British Journal of Pharmacology (2002) 137, 1125–1133

www.nature.com/bjp

The novel antagonist 3-CBW discriminates between kainate receptors expressed on neonatal rat motoneurones and those on dorsal root C-fibres

[1]Julia C.A. More, [1]Helen M. Troop & *,[1]David E. Jane

[1]Department of Pharmacology, MRC Centre for Synaptic Plasticity, School of Medical Sciences, University of Bristol, Bristol BS8 1TD

1 The natural product willardiine is a selective AMPA receptor agonist. We report that an N^3-substituted analogue of willardiine, (*S*)-3-(4-carboxybenzyl)willardiine (3-CBW), antagonizes AMPA and kainate receptors expressed on motoneurones and dorsal root C-fibres, respectively.

2 Reduction of the fast component of the dorsal root-evoked ventral root potential (fDR-VRP) has been used as a novel method to compare AMPA receptor antagonists. 3-CBW, NBQX and GYKI53655 depressed the fDR-VRP with IC_{50} values of 10.3 ± 2.4, 0.214 ± 0.043 and 4.03 ± 0.31 μM, respectively. That 3-CBW depressed the fDR-VRP by acting at AMPA and not metabotropic glutamate receptors was demonstrated by the lack of effect of LY341495 (100 μM).

3 The Schild plot for antagonism of responses to (*S*)-5-fluorowillardiine on motoneurones by 3-CBW had a slope of 1.11 ± 0.13 giving a pA_2 value of 4.48. The Schild plot for antagonism of kainate responses on the dorsal root by 3-CBW had a slope of 1.05 ± 0.05 giving a pA_2 value of 4.96.

4 On neonatal rat motoneurones 3-CBW (200 μM) almost completely abolished responses to AMPA while responses to NMDA, kainate and DHPG were $101.6 \pm 11.6\%$, $39.4 \pm 5.8\%$ and $110.5 \pm 9.0\%$ of control, respectively. 3-CBW can therefore be used to isolate kainate receptor responses from those mediated by AMPA receptors.

5 3-CBW antagonized kainate-induced responses on dorsal root C-fibres with a pA_2 value of 4.96 whereas kainate receptor mediated responses (isolated by including GYKI53655 in the medium) on motoneurones were not completely blocked by 200 μM 3-CBW, substantiating evidence that kainate receptors on neonatal rat motoneurones differ from those on dorsal root C-fibres.
British Journal of Pharmacology (2002) **137,** 1125–1133. doi:10.1038/sj.bjp.0704957

Keywords: Neonatal rat spinal cord; (*S*)-3-(4-carboxybenzyl)willardiine; 3-CBW; GYKI53655; kainate; AMPA; antagonist

Abbreviations: AMPA, (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propanoate; (*R*)-AP5, (*R*)-2-amino-5-phosphonopentanoic acid; 3-CBW, (*S*)-3-(4-carboxybenzyl)willardiine; CNQX, 2-cyano-3-nitroquinoxaline-2,3-dione; DHPG, (*S*)-3,5-dihydroxyphenylglycine; fDR-VRP, fast component of the dorsal root evoked ventral root potential; GYKI53655, (±)-1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-(methylenedioxy)-5*H*-2,3-benzodiazepine; LY293558, (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2*H*)-tetrazole-5-yl)ethyl]decahydro-isoquinoline-3-carboxylic acid; LY294486, (3*SR*,4a*RS*,6*SR*,8a*RS*)-6-((((1*H*-tetrazol-5-yl)methyl)oxy)methyl)-1,2,3,4,4a,5,6,7,8,8a-6-decahydroisoquinoline-3-carboxylic acid); LY341495, (2*S*,1′*S*,2′*S*)-2-(9-xanthylmethyl)-2-(2-carboxycyclopropyl)glycine; LY382884, (3*S*,4a*R*,6*S*,8a*R*)-6-((4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid; NBQX, 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and acts at two main families of receptors, ionotropic glutamate (iGlu) receptors (Watkins *et al*., 1990), which are ligand gated cation channels, and metabotropic glutamate (mGlu) receptors, which are G-protein coupled receptors (Schoepp *et al*., 1999). The iGlu receptors have been separated into *N*-methyl-D-aspartate (NMDA), (*S*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propanoate (AMPA) and kainate receptors based on their pharmacology (for comprehensive reviews see Jane *et al*., 2000; Jane, 2002). AMPA receptors are known to consist of GluR1–4 subunits, while kainate receptors are made up from GluR5–7, KA1 and KA2 subunits (Bleakman & Lodge, 1998). Though selective antagonists for AMPA receptors are known there is a paucity of selective kainate receptor antagonists (for reviews see Chittajallu *et al*., 1999; Jane *et al*., 2000). A range of decahydroisoquinoline analogues including (3*SR*,4a*RS*,6*SR*,8a*RS*)-6-((((1*H*-tetrazol-5-yl)methyl) oxy)-methyl)-1,2,3,4,4a,5,6,7,8,8a-6-decahydroisoquinoline-3-carboxylic acid (LY294486) (Clarke *et al*., 1997; O'Neill *et al*., 1998) and (3*S*,4a*R*,6*S*,8a*R*)-6-((4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid (LY382884) (O'Neill *et al*., 1998; Simmons *et al*., 1998) have been reported to selectively antagonize recombinant human homomeric GluR5 receptors. These antagonists have been used to demonstrate that GluR5-containing kainate receptors are involved in the control of transmitter release (Clarke *et al*., 1997; Chittajallu *et al*., 1999) and in nociceptive signalling (Simmons *et al*., 1998). In addition, LY382884 was shown to

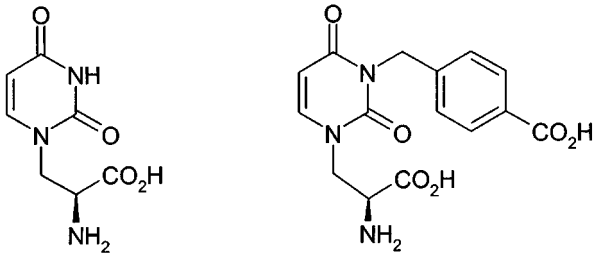
*Author for correspondence: E-mail: david.jane@bris.ac.uk

be an effective neuroprotective agent in animal models of cerebral ischaemia (O'Neill *et al*., 1998).

In 1980, it was demonstrated that the natural product willardiine was a moderately potent agonist at AMPA receptors expressed on motoneurones (Evans *et al*., 1980). More recently, a range of willardiine analogues has been synthesized with selectivity for either AMPA or GluR5-containing kainate receptors depending on the nature of the 5-substituent on the uracil ring (Patneau *et al*., 1992; Wong *et al*., 1994; Jane *et al*., 1997). (*S*)-5-Fluorowillardiine is currently the most potent and selective AMPA receptor agonist and also displays a degree of subunit selectivity as it binds with higher affinity to GluR1 or GluR2 compared to GluR3 or GluR4 (Jane *et al*., 1997; Varney *et al*., 1998). (*S*)-5-Iodowillardiine, on the other hand, is a highly selective GluR5 agonist (Jane *et al*., 1997; Thomas *et al*., 1998), although it has been recently reported to only weakly activate heteromeric assemblies of KA2 with GluR6 or GluR7 (Swanson *et al*., 1998).

