thompson et al 1995, Buccafusco 2004, Porrino 2005). In addition to the behavioural pharmacology understanding, the knowledge of the overall structure of the receptor offers the opportunity to exploit structure-based design opportunities against the extracellular binding domains (Baranovic 2016).

1.3 AMPAR positive modulators – clinical studies

Although there have been many reports of AMPAR positive allosteric modulators in pre-clinical studies, relatively few have been progressed beyond Phase I into patient studies. The initial clinical evaluation was driven by the set of CX molecules originating from the

University of California, subsequently as Cortex, from the pioneering work of Gary Lynch. These molecules initially demonstrated effects in healthy young (Ingvar et al., 1997) and aged volunteers (Lynch et al., 1997), as well as in schizophrenic patients stabilised on the atypical antipsychotic drug clozapine (Goff et al 2001), but were subsequently ineffective in a later study (Goff et al., 2008). Studies with later compounds have generated effects in attention deficit hyperactivity disorder (ADHD) patients (2006, Weisler 2007). The second major class of the phenethylamine sulphonamides produced two clinical stage molecules from Lilly, one of which was reported as negative in a Phase II study of cognition in Alzheimer's disease, as well as molecules from GlaxoSmithKline (Ward 2011, 2010) and Pfizer (Shaffer 2015).

2. Methods

2.1 Compound

The chemical structure of **UoS12258** is shown in Figure 1. The preparation and preliminary characterization of **UoS12258** has been described previously (Ward 2010).

Figure 1

2.2 Intracellular Ca^{2+} influx assay

AMPA receptors: Human Embryonic Kidney (HEK293) sticky cells (293 TAG/hsr-A pCIP4) were grown in a Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM/F-12) supplemented with 10 % foetal bovine serum (FBS), 2 mM glutamine, and 1.5 ug/mL puromycin in a 5 % CO₂ humidified atmosphere at 37 °C. Transient transfection was performed according to the lipofectamine 2000 method. Briefly, a sub-confluent T-175 cm² flask was washed with PBS to remove serum and 20 mL of optemem were added. Separately, in 10 mL optemem, 100 µg DNA were pre-incubated with 300 µL of lipofectamine for 20 min at room temperature and then added to the flask. After 4 h of incubation at 37 °C 5 % CO₂, cells were detached with versene and resuspended in the post transfection medium (DMEM supplemented by 10 % of dialyzed FBS). The confluent monolayer for the functional assay was obtained by seeding 7500cell/well 2 days prior the experiment in 384-well black with clear bottom, poly-D-lysine coated plates (Greiner).

The following homomeric AMPAR subtypes were studied: human GluA1 flip isoform (hGluA1i), hGluA3i and hGluA4i and rat GluA2i using DNAs (all Q-unedited in TM2 region

to allow Ca^{2+} permeability) hGRIA1i, 3i and 4i and rGRIA2i. Human GluA2i was studied using HEK293-hGRIA2i Q-unedited RC17 cell line that was established in house.

NMDA receptors: HEK293 sticky cells, grown at 80-95% confluency in 175cm² T-flask, were transduced by addition of 5% NR1A-BacMam / 25% NR2B-BacMam mixture in 15 mL of medium containing DMEM/F-12, 10% dialyzed FCS, 0.3 mM MgCl_2 , 500 μM ketamine hydrochloride (non-competitive NMDAR antagonist). Cells were incubated with the transduction mixture at 37 °C in 5% CO_2 for 24 h. Then, BacMam was washed away with PBS, and cells were plated onto poly-D-lysine coated black/clear plates at a density of 20,000 cells/well, always in presence of 500 μM ketamine hydrochloride. After additional 24 h cells were tested in FLIPR assay.

Kainate receptors: Transient transfection was performed according to the lipofectamine 2000 method. Briefly, a sub-confluent T-175cm² flask was washed with PBS to remove serum and added by 20 mL of optimem. Separately, in 10 mL optimem, 60 μg pcDNA3.2-human GRIK1 (Q/unedited) were pre-incubated with 300 μL of lipofectamine for 20 min at room temperature and then added to the flask. After 4 h incubation at 37 °C 5% CO_2 , cells were detached with versene and resuspended in the post transfection medium (DMEM supplemented by 10% of dialyzed foetal bovine serum). The confluent monolayer for the functional assay was obtained by seeding 7,500 cells/well one day prior the experiment in 384-well black with clear bottom, poly-D-lysine coated plates.

Intracellular calcium levels through AMPA receptors were measured. Buffer comprised (mM): 20 HEPES, 145 NaCl, 5 KCl, 1 MgCl_2 , 2 CaCl_2 , 5.5 glucose, pH 7.3 with NaOH. On the day of the experiment, cell plates were washed 3-times with the assay buffer using the EMBLA instrument (Skatron) and 20 μL of buffer was left in each well. 20 μL of Fluo-4AM (Molecular Probes) buffer was added to each well to give a final concentration of 2 μM and cells were then incubated at room temperature for 60 min in the dark. After the loading incubation, cell plates were washed 3-times to remove unloaded Fluo-4 and 30 μL of buffer was left in each well. Then cell plates were placed into the Fluorescent Imaging Plated Reader (FLIPR) instrument with 1 W laser power, 10 % gain and 0.4 s exposure and a dual addition protocol of 10 min fluorescence measurement was used. Compounds were received in 384-well plates at a concentration of 10 mM. A serial dilution (1:3) was performed using a Beckman FX. A copy of the serial dilution plates was prepared and diluted with buffer immediately prior to the experiment. The first 10 μL addition into a well was from a buffer

pre-diluted compound plate and it was followed by 10 minutes incubation. AMPA positive modulators did not give rise to detectable calcium increases when applied in the absence of glutamate. Then in the second 10 μ L addition glutamate was applied to give a final concentration of 100 μ M. Glutamate caused a dramatic increase in FLIPR counts when a positive modulator was pre-applied. Cyclothiazide (Sigma Aldrich) 150 μ M, a known AMPA receptor positive modulator, was used as positive control.

Intracellular calcium measurements through NMDA receptors were similar to that described for AMPA receptors except for the addition of 500 μ M ketamine hydrochloride during labelling with FLUO-4. Calcium influx measurements through kainate receptors were also similar to that described for AMPA receptors except for the addition of concanavalin A (Sigma Aldrich) 0.125 mg/mL which reduced the level of receptor desensitisation.

Assay quality was determined by calculating the Z' value: high (150 μ M cyclothiazide and 100 μ M glutamate) and low controls (only 100 μ M glutamate) were always present within each 384 well plate. FLIPR kinetics data were transformed to a single end point using the peak high of the response. Values were then normalized as the specific percent increase from the 100 μ M glutamate effect (0 %) to the maximal cyclothiazide positive modulator effect (150 μ M) in the presence of the 100 μ M glutamate stimulation (100 %). In general, data from plates were only accepted if $Z' > 0.3$ and 2 out of three pharmacological standards had values that were within 3x Standard Deviation of historical values. pEC_{50} for **UoS12258** was calculated using a non-linear, 4 parameter logistic curve-fitting program.

2.3 Recombinant receptor electrophysiology

AMPA receptors: HEK293-hGluA2i unedited RC17 cells were grown in a DMEM medium supplemented with 10% FBS, 2 mM glutamine, 10% non-essential amino acids, 1% penicillin/streptomycin and 500 μ g/mL geneticin (G418) in a 5% CO₂ humidified atmosphere at 37 °C. Cells were plated on glass treated with poly-D-lysine (BD BioCoat coverslips, BD Biosciences Cat.No. 354086) at a density of 50,000-75,000 cells/mL (2mL of cell suspension were applied to 35x10 mm Petri dish containing the glass coverslips), kept at 30°C and used, respectively, 1 and 2 days after plating. Experiments were carried out at room temperature.

NMDA receptors: HEK293-MSR2 cells (passages 19-23) were seeded onto 12 mm poly-D-lysine coated coverslips (100,000 cells per 35 mm petri dish holding 3 coverslips) and the cells were transfected 24 h after seeding. The following DNAs: hNR1A (0.55 μ g), hNR2B (2.8 μ g) and pCMS-EGFP (0.8 μ g) were added to the petri dish in the presence of opti-mem 1

(0.25 mL; Invitrogen) and lipofectamine 2000 (9 μ L) and incubated for 4 h at 37 °C in an atmosphere of 5% CO₂. After rinsing off the transfection medium, the cells were maintained in culture medium containing 0.3 mM MgSO₄ plus 200 μ M D-2-amino-5-phosphonovaleric acid (D-AP5; NMDA receptor antagonist), at 30 °C in an atmosphere of 5% or 10% CO₂ for 24 h prior to commencing electrophysiological recordings.

The external recording solution comprised (in mM), 140 NaCl, 2 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose, and 12 HEPES, pH 7.35 (with NaOH). The intracellular solution comprised (in mM) 150 CsCl, 10 EGTA, and 10 HEPES, pH 7.3 (with CsOH). For perforated patch-clamp recordings, intracellular solution containing 240 μ g/mL amphotericin B was used to backfill the pipette while intracellular solution alone was used to fill the tip. Glutamate (Sigma Aldrich) was dissolved in H₂O to generate a 1 M stock solution. Cyclothiazide (Sigma Aldrich) was dissolved in DMSO to generate a 100 mM stock solution.

