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COMMENTARY

COMPETITIVE ANTAGONISM OF GLYCINE AT THE *N*-METHYL-D-ASPARTATE (NMDA) RECEPTOR

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N-Methyl-D-aspartate (NMDA) was recognized in the late seventies as a selective agonist for a distinct subtype of glutamate receptor (see Ref. 1 for review). Binding of NMDA or glutamate controls the gating of ion channels that are permeable to calcium and monovalent cations [2–6]; physiological concentrations of extracellular Mg^{2+} block the channel in a voltage-dependent manner [6]. In 1987, Johnson and Ascher [7] discovered that glycine, another normal constituent of extracellular fluid, acts as a powerful positive modulator of NMDA receptors. Activation of the NMDA channel leads to depolarization and the calcium that enters is thought to serve as an internal messenger to regulate the strength and stability of excitatory synapses [8–10].

Excessive calcium entry during periods of hyperexcitation has been implicated in excitotoxic cell death [11–14] associated with epilepsy, ischemia and several neurodegenerative conditions. For this reason, antagonists of NMDA receptor function may be clinically useful in the treatment of these disorders and have become the subject of intense interest [15, 16]. Because of its complexity, the NMDA receptor provides several possible targets for chemical intervention including the glutamate or NMDA binding site [1, 17], the ion channel [18] and the glycine modulation site. This paper considers recent work on the pharmacology of glycine modulation.

Action of glycine

In recordings from central neurons in culture, Johnson and Ascher [7] found that glycine potentiates the current evoked by NMDA. At the level of single channels, glycine increases the frequency of channel openings in the presence of NMDA but does not change the unitary conductance or mean lifetime of the open state [7]. Application of glycine alone does not open the channel and the potentiation is specific for NMDA receptors; currents gated by kainate and quisqualate, which are selective agonists for the other subtypes of glutamate receptor [1], are not affected by glycine [7]. The binding site for glycine on the NMDA receptor is clearly different from conventional inhibitory glycine receptors that control the gating of chloride channels [19–22]. Strychnine serves as a potent antagonist of inhibitory glycine receptors [20] but does not block the potentiation of responses to NMDA [7]. Since the initial report of Johnson and Ascher [7], potentiation by glycine has been observed in all of the available assays for NMDA receptor function, including recordings from neurons in tissue slices [23–25] and *in vivo* [26–28], as well as experiments on homogenized membranes [29–34], in which potentiation is measured using radiolabeled compounds, such as dizocilpine (MK-801) and 1-(2-thienyl)cyclohexyl piperidine (TCP), that bind to the open state of the channel [35–38]. NMDA receptors expressed by *Xenopus* oocytes injected with rat brain mRNA show potentiation by glycine [39, 40] that is indistinguishable from potentiation in cultured neurons. This result, together with the rapid kinetics of potentiation [41, 42] and the fact that potentiation is preserved in isolated membrane patches [7], suggests that the binding site for glycine is directly coupled to the channel rather than being linked by an indirect second messenger pathway.

One reason that the action of glycine went undetected for so long is the high affinity of the glycine site. Both glycine binding [23, 43, 44] and physiological potentiation [39–41, 45] reach half-saturation at 100–300 nM. Contaminating levels of glycine in many experimental preparations are sufficient to yield at least partial occupation of the glycine site. Tissue slices [23, 24] as well as cells in culture [7, 41, 45] release substantial quantities of glycine and can effectively "buffer" the local glycine concentration in the range of 0.1 to 1 μM, despite fairly rapid bulk perfusion. Most aqueous solutions are contaminated by 20–40 nM glycine [39], enough to produce significant potentiation, so that even in work on isolated cells it is nearly impossible to apply NMDA in the complete absence of glycine.

When glycine levels are reduced to a minimum, there is, in fact, very little response to NMDA. In experiments on NMDA receptors expressed in *Xenopus* oocytes [39], Kleckner and Dingledine took rigorous measures to exclude contaminating glycine and observed virtually no steady-state current in response to NMDA alone. They have suggested that glycine is absolutely required as a "co-agonist" for channel activation. Recordings from isolated neurons [41, 42, 45] and work with glycine site antagonists (see below) tend to support this proposal but it is difficult to rule out a very low level of channel opening with NMDA alone. For comparison, the channel formed by the nicotinic acetylcholine receptor opens spontaneously, albeit with very low frequency, even when the acetylcholine (ACh) binding

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sites are unoccupied [46]. Agonist binding can be thought of as stabilizing the open state of the channel rather than inducing a conformational change that is otherwise impossible [47]. Therefore, it would not be surprising if the binding of NMDA alone produced a slight increase in the probability of opening compared to the unliganded state; but it seems clear that the frequency of channel opening in the absence of glycine is so low as to be unimportant physiologically.