There have been several examples where glutamate receptor agonists have been converted into antagonists by extending their inter-acidic group chain length (Davies *et al*., 1982; Krogsgaard-Larsen *et al*., 1991; Madsen *et al*., 1996; Jane *et al*., 2000). It was proposed therefore that by increasing the inter-acidic group chain length of willardiine it may be possible to obtain a novel AMPA and/or kainate receptor antagonist. We have recently synthesized a novel willardiine analogue, (*S*)-3-(4-carboxybenzyl)willardiine (3-CBW) (Figure 1) by adding a 4-carboxybenzyl side chain to the N^3-position of the uracil ring. In this study we have examined the selectivity of the antagonist activity of 3-CBW on AMPA, kainate, NMDA and group 1 mGlu receptors (mGlu1 and 5) expressed on motoneurones.

To facilitate the testing of antagonists, a novel method of comparing AMPA receptor antagonism by measuring the reduction of the fast component of the dorsal root-evoked ventral root potential (fDR-VRP) has been developed. It has previously been reported that the fDR-VRP is mediated by AMPA/kainate receptors (Long *et al*., 1990). However, this study made use of the antagonist 2-cyano-3-nitroquinoxaline-2,3-dione (CNQX), which cannot differentiate between AMPA and kainate receptors (Honoré *et al*., 1988; Chittajallu *et al*., 1999). In the present study we have investigated the reduction of the fDR-VRP by the selective AMPA receptor antagonist (±)-1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-(methylenedioxy)-5*H*-2,3-benzodiazepine (GYKI53655) (Bleakman *et al*., 1996) in an effort to determine whether the underlying excitatory postsynaptic potential (epsp) responsible for the fDR-VRP is mediated by AMPA and/or kainate receptors.



(*S*)-Willardiine (*S*)-3-(4-Carboxybenzyl)willardiine

Figure 1 Structures of (*S*)-willardiine and 3-CBW.

As willardiine analogues have been reported to be agonists at GluR5-containing kainate receptors (Wong *et al*., 1994; Jane *et al*., 1997), 3-CBW was assessed for its ability to block kainate-induced responses on the dorsal root preparation which are believed to be mediated by GluR5-containing kainate receptors (Agrawal & Evans, 1986; Bettler *et al*., 1990; Partin *et al*., 1993; Thomas *et al*., 1998).

Preliminary reports of this work have been published (More *et al*., 2001; 2002).

Methods

Reduction of the fDR-VRP by AMPA receptor antagonists

The neonatal rat spinal cord preparation is a convenient source of a number of glutamate receptor subtypes (Watkins & Evans, 1981; Tölle *et al*., 1993; Schoepp *et al*., 1999; Stegenga & Kalb, 2001). Stimulation of the dorsal root allows the dorsal root-evoked ventral root potential (DR-VRP) to be recorded from the corresponding ventral root. The fast component of the DR-VRP (fDR-VRP) is mediated chiefly by activation of postsynaptically expressed AMPA/kainate receptors whilst the slow component is mediated mainly by NMDA receptors.

Hemisected spinal cords from non-anaesthetized 1–5-day-old rats killed by cervical dislocation were prepared and used according to the method of Evans *et al*. (1982). The standard superfusion medium contained (mM): NaCl 118, $NaHCO_3$ 25, KCl 3, $CaCl_2$ 2.5, D-glucose 12, gassed with 95% O_2/5% CO_2, with all solutions being perfused over the preparation at a rate of 1 ml min^{-1}.

AMPA receptor antagonists were tested for their ability to depress the fDR-VRP in the neonatal rat spinal cord preparation. A dorsal root in the lumbar region of the spinal cord was stimulated supramaximally (16× threshold, 2 pulses min^{-1}, pulse width 0.2 ms) and recordings made from the corresponding ventral root. To allow isolation of the non-NMDA receptor-mediated component of the DR-VRP, which includes the fast component mediated *via* AMPA and/or kainate receptors (Long *et al*., 1990), 2 mM $MgSO_4$ and 50 μM (*R*)-2-amino-5-phosphonopentanoic acid ((*R*)-AP5) were included in the standard medium (30 min pre-incubation) to block any NMDA receptor mediated component. The antagonists GYKI53655, 2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(f)quinoxaline (NBQX) and 3-CBW were superfused over the preparation at a range of concentrations and the per cent depression of the fDR-VRP calculated to generate non-cumulative concentration response curves for each antagonist. Averages of the peak amplitudes of two consecutive responses in the absence of the antagonist and when the minimum response size was achieved after antagonist application were used to calculate the per cent reduction in the fDR-VRP. To determine the application time for antagonists, 10 μM 3-CBW was applied for 2, 5, 10 or 15 min and the per cent reduction of the fDR-VRP measured.

It is known that mGlu receptors from both group 2 (mGlu2 and mGlu3) and group 3 (mGlu4, 6, 7 and 8) are

expressed on primary afferent terminals in the neonatal rat spinal cord where they act as autoreceptors (Schoepp *et al*., 1999). Activation of these receptors leads to a depression of the fDR-VRP, most likely due to a depression of glutamate release (Watkins & Collingridge, 1994). As 3-CBW depresses the fDR-VRP it was important to rule out an effect on group 2 or 3 mGlu receptors expressed on primary afferent terminals. To determine whether 3-CBW has an action on group 2 or 3 mGlu receptors concentration response curves for the reduction of the fDR-VRP by 3-CBW were constructed in the absence and presence of the mGlu receptor antagonist (2*S*,1′*S*,2′*S*)-2-(9-xanthylmethyl)-2-(2-carboxycyclopropyl)glycine (LY341495) at a concentration that blocks all known mGlu receptors (100 μM; 15 min pre-incubation) (Fitzjohn *et al*., 1998).

Characterization of 3-CBW on spinal motoneurones

Neonatal rat motoneurones express a range of glutamate receptor subtypes, including NMDA, AMPA, kainate, mGlu1 and mGlu5 receptors, making them useful for testing the selectivity of compounds across a range of receptors. Experiments performed to investigate the effect of 3-CBW on agonist-induced effects at glutamate receptors expressed on motoneurones were carried out in the presence of tetrodotoxin (TTX; 10 μM for 2 min, then 0.1 μM continuously) to block action potential dependent glutamate release. In all experiments described in this section no stimulation was applied and the peak amplitude of agonist-induced depolarization of motoneurones was measured from d.c. shifts in ventral root polarity after agonist application (Long *et al*., 1990). This methodology has been previously used to obtain reliable K_D values for antagonists for a range of glutamate receptor subtypes by constructing Schild plots (Evans *et al*., 1982; Pook *et al*., 1993; Thomas *et al*., 1998).

Two types of experiment were carried out using this protocol. For Schild analysis of the antagonist effect of 3-CBW on AMPA receptors, non-cumulative concentration-response curves were constructed for the AMPA receptor agonist (*S*)-5-fluorowillardiine (1 min applications) in the absence and presence of 3-CBW (50, 100, 150 or 200 μM; 30 min pre-incubation). In preliminary studies it was found that if concentrations above 3 μM (*S*)-5-fluorowillardiine were added to the preparation in the absence of antagonist the tissue showed signs of excitotoxicity (responses using concentrations above 3 μM were smaller in amplitude in some cases) and results were not consistent. For these reasons, 3 μM (*S*)-5-fluorowillardiine was used as the maximum concentration in the absence of antagonist.