Whole-cell currents were recorded from cells using the perforated patch-clamp technique (Hamill et al., 1981; Sherman-Gold, 1993), using the EPC9 or EPC10 patch-clamp system and the Pulse program (HEKA). Patch pipettes were pulled from thin-wall borosilicate glass capillary (1.5 mm outer diameter) using a P-97 pipette puller (Sutter Instruments) and had resistances of 2-5 M Ω . Cells were discarded unless the seal formation permitted low resistance access within 10 min (series resistance \leq 20 M Ω) due to the amphotericin B. Results were not used when the access resistance changed significantly during the experiment. Exchange of solution around the cell was achieved by holding the cell in front of one of 16-48 channels with a constant solution flow and quickly (10-50 ms) moving the chip to place an adjacent channel in front of the cell (Dynaflow).

The cell was clamped at -60 mV. Automatic series resistance and capacity compensation were applied and checked regularly. The current induced by a 1 s application of the agonist was recorded. The agonist application was repeated every 60 s. Control currents elicited by a fixed concentration of agonist (3 mM, approximate EC₅₀) were recorded during the experiment in order to monitor any variations in charge. For the concentration response curve, the area under the curve of the current for the first 500 ms (charge) was measured for the control application of glutamate alone and for glutamate in the presence of increasing concentrations of the positive modulator (5 different concentrations from 10 nM up to 100 μ M) in the same cell. Cyclothiazide (Sigma Aldrich) 30 μ M, a known AMPA receptor positive modulator, was used as positive control.

Concentration response curves for positive modulator were fitted to the equation of the form:

$$Y = \text{Top} / (1 + 10^{(\text{Log EC}_{50} - X) * \text{Hill Slope}})$$

Y is the response in % normalised to 30 μM cyclothiazide, Top is the extrapolated or asymptote maximal % of potentiation, X is the logarithm of concentration. Y starts at 0 and goes to the top with a sigmoid shape. Graphs were constructed by averaging the results from all experiments and fitting a single curve to the pooled data.

2.4 Native receptor electrophysiology

Neuronal cultures were prepared from embryonic rat brains harvested following sacrifice of the pregnant female by CO_2 inhalation in accordance with GlaxoSmithKline animal welfare guidelines and the U.K. Animals (Scientific Procedures) Act 1986. The dissected hippocampi were placed into ice-cold Hank's Balanced Salt Solution (HBSS) comprised of: pyruvate, 1 mM; penicillin, 100 mg/mL; streptomycin, 100 mg/mL; HEPES, 10 mM; NaHCO_3 , 0.035%, Ca^{2+} - and Mg^{2+} -free. The tissue was then trypsinised for 30 min at 37 °C in trypsin/EDTA diluted (final concentration 0.05%) in HBSS with sodium pyruvate (Ca^{2+} - and Mg^{2+} -free). Tissue pieces were physically dissociated and neurons were plated onto poly-D-lysine coated coverslips in plating medium comprised of: Neurobasal Medium + 1 mM sodium pyruvate; penicillin, 100 mg/mL; streptomycin, 100 mg/mL; B27 supplement 1x; L-glutamine, 1 mM. Half of the culture medium was replaced twice weekly and the cells were used for recordings after 5 to 13 days in culture.

AMPA receptors: Extracellular solution comprised (in mM), NaCl 145, KCl 2.5, HEPES 10, glucose 10, CaCl_2 1.5, MgCl_2 1.2; pH 7.3 with NaOH. Intracellular solution comprised (in mM), CsF 80, CsCl 80, HEPES 10, MgATP 14, DiTRIS creatine phosphate 14, creatine phosphokinase 50 U/mL; pH 7.3 CsOH. **UoS12258** was dissolved in DMSO to generate a 100 mM stock solution. Test solutions were diluted to concentrations between 10 nM and 100 μM from this stock using extracellular solution (maximum final DMSO concentration $\leq 0.1\%$). AMPA (Tocris-Cookson) was dissolved in H_2O to generate a 10 mM stock. All experiments were performed at 20-21 °C. Whole-cell voltage-clamp recordings from rat cultured hippocampal neurons were made using standard methods (Hamill et al 1981). Briefly, using an Axopatch 200B amplifier and pClamp 8.0 software (Axon Instruments) each cell was held at -70 mV throughout the recording and solution exchange was achieved using a fast-step 2 tube perfusion system (Biologic RSC 160). Control AMPA receptor-

mediated currents were evoked by rapidly moving from normal extracellular solution to extracellular solution containing 30 μ M AMPA, (previously determined approximate EC_{50}) for 2 s then returning to normal extracellular solution for 30 s. This cycle of solution changes was repeated continuously throughout the baseline recording period. The lowest test concentration of **UoS12258** was added to both perfusion tubes and the solution change cycle repeated until a stable current was measured. Test concentrations were increased sequentially. Inward currents induced by AMPA application were complex in that their magnitude and duration depended on three different mechanisms: (1) opening of the AMPA receptor channel measured in terms of peak amplitude, (2) desensitization of AMPA receptors measured as the degree of relaxation of the inward current from its initial peak amplitude to a reduced steady state plateau level in the continued presence of AMPA and (3) deactivation of AMPA receptors measured as the rate at which the inward current decayed back to baseline following termination of the AMPA application.

Peak current amplitude was measured as the difference between baseline current and the maximum inward current detected following fast perfusion application of either AMPA alone or AMPA + **UoS12258**. Peak current amplitude, measured at each concentration of **UoS12258** in the presence of 30 μ M AMPA, was normalised to the peak current amplitude after application of AMPA alone. Desensitisation of AMPA receptors is a measure of the reduction from peak inward current amplitude to a steady-state current influx in the continued presence of AMPA. AMPA receptor deactivation is the rate at which the inward current returns to baseline after agonist removal and is represented by the decay constant, tau, of a mono-exponential curve fitted to the relaxing inward current after AMPA application has stopped. The tau value, measured after each concentration of **UoS12258** in the presence of 30 μ M AMPA, was normalised to the value of tau after application of AMPA alone. Each of these parameters was measured and analysed using Clampfit 8.0 (Axon Instruments), Excel (Microsoft) and Origin (Microcal). The statistical significance of differences in each of the measured parameters described above was assessed using a Student's paired t-test (Excel).

NMDA receptors: Extracellular solution comprised (in mM): NaCl 145, KCl 2.5, HEPES 10, Glucose 10, CaCl₂ 1.5, Glycine (30 μ M) – (Mg²⁺ Free to prevent NMDA receptor block). Intracellular solution comprised (in mM): K Gluconate 140, HEPES 10, NaCl 17, MgATP 4, NaGTP 0.3; pH 7.3 KOH

Baseline whole cell currents were evoked by rapidly exchanging the perfusing medium from extracellular solution to extracellular solution containing 100 μ M NMDA for a period of 2 s. In contrast, test currents were evoked by rapidly exchanging the perfusing medium from extracellular solution containing 100 μ M **UoS12258** to extracellular solution containing 100 μ M **UoS12258** plus 100 μ M NMDA for a period of 2 s. As with baseline currents each test pulse was separated from the next by a 30 s interval.

Kainate receptors: Extracellular solution comprised (in mM): NaCl 145, KCl 2.5, HEPES 10, glucose 10, CaCl₂ 1.5, MgCl₂ 1.2; pH 7.3 with NaOH. Intracellular solution comprised (in mM): CsF 80, CsCl 80, HEPES 10, MgATP 14, DiTRIS Creatine Phosphate 14, Creatine Phosphokinase 50 U/mL; pH 7.3 CsOH. Baseline whole cell currents were evoked by rapidly exchanging the perfusing medium from extracellular solution containing 100 μ M SYM2206 (a selective non-competitive AMPA receptor antagonist) to extracellular solution containing 100 μ M SYM2206 plus 100 μ M kainate for a period of 2 s. In contrast, test currents were evoked by rapidly exchanging the perfusing medium from extracellular solution containing 100 μ M SYM2206 plus 100 μ M **UoS12258** to extracellular solution containing 100 μ M SYM2206 plus 100 μ M **UoS12258** plus 100 μ M kainate for a period of 2 s. As with baseline currents each test pulse was separated from the next by a 30 s interval.

2.5 In vivo electrophysiology

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. GlaxoSmithKline safety regulations were adhered to at all times.

Adult male Lister Hooded rats (Charles River) weighing 243-340 g were housed under a 12 h light/dark cycle with food (Harlan Maintenance Diet) and water available *ad libitum*.

