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7-Chlorokynurenic acid is a selective antagonist at the glycine modulatory site of the *N*-methyl-D-aspartate receptor complex

(excitatory amino acid receptors/allosteric interaction/electrophysiology/glutamate)

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ABSTRACT **Glycine markedly potentiates *N*-methyl-D-aspartate (*N*-Me-D-Asp) responses in mammalian neurons by an action at a modulatory site on the *N*-Me-D-Asp receptor–ionophore complex. Here we present evidence that 7-chlorokynurenic acid (7-Cl KYNA) inhibits *N*-Me-D-Asp responses by a selective antagonism of glycine at this modulatory site. In rat cortical slices 7-Cl KYNA (10–100 μM) noncompetitively inhibited *N*-Me-D-Asp responses, and this effect could be reversed by the addition of glycine (100 μM) or D-serine (100 μM). Radioligand binding experiments showed that 7-Cl KYNA had a much higher affinity for the strychnine-insensitive [^{3}H]glycine binding site (IC_{50} = 0.56 μM) than for the *N*-Me-D-Asp (IC_{50} 169 μM), quisqualate (IC_{50} = 153 μM), or kainate (IC_{50} > 1000 μM) recognition sites. In whole-cell patch-clamp recordings from rat cortical neurones in culture, the inhibitory effects of 7-Cl KYNA on *N*-Me-D-Asp-induced currents could not be overcome by increasing the *N*-Me-D-Asp concentration but could be reversed by increasing the glycine concentration. 7-Cl KYNA could completely abolish *N*-Me-D-Asp responses, including basal responses in the absence of added glycine, suggesting that it may possess negative modulatory effects at the glycine site. These findings indicate that the glycine modulatory site is functional in intact adult tissue and that 7-Cl KYNA should prove to be a selective tool for elucidating the involvement of this site in physiological and pathological events mediated by *N*-Me-D-Asp receptors.**

Johnson and Ascher (1) recently reported that glycine markedly potentiates *N*-methyl-D-aspartate (*N*-Me-D-Asp) receptor-mediated responses on cultured cerebral neurones. Electrophysiological experiments using isolated outside-out membrane patches (1) and radioligand binding studies (2–4) indicate an allosteric interaction through a strychnine-insensitive glycine recognition site that is part of the *N*-Me-D-Asp receptor/ion channel complex.

It has previously been shown that kynurenic acid (KYNA) is a nonselective excitatory amino acid antagonist (5, 6), having similar pA_2 values (negative logarithm of antagonist concentration producing a 2-fold shift of the agonist concentration–response curve) for the antagonism of *N*-Me-D-Asp, quisqualate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate responses on rat cortical slices (6). These pA_2 values correspond to the potency of KYNA to inhibit L-[^{3}H]glutamate binding to *N*-Me-D-Asp recognition sites and [^{3}H]AMPA binding to quisqualate recognition sites but not [^{3}H]kainate binding to kainate recognition sites (6). However, in contrast to the antagonism of non-*N*-Me-D-Asp receptor-mediated responses, Schild plots for the antagonism of *N*-Me-D-Asp agonists were very steep (slope ≈ 2), suggesting more than a simple competitive interaction with *N*-Me-D-Asp receptors (6, 7).

Subsequently it has been shown that KYNA competitively inhibits strychnine-insensitive [^{3}H]glycine binding (8), and functional studies suggest that part of its antagonism is by an action at the glycine modulatory site on the *N*-Me-D-Asp receptor complex (9). Here we present evidence that 7-chloro-KYNA (7-Cl KYNA), which has increased *N*-Me-D-Asp antagonist activity over KYNA itself,* inhibits *N*-Me-D-Asp responses by a selective antagonism of glycine at its modulatory site.

MATERIALS AND METHODS

Excitatory amino acid-induced depolarizations of rat cortical tissue were recorded by using the greased-gap technique as described (10). Excitatory amino acid receptor agonists were applied for 1-min periods in a Mg^{2+}-free artificial cerebrospinal fluid (124 mM NaCl/2 mM KCl/1.25 mM KH_2PO_4/2 mM $CaCl_2$/25 mM $NaHCO_3$/11 mM glucose) containing 0.1 μM tetrodotoxin. 7-Cl KYNA was perfused for 20–30 min prior to repeating excitatory amino acid concentration–response curves.

