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Pharmacology of 5-Chloro-7-Trifluoromethyl-1,4-Dihydro-2,3-Quinoxalinedione: A Novel Systemically Active Ionotropic Glutamate Receptor Antagonist

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ABSTRACT

5-Chloro-7-trifluoromethyl-1,4-dihydro-2,3-quinoxalinedione (ACEA-1011) has analgesic properties in animal models of tonic pain. To investigate the mechanisms underlying this effect we used electrical recording techniques to characterize the *in vitro* pharmacology of ACEA-1011 at mammalian glutamate receptors. Two preparations were used: *Xenopus* oocytes expressing rat brain receptors and cultured rat cortical neurons. Results showed that ACEA-1011 is a competitive antagonist at NMDA receptor glycine sites. Apparent antagonist affinities (K_b values) were 0.4 to 0.8 μM in oocytes and ~0.6 μM in neurons. IC_{50} values for ACEA-1011 against four binary subunit combinations of cloned rat NMDA receptors (NR1A/NR2A, 2B, 2C or 2D) ranged from 0.4 to 8 μM (1 μM glycine). The 20-fold variation in sensitivity was due to a combination of subunit-dependent differences in glycine and antagonist affinities; EC_{50} values for glycine ranged between 0.08 to 0.8 μM and K_b values for ACEA-1011 between 0.2 to 0.8 μM. In addition, ACEA-1011 inhibited AMPA-preferring non-NMDA receptors by competitive antagonism at glutamate binding sites. K_b values were 4 to 9 μM in oocytes and 9 to 10 μM in neurons. The ED_{50} for ACEA-1011 in a mouse maximum electroshock-induced seizure model was ~12 mg/kg i.v.. Our results indicate that ACEA-1011 is a systemically active broad selectivity ionotropic glutamate receptor antagonist.

NMDA receptor antagonists fall into four main classes according to their site of action: PCP site channel blockers, competitive (glutamate site) inhibitors, glycine coagonist site antagonists and ligands for polyamine binding sites (see Bigge, 1993; Leeson and Iversen, 1994, Williams *et al.*, 1991 for reviews). Interest in NMDA receptor glycine site antagonists as therapeutic agents has intensified in recent years due to evidence suggesting that this type of inhibitor has a better side effects profile than channel blockers and competitive antagonists (Kemp and Leeson, 1993). For example, glycine site antagonists do not appear to induce PCP-like behaviors (Koek and Colpaert, 1990; Tricklebank *et al.*, 1989), indeed, the glycine site antagonist R-(+)-HA-966 actually suppresses PCP- and dizocilpine-induced elevations in mesolimbic dopamine turnover (Bristow *et al.*, 1993). In addition, glycine site antagonists do not induce neuronal vacuolization (Hargreaves *et al.*, 1993), a cytotoxic effect of NMDA receptor antagonists that has compromised the clinical development of some channel blockers and competitive antagonists (Olney *et al.*, 1989).

Clinical indications for NMDA antagonists include cerebral ischemia (McCulloch, 1992), tonic pain (Wilcox, 1991) and convulsions (Rogawski, 1992). Glycine site antagonists have therapeutic potential in all three areas, but at present most of the high potency ligands show poor systemic bioavailability (Kemp and Leeson, 1993; but see also Carling *et al.*, 1993; Kulagowski *et al.*, 1994). ACEA-1011 was synthesized as part of a drug discovery program looking for novel excitatory amino acid receptor antagonists. The compound attracted attention when it was found to be a systemically active analgesic in the mouse formalin test (Lutfy *et al.*, 1995, in press; Vaccarino *et al.*, 1993). Interestingly, unlike opiate-induced analgesia, the antinociceptive effects of ACEA-1011 were not associated with development of tolerance (Lutfy *et al.*, 1995, in press). In addition, behaviorally active doses of ACEA-1011 were found to be devoid of PCP-like discriminative stimulus effects in rats (Balster *et al.*, 1995).

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ABBREVIATIONS: 1S, 3R-ACPD, (1S, 3R)-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; DMSO, dimethylsulphoxide; MES, maximum electroshock-induced seizure; NMDA, N-methyl-D-aspartic acid; PCP, phencyclidine; ACEA-1011, 5-chloro-7-trifluoromethyl-1,4-dihydro-2,3-quinoxalinedione.

To help understand the mechanisms involved in the *in vivo* actions of ACEA-1011 we have used electrophysiological techniques to characterize the pharmacology of this compound at NMDA, non-NMDA and metabotropic glutamate receptors expressed in *Xenopus* oocytes by rat brain poly(A)$^+$ RNA (Gundersen *et al.*, 1984; Verdoorn *et al.*, 1989), at four cloned NMDA receptor subunit combinations expressed in oocytes (Monyer *et al.*, 1992; Monyer *et al.*, 1994; Moriyoshi *et al.*, 1991), and at NMDA and non-NMDA receptors present in cultured rat cortical neurons (Swartz *et al.*, 1992). In addition, systemic bioavailability was assessed by measuring anticonvulsant activity of ACEA-1011 in a mouse maximum electroshock-induced seizure model. For comparison, effects of ACEA-1011 in oocyte and mouse maximum electroshock assays were assayed in parallel with the structurally related ligands ACEA-1021 (5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione), a high potency glycine site antagonist (Woodward *et al.*, 1995), and CNQX, an antagonist that shows selectivity toward non-NMDA receptors (Honore *et al.*, 1988) (fig. 1).

Methods and Materials

Preparation of RNA. Polyadenylated mRNA from whole rat brain (including cerebellum and a portion of the brain stem) was prepared using methods described in Woodward *et al.* 1992. cDNA clones encoding the rat NR1A and NR2A-D NMDA receptor subunits were generously provided by Dr. Peter Seeburg (see Kutsuwada *et al.*, 1992; Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992; Monyer *et al.*, 1994 for details of rat and mouse clones). The clones were prepared using conventional techniques and cRNA was synthesized with T3 RNA polymerase.

Expression of receptors in Xenopus oocytes. Surgeries on *Xenopus laevis*, preparation, micro-injection and storage of oocytes, were all as described previously (Woodward *et al.*, 1992). Briefly, oocytes were injected with ~50 ng of whole brain poly(A)$^+$ RNA, or with 2 to 20 ng of cRNA encoding each cloned NMDA receptor subunit. cRNA encoding NR1A and NR2A-D subunits were injected in a 1:1 ratio. Membrane current responses were recorded using a two-electrode voltage clamp (Dagan TEV-200). Recordings were made in frog Ringer's solution containing (in mM): NaCl, 115; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; pH 7.4, or in a nominally zero-Ca^{2+} Ringer in which $CaCl_2$ was replaced by equimolar $BaCl_2$. Drugs were applied by bath perfusion.

Neuronal recordings. Preparation and maintenance of rat cerebral cortical neuron cultures were as in Huettner and Baughman, 1986. Whole-cell currents were recorded in a solution containing (in mM): NaCl, 160 mM; $CaCl_2$, 2 mM; tetrodotoxin, 0.5 μM; HEPES, 10 mM; pH adjusted to 7.4 with NaOH. Drugs dissolved in this solution were applied from a linear array of microcapillary tubes. Internal pipette solutions contained (in mM): CsCl, 5; EGTA, 10; HEPES, 10; and either $CsCH_3SO_3$, 140 or CsF, 140; pH adjusted to 7.4 with CsOH. The recording chamber was perfused at 1 to 2 ml/min with Tyrode's solution containing (in mM): NaCl, 150; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; HEPES, 10; pH 7.4.