For electrophysiological recordings rats were prepared individually. Specifically, once removed from their home cage they were placed in an induction chamber and anaesthetised with 5% isoflurane in a 1:1 mix of oxygen and nitrous oxide which was adjusted to 2.5% isoflurane to maintain anaesthesia throughout the surgical procedure. Surgery involved cannulating the jugular and femoral veins using Portex tubing such that the cannula projected externally from the animal. Each rat was then placed in a stereotaxic frame and secured with atraumatic ear bars. Body temperature was monitored and maintained at appropriate levels by means of a thermostatically-controlled heated blanket and rectal probe. A midline incision

was made to expose the dorsal aspect of the skull and a burr hole made to allow implantation of a multi-barrelled electrode (Kation Scientific, Hungary) into the CA1 region of the hippocampus (from Bregma: AP -4.2 mm, ML 2.4 mm, depth 1.8-2.2 mm). The multi-barrelled electrode consisted of a carbon fibre recording electrode and 3 glass barrels two of which were filled with the following solutions: one barrel contained 2 % w/v methylene blue dissolved in 0.5 M sodium acetate which acted as the balance for the current ejection and the second barrel contained 5 mM AMPA. A silver ground electrode was also positioned under the skin margin. Isoflurane levels were lowered to 1.75% prior to commencing recordings. Neuronal activity was recorded using a Neurolog NL100 AK head stage connected to a NL104A AC preamplifier. The signal was filtered (0.5 - 5 kHz), passed through a Humbug Noise eliminator and fed into an audio amplifier and a CED 1401 digital analogue interface. Spike 2 software (CED) was used to record neuronal activity. AMPA was ejected via iontophoresis (currents of 4-40 nA for periods of 40 s, repeated at intervals of 40-60 s).

UoS12258 was prepared as a nano-milled formulation in 1 % w/v HPMC (Pharmacoat 603) / 0.1 % sodium lauryl sulphate (Sigma-Aldrich) in purified water. The diluent and vehicle was 1 % w/v HPMC / 0.1 % sodium lauryl sulphate and all i.v. doses of **UoS12258** were administered as 0.05 mL per 100 g. For oral dosing the same batch of **UoS12258** that was used for i.v. dosing was administered (Batch number: VNAA\6768\149\4), vehicle was 1% methylcellulose and dose volume was 2 mL/kg in all cases. AMPA (Sigma-Aldrich) was prepared as 5 mM stock solution in 0.9% saline (pH8).

For acute administration animals were divided into two groups, **UoS12258** (n=4) and saline (n=3). Once a minimum of 5 consistent and consecutive responses to AMPA application had been achieved either **UoS12258** or saline were dosed. In this respect, rats in group one were intravenously dosed with **UoS12258** in a cumulative dosing paradigm consisting of 0.5, 1.0, and 1.5 mg/kg doses delivered at 20 minute intervals. A similar procedure was used for the second group of rats except that 3 doses of saline were administered intravenously at 20 minute intervals. For sub-chronic administration of **UoS12258** animals were divided into four groups: vehicle-vehicle (n=5); vehicle-**UoS12258** (n=5); **UoS12258-UoS12258** (n=5) and **UoS12258**-vehicle (n=5) where the first treatment term indicates how the animals were dosed for the seven day period leading up to the test treatment on day 8; this latter treatment being indicated by the second treatment term. The dose of **UoS12258** used for sub-chronic dosing was 0.03 mg/kg (p.o.) in a dose volume of 2 mL/kg and the vehicle was 1 % methylcellulose (p.o.). All animals were dosed at approximately 9 am every day for 7 days.

Animals receiving **UoS12258** on the test day were dosed intravenously using a cumulative dosing paradigm comprising of 0.1, 0.5 and 1.0 mg/kg at 20 minute intervals whereas animals receiving vehicle on the test day received 3 doses of saline at 20 minute intervals.

Brains and blood samples (50 µL) were collected at the end of each experiment and frozen at -80 °C in brain and EDTA tubes, respectively, until analyzed. **UoS12258** content was determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using a Quattro Premier (Micromass) mass spectrometer with positive-ion electrospray ionisation.

2.6 Novel object recognition

Adult male Lister Hooded rats (Charles River) weighing 250-320 g on the day of testing were housed in groups of 4 in the absence of environmental enrichment (sawdust and bedding only) and maintained on a 12 h light/dark cycle with lights on at 06:00 GMT. Food (Harlan Maintenance Diet) and water were available *ad libitum*. For all experiments rats were habituated to Perspex test arenas (42 l x 21 w x 20 h cm) for 60 min (am) and 3 min (pm) on the day prior to testing. For experiments 1 and 2 rats were sham dosed with vehicle on four occasions prior to testing. On the first test day, rats were placed in a test arena for a 3 min habituation period and then presented with 2 identical objects (T1, 3 min). 24 h later rats were placed back in the test arena and presented one of the previous (familiar) objects and a novel object (T2, 3 min). Objects used were similar sized black plastic 'kong' and cylinder shapes (approximately 6.5 h x 6 w cm). Objects were cleaned with 70% ethanol between animals to remove any odour trace. The novel object and novel object side (left or right) was randomised equally across all treatment groups. The time spent exploring the objects during T1 and T2 was recorded by an observer blind to the novel object. Rats received **UoS12258** or vehicle by oral gavage (p.o.). Treatment group sizes were n = 12 unless stated otherwise. For acute studies rats were dosed 4 h prior to both T1 and T2 at 0.1, 0.3 and 1 mg/kg p.o. For sub-chronic studies rats were dosed once daily (8-10 am) for 7-days followed by a single dose 4 h prior to T1 (day 8) and T2 (day 9) at 0.003, 0.01 and 0.03 mg/kg p.o.

In a separate study male Lister Hooded rats were administered with two doses, 24 h apart, of **UoS12258** (0.1, 0.3 and 1 mg/kg p.o.) separated by 24 h to simulate the dosing conditions used in acute behavioural studies. Animals were culled 4 h after the final dose following schedule 1 procedures, blood and brain samples were taken for analysis. For sub-chronic experiments animals were culled immediately following testing in T2 (n = 3) and blood and brains removed for analysis (as detailed for *in vivo* electrophysiology).

2.7 Water maze

This paradigm is used to investigate spatial learning and memory where learning and subsequent retention of the location of a hidden platform are measured. Aged rats show an inherent deficit in this paradigm such that they do not show an improvement in acquisition of the platform position over the course of training and therefore fail to learn where the platform is located as assessed during the probe tasks performed at the end of training. The effects of sub-chronic administration of **UoS12258** (0.3, 1, 3, 10 mg/kg p.o.) upon age-induced cognitive deficits were determined in aged (22 month old) male Wistar rats. **UoS12258** was administered once daily in 1% methylcellulose for 8 days prior to training and then on each training day (3 h prior to session) and also during the recall period, consistent with previous dosing protocols. Plasma samples were not taken from this study. During each trial, the latency to find the hidden platform (to a maximum of 90 s) was recorded. Animals received 5 trials per day (inter-trial interval of 300 s) for 4 days. Recall of platform position was assessed by a probe test at 1, 3 and 7 days following the final training session, in which the platform was removed and animals were allowed to explore the maze for 30 s. The time spent in each quadrant was recorded.

2.8 Passive avoidance

The passive avoidance (PA) procedure is based on the ability of rats to associate a specific context (dark chamber) with an aversive conditioned stimulus (0.75 mA, 0.5 s scrambled foot shock). This experiment investigated the effects of acute **UoS12258** on a scopolamine (0.08 mg/kg i.p.)-induced deficit in PA in male Wistar rats. **UoS12258** (0.1, 1, 3, 10 mg/kg p.o.) with the 5-HT₆ receptor antagonist, SB-399885T (10 mg/kg p.o.) used as a positive control. Test drugs were administered in 1% methylcellulose 3 h prior to the onset of training. Scopolamine hydrobromide (0.8 mg/kg i.p.) was dissolved in saline and administered 6 h post training. Plasma samples were not taken from this study. On the training day rats were treated with vehicle or **UoS12258** 3 hours prior to assessment of spontaneous activity in the open field apparatus. Immediately afterwards the rats were placed in the light compartment and the latency to enter the dark chamber was recorded. Upon entering the dark compartment, rats received a foot shock (0.75 mA, 0.5 s) and returned to the light chamber whereupon they were returned to their home cage. Recall of this inhibitory stimulus was evaluated 24 h post-training by returning the animal into the light chamber and recording the latency to enter the

dark chamber up to a maximum of 600 s. At no point during the test period was foot shock given.

2.9 Microdialysis.

Male Lister Hooded rats (250-275 g) were housed in groups of four, kept under a 12h light/dark schedule, lights on at 07:00 with food and water available *ad libitum*.