Voltage-clamp experiments were performed on primary cultures of cortical neurones prepared from 1-day-old neonatal rats essentially as described by Kaplan *et al.* (11). Recordings were made from cells between 1 and 4 weeks in culture using the whole-cell patch configuration (12) with a List EPC-7 amplifier. Patch pipettes had resistances of ≈4 MΩ when filled with the standard intracellular solution (120 mM CsF/10 mM CsCl/ 10 mM EGTA/10 mM Hepes/0.5 mM $CaCl_2$/4 mM NaCl, pH 7.25, adjusted with CsOH). The external solution had the following composition: 139 mM NaCl/2 mM KCl/1.25 mM KH_2PO_4/2 mM $CaCl_2$/10 mM Hepes/11 mM D-glucose, 0.1 μM tetrodotoxin, pH 7.4. The technique used for rapid solution changes from a double-barreled micropipette was the same as that described by Johnson and Ascher (1).

Radioligand binding assays were carried out as follows: *N*-Me-D-Asp-sensitive L-[^{3}H]glutamate binding to crude postsynaptic densities from rat cerebral cortex was determined as described by Foster and Fagg (13). The same membrane preparation was used to measure [^{3}H]AMPA and [^{3}H]kainate binding by modifications of the methods of Honore and Nielsen (14) and Simon *et al.* (15). For each binding assay, crude postsynaptic densities (50 μg of mem-

Abbreviations: *N*-Me-D-Asp, *N*-methyl-D-aspartate; KYNA, kynurenic acid; 7-Cl KYNA, 7-chloro-KYNA; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; pA_2, negative logarithm of antagonist concentration producing a 2-fold shift of the agonist concentration–response curve.

*Frey, P., Berney, D., Mueller, W., Herrling, P. & Urwyler, S., International Union of Pharmacology Satellite Symposium, Aug. 19–21, 1987, Canberra, Australia, abstr. p. 12.

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brane protein) were incubated at 32°C for 30 min with 50 mM Tris/acetate buffer (pH 7.0) and either 50 nM L-[^{3}H]glutamate (40–60 Ci/mmol; 1 Ci = 37 GBq), 36 nM [^{3}H]AMPA (5–15 Ci/mmol), or 16 nM [^{3}H]kainate (30–60 Ci/mmol; each radioligand was from DuPont–New England Nuclear) in a final volume of 0.5 ml. In the [^{3}H]AMPA binding assays, 100 mM KSCN was included (14). Nonspecific binding was determined by the inclusion of 1 mM L-glutamate. For L-[^{3}H]glutamate and [^{3}H]AMPA, bound and free radioactivities were separated by centrifugation in an Eppendorf microcentrifuge, and filtration through Whatman GF/C filters using a Brandell cell harvester was used in the case of [^{3}H]kainate binding.

For [^{3}H]glycine binding experiments, synaptic plasma membranes were prepared from rat cerebral cortex as described by Foster and Fagg (13). However, they were not treated with Triton X-100 but were frozen at −20°C for at least 18 hr before washing four times by resuspension in 50 vol (with respect to original tissue weight) of 5 mM Tris/acetate buffer (pH 7.0) with a glass/Teflon homogenizer and centrifugation at 50,000 × *g* for 60 min. The final pellet was resuspended in a small volume of 5 mM Tris/acetate buffer (pH 7.0) to give a membrane protein concentration of ≈3 mg/ml and stored at −80°C. For the binding assay, synaptic plasma membranes (50–100 μg of membrane protein) were incubated at 4°C for 30 min with 50 mM Tris/acetate buffer (pH 7.0) and 50 nM [^{3}H]glycine (40–60 Ci/mmol; Dupont–New England Nuclear) in a final volume of 0.5 ml. Nonspecific binding was determined by the inclusion of 1 mM glycine, and bound radioactivity was separated by centrifugation in an Eppendorf microcentrifuge.

7-Cl KYNA was prepared as described (16). All other drugs and reagents were obtained from commercial sources.

RESULTS

Chloro substitution at the 7-position of KYNA resulted in a selective increase in *N*-Me-D-Asp antagonist potency on rat cortical slices. Calculated from the concentration ratios produced by 10 and 30 μM (Fig. 1), 7-Cl KYNA had an apparent pA_2 of 5.2 against *N*-Me-D-Asp responses. Thus, 7-Cl KYNA was 20 times more potent as an antagonist of *N*-Me-D-Asp responses on rat cortical slices than KYNA itself (6). In comparison, there was little difference in potency between 7-Cl KYNA and KYNA as antagonists of quisqualate and kainate responses on rat cortical slices. Apparent pA_2 values (mean ± SEM) of 4.2 ± 0.09 (n = 5) and 4.3 ± 0.02 (n = 5) were obtained from the concentration ratios produced by 100 μM 7-Cl KYNA against quisqualate and kainate, respectively, which are similar to the previously reported values for KYNA (6).

