BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 1417–1429

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

A series of structurally novel heterotricyclic α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor-selective antagonists

MB Gill[1], S Frausto[1], M Ikoma[2], M Sasaki[2], M Oikawa[2,3], R Sakai[4] and GT Swanson[1]

[1]*Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Feinberg School of Medicine, Chicago, IL, USA,* [2]*Graduate School of Life Sciences, Tohoku University, Aoba-ku, Sendai, Japan,* [3]*Graduate School of Nanobioscience, Yokohama City University, Kanazawa-ku, Yokohama, Japan and* [4]*Graduate School of Fisheries Sciences, Hokkaido University, Minato-cho, Hakodate, Japan*

Background and purpose: A new class of heterotricyclic glutamate analogues recently was generated by incorporating structural elements of two excitotoxic marine compounds, kainic acid and neodysiherbaine A. Rather than acting as convulsants, several of these 'IKM' compounds markedly depressed CNS activity in mice. Here, we characterize the pharmacological profile of the series with a focus on the most potent of these molecules, IKM-159.

Experimental approach: The pharmacological activity and specificity of IKM compounds were characterized using whole-cell patch clamp recording from neurons and heterologous receptor expression systems, in combination with radioligand binding techniques.

Key results: The majority of the IKM compounds tested reduced excitatory synaptic transmission in neuronal cultures, and IKM-159 inhibited synaptic currents from CA1 pyramidal neurons in hippocampal slices. IKM-159 inhibited glutamate-evoked whole-cell currents from recombinant GluA2- and GluA4-containing α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors most potently, whereas kainate and NMDA receptor currents were not reduced by IKM-159. Antagonism of steady-state currents was agonist concentration dependent, suggesting that its mechanism of action was competitive, although it paradoxically did not displace [^{3}H]-AMPA from receptor binding sites. IKM-159 reduced spontaneous action potential firing in both cultured hippocampal neurons in control conditions and during hyperactive states in an *in vitro* model of status epilepticus.

Conclusions and implications: IKM-159 is an AMPA receptor-selective antagonist. IKM-159 and related nitrogen heterocycles represent structurally novel AMPA receptor antagonists with accessible synthetic pathways and potentially unique pharmacology, which could be of use in exploring the role of specific populations of receptors in neurophysiological and neuropathological processes.

British Journal of Pharmacology (2010) **160,** 1417–1429; doi:10.1111/j.1476-5381.2010.00784.x

Keywords: glutamate receptors; antagonist; subunit selective; AMPA receptor; electrophysiology; kainite

Abbreviations: 4-AP, 4-aminopyridine; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; AP, action potential; Bic, bicuculline methiodide; CA, cornu ammonis; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CTZ, cyclothiazide; D-APV, D-(–)-2-amino-5-phosphonopentanoic acid; D,L-APV, DL-2-amino-5-phosphonopentanoic acid; DOS, diversity-oriented synthesis; DPBS, Dulbecco's phosphate-buffered saline; EB, Evans blue; eGFP, enhanced green fluorescent protein; EPSC, excitatory post-synaptic current; GluA, glutamate receptor, AMPA-type; GluK, glutamate receptor, KA-type; GluN, glutamate receptor, NMDA-type; HEK, human embryonic kidney; iGluR, ionotropic glutamate receptor; KA, kainate; MK-801, dizocilpine; neoDH, neodysiherbaine; NMDA, *N*-methyl-D-aspartate; PTX, picrotoxin; QX-314, lidocaine *N*-ethyl bromide; SE, status epilepticus; TEA, tetraethylammonium chloride; V_m, membrane voltage

Introduction

Physiological brain function relies on a delicate balance of excitatory and inhibitory neurotransmission acting on the dynamic intrinsic excitability of neurons (Nelson and Turrigiano, 1998; 2008). Excitatory synaptic transmission is

Correspondence: Geoffrey T. Swanson, Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Feinberg School of Medicine, Chicago, IL 60611, USA. E-mail: gtswanson@northwestern.edu
Received 5 October 2009; revised 1 February 2010; accepted 22 February 2010

critically mediated by ionotropic glutamate receptors (iGluRs). Three subfamilies of receptors comprise the iGluRs: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate (KA) receptors (Mayer and Westbrook, 1987; Hollmann and Heinemann, 1994). Disruption of the equilibrium between these excitatory receptors and their inhibitory counterparts contributes to many CNS injury and disease states, particularly epilepsy (Ben-Ari, 2001; Jensen, 2002).

Antagonists acting on all three iGluR subtypes are efficacious in a variety of laboratory models of disease (Namba *et al*., 1994; O'Neill *et al*., 1998; Smith *et al*., 2000; Gigler *et al*., 2007), but very few successful outcomes have been achieved in clinical trials with these molecules (Hoyte *et al*., 2004; Walters *et al*., 2005; Wood, 2005). Currently, only one iGluR antagonist is used clinically, the rapidly dissociating uncompetitive NMDA receptor antagonist, memantine (Parsons *et al*., 2007), although selective antagonists of NMDA receptors containing the GluN2B subunit are in clinical studies (Gogas, 2006; Mony *et al*., 2009). The generation of molecules with enhanced pharmacological specificity for a subset of iGluRs in the brain therefore represents a promising treatment strategy that remains largely unexplored, particularly with respect to AMPA receptors.

Current rational drug design to generate new pharmacological and clinical tools within small molecule libraries employs several synthesis paradigms, including diversity-oriented synthesis (DOS), which typically produces a structurally complex and diverse library that is screened for activity on a variety of molecular targets (Tan, 2005; Cordier *et al*., 2008). Recently, we pursued a modest DOS approach aimed at creating a family of novel heterocyclic scaffolds referred to as 'IKM' compounds (Ikoma *et al*., 2008; Oikawa *et al*., 2009). The IKM molecules were inspired by two naturally occurring convulsants, kainic acid (KA) and neodysiherbaine A (neoDH), which are potent glutamate receptor agonists (Swanson and Sakai, 2009). Despite their structural resemblance to KA and neoDH, however, several of the IKM molecules elicited pronounced hypoactivity and muscle rigidity characteristic of catalepsy, instead of seizure behaviours, in preliminary *in vivo* mouse bioassays (Ikoma *et al*., 2008; Oikawa *et al*., 2009).

In the current study, we determined the pharmacological activity underlying this apparent depression of CNS function. The majority of the IKM compounds reduced synaptic excitation and excitability in cultured hippocampal neurons through antagonism of neuronal AMPA receptors. The most potent molecule in behavioural studies, IKM-159, inhibited AMPA receptors, but did not alter KA or NMDA receptor currents. Action potential (AP) firing in *in vitro* basal and seizure-like states was reduced in the presence of the IKM compounds, which may in part underlie their *in vivo* behavioural properties. IKM-159 therefore could serve as a template for a series of more potent AMPA receptor-selective antagonists with greater therapeutic potential.

Experimental procedures

Cell culture and transfection

Human embryonic kidney cells expressing T-antigen, clone 17 (HEK293-T/17) from ATCC (CRL-11268) were cultured at 37°C with 5% CO_2 in Dulbecco's minimal essential medium, including 100 $\mu g \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 10% heat-inactivated fetal bovine serum. Cell cultures were divided twice per week. For transfection, HEK293-T/17 cells were plated at 3×10^4 cells$\cdot cm^{-2}$ for pharmacology experiments, and 5×10^3 cells$\cdot cm^{-2}$ for physiology experiments in fresh medium 24 h prior to transfection. Cells were transfected with AMPA or KA receptor plasmid cDNAs using Mirus Trans-IT transfection reagent (Mirus Bio Corporation, Madison, WI, USA) at a ratio of 1 μg cDNA : 3 μL Trans-IT reagent, and maintained in transfection medium overnight followed by a media change the next day. Transfected cells were maintained in this medium until the experiment was conducted (48–72 h total post-transfection).

Electrophysiology

For analysis of recombinant receptors, HEK293-T/17 cells were co-transfected with 0.2 μg of iGluR cDNA and 0.05 μg of enhanced green fluorescent protein (eGFP). The cDNAs were kindly provided by Stephen Heinemann (The Salk Institute, La Jolla, CA, USA) and Peter Seeburg (Max-Planck Institute, Heidelberg, Germany). For heterologous GluA1/GluA2 receptors, the GluA1 : GluA2 cDNA ratio was 1:6. Two to three days post-transfection, eGFP-expressing cells were lifted from the coverslip and voltage clamped in whole-cell mode. The internal solution contained (in mM) 30 CsF, 110 CsCl, 10 HEPES, 4 NaCl, 5 EGTA and 0.5 $CaCl_2$ (pH 7.3), while the extracellular solution contained (in mM) 140 NaCl, 10 glucose, 10 Cs-HEPES, 3 KCl, 2 $CaCl_2$ and 1 $MgCl_2$ (pH 7.3). Borosilicate patch electrodes were pulled and fire polished to 2–3 MΩ resistance. Lifted cells were maintained in a laminar stream of extracellular solution from a triple-barrelled flow pipe, which was rapidly translated using a piezoceramic bimorph for fast application of glutamate or KA alone, or in combination with various antagonists (the 10–90% rise time of glutamate-evoked currents was ~1 ms). IKM compounds were applied for 3 min with analysis performed on the final 1 min of drug application.

