Neuron, Vol. 10, 51-59, January, 1993, Copyright © 1993 by Cell Press

GYKI 52466, a 2,3-Benzodiazepine, Is a Highly Selective, Noncompetitive Antagonist of AMPA/Kainate Receptor Responses

Sean D. Donevan and Michael A. Rogawski
Neuronal Excitability Section
Epilepsy Research Branch
National Institute of Neurological Disorders and Stroke
National Institutes of Health
Bethesda, Maryland 20892

Summary

In whole-cell voltage-clamp recordings from cultured rat hippocampal neurons, the 2,3-benzodiazepine GYKI 52466 was a potent antagonist of kainate- and AMPA-activated currents (IC_{50} values, 7.5 and 11 μM, respectively), but was inactive against N-methyl-D-aspartate (NMDA) or γ-aminobutyric acid responses. The block produced by GYKI 52466 occurred in a noncompetitive fashion, was voltage independent, and failed to show use dependence, indicating an allosteric blocking mechanism. In kinetic experiments with kainate as the agonist, the GYKI 52466 binding and unbinding rates were 1.6×10^5 M^{-1} s^{-1} and 3.2 s^{-1}, respectively. GYKI 52466 also suppressed non-NMDA receptor-mediated spontaneous synaptic currents via a postsynaptic action. Noncompetitive AMPA/kainate antagonists such as GYKI 52466 could offer advantages over competitive antagonists in the treatment of glutamate-associated neurological disorders, particularly under conditions in which high levels of the amino acid would render the competitive antagonists relatively ineffective. Moreover, the results demonstrate the existence of a novel recognition site for an atypical benzodiazepine on non-NMDA receptors.

Introduction

Excessive activation of glutamate receptors is believed to play a role in the pathogenesis of a diverse group of neurological disorders including epilepsy and various forms of central nervous system injury and degeneration (Rothman and Olney, 1986; Choi, 1988). Consequently, antagonists of glutamate-induced neuronal excitation could be useful therapeutically in these disorders (Meldrum and Garthwaite, 1990). Although there has been intense interest in N-methyl-D-aspartate (NMDA) receptor antagonists as antiepileptic and neuroprotective agents, non-NMDA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate [AMPA]/kainate) receptor blockers also appear to protect against certain forms of epileptic activity (Gean, 1990; Hwa and Avoli, 1991; Smith et al., 1991; Chapman et al., 1991; Turski et al., 1992) and glutamate-mediated neurotoxicity (Sheardown et al., 1990; Koh et al., 1990; Buchan et al., 1991; Le Peillet et al., 1992; Nellgård and Wieloch, 1992) and may be of benefit in Parkinson's disease (Klockgether et al., 1991). Moreover, under some circumstances, blockade of both NMDA and non-NMDA receptors may be necessary to prevent completely glutamate-induced cell destruction (Frandsen et al., 1989; Mosinger et al., 1991; Sucher et al., 1991; Kaku et al., 1991). Thus, increasing attention is being directed toward the therapeutic potential of non-NMDA receptor antagonists. Most recent studies have utilized quinoxalinediones (Honoré et al., 1988; Davies and Collingridge, 1990), such as NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline) (Sheardown et al., 1990), which are selective and potent competitive non-NMDA receptor antagonists. However, the 2,3-benzodiazepine muscle relaxant GYKI 52466 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine HCl] (Tarnawa et al., 1989) has also been reported to antagonize non-NMDA receptor responses (Tarnawa et al., 1990a; Ouardouz and Durand, 1991). In the present study, we demonstrate that GYKI 52466 is a highly selective non-NMDA antagonist that appears to act by a novel noncompetitive (allosteric) mechanism. Such allosteric antagonists could offer certain advantages over competitive blockers in the treatment of neurological disorders.

Results

Amino acids were applied to cultured rat hippocampal neurons under voltage clamp using a rapid perfusion system. As previously described (Tang et al., 1989; Trussell and Fischbach, 1989; Patneau and Mayer, 1991), AMPA evoked inward current responses that rapidly desensitized to a steady-state level, whereas kainate-induced currents showed little desensitization.

GYKI 52466 Block of AMPA and Kainate Currents

GYKI 52466 (0.3-100 μM) caused a concentration-dependent block of the sustained responses to 10 μM AMPA and 150 μM kainate (Figure 1); the IC_{50} values of the concentration-response isotherms were 7.5 ± 0.4 μM and 11 ± 1 μM, respectively. The peak response to AMPA was blocked to an equivalent degree as the steady-state response, and the drug had no effect on the rate of AMPA current desensitization (Figure 2; Table 1). Because solution exchange times for perfusion of whole neurons may be slower than the time course of AMPA current desensitizaton (Tang et al., 1989), we also examined the effects of GYKI 52466 on the desensitization of AMPA currents in excised outside-out patches brought in close proximity to the tip of the perfusion pipette. In this experimental configuration, the speed of solution exchange should approach the maximal exchange speed of the perfusion system (time constant, <1.5 ms; see Experimental