Mouse MES assays. Procedures for MES assays were as described previously (Swinyard, 1972). Seizures were induced in Swiss Webster mice (body weight 20–30 g) *via* corneal electrodes. Electroshock parameters were: rectangular positive pulses, amplitude—50 mA, frequency—60 to 75 Hz, pulse width—0.8 msec, duration—200 msec.

Data analysis. Agonist concentration-response curves and antagonist concentration-inhibition curves were analyzed as described in Swartz *et al.*, 1992. Briefly, concentration response curves were fit to the logistic equation (equation 1):

$$I/I_{max} = 1/(1 + (10^{-pEC50}/[agonist])^n) \quad \text{(Equation 1)}$$

where n is the slope factor, and $pEC_{50} = -\log EC_{50}$.

Concentration-inhibition curves were fit with equation 2.

$$I/I_{control} = 1/(1 + ([antagonist]/10^{-pIC50})^n) \quad \text{(Equation 2)}$$

where $pIC_{50} = -\log IC_{50}$.

Apparent dissociation constants of antagonists (K_b values) were determined by two approaches.

(i) From simultaneous fits of concentration-response data in the presence or absence of fixed concentrations of antagonist using equation 3; a Gaddum/Schild-type of analysis (Swartz, *et al.*, 1992):

$$I/I_{max} = 1/(1 + ((10^{-pEC50})(1 + ([antagonist]/(10^{-pKb})))/[agonist])^n) \quad \text{(Equation 3)}$$

(ii) From concentration-inhibition data by applying equation 4; a generalized form of Cheng-Prusoff analysis (Leff and Dougall, 1993):

$$K_b = IC_{50}/((2 + ([agonist]_f/EC_{50})^b)^{1/b} - 1) \quad \text{(Equation 4)}$$

where $[agonist]_f$ is the inhibition curve agonist concentration, EC_{50} and b (slope factor) refer to the agonist concentration-response relation. In practice, this was accomplished by replacing the parameter 10^{-pIC50} in equation 2 by: $(10^{-pKb})((2 + ([agonist]_f/EC_{50})^b)^{1/b}) - 1)$.

Consistency with the simple competitive model of inhibition was assessed by calculating ratios of residual variance using equation 5:

$$F_{df2-df1,df1} = ((SS_2 - SS_1)/(df_2 - df_1))/(SS_1/df_1) \quad \text{(Equation 5)}$$

where SS_1 is the sum of squared deviations for individual fits (equation 1), SS_2 is the sum of squared deviations for the simultaneous fit (equation 3), df_1 and df_2 are the degrees of freedom for individual and simultaneous fits, respectively.

Drugs. ACEA-1011 (mp. 334–336°C) was synthesized by condensation of 1,2-diamino-3-chloro-5-trifluoromethylbenzene with diethyl oxalate, and isolated as an off-white powder (Keana *et al.*, 1995, in press). CNQX was purchased from Research Biochemicals Inc. (Natick, MA), 1S, 3R-ACPD was from Tocris Neuramin (Bristol, U.K.).

Tris(hydroxymethyl)-amino-methane and glucose were from J. T. Baker (Philipsburg, NJ). All other drugs were from Sigma Chemical Co. (St. Louis, MO). Quinoxalinediones were made up as a series of DMSO stock solutions over the concentration range 0.0003 to 30 mM. Stocks stored for up to 2 wk in the dark at 4°C with no discernible changes in potency. Working solutions were made by 1000 to 3000-fold dilution of stocks into Ringer. Applied alone, 0.03 to 0.1% DMSO had no appreciable effects on membrane current responses. For i.v. injection ACEA-1011 and CNQX were dissolved at 5 mg/ml in 0.1 M arginine. ACEA-1021 was formulated at 5 mg/ml in 0.05 M tris(hydroxymethyl)-amino-methane with 5% glucose. Neither vehicle had any protective effects in MES assays when administered alone.

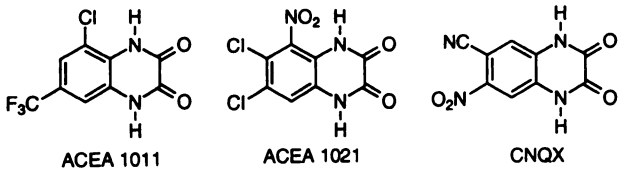

Fig. 1. Structures of ACEA-1011, ACEA-1021 and CNQX.

Results

Electrical Recordings from Xenopus Oocytes

NMDA responses expressed by rat whole brain poly(A)$^+$ RNA. Membrane current responses elicited by co-application of NMDA and glycine were inward at a holding potential of −70 mV and followed a multiphasic time course