Experiments involving primary hippocampal neurons were carried out as described previously (Vivithanaporn *et al*., 2007). Dissociated neurons were cultured from E18 prenatal hippocampi and were used for analysis between 17 and 28 days *in vitro*. The extracellular solution was the same as used for recombinant receptor analysis, while the internal solution for voltage clamp recordings contained (in mM) 95 CsF, 25 CsCl, 10 Cs-HEPES, 10 EGTA, 2 NaCl, 2 Mg-ATP, 10 lidocaine *N*-ethyl bromide (QX-314), 5 tetraethylammonium chloride (TEA) and 5 of 4-aminopyridine (4-AP) (pH 7.3), and the internal solution for current clamp recordings contained (in mM) 120 $KMeSO_4$, 5 KCl, 5 NaCl, 1 $MgCl_2$, 11 Na-HEPES, 10 phosphocreatine, 4 Na-ATP and 0.3 Na-GTP (pH 7.0). Borosilicate patch electrodes were pulled and fire polished to 3–5 MΩ resistance. Both whole-cell current clamp and voltage clamp recordings were performed using an Axopatch 200B amplifier (MDS, Sunnyvale, CA, USA) and filtered at 20 kHz. Gigaohm seals were established in voltage clamp mode, and, after membrane rupture, the recordings were carried out either in voltage clamp (clamped at V_m = –70 mV) with a 5–15 mΩ series resistance, which was compensated to ~60%,

or in current clamp mode (V_m = 60–65 mV) with the bridge balance was corrected and pipette resistance neutralized. *In vitro* status epilepticus (SE) was induced as described previously (Pal *et al*., 1999; Deshpande *et al*., 2007), and APs were recorded in current clamp mode. Briefly for *in vitro* SE, Mg^{2+}-containing external solution was exchanged for external solution without Mg^{2+} and supplemented with 2 μM glycine. This solution exchange produced a period of rapid depolarization and increased AP firing, followed by a period of channel inactivation and reduced AP firing. After channel inactivation, neurons characteristically would hold at a membrane voltage (V_m) which was depolarized relative to control V_m, with an increase in AP firing frequency. Neurons were excluded from analysis if the AP firing frequency did not hold at ~2 Hz for at least 2 min or demonstrated a significant rundown in AP firing frequency prior to administration of experimental compounds. External solutions were bath-applied at a flow rate of 1.5–2 mL·min^{-1}. After recording a basal control period, IKM compounds were applied for 5 min (10 min for *in vitro* SE experiments), and changes in excitatory post-synaptic current (EPSC) charge transfer or AP frequency were analysed during the last 2 min of IKM application.

For recordings from acute hippocampal slices, we used brain slices from postnatal day 15–21 C57Bl/6 mice of both sexes that were bred in an in-house colony. All animal care and experimental procedures complied with the guidelines from the American Association for Laboratory Animal Care and were approved by the Northwestern University Institutional Animal Care and Use Committee. The animals were deeply anaesthetized with inhaled isoflurane before rapid decapitation; brains were removed and sliced transversely (350 μm thickness) in a sucrose slicing solution containing (in mM) 85 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 75 sucrose, 0.5 $CaCl_2$, 4 $MgCl_2$, 0.5 Na ascorbate, 10 μM DL-2-amino-5-phosphonopentanoic acid (DL-APV) and 100 μM kynurenic acid (pH 7.3). After slicing, the solution was exchanged with an incubation solution containing (in mM) 125 NaCl, 2.4 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 25 glucose, 1 $CaCl_2$, 2 $MgCl_2$, 0.5 Na ascorbate, 10 μM DL-APV and 100 μM kynurenic acid (pH 7.3) that was bubbled continuously with CO_2-balanced O_2 (carbogen). During this solution exchange, the temperature of the bath was slowly increased to 32°C and then allowed to return to ~23°C, after which slices were transferred to the recording bath for experiments. Recordings in voltage clamp mode used the CsF/CsCl internal solution and carbogenated external recording solution containing (in mM) 120 NaCl, 2.4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 25 glucose, 10 μM bicuculline methiodide, 50 μM picrotoxin (PTX) and 50 μM D-APV (pH 7.3). For NMDA EPSCs, $MgCl_2$ was omitted and glycine (10 μM)/6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50 μM) added to the external solution. NMDA EPSCs were validated by addition of D-APV (50 μM) at the end of each recording. Hippocampal CA1 or CA3 pyramidal cells were voltage clamped at −70 mV and stimulated with a monopolar electrode in either the stratum radiatum or stratum lucidum respectively. IKM compounds were applied for 10 min, and changes in the peak amplitudes of EPSCs and paired-pulse ratios were analysed during the last 2 min of drug application. EPSC charge transfer, AP frequency, paired-pulse ratio and EPSC peak amplitude were analysed with Mini-Analysis v6.03 (Synaptosoft, Decatur, GA, USA) and Clampfit v10.0 (MDS) software.

Radioligand binding

HEK293-T/17 cells were transfected with 5 μg of iGluR cDNA in 100 mm^2 culture dishes. Two days post-transfection, the plates were washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS) and then incubated with 50 mM Tris (pH 7.4) and 1 mM EDTA for 3 min on ice. Cells were collected and centrifuged for 5 min at 800× *g* at room temperature. The cell pellet was homogenized in 50 mM Tris (pH 7.4) containing 320 mM sucrose [homogenization buffer (HB)] on ice using a glass Dounce homogenizer and centrifuged at 4°C for 10 min at 800× *g*. The supernatant, containing the membrane fragments, was saved, and the remaining pellet containing nuclei and unbroken cells was resuspended in HB. This homogenization process was repeated two more times and after collection of the final resulting supernatant, the remaining pellet was discarded. The collected supernatants were centrifuged at 4°C for 20 min at 20 000× *g*, and the resulting supernatant was discarded leaving a membrane-containing pellet. The pellets were resuspended in freshly prepared binding buffer containing (in mM) 50 Tris (pH 7.2), 2.5 $CaCl_2$, 10% glycerol and 100 KSCN (for AMPA receptors), or exclusively 50 Tris (pH 7.4) (for KA receptors). Membrane protein (50–100 μg) was combined with radiolabelled ligand (10 nM [^{3}H]-KA or 20 nM [^{3}H]-AMPA for KA receptor and AMPA receptor binding, respectively), alone or with 100 μM of IKM-159, and then incubated for 1 h at 4°C. Non-specific binding was determined as the amount of radioligand bound in the presence of a saturating concentration of glutamate (1 mM). Total binding was determined as the amount of radioligand bound in the absence of any competing ligands minus the non-specific binding. Experimental variations included a 1 h pre-incubation with IKM-159, an increase in the total binding period to 2 h and an incubation with a saturating concentration of a known AMPA/KA antagonist, CNQX (10 μM). Data are presented as a percentage of the total binding. All experimental conditions were performed in duplicate in each assay and then averaged for each trial.

Statistical analysis

Data are presented as mean ± SEM. At least three separate experiments were performed for each assay. For data involving three or more groups, a one-way ANOVA was performed with a Tukey–Kramer *post hoc* test for comparison among the groups. For data involving two groups, an unpaired Student's *t*-test was performed; if the standard deviations between the two groups were statistically significant, a Welch correction was added. If the data involving three or more groups or data involving two groups where multiple treatments were performed on the same sample, a repeated measures ANOVA and paired *t*-test were used, respectively, for analysis of significance. Dose–inhibition response curves were fitted, with the maximum value constrained to 100 and the minimum value constrained to values greater than or equal to zero, using the following equation in order to calculate the IC_{50} values: response = minimum + (maximum − minimum)/[1 + (IC_{50}^{hill}

$_{slope}$/dose$^{hill\ slope}$)]. All calculations for statistical analysis were carried out with Graphpad Prism4 software (La Jolla, CA, USA), with statistical significance set at $P < 0.05$.

Materials

All salts, buffers, potassium channel antagonists, TEA (T2265), 4-AP (275875) and lidocaine *N*-ethyl bromide (QX-314; L5783) were purchased from Sigma-Aldrich (St Louis, MO, USA). Kynureic acid (0223), D-(–)-2-amino-5-phosphonopentanoic acid (D-APV; 0106), DL-2-amino-5-phosphonopentanoic acid (D,L-APV; 0105), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 0190), PTX (1128) and bicuculline methiodide (Bic; 2503] were purchased from Tocris Bioscience (Ellisville, MO, USA). Rat GluA1-4 flip cDNAs within the pRK plasmids were provided by Peter Seeburg (University of Heidelberg, Heidelberg, Germany), while myc-tagged rat GluK1-2b and GluK2a cDNAs within pcDNA3.1 plasmids were provided by John Marshall (Brown University, Providence, RI, USA) and Christophe Mulle (Université Bordeaux II, Bordeaux Cedex, France).

Nomenclature of drugs, receptors and splice variants follows Alexander *et al*. (2009).

Results

IKM compounds reduce excitatory transmission in cultured neurons

Domino metathesis schemes (ring-opening metathesis/cross metathesis/ring-closing metathesis) were used to synthesize a family of 'IKM' compounds inspired by the naturally occurring convulsant toxins, KA and neoDH (Figure 1) (Ikoma *et al*., 2008; Oikawa *et al*., 2009; Oikawa *et al*., 2010). The IKM molecules are all heterotricyclic hexahydro-2*H*-furo[2,3-c]pyrrole-dicarboxylic acids with variable third ring components that include substituted pyrans (IKM-86 and -110), oxepanes (IKM-98 and -107) and tetrahydropyridines (IKM-27 and -159). In mouse behavioural assays, IKM-98, IKM-110 and IKM-107 elicited varying degrees of hyperactivity (Oikawa *et al*., 2009). In contrast, IKM-27, IKM-86 and IKM-159 induced hypoactive phenotypes similar to cataleptic states (i.e. with marked muscle rigidity), suggesting that their pharmacological activity differed fundamentally from the parent convulsants (Ikoma *et al*., 2008; Oikawa *et al*., 2009; Oikawa *et al*., 2010). Here, we have determined if molecular mechanisms underlying this distinctive behavioural phenotype involved actions on AMPA or KA receptors.