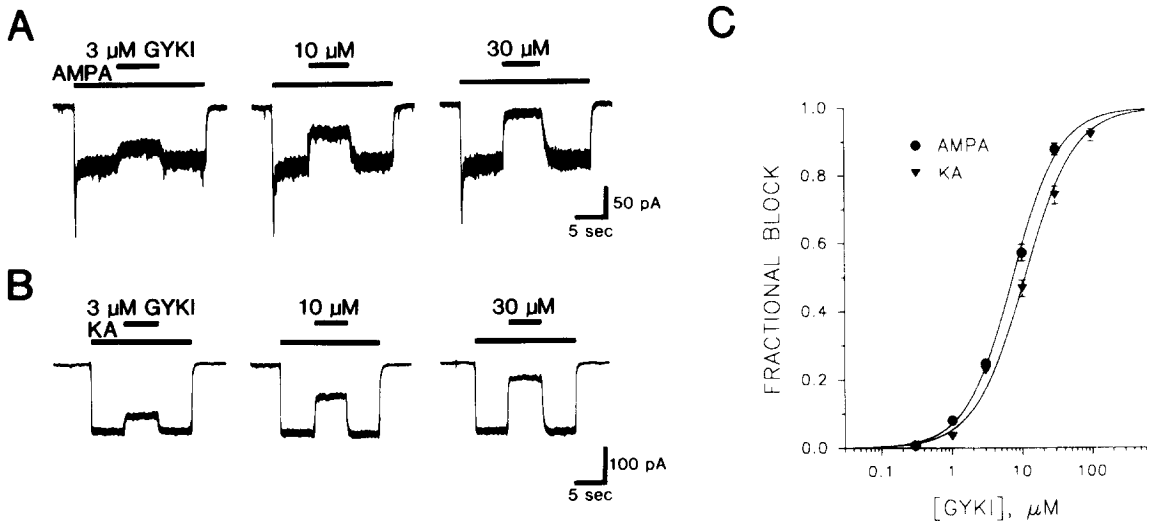

Figure 1. GYKI 52466 Inhibits Inward Currents Activated by AMPA and Kainate

Responses to 10 μM AMPA (A) and 150 μM kainate (B) were recorded in the absence and presence of various concentrations of GYKI 52466. The traces in (A) and (B) are from different neurons. (C) Concentration-response relationships for block of steady-state AMPA and kainate currents. Fractional block values were calculated according to the formula $B = 1 - I_{GYKI}/I$, where I is the steady-state current evoked by the agonist and I_{GYKI} is the current evoked by the agonist in the presence of GYKI 52466. Data points indicate the mean ± SEM of the fractional block values from 5 experiments with AMPA and 13 experiments with kainate; the curves indicate the nonlinear best fits according to the logistical function $1/[1 + (IC_{50}/[GYKI])^{n_H}]$, where [GYKI] is the concentration of GYKI 52466, IC_{50} is the 50% blocking concentration, and n_H is the slope factor (1.2–1.3).

Procedures). As illustrated in Figure 3, 10 μM GYKI 52466 substantially reduced the amplitude of the patch current, but did not alter its time course (see also Table 1, which summarizes data from seven patches). The desensitization time constants obtained in the patch recordings were within the range of those reported previously by others (Tang et al., 1989; Trussell and Fischbach, 1989), as was also the case with the time constants for desensitization of the whole-cell currents (Mayer and Vyklicky, 1989). The difference in the desensitization rates in the whole-cell and excised patch experiments may be attributable to nonuniformity of the whole-cell perfusion (Mayer and Vyklicky, 1989) and to the presence of two components to the decay of the current (Trussell and Fischbach, 1989), the faster of which would not be detected in the whole-cell experiments.

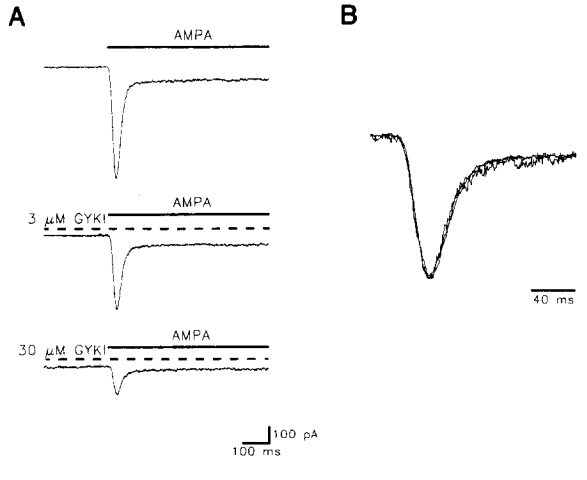

Figure 2. GYKI 52466 Fails to Alter the Rate of Desensitization of AMPA-Evoked Whole-Cell Currents

(A) Initial desensitizing response at the onset of 100 μM AMPA under control conditions and in the presence of 3 and 30 μM GYKI 52466. In (B), the initial current transients have been scaled to demonstrate that the residual current in the presence of GYKI 52466 has a similar time course to the control current.

Pharmacology of the GYKI 52466 Blocking Site

Unlike GYKI 52466, the 1,4-benzodiazepine diazepam failed to affect kainate responses (fractional block <0.03 with 0.1, 1, 10, and 100 μM diazepam; n = 5 for each concentration). Moreover, the benzodiazepine receptor antagonist flumazenil (RO 15-1788) did not alter the blocking action of 30 μM GYKI 52466 (fractional block 0.76 ± 0.03 in the absence of flumazenil and 0.76 ± 0.03, 0.75 ± 0.03, and 0.74 ± 0.03, respectively, in the presence of 1, 10, and 100 μM flumazenil; n = 5).

In contrast to the potent blocking action of GYKI 52466 against kainate and AMPA responses, the drug had a negligible effect on currents evoked by NMDA (Figures 4A and 4B). Moreover, in experiments using single-cell microspectrofluorimetry with the Ca^{2+} indicator dye fura-2, 50 μM GYKI 52466 had no effect on elevations in intracellular Ca^{2+} induced by 200 μM trans-1-amino-cyclopentyl-1,3-dicarboxylic acid, a specific agonist of metabotropic glutamate receptors (S. D. Donevan, R. P. Irwin, and M. A. Rogawski, unpub-

Table 1. Effects of GYKI 52466 on AMPA Response Amplitude and Desensitization Rate

	Concentration (μM)	Fractional Block		τ (ms)
		Peak	Steady State	
Whole cell	0			26.8 ± 2.0
	3	0.28 ± 0.04	0.24 ± 0.01	26.9 ± 1.8
	30	0.85 ± 0.02	0.88 ± 0.02	27.2 ± 3.1
Excised patch	0			7.5 ± 1.2
	10	0.64 ± 0.06		7.1 ± 1.4

Peak response fractional block and decay time constants (τ) were determined from currents evoked with 100 μM AMPA; steady-state fractional block was determined in whole-cell recordings with responses to 10 μM AMPA. Fractional block was calculated according to the formula given in the legend to Figure 1. The time constants were derived from the best single exponential fits to the falling phase of the transient current response (see Figure 2). In the excised patch experiments, currents from 10–15 AMPA applications were averaged. The values shown are the means ± SEM of data from six whole-cell and seven excised patch experiments. Control peak and steady-state whole-cell currents were 917 ± 136 pA and 175 ± 48 pA. Control peak average patch currents were 18 ± 4 pA. Channel openings following the fast desensitizing current response in the patch experiments were too infrequent to allow slowly desensitizing or steady-state current to be quantitated.