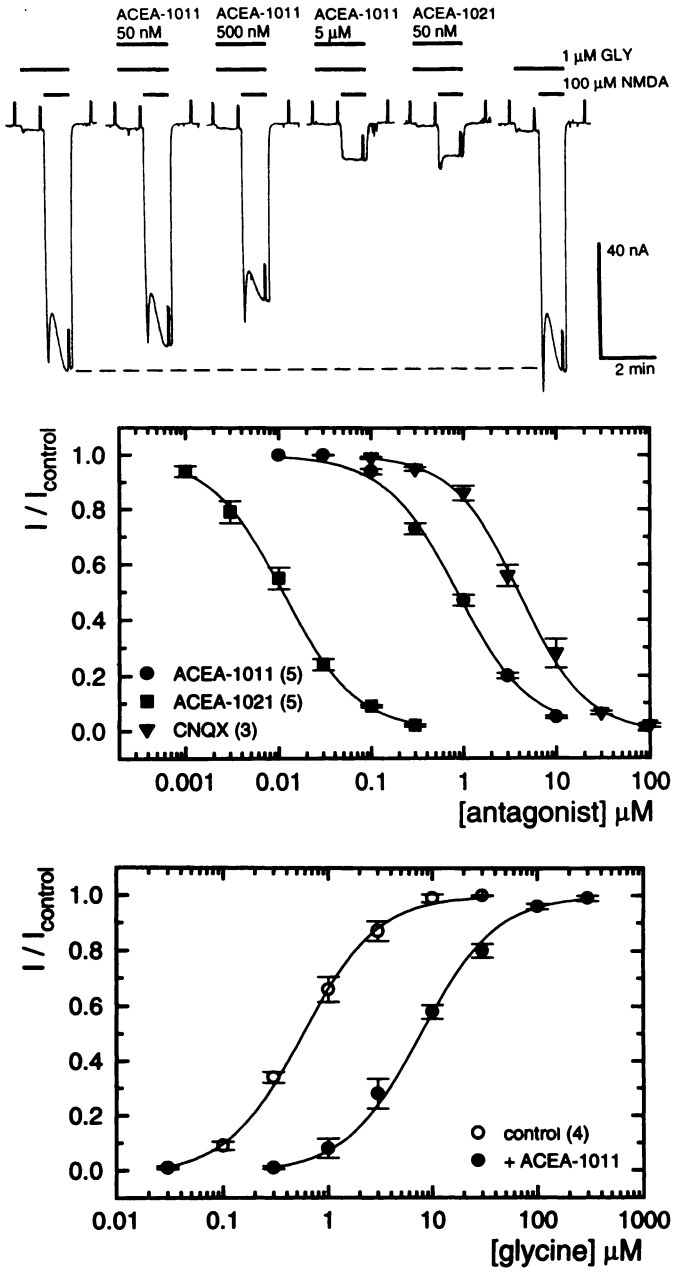

Fig. 2. Inhibition of NMDA responses expressed in *Xenopus* oocytes by rat brain poly(A)$^+$ RNA. Records, Sample concentration-inhibition experiments comparing potencies of ACEA-1011 and ACEA-1021. Holding potential was −70 mV, pulsed at ~50-sec intervals to −60 mV as a monitor of membrane conductance and cue for solution changes. Downward deflection denotes inward current, drug applications indicated by bars. Responses were separated by ~5 min intervals of wash (not shown). Broken line is the estimated level of control current measured at the second phase of the response, as used for all pharmacological assays. Middle panel, Concentration-inhibition curves comparing potencies of ACEA-1011, ACEA-1021 and CNQX (glycine = 1 μM, NMDA = 100 μM). Data are plotted as the mean ±SEM expressed as a fraction of control responses. Number of experiments (cells) given in parentheses. Smooth curves, Best fits of equation 2 to data; IC_{50} and slope values (curve parameters) are given in table 1. Lower panel, Effect of ACEA-1011 (10 μM) on the glycine steady-state concentration-response relationship (NMDA = 100 μM). Data expressed as a fraction of I_{max}. Smooth curves, Best fits of equation 3 to data for each drug. EC_{50} for control curves and optimal slope values for paired curves are given in table 1. Data presentation is the same in all following graphs.

(*e.g.*, fig. 2 records) (*e.g.*, Verdoorn *et al.* 1989; Williams, 1993). For all pharmacological assays the initial spike of current, due to secondary activation of Ca^{2+}-gated Cl^- channels (see Leonard and Kelso, 1990), was ignored. Current amplitudes were measured at the peak or plateau of the second phase, which corresponds more closely to currents flowing directly through NMDA receptors (Woodward *et al.*, 1995). As an indication of expression levels, responses elicited by 100 μM NMDA and 10 μM glycine (I_{max}) ranged from 40 to 545 nA (mean ± SEM = 183 ± 121 nA, n = 4).

Antagonist potencies were initially gauged from concentration-inhibition curves measured on responses elicited by 100 μM NMDA and 1 μM glycine. The standard protocol is illustrated in figure 2. Cells were pretreated for 30 to 60 sec with glycine plus antagonist and receptors then activated by co-application of NMDA. Levels of control responses were tested every 2 to 4 drug applications. In all cases antagonism reversed fully after 2 to 5 min wash. The IC_{50} for ACEA-1011 was 850 nM, approximately 80-fold higher than that for ACEA-1021 and 5-fold lower than that for CNQX (fig. 2, middle panel; table 1, upper panel). Slopes of inhibition curves were in the range −1.2 to −1.1. Potency of ACEA-1011 in Ringer where Ca^{2+} was substituted by Ba^{2+} was not appreciably different from that measured in normal Ringer. This indicated that any residual Cl^- current activation in normal Ringer was not compromising pharmacological measurements. Inhibition of NMDA responses by ACEA-1011 was fully surmounted by increasing glycine concentrations. At 10 μM, ACEA-1011 induced a parallel rightward transposition of the concentration-response relation resulting in a ~9-fold decrease in apparent affinity for glycine (fig. 2, lower panel). The effect was consistent with competitive inhibition at the glycine binding site ($F_{1,67}$ = 1.2). K_b values for ACEA-1011, ACEA-1021 and CNQX were estimated from inhibition curves (Leff and Dougall, 1993) (table 1, upper panel). For comparison, values for ACEA-1011 were also estimated using the more conventional Gaddum-Schild method (Swartz *et al.* 1992) (table 1, lower panel).

To determine whether ACEA-1011 had any inhibitory effects at glutamate binding sites on NMDA receptors experiments were set up using a high concentration of glycine (1 mM), to saturate glycine sites, and low concentrations of NMDA (1–3 μM), to maximize the chances of detecting inhibition at glutamate sites. At concentrations up to 20 μM, ACEA-1011 caused <25% inhibition of NMDA responses (not illustrated). Assuming competitive inhibition, and an EC_{50} and slope for NMDA of ~30 μM and 1.4 respectively (Woodward *et al.* 1995), these assays indicate that the K_b for ACEA-1011 at glutamate biding sites is >30 μM. Currents mediated by coexpressed strychnine-sensitive glycine receptors were minimized by coapplication of 10–30 μM strychnine.

Cloned rat NMDA receptors expressed by subunit-encoding cRNA. Potency of ACEA-1011 was assayed at four binary subunit combinations; NR1A expressed together with either NR2A, 2B, 2C or 2D. Currents elicited by activation of cloned receptors were similar to those observed with whole brain poly(A)$^+$ RNA (Kutsuwada *et al.*, 1992; Williams, 1993; Woodward *et al.*, 1995).

Potency of ACEA-1011 at cloned rat NMDA receptors was first assessed by measuring concentration-inhibition curves. Using a fixed concentration of agonist (1 μM glycine) the four subunit combinations showed clear variations in sensitivity

TABLE 1
Inhibition of rat glutamate receptors expressed in *Xenopus* oocytes

Receptor/Agonist[a]	Antagonist	IC_{50} (μM)	Slope C/I Curve	K_b (μM) (Leff/Dougall)	n
Rat brain poly(A)⁺ RNA					
NMDA/glycine	ACEA-1011	0.85 (0.79, 0.92)[b]	−1.1 (−1.2, −1)	0.37 (0.35, 0.4)	5
(1 μM glycine)[c]	ACEA-1021	0.011 (0.01, 0.013)	−1.1 (−1.2, −1)	0.005 (0.0045, 0.0055)	5
	CNQX	4 (3.6, 4.5)	−1.2 (−1.3, −1.1)	1.9 (1.7, 2.1)	3
Non-NMDA/AMPA	ACEA-1011	8 (7.4, 8.6)	−1.1 (−1.2, −1)	5.8 (5.4, 6.3)	3
(10 μM AMPA)[c]	CNQX	0.37 (0.35, 0.38)	−1.3 (−1.3, −1.2)	0.38 (0.36, 0.4)	3
Non-NMDA/kainate	ACEA-1011	3.2 (3.0, 3.5)	−1.3 (−1.3, −1.1)	4.7 (4.4, 5.1)	5
(20 μM KAINATE)[c]	CNQX	0.16 (0.15, 0.17)	−1.2 (−1.4, −1.1)	0.31 (2.8, 3.4)	3
NMDA receptor clones					
NR1A/2A	ACEA-1011	0.4 (0.37, 0.44)	−1 (−1.1, −0.95)	0.33 (0.3, 0.36)	4
NR1A/2B	ACEA-1011	1.5 (1.4, 1.6)	−1.3 (−1.4, −1.2)	0.46 (0.43, 0.49)	4
NR1A/2C	ACEA-1011	1.1 (1, 1.1)	−1.3 (−1.4, −1.2)	0.21 (0.19, 0.22)	4
NR1A/2D	ACEA-1011	7.8 (7.5, 8.1)	−1.6 (−1.7, −1.5)	0.74 (0.71, 0.77)	3
(1 μM glycine)[c]					