We first measured the actions of the IKM compounds on the charge transfer during spontaneously occurring, polysynaptic bursts of EPSCs mediated by AMPA receptors in cultured hippocampal neurons (recorded in the presence of bicuculline methiodide and PTX to inhibit $GABA_A$ receptors and D-APV to inhibit NMDA receptors). The charge transfer during AMPA EPSCs before IKM application, which had a mean of 6.3 ± 1.0 μA·ms (n = 26), was reduced to varying degrees by the compounds. IKM-159 had the greatest inhibitory effect at a fixed concentration of 30 μM, producing a reduction in charge transfer of 65 ± 5% (n = 6, $P < 0.01$). The other compounds were less effective, although most showed some

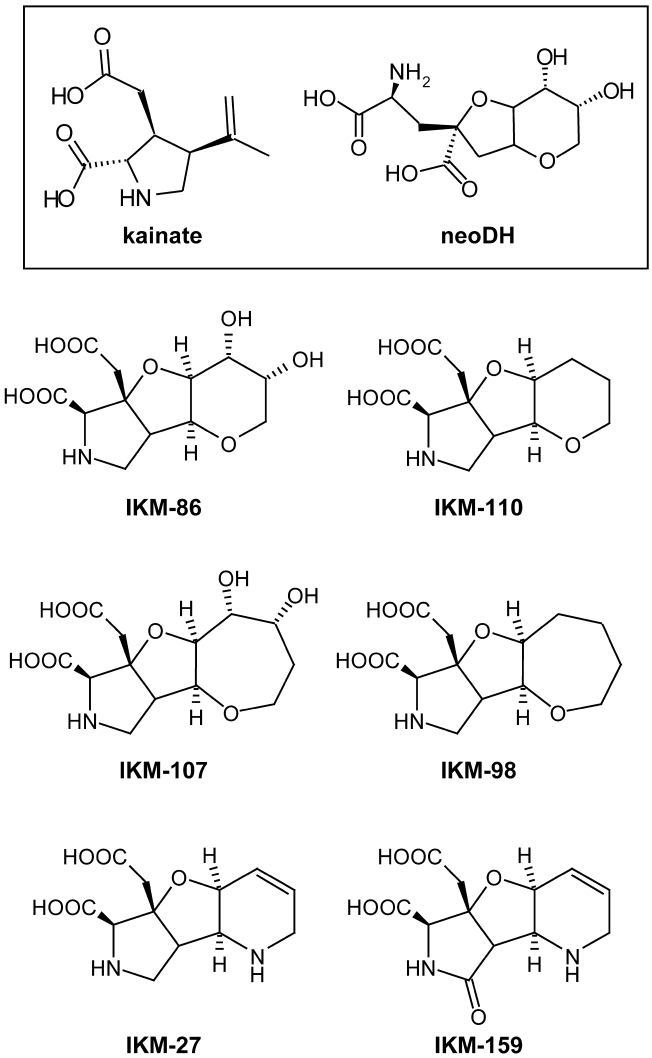

Figure 1 Chemical structures of parent convulsant toxins (KA and neoDH) and synthetic IKM compounds.

degree of inhibitory activity (reduction from control charge transfer: IKM-27, 31 ± 9%; IKM-86, 42 ± 8%; IKM-107, 39 ± 12%; IKM-110, 34 ± 12%, n = 3–5, $P < 0.05$ for all except IKM-110: $P = 0.052$, paired t-test) (Figure 2B). IKM-98 did not significantly affect charge transfer (reduction by 11 ± 14%), but did elicit a robust whole-cell current (380 ± 160 pA, $n = 3$) that was only observed to a lesser extent with IKM-107 (47 ± 11 pA, n = 3) (data not shown). Despite the polar chemical nature of the compounds, recoveries to basal EPSC charge transfers were minimal. In summary, these data provided the first indication that AMPA receptors could be a target for IKM activity.

Subsequent pharmacological characterization was focused predominantly on IKM-159 because this compound was the most effective in our initial neuronal recordings. The action of IKM-159 was next tested on AMPA, KA and NMDA receptor-mediated EPSCs in a more intact preparation, acute hippocampal slices from mice. As shown in the normalized data in Figure 3A, IKM-159 (20 μM) reduced the mean amplitude of pharmacologically isolated AMPA receptor EPSCs evoked

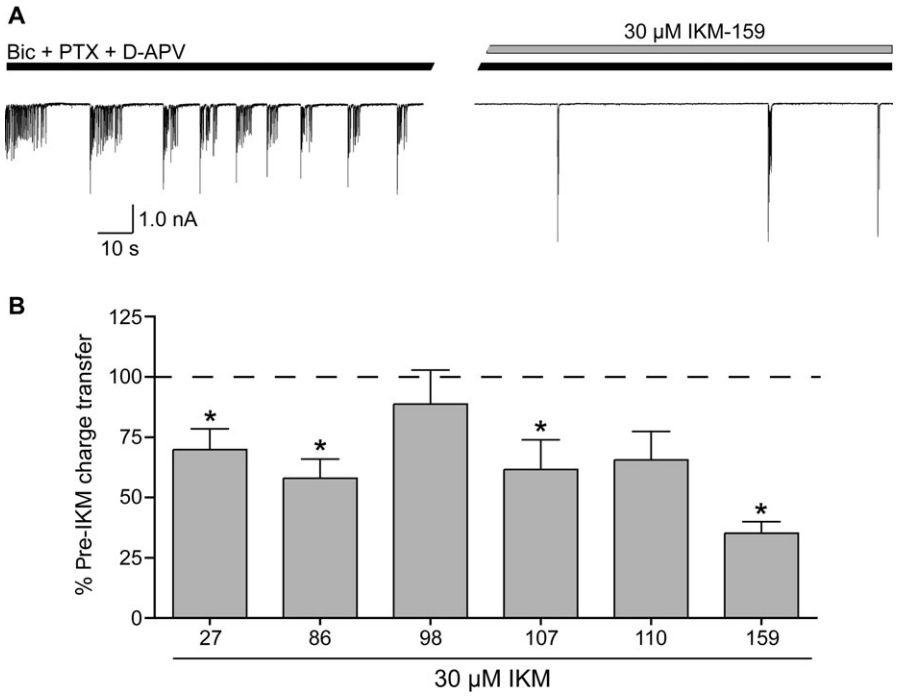

Figure 2 IKM-159 reduces hippocampal AMPA receptor excitatory drive. (A) Representative whole-cell current recording from a primary hippocampal neuron demonstrating a reduction in AMPA receptor-mediated EPSCs during application of 30 μM IKM-159 (right) when compared to the control period (left). (B) The effect of IKM molecules was quantified by measuring the charge transfer during bursts of AMPA receptor-mediated synaptic events. The column graph shows reductions in charge transfer in the presence of 30 μM of each IKM compounds as a percent of the charge transfer during the control period (n = 3–6 for the compounds tested, $P < 0.05$ for all except IKM-110: $P = 0.052$ and IKM-98, paired t-test). Average initial charge transfer: 6.3 ± 1.0 μA·ms, n = 26. Cultured hippocampal neurons were voltage clamped at –70 mV.

from Schaffer collateral–CA1 pyramidal cell synapses by 34 ± 5% (control: 560 ± 20 pA; IKM-159, 370 ± 40 pA, n = 3, $P <$ 0.05). Similar to its effect on bursts of AMPA EPSCs in cultured neurons, the synaptic amplitudes remained depressed for >10 min after application of IKM-159. In contrast, IKM-159 did not alter the mean amplitude of mossy fibre–CA3 pyramidal cell KA receptor-mediated EPSCs, which exhibit a marked time-dependent run-down of approximately 20–40% from initial peak amplitudes (Hirbec *et al*., 2003; Ito *et al*., 2004) (Figure 3B). IKM-159 (20 μM) also did not alter the amplitude of NMDA receptor-mediated EPSCs evoked from Schaffer collateral–CA1 pyramidal cell synapses (99 ± 3% of control amplitudes following a 5 min treatment, n = 3, data not shown). Together, these data demonstrate that IKM-159 selectively inhibits AMPA receptors in both acute mouse hippocampal slices and cultured primary hippocampal neurons.

IKM-159 may exhibit subunit-selective antagonism of AMPA receptors

We next examined the efficacy of IKM compounds on recombinant AMPA and KA receptors to determine if the analogues were selective for a particular subset of receptor stoichiometries. As an initial survey of the six IKM compounds, inhibition of recombinant GluA4 receptor activation was measured in whole-cell patch clamp recordings from transfected HEK-293 cells. Currents were elicited rapidly with a 100 ms application of 10 mM glutamate alone and in the presence of the compounds shown in Figure 1 (30 μM for each IKM analogue, pre-applied for at least 1 min before glutamate). The IKM compounds inhibited GluA4 receptor peak glutamate currents to differing degrees, with IKM-159 again exhibiting the greatest effect (reduction from control peak amplitudes: IKM-27, 20 ± 6%; IKM-86, 19 ± 2%; IKM-98, 12 ± 2%; IKM-107, 5 ± 2%; IKM-110, 14 ± 2%; IKM-159, 40 ± 4%; n = 3–8 cells for each compound, $P < 0.05$ for IKM-86, -98, -110 and -159; $P = 0.07$ for IKM-27, paired t-test). Only the inhibition by IKM-159 was significantly greater relative to the other IKM compounds ($P < 0.05$); conversely, the oxepane analog, IKM -107, was relatively ineffective (Figure 4A,B).

AMPA receptors comprised of other subunit combinations were tested next. IKM-159 (30 μM) inhibited heteromeric GluA1/GluA2 receptors and GluA4 homomeric AMPA receptors to roughly an equivalent degree (44 ± 5% and 40 ± 4% reduction in peak current amplitude, n = 5 and 8, respectively, $P < 0.05$), had weaker, yet still significant, activity on homomeric GluA3 receptors (17 ± 2% reduction in peak current amplitude, n = 4, $P < 0.05$), and did not inhibit activation of homomeric GluA1 receptors (92 ± 3% of glutamate alone peak, n = 3, n.s.) (Figure 5A). Inhibition–response curves were generated for the action of IKM-159 on GluA1/GluA2 and GluA4 AMPA receptors, revealing a slightly higher potency for the heteromeric receptors (IC_{50} values: GluA1/GluA2, 26 ± 2 μM; GluA4 IC_{50}, 60 ± 8 μM; currents evoked by 10 mM glutamate) (Figure 5B). In contrast to its actions on AMPA receptors, IKM-159 did not reduce currents mediated by either homomeric GluK1-2b or GluK2a KA receptors (90 ± 10% and 93 ± 2% of peak glutamate current, n = 3, n.s.) (Figure 5A).