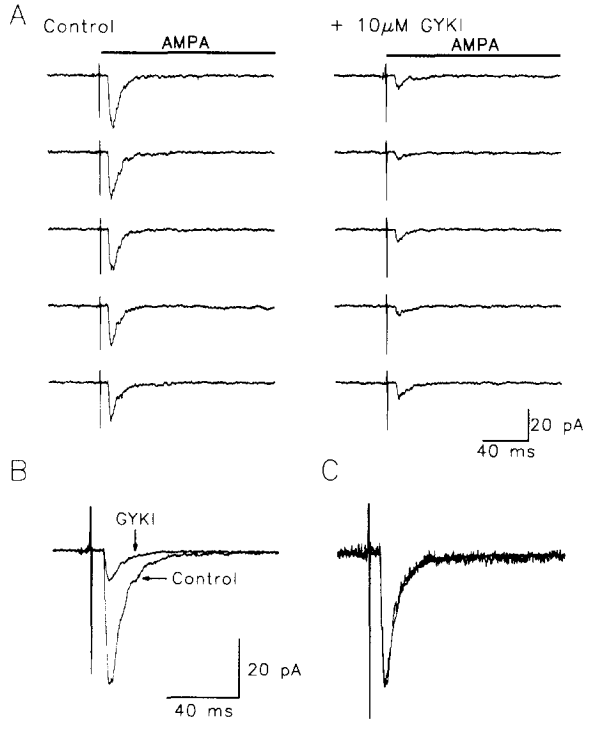

Figure 3. GYKI 52466 Blocks AMPA Current in Excised Outside-Out Patches

(A) Inward currents evoked by 100 μM AMPA in the absence and presence of 10 μM GYKI 52466. In (B), ensemble averages of ten successive control and GYKI 52466-attenuated currents are superimposed. The time course of the patch current was unaffected by GYKI 52466, as demonstrated in (C), where the peak of the GYKI 52466-attenuated current from (B) is shown scaled to match the peak of the control current. In this experiment, there is a 6.5 ms delay between valve actuation (marked by artifact) and onset of the solution change.

lished data). We also investigated whether GYKI 52466 would, like other benzodiazepines, potentiate γ-aminobutyric acid (GABA)-activated Cl^- current responses. GABA-evoked currents in the presence of 1, 10, and 50 μM GYKI 52466 were, respectively, 1.00 ± 0.02 (n = 4), 1.02 ± 0.04 (n = 5), and 0.95 ± 0.03 (n = 5) of control, indicating that the drug is essentially inactive as a modulator of $GABA_A$ receptor responses.

GYKI 52466 Is a Noncompetitive Antagonist

To investigate the mechanism of block by GYKI 52466, we characterized its effect on the concentration-response relationship for kainate. As illustrated in Figure 5A, 10 and 30 μM GYKI 52466 caused a concentration-dependent reduction in the maximal

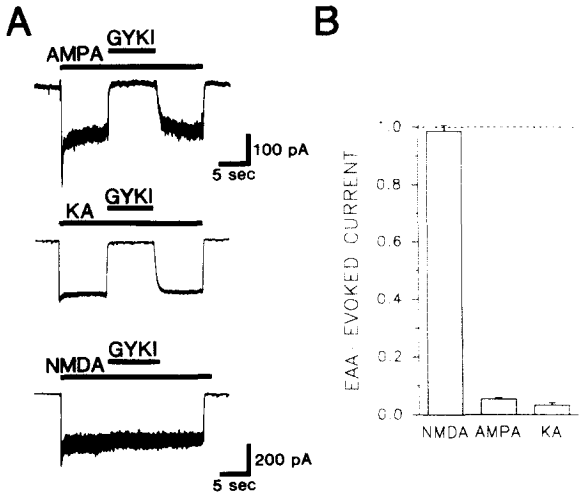

Figure 4. GYKI 52466 Selectively Blocks AMPA- and Kainate- but Not NMDA-Activated Currents

(A) Comparison of the effect of 50 μM GYKI 52466 on inward currents evoked by 10 μM AMPA, 100 μM kainate (KA), and 10 μM NMDA (+ 10 μM glycine). Top two traces are from the same neuron. (B) Fractional current values (normalized to control) for experiments similar to those illustrated in (A) (AMPA, n = 4; KA, n = 4; NMDA, n = 7). In experiments examining the response to NMDA, Mg^{2+} and MK-801 were omitted from the bathing solutions, 10 μM glycine was added to saturate fully its site on the NMDA receptor, and Ca^{2+} was lowered to 0.1 mM to prevent Ca^{2+}-induced desensitization.

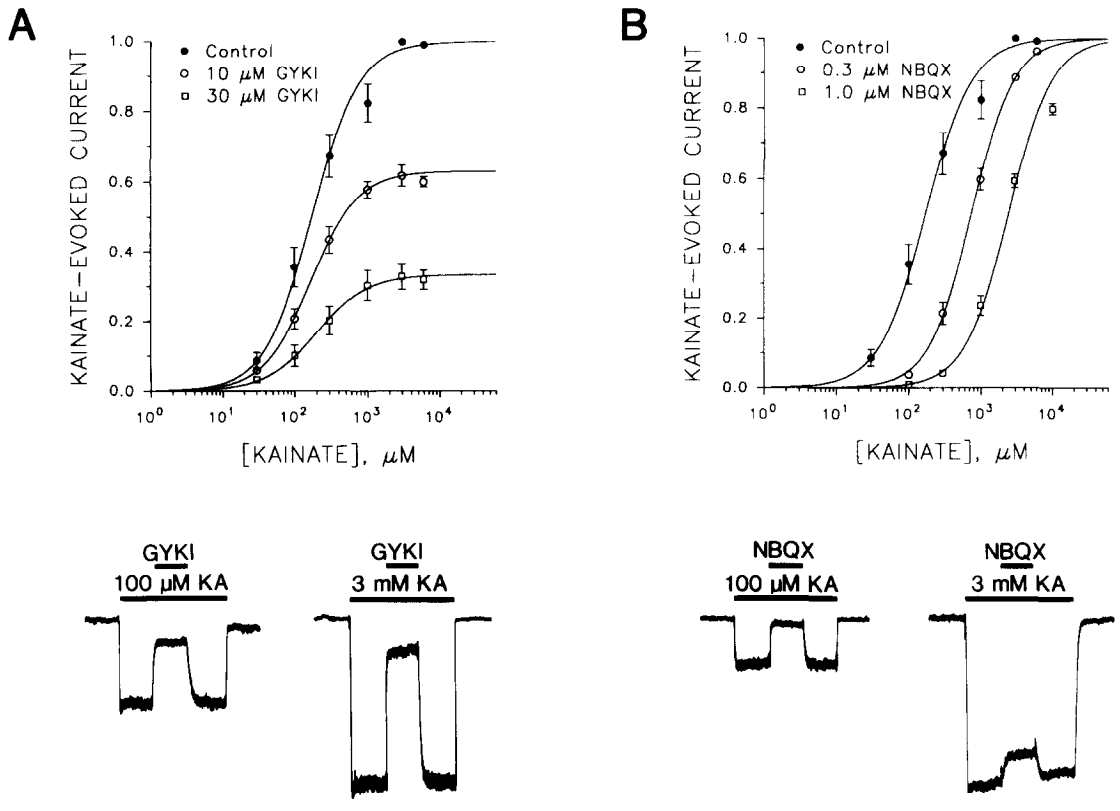

Figure 5. Comparision of the Effects of GYKI 52466 and NBQX on Kainate Concentration-Response Curves

