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ORIGINAL INVESTIGATION

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In vitro and in vivo studies in rats with LY293558 suggest AMPA/kainate receptor blockade as a novel potential mechanism for the therapeutic treatment of anxiety disorders

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Abstract *Rationale:* Although convergent evidence exists for a role of glutamate in the regulation of anxiety, the involvement of specific glutamate receptor subtypes has yet to be defined. *Objective:* To evaluate the potential for blockade of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA)/kainate receptors to produce anxiolytyic-like effects with the AMPA/GLU_{K5} (kainate) antagonist (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazole-5yl)ethyl]decahydroisoquinoline-3carboxylic acid (LY293558). *Materials and methods:* Punished responding of rats was used to determine the efficacy of LY293558. Other in vivo and in vitro studies further characterized the specificity of LY293558 for AMPA/kainate receptors. *Results:* LY293558 had a rank order of potency of $GLU_{K5} \geq GLU_{K5/6} \approx GLU_{A2i} \approx GLU_{K2/5} \approx GLU_{A1i} \approx GLU_{A2o} \approx GLU_{A3i} \approx GLU_{A1o} \geq GLU_{A3o} \geq GLU_{A4i} \approx GLU_{A4o}$ and >100 μM affinity for rat cortical $GABA_A$ receptors. Comparison of the blockade of AMPA- vs *N*-methyl-D-aspartate (NMDA)-induced inward currents demonstrated that LY293558 was fivefold more potent as an antagonist at AMPA vs NMDA receptors in vitro. In keeping with the low affinity of LY293558 for NMDA receptors, LY293558 was not effective in preventing NMDA-induced seizures in mice. LY293558 increased punished responding, a preclinical predictor of anxiolytic efficacy, at a dose that decreased unpunished responding (10 mg/kg, i.p.). Chlordiazepoxide produced comparable increases in both punished and unpunished responding. The NMDA antagonist dizocilpine [(+)-MK-801] also increased both punished and unpunished responding. *Conclusions:* These data along with those in the literature suggest that AMPA and/or kainate receptor blockade may be an important component to producing anxiolytic-like effects and may therefore be a target for compounds with efficacy in the therapeutic treatment of anxiety disorders.

Keywords Kainate receptors · AMPA receptors · LY293558 · Chlordiazepoxide · (+)-MK-801 · Anxiolytic drugs · Rats

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Data from convergent sources have suggested that dysregulation of glutamatergic neurotransmission contributes to the pathophysiological states of epilepsy, Parkinson's and Huntington's diseases, schizophrenia, and dependence disorders (c.f., Parsons et al. 1998; Krystal et al. 1999). Mood and anxiety disorders are also likely to involve perturbations in glutamatergic neurotransmission (c.f. Chojnacka-Wojcik et al. 2001; Kent et al. 2002; Moghaddam 2002). Preclinical models of anxiety and depression have documented that glutamatergic blockade can result in antidepressant- and anxiolytic-like effects (Witkin 1995; Chojnacka-Wojcik et al. 2001; Skolnick 2002), but the potential for glutamatergic blockade as a therapeutic treatment for anxiety disorders remains uncertain. Although competitive blockade of *N*-methyl-D-aspartate (NMDA) receptors has been associated with anxiolytic-like effects in animal models, the data are less clear for compounds that antagonize the NMDA receptor ion channel (see review by Witkin 1995).

The involvement of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors in anxiolytic drug action is even less well described. The AMPA/kainate antagonist LY326325 produced increases in drinking suppressed by electric shock (Kotlinska and Liljequist 1998). However, there are data that are not supportive of an anxiolytic profile; LY326325 has been shown to produce anxiogenic rather than anxiolytic-like effects on the elevated plus maze (Karcz-Kubicha and Liljequist 1995). LY326325 is the monohydrate salt of LY293558, (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazole-5yl)ethyl]decahydroisoquinoline-3carboxylic acid (Ornstein et al. 1993), a competitive antagonist with high affinity

for GLU_{K5} kainate receptors (Schoepp et al. 1995; Bleakman et al. 1996). LY293558 produced increases in punished responding of pigeons (Benvenga et al. 1995).

As punished responding in pigeons may be more sensitive to certain anxiolytic agents than in other species (c.f. Barrett and Witkin 1991), the present study was designed to compare LY293558 with the benzodiazepine anxiolytic chlordiazepoxide and the NMDA receptor ion-channel blocker (+)-MK-801 on responding suppressed by punishment of rats. Responding suppressed by punishment is widely used as a method for detecting anxiolytic activity of compounds, based upon the established findings that clinically effective agents like benzodiazepines and other less used agents can be detected with these procedures (c.f. Geller 1964; Cook and Davidson 1973; Barrett and Witkin 1991). As both chlordiazepoxide and LY293558 increased food-maintained responding suppressed by punishment in the present experiment and as chlordiazepoxide can also increase food intake (Bainbridge 1968; Witkin and Leander 1982), we also studied the effects of both chlordiazepoxide and LY293558 on food intake at doses that were effective in increasing punished responding. An additional aim of the present study was to broadly characterize LY293558 for its affinities and selectivity for different AMPA receptor subunits using specific cell lines expressing recombinant human receptors in vitro.

Materials and methods

Cell culture All cell and tissue culture reagents were from Invitrogen (Grand Island, New York). Recombinant human AMPA receptors (GLU_{A1-4}, both flip (i) and flop (o) variants) were stably expressed in human embryonic kidney (HEK) 293 cell lines, with the exception of GLU_{A3o} which was stably expressed in AV12 cells. Cells were grown as monolayers under 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium with 4.5 g/l D-glucose, L-glutamine, and pyridoxine HCl (Invitrogen, catalog #11965-084), with 10% fetal bovine serum and 250 μg/ml geneticin added. Medium for GLU_{A3o} cells contained 5% fetal bovine serum and 700 μg/ml geneticin. It also contained 10 mM HEPES, 1 mM sodium pyruvate, 50 μg/ml streptomycin, 50 units/ml penicillin, and 300 μg/ml hygromycin.