General anaesthesia was induced using isoflurane. Once deep anaesthesia had been obtained rats were administered synulox (antibiotic; s.c.) and rimadyl (analgesic; s.c.), and the rat transferred to a stereotaxic frame. The skull was exposed and holes drilled relative to the Bregma, four anchor screws (1.6x3 mm stainless steel cheese head screws, Wood and Hughs), and additional holes drilled at 12 degrees to the dura surface for placement of microdialysis guide cannula (CMA 11, CMA, UK) into the dorsal hippocampus (AP: + 3.5 mm, ML -2.0 mm, and DV - 2.0) and anterior cingulate cortex (AP: + 2.7 mm, ML -1.6mm, and DV - 2.0 mm) according to Paxinos and Watson (1986). Probe and anchor screws are secured in place with dental cement (Poly-F-plus, zinc polycarboz cement, Cladius Ashe) and the wound sealed. Postoperative fluids (5 mL saline, 2.5mL per side; s.c.) and analgesic (Nubain 0.4 mL/100 g; s.c.) were administered to aid recovery. The rats were monitored until they regain their righting reflex and received at least 5 days post-operative care.

18 h prior to the start of the dialysis experiment rats were moved into microdialysis cages (285 mm diameter, 355 mm high; manufactured in-house) to allow for overnight acclimatisation to the dialysis procedure room. On the morning of each experiment, microdialysis probes (CMA 11, 14/02, Linton Instruments; 2 mm active membrane) were perfused with a buffered artificial cerebral spinal fluid (aCSF) solution (comprising in mM: 145 NaCl, 2.7 KCl, 1 MgCl₂, 1.2 CaCl₂ and 2 Na₂HPO₄) at 1µL / minute via a dual-channel liquid swivel (Instech 375/D/22QE microdialysis swivel, BAS). Microdialysis probes were inserted into the implanted guide cannulae and perfused with aCSF for 2 h before sampling began to allow for equilibration of neurotransmitter levels, following which samples were collected every 30 min. 4 dialysate samples were collected to generate baseline readings before administration of either test compound **UoS12258** was dissolved in 1% methylcellulose and sonicated prior to administration. Doses of **UoS12258**, 0.1, 0.3, and 1 mg/kg or vehicle (1% methylcellulose) were administered in a volume of 2ml/kg; p.o. Following administration of compound, a further 10 dialysate samples were collected until the end of sampling. Dialysate samples were analysed for 5-HT, DA and NA using high

performance liquid chromatography (HPLC) with electrochemical detection (ECD) and ACh using mass spectrometry. At the end of each experimental day, animals were returned to their home cage and re-used in a randomised cross-over design, allowing at least 7 days drug washout before subsequent uses. After the final experiment brains were removed and stored in formalin solution for probe placement verification.

All dialysate samples were calculated as a subsequent percentage of the average absolute levels of neurotransmitters from the four baseline samples prior to administration of compound. Significant differences between groups of the same compound were calculated by repeated measures analysis of variance (ANOVA) followed by Fischer's least significance difference (LSD) post-hoc test where appropriate with significance set at $P < 0.05$ (Statistica v6.0).

2.10 Maximal electroshock threshold (MEST) test

Adult male Sprague Dawley or Lister Hooded rats (Charles River) weighing 95 to 160 g on the day of testing were housed in groups of 6 or less and maintained on a 12 h light/dark cycle with lights on at 06:00 GMT. Food (Harlan Maintenance Diet) and water were available *ad libitum*. Treatment groups of 12 animals were administered **UoS12258** at 10, 30 or 100 mg/kg by oral gavage (p.o.) two hours before testing (expt 1) or 30 mg/kg p.o. two, five or seven hours before testing in Sprague Dawley rats (expt 2) or 1.0, 3.0, 10 mg/kg four hours before testing in Lister Hooded rats (expt 3). In all experiments the known pro-convulsant, picrotoxin (2.0 mg/kg as a positive control) was administered i.p. 30 min before testing. After dosing the animals were returned to their home cages. Testing occurred in a separate room and consisted of assessing the induction of a tonic hind limb extensor seizure following a 0.1 s shock administered via corneal electrodes according to the 'up and down' method (Kimball et al., 1957). In all experiments a separate group of animals (n=3 per dose) were dosed and blood and brain samples taken at the appropriate pre-treatment time for analysis of compound levels. The threshold for tonic hind limb extensor seizures was determined using a Hugo Sachs Elektronik stimulator which delivered a constant current of 0.1 second duration, 50 Hz, sine wave form, fully adjustable between 1 to 300 mA, via corneal electrodes. The electrodes were briefly immersed in saline before application of the electroshock in order to achieve good electrical contact.

3. Results

3.1 Functional activity at recombinant human GluA2i homomeric AMPARs.

The functional activity of **UoS12258** at the hGluA2i homomeric AMPAR stably expressed in HEK293 cells was assessed using FLIPR/Ca²⁺ influx methodology. Application of glutamate (100 μ M) alone did not elicit a signal due to rapid AMPAR desensitisation. However, in the presence of **UoS12258**, application of glutamate consistently increased intracellular Ca²⁺ levels such that an increase in the concentration of **UoS12258** produced a progressive potentiation of glutamate-induced responses. A non-linear, 4-parameter logistic curve-fit of the data generated pEC₅₀ and maximum potentiation values (relative to the established AMPAR positive modulator cyclothiazide (150 μ M)) of 5.57 ± 0.07 and 100.7 ± 3.9 % (mean \pm sem), respectively ($n = 6$; Figure 1). When applied alone, in the absence of glutamate, **UoS12258** did not affect intracellular Ca²⁺ levels indicating that it possessed no intrinsic agonist activity.

Figure 1

3.2 Electrophysiological Activity at human recombinant GluA2i homomeric AMPARs.

Using this approach, **UoS12258** potentiated glutamate-induced whole-cell, AMPAR-mediated currents producing a maximal response that amounted to $112 \pm 32\%$ of the maximal response induced by the reference AMPAR positive modulator cyclothiazide (30 μ M). The pEC₅₀ value (5.19 ± 0.02 (mean \pm sem; $n = 4$) Figure 2) for this positive modulatory effect of **UoS12258** was in close agreement to that generated using the FLIPR/Ca²⁺ influx assay described above.

Figure 2

3.3 Functional activity at recombinant human GluA1i, GluA3i, GluA4i and rat GluA2i homomeric AMPARs

To demonstrate (a) the broad spectrum positive modulatory activity of **UoS12258** across AMPARs formed from different GluA subunits, and (b) lack of species dependent pharmacology, FLIPR-based assays were used to assess its activity at human AMPARs formed by homomeric assembly of GluA1i, 3i or 4i subunits as well as homomeric assembly of rat GluA2i subunits transiently transfected into HEK293 cells (Table 1). There was less than a 10 fold difference between pEC₅₀ values for all hGluAi homomeric AMPARs and

between rat and human GluA2i indicating that **UoS12258** possesses little specificity for individual GluA subunits or across species. (Supplementary Table 1).

3.4 Potentiation of rat native AMPA receptor-mediated responses.

Repeated (2 s duration, every 30 s) applications of 30 μ M AMPA in rat cultured hippocampal neurons produced inward currents, the peak amplitude and waveform of which were consistent in magnitude between successive applications. Measurements taken reflected the three different mechanisms which occurred during this period: (1) opening of the AMPAR channel measured in terms of peak amplitude, (2) desensitization of AMPARs measured as the degree of relaxation of the inward current from its initial peak amplitude to a reduced steady state plateau level in the continued presence of AMPA and (3) deactivation of AMPARs measured as the rate at which the inward current decayed back to baseline following termination of the AMPA application.

Whilst **UoS12258** alone did not evoke a whole-cell inward current at any concentration tested (10 nM - 100 μ M), application of 30 μ M AMPA in the presence of **UoS12258** produced inward currents that exhibited larger peak amplitudes, reduced desensitization and slowed deactivation which all contributed to an increase in charge transfer (area under the inward current), relative to control responses in the absence of **UoS12258**. The effect of **UoS12258** on each of these electrophysiological parameters was clearly concentration-dependent and was first evident in the nM concentration range.

Thus, although the pEC₅₀ and maximal potentiation of charge transfer induced by **UoS12258** were 5.1 and a 4.1 ± 0.97 fold increase, respectively, 10 nM **UoS12258** produced a statistically significant 1.4-fold increase in charge transfer when compared to AMPA alone (* n = 8; $P < 0.01$, paired t-test, Figure 3A).

UoS12258 produced a concentration-dependent increase in the rate constant of AMPAR deactivation (pEC₅₀ 5.3) such that at 100 μ M the rate of deactivation was 5.9 ± 1.5 -fold greater than that produced by AMPA application alone. As with charge transfer **UoS12258** also potentiated this component of the AMPAR-mediated response at nM concentrations such that at 10 nM it produced a $44 \pm 6\%$ decrease in the rate of deactivation (* $P < 0.05$, paired t-test n = 7; Figure 3B).

In contrast to the significant increases in both charge transfer and rate of deactivation, inhibition of AMPAR desensitisation by **UoS12258** was more variable and only statistically

significant at concentrations of 10 μ M and above where a greater than $52 \pm 17\%$ reduction in AMPA receptor desensitisation was observed ($n = 7$, * $P < 0.05$, paired t-test, Figure 3C).

Notably, since the minimum effective concentration of **UoS12258** on desensitization was 10 μ M the previously described potentiation of charge transfer at 10 nM reflects **UoS12258**-mediated potentiation due to changes in peak amplitude and deactivation (Supplementary Table 2).