(A) Concentration-response relationships for kainate (30 μM to 6 mM) under control conditions and in the presence of 10 and 30 μM GYKI 54266. For each cell, the data were normalized to the current evoked by a saturating concentration of kainate (3 mM) in the absence of antagonists. Points indicate the mean ± SEM of the peak current amplitude values from five to six cells; the curves indicate the best fits to the data according to the logistical function $1/[1 + (EC_{50}/[KA])^{n_H}]$, where EC_{50} is the concentration causing a 50% maximal response and n_H is the slope factor (1.3–1.5). The representative current traces shown below the graph illustrate a similar fractional block by 30 μM GYKI 52466 for low and high concentrations of kainate; both traces are from the same neuron. (B) A similar series of experiments to that of (A), except that 300 nM and 1 μM NBQX were used. The control data from (A) are repeated for comparison. The representative traces shown below (both from the same neuron) demonstrate the ability of high concentrations of kainate to overcome the 300 μM NBQX block. Each data point represents the results of experiments with five to six cells.

current; however, the EC_{50} values in the presence of drug (169 ± 2 μM, n = 6 and 202 ± 18 μM, n = 6, respectively) were similar to the control value of 173 ± 16 μM (n = 5). In contrast, 0.3 and 1 μM NBQX caused a concentration-dependent rightward shift in the concentration-response curve with no change in the maximal response amplitude (Figure 5B); the EC_{50} values for kainate in the presence of 0.3 and 1 μM NBQX were 749 ± 15 μM (n = 5) and 2450 ± 238 μM (n = 5), respectively. Thus, unlike NBQX, which interacts competitively at the kainate-binding site, GYKI 52466 blocks kainate responses by a noncompetitive mechanism.

Voltage Dependence and Kinetics of GYKI 52466 Block

Barbiturates and certain toxins may block non-NMDA receptor responses by an open channel-type noncompetitive mechanism (Miljkovic and MacDonald, 1986; Akaike et al., 1987; Jones et al., 1990). To investigate the possibility that GYKI 52466 acts in a similar fashion, we first characterized the voltage dependence of the block. Figure 6A shows the mean kainate-(100 μM) evoked current (normalized to control at −60 mV) for four cells at various holding potentials, in the presence and absence of 10 μM GYKI 52466. The current-voltage relationships under both conditions were linear, indicating that the GYKI 52466 block is voltage independent. Furthermore, as shown in Figure 6B, the rates of block and unblock of kainate-evoked currents by GYKI 52466 (30 μM) were similar at positive and negative potentials. These results suggest that GYKI 52466, a cation at physiological pH, is not an open-channel blocker. Nevertheless, it is conceivable that the drug binds to a site in the channel that is located superficially or outside the transmembrane electric field. To rule out further an open-channel blocking mechanism, we determined whether the drug could block closed channels. With 30 μM GYKI 52466, the block of kainate-evoked current developed over several hundred milliseconds, and recovery occurred with a similar time course (Fig-

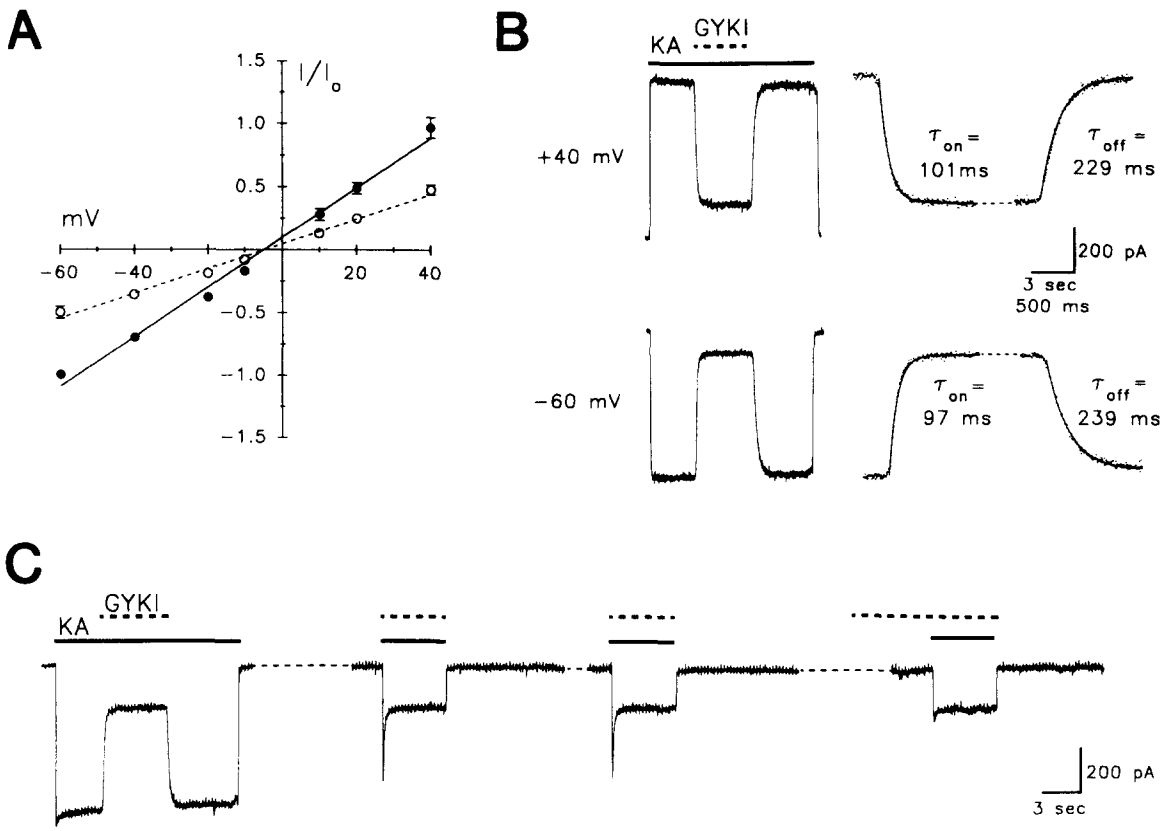

Figure 6. The GYKI 52466 Block Is Voltage Independent and Does Not Require Open Channels