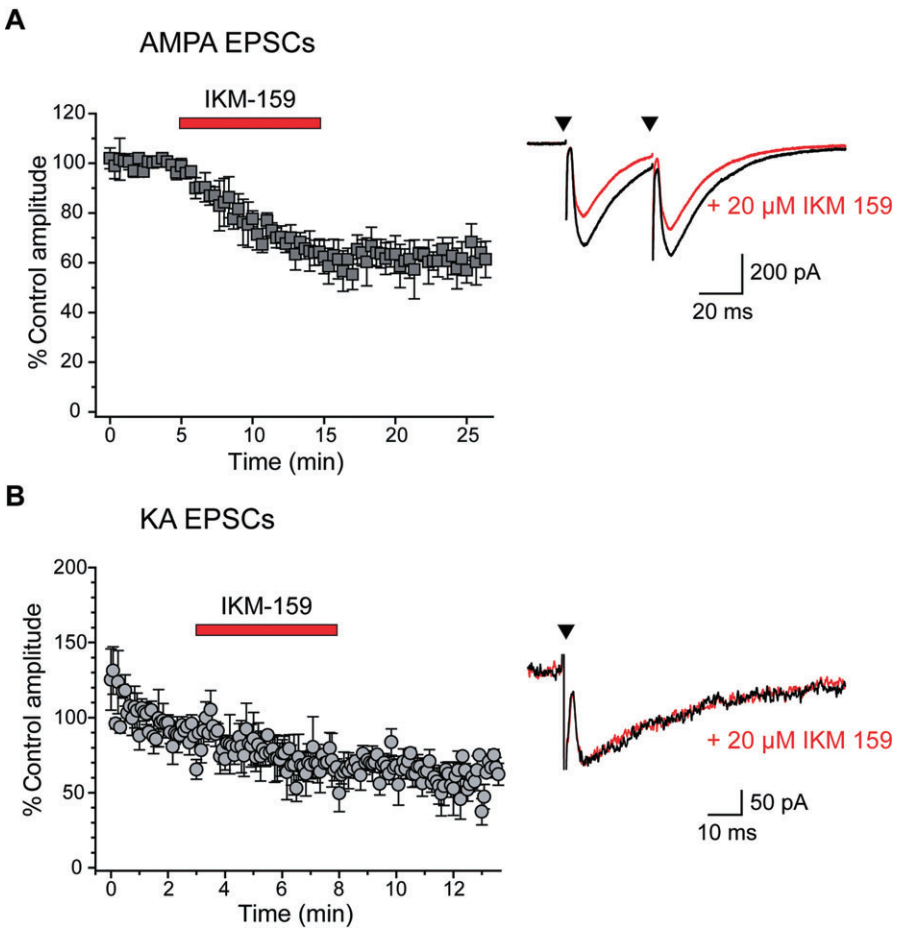

Figure 3 IKM-159 reduces synaptic AMPA receptor EPSCs amplitudes, but does not inhibit KA receptor EPSCs. (A) EPSCs mediated by AMPA receptors were elicited by stimulation of Schaffer collateral inputs to CA1 pyramidal neurons during whole-cell voltage clamp recording from mouse acute brain slices. Left: Summary data of the percent control AMPA EPSC peak amplitudes elicited by Schaffer collateral stimulation during the control period and during application of 20 μM IKM-159 demonstrate a reduction in EPSC peak amplitudes with application of IKM-159 (expressed as a percent of control peak amplitude: IKM-159; 66 ± 5%, $n = 3$, $P < 0.05$, paired *t*-test). Average control peak AMPA EPSC amplitude: 560 ± 20 pA, $n = 3$. Right: Representative paired-pulse trace from a CA1 pyramidal neuron after stimulation of Schaffer collateral fibres during a control period and during application of 20 μM IKM-159. (B) EPSCs mediated by KA receptors were elicited by stimulation of mossy fibre inputs to CA3 pyramidal neurons during whole-cell voltage clamp recording from mouse acute brain slices. Left: Summary data of the per cent control KA EPSC peak amplitudes. Application of 20 μM IKM-159 did not alter the typical run-down in amplitude observed with these synaptic currents. Average control peak KA EPSC amplitude: 170 ± 40 pA, $n = 3$. Right: Representative single pulse trace from a CA3 pyramidal neuron after stimulation of mossy fibres during a control period and during application of 20 μM IKM-159.

Thus, these results demonstrate that IKM-159 acts as an AMPA receptor-selective antagonist with preference for GluA1/GluA2 at the concentrations tested in these studies.

IKM-159 inhibition: competitive or non-competitive?

The IKM molecules failed to displace radioligand from iGluR proteins in rat brain membranes in earlier studies (Ikoma *et al*., 2008; Oikawa *et al*., 2009), leading to the conclusion that they did not directly interact with AMPA, KA or NMDA receptors. Given that our physiological studies clearly supported antagonist activity on AMPA receptors, we further explored the nature of this activity in additional radioligand binding assays with recombinant receptor subunits expressed in HEK-293 cells. Consistent with the earlier results, a physiologically active concentration of IKM-159 (100 μM) failed to displace either [^{3}H]-AMPA from GluA1, GluA2 or GluA4 receptors or [^{3}H]-KA from recombinant GluK1 or GluK2 KA receptors ($n = 3$–4 trials for each cDNA construct; Figure 6). As a positive control for the displacement assay conditions, we confirmed in parallel experiments that 10 μM CNQX, a competitive antagonist for AMPA and KA receptors, effectively competed for GluA2 receptor binding (82% displacement of the radioligand; data not shown). Two other reaction conditions were also tested to determine if the absence of displacement occurred because binding of IKM-159 failed to reach equilibrium during the incubation with membrane preparations. We measured the percent of [^{3}H]-AMPA bound after: (i) a 1 h pre-incubation with 100 μM IKM-159 prior to addition of the radioligand; and (ii) a 2 h incubation of 100 μM IKM-159 with [^{3}H]-AMPA. Neither condition resulted in any significant displacement of [^{3}H]-AMPA from GluA4 homomeric receptors (100 and 113% bound radioligand, respectively; data not shown). At face value, these results suggest that

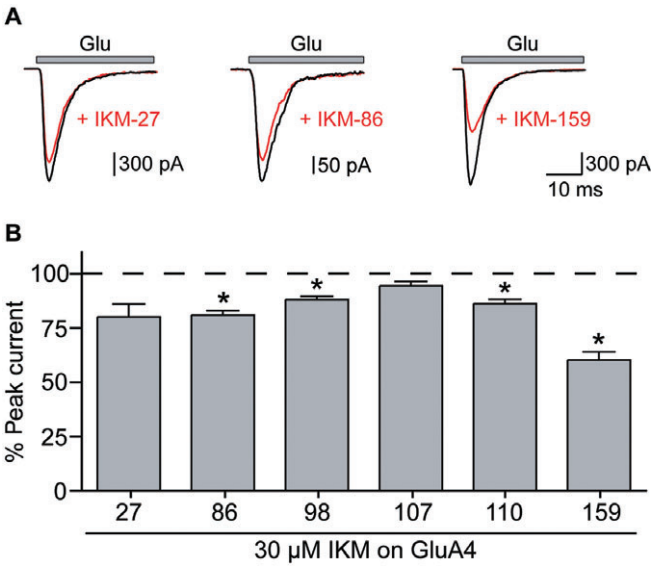

Figure 4 IKM molecules inhibit GluA4 receptor activation. (A) Representative traces from recombinant GluA4 receptors before and during co-application of 30 μM IKM-27 (left), 30 μM IKM-86 (middle) and 30 μM IKM-159 (right) with glutamate reveal that IKM-159 inhibits GluA4 currents to a greater degree than IKM-27 or IKM-86. Grey bar denotes a 100 ms application of 10 mM glutamate. All IKM molecules were pre-applied to the receptor-expressing HEK293 cells for at least 1 min prior to glutamate application. (B) The graph shows the per cent reduction in peak GluA4 currents in the presence 30 μM of each IKM molecule. IKM-159 inhibits GluA4 peak current amplitudes most effectively ($n = 3$–8 cells for each compound, $P < 0.05$ for IKM-86, -98, -110 and -159, $P = 0.07$ for IKM-27, paired *t*-test).