Measurement of ion influx using Fluo-3 Cells were seeded into poly-D-lysine-coated 96-well plates (Becton Dickinson Labware, Bedford, MA) 1 day prior to experiments at a density of 60,000 cells/well. Cells were washed three times with 100 μl assay buffer composed of Hanks Balanced Salt Solution (HBSS) without phenol red (Invitrogen) with 20 mM HEPES and 3.7 mM $CaCl_2$ added (final [$CaCl_2$] =5 mM). Plates were then incubated for 2 h in the dark at room temperature in 40 μl assay buffer with 8 μM Fluo3-AM dye (Molecular Probes, Eugene, OR). After dye incubation, cells were rinsed once with 100 μl assay buffer. Finally, 50 μl assay buffer that included the AMPA receptor potentiator LY392098 (10 μM, to prevent desensitization of AMPA receptors) was added to wells and fluorescence measured using a fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA). A first addition of 50 μl of LY392098-containing assay buffer was followed by a second addition of 100 μl LY392098-containing buffer 3 min later. LY293558 was added in the absence of agonist during the first addition and in the presence of 100 μM glutamate during the second addition. Because of the high potency of glutamate at GLU_{A4i} receptors (EC_{50}=2 μM, determined from pilot experiments), only 1 μM glutamate was used when testing antagonists at these receptors to provide more accurate estimations of IC_{50} and K_b values.

$GABA_A$ receptor binding The affinity of LY293558 for $GABA_A$ receptors was assessed in membranes prepared from frozen rat brain cortex. Membranes were prepared using freezing, thawing, and Triton X-100 extraction to remove endogenous GABA (Williams and Risley 1979). The final membrane pellets were resuspended in 50 mM Tris buffer at pH 7.4. [^{3}H]Muscimol (2 nM) was used to label $GABA_A$ receptors and non-specific binding was determined in the presence of 10 μM GABA.

Increasing concentrations of compound (10 μM to 0.1 nM) were incubated with radiolabel and membrane aliquots in a final volume of 1.0 ml and incubated under refrigeration for ≥2 h. The assay was terminated by filtration over GF/C filters on a Packard Filtermate Cell Harvester, followed by an ice-cold 10-ml-saline wash. Radioactivity bound was determined after a period of equilibration in Micoscint-20 scintillation cocktail using a Packard Microplate scintillation counter.

Primary culture of hippocampal pyramidal neurons Hippocampal pyramidal neurons were cultured via modification of the method of Brewer et al. (1993) from E17 Sprague–Dawley rat fetuses. Neurons were mechanically dispersed in 1 ml HBSS without Mg^{2+} or Ca^{2+} supplemented with 1 mM sodium pyruvate and 5 mM HEPES utilizing a fire-polished glass Pasteur pipette. The suspension was then diluted with HBSS with Mg^{2+} and Ca^{2+} supplemented as above and non-dispersed tissue was allowed to settle for 3 min. The supernatant was transferred to a 15-ml conical tube and centrifuged for 1 min at 200×*g*. The pellet was resuspended in Neurobasal Media (Invitrogen) supplemented with B27, 0.5 mM glutamine, and 25 μM glutamic acid. Neurons were plated on collagen/laminin-coated glass coverslips (15 mm) at 2×10^5 cells per coverslip. Cultures were maintained in a humidified 5% CO_2 incubator at 37°C. On day 4 and every seventh day after, media were half exchanged with fresh plating media lacking glutamic acid. Neurons were used for electrophysiological recording after 12–15 days in vitro.

Electrophysiological recordings and drug application Whole-cell voltage clamp recordings (V_h=−70 mV) were made from single cells with the use of tight-seal whole-cell configuration of the patch-clamp technique (Hamill et al.

1981). Glass fragments of coverslips with adherent cells were placed in a perfusion chamber and rinsed with buffer of composition: 140 mM NaCl, 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4 with NaOH (osmolality, 315 mosM/kg). Pipette solutions contained 140 mM CsCl, 1 mM $MgCl_2$, 14 mM diTris creatine phosphate, 50 U/ml creatine phosphokinase, 14 mM MgATP, 10 mM HEPES, and 15 mM BAPTA, pH 7.2 with CsOH (osmolality, 295 mosM/kg). Experiments were performed at room temperature (20–22°C) and recorded on an Axopatch 200 A amplifier using pClamp6 software (Axon Instruments, Foster City, CA). Pipette resistances were typically 1.5–2.5 MΩ. Drug application was via bath perfusion and occurred within approximately 5 s of initiation of flow. The recording buffer used when measuring NMDA-evoked currents excluded $MgCl_2$ and was supplemented with 10 μM glycine to maximize NMDA-evoked responses. Tetrodotoxin (1 μM) was also included in all recording solutions to prevent synaptic activity in these cultures. Concentration–effect curves were generated using data from a minimum of three separate cells.

Animals Experimentally naïve, male Fischer 344 rats (Harlan–Sprague–Dawley, Indianapolis, IN) were individually housed in a temperature-, humidity-, and light-controlled vivarium (lights on 0600–1800 hours). The rats for the punished responding experiments were maintained at about 85 to 90% of their free-feeding body weights by post-session feeding. Rats for the food intake experiments were given continuous access to food except prior to the feeding test as described below. Non-food-deprived, male NIH Swiss mice (Harlan–Sprague–Dawley, Indianapolis, IN) were used for the NMDA seizure and rotorod experiments. The mice were housed in large cages in groups of 12. Water was continuously available for both species except during experimental sessions. The facilities in which the animals were maintained are fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and the studies described herein were conducted in accordance with the Guide for Care and Use of Laboratory Animals under protocols approved by local institutional animal care and use committees.