Receptor/Agonist[d]	Antagonist	EC_{50} (μM) (Control curve)	Slope C/R Curve	K_b (μM) (Gaddum/Schild)	n
Rat brain poly(A)⁺ RNA					
NMDA/glycine	ACEA-1011	0.56 (0.5, 0.63)	1.2 (1.1, 1.3)	0.79 (0.65, 0.96)	4
Non-NMDA/AMPA	ACEA-1011	5.8 (5.4, 6.3)	1.8 (1.6, 1.9)	8.5 (7.4, 9.8)	3
Non-NMDA/KAIN	ACEA-1011	87 (76, 99)	1.4 (1.3, 1.6)	7.5 (5.9, 9.4)	4
NMDA receptor clones					
NR1A/2A	ACEA-1011	0.7 (0.64, 0.76)	1.5 (1.4, 1.6)	0.4 (0.36, 0.45)	3
NR1A/2B	ACEA-1011	0.27 (0.25, 0.3)	1.6 (1.4, 1.7)	0.43 (0.37, 0.50)	3
NR1A/2C	ACEA-1011	0.17 (0.17, 0.19)	1.5 (1.4, 1.6)	0.22 (0.2, 0.24)	3
NR1A/2D	ACEA-1011	0.089 (0.083, 0.097)	1.6 (1.5, 1.7)	0.72 (0.64, 0.81)	3

[a] Analysis of concentration-inhibition (C/I) experiments. IC_{50} and slope values are the best fits of data to equation 2. K_b values were estimated using the Leff/Dougall approach. *n*, indicates the number of separate experiments (cells examined). Curves plotted in figures 2, 3 and 5.
[b] Data are given as the mean, quoted to two significant figures, numbers in parentheses are 95% confidence intervals.
[c] Fixed agonist concentrations used in C/I experiments. For NMDA receptor glycine site assays, NMDA = 100 μM.
[d] Analysis of concentration-response (C/R) experiments. EC_{50} for control curves (in the absence of antagonist) and slope values are the best fits of data to equation 3. K_b values were estimated using the Gaddum/Schild approach. Curves plotted in figures 2, 4 and 5. General presentation also applies to table 2.

to ACEA-1011 (fig. 3). NR1A/2A receptors were most sensitive to inhibition (IC_{50} 0.4 μM), NR1A/2B and 2C receptors had intermediate sensitivity (IC_{50} 1–2 μM) and NR1A/2D receptors were least sensitive to inhibition (IC_{50} ~8 μM). Slopes for the inhibition curves ranged between −1.6 to −1 (table 1, upper panel).

Previous characterization of cloned rat and mouse NMDA receptors indicated that different subunit combinations vary in their apparent affinities for glycine (Kutsuwada *et al.*, 1992, Monyer *et al.*, 1992; Woodward *et al.*, 1995). In the present experiments, NR1A/2A combinations showed the lowest glycine affinity (EC_{50} 0.7 μM), NR1A/2B and NR1A/2C intermediate affinities (EC_{50} 0.17–0.27 μM), and NR1A/2D the highest affinity (EC_{50} 0.09 μM) (table 1, lower panel). For an indication of expression levels, current ranges and mean I_{max} in these experiments were: NR1A/2A, 106 to 248 nA (155 ± 57 nA); NR1A/2B, 140 to 545 nA (284 ± 200 nA); NR1A/2C, 70 to 470 nA (221 ± 154 nA); NR1A/2D, 35 to 151 nA (82 ± 58 nA) (n = 3 for all).

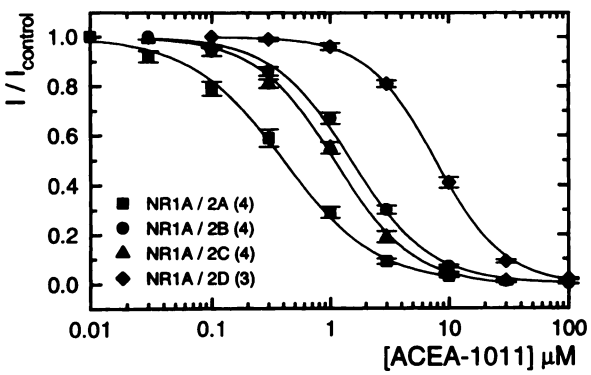

Fig. 3. Inhibition of cloned rat NMDA receptors expressed in *Xenopus* oocytes. Concentration-inhibition curves showing differential sensitivity of four binary NMDA receptor subunit combinations to ACEA-1011 (glycine = 1 μM, glutamate = 100 μM). Smooth curves, Best fits of equation 2 to data for each subunit combination. Curve parameters given in table 1.

As described for NMDA receptors expressed by whole brain mRNA, ACEA-1011 caused parallel rightward shifts in glycine concentration-response curves in all four subunit combinations (fig. 4). For NR1A/2A, NR1A/2B and NR1A/2D transposition of curves induced by 10 μM ACEA-1011 were consistent with competitive inhibition at the glycine site ($F_{1,33}$ = 0.053, $F_{1,34}$ = 0.2, and $F_{1,44}$ = 0.71, respectively) (fig. 4, upper panel). The NR1A/2C receptors were used for a more stringent test of the competitive model. In this case shifts in apparent glycine affinity were measured for three concentrations of ACEA-1011: 0.3, 3 and 30 μM (fig. 4, middle panel). Dose ratios were calculated from shifts in glycine EC_{50} and were plotted as a function of antagonist concentration. Schild regression of this data gave a slope of 0.92 ± 0.01 (n = 3), suggesting a predominantly competitive mechanism (fig. 4, lower panel). The simultaneous fit of the data did, however, show significant deviation from the competitive model ($F_{5,83}$ = 4.7); note that the predicted curve in 0.3 μM ACEA-1011 is displaced slightly to the left of the data. Calculation of K_b