(A) Current-voltage curves of 100 μM kainate responses in the absence (closed circles) and presence (open circles) of 10 μM GYKI 52466 (n = 4) demonstrate the lack of voltage dependence of the GYKI 52466 block. Currents were normalized to the control current at −60 mV. (B) The rates of onset and recovery from block are voltage independent (100 μM kainate and 30 μM GYKI 52466). Traces to the right show the onset and recovery on an expanded time scale; the best single exponential fits to the raw data are shown by the smooth curves (time constants as noted). All traces are from the same neuron. (C) The onset and recovery from block of kainate-evoked currents by GYKI 52466 are not use dependent. As shown in left and middle two traces, the block of the 100 μM kainate-evoked current by 30 μM GYKI 52466 developed slowly over several hundred milliseconds. (In the middle two traces, kainate and GYKI 52466 were applied simultaneously; the interval between the two traces was 15 s.) Pretreatment with GYKI 52466 prevented the slow onset of the block (right trace), indicating that the drug can block closed channels. (The slight inactivation of the kainate response in the right trace is similar to that observed with kainate alone in the left trace.) Similarly, recovery from block occurred in the absence of agonist, such that the current responses to consecutive applications of GYKI with kainate were identical (middle two traces). These results were replicated in two other neurons.

ures 6B and 6C). However, the slow onset and recovery from block appeared not to be due to a use-dependent action of the drug because in recordings in which the GYKI 52466 application was begun prior to the kainate perfusion, the current was fully blocked at the onset of the kainate perfusion (Figure 6C, last trace). Moreover, as illustrated in the middle two traces of Figure 6C, the response to the second of two consecutive coapplications of kainate plus GYKI 52466 was identical to the first, indicating that trapping of GYKI 52466 in closed channels does not occur.

We used the slow onset and recovery of block to determine the rate constants for binding and unbinding of GYKI 52466. The apparent rate (k_{app}) for onset of block was taken as the reciprocal of the time constant of the best single exponential fit to the trajectory of the current at the initiation of GYKI 52466 perfusion (see Figure 6B, lower panel). As illustrated in Figure 7, k_{app} increased in a linear fashion with GYKI 52466 concentration. Assuming a 1:1 bimolecular binding reaction between the drug and its acceptor site, $k_{app} = k_1[GYKI] + k_{-1}$, where [GYKI] is the concentration of GYKI 52466 and k_1 and k_{-1} are the forward and reverse rate constants, respectively. The k_1 and k_{-1} values determined from the best fit straight line to the k_{app} values of Figure 7 were $1.6 \times 10^5\ M^{-1}\ s^{-1}$ and $3.2\ s^{-1}$, respectively. The kinetically determined dissociation constant $K_D = k_{-1}/k_1$ was 19 μM, which compares favorably with the IC_{50} value determined from the concentration-response isotherm (Figure 1). Figure 7 also plots the off rates determined from the best single exponential fits to the time courses for recovery of kainate responses upon termination of the GYKI 52466 perfusion. The best straight line fit to these data was not significantly different from a flat line, indicating the expected lack of concentration dependence of

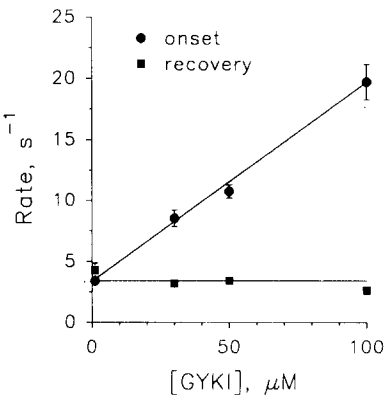

Figure 7. Concentration Dependence of the GYKI 52466 Blocking and Unblocking Rates

Various concentrations of GYKI 52466 were applied during continuous perfusion with 100 μM kainate as in the experiment of Figure 6B (bottom). Apparent rates were calculated as the reciprocal of the time constants of best single exponential fits. Each point represents the mean ± SEM of data from six to eight cells (time constants from one to four drug applications per cell were averaged). The slope and intercept values of the best fit straight line to the onset rate data were 1.6 ± 0.1 × 10^5 M^{-1} s^{-1} and 3.2 ± 0.4 s^{-1}. The slopes of the onset and recovery rate fitted lines were significantly different by the test for parallelism ($t = 19.5$; $p < 0.01$). The 95% confidence limits for the slope of the fit to the recovery rate values spanned 0, indicating that the fit is not significantly different from a flat line. The mean of the values at the different concentrations was 3.4 ± 0.3 s^{-1}.

the unbinding rates. The mean of the values at the different concentrations was 3.4 s^{-1}. This value closely matches the value determined from extrapolation of the fit to the k_{app} data.

GYKI 52466 Attenuates Spontaneous Excitatory Postsynaptic Currents

In the absence of tetrodotoxin, spontaneous synaptic currents are recorded in a high percentage of cultured hippocampal neurons. Fast non-NMDA receptor-mediated synaptic currents were isolated by inclusion of bicuculline, which eliminates $GABA_A$ receptor-mediated inhibitory synaptic currents, and by the MK-801 and Mg^{2+} normally present in the bath solution (see Experimental Procedures), which minimize the contribution of NMDA receptor currents to the excitatory postsynaptic currents (EPSCs) (see Tang et al., 1991; Vyklicky et al., 1991). As illustrated in Figure 8A, 10 μM GYKI 52466 caused a reversible reduction in the amplitude of spontaneous EPSCs recorded under these conditions. In four separate experiments, the mean EPSC amplitude was decreased by 56% ± 5% (Figure 8C, top). Decay rates of the EPSCs were determined by fitting the falling phase of the synaptic current to an exponential function. Despite the marked depression in the size of EPSCs, decay rates were unaffected (Figure 8B). In experiments with four patches in which ten EPSCs were analyzed per patch, the control decay rate was 2.08 ± 0.03 ms, whereas in the presence of 10 μM GYKI 52466 the decay rate was 2.13 ± 0.10 ms. We used the coefficient of variation (CV) method to confirm that the reduction in EPSC amplitude produced by GYKI 52466 occurred at a postsynaptic site of action. CV values (calculated according to the formula given in the legend to Figure 8) were not significantly affected by GYKI 52466, indicating that it predominantly suppresses EPSCs by affecting postsynaptic responsiveness without altering synaptic transmitter release (Figure 8C).