These observations raise the question of whether the glycine site is saturated, or nearly so, by the normal levels of extracellular glycine in intact tissue. Neurons in most regions of the central nervous system respond briskly to iontophoretic application of NMDA [1, 17]; if glycine is required for channel activation, this result suggests that glycine is chronically present at significant levels. Human cerebrospinal fluid contains roughly 5–10 μM glycine [48, 49], but it is possible that the local concentration near receptors is maintained at a lower level. A few recent studies on neurons *in vivo* [26–28] and on cortical slices [25] have obtained potentiation by addition of exogenous glycine, which indicates that the receptors were not saturated; however, in several other cases [23, 24] there was no effect of glycine application unless antagonists were used to compete with endogenous glycine. Glycine is released and taken up at some inhibitory synapses [50], but the inhibitory glycine receptor has a much lower affinity (EC_{50} = 90–100 μM) [22, 51], so that it would not be affected by a maintained level of glycine below 1 μM. For now, it remains an open question whether the concentration of extracellular glycine is temporally regulated *in vivo* in order to modulate current through the NMDA receptor.

The exact mechanism of the action of glycine is also still unclear. As mentioned above, the main effect of glycine is to increase the frequency of channel openings once glutamate or NMDA has bound to the receptor [7]. In physiological experiments [7, 39–41, 45], glycine does not shift the concentration–response relation for NMDA, but simply scales up the current obtained for each NMDA concentration. Mayer *et al.* [52] have suggested that glycine inhibits one component of desensitization of the NMDA receptor. Under conditions that minimize Ca^{2+}-dependent desensitization, they found that saturating glycine completely overcomes the rapid phase of desensitization ($\tau \sim 250$ msec) seen in whole-cell recordings. Ascher and colleagues [41, 53], using similar recording methods and also studying single channels in outside-out patches, have observed that a rapid component of desensitization persists even with saturating concentrations of glycine. At present it is not clear how to resolve these conflicting results.

One possible interpretation that is consistent with the results of Mayer *et al.* [52] is that the binding of NMDA reduces the affinity of the glycine site for glycine. A number of recent radioligand binding studies [43, 54–56] have detected subtle interactions between glycine and glutamate binding, but in these experiments [43, 54] agonists of the NMDA receptor enhanced the binding of glycine. Similarly, agonists of the glycine site increase the binding of glutamate in some studies [55, 56; but see 44]. It is difficult to compare the physiological results and radioligand binding data because of the different experimental conditions and the very different time scales of observation in each kind of experiment. Further work is clearly needed to better understand the allosteric interactions between agonist binding to the glycine and NMDA sites and the control of channel gating.

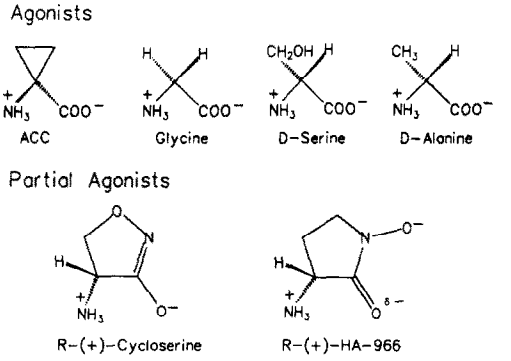

Fig. 1. Agonists and partial agonists of the glycine potentiation site.

Glycine pharmacology: Agonists

A number of compounds are known to act as agonists at the glycine potentiation site but none is significantly more potent than glycine and most have a much lower affinity. Dingledine and colleagues [57] have conducted the most thorough evaluation of agonist candidates and have drawn several conclusions about the structural requirements of the glycine site. All of the most potent agonists are α-amino acids with ionized carboxy and amino groups, and substituents on the α-carbon must be small in order to preserve significant activity [57]. Figure 1 shows the structures of the most effective agonists. After glycine, the first compounds identified were D-serine and D-alanine [7, 39, 58; see also 59], both of which produce maximal potentiation nearly equivalent to that of glycine [57, 58]. Half-saturation is achieved with approximately 0.2 to 1 μM D-serine and 0.5 to 2 μM D-analine [57, 58]; the L-isomers have roughly 30-fold lower affinity. Thus, the binding site shows stereoselectivity even though glycine itself is not a chiral molecule. To account for the enhanced activity of D-serine relative to D-alanine, despite the larger volume taken up by serine, McBain *et al.* [57] have proposed that the β-OH of serine may form a hydrogen bond to the receptor. Consistent with this hypothesis, compounds with a β-halogen, which can accept a proton in forming a hydrogen bond, preserve substantial activity at the glycine site, whereas molecules with β-methyl, -SH or $-NH_3^+$ substitutions are inactive [57, 58].