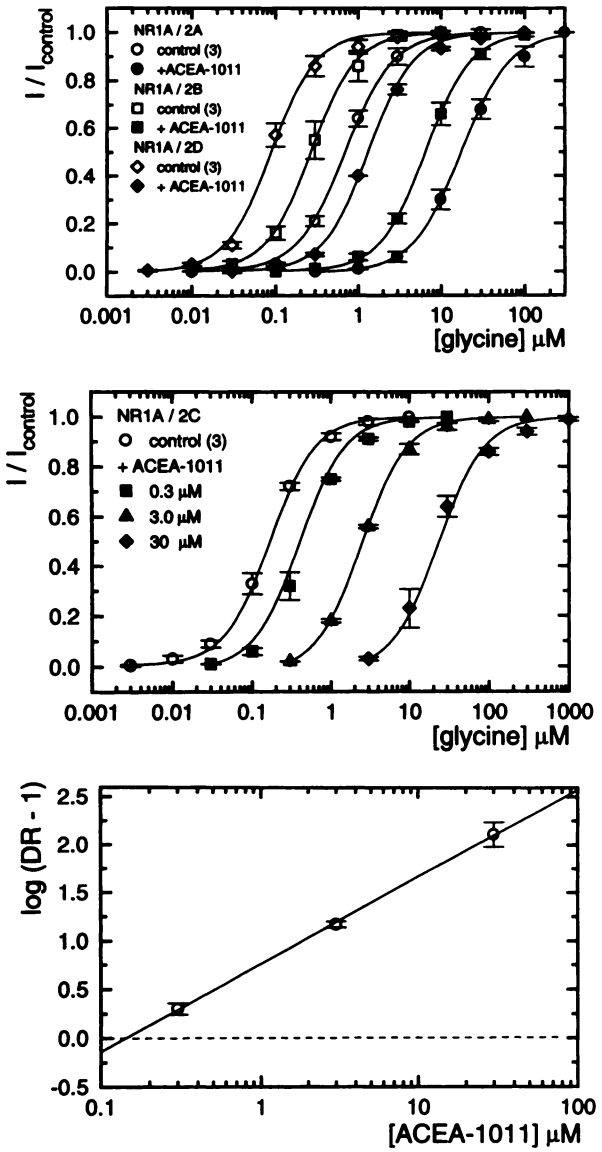

Fig. 4. Mechanism of inhibition at cloned rat NMDA receptors expressed in *Xenopus* oocytes. Upper panel, Effect of ACEA-1011 (10 μM) on glycine concentration-response curves for three binary subunit combinations (glutamate = 100 μM). Smooth curves, Best fits of equation 3, curve parameters given in table 1. Middle panel, Effects of ACEA-1011 (0.3, 3 and 30 μM) on steady-state concentration-response relationships for glycine at NR1A/NR2C subunit combinations (glutamate = 100 μM). Smooth curves, Best fits of equation 3, curve parameters (EC_{50} for control and optimal slope for simultaneous fit of the four curves) are given in table 1. Lower panel, Schild regression of the same data. Dose-ratios calculated from EC_{50} for each curve were fitted independently with equation 1. Line fit by linear regression; slope = 0.92.

values indicated that, in terms of antagonist affinity, ACEA-1011 showed a maximum of 3.4-fold selectivity between the four subunit combinations (table 1). This occurred between NR1A/2C and NR1A/2D. K_b values calculated from inhibition curves and from shifts in glycine concentration-response curves showed good correlation for each subunit combination.

As described for the rat brain NMDA receptors, ACEA-1011 was also tested for inhibition at glutamate binding sites on the cloned NMDA receptors. Inhibition of responses was <30% using 30 μM ACEA-1011, implying that K_b values for ACEA-1011 at glutamate binding sites on all four subunit combinations are >30 μM. Due to poor levels of expression (I_{max} 5–15 nA), ACEA-1011 was not assayed in detail at putative homooligomeric NR1A receptors expressed in oocytes. Nevertheless, preliminary experiments suggested that potency was similar to that described for the heterooligomeric receptors (not illustrated).

AMPA/kainate responses expressed by rat whole brain poly(A)$^+$ RNA. Non-NMDA glutamate receptors were activated using AMPA or kainic acid (Gundersen *et al.*, 1984). When assayed in the same oocyte, steady-state maximum AMPA responses were usually <5% of maximum kainate currents. Coapplying AMPA on established kainate responses resulted in concentration-dependent reductions in net current (fig. 5, records). This implies that AMPA and kainate activate a common population of non-NMDA gluta-

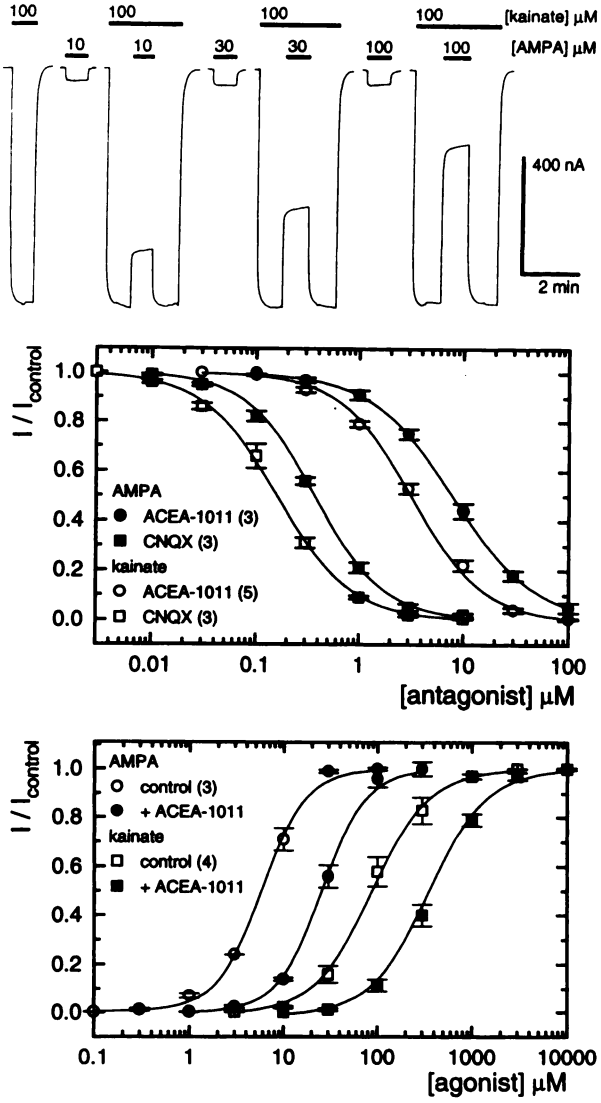

Fig. 5. Inhibition of AMPA and kainate responses expressed in Xenopus oocytes by rat brain poly(A)$^+$ RNA. Records, Sample records illustrating dose-dependent occlusion of established kainate responses by coapplication of AMPA. Holding potential fixed at −70 mV. Middle panel, Concentration-inhibition curves comparing potency of ACEA-1011 and CNQX on AMPA and kainate responses. Smooth curves, Best fits of equation 2, parameters given in table 1. Lower panel, Effect of ACEA-1011 (30 μM) on steady-state concentration-response relationships for AMPA and kainic acid. Smooth curves, Best fits of equation 3, parameters given in table 1.

mate receptors expressed by the poly(A)$^+$ RNA. The majority of these receptors have higher affinity for AMPA than kainate (see below) and therefore should be considered "AMPA preferring" (Hollmann and Heinemann, 1994). Kainate is known to produce less desensitization of AMPA receptors and therefore evokes a larger steady-state current than AMPA itself. Due to the slow exchange of solutions bathing the oocyte it was not possible to detect the rapid (msec) onset of desensitization to AMPA (*e.g.*, Patneau and Mayer, 1991).

Potency of ACEA-1011 at AMPA-prefering non-NMDA receptors was first assessed from concentration-inhibition curves using fixed concentrations of agonist (10 μM AMPA, ~50% I_{max}; 20 μM kainate, ~10% I_{max}). As with other substituted quinoxaline-2,3-diones (Honore *et al.*, 1988), ACEA-1011 showed inhibition of AMPA and kainate responses. In this case potency was ~20-fold weaker than the reference compound CNQX (fig. 5, middle panel). Depending on whether the agonist was AMPA or kainate, IC_{50} were, respectively: 0.37 and 0.16 μM for CNQX, and 8 and 3.2 μM for ACEA-1011 (table 1, upper panel).