Figure 3

3.5 Selectivity versus recombinant NMDA and kainate receptors.

In the NMDA receptor FLIPR assay **UoS12258** (tested up to 50 μ M, $n=4$) did not show any activity both as opener and blocker at human GluN1/2B receptors. Similarly, in electrophysiology experiments **UoS12258** did not potentiate GluN1/2B receptor mediated activity at either 10 nM, the minimum concentration ($P=0.87$, paired t-test, $n=5$) or 1 μ M, 100 times the minimum concentration ($P=0.18$, paired t-test, $n=5$) shown to potentiate AMPA-mediated currents; although there was a partial inhibition ($18 \pm 1\%$ at 100 μ M, $P = 0.049$, paired t-test, $n=5$) when tested at 100 μ M.

In FLIPR experiments on transiently expressed GluK1 (Q-edited) homomeric kainate receptors **UoS12258** (up to 50 μ M, $n=3$) did not show any activity either as an agonist, antagonist or positive modulator. Identical results were obtained using electrophysiological analysis.

3.6 Functional selectivity versus rat native NMDA and Kainate receptors.

In separate neurons, repeated applications of either NMDA, in the presence of NMDA receptor co-agonist glycine, or kainate, in the presence of the selective AMPA receptor antagonist SYM2206, generated reproducible inward currents that were mediated by activation of NMDA receptors and kainate receptors, respectively and that were not significantly affected by **UoS12258** (100 μ M) ($n = 4$ for both; $P = 0.06$ for NMDAR-mediated responses and $P = 0.89$ for kainate receptor-mediated responses, paired t-test). There were also no significant changes in NMDA receptor-mediated charge transfer with **UoS12258** at either 10 nM, the minimum concentration ($P=0.16$, paired t-test, $n=4$) or 1 μ M, 100 times the concentration ($P=0.69$, paired t-test, $n=4$) shown to potentiate AMPA-mediated currents.

3.7 Potentiation of AMPA receptor-mediated synaptic responses *in vivo*.

The effect of **UoS12258** was evaluated *in vivo* in an electrophysiological model where synaptic connectivity is intact, specifically the potentiation of electrically evoked AMPAR-mediated synaptic potentials recorded from the dentate gyrus of the anaesthetised rat. Electrical stimulation of the medial perforant pathway evoked a population spike recorded in the hippocampal dentate gyrus granule cell layer. **UoS12258** (0.1 mg/kg i.v.) significantly increased population spike amplitude by 18 ± 3 % compared to vehicle (Figure 4, $P < 0.05$, $n=4$ ANCOVA, univariate test of significance. Analysis of brain samples taken immediately after the experiment generated a mean **UoS12258** concentration of 198 ± 40 ng/g). Brain tissue binding for **UoS12258** was measured as 97.4 % resulting in an estimated mean unbound concentration of 5.1 ng/g, which equates to an approximate free concentration of 15 nM, which is in close agreement with the MEC (10 nM) for **UoS12258** induced potentiation of AMPAR-mediated charge transfer and deactivation in rat hippocampal neurons.

Figure 4

3.8 Novel Object Recognition (NOR Test).

The NOR test is a two-trial recognition memory test of the ability of rats to discriminate between novel and familiar objects. NOR can be impaired by inserting a time delay (24 h) between presentations of the objects.

Data are expressed both as total time spent exploring the novel and familiar objects in T2 and as the d2 index (proportion of time exploring the novel object in T2). In the first study acute **UoS12258** (0.1, 1, 10 mg/kg) produced a bell-shaped dose response curve (i.e. improvement in object recognition at 1 mg/kg with a loss of effect at 10 mg/kg). At the higher dose 1 rat out of 12 had a seizure and was excluded from the test. Based on an efficacious dose of 1 mg/kg, a follow up experiment, was conducted to assess a lower dose range (i.e. 0.1-1 mg/kg p.o.). Following acute administration, **UoS12258** significantly increased novel object exploration compared to vehicle treated rats at 0.3 mg/kg ($P < 0.05$) and 1 mg/kg ($P < 0.01$, Figure 5A) and the d2 index ($P < 0.01$, Figure 5A). In a separate study, mean blood and brain levels of **UoS12258** were determined in rats dosed under identical conditions ($n = 3$) and are shown in Supplementary Table 3.

Following 7-days sub-chronic dosing plus treatment 4 h prior to T1 and T2, **UoS12258** significantly increased novel object exploration compared to vehicle treated rats at 0.03 mg/kg ($P < 0.05$, Figure 5B). The d2 index was significantly increased at both 0.01 and 0.03

mg/kg ($P < 0.01$, Figure 5B). The sub-chronic MED was determined as 0.03 mg/kg based on significant changes in both measures. Mean blood and brain levels of **UoS12258** in rats sampled immediately after testing are presented in Supplementary Table 4 ($n = 3$). A previous sub-chronic NOR study with **UoS12258** (0.03, 0.1, 0.3 mg/kg) also exhibited a bell shaped dose-response curve with efficacy at 0.03 and 0.1 mg/kg and no effect at 0.3 mg/kg. Thus, two studies confirm that sub-chronic dosing of **UoS12258** results in increased potency to facilitate novel object recognition learning, in accordance with data reported in the literature for compounds of this class.

Figure 5

3.9 Passive Avoidance.

Neither **UoS12258** nor SB-399885T (5-HT₆ receptor antagonist) had any effect on spontaneous open field activity (data not shown), suggesting a lack of confounding motor effects. Scopolamine impaired passive avoidance ($P < 0.0001$, Figure 6). **UoS12258** (10 mg/kg) did not impair performance when given alone, and prevented the scopolamine-induced impairment in a dose-dependent manner ($P < 0.0001$), with significant effects observed at 3 and 10 mg/kg ($P < 0.05$). The positive control SB-399885T also prevented the scopolamine-induced memory deficit ($P = 0.0003$).

Figure 6

3.10 Morris Water Maze in Aged Rats.

UoS12258 significantly reduced escape latencies following 3 and 10 mg/kg (Figure 7; $F[1,180] = 14.1$ and 5.9 , $P = 0.0002$ and 0.0156 for 3 and 10 mg/kg, respectively, vs. vehicle-treated controls).

UoS12258 significantly reduced swim angle, the angle between start position and the position of the hidden platform in the water maze, over the 4 training sessions ($F[1,36] = 19.7$ and 5.1 ; $P < 0.0001$ and $P = 0.03$ for 3 and 10 mg/kg, respectively, vs. vehicle-treated), suggesting an improved search strategy (data not shown). **UoS12258** had variable effects upon swim speed, i.e. no consistent increase in swim speed at doses shown to be efficacious at improving acquisition, which therefore does not explain the reduced latency to find the hidden platform.

Figure 7

Recall of the hidden platform position was assessed by probe trials carried out 1, 3 and 7 days following the final training session. **UoS12258** (3 mg/kg) improved recall of the task in all 3 probe trials (Figure 7, $P < 0.05$, Mann-Whitney U-test). **UoS12258** (10 mg/kg) significantly improved recall in the 1 day post-training probe trial only.

Figure 8

3.11 Microdialysis

UoS12258 significantly ($F_{3,23}=5.94$, $P < 0.01$) increased extracellular levels of acetyl choline (ACh) in both the anterior cingulate cortex at all 3 doses (0.1 mg/kg, $P < 0.001$, 0.3 mg/kg, $p < 0.05$ and 1.0 mg/kg, $P < 0.01$) and a significant ($F_{3,22}=5.076$, $P < 0.001$) increase in the dorsal hippocampus again at all 3 doses (0.1 mg/kg, $P < 0.05$, 0.3 mg/kg, $P < 0.001$ and 1.0 mg/kg, $P < 0.005$, Figure 9).

Figure 9

UoS12258 also induced a significant ($F_{3,24}=4.47$, $P < 0.05$) increase in extracellular dopamine levels at both 0.3 mg/kg ($P < 0.05$) and 1 mg/kg ($P < 0.01$) (Figure 9). There was no significant effect of AMPA positive modulation on extracellular levels of 5-HT or noradrenaline (NA) in any of the brain structures studied.

3.12 Side effect profiling using maximal electroshock seizure threshold (MEST) test.

In order to assess the potency of **UoS12258** to potentiate induced seizure activity, rats were pre-treated with the compound prior to induction of seizure. Corneal application of electrical current (CC_{50} approx. 60-70 mA, 0.1 ms duration) in the rat induces tonic and full tonic-clonic seizures. In separate experiments **UoS12258** was administered 10, 30, 100 mg/kg, p.o., 2 h ptt; 30 mg/kg, p.o., 2, 5, 7 h ptt to Sprague Dawley (SD) rats; 1.0, 3.0 10 mg/kg, p.o., 4 h ptt to Lister Hooded (LH) rats before testing for pro-convulsant activity. Lister Hooded rats were chosen to provide a comparison with the NOR potency data.