This increase in potency of 7-Cl KYNA against *N*-Me-D-Asp responses was not, however, paralleled by an increase in affinity for *N*-Me-D-Asp-sensitive L-[^{3}H]glutamate binding sites. The ability to inhibit *N*-Me-D-Asp-sensitive L-[^{3}H]glutamate binding was little changed, with an IC_{50} of 184 (176, 193) μM for KYNA and 169 (115, 250) μM for 7-Cl KYNA [geometric mean (−SEM, +SEM), n = 3 each]. The inhibition of [^{3}H]AMPA binding was also largely unaffected by the 7-chloro substitution; the IC_{50} was 101 (93, 110) μM for KYNA and 153 (127, 185) μM for 7-Cl KYNA (n = 3 or 4). 7-Cl KYNA was a weak inhibitor of [^{3}H]kainate binding, with an IC_{50} > 1000 μM (n = 3).

In agreement with these results was the finding that the antagonism of *N*-Me-D-Asp responses in rat cortical slices by 7-Cl KYNA was clearly noncompetitive in nature, producing a progressive flattening of the *N*-Me-D-Asp concentration–response curve with increasing concentrations from 10 to 100 μM (Fig. 1). At a concentration of 100 μM, 7-Cl KYNA completely flattened the *N*-Me-D-Asp concentration–response curve.

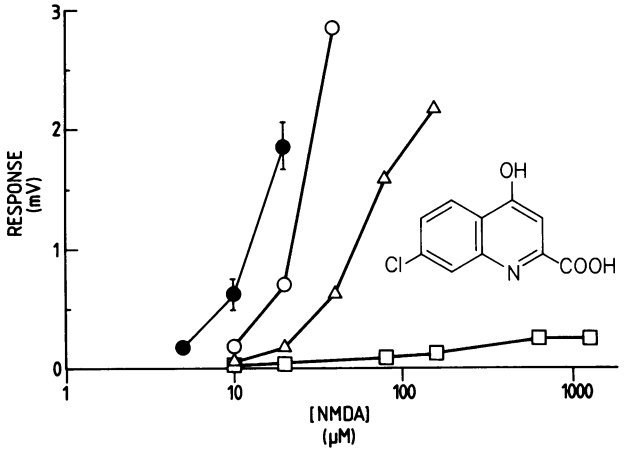

FIG. 1. Antagonist effect of 7-Cl KYNA on *N*-Me-D-Asp (NMDA)-induced depolarizations of rat cortical slices. Concentration–response curves to *N*-Me-D-Asp in the absence (●) and presence of 7-Cl KYNA at 10 μM (○), 30 μM (△), 100 μM (□). The noncompetitive nature of the antagonism is apparent from the progressive flattening of the *N*-Me-D-Asp concentration–response curves produced by increasing the concentration of 7-Cl KYNA. The curves in the presence of 7-Cl KYNA are from three different slices, and the control curve is the mean ± SEM of the control curves from these three slices. The mean logarithmic concentration ratios (±SEM) calculated from the midpoint of the control curves produced by 10 and 30 μM 7-Cl KYNA were 0.40 ± 0.03 (n = 11) and 0.74 ± 0.03 (n = 12), respectively. No concentration ratio could be calculated for 100 μM 7-Cl KYNA because of the profound flattening of the *N*-Me-D-Asp concentration–response curve produced by this concentration (n = 7). (*Inset*) Structure of 7-Cl KYNA.