Assaying ACEA-1011 (30 μM) on concentration-response relationships for AMPA and kainate showed that inhibition was surmountable for both agonists (fig. 5, lower panel). For each agonist the parallel rightward shifts in concentration-response curves were consistent with competitive inhibition at glutamate binding sites; $F_{1,31} = 0.72$ for AMPA, and $F_{1,43} = 0.19$ for kainate. K_b values were again calculated and cross-checked using the two methods of analysis. There was tight correlation of values for AMPA (K_b = ~8.3 μM), and the values for kainate agreed within a factor of two (K_b = 3.2 and 7.5 μM) (table 1). In these experiments current ranges and mean I_{max} for AMPA and kainate responses were: 50 to 75 nA (62 ± 9 nA, $n = 3$), and 1200 to 1650 nA (1340 ± 120 nA, $n = 4$), respectively.

As reported previously (Geoffroy *et al.*, 1991), maximum non-NMDA responses declined at high concentrations of agonist, an effect that was not reversed in a parallel manner by antagonists. For purposes of curve fitting currents in figure 5 were normalized to take account of this phenomena.

Metabotropic responses expressed by rat whole brain poly (A)$^+$RNA. Metabotropic glutamate receptors were selectively activated using 1S,3R-ACPD (Manzoni *et al.*, 1990). Metabotropic responses were fluctuating Cl^- currents elicited by receptor-mediated stimulation of the phosphoinositide/Ca^{2+} pathway (Oron *et al.*, 1985). ACEA-1011 (10–30 μM) caused no discernible inhibition of responses elicited by 5 to 10 μM 1S,3R-ACPD. Using an affinity and slope for 1S,3R-ACPD of ~30 μM and 1.8, respectively (Woodward *et al.*, 1995), and assuming competitive inhibition, these experiments suggest that the K_b for ACEA-1011 at metabotropic glutamate receptors is >50 μM.

Electrical Recordings from Cultured Rat Cortical Neurons

NMDA receptors. Current through NMDA receptors was recorded from rat cortical neurons under whole cell voltage clamp after 1 to 3 wk in culture. For pharmacological assays NMDA and glycine were coapplied in the absence of Mg^{2+} at a holding potential of −70 mV. Currents evoked by steady application of NMDA/glycine rose rapidly to a peak and then decayed over a period of several seconds to reach a stable plateau (Swartz *et al.*, 1992). Current amplitudes were measured during the plateau phase. Maximum responses ranged between 327 to 2470 pA, mean = 1097 ± 179 ($n = 9$).

Preliminary experiments using fixed concentrations of agonist (1 μM glycine, 100 μM NMDA) demonstrated moderate potency inhibition by ACEA-1011. The level of antagonism did not depend on membrane potential. At 10 μM, ACEA-1011 produced 84.3 ± 1% inhibition at a holding potential of −60 mV, as compared to 84.0 ± 2.9% inhibition while holding at +40 mV (n = 3).

As described for NMDA receptors expressed in oocytes, inhibition by ACEA-1011 was completely overcome by increasing the glycine concentration. Potency at neuronal receptors was determined from the displacement of the concentration-response relationship for glycine (fig. 6, upper panel). The EC_{50} increased >18-fold in the presence of 10 μM ACEA-1011 without any significant change in slope ($F_{1,45} = 2.7$), predicting a K_b of 0.58 μM (table 2).

Non-NMDA receptors. Antagonism by ACEA-1011 was also studied at AMPA-preferring receptors expressed by cultured cortical neurons. As discussed above, AMPA serves at a potent agonist for these receptors (EC_{50} ~4 μM) but causes strong desensitization (steady-state currents ranged between 71 to 1700 pA, mean = 834 ± 1650 pA, $n = 9$). Kainate, by contrast, is a less potent agonist (EC_{50} ~150 μM), but induces less desensitization (steady-state currents ranged between 138 to 1788 pA, mean = 736 ± 115 pA, $n = 9$) (Pateneau and Mayer, 1991).

ACEA-1011 was appreciably less potent at AMPA recep-

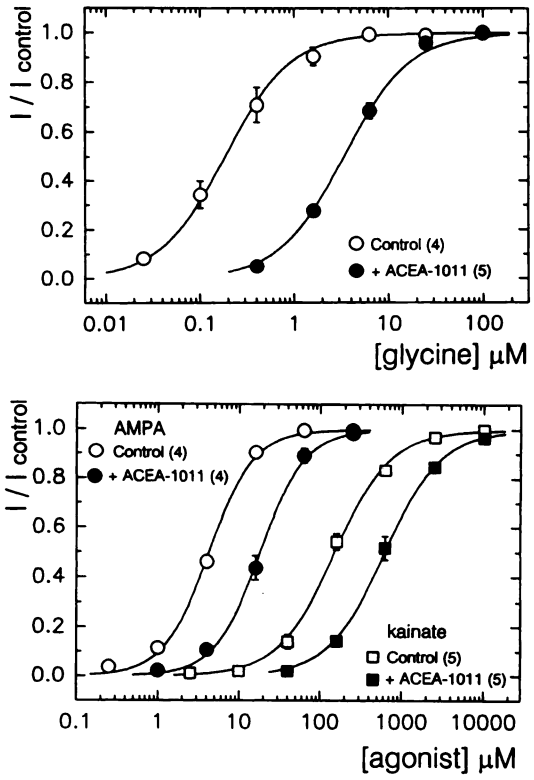

Fig. 6. Inhibition of whole-cell currents in cultured rat cortical neurons. Upper panel, Effect of ACEA-1011 (10 μM) on the glycine concentration-response curve (NMDA = 100 μM). Smooth curves, Best fit of equation 2, curve parameters are given in table 2. Lower panel, Effects of ACEA-1011 (30 μM) on the steady-state concentration-response relations for AMPA and kainate. Smooth curves, Best fits of equation 2, curve parameters given in table 2.

TABLE 2
Inhibition of NMDA and non-NMDA receptors in cultured rat cortical neurons

Receptor/Agonist[a]	EC_{50} (μM) (control curve)	Slope C/R Curve	K_b (μM) (Gaddum/Schild)	n
NMDA/glycine[b]	0.18 (0.16, 0.21)	1.3 (1.1, 1.4)	0.58 (0.47, 0.71)	5
Non-NMDA/AMPA	4.2 (3.9, 4.6)	1.6 (1.4, 1.7)	9.2 (7.8, 11)	5
Non-NMDA/kainate	150 (130, 170)	1.3 (1.1, 1.4)	9.6 (7.5, 12)	4

[a] Analysis of concentration-response (C/R) experiments. EC_{50} for control curves and slope values are the best fits of data to equation 3. K_b values estimated using the Gaddum/Schild approach.
[b] NMDA = 100 μM. Curves plotted in figure 6.

tors than at NMDA glycine sites. K_b values were determined from the displacement of steady-state concentration-response relations for both AMPA and kainate (fig. 6, lower panel). Potency of ACEA-1011 was similar on AMPA and kainate responses, consistent with the idea that these agonists act on a common pool of receptors. At 30 μM, ACEA-1011 produced a slightly greater than 4-fold increase in the EC_{50} values for both AMPA and kainate, indicating K_b of 9.2 and 9.6 μM, respectively. Departure from parallel displacement was not significant for either agonist ($F_{1,40}$ = 0.04, AMPA; $F_{1,56}$ = 0.14, kainate).