In SD rats, **UoS12258**, significantly decreased seizure threshold at 30 and 100 mg/kg by -41% (3390 ng/g in brain) and -58% (4961 ng/g in brain), respectively 2 h post dose (Table 1). A subsequent DMPK study showed that concentration was equivalent at 2 h and 5 h. Therefore, in a repeat study, **UoS12258** (30 mg/kg p.o.) was tested 2, 5 and 7 h post dose. Seizure threshold compared to vehicle (5 h ptt) was reduced at all time points by -54%, -43% and -57% (2, 5 and 7 h ptt respectively). At 2 h 1/13 animals and at 7 h 1/15 animals showed

spontaneous convulsions and were culled just before testing. In LH rats (same strain as NOR studies), **UoS12258** significantly reduced seizure threshold by -38% at 10 mg/kg (Supplementary Table 5). Both rat strains show similar brain exposure (2446 ng/g in LH vs. 2283 ng/g SD at 10 mg/kg, respectively).

Table 1

4. Discussion

In this article we present data from experiments that describe the pharmacological properties of **UoS12258**, a novel and selective AMPAR positive modulator. Although, as stated earlier, many molecules have been investigated pre-clinically and several progressed into clinical trials, there is limited full disclosure of the enabling pre-clinical data sets – particularly regarding behavioural and tolerability data. To address this situation, we disclose the full data set generated for this molecule which has progressed into clinical evaluation, including details of the differences observed in behavioural studies between acute and repeat dosing and also the clear side effects seen at elevated concentrations. In addition to the studies reported below, a wider investigation was conducted of **UoS12258** in additional behavioural pharmacology models, for which the data were either inconclusive: reversal of ketamine or amphetamine-induced hyperactivity in rat and reversal of quinelorane (D2/3 receptor agonist)-induced hypoactivity in rat, or only effective at high doses: mouse forced swim test (10 mg/kg po administered 240 min prior to test gave efficacy of imipramine dosed ip 30 min prior to test). Full data for these studies are not included in this manuscript.

4.1 Characterisation using recombinant systems

We investigated the effect of **UoS12258** on intracellular Ca^{2+} influx using FLIPR based assays and the results suggest that **UoS12258** shows no clear subunit specificity and should act equipotently at heteromeric AMPARs with different subunit compositions, which exist in native tissues. Also, given that pEC_{50} values for **UoS12258** were similar at rat and human there appears to be little evidence of species selectivity. Using the same recombinant system, HEK293-hGluA2i stable cell line with whole-cell voltage clamp electrophysiology the pEC_{50} for **UoS12258** was again similar to the FLIPR values with this AMPAR subunit.

4.2 Characterisation using native systems

Although rates of receptor activation and deactivation are comparable across all homomeric AMPARs and are therefore independent of AMPAR GluA subunit complement, desensitisation kinetics differ depending upon AMPAR subunit stoichiometry (see Schlesinger *et al.*, 2005). As whole cell currents in neurons sample activation of a heterogeneous population of AMPARs that will differ from neuron to neuron it is likely that the observed variation in desensitization is due to the heterogeneity of the AMPAR populations assessed. Activation (charge transfer) and deactivation of AMPA receptors are

measures that define the onset and offset of glutamatergic activity and are therefore directly relevant to the propagation of synaptic transmission and synaptic plasticity. Receptor desensitisation, during which the receptor becomes inactive in the continued presence of the ligand, has been more closely associated with excessive or pathological glutamatergic activity. In native hippocampal neurons using whole-cell voltage clamp electrophysiology it was possible to measure AMPA receptor-mediated charge transfer and deactivation which were potentiated significantly by concentrations of **UoS12258** as low as 10 nM. It is interesting however, that the pEC₅₀ of **UoS12258** on charge transfer, desensitisation and deactivation of AMPA-mediated currents in native neurons are all in the same range as the pEC₅₀ values in recombinant systems. There are clearly many physiological differences between recombinant and native systems for example increased levels of phosphorylation, the presence of transmembrane AMPA regulatory proteins (TARPs) or increased AMPAR expression at the cell membrane, which could explain the observed low concentration-mediated AMPAR potentiation by **UoS12258** in the native system. Perhaps more interestingly this concentration is comparable to the concentrations measured in the brain immediately after *in vivo* electrophysiological studies, in which **UoS12258** increased AMPA receptor-mediated synaptic activity. Together these data mutually support the observation that, at equivalent concentrations, **UoS12258** evoked significant potentiation of AMPAR-mediated neuronal activity both *in vitro* and *in vivo*.

Selectivity of **UoS12258** for AMPA subtype glutamate receptors could however be a desirable property since activation and/or potentiation of other ionotropic glutamate receptor subtypes (i.e. NMDA and kainate) could cause excessive glutamate channel opening, increasing Ca²⁺ influx and leading to excitotoxic damage. To establish its selectivity for the AMPAR the functional activity (agonism, antagonism and positive modulation) of **UoS12258** was assessed at the human NMDAR1/2B heteromeric NMDA receptor and the human GRIK1 (Q) homomeric kainate receptor using both FLIPR/Ca²⁺ influx and electrophysiological methodologies.

UoS12258 did not potentiate NMDAR1/2B receptor mediated activity at any concentration tested whether tested by Ca²⁺ influx or whole-cell patch clamp. Similarly, in FLIPR experiments on transiently expressed kainate **UoS12258** did not show any activity either as an agonist, antagonist or positive modulator. Identical results were obtained using electrophysiological analysis. **UoS12258** also produced no significant potentiation in activity

at rat NMDA or kainate receptors suggesting that this compound exhibits selectivity for AMPA compared to other glutamate receptor subtypes.

4.3 Cognition

UoS12258 improved performance in three different cognition paradigms using a variety of approaches to cause impairments (i.e. delay, scopolamine and age).

The NOR test was the most sensitive paradigm, particularly following sub-chronic dosing. **UoS12258** reversed a delay-induced deficit in novel object recognition in rats after both acute dosing and sub-chronic dosing. However, behavioural studies in the water maze showed that although **UoS12258** was effective at improving aged-induced deficits in learning and retention the MED, 3 mg/kg, was higher than in the NOR study. This difference could be a reflection of young and aged rats' performance in particular cognitive tasks or it could be due to the underlying mechanisms involved in performing the task. NOR is dependent on the innate curiosity of the rat to explore novelty and might be more sensitive to modulation of AMPAR activity than in a model which involves the complex physiological processes which are altered during cognitive impairment associated with aging. Similarly the higher MED, 3 mg/kg in the passive avoidance cognition model could reflect the increased difficulty in overcoming a scopolamine-induced impairment compared to a time-induced deficit used in the NOR model.

Interestingly, a bell-shaped dose-response relationship (i.e., loss of, or reduced, effect at higher doses) was apparent in both the NOR and water maze paradigms.

In addition to **UoS12258** being an AMPAR positive modulator microdialysis studies have shown that it also enhances cholinergic neurotransmission in brain structures, the dorsal hippocampus and anterior cingulate cortex, which are implicated in cognitive functioning.

4.4 Safety and tolerability

Although **UoS12258** decreased seizure threshold at doses greater than 10 mg/kg it should be noted brain concentrations in the MEST test were approximately 20 fold higher than concentrations measured after improved performance of NOR after acute dosing. Further research is ongoing to identify molecules that exhibit a greater margin between efficacious exposures and those which evoke pro-convulsant activity.

5. Conclusions

UoS12258 is a selective positive allosteric modulator of AMPA-type ionotropic glutamate receptors exhibiting equivalent potency at all AMPA receptor subtypes, with no measurable activity on other glutamate receptors. At comparable concentrations (10-15 nM), **UoS12258** has been shown to potentiate AMPA receptor currents *in vitro*; enhance synaptic AMPA receptor-mediated activity *in vivo* as well as demonstrating an excellent *in vivo* efficacy profile. It improved novel object recognition task performance in the rat at low brain concentrations which were consistent with its activity on native tissue *in vitro*, importantly efficacious concentrations were significantly lower than those required for efficacy with compounds such as CX516 in a similar assay (Damgaard 2010). **UoS12258** was also effective at improving rats' performance of water maze and passive avoidance suggestive of enhanced learning and memory, although at higher concentrations. On the basis of the preclinical studies reported in this article, **UoS12258** would be expected to be highly efficacious at improving cognitive measures in schizophrenic patients as well as other diseases with cognitive impairments, and is therefore a worthy candidate for clinical evaluation.

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Author contributions

SW and MH conceived the project design and were responsible for the interpretation of all data. MH, NC, JG, LL, MHSM, BO, JP, DJ, LD, MW and KS planned, performed, analysed and interpreted individual studies within the manuscript. SW, and MH wrote the manuscript, PB revised the document.