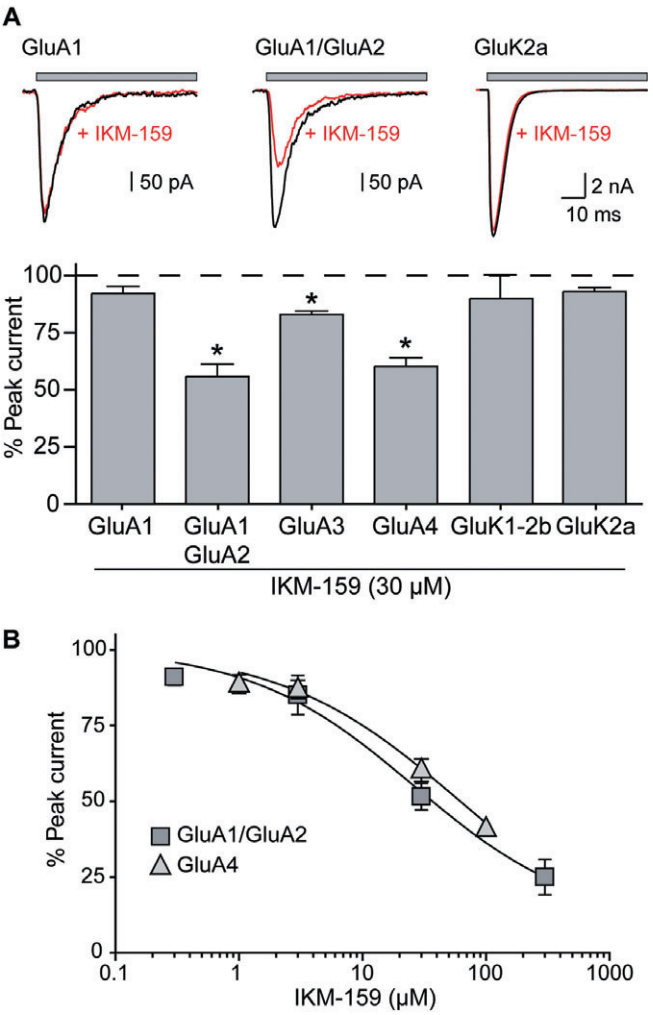

Figure 5 IKM-159 is more potent on GluA2- and GluA4-containing AMPA receptors. (A) Top: Representative traces from AMPA and KA receptor currents in the absence and presence of IKM-159. Glutamate (100 ms, 10 mM) was applied to recombinant homomeric GluA1 receptors (left), heteromeric GluA1/GluA2 receptors (middle) and homomeric GluK2a receptors (right) in the absence and presence of 30 μM IKM-159. IKM-159 was pre-applied to the receptor-expressing HEK293 cells for at least 1 min prior to the test glutamate application. Bottom: The graph shows the reduction in mean peak amplitude as a per cent of the control current amplitude. At this concentration, IKM-159 inhibits GluA2-containing and GluA4 receptors; modestly inhibits GluA3 receptors; and has no effect on homomeric GluA1, GluK1-2b or GluK2a receptors ($n = 3$–8 cells for each receptor; $P < 0.05$ for GluA1/GluA2, GluA3 and GluA4, paired *t*-test). (B) Fitting of concentration–inhibition data with logistic curves yields IC_{50} values of 26 ± 2 μM for GluA1/GluA2, and 60 ± 8 μM for GluA4 receptors. For these curves, the maximum peak current was constrained to 100% and the minimum current constrained to values greater than or equal to zero.

IKM-159 acts as a non-competitive antagonist of AMPA receptors.

These results of the binding assays were surprising, however, because it seemed rather improbable that AMPA receptor antagonists containing cyclized glutamate backbones (albeit with distinct stereochemistry) would interact serendipitously with an allosteric domain removed from the receptor ligand-binding site. For that reason, we carried out additional physiological experiments designed to test the mechanistic nature of IKM-159 antagonism. We tested if the magnitude of IKM-159 inhibition of GluA4 receptor activation varied dependent upon agonist concentration, which would be consistent with a competitive mechanism of action. Steady-state GluA4 receptors currents were elicited with two concentrations of glutamate (100 μM and 10 mM) in the presence of 100 μM cyclothiazide (CTZ), which prevented desensitization of these receptors (Wong and Mayer, 1993; Fletcher and Lodge, 1996). We found that 30 μM IKM-159 reduced steady-state currents elicited by 100 μM glutamate to a greater degree than those with 10 mM glutamate ($n = 4$; $P < 0.05$; Figure 7A). We confirmed the agonist concentration dependence of inhibition in an additional experiment with KA, a partial agonist that predominantly activates a small equilibrium current from GluA4 and other AMPA receptors (Patneau *et al*., 1993; Stern-Bach *et al*., 1998). Similar to the findings from glutamate application, 30 μM IKM-159 inhibited KA-evoked steady-state currents to greater degree at a lower (50 μM KA) agonist concentration than at a higher (500 μM KA) agonist concentration ($n = 5$; $P < 0.05$; Figure 7B). However, when we measured the inhibition of GluA4 non-equilibrium peak currents elicited by application of 500 μM and 10 mM glutamate, we observed no significant difference in inhibition with application of 30 μM IKM-159 ($n = 9$; n.s.; Figure 7C). In summary, reduction of steady-state currents by IKM-159 was agonist concentration dependent, in contrast to inhibition of peak, non-desensitized currents. Taken together,

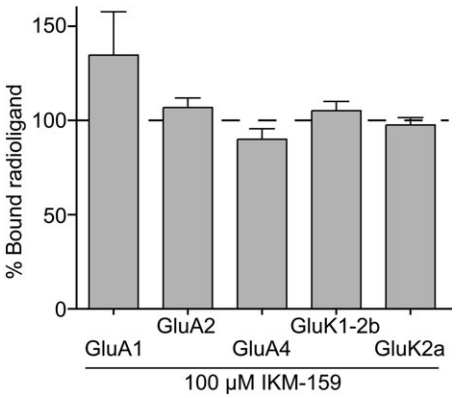

Figure 6 IKM-159 does not displace radiolabelled ligand from AMPA or KA receptors. IKM-159 (100 μM) competed with either [^{3}H]AMPA or [^{3}H]KA for binding to membranes prepared from HEK293-T/17 cells expressing AMPA or KA receptor subunits respectively. No displacement was observed for any receptors (n = 3–4 trials for each receptor).

the results of the electrophysiology and binding studies supported a complex mechanism of action for IKM-159 that was not consistent with either simple competitive or state-independent allosteric inhibition.

Effects of IKM-159 on neuronal excitability

The behavioural response to IKM-159 and IKM-86 is one of marked hypoactivity and immobility, which differs from behaviours associated with non-selective AMPA receptor antagonists, such as CNQX, and NMDA antagonists such as MK-801 and memantine (Maj *et al*., 1995; Czlonkowska *et al*., 1997; Fredriksson and Archer, 2002; Ikoma *et al*., 2008). To determine if altered neuronal excitability could contribute to this behavioural phenotype, we examined the effect of IKM-86 and IKM-159 on AP firing in cultured hippocampal neurons. Spontaneous APs were recorded in current clamp mode before, during and after application of 20 μM IKM-86 and IKM-159. The firing frequency was reduced modestly by IKM-86 (n = 6, P < 0.05; Figure 8B) and to a greater extent, by IKM-159 (n = 3, P < 0.05, Figure 8A,C). Similar to the effect of IKM compounds on neuronal AMPA EPSC charge transfer, this reduction in AP firing frequency was prolonged after removal of IKM-86 and IKM-159.

Several AMPA receptor-selective antagonists reduce neuronal hyperactivity in both acute brain slice preparations and in *in vitro* models of epilepsy (Grasso *et al*., 1999; Szabados *et al*., 2001). Non-competitive AMPA receptor antagonists also have demonstrated therapeutic potential for epilepsy in interim analysis of pre-clinical trials (Bialer *et al*., 2002; Howes and Bell, 2007). We therefore tested the efficacy of IKM-159 in reducing the hyperactive neuronal phenotype in dissociated hippocampal neurons using an *in vitro* model of SE (Pal *et al*., 1999; Deshpande *et al*., 2007). AP firing frequencies were elevated and membrane potentials depolarized following removal of extracellular Mg^{2+} from the external bath and concomitant application of 2 μM glycine (Figure 9A, top left). A period of depolarization-induced channel inactivation ensued, followed by a slow hyperpolarization and a return of AP firing to an average frequency of >2 Hz (Pal *et al*., 1999; Deshpande *et al*., 2007) (Figure 9A, top centre). Addition of IKM-159 (20 μM) to the bath significantly reduced the hyperactive firing frequencies to near basal levels (n = 3, P < 0.05; Figure 9A, top right, 9B). We observed no return in AP firing frequency to hyperactive frequencies upon wash-out of IKM-159 (Figure 9B). A lower concentration of IKM-159 (3 μM) was less effective (n = 3; n.s., Figure 9C), suggesting that the reduction in neuronal excitability could be accounted for predominantly by AMPA receptor inhibition. Thus, IKM-159 effectively reduces the seizure-like phenotype in an *in vitro* SE model, in a concentration range similar to that which inhibited AMPA receptors. In parallel experiments with the SE model, the non-selective AMPA and KA receptor antagonist CNQX (1 μM) similarly reduced AP firing frequency (n = 4, P < 0.05; Figure 9D). These results are consistent with AMPA receptor inhibition as the principal pharmacological activity of IKM-159 that underlies its effects on neuronal excitability, although they do not preclude contributions by other receptor signalling systems to the behavioural phenotype.

Discussion

Aberrant iGluR signalling is central to a variety of neurological diseases (e.g. epilepsy, stroke and pain) (Ben-Ari, 2001; Jensen, 2002), and therefore antagonists with subunit selectivity could be of substantive pharmacological and clinical utility. The unusual behavioural activity observed previously with some of the IKM compounds led us to examine their pharmacological activities in detail. Several intriguing points emerged from this analysis. Most notably, we found that the tetrahydropyridine analogue IKM-159 acts as the first AMPA receptor-selective antagonist that demonstrates partial subunit specificity for GluA2-, GluA3- and GluA4-containing receptors. Furthermore, application of IKM-159 reduced neuronal hyperexcitability in an *in vitro* model of SE, most likely through inhibition of AMPA receptors. In addition to the novel pharmacological profile, the potential utility of this and related compounds resides in the high-efficiency, relatively simple synthetic pathways that have been established previously (Ikoma *et al*., 2008; Oikawa *et al*., 2009). We therefore propose that IKM-159 could serve as a structural template for a series of more potent AMPA receptor-selective antagonists possessing greater therapeutic potential.