In experiments to determine the antagonist selectivity of 3-CBW medium containing approximately equi-effective concentrations of either (*S*)-AMPA (0.5 – 1.5 μM), kainate (2 μM), NMDA (10 μM), or (*S*)-3,5-dihydroxyphenylglycine (DHPG) (20 μM) was applied for 1 min, in the absence and presence of 3-CBW (200 μM; 30 min pre-incubation).

To investigate the ability of 3-CBW to block kainate receptors on motoneurones, approximately equi-effective concentrations of (*S*)-AMPA (0.8 μM) and kainate (2 μM) were applied for 1 min in the absence of antagonist, in the presence of 50 μM GYKI53655 and in the combined presence of 50 μM GYKI53655 and 200 μM 3-CBW (antagonists were pre-incubated for 30 min).

Antagonism of kainate responses on dorsal root C-fibres by 3-CBW

Experiments to test the antagonistic effect of 3-CBW on GluR5 containing kainate receptors were conveniently carried out on kainate-induced responses on isolated dorsal roots. The dorsal root (L3 – L5) was dissected from the point of exit from the spinal cord to just proximal to the dorsal root ganglion (DRG) of 1 – 5-day-old rats, as reported previously (Agrawal & Evans, 1986). In order to record the shift in d.c. potential, the peripheral end of the dorsal root was electrically insulated from the rest of the preparation by a grease seal. To prevent desensitization of kainate receptors, the dorsal root was superfused with 1 mg ml^{-1} concanavalin A for 20 min after a 20 min exposure to glucose free superfusion medium. Standard superfusion medium was then applied throughout the experiments. This protocol allowed measurement of depolarizations evoked by the exogenously applied agonist, kainate (1 min applications) that were measured as the area of the response. For Schild analysis, non-cumulative concentration-response curves were constructed for kainate in the absence and presence of 3-CBW (50, 100, 150 or 200 μM; 30 min pre-incubation). In preliminary studies it was found that 30 μM kainate was the maximum concentration that could be used in the absence of antagonist to maintain repeatable results, therefore this concentration was used as the maximum kainate dose in the absence of antagonist. Using this methodology EC_{50} values for kainate were obtained that were similar to previously reported values (Agrawal & Evans, 1986; Pook *et al*., 1993; Thomas *et al*., 1998).

Data analysis

Concentration-response curves were analysed by iterative non-linear regression (GraphPAD Prism). IC_{50} values for the antagonists were measured as the concentration required to obtain a 50% reduction of the fDR-VRP. The pA_2 values were calculated following Schild analysis for the antagonism by 3-CBW of (*S*)-5-fluorowillardiine-induced responses on motoneurones and kainate-induced responses on dorsal root C-fibres. A paired student's *t*-test was used to test for statistical significance.

Materials

3-CBW was synthesized in our own laboratories by a method that will be reported elsewhere. Stock solutions of 3-CBW were made up in one equivalent of 100 mM aqueous sodium hydroxide. Concanavalin A (type VI) was obtained from Sigma, U.K. AMPA, NMDA, kainate, (*S*)-5-fluorowillardiine, GYKI53655, DHPG, LY341495, TTX and (*R*)-AP5 were obtained from Tocris Cookson, Bristol, U.K. All other chemicals were of analytical grade or above.

Results

Depression of the fDR-VRP by AMPA receptor antagonists

To investigate whether the reduction of the fDR-VRP is a useful method of comparing AMPA receptor antagonists, the

following experiments were carried out. In an initial study to find an application time for antagonists that reached a steady state while using the minimum amount of novel compound, it was found that responses reached a plateau after a 5 min application time (Figure 2). This time course was therefore used for all other experiments that looked at the reduction of the fDR-VRP by antagonists. The selective AMPA receptor antagonist GYKI53655 blocked the fDR-VRP with an IC_{50} value of 4.03 ± 0.31 μM ($n=3$) and a 100% depression of the response was observed at a concentration of 30 μM (Figure 3A,B). The novel willardiine analogue 3-CBW reduced the fDR-VRP with an IC_{50} value of 10.3 ± 2.4 μM ($n=3$). The selective AMPA/kainate receptor antagonist NBQX had an IC_{50} value of 214 ± 43 nM ($n=3$) for depression of the fDR-VRP (see Figure 3B).

The fDR-VRP can also be reduced by activation of group 2 or 3 mGlu autoreceptors expressed on primary afferent terminals. In order to rule out a contribution of these receptors to the 3-CBW induced depression of the fDR-VRP the antagonist LY341495 was used at a concentration that was sufficient to block all eight mGlu receptors. The ability of 3-CBW to reduce the fDR-VRP was measured in the absence and presence of LY341495 (100 μM). In these experiments 3-CBW depressed the fDR-VRP with IC_{50} values of 14.7 ± 2.31 μM ($n=3$) in the absence and 12.8 ± 1.77 μM ($n=3$) in the presence of LY341495. A paired student's *t*-test showed that these results were not significantly different ($P=0.10$).

3-CBW is a competitive antagonist of AMPA receptors on spinal motoneurones

In the continuous presence of TTX approximately parallel concentration-response curves were obtained for the concentration dependent shifts in d.c. potential of ventral roots induced by the selective AMPA receptor agonist (*S*)-fluorowillardiine in the absence and presence of 3-CBW (100 μM; Figure 4A). Similar experiments were carried out using 50, 150 and 200 μM 3-CBW in order to generate a Schild plot (Figure 4B). The Schild plot for 3-CBW against (*S*)-5-fluorowillardiine responses on spinal cord motoneurones had a slope of 1.11 ± 0.13 and gave a pA_2 value of 4.48.

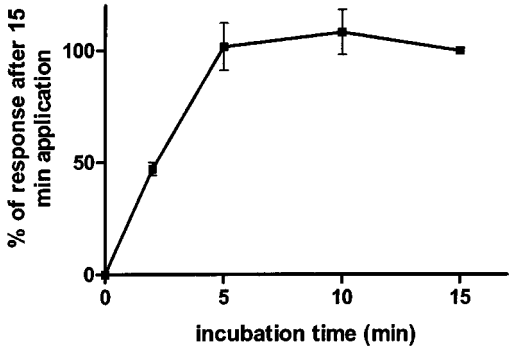


Figure 2 Effect of different antagonist application times on the reduction of the fDR-VRP. Comparison of the reduction of the fDR-VRP by 10 μM 3-CBW after 2, 5, 10 and 15 min application times. Results are expressed as per cent of the reduction seen after a 15 min application. Each point represents the mean$\pm$s.e.mean from three preparations.

3-CBW is a competitive antagonist of kainate receptors expressed on rat dorsal root C-fibres

Approximately parallel concentration-response curves were obtained for kainate-induced shifts in d.c. potential of isolated dorsal root in the absence and presence of 3-CBW (100 μM; Figure 5A). Similar experiments were carried out using 50, 150 and 200 μM 3-CBW in order to generate a Schild plot (Figure 5B). The Schild plot for antagonism of kainate-induced responses on dorsal root by 3-CBW had a slope of 1.05 ± 0.05 and gave a pA_2 value of 4.96.