Conflicts of Interest

All authors are or have been employees and shareholders of GlaxoSmithKline. SW is grant holder of Wellcome Trust funded project *Transforming the treatment of schizophrenia:*

Design and development of AMPA receptor modulators with a much improved safety profile

as novel drugs for treating the cognitive dysfunction associated with schizophrenia and other CNS disorders grant WT-103096/Z/13/Z. Some authors are currently employed by other pharmaceutical companies: NC is currently an employee of Roche, Switzerland; JC is currently an employee of Eisai, USA; BO is currently an employee of Aptuit, Italy; LD is currently an employee of Astex Pharmaceuticals; MW is currently an employee of GWPharma; DJ is currently an employee of Johnson & Johnson, UK.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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FIGURES & TABLES

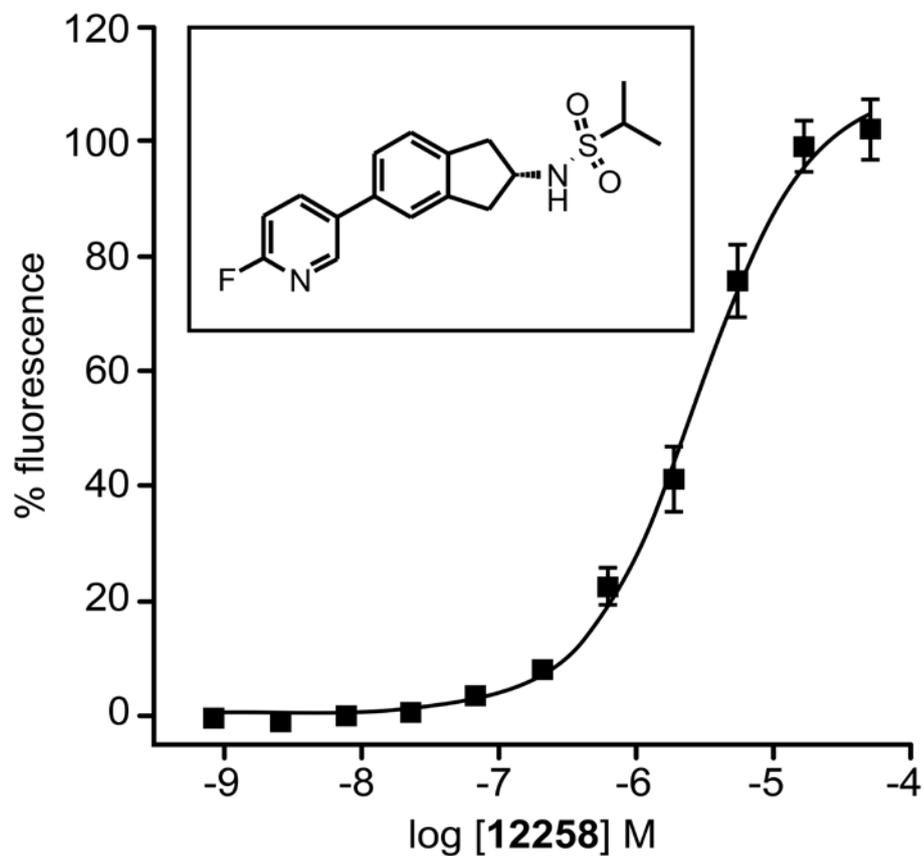


Figure 1. Concentration-response curve and chemical structure for UoS12258 showing its potentiation of glutamate-induced rises in intracellular calcium in HEK293 cells stably expressing the hGluA2i homomeric AMPAR, $pEC_{50} = 5.57$. Responses are normalised to 150 μ M cyclothiazide. Mean \pm sem, $n = 6$ independent experiments.

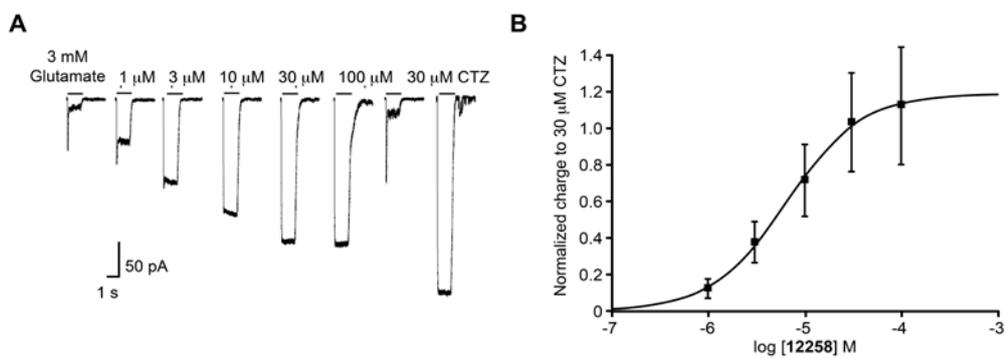


Figure 2. A, Representative whole cell current traces recorded from a single HEK293 cell expressing hGluA2i homomeric AMPARs. UoS12258 (1-100 μ M, upper concentration limited by solubility) produced a concentration-dependent and reversible increase in charge transfer. Positive modulator, cyclothiazide 30 μ M, also increased charge transfer following application of glutamate (line 7). B, Concentration-response curve for the potentiation of hGluA2i-mediated inward currents by UoS12258, $pEC_{50} = 5.19$. Responses are normalized to 30 μ M cyclothiazide. $n = 4$ independent experiments.

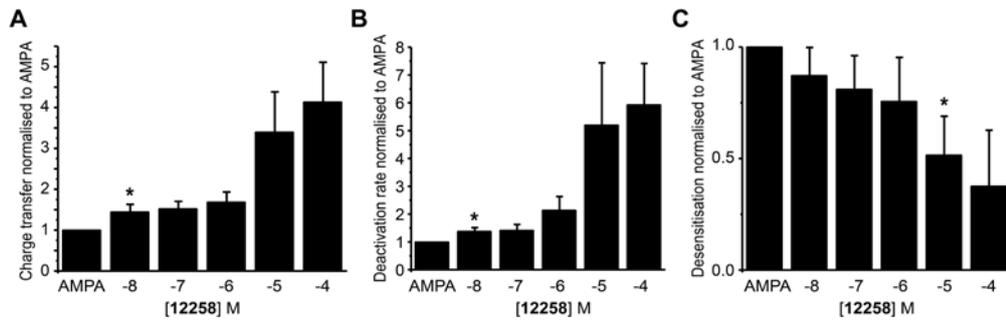


Figure 3. Rat native neuronal data. *A*, Concentration-response plot for UoS12258-mediated potentiation of charge transfer. *B*, Concentration-response plot for UoS12258-mediated potentiation of AMPAR deactivation. *C*, Concentration-response plot for the inhibition of AMPAR desensitisation induced by UoS12258. Responses were normalised to 30 μ M AMPA control. Lowest concentration to cause a significant ($*P < 0.05$) difference.

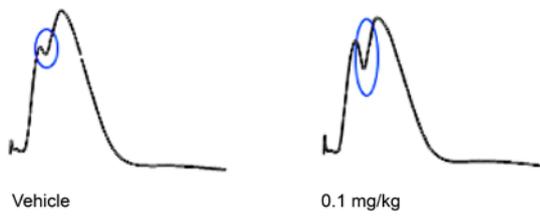


Figure 4. Representative traces recorded from the dentate gyrus of the anaesthetised rat following vehicle (left trace) and 0.1 mg/kg i.v. administration of UoS12258 (right trace). Synaptic traces are an average of 10 consecutive responses; the population spike is ringed in blue.

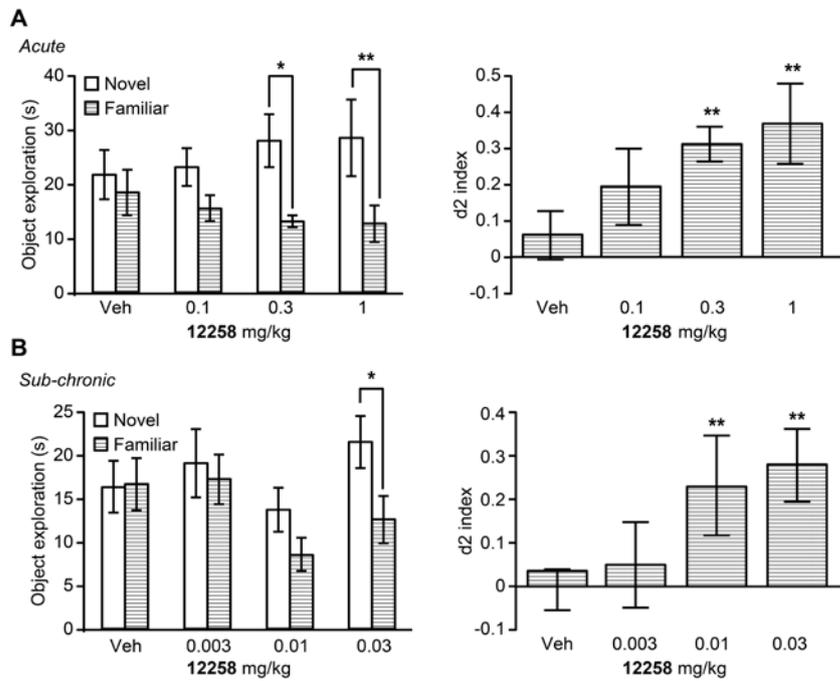


Figure 5. A Effects of UoS12258 on NOR in rats after acute administration (0.1-1 mg/kg p.o., 4 h prior to T1 and T2); **B** Effects of UoS12258 on NOR in rats after 7-days sub-chronic administration (0.003-0.03 mg/kg p.o. dosed daily for 7 days and then 4 h prior to T1 and T2). Significant differences (* $P < 0.05$, ** $P < 0.01$) compared to vehicle treated animals.