Pharmacological activity of IKM-159

While their synthesis was inspired by the high-affinity KA receptor agonists KA and neoDH, the structure of IKM-159 and other analogues differs substantially from the parent convulsant, and it was therefore possible that their mechanism of action diverged from actions on iGluRs (particularly in the case of those that induced hypoactivity). Indeed, we found that synaptic KA receptors at mossy fibre synapses, which are a heteromeric combination of the GluK2, GluK4 and GluK5 subunits (Mulle *et al*., 1998; Contractor *et al*., 2003; Fernandes *et al*., 2009), were unaffected by the compound. IKM-159 instead acts directly on AMPA receptors in neuronal and recombinant systems to reduce channel activation, and,

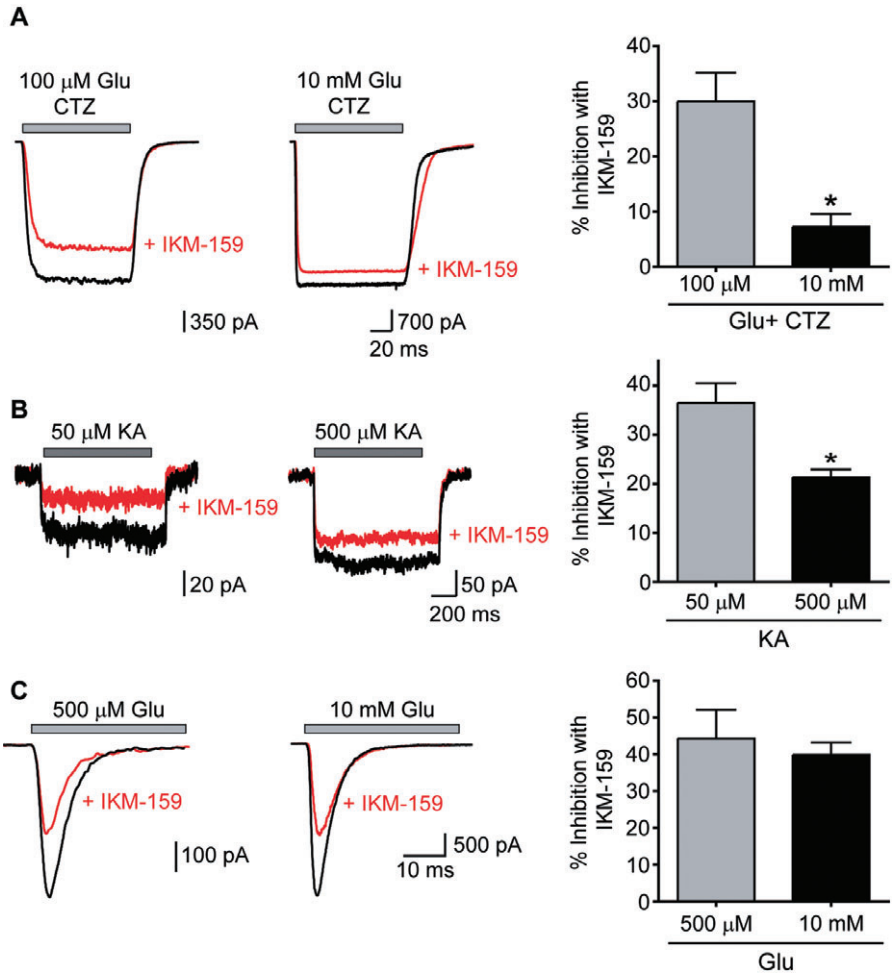

Figure 7 Inhibition of GluA4 receptors by IKM-159 is dependent upon the agonist concentration. (A) Representative steady-state currents evoked from GluA4 AMPA receptors by two concentrations of glutamate (100 μM and 10 mM) in the presence of CTZ (100 μM). The graph shows that IKM-159 inhibited currents evoked by the lower concentration to a greater degree. (B) A similar experiment in which the agonist KA was used at two different concentrations (50 and 500 μM). The graph shows that currents evoked by 50 μM KA were inhibited to a greater degree by IKM-159. (C) In contrast to the preceding experiments, IKM-159 inhibition of peak glutamate-evoked currents did not vary with agonist concentration (in this case, 500 μM and 10 mM). The antagonist IKM-159 was pre-applied to the receptor-expressing HEK293-T/17 cells for at least 1 min prior to the test agonist in the experiments shown in (A–C).

indeed, has a substantially altered pharmacological profile relative to KA and neoDH. In acute hippocampal slices, application of IKM-159 inhibited Schaffer-collateral CA1 EPSCs, which are thought to be predominantly mediated by heteromeric GluA1/GluA2 or GluA2/GluA3 AMPA receptors (Malinow and Malenka, 2002; Lu *et al.*, 2009). The degree of CA1 EPSC inhibition was generally consistent with what was observed with GluA1/GluA2 receptors in heterologous systems.

It will be of interest to determine if this apparent subunit selectivity extends to differential activity at a variety of excitatory synapses. If so, IKM-159 could prove to be a valuable pharmacological tool for dissecting fundamental contributions to excitatory transmission by receptors of different subunit composition, which is not possible with the current collection of AMPA receptor antagonists. To date, few AMPA receptor-selective antagonists are commercially available, with GYKI 52466 and the related, more potent non-competitive antagonist, GYKI 53655, being often employed (Wilding and Huettner, 1995; Bleakman *et al.*, 1996). Both GYKI compounds inhibit AMPA receptors in the low micromolar range, but neither demonstrates any significant selectivity between AMPA receptor subunits (Bleakman *et al.*, 1996). Evans Blue (EB) dye is one of the few AMPA receptor antagonists that exhibits subunit selectivity. Like GYKI 53655, EB is a non-competitive AMPA receptor antagonist with an IC_{50} in the high nanomolar–low micromolar range, but, unlike GYKI 53655, EB selectively inhibits recombinant homomeric GluA1, GluA2 and GluA4, and heteromeric GluA1/GluA2 AMPA receptors (Keller *et al.*, 1993; Weigand and Keller, 1998). Further analysis of IKM-159 activity at higher concentrations was precluded in the current studies by limitations in the availability of the compound, but additional syntheses in the future will provide rigorous and quantitative comparison of the pharmacological selectivity and determination of the maximal efficacy of the antagonist on GluA1/GluA2, GluA3 and GluA4 receptors. As well, we will determine how other IKM compounds might

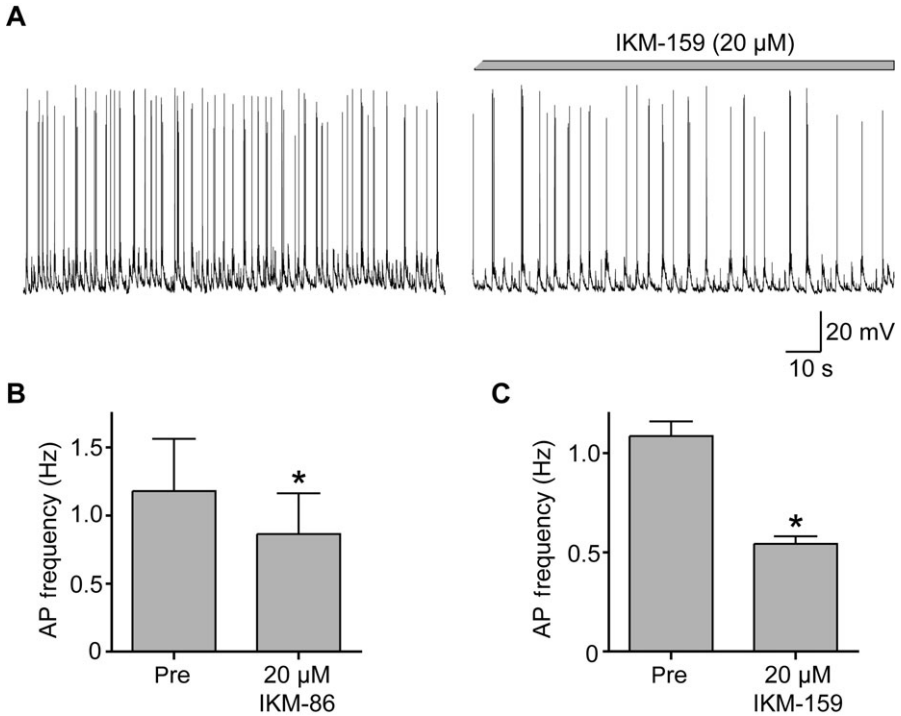

Figure 8 IKM-159 reduces basal neuronal excitability. (A) Representative current clamp recording from a primary hippocampal neuron in the absence and presence of 20 μM IKM-159 illustrating the reduction in basal AP firing frequency. (B,C) AP frequencies during the control period and in the presence of 20 μM IKM-86 (B) and 20 μM IKM-159 (C) The basal firing frequency was significantly reduced by the antagonists (B, $n = 6$, $P < 0.05$, paired *t*-test) (C, $n = 3$, $P < 0.05$, paired *t*-test).

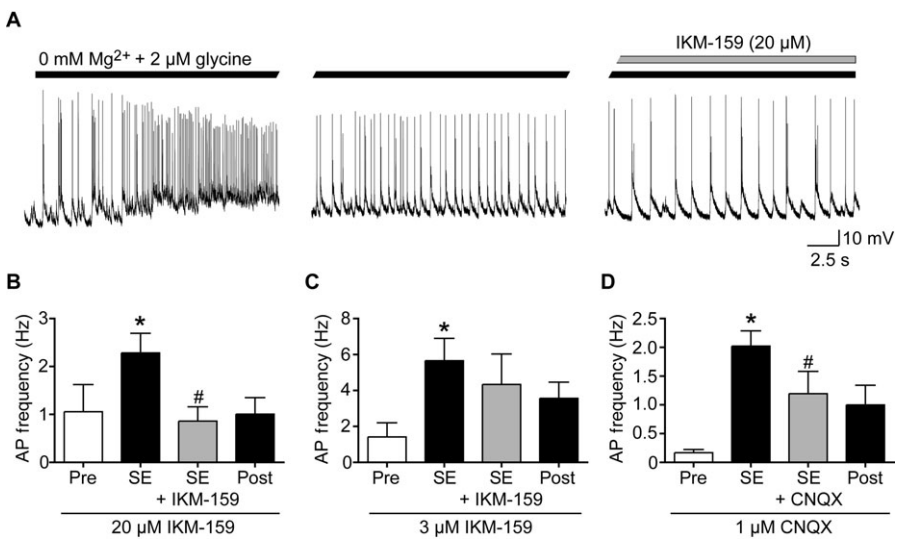

Figure 9 IKM-159 reduces neuronal hyperexcitability in an *in vitro* model of SE. (A) Representative current clamp recording from a primary hippocampal neuron upon induction of SE (by removal of Mg^{2+} and addition of 2 μM glycine), following stabilization of high-frequency AP firing, and in the presence of IKM-159 (20 μM). (B–D) AP frequencies before SE induction, during a stable SE state (>2 Hz), in the presence of IKM-159 or CNQX and 10 min after removal of the compound. IKM-159 was tested at concentrations of 20 μM (B) and 3 μM (C), whereas CNQX was used at 1 μM (D). Mean AP frequencies in each condition are given in the text. All data were analysed using a repeated measures ANOVA with Tukey–Kramer *post hoc* test. *Significantly different from control AP frequency; #significantly different from AP frequency during SE.

overlap in their pharmacological activity and subunit selectivity. Clearly, IKM-127, IKM-86 and IKM-159 share overlapping actions on AMPA receptors, while other IKM molecules (-98 and -107) elicit whole-cell currents from hippocampal neurons, but not in recombinant systems, which suggests activity on unknown population of channels or receptors in addition to their weak activity on AMPA receptors.