Discussion

These results demonstrate that GYKI 52466 selectively inhibits AMPA/kainate receptor responses by an allosteric blocking mechanism. GYKI 52466 is the first noncompetitive non-NMDA antagonist to be described that is inactive against NMDA responses, and it is therefore a potentially important pharmacological tool. Moreover, GYKI 52466 appears to act via a novel blocking mechanism that is distinct from the open-channel block produced by other noncompetitive non-NMDA antagonists that have been examined to date (Miljkovic and MacDonald, 1986; Akaike et al., 1987; Jones et al., 1990). Since GYKI 52466 was equally effective in whole-cell and excised patch recordings, a cytoplasmic second messenger is unlikely to mediate the blocking effect of the drug. Rather, block presumably takes place by drug binding to the AMPA/kainate receptor-channel or to a closely associated molecule. The lack of voltage dependence suggests that the GYKI 52466 acceptor site is largely outside the transmembrane electric field.

Modification of desenzitization is one means by which AMPA/kainate receptor responses can be altered by pharmacological agents (Tang et al., 1991; Vyklicky et al., 1991; Isaacson and Nicoll, 1991). This mechanism cannot be implicated in the GYKI 52466 block of AMPA currents, since the drug had no effect on the rate of AMPA current desensitization in either whole-cell or patch recordings. In the case of kainate, the slow onset of the GYKI 52466 block (Figures 6B and 6C) could be interpreted as indicating that the drug induces desensitization. However, several observations strongly argue against this notion. First, when GYKI 52466 was allowed time to equilibrate before the kainate application (Figure 6C, final coapplication), there was negligible decay of the current, indicating that the drug can bind and block in the absence of agonist, i.e., to nondesensitized channels. Second, the linear increase in decay rate with increasing GYKI 52466 concentration (Figure 7) is more consistent with a blocking model in which drug-bound channels are nonconducting than with a model in which drug binding induces desensitization. Finally, the time course of fast EPSCs, which may in part be determined by desensitization (Trussell and Fischbach, 1989; Tang et al., 1991; Isaacson and Nicoll, 1991), was unaffected by GYKI 52466.

Studies of spontaneous synaptic currents con-

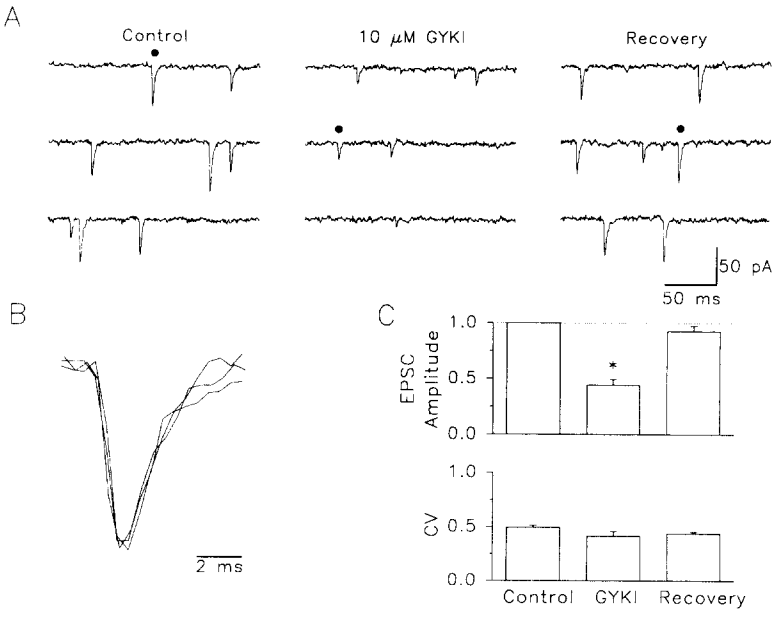

Figure 8. GYKI 52466 Depresses Spontaneous Excitatory Synaptic Currents by a Postsynaptic Action

Whole-cell voltage-clamp recordings were carried out in tetrodotoxin-free bath solution containing 5 μM bicuculline, 500 nM MK-801, and 1 mM Mg^{2+}. (A) Representative EPSCs before, during, and 10 sec after perfusion with 10 μM GYKI 52466. (B) The synaptic currents marked by closed circles in (A) are shown scaled to similar peak amplitudes to demonstrate that GYKI 52466 does not affect the time course of the synaptic responses. (C) Summary of data from four experiments including the one shown in (A). In each experiment, EPSCs were acquired in three epochs before, during, and after drug application; an average of 195 events were acquired in each epoch. The mean amplitudes of the drug-attenuated and recovery EPSCs normalized to the mean control amplitudes are plotted in the upper bar chart. Paired comparisons of the GYKI 52466 mean amplitude values with the control or recovery values indicated significance at the $p < 0.05$ level (Wilcoxin signed rank test). The CV was calculated according to $CV = \sqrt{\sigma^2 - \sigma_n^2}/\mu$, where σ^2 and μ are the variance and mean of the EPSC amplitude data sets and σ_n^2 is the variance of the noise of the corresponding epoch. The means ± SEM of the CV values are shown in the lower bar chart. In paired comparisons, the GYKI 52466 CV values were not significantly different from the control or recovery values.

firmed that GYKI 52466 can block the response of non-NMDA receptors to synaptically released transmitter. The CV technique was used to determine whether a presynaptic action of GYKI 52466 contributes to the observed depression in the EPSC amplitude (Martin, 1966). Although usually applied to synaptic potential distributions that conform to the assumptions of a Poisson model, the CV is a useful statistical measure even in situations in which quantal release deviates from these assumptions, as may be the case for central neuron synapses (Clements, 1990). Our failure to observe a significant change in CV in the face of a profound reduction in EPSC amplitude indicates that the depression of synaptic currents by GYKI 52466 results predominantly from a postsynaptic action of the drug. This is supported by the close correspondence between the magnitude of the effects of the drug on EPSC amplitude and agonist-evoked currents. Recently Arvin et al. (1992) reported that GYKI 52466 attenuates the increase in extracellular glutamate induced by brain ischemia and proposed that the drug may inhibit glutamate release. Our results suggest that this does not occur by a presynaptic action on glutamate-containing nerve terminals.

The pharmacological specificity of the 2,3-benzodiazepine recognition site at which GYKI 52466 acts to block non-NMDA receptors is distinct from the benzodiazepine site associated with $GABA_A$ receptors. Thus, the $GABA_A$ receptor active 1,4-benzodiazepine diazepam failed to affect non-NMDA receptor responses, and the benzodiazepine antagonist flumazenil did not alter the GYKI 52466 block. Conversely, GYKI 52466 did not affect GABA responses, indicating that it does not have conventional benzodiazepine receptor agonist activity.