Punished responding Experiments were conducted in standard operant conditioning chambers (Coulbourn Instruments, Allentown, PA) containing a single response lever. A group of twenty-four rats was used in this experiment wherein each individual rat was used on several occasions to construct dose–effect curves. Lever press training was accomplished by arranging each lever press to produce 45 mg food pellet (BioServe, Frenchtown, NJ). Responding was subsequently maintained under a multiple schedule of food delivery. In the presence of one set of stimulus lights (houselight and lever light illuminated), responding was maintained under a variable interval 30 s (VI 30 s) schedule in which lever presses produced food on an average of every 30 s. In the presence of an alternating stimulus condition (houselight + flashing lever light), the VI 30 s schedule remained in effect but, in addition, every tenth response produced a brief electrification of the grid floor (0.3–0.5 mA for 100 ms). Shock intensities were individually set to produce about 90% suppression of responding relative to behavior in the nonpunished schedule component. Punishment and no punishment schedule components were separated by a 60s period during which all lights were turned off and responding had no scheduled consequences. Training to final baseline levels was achieved after 8–10 weeks.

Once responding had stabilized such that there was no more than 15% variation in rates of responding from day to day for a 4-day period, drug experiments were initiated. Compounds and compound vehicles were generally administered on Tuesdays and Fridays given that responding on the preceding control sessions were within tolerance for the individual rat. Dose–effect curves were constructed using four to six rats per dose for each compound with vehicle control information coming from at least 10–15 rats. Chlordiazepoxide (i.p.), and LY293558 (i.p.) (+)-MK-801 (s.c. injection) were given 30 min prior to testing. Dose–effect curves were determined in the order listed above with chlordiazepoxide given at a number of different time points in the experiment to determine stability and durability of the response.

Food intake Rats were deprived of food for 18 h prior to testing. Chlordiazepoxide (15 mg/kg, i.p.) and LY293558 (10 mg/kg, i.p.) were administered 30 min prior to testing in individual test chambers (17.6×28.2×12.6-cm high) with sawdust bedding. Food (the same as used for daily maintenance, Rodent Diet 5001, PMI Nutrition International, Brentwood, MO) was weighed before and after a 90-min access. Seven rats were studied under each condition.

NMDA-induced seizures To determine whether the low-affinity binding of LY293558 to NMDA receptors observed in vitro could be responsible for the anxiolytic-like efficacy of LY293558, the ability of LY293558 to prevent convulsions induced by NMDA were compared to that of (+)-MK-801. The NMDA seizure/lethality test has been used as an in vivo predictor of NMDA receptor blockade (c.f., Leander et al. 1998). Mice were pretreated by s.c. injection with test compound or vehicle. Thirty minutes after pretreatment, mice were injected with NMDA (200 mg/kg, i.p.) and observed for the presence or absence of seizures and lethality as previously described (Leander et al. 1988; Geter-Douglass and Witkin 1999). Prior to treatment with NMDA, mice were tested on the rotorod to test for possible motor effects of test compounds.

Rotorod performance Mice were injected with vehicle or compound and, after 30 min, were placed on a rotorod (Ugo Basile 7650) operating at a speed of 6 rpm and observed for falling. Mice that fell off the rotorod on two occasions during 2 min were scored as failing. Mice were

not pretrained on this task. After 2 min on the rotorod, they were given NMDA and observed for seizures.

Compounds Chlordiazepoxide HCl and (+)-MK-801 maleate (dizocilpine) were obtained from Sigma Chemical Co.(St. Louis, MO), and LY293558 (synthesized in-house) were dissolved in 0.9% NaCl. All compounds were prepared just prior to dosing and administered in a volume of 1 ml/kg (rats) or 10 ml/kg (mice) body weight. Chlordiazepoxide (i.p.) and LY293558 (i.p.) were given 30 min prior to testing; (+)-MK-801 (s.c.) was administered 20 min prior to testing. Drug doses are expressed as drug forms noted above.

Data analysis Affinities of LY293558 in transfected cell lines were analyzed from concentration–response curves for LY293558 antagonism of glutamate effects. The curves were analyzed using GraphPad Prism 3.02 software (San Diego, CA), with the slope factor not fixed and the top and bottom fixed at 100 and 0% inhibition, respectively. The dissociation constant (K_b) was calculated from the IC_{50} value for inhibiting 100 μM glutamate-induced calcium influx (or 1 μM glutamate for GLU_{A4i}) according to the Cheng–Prusoff equation (Cheng and Prusoff 1973): $K_b = IC_{50}/(1+[Glu]/EC_{50\ Glu})$ where [Glu] is the concentration of glutamate (1 or 100 μM). $EC_{50\ Glu}$ is the EC_{50} value of glutamate for evoking calcium influx in a given cell line, determined from glutamate concentration–response curves run in the same plates as the antagonist concentration–response curves.

Rates of responding (responses per minute) in the punishment experiment were calculated for each component of the multiple schedule. Dose–effect curves were analyzed by one-way ANOVA for unpunished and punished responding separately. Given significant dose–effect changes, rates of responding under drug were evaluated for significance in comparison to vehicle by post-hoc Dunnett's test. Food intake in grams was compared by Student's *t* test. Seizure/lethality data and rotorod performances were analyzed by Fisher's exact probability test. All results were considered significant if $p<0.05$.