3-CBW selectively antagonizes AMPA receptors expressed on spinal motoneurones

Figure 6 shows a representative trace of experiments carried out to investigate the selectivity of 3-CBW on spinal motoneurones. The experiments looked at the ability of 3-CBW to block equi-effective concentrations of AMPA,

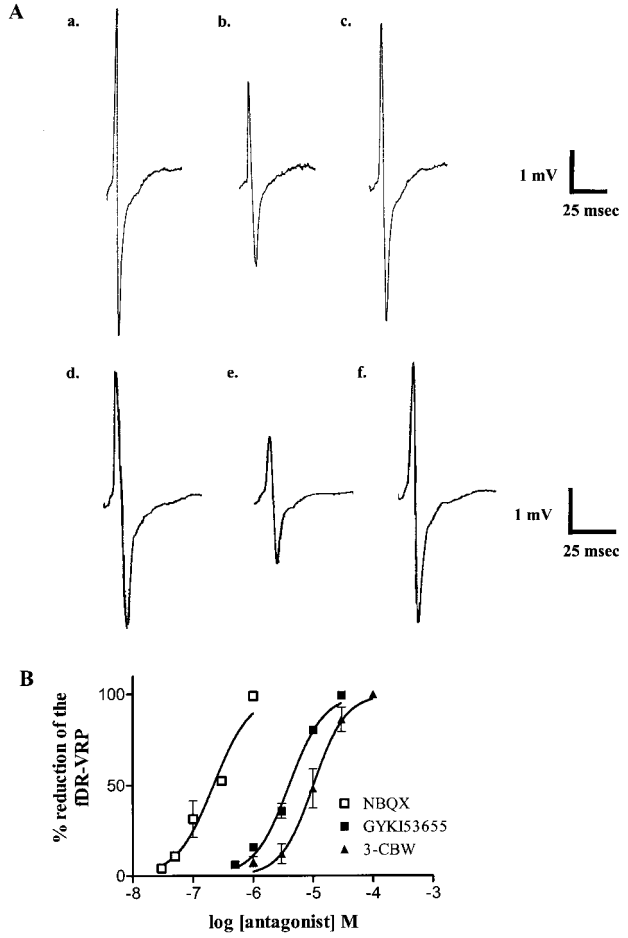


Figure 3 (A) Effect of GYKI53655 and 3-CBW on the fDR-VRP. (a) to (f) represent chart recordings from sample experiments. (a) and (d) represent control traces before antagonist application, (b) and (e) represent the minimum responses recorded after application of 3 μM GYKI53655 (b) and 10 μM 3-CBW (e) and (c) and (f) show responses after the antagonists have been washed off. (B) Graph to compare the ability of NBQX, GYKI53655 and 3-CBW to reduce the fDR-VRP. The IC_{50} values were 214 ± 43 nM, 4.03 ± 0.31 μM and 10.3 ± 2.4 μM, respectively. Each point represents the mean$\pm$s.e.mean from three preparations.

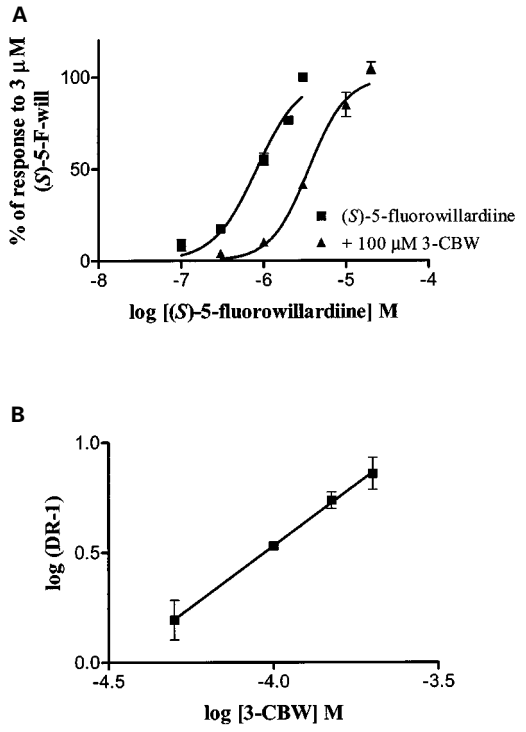


Figure 4 Effect of 3-CBW on (*S*)-5-fluorowillardiine responses on motoneurones. (A) Concentration-response curves for (*S*)-5-fluorowillardiine in the absence and presence of 100 μM 3-CBW. Each point represents the mean$\pm$s.e.mean from three preparations. To normalize the data, responses were analysed as the per cent of the response to 3 μM (*S*)-5-fluorowillardiine in the absence of antagonist. (B) Schild plot for 3-CBW against (*S*)-5-fluorowillardiine. Each point represents the mean response$\pm$s.e.mean from at least three preparations.

NMDA, DHPG (mGlu1 and mGlu5 receptor agonist) and kainate (kainate/AMPA receptor agonist). In the presence of 3-CBW (200 μM), responses to AMPA were almost completely abolished while responses to NMDA, kainate and DHPG were 101.6$\pm$11.6%, 39.4$\pm$5.8% and 110.5$\pm$9.0%, respectively, of those in the absence of antagonist (n=4). It was also found that in the presence of 50 μM GYKI53655 in combination with 200 μM 3-CBW a residual kainate response could still be evoked. In the presence of 50 μM GYKI53655 control responses to AMPA were abolished while kainate responses were reduced to 57.3$\pm$6.8%. However, in the presence of both 50 μM GYKI53655 and 200 μM 3-CBW the kainate responses were further reduced to 39.7$\pm$9.2% of the control values (n=3, mean$\pm$s.e.mean; Figure 7 shows a typical trace).

Discussion

The main objective of this study was to convert the AMPA receptor agonist willardiine into an agonist whilst maintaining selectivity for these receptors. 3-CBW was indeed found to be an antagonist of non-NMDA ionotropic glutamate receptors in assays carried out on AMPA receptors on motoneurones and kainate receptors on isolated dorsal root. However, unlike willardiine, 3-CBW was found to show selectivity for kainate receptors expressed on dorsal roots over kainate receptors on motoneurones. In addition, we have demonstrated that 3-CBW potently antagonizes responses mediated by AMPA receptors but only weakly antagonized those due to kainate receptors on motoneurones. Thus, 3-CBW is an antagonist that can discriminate kainate receptors expressed on dorsal root C-fibres from those expressed on motoneurones. The following sections discuss these findings in more detail.

Depression of the fDR-VRP is a convenient method for assessing the potency of AMPA receptor antagonists

In order to obtain a preliminary assessment of the potency of novel antagonists on AMPA receptors expressed on motoneurones a convenient assay was required. Previous studies using CNQX and kynurenate have indicated that the fast component of the dorsal root evoked ventral root potential (fDR-VRP) is evoked due to the activation of AMPA and/or kainate receptors (Long *et al*., 1988; 1990). However, these antagonists do not show selectivity between AMPA and kainate receptors and the receptor type(s) involved in mediating the underlying epsp responsible for the fDR-VRP have not been further differentiated. In this study it was found that the AMPA receptor selective antagonist GYKI53655 blocked the fDR-VRP with an IC_{50} value of 4.03$\pm$0.31 μM, which correlates well with its activity at other native and recombinant AMPA receptors (Wilding & Huettner, 1995; Bleakman *et al*., 1996). In the present study we have found that the fDR-VRP was completely abolished by 30 μM GYKI53655, a concentration at which this antagonist has been reported to block AMPA but not kainate receptors (Wilding & Huettner, 1995; Bleakman *et al*., 1996). We have also noted that GYKI53655 (50 μM) reduces kainate receptor-mediated responses on motoneurones by less than 50% (Figure 7 and More & Jane, unpublished observation). This suggests that if the kainate receptors present in this preparation played a major part in mediating the fDR-VRP, GYKI53655 (30 μM) would not completely abolish the response, as at this concentration it does not fully block the kainate receptors on motoneurones. The IC_{50} value obtained for the reduction of the fDR-VRP by NBQX also correlates with the reported activity of this antagonist at AMPA receptors (Sheardown *et al*., 1990; Bleakman *et al*., 1999). Thus, there is strong evidence that the majority of the fDR-VRP is evoked by stimulation of AMPA receptors and can therefore be used to assess AMPA receptor antagonist activity. This suggests that the ability of antagonists to block AMPA receptors can be examined under close to physiological conditions, as the fDR-VRP is a response to stimulation-evoked neurotransmitter release as opposed to exogenously applied agonist.