Strychnine-insensitive [^{3}H]glycine binding to synaptic plasma membranes from rat cerebral cortex was used to label the glycine site associated with *N*-Me-D-Asp receptors. Under the assay conditions employed, [^{3}H]glycine binding had similar pharmacological characteristics to those reported by Kishimoto *et al.* (17) and Bristow *et al.* (18). IC_{50} values for inhibition of [^{3}H]glycine binding were as follows: glycine = 0.18 (0.16, 0.20) μM (n = 4); D-serine = 0.57 (0.42, 0.77) μM; L-serine = 30.3 (20.3, 45.1) μM (n = 3 each). 7-Cl KYNA exhibited a marked increase in affinity for this site relative to KYNA (Fig. 2). The IC_{50} for inhibition of [^{3}H]glycine binding was increased from 41 (27, 61) μM for KYNA (n = 3) to 0.56 (0.38, 0.75) μM for 7-Cl KYNA (n = 4, Fig. 2). This led us to investigate the possibility that the noncompetitive antagonism of *N*-Me-D-Asp responses by 7-Cl KYNA was mediated by an action at the glycine modulatory site.

Our initial studies failed to show any potentiating effect of exogenously applied glycine on *N*-Me-D-Asp responses from rat cortical slices. However, we found that glycine (100 μM) was able to almost completely reverse the antagonist effects of 7-Cl KYNA (10 μM), restoring the *N*-Me-D-Asp responses back toward control levels (Fig. 3 *Upper*). The mean logarithmic concentration ratio (±SEM) produced by 7-Cl KYNA (10 μM) was reduced from 0.43 ± 0.029 to 0.086 ± 0.024 (n = 8) by glycine (100 μM). Furthermore, D-serine (100 μM), which is also an agonist at the glycine site on the *N*-Me-D-Asp receptor (2, 4), was able to reverse the antagonist effects of 7-Cl KYNA, including the unsurmountable block produced by 100 μM 7-Cl KYNA (Fig. 3 *Lower*). The ability of glycine and D-serine to reverse *N*-Me-D-Asp antagonism was specific for 7-Cl KYNA. D-Serine (100 μM) had no effect on the competitive antagonism produced by 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (19) (6 μm, n = 3 slices) or the uncompetitive antagonism produced by MK-801 (10) (1 μM, n = 3 slices; data not shown).

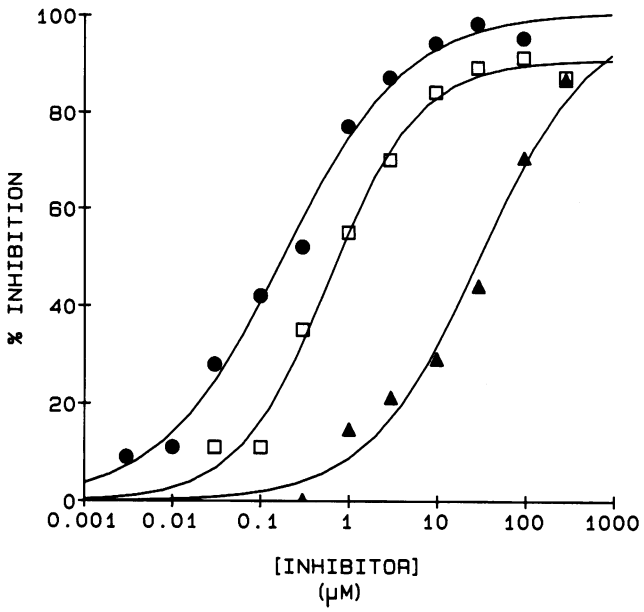

FIG. 2. Inhibition of [^{3}H]glycine binding to rat cerebral cortex synaptic plasma membranes by glycine (●), 7-Cl KYNA (□), and KYNA (▲). Each curve is from a single experiment that was repeated at least twice with similar results (see text for averaged IC_{50} values); individual points represent averages of triplicate determinations. Lines were fitted and IC_{50} values were determined by an iterative curve-fitting program using Research System 1 (Bolt, Beranek and Newman, Cambridge, MA). Typical values from the binding assay were total binding = 20,388 ± 426 dpm and nonspecific binding (in presence of 1 mM L-glycine) = 12,008 ± 520 dpm (n = 7).