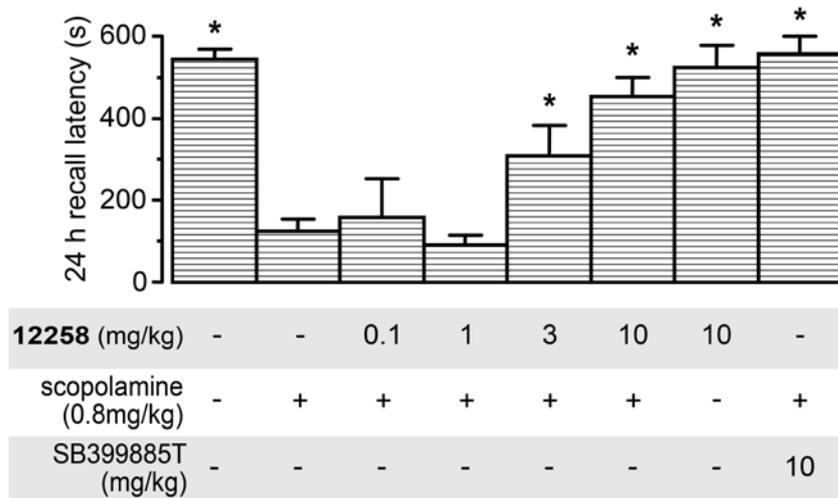


Figure 6. Effects of acute UoS**12258** and SB-399885T on scopolamine-induced impairment of a PA response in adult Wistar rats. Data represent mean \pm SEM avoidance latency at the 24 hour recall time. Significant difference (* $p < 0.05$) compared to scopolamine-treated animals (Mann-Whitney *u*-test, $n = 6$ per group).

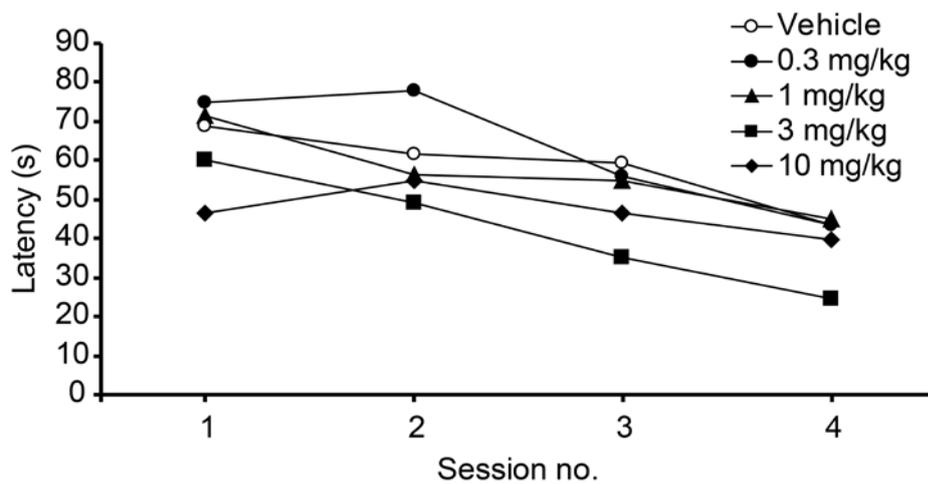


Figure 7. Effect of sub-chronic UoS12258 on acquisition of a water maze spatial learning task by aged Wistar rats. Data represents mean \pm SEM escape latency (sec) averaged over the 5 trials of each training session. By session 3 there was a significant, $P < 0.05$, difference between groups administered with either 3 mg/kg or 10 mg/kg compared to vehicle treated animals ($n = 6$ per group).

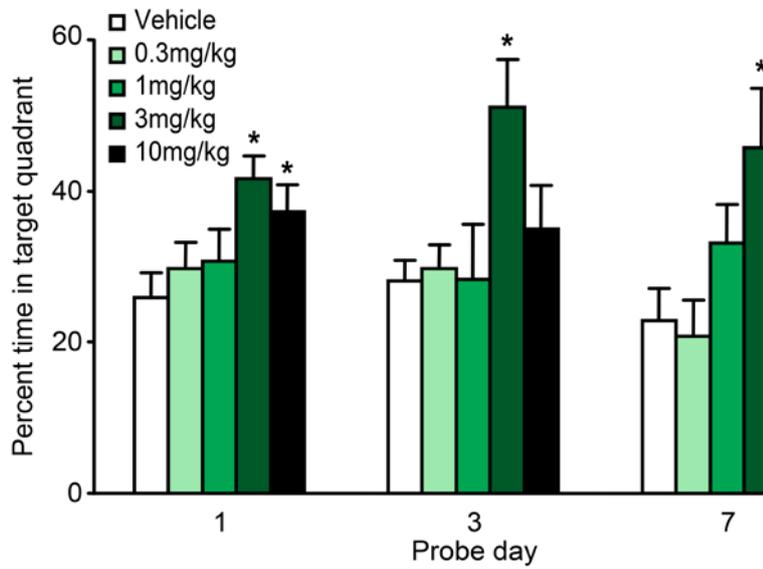


Figure 8. Effect of sub-chronic UoS12258 on recall of a water maze spatial task by aged rats. Data represents mean \pm SEM time (s) swimming in the target quadrant of the water maze during probe trials 1, 3 and 7 days post-training. Significant difference (* $P < 0.05$) compared to vehicle treated animals.

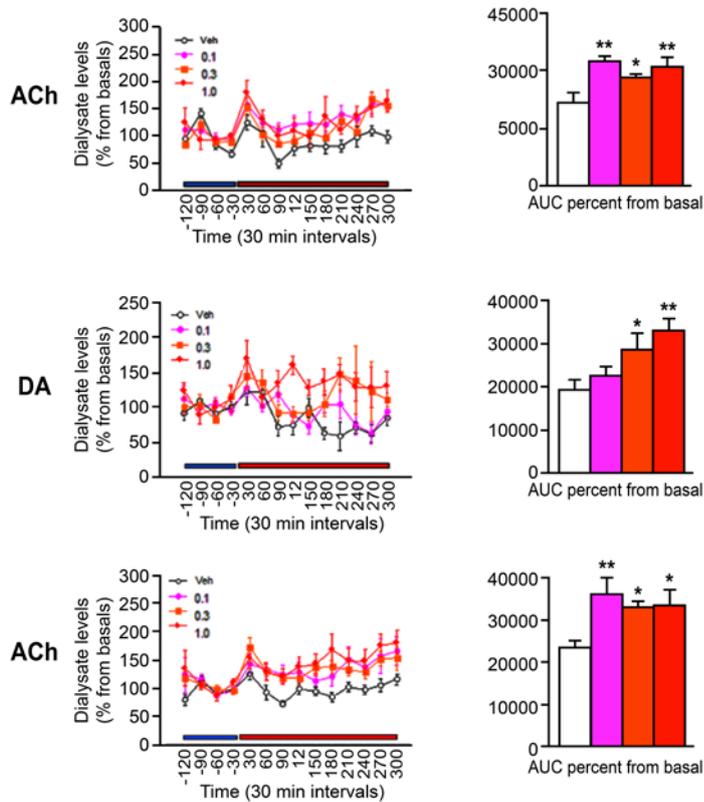


Figure 9. Acetyl choline (ACh) and dopamine (DA) percent changes from basal (line graphs) and total efflux (bar chart - calculated as area under the curve (AUC) of percent changes from basal levels) in the anterior cingulate cortex (left panel) and dorsal hippocampus (right panel) following administration of UoS12258 (0.1, 0.3 and 1.0 mg/kg, p.o). Significant differences (* $P < 0.05$ and ** $P < 0.01$) compared to vehicle treated animals.

| UoS12258 (mg/kg) | CC50 (mA) | + sem | % change compared to vehicle | Mean Blood levels (ng/mL) | Mean Brain levels (ng/g) |
|----------------------------|--------------|-------|------------------------------------|---------------------------------|--------------------------------|
| Saline | 58.5 | 3.4 | - | - | - |
| Picrotoxin | 25.8 | 1.9 | -56% *** | - | - |
| 1% MeCell | 55.5 | 3.7 | - | - | - |
| 10 | 50.0 | 4.9 | -10% | 1285 | 2283 |
| 30 | 32.5 | 3.2 | -41% *** | 2136 | 3390 |
| 100 | 23.5 | 2.1 | -58% *** | 2151 | 4961 |

Table 1 (10, 30, 100 mg/kg, p.o., 2h ptt) in CD rats in the pro-convulsant MEST test.
Significant difference (***)P<0.001) compared to vehicle treated animals.