Mouse Maximum Electroshock-Induced Seizure Studies

Anticonvulsant effects and systemic bioavailability of ACEA-1011 were assessed using a mouse MES model (Swinyard, 1972), and were compared with effects of ACEA-1021 and CNQX. Time courses of protection were established after i.v. injection of 20 mg/kg ACEA-1011, 10 mg/kg ACEA-1021 and 20 mg/kg CNQX (fig. 7, upper panel). The protective effect of ACEA-1011 peaked at ~15 min and subsided slowly, such that there was still ~60% protection 1 hr after drug administration. Effects of ACEA-1021 had a faster onset, peaking at 2 min, and fell to ~40% after 1 hr. CNQX had only weak protective effects, maximum ~25%, that appeared to peak 5 to 15 min after injection. Potency measurements for the three drugs were taken at maximum efficacy points during the time course (fig. 7, lower panel). The ED_{50} value for ACEA-1011 was ~12 mg/kg, with 90% protection at 25 mg/kg. ACEA-1021 was approximately twice as potent, ED_{50} ~5 mg/kg with full protection at 7.5 mg/kg. We were unable to measure an ED_{50} value for CNQX due to weak activity and limited solubility in formulation buffers. The dose response curve for CNQX had a low slope over the range 10 to 25 mg/kg and extrapolation suggested an ED_{50} >40 mg/kg.

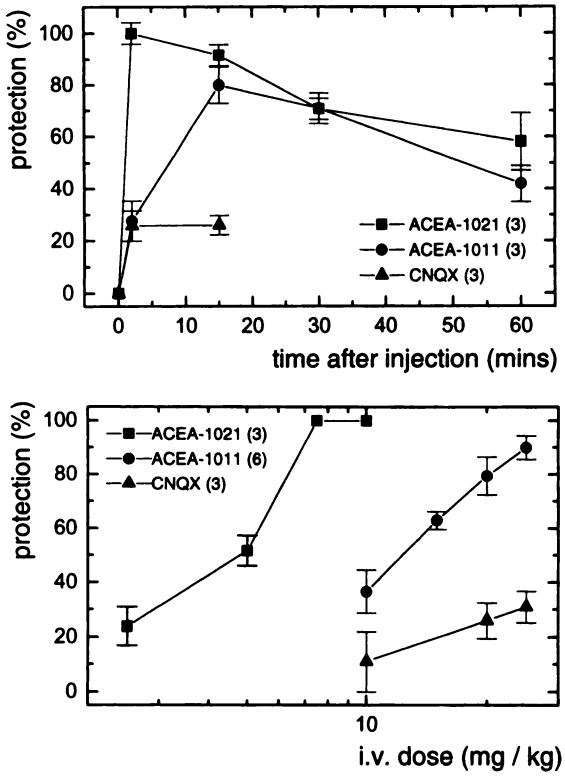

Fig. 7. Anticonvulsant effects of ACEA-1011, ACEA-1021 and CNQX in a mouse MES model. Upper panel, Time course of seizure protection after i.v. injection of 20 mg/kg ACEA-1011, 10 mg/kg ACEA-1021 and 20 mg/kg CNQX. Lower panel, Dose-response relationships for ACEA-1011, ACEA-1021 and CNQX. Protection was assayed at peak efficacy in the time course; 15 min for ACEA-1011, 2 min for ACEA-1021 and 15 min for CNQX. Data are plotted as the mean ± SEM with number of separate experiments given in parentheses. For each experiment percentage protection was determined from groups of between 5 to 10 mice.

Discussion

ACEA-1011 shows activity as an analgesic in rodent models of chronic pain (Lutfy *et al.*, 1995, in press; Vaccarino *et al.*, 1993). ACEA-1011 also blocks clonic seizure-like effects induced by intrathecal morphine (Lutfy *et al.*, 1994), and is efficacious as a neuroprotectant in rat models of focal cerebral ischemia (P. Marek and E. Weber, unpublished data). To investigate mechanisms involved in these effects we characterized the pharmacology of ACEA-1011 at mammalian excitatory amino acid receptors studied *in vitro*, and the anticonvulsant effects of the compound in a mouse MES model. Our results indicate that ACEA-1011 is a systemically active ionotropic glutamate receptor antagonist with moderate potency at NMDA receptor glycine sites and only 10-fold selectivity for NMDA as compared to non-NMDA receptors.

Antagonism at NMDA and non-NMDA receptors. Contrary to early binding experiments, which suggested that ACEA-1011 was a highly selective NMDA receptor antagonist (Vaccarino *et al.*, 1993), the present study indicates that ACEA-1011 is an inhibitor at both NMDA and AMPA-preferring non-NMDA receptors. Antagonism at NMDA receptors is *via* competitive inhibition at glycine co-agonist sites, and antagonism at AMPA receptors is *via* competitive inhibition at glutamate binding sites.

K_b values for ACEA-1011 at NMDA receptors expressed in oocytes by rat brain poly(A)$^+$ RNA varied between 0.4 to 0.8 μM, depending upon method of measurement, and the K_b value at neuronal NMDA receptors was approximately 0.6 μM. The close correlation between these results confirms that glycine site pharmacology has been accurately reproduced in the oocyte expression system. Cloned receptors ex-

pressed in oocytes showed a similarly close correlation (K_b values 0.2–0.7 μM), suggesting that the glycine site pharmacology of the simple binary subunit combinations corresponds closely to that of neuronal NMDA receptors. Potencies of the reference compound ACEA-1021 and CNQX were similar to those reported previously (Honore *et al.*, 1988; Verdoorn *et al.*, 1989; Woodward *et al.*, 1995). Taken together, these results underscore that ACEA-1011 is only a moderate potency NMDA antagonist, ~100-times weaker than ACEA-1021 and only 5 times more potent than CNQX.

Potency of ACEA-1011 at AMPA receptors was also consistent between assay systems. K_b values varied between 4 to 9 μM in the oocyte assays, depending on method of measurement, and between 9 to 10 μM for neuronal assays. Values for CNQX at non-NMDA receptors were approximately 15 times higher (K_b between 0.3–0.4 μM); in line with previous estimates for this compound (Verdoorn *et al.*, 1989). Potency of antagonism was similar whether using AMPA or kainate as agonists, consistent with evidence that in both the oocyte and neuronal systems these two ligands activate a common population of receptors.

In oocytes expressing brain poly(A)$^+$ RNA slopes of NMDA and non-NMDA receptor inhibition curves were between -1.3 and -1.1, giving no indication that ACEA-1011 discriminates strongly between subtypes within the presumably heterogeneous population of receptors. The present experiments leave open the question of selectivity between AMPA-preferring and kainate-preferring subtypes of non-NMDA receptors (Huettner, 1990). Comparison of ACEA-1011, ACEA-1021 and CNQX illustrates how potency and selectivity of quinoxaline-2,3-diones at NMDA and AMPA receptors are critically dependent on benzene ring substitutions (see also Keana *et al.*, 1995, in press; Woodward *et al.*, 1995).