Results

The in vitro pharmacological profile of LY293558 at recombinant human AMPA and kainate receptors is shown in Table 1 including data on some recombinant receptors previously studied (Alt et al. 2004). LY293558 exhibited antagonist activity at both AMPA and kainate receptors in vitro, with a rank order of potency: $GLU_{K5} \geq GLU_{K5/6} \approx GLU_{A2i} \approx GLU_{K2/5} \approx GLU_{A1i} \approx GLU_{A2o} \approx GLU_{A3i} \approx GLU_{A1o} \geq GLU_{A3o} \geq GLU_{A4i} \approx GLU_{A4o}$. LY293558 had no effect at GLU_{K6} or heteromeric $GLU_{K2/6}$ kainate receptors.

The affinity of LY293558 was also determined at rat cortical $GABA_A$ receptors using [^{3}H]muscimol binding. At the maximum concentration tested, no affinity for the $GABA_A$ receptor was detected and the K_i was reported to be >100 μM.

To determine the selectivity of LY293558 for AMPA vs NMDA receptors in vitro, the ability of LY293558 to antagonize inward currents evoked by AMPA or NMDA was measured using whole-cell electrophysiological recordings in primary cultures of rat hippocampal pyramidal neurons. Figure 1 shows the ability of LY293558 to antagonize currents evoked by either 30 μM AMPA or 10 μM NMDA. These concentrations were chosen because they represent approximate EC_{80} concentrations for the agonists (data not shown). LY293558 displayed moderate (approximately five-fold) selectivity for AMPA vs NMDA receptors, antagonizing AMPA-evoked currents with an IC_{50} of 3 μM (95% confidence interval 2–3 μM) vs an IC_{50} of 13 μM (7–27 μM) for antagonizing NMDA-evoked currents.

Responding maintained under the multiple schedule was characterized by relatively constant rates of responding

Table 1 Potency of LY293558 for inhibition of glutamate-induced calcium influx in HEK293 cells expressing recombinant human AMPA and kainate receptors in vitro

AMPA receptors								
	1i	1o	2i	2o	3i	3o[a]	4i[b]	4o
K_b	0.8	1.1	0.6	0.8	1.0	5.9	17.0	32.8
95% CI	(0.6–1.0)	(0.3–5.0)	(0.5–0.7)	(0.6–1.1)	(0.9–1.0)	(2.6–13.6)	(12.1–23.9)	(8.0–133.6)
IC_{50}	31.0	18.5	20.9	15.0	55.0	66.7	27.2	129.2
95% CI	(18.0–53.3)	(6.7–50.9)	(15.8–27.6)	(10.2–22.0)	(47.9–63.2)	(21.9–203.1)	(20.3–36.4)	(84.4–197.9)
Kainate receptors[c]								
	5	5/6	2/5	6	2/6			
K_b	0.2	0.5	0.7	NE	NE			
IC_{50}	0.7	1.0	3.2	NE	NE			

Values are in micromolar (μM)
NE No effect (<20% inhibition at 100 μM)
[a]Expressed in AV12 cells
[b]Data listed for 4i cell line are from experiments using 1 μM glutamate (rather than 100 μM, as used for other cell lines)
[c]Values for LY293558 at kainate receptors are reported from Alt et al. (2004)

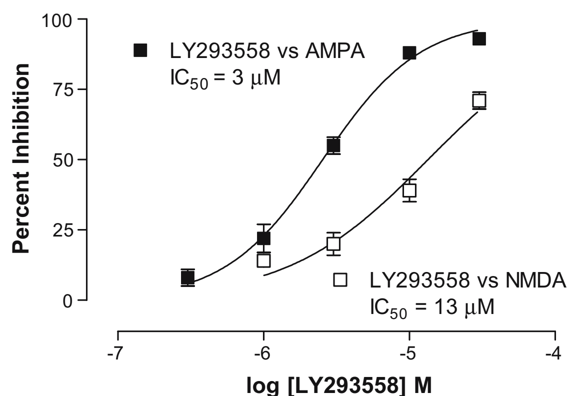

Fig. 1 Inhibition of AMPA- and NMDA-evoked inward currents by LY293558 in cultured rat hippocampal neurons in vitro using whole-cell voltage clamp electrophysiology. Inhibition of inward current evoked by 30 µM AMPA resulted in an estimated IC_{50} value of 3 µM (*filled squares*). Inhibition of inward current evoked by 10 µM NMDA resulted in an estimated IC_{50} value of 13 µM (*open squares*)

with rates in the punishment component being suppressed to about 6% of the rates of unpunished responding. Mean non-injection control rates of responding (n=11) were 22.4±4.2 and 1.44±0.09 responses per min for unpunished and punished responding, respectively.

Chlordiazepoxide (15 mg/kg) increased punished responding to 208% of control levels with corresponding increases in rates of unpunished response rates (172%) (Fig. 2). LY293558 also increased punished responding to about the same extent as chlordiazepoxide at 10 mg/kg (190% of control); this dose decreased unpunished responding to 67% of control values. The uncompetitive NMDA receptor antagonist (+)-MK-801 produced increases in both punished (171%) and unpunished responding (131%) at lower doses while higher doses produced dose-dependent decreases in rates.

Food intake was not significantly affected by either chlordiazepoxide (15 mg/kg) or LY293558 (10 mg/kg) at the doses that increased punished responding. Under vehicle, rats consumed 5.8±0.5 g food. After chlordiazepoxide, rats ate 7.1±1.2 g food and, after LY293558, rats consumed 4.9±0.6 g food over the 90-min session.