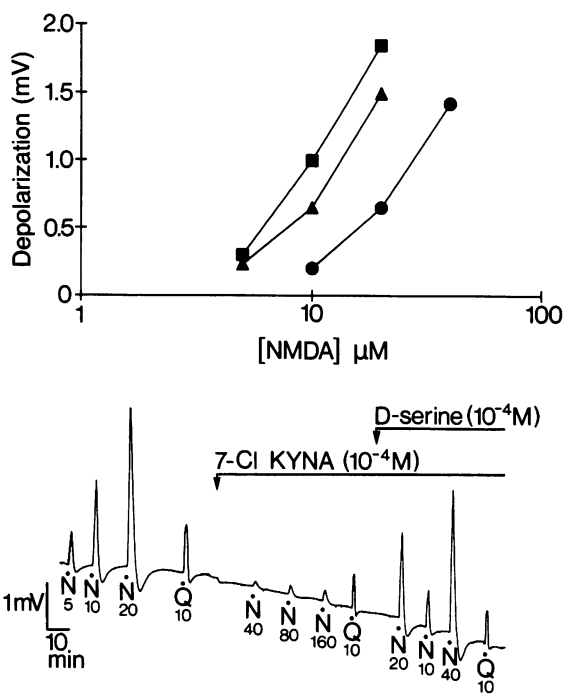

FIG. 3. Reversal of the *N*-Me-D-Asp (NMDA) antagonist effect of 7-Cl KYNA by glycine and D-serine on rat cortical slices. (*Upper*) Control responses to *N*-Me-D-Asp (■) were antagonized in the presence of 7-Cl KYNA (10 μM), resulting in a shift to the right of the *N*-Me-D-Asp concentration–response curve (●). Addition of glycine (100 μM) produced a marked reversal of the antagonist effect of 7-Cl KYNA (▲). Responses shown are from a single slice. (*Lower*) Reversal by D-serine (100 μM) of the pronounced *N*-Me-D-Asp antagonism produced by 100 μM 7-Cl KYNA. The trace shows depolarizing responses to *N*-Me-D-Asp (N) (numbers indicate concentration in μM) and quisqualate (Q). Following continuous perfusion with 7-Cl KYNA (100 μM, from arrow) the responses to *N*-Me-D-Asp were depressed in an insurmountable fashion, and at this concentration there was also a reduction in the quisqualate response. Continuous perfusion with D-serine (100 μM, from second arrow) reversed, to a large extent, the block produced by 7-Cl KYNA on *N*-Me-D-Asp responses, but the antagonism of the quisqualate response remained unaffected.

To investigate the action of 7-Cl KYNA in greater detail and under conditions where the extracellular glycine concentration could be controlled, whole-cell patch-clamp recordings (12) from primary cell cultures of rat cortical neurones were used. By using the fast-perfusion technique employed by Johnson and Ascher (1) we were able to reproduce their demonstration of the marked potentiating effect of glycine on *N*-Me-D-Asp receptor-mediated responses (Fig. 4). 7-Cl KYNA was able to completely abolish basal *N*-Me-D-Asp and glycine (1 μM)-potentiated *N*-Me-D-Asp responses (Fig. 4), and this effect could not be overcome by increasing the *N*-Me-D-Asp concentration (not shown). It could, however, be reversed by increasing the concentration of glycine (10 and 30 μM), again demonstrating competition between these compounds (Fig. 4). Nevertheless, it can be seen (Fig. 4) that in the presence of 30 μM 7-Cl KYNA, raising the glycine concentration to 30 μM failed to completely return the *N*-Me-D-Asp response back to the control size obtained in the presence of 1 μM glycine. This suggests that 7-Cl KYNA (30 μM) produced a large shift to the right of the glycine concentration–response curve. Preliminary experiments indicate that 10 μM 7-Cl KYNA produces an ≈30-fold shift to the right of the glycine concentration–response curve for potentiation of the *N*-Me-D-Asp (30 μM) response.

DISCUSSION

These results suggest that the noncompetitive *N*-Me-D-Asp antagonist effects of 7-Cl KYNA are mediated by an action

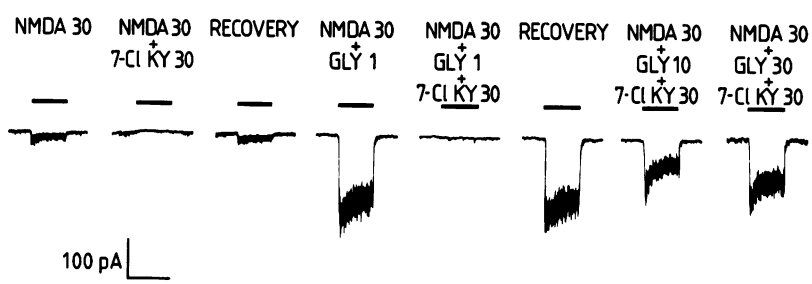

FIG. 4. Antagonist effect of 7-Cl KYNA on whole-cell currents induced by *N*-Me-D-Asp (NMDA) in the presence and absence of glycine and its reversal by increased glycine concentration. Holding potential, −60 mV. 7-Cl KYNA (30 μM) reversibly abolished the *N*-Me-D-Asp (30 μM) response obtained in the absence of glycine. Addition of glycine (1 μM) produced a marked potentiation of the *N*-Me-D-Asp response, which was also completely blocked by 7-Cl KYNA (30 μM). Increasing the glycine concentration (10 and 30 μM) reversed, to a large extent, the antagonism produced by 7-Cl KYNA.