3-CBW is a novel competitive AMPA/kainate receptor antagonist

We have demonstrated that the AMPA receptor agonist activity of willardiine can be converted into antagonism by increasing the inter-acidic group chain length by adding a carboxybenzyl group to the N^3-position of the uracil ring. Thus, 3-CBW is a novel AMPA receptor antagonist.

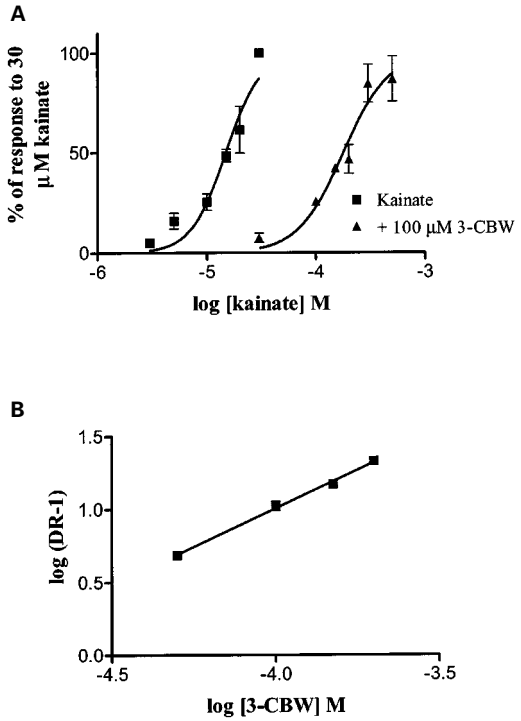


Figure 5 Effect of 3-CBW on kainate responses on dorsal root fibres. (A) Concentration-response curves for kainate in the absence and presence of 100 μM 3-CBW. Each point represents the mean$\pm$ s.e.mean from three preparations. To normalize the data, responses were analysed as the per cent of the response to 30 μM kainate in the absence of antagonist. (B) Schild plot for 3-CBW against kainate. Each point represents the mean response$\pm$s.e.mean from three preparations.

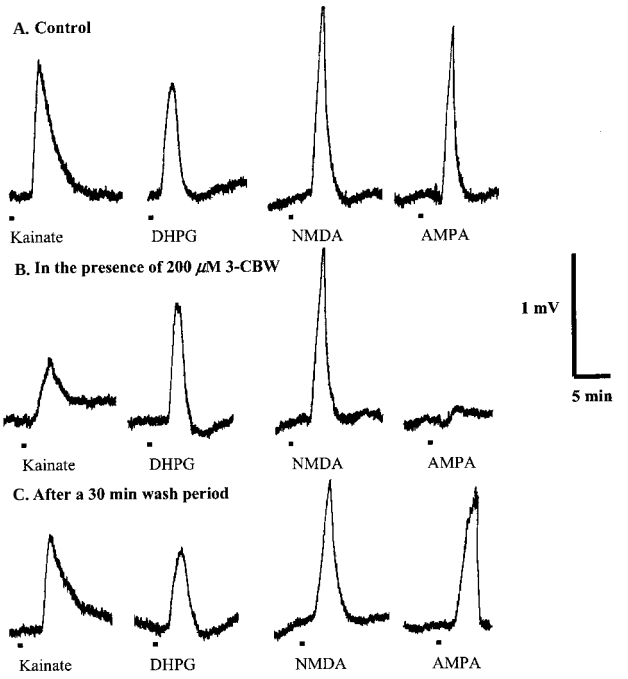


Figure 6 Effect of 3-CBW treatment on agonist-induced depolarization of motoneurones. Responses to kainate (2 μM), DHPG (20 μM), NMDA (7 μM) and (*S*)-AMPA (0.7 μM) are shown in the absence (A) and presence (B) of 200 μM 3-CBW. Responses were recovered after a 30 min wash off period (C).

However, 3-CBW also has antagonist activity at the GluR5-containing kainate receptors on the dorsal root. Concentration-response curves to kainate on isolated dorsal roots and (*S*)-5-fluorowillardiine on motoneurones in the absence and presence of 3-CBW were approximately parallel and the Schild plots have a slope close to 1, suggestive of a competitive mode of antagonism. In support of this theory, it has been reported that 3-CBW can displace the binding of the AMPA receptor agonist [^{3}H]-fluorowillardiine to rat brain membranes (Jones *et al*., 2001). It must be pointed out, however, that large doses of agonist had to be avoided to prevent toxic effects and therefore the truly competitive nature of the 3-CBW antagonism cannot be ascertained from our results, as we cannot be certain that the same maximal response is attained in the absence and presence of the antagonist. In addition, the slow agonist application method used in these studies is likely to cause receptor desensitization and the extent of the desensitization will depend on agonist concentration (being greater at higher agonist concentrations). As a result of the slow penetration of agonist to cells at various depths within the tissue, some populations of receptors will be only partially desensitized and therefore partly responsive rather than totally inactivated. Thus, the signal recorded extracellularly is an average of the signal generated by populations of partially desensitized receptors, which are continuously switching between the active and desensitized states.

3-CBW is a selective antagonist of AMPA receptors expressed on motoneurones and kainate receptors on dorsal root C-fibres

At a concentration of 100 μM, LY341495 is known to block all the metabotropic glutamate receptor subtypes (mGlu1-8) (Fitzjohn *et al*., 1998). As LY341495 did not affect the ability of 3-CBW to depress the fDR-VRP, it was concluded that 3-CBW does not act at the presynaptic group 2 and 3 mGlu autoreceptors present on primary afferent terminals (Jane *et al*., 1994; Schoepp *et al*., 1999).

In the present study we have shown that 3-CBW, at a concentration of 200 μM, blocked AMPA evoked depolarizations on motoneurones while responses to equi-effective doses of NMDA and DHPG were relatively unaffected. This suggests that 3-CBW does not antagonize NMDA or group 1 mGlu receptors as DHPG is known to activate mGlu1 and mGlu5 receptors on neonatal rat motoneurones (Schoepp *et al*., 1999; Miller *et al*., 2000). In each experiment there was a reduction in size of kainate-evoked depolarizations in the presence of 3-CBW. It is likely that this reduction is in part due to a proportion of the kainate response being mediated by AMPA receptors, as suggested previously (Zeman & Lodge, 1992; Thomas *et al*., 1998). It is also evident from Figure 6 that in the presence of 200 μM 3-CBW, a concentration that completely abolishes AMPA-evoked depolarizations on motoneurones and kainate-evoked responses on dorsal root, there is still a noticeable depolarization evoked by kainate on motoneurones. This observation suggests that there are separate populations of AMPA and kainate receptors expressed on neonatal rat motoneurones, which can be differentiated by 3-CBW. Nevertheless, it is a