To determine whether the low-affinity binding of LY293558 to NMDA receptors observed in vitro could be responsible for the anxiolytic-like efficacy of LY293558, the ability of LY293558 to prevent convulsions induced by NMDA were compared to that of (+)-MK-801. The NMDA seizure/lethality test has been used as an in vivo predictor of NMDA receptor blockade (c.f., Leander et al. 1998). NMDA-induced seizures/lethality in all of the vehicle-treated mice (Table 2). (+)-MK-801 fully protected mice from seizures and lethality. LY293558 (10 and 30 mg/kg) was not efficacious against NMDA-induced seizures/lethality.

Table 2 Effects of LY293558 and (+)-MK-801 on seizures/lethality induced by *N*-methyl-D-aspartic acid compared to effects on the rotorod

Compound	Seizures/lethality[a]	Rotorod failures[a]
Vehicle	6/6	1/6
(+)-MK-801 (0.3 mg/kg)	0/6*	6/6*
LY293558 (10 mg/kg)	6/6	0/6
LY293558 (30 mg/kg)	5/6	3/6

*p<0.05 represents effects significantly different than vehicle control values by Fisher's exact probability test
[a]Data are expressed as number of mice affected/mice tested

Rotorod performance of mice was studied to determine if the doses of compounds that protect against NMDA-induced seizures produced motor side effects. (+)-MK-801 disrupted motor performance on the rotorod in all mice at the dose studied against NMDA-driven seizures (0.3 mg/kg) (Table 2). LY293558 showed a trend toward rotorod disruption that did not reach statistical significance up to 30 mg/kg.

Discussion

The increase in punished responding produced by LY293558 was of comparable magnitude to that produced by the benzodiazepine anxiolytic chlordiazepoxide. The results with LY293558 substantiate the anxiolytic-like effects of this compound and its salt form, LY326325, that have been reported using punished responding in pigeons (Benvenga et al. 1995) and Vogel conflict responding in rats, and a modest effect in the rat elevated plus maze (Kotlinska and Liljequist 1998). In one report, however, LY326325 produced an anxiogenic rather than an anxiolytic effect in the elevated plus maze (Karcz-Kubicha and

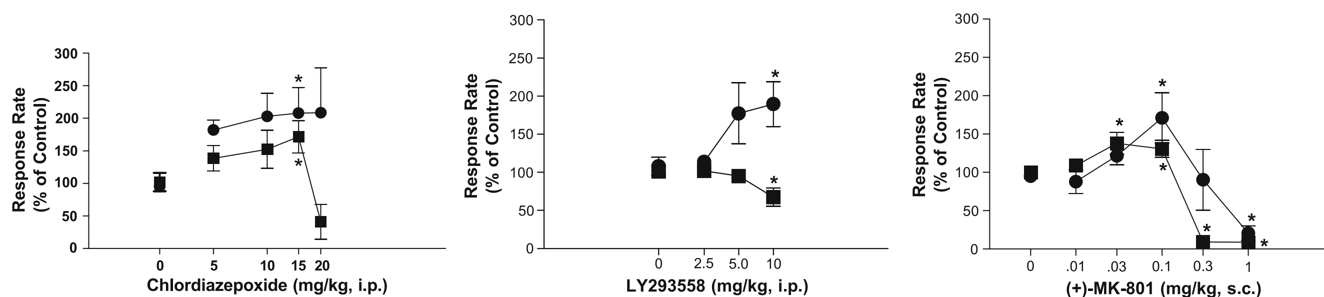

Fig. 2 Effects of chlordiazepoxide, LY293558, and (+)-MK-801 on punished and unpunished responding of rats. Nonpunished responding (*squares*), punished responding (*circles*). Each point represents the mean±SEM of four to six rats; n=10–15 for vehicle control. Significant differences (p<0.05, Dunnett's test) from vehicle control values (points above 0) are represented by *asterisks*

Liljequist 1995), a finding that may be species-related (mouse). The results of the present study using the Geller conflict procedure (Geller 1964) provide support for the potential of AMPA/kainate antagonism as a novel approach to the therapeutic management of anxiety disorders. As functional agonists of $GABA_A$ receptors can also increase punished responding (e.g., chlordiazepoxide as shown here), we evaluated the affinity of LY293558 to bind to $GABA_A$ receptors. Both the low affinity of this compound (>100 μM) compared to its higher affinity at AMPA/kainite receptors along with the lack of chlordiazepoxide-like effects on unpunished responding (increases) support the conclusion that $GABA_A$ receptors are not relevant to the in vivo effects of LY293558 reported here. Nonetheless, in vivo experiments with selective pharmacological tools would also be needed to substantiate this point.

The increases in punished responding produced by LY293558 did not appear to be due to increases in food motivation per se, as LY293558 did not increase rates of nonpunished responding or increase food consumption at the dose efficacious in the punishment experiment. Chlordiazepoxide increased rates of nonpunished responding, an effect reported previously by a number of investigators (see Witkin and Katz 1990 for a summary and discussion). In addition, chlordiazepoxide has been reported to increase feeding in rats (Bainbridge 1968; Witkin and Leander 1982), although chlordiazepoxide did not significantly increase food consumption in the present study at the dose that was active in the punishment experiment. It must be noted, however, that the conditions of food deprivation were not identical in the food intake experiments and the experiments on punished responding.

In contrast to the effects produced by LY293558 in the current study, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F) quinoxaline, a competitive AMPA/kainate receptor antagonist without the degree of specificity shown by LY293558 for GLU_{K5} receptors (c.f., Bleakman et al. 1999; Alt et al. 2004), did not have efficacy in the Vogel conflict test in rats (Członkowska et al. 1997) or in the pentylenetetrazol discrimination test (Swedberg et al. 1995) but did show small anxiolytic-like effects in the elevated plus maze when given directly into the nucleus accumbens of rats (Martínez et al. 2002). Pharmacological distinctions between AMPA and AMPA/kainate receptor antagonists are beginning to emerge in other areas of potential psychiatric application as well (e.g., psychosis, Vanover 1998).