at the glycine modulatory site on the *N*-Me-D-Asp receptor complex. The binding studies indicate that the affinity of 7-Cl KYNA for the strychnine-insensitive glycine site is 273-, 302- and >1000-fold higher than its affinity for quisqualate, *N*-Me-D-Asp, or kainate recognition sites, respectively, equivalent values for KYNA being 2.5, 4.5, and >1000, respectively. Thus, 7-chloro substitution of KYNA results in a selective 70-fold increase in affinity for the glycine site.

An accurate estimation of its affinity for the glycine site from the electrophysiological studies in rat cortical slices is complicated by the noncompetitive nature of the antagonism and by the presence of unknown concentrations of endogenous extracellular glycine with which 7-Cl KYNA would be competing. In agreement with the studies of Johnson and Ascher (1) we found that the augmentation of *N*-Me-D-Asp responses by glycine in neuronal cultures was near maximal at 1–3 μM. The inability of added glycine to potentiate *N*-Me-D-Asp responses on rat cortical slices suggests that during *N*-Me-D-Asp applications the extracellular concentration of glycine must be at least equal to or in excess of this. However, it has been shown that L-glutamate and L-aspartate cause a Ca^{2+}-independent release of glycine from retina (20). Thus, it is possible that application of *N*-Me-D-Asp evokes the release of glycine within the slices, which then potentiates the *N*-Me-D-Asp response, making it difficult, under normal circumstances, to observe a potentiation of an *N*-Me-D-Asp response with exogenously applied glycine. Nevertheless, 7-Cl KYNA was still 10 times more potent as an antagonist of *N*-Me-D-Asp responses than of quisqualate or kainate responses on rat cortical slices. The shift to the right of the glycine concentration–response curve produced by 7-Cl KYNA (10 μM) in the patch-clamp experiments suggests it has an affinity $\approx$ 0.3 μM for the glycine site, which would be in good agreement with the binding data.

The finding that in the neuronal culture experiments 7-Cl KYNA, at concentrations (10 and 30 μM) below those interacting competitively with *N*-Me-D-Asp receptors, could completely inhibit *N*-Me-D-Asp responses suggests two interesting possibilities. One is that even in the absence of any added glycine, some activation of the glycine site occurs and that this is antagonized by 7-Cl KYNA. If this is the case, it suggests that rather than simply potentiating responses at *N*-Me-D-Asp receptors, the glycine modulatory site has a completely permissive role over the activation of *N*-Me-D-Asp receptors by an agonist. A second explanation is that binding of 7-Cl KYNA to the glycine site has negative modulatory effects, opposite to the positive modulatory effects of glycine and D-serine, and is able to reduce *N*-Me-D-Asp receptor responses below those produced in the absence of any glycine site activation. This situation would be similar in some respects to benzodiazepine receptors on the γ-aminobutyric acid receptor/Cl^--ion channel complex, where ligands with agonist (positive modulators), inverse agonist (negative modulators), and antagonist (no intrinsic activity) properties exist (21–23). Thus, 7-Cl KYNA could be regarded as an inverse agonist rather than a simple antagonist at the glycine modulatory site. If this is the case then the affinity of 7-Cl KYNA assessed from the shift of the glycine concentration–response curve is likely to be an overestimate of its true affinity.

These observations with 7-Cl KYNA have provided evidence that the glycine modulatory site on the *N*-Me-D-Asp receptor is functional in intact adult neuronal tissue. Indeed, under the conditions of the cortical slice experiments it would appear that this allosteric site is maximally activated. Future experiments can be expected to elucidate the mechanisms that regulate the levels of glycine in the synapse *in vivo*. In addition, the identification of 7-Cl KYNA as a potent and selective antagonist of the glycine site opens the way for studies of the contribution of this regulatory site to *N*-Me-D-Asp receptor function under physiological and pathological conditions.

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