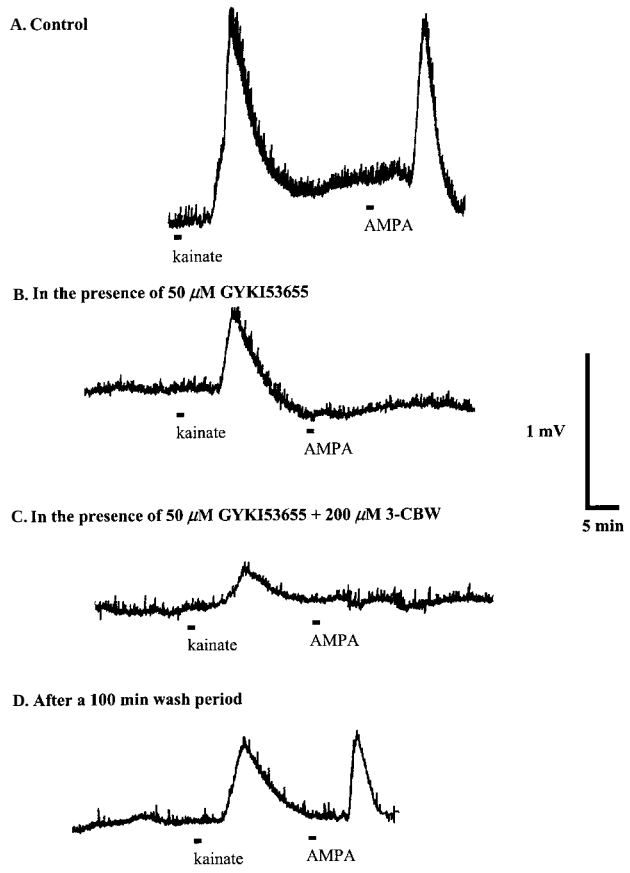


Figure 7 Effect of GYKI53655 and 3-CBW on AMPA and kainate-induced depolarization of motoneurones. Responses to kainate (2 μM) and (*S*)-AMPA (0.8 μM) are shown (A) in the absence of antagonists (B) in the presence of 50 μM GYKI53655 and (C) in the combined presence of 50 μM GYKI53655 + 200 μM 3-CBW. (D) shows the recovery of responses after a 100 min wash off period.

possibility that the residual kainate response in the presence of 3-CBW is due to kainate being non-desensitizing at AMPA receptors. This explanation seems unlikely, however, as a residual kainate response can still be evoked in the presence of both 3-CBW and a high concentration (50 μM) of the selective AMPA receptor antagonist GYKI53655 (see Figure 7 and Thomas *et al*., 1998). Until recently it was unclear whether or not there was a population of kainate receptors present on motoneurones. However, several pharmacological experiments have provided evidence for separate AMPA and kainate receptors. Zeman & Lodge (1992) demonstrated that AMPA- and kainate-mediated responses are differentially sensitive to NBQX. In addition, it has been reported that kainate and AMPA responses were differentially sensitive to the selective non-competitive AMPA receptor antagonist GYKI53655 (Thomas *et al*., 1998) and the competitive AMPA receptor antagonists (*R*)-3,4-dicarboxyphenylglycine (3,4-DCPG) and (*RS*)-3,5-dicarboxyphenylglycine (3,5-DCPG) (Thomas *et al*., 1997). Thus, there is mounting evidence for separate populations of AMPA and kainate receptors on motoneurones.

The antagonism of kainate-induced depolarizations of dorsal root C-fibres by 3-CBW suggests that it is a relatively potent antagonist of the kainate receptors expressed on these fibres. Kainate and certain 5-substituted analogues of (*S*)-willardiine induce depolarizations of dorsal roots isolated from neonatal rats while NMDA, AMPA or (*S*)-willardiine are virtually inactive (Agrawal & Evans, 1986; Blake *et al*., 1991) suggesting the presence of a pure population of kainate receptors. Previous studies have shown that this preparation contains predominantly kainate receptors of the GluR5 subtype (Bettler *et al*., 1990; Partin *et al*., 1993) although possibly combined with KA1 or KA2 (Fletcher & Lodge, 1996), making it useful for the examination of kainate receptor responses.

The presence of GluR5 subunits in kainate receptors expressed on DRG cells has been confirmed by the observation that the previously reported AMPA receptor antagonist (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2*H*)-tetrazole-5-yl)ethyl]-decahydro-isoquinoline-3-carboxylic acid (LY293558) (Schoepp *et al*., 1995), is an antagonist at homomeric GluR5 but not GluR6 and also antagonizes the effects of kainate on DRG neurones (Bleakman *et al*., 1996). GluR5-containing kainate receptors are not just expressed in the spinal cord, low levels of GluR5 expression are observed throughout the adult CNS with it being particularly abundant in the CA1 region of the hippocampus, the Purkinje cell layer in the cerebellum and in the subiculum (Bettler *et al*., 1990; Bahn *et al*., 1994). GluR5 selective antagonists are therefore useful pharmacological tools to study the functional roles of kainate receptors containing the GluR5 subunit in the CNS.

The combined results of these experiments indicate that 3-CBW is an antagonist that is selective for AMPA receptors and the GluR5-containing kainate receptors present in the dorsal root over the kainate receptors present on motoneurones. It is a possibility that the use of concanavalin A in the dorsal root preparation but not in experiments using the motoneurone preparation may have altered the sensitivity of kainate receptors to 3-CBW. To achieve full concentration response curves on the dorsal root it is necessary to pre-incubate the tissue with concanavalin A. However, in the motoneurone preparation concanavalin A has no enhancing effect on kainate responses, most likely due to poor tissue penetration (Thomas *et al*., 1998; Chittajallu *et al*., 1999). Thus, it was not possible in these studies for us to compare both kainate receptor populations without involving the use of concanavalin A. It should however be noted that previous findings on the dorsal root preparation using competitive antagonists have shown no significant differences between pA_2 values obtained from 3-point assays in the absence or presence of concanavalin A (Pook *et al*., 1993) suggesting that it does not alter the pharmacology of kainate receptors in this tissue.

Previously reported evidence supports the existence of a population of kainate receptors on motoneurones that has a different subunit composition from the kainate receptors present on dorsal root C-fibres. For example, (*RS*)-3,5-DCPG antagonizes kainate responses on dorsal root C-fibres and AMPA responses on motoneurones, but enhances kainate responses on motoneurones (Thomas *et al*., 1997; 1998). The ratio of EC_{50} values for the GluR5 selective agonist (*S*)-5-iodowillardiine when tested on neonatal rat motoneurones and dorsal root is close to the ratio of EC_{50}s for activation of AMPA receptors on hippocampal neurones and kainate receptors on DRG neurones suggesting that this agonist is activating AMPA rather than kainate receptors on motoneurones (Wong *et al*., 1994; Thomas *et al*., 1998). In

support of this conclusion, the selective AMPA receptor antagonists (*RS*)-3,4- and 3,5-DCPG antagonize 5-iodowillardiine-induced responses on motoneurones with K_D values similar to those obtained for blocking AMPA and (*S*)-5-flurowillardiine responses (Thomas *et al*., 1997; 1998; Thomas and Jane, unpublished observations).