Mechanism of antagonism of IKM-159

Determining the mechanism of IKM-159 antagonism proved more complex than first anticipated. Competitive AMPA receptor antagonists such as CNQX readily displace radiolabelled ligands from binding sites on AMPA receptors (see Dev *et al*., 1996), but a high concentration of IKM-159 did not displace [^{3}H]-AMPA from any recombinant non-NMDA receptors. These data, in combination with earlier results noting an absence of radioligand displacement by IKM molecules from rat brain membranes (Ikoma *et al*., 2008; Oikawa *et al*., 2009), suggested that compounds act as a non-competitive antagonists. However, in our current studies, we observed that the degree of inhibition of steady-state currents elicited by application of IKM-159 was dependent on agonist concentration. It remains unclear to us why results from the displacement assays and the physiological measures do not correlate; this dichotomy was not observed previously in similar analyses of natural or synthetic analogues of neoDH (Sanders *et al*., 2005; Lash *et al*., 2008). It is possible that IKM-159 inhibits AMPA receptors in a state-dependent manner, and that the conformational state present in equilibrium conditions during displacement assays is not the functionally relevant state accessed by IKM-159, resulting in an absence of detectable binding affinity. State dependence of the antagonist activity is consistent with our analysis of the inhibition of steady-state and peak currents by IKM-159; we observed a significantly greater degree of inhibition for peak glutamate currents (40%) when compared to inhibition of steady-state currents evoked by the partial agonist, KA (21%), or glutamate in the presence of CTZ (7%). Indeed, the latter set of data demonstrated that the antagonist was largely ineffective if the receptors were activated with saturating glutamate concentrations while desensitization was eliminated with CTZ. Resolution of these questions regarding the mechanism of action of IKM-159 will be pursued using more rigorous pharmacological analyses, including a Schild analysis, which will be possible following additional synthetic efforts. Despite the lack of a clearly elucidated mechanism of antagonism, these data underscore the importance of carrying out multiple functional tests in addition to radioligand binding assays when assessing the competitive or non-competitive nature of an antagonist.

Structural contributions to IKM activity

The similarity in structure of the IKM molecules studied here, and their varying pharmacological activities, allow us to draw some limited conclusions regarding the importance of defined chemical elements to their biological properties. For example, molecules that differed in the substitution of a pyran oxygen (IKM-110) for a tetrahydropyridine nitrogen (IKM-27) in the variable third ring largely overlap in their inhibitory action on neuronal and recombinant AMPA receptors. Substitution of the hydroxyl groups on the pyran ring (IKM-86 vs. IKM-110) clearly increased both *in vivo* and *in vitro* AMPA receptor antagonist activity, although these functional groups clearly do not appear to play the critically important role in receptor selectivity and affinity observed for the analogous moieties in neoDH (Sanders *et al*., 2005). Addition of a carbonyl oxygen within the pyrrolidine ring (IKM-27 vs. IKM-159) significantly increased inhibition of both recombinant GluA4 glutamate-evoked currents and *in vitro* neuronal AMPA receptor EPSC charge transfer, suggesting that this functional group plays an important role in determining potency of the antagonism. Finally, modification of the ring size produced heterogeneous effects on AMPA receptor inhibition by the IKM compounds. Increasing the size of the third ring, from a six-membered pyran ring to a seven-membered oxepane ring, had minimal effect when comparing the actions of IKM-110 and IKM-98 on recombinant glutamate-evoked GluA4 currents, but clearly was unfavourable in the case of IKM-107, which was less potent than IKM-86. As well, both oxepanes, IKM-98 and IKM-107, elicited a whole-cell current in the presence of $GABA_A$ and NMDA receptor antagonists, which could underlie the hyperactivity elicited by injection of these two IKM compounds in mice (Ikoma *et al*., 2008). This activity is unlikely to be mediated by AMPA receptors, because IKM-98 had no effect on recombinant AMPA receptors and a very modest impact on charge transfer during bursts of AMPA receptor EPSCs in neurons. The data therefore suggest that enlarging the third ring size promotes associated with a new, uncharacterized target that depolarizes neurons and thereby promotes hyperactivity. In conclusion, the two molecular constituents critical for AMPA receptor antagonism within this group of molecules are the tetrahydropyridine third ring and the presence of an oxo group in the first pyrrolidine ring.

AMPA receptor antagonism as a therapeutic strategy

Over-activation of iGluRs and inflammatory signalling has been implicated in several CNS excitotoxic diseases (Ben-Ari, 2001; Jensen, 2002). iGluR inhibition effectively alleviates detrimental outcomes in both *in vivo* and *in vitro* disease models (Namba *et al*., 1994; O'Neill *et al*., 1998; Smith *et al*., 2000; Gigler *et al*., 2007). However, the therapeutic promise of non-selective iGluR antagonists has fallen short in therapeutic efficacy in clinical trials (Hoyte *et al*., 2004; Wood, 2005). This failure, in part, could result from inhibition of iGluRs involved in physiological signalling by very potent, non-selective iGluR antagonists. Therefore, therapeutic strategies based on iGluR antagonists with a higher degree of selectivity may prove more successful by sparing excitatory transmission while reducing excitotoxicity.

The pursuit of iGluR subunit-selective agents has been most successful for the NMDA receptor, particularly for the GluN2B subunit [e.g. ifenprodil, traxoprodil (CP 101166) and Ro 25-6981] with the GluN2B subunit-selective NMDA receptor antagonist, CP 101606, recently demonstrating efficacy in modestly reducing dyskinesia and Parkinsonism (Gogas, 2006; Nutt *et al*., 2008). As discussed earlier, however, analogous highly subunit-selective antagonists for AMPA receptors remain to be developed. AMPA receptors have shown efficacy in a range of animal CNS injury models, and early interim results from pre-clinical trials support the targeting of AMPA receptors for reducing the number and severity of epileptic episodes (Howes and Bell, 2007). In this current study, we observed that IKM-159, an AMPA receptor-selective antagonist, significantly reduced increased neuronal AP firing frequency in an *in vitro* model of SE to near basal AP firing frequency, suggesting that this compound could serve as a useful starting point for the development of potentially clini-

cally relevant subunit-selective antagonists. Furthermore, selective AMPA receptor antagonism could be employed not only for epilepsy, but also for the treatment of viral encephalomyelitis (Greene *et al*., 2008), cerebral ischaemia (Gigler *et al*., 2007) and glioblastomas (Grossman *et al*., 2009), which have all been ameliorated with application of the AMPA receptor antagonist talampanel.

Here, we have detailed the pharmacological profile of a novel series of heterotricyclic glutamate analogues, whose generation was inspired by DOS techniques designed to increase structural diversity. IKM-159, the most potent of this first generation of IKM compounds, acts as an AMPA receptor-selective antagonist with no inhibitory action on KA receptors. These molecules could prove to be useful tools for the mechanistic dissection of AMPA receptor diversity, and could serve as structural templates for the design of a second generation of antagonists,with higher potency.

Acknowledgements

We would like to thank Dr William Marszalec for preparation of neuronal cultures. G.T.S. was supported by NIH R01 NS44322. M.O. was supported by a Grant-in-Aid for Scientific Research (21603004) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and M.I. was funded by a research fellowship from the Japan Society for the Promotion of Science.

Conflicts of interest

The authors have no conflicts of interest.

References

Alexander SPH, Mathie A, Peters JA (2009). *Guide to Receptors and Channels (GRAC)*, 4th edn. *Br J Pharmacol* **158** (Suppl. 1): S1–S254.

Ben-Ari Y (2001). Cell death and synaptic reorganizations produced by seizures. *Epilepsia* **42** (Suppl. 3): 5–7.

Bialer M, Johannessen SI, Kupferberg HJ, Levy RH, Loiseau P, Perucca E (2002). Progress report on new antiepileptic drugs: a summary of the Sixth Eilat Conference (EILAT VI). *Epilepsy Res* **51**: 31–71.

Bleakman D, Ballyk BA, Schoepp DD, Palmer AJ, Bath CP, Sharpe EF *et al*. (1996). Activity of 2,3-benzodiazepines at native rat and recombinant human glutamate receptors *in vitro*: stereospecificity and selectivity profiles. *Neuropharmacology* **35**: 1689–1702.

Contractor A, Sailer AW, Darstein M, Maron C, Xu J, Swanson GT *et al*. (2003). Loss of kainate receptor-mediated heterosynaptic facilitation of mossy- fiber synapses in KA2$^{-/-}$ mice. *J Neurosci* **23**: 422–429.

Cordier C, Morton D, Murrison S, Nelson A, O'Leary-Steele C (2008). Natural products as an inspiration in the diversity-oriented synthesis of bioactive compound libraries. *Nat Prod Rep* **25**: 719–737.

Czlonkowska A, Siemiatkowski M, Plaznik A (1997). Some behavioral effects of AMPA/kainate receptor agonist and antagonists. *J Physiol Pharmacol* **48**: 479–488.

Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S, DeLorenzo RJ (2007). *In vitro* status epilepticus but not spontaneous recurrent seizures cause cell death in cultured hippocampal neurons. *Epilepsy Res* **75**: 171–179.

Dev KK, Petersen V, Honore T, Henley JM (1996). Pharmacology and regional distribution of the binding of 6-[^{3}H]nitro-7-sulphamoylbenzo[f]-quinoxaline-2,3-dione to rat brain. *J Neurochem* **67**: 2609–2612.

Fernandes HB, Catches JS, Petralia RS, Copits BA, Xu J, Russell TA *et al*. (2009). High affinity subunits are necessary for ionotropic, but not metabotropic signaling by neuronal kainate receptors. *Neuron* **63**: 818–829.

Fletcher EJ, Lodge D (1996). New developments in the molecular pharmacology of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate receptors. *Pharmacol Ther* **70**: 65–89.

Fredriksson A, Archer T (2002). Functional alteration by NMDA antagonist: effects of L-dopa, neuroleptics drug and postnatal administration. *Amino Acids* **23**: 111–132.

Gigler G, Moricz K, Agoston M, Simo A, Albert M, Benedek A *et al*. (2007). Neuroprotective and anticonvulsant effects of EGIS-8332, a non-competitive AMPA receptor antagonist, in a range of animal models. *Br J Pharmacol* **152**: 151–160.

Gogas KR (2006). Glutamate-based therapeutic approaches: NR2B receptor antagonists. *Curr Opin Pharmacol* **6**: 68–74.

Grasso S, De Sarro G, De Sarro A, Micale N, Zappala M, Puia G *et al*. (1999). Synthesis and anticonvulsant activity of novel and potent 2,3-benzodiazepine AMPA/kainate receptor antagonists. *J Med Chem* **42**: 4414–4421.

Greene IP, Lee EY, Prow N, Ngwang B, Griffin DE (2008). Protection from fatal viral encephalomyelitis: AMPA receptor antagonists have a direct effect on the inflammatory response to infection. *Proc Natl Acad Sci USA* **105**: 3575–3580.

Grossman SA, Ye X, Chamberlain M, Mikkelsen T, Batchelor T, Desideri S *et al*. (2009). Talampanel with standard radiation and temozolomide in patients with newly diagnosed glioblastoma: a multicenter phase II trial. *J Clin Oncol* **27**: 4155–4161.

Hirbec H, Francis JC, Lauri SE, Braithwaite SP, Coussen F, Mulle C *et al*. (2003). Rapid and differential regulation of AMPA and kainate receptors at hippocampal mossy fibre synapses by PICK1 and GRIP. *Neuron* **37**: 625–638.

Hollmann M, Heinemann S (1994). Cloned glutamate receptors. *Annu Rev Neurosci* **17**: 31–108.

Howes JF, Bell C (2007). Talampanel. *Neurotherapeutics* **4**: 126–129.

Hoyte L, Barber PA, Buchan AM, Hill MD (2004). The rise and fall of NMDA antagonists for ischemic stroke. *Curr Mol Med* **4**: 131–136.

Ikoma M, Oikawa M, Gill MB, Swanson GT, Sakai R, Shimamoto K *et al*. (2008). Regioselective domino metathesis of 7-oxanorbornenes and its application to the synthesis of biologically active glutamate analogues. *Eur J Org Chem* **2008**: 5215–5220.

Ito K, Contractor A, Swanson GT (2004). Attenuated plasticity of postsynaptic kainate receptors in hippocampal CA3 pyramidal neurons. *J Neurosci* **24**: 6228–6236.

Jensen FE (2002). Relationship between encephalopathy and abnormal neuronal activity in the developing brain. *Int Rev Neurobiol* **49**: 23–35.

Keller BU, Blaschke M, Rivosecchi R, Hollmann M, Heinemann SF, Konnerth A (1993). Identification of a subunit-specific antagonist of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate/kainate receptor channels. *Proc Natl Acad Sci USA* **90**: 605–609.

Lash LL, Sanders JM, Akiyama N, Shoji M, Postila P, Pentikainen OT *et al*. (2008). Novel analogs and stereoisomers of the marine toxin neodysiherbaine with specificity for kainate receptors. *J Pharmacol Exp Ther* **324**: 484–496.

Lu W, Shi Y, Jackson AC, Bjorgan K, During MJ, Sprengel R *et al*. (2009). Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. *Neuron* **62**: 254–268.

Maj J, Rogoz Z, Skuza G, Jaros T (1995). Some behavioral effects of CNQX AND NBQX, AMPA receptor antagonists. *Pol J Pharmacol* **47**: 269–277.

Malinow R, Malenka RC (2002). AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* **25**: 103–126.

Mayer ML, Westbrook GL (1987). The physiology of excitatory amino acids in the vertebrate central nervous system. *Prog Neurobiol* **28**: 197–276.

Mony L, Kew JN, Gunthorpe MJ, Paoletti P (2009). Allosteric modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic potential. *Br J Pharmacol* **157**: 1301–1317.

Mulle C, Sailer A, Pérez-Otaño I, Dickinson-Anson H, Castillo PE, Bureau I *et al*. (1998). Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice. *Nature* **392**: 601–605.

Namba T, Morimoto K, Sato K, Yamada N, Kuroda S (1994). Antiepileptogenic and anticonvulsant effects of NBQX, a selective AMPA receptor antagonist, in the rat kindling model of epilepsy. *Brain Res* **638**: 36–44.

Nelson SB, Turrigiano GG (1998). Synaptic depression: a key player in the cortical balancing act. *Nat Neurosci* **1**: 539–541.

Nelson SB, Turrigiano GG (2008). Strength through diversity. *Neuron* **60**: 477–482.

Nutt JG, Gunzler SA, Kirchhoff T, Hogarth P, Weaver JL, Krams M *et al*. (2008). Effects of a NR2B selective NMDA glutamate antagonist, CP-101,606, on dyskinesia and Parkinsonism. *Mov Disord* **23**: 1860–1866.

Oikawa M, Ikoma M, Sasaki M, Gill MB, Swanson GT, Shimamoto K *et al*. (2009). Regioselective domino metathesis of unsymmetrical 7-oxanorborenes with electron-rich vinyl acetate toward biologically active glutamate analogues. *Eur J Org Chem* **2009**: 5531–5548.

Oikawa M, Ikoma M, Sasaki M, Gill MB, Swanson GT, Shimamoto K *et al*. (2010). Improved synthesis and in vitro/in vivo activities of natural product-inspired, artificial glutamate analogs. *Bioorg Med Chem* (in press).

O'Neill MJ, Bond A, Ornstein PL, Ward MA, Hicks CA, Hoo K *et al*. (1998). Decahydroisoquinolines: novel competitive AMPA/kainate antagonists with neuroprotective effects in global cerebral ischaemia. *Neuropharmacology* **37**: 1211–1222.

Pal S, Sombati S, Limbrick DD Jr, DeLorenzo RJ (1999). *In vitro* status epilepticus causes sustained elevation of intracellular calcium levels in hippocampal neurons. *Brain Res* **851**: 20–31.

Parsons CG, Stoffler A, Danysz W (2007). Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system – too little activation is bad, too much is even worse. *Neuropharmacology* **53**: 699–723.

Patneau DK, Vyklicky L Jr, Mayer ML (1993). Hippocampal neurons exhibit cyclothiazide-sensitive rapidly desensitizing responses to kainate. *J Neurosci* **13**: 3496–3509.

Sanders JM, Ito K, Settimo L, Pentikainen OT, Shoji M, Sasaki M *et al*. (2005). Divergent pharmacological activity of novel marine-derived excitatory amino acids on glutamate receptors. *J Pharmacol Exp Ther* **314**: 1068–1078.

Smith T, Groom A, Zhu B, Turski L (2000). Autoimmune encephalomyelitis ameliorated by AMPA antagonists. *Nat Med* **6**: 62–66.

Stern-Bach Y, Russo S, Neuman M, Rosenmund C (1998). A point mutation in the glutamate binding site blocks desensitization of AMPA receptors. *Neuron* **21**: 907–918.

Swanson GT, Sakai R (2009). Ligands for ionotropic glutamate receptors. *Prog Mol Subcell Biol* **46**: 123–157.

Szabados T, Gigler G, Gacsalyi I, Gyertyan I, Levay G (2001). Comparison of anticonvulsive and acute neuroprotective activity of three 2,3-benzodiazepine compounds, GYKI 52466, GYKI 53405, and GYKI 53655. *Brain Res Bull* **55**: 387–391.

Tan DS (2005). Diversity-oriented synthesis: exploring the intersections between chemistry and biology. *Nat Chem Biol* **1**: 74–84.

Vivithanaporn P, Lash LL, Marszalec W, Swanson GT (2007). Critical roles for the M3-S2 transduction linker domain in kainate receptor assembly and postassembly trafficking. *J Neurosci* **27**: 10423–10433.

Walters MR, Kaste M, Lees KR, Diener HC, Hommel M, De Keyser J *et al*. (2005). The AMPA antagonist ZK 200775 in patients with acute ischaemic stroke: a double-blind, multicentre, placebo-controlled safety and tolerability study. *Cerebrovasc Dis* **20**: 304–309.

Weigand E, Keller BU (1998). Functional diversity of synaptic AMPA/KA receptors from rat as revealed by subtype-specific antagonists. *Eur J Neurosci* **10**: 64–70.

Wilding TJ, Huettner JE (1995). Differential antagonism of alpha-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid-preferring and kainate-preferring receptors by 2,3-benzodiazepines. *Mol Pharmacol* **47**: 582–587.

Wong LA, Mayer ML (1993). Differential modulation by cyclothiazide and concanavalin A of desensitization at native alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid- and kainate-preferring glutamate receptors. *Mol Pharmacol* **44**: 504–510.

Wood PL (2005). The NMDA receptor complex: a long and winding road to therapeutics. *IDrugs* **8**: 229–235.