Since GYKI 52466 is orally active and has good blood brain barrier permeability, it or a related compound could be useful clinically. The concentrations of GYKI 52466 that significantly reduced currents evoked by kainate and AMPA in this study are similar to those found in the brain after the intraperitoneal administration of an anticonvulsant dose of the drug (Tarnawa et al., 1990b), suggesting that the anticonvulsant action of GYKI 52466 is mediated by its interaction with non-NMDA receptors. Noncompetitive blockers like GYKI 52466 could be particularly advantageous as therapeutic agents in pathological states associated with high levels of glutamate, as may occur during a seizure in which there is intense activity of glutamate synapses (Carlson et al., 1992), or during an ischemic event or other brain injury associated with massive extracellular glutamate release (Rothman and Olney, 1986). Under these conditions, competitive antagonists would be relatively less effective because their blocking action could be overcome by high levels of glutamate. Our results also raise the intriguing possibility that there may be an endogenous ligand that modulates the function of non-NMDA receptor-channels via its action at the GYKI 52466 site.

Experimental Procedures

•

Hippocampal neurons from 19-day-old Sprague-Dawley rat embryos were grown in primary culture as described previously (Donevan et al., 1992) and were used 7–12 days after plating. Recordings were carried out in a bathing solution containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 10 mM HEPES. The bathing

medium also contained 1 μM tetrodotoxin to block voltage-activated Na^+ channels, 1 μM strychnine to block glycine-activated Cl^- channels, and 500 nM MK-801 and 1 mM Mg^{2+} to prevent activation of NMDA channels. In recordings of spontaneous EPSCs, tetrodotoxin was omitted, and 5 μM bicuculline methiodide was added to suppress GABA-mediated synaptic currents. Whole-cell voltage-clamp recordings were obtained with an Axopatch 1C amplifier (Axon Instruments, Burlingame, CA) using patch electrodes (2–4 MΩ) filled with an intracellular solution containing 145 mM CsCl, 2 mM $MgCl_2$, 5 mM HEPES, 0.1 mM $CaCl_2$, and 1 mM EGTA. Unless otherwise noted the holding potential was −60 mV. All experiments were performed at room temperature (23°C). Membrane currents were filtered at 1 kHz (−3 dB; 4-pole, low pass Bessel filter), digitally sampled at 10 kHz, and acquired on a microcomputer using the pClamp (CLAMPEX) or Axotape software packages (Axon Instruments). NFIT (Island Products, Galveston, TX) was used for nonlinear curve fitting. Data are presented as the mean ± SEM; n is the number of cells or membrane patches tested.

The rapid perfusion system consisted of a seven barrel array of fine glass capillary tubes that emptied via a common ~200 μm diameter orifice in a conical-shaped tip pulled from glass theta tubing. The capillary tubes passed nearly to the end of the tip, which was divided into two compartments by the septum of the theta tubing. Flow through each barrel was gravity fed and was regulated by solenoid microvalves controlled by a microprocessor-based timing system (Donevan et al., 1992). One barrel contained bathing solution while the others contained the agonists and antagonists either alone or in combination. Only one valve was open at any one time, and bathing solution flowed continuously between drug applications. To minimize mixing, solution changes were made by switching between capillary tubes in opposite compartments of the solution delivery tip. After actuating the valves, there was a 4–9 ms delay before the solution change was detected at the orifice of the perfusion pipette. In estimating time constants, we accounted for the actual delay obtained in each experiment. The exchange time constant (determined from the current transient obtained when switching an open patch electrode between normal and 10-fold dilute bath solution) was 1–1.5 ms. GYKI 52466 was kindly provided by Dr. I. Tarnawa (Institute for Drug Research, Budapest, Hungary) and NBQX by Drs. T. Honoreí and L. Nordholm (Novo Nordisk A/S, Måløv, Denmark). AMPA was from Tocris Neuramin (Essex, England). MK-801 was from Research Biochemicals, Inc. (Natick, MA). Flumazenil was a gift of Dr. J. Crawley (National Institute of Mental Health, Bethesda, MD). All other drugs and chemicals were from Sigma Chemical Co. (St. Louis, MO). Stock solutions of drugs were prepared in distilled water and stored frozen at −20°C.

Acknowledgments

We thank Dr. B. Meldrum for bringing GYKI 52466 to our attention and Dr. C.-M. Tang for discussions.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received June 24, 1992; revised September 9, 1992.

References

Akaike, N., Kawai, N., Kiskin, N. I., Kljuchko, E. M., Krishtal, O. A., and Tsyndrenko, A. Y. (1987). Spider toxin blocks excitatory amino acid responses in isolated hippocampal pyramidal neurons. Neurosci. Lett. *79*, 326–330.

Arvin, B., Moncada, C., Le Peillet, E., Chapman, A., and Meldrum, B. S. (1992). GYKI 52466 blocks the increase in extracellular glutamate induced by ischaemia. Neuroreport *3*, 235–238.

Buchan, A. M., Li, H., Cho, S., and Pulsinelli, W. A. (1991). Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats. Neurosci. Lett. *132*, 255–258.

Carlson, H., Ronne-Engstrom, E., Ungerstedt, U., and Hillered, L. (1992). Seizure related elevations of extracellular amino acids in human focal epilepsy. Neurosci. Lett. *140*, 30–32.

Chapman, A. G., Smith, S. E., and Meldrum, B. S. (1991). The anticonvulsant effect of the non-NMDA antagonists, NBQX and GYKI 52466, in mice. Epilepsy Res. *9*, 92–96.

Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. Neuron *1*, 623–634.

Clements, J. D. (1990). A statistical tool for demonstrating a presynaptic site of action for a modulator of synaptic transmission. J. Neurosci. Meth. *31*, 75–88.

Davies, S. N., and Collingridge, G. L. (1990). Quinoxalinediones as excitatory amino acid antagonists in the vertebrate central nervous system. Int. Rev. Neurobiol. *32*, 281–303.

Donevan, S. D., Jones, S. M., and Rogawski, M. A. (1992). Arcaine blocks *N*-methyl-D-aspartate receptor responses by an open channel mechanism: whole-cell and single-channel recording studies in cultured hippocampal neurons. Mol. Pharmacol. *41*, 727–735.

Frandsen, A., Drejer, J., and Schousboe, A. (1989). Direct evidence that excitotoxicity in cultured neurons is mediated via *N*-methyl-D-aspartate (NMDA) as well as non-NMDA receptors. J. Neurochem. *53*, 297–299.