In situ hybridization studies have revealed that young motoneurones express GluR5, GluR6 and KA2 (Stegenga & Kalb, 2001) whilst mRNA for these subunits cannot be detected in older animals (Tölle *et al*., 1993; Stegenga & Kalb, 2001). Thus, kainate receptors on neonatal rat motoneurones may be comprised of GluR5, GluR6 and KA2 (or there may be a population of GluR6+KA2 receptors) whilst those on dorsal root C-fibres are comprised of GluR5, possibly in combination with KA1 or KA2 (Bettler *et al*., 1990; Partin *et al*., 1993; Fletcher & Lodge, 1996). This difference in subunit composition may underlie the difference in pharmacology observed for kainate receptors expressed on motoneurones and dorsal root C-fibres.

Conclusion

The novel antagonist 3-CBW, constructed by increasing the inter-acidic group chain length of willardiine, has been identified as a selective AMPA and kainate receptor antagonist. 3-CBW has been shown to selectively antagonize AMPA receptors over the NMDA, kainate and mGlu receptors expressed on neonatal rat motoneurones and is therefore a useful tool for the isolation of kainate from AMPA receptor mediated responses in this preparation. We have further substantiated evidence that there is a population of kainate receptors on the motoneurones in this preparation that differs from the GluR5-containing kainate receptors present in the dorsal root preparation. New willardiine analogues based on the structure of 3-CBW are likely to prove useful tools for the characterization of the physiological roles of kainate receptors and it is envisaged that further structure-activity analysis may lead to antagonists with selectivity for individual AMPA or kainate receptor subunits.

This work was funded by the Medical Research Council and Tocris Cookson Ltd.

References

AGRAWAL, S.G. & EVANS, R.H. (1986). The primary afferent depolarizing action of kainate in the rat. *Br. J. Pharmacol.,* **87,** 345–355.

BAHN, S., VOLK, B. & WISDEN, W. (1994). Kainate receptor gene expression in the developing rat brain. *J. Neurosci.,* **14,** 5525–5547.

BETTLER, B., BOULTER, J., HERMANS-BORGMEYER, I., O'SHEA-GREENFIELD, A., DENERIS, E.S., MOLL, C., BORGMEYER, U., HOLLMAN, M. & HEINEMANN, S. (1990). Cloning of a novel glutamate receptor subunit, GluR5: expression in the central nervous system during development. *Neuron,* **5,** 583–595.

BLAKE, J.F., JANE, D.E. & WATKINS, J.C. (1991). Action of willardiine analogues on immature rat dorsal roots. *Br. J. Pharmacol. Proc.,* **104,** (Suppl) P334.

BLEAKMAN, D., BALLYK, B.A., SCHOEPP, D.D., PALMER, A.J., BATH, C.P., SHARPE, E.F., WOOLLEY, M.L., BUFTON, H.R., KAMBOJ, R.K., TARNAWA, I. & LODGE, D. (1996). Activity of 2,3-benzodiazepines at native rat and recombinant human glutamate receptors in vitro: stereospecificity and selectivity profiles. *Neuropharmacology,* **35,** 1689–1702.

BLEAKMAN, D. & LODGE, D. (1998). Neuropharmacology of AMPA and kainate receptors. *Neuropharmacology,* **37,** 1187–1204.

BLEAKMAN, D., OGDEN, A., ORNSTEIN, P. & HOO, K. (1999). Pharmacological characterization of a GluR6 kainate receptor in cultured hippocampal neurones. *Eur. J. Pharmacol.,* **378,** 331–337.

CHITTAJALLU, R., BRAITHWAITE, S.P., CLARKE, V.R. & HENLEY, J.M. (1999). Kainate receptors: subunits, synaptic localization and function. *Trends Pharmacol. Sci.,* **20,** 26–35.

CLARKE, V.R., BALLYK, B.A., HOO, K.H., MANDELZYS, A., PELLIZZARI, A., BATH, C.P., THOMAS, J., SHARPE, E.F., DAVIES, C.H., ORNSTEIN, P.L., SCHOEPP, D.D., KAMBOJ, R.K., COLLINGRIDGE, G.L., LODGE, D. & BLEAKMAN, D. (1997). A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. *Nature,* **389,** 599–603.

DAVIES, J., EVANS, R.H., JONES, A.W., SMITH, D.A. & WATKINS, J.C. (1982). Differential activation and blockade of excitatory amino acid receptors in the mammalian and amphibian central nervous systems. *Comp. Biochem. Physiol.,* **72** (Suppl), C211–C224).

EVANS, R.H., JONES, A.W. & WATKINS, J.C. (1980). Willardiine: A potent quisqualate-like excitant. *J. Physiol.,* **308,** 71–72P.

EVANS, R.H., FRANCIS, A.A., JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1982). The effects of a series of ω-phosphonic α-carboxylic amino acids on electrically evoked and excitant amino acid-induced responses in isolated spinal cord preparations. *Br. J. Pharmacol.,* **75,** 65–75.

FITZJOHN, S.M., BORTOLOTTO, Z.A., PALMER, M.J., DOHERTY, M.J., DOHERTY, A.J., ORNSTEIN, P.L., SCHOEPP, D.D., KINGSTON, A.E., LODGE, D. & COLLINGRIDGE, G.L. (1998). The potent mGlu receptor antagonist LY341495 identifies roles for both cloned and novel mGlu receptors in hippocampal synaptic plasticity. *Neuropharmacology,* **37,** 1445–1458.

FLETCHER, E.J. & LODGE, D. (1996). New developments in the molecular pharmacology of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate receptors. *Pharmacology and Therapeutics,* **70,** 65–89.

HONORÉ, T., DAVIES, S.N., DREJER, J., FLETCHER, E.J., JACOBSEN, P., LODGE, D. & NIELSEN, F.E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science,* **241,** 701–703.

JANE, D.E. (2002). Antagonists acting at the NMDA receptor complex: potential for therapeutic applications. In *Glutamate and GABA receptors and transporters.* Eds Krogsgaard-Larsen, P., Egebjerg, J., Schousboe, A. pp. 69–98. London: Taylor & Francis.

JANE, D.E., HOO, K., KAMBOJ, R., DEVERILL, M., BLEAKMAN, D. & MANDELZYS, A. (1997). Synthesis of willardiine and 6-azawillardiine analogs: Pharmacological characterization on cloned homomeric human AMPA and kainate receptor subtypes. *J. Med. Chem.,* **40,** 3645–3650.

JANE, D.E., JONES, P.L., ST. J., POOK, P.C.-K., TSE, H.-W. & WATKINS, J.C. (1994). Actions of two new antagonists showing selectivity for different sub-types of metabotropic glutamate receptor in the neonatal rat spinal cord. *Br. J. Pharmacol.,* **112,** 809–816.

JANE, D.E., TSE, H.-W., SKIFTER, D.A., CHRISTIE, J.M. & MONAGHAN, D.T. (2000). Glutamate receptor ion channels: Activators and inhibitors. In: *Pharmacology of Ionic Channel Function: Activators and Inhibitors.* Eds Endo, M., Kurachi, Y., Mishina, M. pp. 415–459. Berlin: Springer-Verlag.

JONES, S.C., TROOP, H.M., JANE, D.E. & ROBERTS, P.J. (2001). Pharmacological characterization of novel AMPA receptor ligands in rat brain. *Br. J. Pharmacol.,* **134,** 145P.

KROGSGAARD-LARSEN, P., FERKANY, J.W., NIELSON, E.Ø., MADSEN, U., EBERT, B., JOHANSEN, J.S., DIEMER, N.H., BRUHN, T., BEATTIE, D.T. & CURTIS, D.R. (1991). Novel class of amino acid antagonists at non-*N*-methyl-D-aspartic acid excitatory amino acid receptors. Synthesis, in vitro and in vivo pharmacology, and neuroprotection. *J. Med. Chem.,* **34,** 123–130.