There have been a number of reports that NMDA receptor antagonists that block the ion channel do not always produce anxiolytic-like effects in animal models (see summary by Witkin 1995). However, increases in punished responding of rats with phencyclidine and a competitive NMDA receptor antagonist, NPC 12626, have been reported previously (e.g., Wiley et al. 1992), and (+)-MK-801 produced significant increases in punished responding in the present experiment as noted in the original report on this compound by Clineschmidt et al. (1982). The ability of LY293558 to antagonize NMDA-evoked responses in vitro, as demonstrated in the present study in a functional electrophysiological assay, and its affinity for [^{3}H]CGS 19755 binding sites (IC_{50}=12 μM vs 28 μM for [^{3}H]kaiante) (Lodge and Schoepp 1997) suggested the possibility that NMDA receptor blockade may account for the anxiolytic-like effects of this compound. However, the lack of stimulation in unpunished responding by LY293558 (that was seen with (+)-MK-801) along with the absence of significant impairment in rotorod performance as seen with (+)-MK-801 (c.f., Ginski and Witkin 1994) provide evidence that LY293558 and related compounds (Benvenga et al. 1995) do not functionally block NMDA receptors in vivo at doses that produce anxioltyic-like activity.

As the high-affinity NMDA receptor antagonist (+)-MK-801 increased punished responding in the present experiment, we further evaluated whether LY293558 produces blockade of the NMDA receptor in vivo by examining its effects on NMDA-induced seizures/lethality, a test of in vivo NMDA receptor antagonism (Leander et al. 1988).

It should be noted that the species used for the NMDA-induced seizures/lethality experiments (mouse) differs from that used for measuring anxiolytic-like effects (rat). However, the inability of LY293558 to prevent NMDA-evoked convulsions in this model differentiates it from other low-affinity NMDA receptor ligands. LY293558 blocked NMDA currents with an IC_{50} of 13 μM in the present study. Ibogaine and 5-aminocarbonyl-10,11- dihydro-5h-dibenzo[a,d]cyclohepen-5,10-imine, which have been shown to bind to NMDA receptors with K_i values of 1 and 11 μM, respectively (Monn et al. 1990; Popik et al. 1994), both inhibit NMDA-induced seizures in mice (Geter-Douglass and Witkin 1999), suggesting that affinity is not the only determinant of the anticonvulsant activity of these agents. The specificity of action of LY293558 as an antagonist for AMPA/kainate receptors over NMDA receptors has been reported previously using rat spinal neuron electrophysiological methods in vitro and in vivo (Lodge and Schoepp 1997). The lack of effect of LY293558 in an in vivo assay of NMDA receptor blockade combined with its low functional affinity against NMDA-induced currents and its profile as an AMPA/kainate antagonist in vitro reinforces the hypothesis that blockade of AMPA, kainate, or AMPA/kainate receptors without blockade of NMDA receptors is sufficient to produce anxioltyic-like efficacy in an established animal model of anxiolytic activity. These findings are thus consistent with the data reported using pigeons in which similar conclusions were rendered (Benvenga et al. 1995).

LY293558 demonstrates high affinity binding to kainate GLU_{K5} receptors (Bleakman et al. 1999). As these receptors have been shown pharmacologically (Procter et al. 1998; Simmons et al. 1998; Blackburn-Munro et al. 2004; Palecek et al. 2004), clinically (Gilron et al. 2000), and through the use of transgenic mice (Ko et al. 2005; however, see Youn and Randic 2004) to be related to the control of pain pathways, the question of whether the anxiolytic-like effects of LY293558 are mediated through an analgesic effect can be raised. Although specific measurements of reactivity or detection thresholds for the electric foot current used as a punisher in the present experiment were not

established, ample evidence has long been available to document that analgesia per se is not a driver of anxiolytic-like activity in the punishment assay used here (c.f., Kelleher and Morse 1968). Nonetheless, the comorbidity of pain and anxiety states (c.f., Ploghaus et al. 2001; Sheftell and Atlas 2002; Huckle 2004) may make such a dual-acting molecule appealing for the treatment of chronic pain and/or anxiety states. Other potential behavioral effects of LY293558 that may be related to the increases in punished responding should be investigated to further characterize the behavioral pharmacology of this compound. However, effects of compounds on punished responding are generally related to their in vivo pharmacology and the baseline behaviors rather than to effects on ancillary behavioral processes such as analgesia, discrimination, vigilance, etc. (c.f., Kelleher and Morse 1968).

GLU_{K5} receptors are localized in brain areas regulating anxiety (e.g., basolateral amygdala; c.f., Li et al. 2001), providing additional support to the possibility that targeting specific glutamate receptor subtypes may prove more reliable for generating anxiolytic activity. A recent report using mice devoid of GLU_{K5}- or GLU_{K6}-containing receptors showed through electrophysiological and behavioral means that GLU_{K6} modulates the memory of aversive events in fear conditioning whereas GLU_{K5} does not (Ko et al. 2005). Although memory of aversive events associated with environmental cues is not isomorphic with anxiety, one must remain cautious about the exclusive assignment at present a specific receptor isoform to control of anxiety states. LY293558, despite its lack of significant interaction with other kainate receptors, GLU_{K6}, GLU_{K7}, or GLU_{K2} (Bleakman et al. 1999; Alt et al. 2004), also has affinity for $GLU_{A1\text{-}4}$ (AMPA) as fully documented here (see also Bleakman et al. 1999). It is also important to recognize that the affinities and selectivities of LY293558 have largely been defined in systems using recombinant receptors, which may have important differences from those in native tissues. For instance, while the affinity of LY293558 for AMPA receptor subunits was measured using homomeric AMPA receptors in the current study, natively expressed AMPA receptors are likely to form heteromeric channel assemblies (Mansour et al. 2001; Brorson et al. 2004). Work to further define the mechanism of action of LY293558 in vivo, along with investigation of the effects of compounds with greater selectivity for GLU_{K5}, will be necessary to determine the potential utility of GLU_{K5} antagonists as anxiolytic compounds.