Gean, P.-W. (1990). The epileptiform activity induced by 4-aminopyridine in rat amygdala slices: antagonism by non-*N*-methyl-D-aspartate receptor antagonists. Brain Res. *530*, 251–256.

Honoré, T., Davies, S. N., Drejer, J., Fletcher, E. J., Jacobsen, P., Lodge, D., and Nielsen, F. E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. Science *241*, 701–703.

Hwa, G. G., and Avoli, M. (1991). The involvement of excitatory amino acids in neocortical epileptogenesis: NMDA and non-NMDA receptors. Exp. Brain Res. *86*, 248–256.

Isaacson, J. S., and Nicoll, R. A. (1991). Aniracetam reduces glutamate receptor desensitization and slows the decay of fast excitatory synaptic currents in the hippocampus. Proc. Natl. Acad. Sci. USA *88*, 10936–10940.

Jones, M. G., Anis, N. A., and Lodge, D. (1990). Philanthotoxin blocks quisqualate-, AMPA- and kainate-, but not NMDA-, induced excitation of rat brainstem neurones in vivo. Br. J. Pharmacol. *101*, 968–970.

Kaku, D. A., Goldberg, M. P., and Choi, D. W. (1991). Antagonism of non-NMDA receptors augments the neuroprotective effect of NMDA receptor blockade in cortical cultures subjected to prolonged deprivation of oxygen and glucose. Brain Res. *554*, 344–347.

Klockgether, T., Turski, L., Honoré, T., Zhang, Z. M., Gash, D. M., Kurlan, R., and Greenamyre, J. T. (1991). The AMPA receptor antagonist NBQX has antiparkinsonian effects in monoamine-depleted rats and MPTP-treated monkeys. Ann. Neurol. *30*, 717–723.

Koh, J. Y., Goldberg, M. P., Hartley, D. M., and Choi, D. W. (1990). Non-NMDA receptor-mediated neurotoxicity in cortical culture. J. Neurosci. *10*, 693–705.

Le Peillet, E., Arvin, B., Moncada, C., and Meldrum, B. S. (1992). The non-NMDA antagonists, NBQX and GYKI 52466, protect against cortical and striatal cell loss following transient global ischaemia in the rat. Brain Res. *571*, 115–120.

Martin, A. R. (1966). Quantal nature of synaptic transmission. Physiol. Rev. *46*, 51–66.

Mayer, M. L., and Vyklicky, L., Jr. (1989). Concanavalin A selectively reduces desensitization of mammalian neuronal quisqualate receptors. Proc. Natl. Acad. Sci. USA *86*, 1411–1415.

Meldrum, B., and Garthwaite, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol. Sci. *11*, 379–387.

Miljkovic, Z., and MacDonald, J. F. (1986). Voltage-dependent block of excitatory amino acid currents by pentobarbital. Brain Res. *376*, 396–399.

Mosinger, J. L., Price, M. T., Bai, H. Y., Xiao, H., Wozniak, D. F., and Olney, J. W. (1991). Blockade of both NMDA and non-NMDA receptors is required for optimal protection against ischemic neuronal degeneration in the *in vivo* adult mammalian retina. Exp. Neurol. *113*, 10–17.

Nellgård, B., and Wieloch, T. (1992). Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. J. Cereb. Blood Flow Metab. *12*, 2–11.

Ouardouz, M., and Durand, J. (1991). GYKI 52466 antagonizes glutamate responses but not NMDA and kainate responses in rat abducens motoneurones. Neurosci. Lett. *125*, 5–8.

Patneau, D. K., and Mayer, M. L. (1991). Kinetic analysis of interactions between kainate and AMPA: evidence for activation of a single receptor in mouse hippocampal neurons. Neuron *6*, 785–798.

Rothman, S. M., and Olney, J. W. (1986). Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann. Neurol. *19*, 105–111.

Sheardown, M. J., Nielsen, E.Ø., Hansen, A. J., and Honoré, T. (1990). 2,3- Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. Science *247*, 571–574.

Smith, S. W., Dürmuller, N., and Meldrum, B. S. (1991). The non-N-methyl-D-aspartate receptor antagonists, GYKI 52466 and NBQX are anticonvulsant in two animal models of reflex epilepsy. Eur. J. Pharmacol. *201*, 179–183.

Sucher, N. J., Aizenman, E., and Lipton, S. A. (1991). N-Methyl-D-aspartate antagonists prevent kainate neurotoxicity in rat retinal ganglion cells in vitro. J. Neurosci. *11*, 966–971.

Tang, C.-M., Dichter, M., and Morad, M. (1989). Quisqualate activates a rapidly inactivating high conductance ionic channel in hippocampal neurons. Science *243*, 1474–1477.

Tang, C.-M., Shi, Q.-Y., Katchman, A., and Lynch, G. (1991). Modulation of the time course of fast EPSCs and glutamate channel kinetics by aniracetam. Science *254*, 288–290.

Tarnawa, I., Farkas, S., Berzsenyi, P., Pataki, A., and Andrasi, F. (1989). Electrophysiological studies with a 2,3-benzodiazepine muscle relaxant: GYKI 52466. Eur. J. Pharmacol. *167*, 193–199.

Tarnawa, I., Engberg, I., and Flatman, J. A. (1990a). GYKI 52466, an inhibitor of spinal reflexes is a potent quisqualate antagonist. In Amino Acids, Chemistry, Biology and Medicine, G. Lubec and G. A. Rosenthal, eds. (Leiden, Netherlands: ESCOM Scientific Publishers), pp. 538–546.

Tarnawa, I., Farkas, S., Berzsenyi, P., Pátfalusi, M., and Andrási, F. (1990b). Reflex inhibitory action of a non-NMDA type excitatory amino acid antagonist, GYKI 52466. Acta Physiol. Hung. (Suppl.) *75*, 277–278.

Trussell, L. O., and Fischbach, G. D. (1989). Glutamate receptor desensitization and its role in synaptic transmission. Neuron *3*, 209–218.

Turski, L., Jacobsen, P., Honoré, T., and Stephens, D. N. (1992). Relief of experimental spasticity and anxiolytic/anticonvulsant actions of the α-amino-3- hydroxy-5-methyl-4-isoxazolepropionate antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline. J. Pharmacol. Exp. Ther. *260*, 742–747.

Vyklicky, L., Jr., Patneau, D. K., and Mayer, M. L. (1991). Modulation of excitatory synaptic transmission by drugs that reduce desensitization at AMPA/kainate receptors. Neuron *7*, 971–984.