Subunit-selectivity at cloned NMDA receptors. In terms of IC_{50} values, the four putative subtypes of NMDA receptor showed appreciable, but not pronounced, differences in sensitivity to ACEA-1011. NRIA/2A receptors were approximately 2 to 4 times more sensitive than NR1A/2B and NR1A/2C, and were 20 times more sensitive than NR1A/2D. With respect to antagonist affinity (K_b value), however, ACEA-1011 showed <4-fold levels of subunit selectivity. The 20-fold range in IC_{50} values arises through the combination of modest (~1 to 4-fold) variations in antagonist affinity and the up to 8-fold differences in affinity for glycine (Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992). The true *in vivo* subtype selectivity of drugs such as ACEA-1011 will depend on levels and dynamics of local agonist concentrations, where the agonist could be either glycine or D-serine (*e.g.*, Refs. D'Angelo *et al.*, 1990; Hashimoto *et al.*, 1995; Leeson and Iversen, 1994). Regional variation in agonist pools could either enhance or erode the pattern of selectivity recorded under steady-state conditions using a uniform concentration of agonist.

Binding studies on cloned receptor subunits expressed in mammalian cell lines indicate that the glycine site is associated with NR1 subunits (Lynch *et al.*, 1994;). Site-directed mutagenesis of NR1 confirms this result (Kuryatov *et al.*, 1994; Wafford *et al.*, 1995). To date, studies testing glycine site antagonists at the binary subunit combinations NR1A/2A, 2B, 2C or 2D have revealed only modest levels of subtype-selectivity (Kutsuwada *et al.*, 1992; Woodward *et al.*, 1995). This raises the question whether glycine sites show any heterogeneity between the NR1 splice variants (Hollmann and Heinemann, 1994). Detailed studies using heterooligomeric receptors have not yet been reported, but it is worth noting that the N-terminal (extracellular) insert, and other splice varients of NR1, did not alter sensitivity to 7-chlorokynurenic acid (Sugihara *et al.*, 1992). Taken together, these results begin to suggest that designing glycine site antagonists with high levels of subunit-selectivity may be problematic.

Selectivity of antagonism *in vivo*. In terms of steady-state selectivity, ACEA-1011 is approximately 10 times more potent at NMDA than at AMPA receptors; values ranged between 9- to 17-fold depending on preparation and type of analysis. A steady-state selectivity index presupposes that inhibition at both types of receptor is under equilibrium conditions. This may be true for NMDA receptor glycine sites *in vivo*, where levels of agonist are generally thought to be stable, but it is unlikely for AMPA receptor glutamate sites, which rapidly bind and unbind agonist during synaptic transmission (Clements *et al.*, 1992). At AMPA receptors ACEA-1011 presumably equilibrates with a low background level of glutamate. The functional potency of ACEA-1011 for inhibiting excitatory transmission will then depend on unbinding kinetics. If the dissociation rate of ACEA-1011 is sufficiently slow, synaptically released glutamate will not be present in the cleft long enough to fully equilibrate with the antagonist. A slow rate of unbinding would therefore diminish the already modest selectivity indicated by the steady-state measurements (see Randle *et al.*, 1992). Alternatively, if unbinding is rapid, the antagonist will compete against high concentrations of agonist, effectively increasing the functional selectivity. Characterizing the synaptic pharmacology of ACEA-1011 will be necessary to resolve these uncertainties. At present, and mainly from the perspective of steady-state values, we think it appropriate to class ACEA-1011 as a "broad selectivity" ionotropic glutamate receptor antagonist, rather than simply as a glycine site antagonist. Considered as such, ACEA-1011 is comparable to the most potent systemically active compounds described to date (Carling *et al.*, 1993).

Anticonvulsant effects. The ED_{50} for ACEA-1011 in protecting against electroshock-induced seizures was ~12 mg/kg i.v., similar to a previously reported broad selectivity NMDA/AMPA receptor antagonist L-698, 544 (Carling *et al.*, 1993). Activity in the MES model clearly confirms that ACEA-1011 has CNS bioavailability after systemic administration. Indeed, potency of ACEA-1011 in the mouse MES model is in the same range as previously reported analgesic potency (9–16 mg/kg i.p.) in the mouse formalin test (Lutfy *et al.*, 1995, in press; Vaccarino *et al.*, 1993), suggesting that both effects occur through a common mechanism. *In vitro*, ACEA-1011 is ~100 times less potent than ACEA-1021 as a glycine site antagonist and ~10-fold less potent as an AMPA receptor antagonist. Still, ACEA-1011 is only ~2-times less potent than ACEA-1021 in the MES model. Either ACEA-1011 has appreciably better bioavailability than ACEA-1021, or the anticonvulsant effects show dependence on AMPA receptor inhibition. Resolving this issue will require further *in vivo* studies. Nevertheless, a recent report indicates that highly selective systemically active glycine site antagonists have potent (1 mg/kg i.p.) anticonvulsant effects in a mouse audiogenic seizure model (Kulagowski *et al.*, 1994), indicating that

glycine site antagonism alone is quite sufficient to explain the anticonvulsant effects of ACEA-1011 and ACEA-1021. In this case, the apparent discrepancy between the *in vitro* and *in vivo* data for ACEA-1011 and ACEA-1021 is most readily explained by differences in bioavailability. CNQX was distinctly weak in the MES model (ED_{50} >40 mg/kg). Separate studies indicate that many quinoxalinediones, particularly 6,7 substituted molecules, are weak or inactive *in vivo*. The major problem would appear to be inadequate penetration of the blood brain barrier, augmented in some cases by strong binding to plasma proteins. As described for potency at the receptor level, bioavailability of quinoxalinediones is critically dependent on the type and pattern of benzene ring substitutions.

Analgesia. A role for NMDA receptors in nociception was first suggested in studies monitoring the behavioral effects of intrathecally applied agonists and antagonists (Cahusac *et al.*, 1984). From a mechanistic perspective, NMDA receptors are involved in "wind-up" (Mendell, 1966), a frequency-dependent plasticity phenomenon wherein, after repeated C-fiber stimulation, nociceptive neurons in the spinal cord show an increased propensity to generate action potentials (Davies and Lodge, 1987; Dickenson and Sullivan, 1987). As described above, previous studies have shown that ACEA-1011 is a potent analgesic in the mouse formalin test, a model of tonic pain (Lutfy *et al.*, 1995, in press; Vaccarino *et al.*, 1993). The current results raise the possibility that dual antagonism, at NMDA and AMPA receptors, could contribute to these antinociceptive effects. Arguing against this notion, however, are reports of potent late phase analgesia induced by R(+)-HA-966, dizocilpine and 7-chloro-kynurenate, all of which are selective NMDA antagonists (*e.g.*, Dickenson and Aydar, 1991; Millan and Seguin, 1993; Vaccarino *et al.*, 1993). These studies imply that antinociceptive effects of broad spectrum drugs such as ACEA-1011 can, like the anticonvulsant effects, be wholly explained by inhibition at NMDA receptors. To test if inhibition at non-NMDA receptors in some way contributes to the analgesia induced by ACEA-1011 it will be necessary to characterize actions of selective non-NMDA receptor antagonists in animal pain models (Nasstrom *et al.*, 1992). It will also be important to determine what roles the AMPA and kainate subtypes of non-NMDA receptor play in mediating nociceptive stimuli, and whether drugs such as ACEA-1011 discriminate between these receptors.

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