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References

Alt A, Weiss B, Ogden AM, Knauss JL, Oler J, Ho K, Large TH, Bleakman D (2004) Pharmacological characterization of glutamatergic agonists and antagonists at recombinant human homomeric and heteromeric kainate receptors in vitro. Neuropharmacology 46:793–806

Bainbridge JG (1968) The effect of psychotropic drugs on food reinforced behavior and on food consumption. Psychopharmacologia 12:204–213

Barrett JE, Witkin JM (1991) Buspirone in animal models of anxiety. In: Tunnicliff G, Eison AS Taylor DP (eds) Buspirone: mechanisms and clinical aspects. Academic, New York, pp 37–79

Benvenga MJ, Ornstein PL, Leander JD (1995) Schedule-controlled behavioral effects of the selective 2-amino-3-(5-methyl-3-hydroxyisoxazol-4-yl)propanoic acid antagonist LY293558 in pigeons. J Pharmacol Exp Ther 175:164–170

Blackburn-Munro G, Bomholt SF, Erichsen HK (2004) Behavioral effects of the novel AMPA/GluR5 selective receptor antagonist NS1209 after systemic administration in animal models of experimental pain. Neuropharmacology 47:351–362

Bleakman D, Schoepp DD, Ballyk B, Bufton H, Sharpe EF, Thomas K, Ornstein PL, Kamboj RK (1996) Pharmacological discrimination of GluR5 and GluR6 kainate receptor subtypes by (3*S*, 4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazole-5-yl)ethyl]decahydroisdoquin oline-3 carboxylic-acid. Mol Pharmacol 49:581–585

Bleakman D, Ogden AM, Ornstein PL, Hoo K (1999) Pharmacological characterization of a GluR6 receptor in cultured hippocampal neurons. Eur J Pharmacol 378:331–337

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented neurobasal, a new serum-free medium combination. J Neurosci Res 35:567–576

Brorson JR, Li D, Suzuki T (2004) Selective expression of heteromeric AMPA receptors driven by flip-flop differences. J Neurosci 24:3461–3470

Cheng YC, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Chojnacka-Wojcik E, Klodzinska A, Pilc A (2001) Glutamate receptor ligands as anxiolytics. Curr Opin Investig Drugs 2:1112–1119

Clineschmidt BV, Martin GE, Bunting PR, Papp NL (1982) Central sympathomimetic activity of (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), a substance with potent anticonvulsant, central sympathomimetic, and apparent anxiolytic properties. Drug Dev Res 2:135–145

Cook L, Davidson A (1973) Effects of behaviorally-active drugs in a conflict–punishment procedure in rats. In: Garratini S, Mussini E, Randall LO (eds) The benzodiazepines. Raven, New York, pp 327–345

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

Geller I (1964) Relative potencies of benzodiazepines as measured by their effects on conflict behavior. Arch Int Pharmacodyn Ther 149:243–247

Geter-Douglass B, Witkin JM (1999) Behavioral effects and anticonvulsant efficacies of low-affinity, uncompetitive NMDA antagonists in mice. Psychopharmacology 146: 280–289

Gilron I, Max MB, Lee G, Booher SL, Sang CN, Chappell AS, Dionne RA (2000) Effects of the 2-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid/kainate antagonist LY293558 on spontaneous and evoked postoperative pain. Clin Pharmacol Ther 68:320–327

Ginski MJ, Witkin JM (1994) Sensitive and rapid behavioral differentiation of *N*-methyl-D-aspartate receptor antagonists. Psychopharmacology 114:573–582

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) An improved patch-clamp technique for high-resolution current recordings from cell and cell-free membrane patches. Pflügers Arch 391:85–100

Huckle R (2004) Pregabalin (Pfizer). Curr Opin Investig Drugs 5:82–89

Karcz-Kubicha M, Liljequist S (1995) Evidence for an anxiogenic action of AMPA receptor antagonists in the plus-maze test. Eur J Pharmacol 279:171–177

Kelleher RT, Morse WH (1968) Determinants of the specificity of the behavioral effects of drugs. Ergeb Physiol 60:1–56

Kent JM, Mathew SJ, Gorman JM (2002) Molecular targets in the treatment of anxiety. Biol Psychiatry 52:1008–1030

Ko S, Zhao M-G, Toyoda H, Qiu C-S, Zhuo M (2005) Altered behavioral responses to noxious stimuli and fear in glutamate receptor 5 (GluR5)- or GluR6-deficient mice. J Neurosci 25:977–984

Kotlinska J, Liljequist S (1998) The putative AMPA receptor antagonist, LY326325, produces anxiolytic-like effects without altering locomotor activity in rats. Pharmacol Biochem Behav 60:119–124

Krystal JH, D'Souza DC, Petrakis IL, Belger A, Berman RM, Charney DS, Abi-Saab W, Madonick S (1999) NMDA agonists and as probes of glutamatergic dysfunction and pharmacotherapies in neuropsychiatric disorders. Harv Rev Psychiatry 7: 125–143

Leander JD, Lawson RR, Ornstein PL, Zimmerman DM (1988) *N*-Methyl-D-aspartatic acid-induced lethality in mice: selective antagonism by phencyclidine-like drugs. Brain Res 448: 115–120