LONG, S.K., EVANS, R.H., CULL, L., KRIJZER, F. & BEVAN, P. (1988). An *in vitro* mature spinal cord preparation from the rat. *Neuropharmacology,* **27,** 541–546.

LONG, S.K., SMITH, D.A.S., SIAREY, R.J. & EVANS, R.H. (1990). Effect of 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX) on dorsal root-, NMDA-, kainate- and quisqualate-mediated depolarization of rat motoneurones *in vitro*. *Br. J. Pharmacol.,* **100,** 850–854.

MADSEN, U., BANG-ANDERSEN, B., BREHM, L., CHRISTENSEN, I.T., EBERT, B., KRISTOFFERSEN, Y.L. & KROGSGAARD-LARSEN, P. (1996). Synthesis and pharmacology of highly selective carboxy and phosphono isoxazole amino acid AMPA receptor antagonists. *J. Med. Chem.,* **39,** 1682–1691.

MILLER, J.C., MORE, J.C.A., HOWSON, P.A. & JANE, D.E. (2000). Pharmacological identification of mGlu1 and mGlu5 group I metabotropic receptor subtypes on neonatal rat motoneurones. *Br. J. Pharmacol.,* **129,** 103P.

MORE, J.C.A., TROOP, H.M. & JANE, D.E. (2001). The N^3-substituted willardiine analogue (*S*)-3-(4-carboxybenzyl)willardiine selectively antagonises AMPA over kainate receptors on neonatal rat spinal motoneurones. *Br. J. Pharmacol.,* **133,** 152P.

MORE, J.C.A., TROOP, H.M. & JANE, D.E. (2002). N^3-substituted willardiine analogues act as kainate receptor antagonists in the neonatal rat dorsal root preparation. *Br. J. Pharmacol.,* **135,** 86P.

O'NEILL, M.J., BOND, A., ORNSTEIN, P.L., WARD, M.A., HICKS, C.A., HOO, K., BLEAKMAN, D. & LODGE, D. (1998). Decahydroisoquinolines: novel competitive AMPA/kainate antagonists with neuroprotective effects in global cerebral ischaemia. *Neuropharmacology,* **37,** 1211–1222.

PARTIN, K.M., PATNEAU, D.K., WINTERS, C.A., MAYER, M.L. & BUONANNO, A. (1993). Selective modulation of desensitisation at AMPA versus kainate receptors by cyclothiazide and concanavlin A. *Neuron,* **11,** 1069–1082.

PATNEAU, D.K., MAYER, M.L., JANE, D.E. & WATKINS, J.C. (1992). Activation and desensitization of AMPA/kainate receptors by novel derivatives of willardiine. *J. Neurosci.,* **12,** 595–606.

POOK, P., BURGGER, F., HAWKINS, N.S., CLARK, K.C., WATKINS, J.C. & EVANS, R.H. (1993). A comparison of the actions of agonists and antagonists at non-NMDA receptors of C fibres and motoneurones of the immature rat spinal cord *in vitro*. *Br. J. Pharmacol.,* **108,** 179–184.

SCHOEPP, D.D., JANE, D.E. & MONN, J.A. (1999). Pharmacological agents acting at subtypes of metabotropic glutamate receptors. *Neuropharmacology,* **38,** 1431–1476.

SCHOEPP, D.D., LODGE, D., BLEAKMAN, D., LEANDER, J.D., TIZZANO, J.P., WRIGHT, R.A., PALMER, A.J., SALHOFF, C.R. & ORNSTEIN, P.L. (1995). *In vitro* and *in vivo* antagonism of AMPA receptor activation by (3S,4aR,6R,8aR)-6-[2-(1-2H-tetrazole-5-yl)ethyl]decahydroisoquinoiline-3-carboxylic acid. *Neuropharmacology,* **34,** 1159–1168.

SHEARDOWN, M.J., NIELSEN, E.Ø., HANSEN, A.J., JACOBSEN, P. & HONORE, T. (1990). 2,3-Dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline: A neuroprotectant for cerebral ischemia. *Science,* **247,** 571–574.

SIMMONS, R.M.A., LI, D.L., HOO, K.H., DEVERILL, M., ORNSTEIN, P.L. & IYENGAR, S. (1998). Kainate GluR5 receptor mediates the nociceptive response to formalin in the rat. *Neuropharmacology,* **37,** 25–36.

STEGENGA, S.L. & KALB, R.G. (2001). Developmental regulation of N-methyl-D-aspartate- and kainate-type glutamate receptor expression in the rat spinal cord. *Neuroscience,* **105,** 499–507.

SWANSON, G.T., GREEN, T. & HEINEMANN, S.F. (1998). Kainate receptors exhibit differential sensitivities to (*S*)-5-iodowillardiine. *Mol. Pharmacol.,* **53,** 942–949.

THOMAS, N.K., CLAYTON, P. & JANE, D.E. (1997). Dicarboxyphenylglycines as selective antagonists of AMPA-induced depolarizations in neonatal rat motoneurones. *Eur. J. Pharmacol.,* **338,** 111–116.

THOMAS, N.K., HAWKINS, L.M., MILLER, J.C., TROOP, H.M., ROBERTS, P.J. & JANE, D.E. (1998). Pharmacological differentiation of kainate receptors on neonatal rat spinal motoneurones and dorsal roots. *Neuropharmacology,* **37,** 1223–1237.

TÖLLE, T.R., BERTHELE, A., ZIEGLGANSBERGER, W., SEEBURG, P.H. & WISDEN, W. (1993). The differential expression of 16 NMDA and non-NMDA receptor subunits in the rat spinal cord and in periaqueductal gray. *J. Neurosci.,* **13,** 5009–5028.

VARNEY, M.A., RAO, S.P., JACHEC, C., DEAL, C., HESS, S.D., DAGGETT, L.P., LIN, F.F., JOHNSON, E.C. & VELIÇELEBI, G. (1998). Pharmacological characterization of the human ionotropic glutamate receptor subtype GluR3 stably expressed in mammalian cells. *J. Pharmacol. Exp. Ther.,* **285,** 358–370.

WATKINS, J.C. & COLLINGRIDGE, G.L. (1994). Phenylglycine derivatives as antagonists of metabotropic glutamate receptors. *Trends Pharmacol. Sci.,* **15,** 333–342.

WATKINS, J.C. & EVANS, R.H. (1981). Excitatory amino acid transmitters. *Ann. Rev. Pharmacol.,* **21,** 165–204.

WATKINS, J.C., KROGSGAARD-LARSEN, P. & HONORÉ, T. (1990). Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive agonists. *Trends Pharmacol. Sci.,* **11,** 25–33.

WILDING, T.J. & HUETTNER, J.E. (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, L.A., MAYER, M.L., JANE, D.E. & WATKINS, J.C. (1994). Willardiines differentiate agonist binding sites for kainate- versus AMPA-preferring glutamate receptors in DRG and hippocampal neurones. *J. Neurosci.,* **14,** 3881–3897.

ZEMAN, S. & LODGE, D. (1992). Pharmacological characterization of non-NMDA subtypes of glutamate receptor in the neonatal rat hemisected spinal cord *in vitro*. *Br. J. Pharmacol.,* **186,** 367–372.

(Received August 22, 2002
Revised August 28, 2002
Accepted September 5, 2002)