Li H, Chen A, Xing G, Wei M-L, Rogawski MA (2001) Kainate receptor-mediated heterosynaptic facilitation in the amygdala. Nat Neurosci 4:612–620

Lodge D, Schoepp DD (1997) (3*S*,4a*R*,6*R*,8a*R*)-6-[2-(1(2)*H*-Tetrazole-5-yl) ethyl]decahydroisoquinoline-3-carboxylic acid (LY293558) and its racemate (LY215490): a selective and competitive AMPA/kainate receptor antagonist. In: Herling P (ed) Excitatory amino acids: clinical results with antagonists. Academic, San Diego, pp 81–87

Mansour M, Nagarajan N, Nehring RB, Clements JD, Rosenmund C (2001) Heteromeric AMPA receptors assemble with a preferred subunit stoichiometry and spatial arrangement. Neuron 32: 841–853

Martínez G, Ropero C, Funes A, Flores E, Blotta C, Landa AI, Gargiulo PA (2002) Effects of selective NMDA and non-NMDA blockade in the nucleus accumbens on the plus-maze test. Physiol Behav 76:219–224

Moghaddam B (2002) Stress activation of glutamate neurotransmission in the prefrontal cortex: implications for dopamine-associated psychiatric disorders. Biol Psychiatry 51:775–787

Monn JA, Thurkauf A, Mattson MV, Jacobsen AE, Rice KC (1990) Synthesis and structure–activity relationship of C5-substituted analogues of (±)-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine[(±)-desmethyl-MK801]: ligands for the NMDA receptor-coupled phencyclidine binding site. J Med Chem 33:1069–1076

Ornstein PL, Arnold MB, Augenstein NK, Lodge D, Leander JD, and Schoepp DD (1993) 3*SR*,4a*RS*,6*RS*,8a*RS*)-6-[2-(1*H*-Tetrazol-5-yl)ethyl]decahydroisoquinoline-3-carboxylic acid: a structurally novel, systemically active, competitive AMPA receptor antagonist. J Med Chem 36:2046–2048

Palecek J, Neugebauer V, Carlton SM, Iyengar S, Willis WD (2004) The effect of a kainate GluR5 receptor antagonist on responses of spinothalamic tract neurons in a model of peripheral neuropathy in primates. Pain 111:151–161

Parsons CG, Danysz W, Quack G (1998) Glutamate in CNS disorders as a target for drug development: an update. Drug News Perspect 11:523–569

Ploghaus A, Narain C, Beckmanhn CF, Clare S, Bantick S, Wise R, Matthews PM, Rawlins JNP, Tracey I (2001) Exacerbation of pain by anxiety is associated with activity in a hippocampal network. J Neurosci 21:9896–9903

Popik P, Layer RT, Skolnick P (1994) The putative anti-addictive drug ibogaine is a competitive inhibitor of [3H]MK-801 binding to the NMDA receptor complex. Psychopharmacology 114:672–674

Procter MJ, Houghton AK, Faber ES, Chizh BA, Ornstein PL, Lodge D, Headley PM (1998) Actions of kainate and AMPA selective glutamate receptor ligands on nociceptive processing in the spinal cord. Neuropharmacology 37:1287–1297

Schoepp DD, Lodge D, Bleakman D, Leander JD, Tizzano JP, Wright RA, Palmer AJ, Salhoff CR, Ornstein PL (1995) In vitro and in vivo antagonism of AMPA receptor activation by (3*S*,4a*R*, 6*R*,8a*R*)-6-[2-(1(2)*H*-tetrazole-5-yl)ethyl] decahydroisoquinoline-3-carboxylic acid. Neuropharmacology 34: 1159–1168

Sheftell FD, Atlas SJ (2002) Migraine and psychiatric comorbidity: from theory and hypotheses to clinical application. Headache 42:934–944

Simmons RMA, Li DL, Ho KH, Deverill M, Ornstein PL, Iyengar S (1998) Kainate GluR5 receptor subtype mediates the nociceptive response to formalin in the rat. Neuropharmacology 37: 25–36

Skolnick P (2002) Modulation of glutamate receptors: strategies for the development of novel antidepressants. Amino Acids 23:153–159

Swedberg MD, Jocobsen P, Honore T (1995) Anticonvulsant, anxiolytic and discriminative effects of the AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX). J Pharmacol Exp Ther 274:1113–1121

Vanover KE (1998) Effects of AMPA receptor antagonists on dopamine-mediated behaviors in mice. Pyschopharmacology 136:123–131

Wiley JL, Porter JH, Compton AD, Balster RL (1992) Antipunishment effects of acute and repeated administration of phencyclidine and NPC 12626 in rats. Life Sci 50:1519–1528

Williams M, Risley EA (1979) Characterization of the binding of [^{3}H]muscimol, a potent g-aminobutyric acid agonist, to rat brain synaptosomal membranes using a filtration assay. J Neurochem 32:713–718

Witkin JM (1995) Role of *N*-methyl-D-aspartate receptors in behavior and behavioral effects of drugs. In: Stone TW (ed) CNS neurotransmitters and neuromodulators, vol 1. Glutamate. CRC, Boca Raton, pp 323–350

Witkin JM, Katz JL (1990) Analysis of behavioral effects of drugs. Drug Dev Res 20:389–409

Witkin JM, Leander JD (1982) Effects of the appetite stimulant chlordimeform on food and water consumption or rats: comparison with chlordiazepoxide. J Pharmacol Exp Ther 223:130–134

Youn D-H, Randic M (2004) Modulation of excitatory synaptic transmission in the spinal substantia gelatinosa of mice deficient in the kainate receptor GluR5 and/or GluR6 subunit. J Physiol 555:683–698