The agonist with the highest affinity for the glycine site is the cyclic compound, 1-aminocyclopropane carboxylic acid (ACC, Fig. 1) [57, 60, 61]. ACC potentiates the binding of [^{3}H]MK-801 to rat forebrain membranes with an EC_{50} of 135 nM compared to 206 nM for glycine [61]. In these experiments [61], ACC appeared to function as a partial agonist,

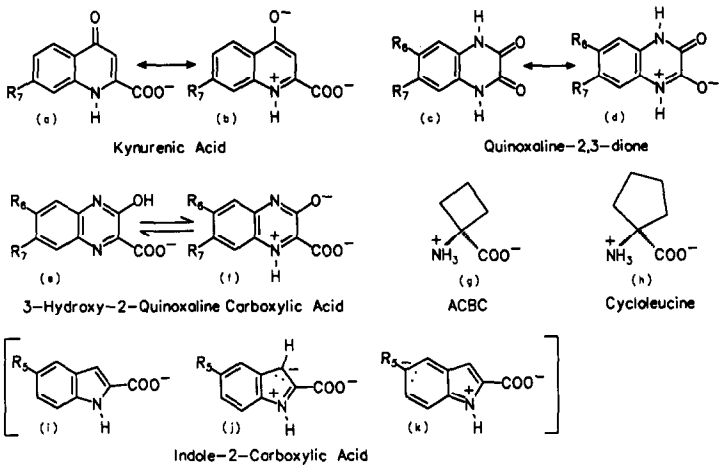

Fig. 2. Antagonists of the glycine potentiation site. (a) and (b) Canonical forms of kynurenic acid (R_7 = H) and 7-chlorokynurenic acid (R_7 = Cl). (c) and (d) Canonical forms of quinoxaline-2,3-dione (R_6,R_7 = H), CNQX (R_6 = CN, R_7 = NO_2), DNQX (R_6, R_7 = NO_2), and dichloro-quinoxaline-2,3-dione (R_6, R_7 = Cl). (e) and (f) Tautomers of 3-hydroxy-2-quinoxaline carboxylic acid (HQC) (R_6, R_7 = H) and dichloro-HQC (R_6, R_7 = Cl). (g) ACBC. (h) Cycloleucine, (i), (j) and (k) Three of the structures that contribute to indole-2-carboxylic acid (I2CA) (R_5 = H) and its 5-substituted derivatives.

producing only 40% of the maximal increase in MK-801 binding observed with glycine. Electrophysiological recordings in the *Xenopus* oocyte system have confirmed the high affinity of ACC [57], but have indicated a high efficacy. McBain *et al.* [57] found that ACC enhanced current gated by NMDA to within 90% of the maximal potentiation obtained with glycine. Opening the cyclopropane ring to yield 2-aminoisobutyric acid greatly reduces agonist activity at the glycine site [57], while molecules with larger rings of 4-(1-amino-cyclobutane carboxylic acid, ACBC, Fig. 2g) [62] or 5 carbons (cycloleucine, Fig. 2h) [63, 64] function as antagonists.

Antagonists and partial agonists

When Johnson and Ascher [7] discovered the potentiating action of glycine, several compounds known to inhibit responses to NMDA were reconsidered as possible glycine site antagonists. Both kynurenic acid (Fig. 2, a and b) [65] and 1-hydroxy-3-aminopyrrolid-2-one (HA-966, Fig. 1) [66], which had been shown to produce non-competitive antagonism of NMDA [66, 67], were subsequently found to act primarily at the glycine potentiation site [24, 41, 68–74]. Antagonism by these compounds is overcome with the addition of glycine [24, 41, 68–74], and they both inhibit [^{3}H]glycine binding to strychnine-insensitive sites in rat brain membranes [23, 43, 72].

Kynurenic acid, as well as several other compounds considered below, produce complete antagonism of responses to NMDA [23, 41, 68]. In contrast, HA-966, even at saturating concentrations, only blocks about 90% of the response obtained with glycine [72, 74–77]. This difference between HA-966 and kynurenic acid was initially taken as evidence that kynurenic acid functioned as an inverse agonist to actively suppress responses to NMDA in the absence of glycine [23, 68, 72]; HA-966 was assumed to be a simple antagonist, which would not block the basal response to NMDA alone. An alternative possibility [23, 41, 72, 74, 77], however, is that responses obtained to NMDA in nominally glycine-free media are, in fact, dependent on the low concentrations of glycine that contaminate nearly all solutions. Under this hypothesis, blockade by kynurenic acid would be due to displacement of the contaminating glycine, whereas HA-966 would serve as a partial agonist, producing about 5–10% of the maximal response observed with glycine.

It has been difficult to resolve these two possibilities because of the serious problem of eliminating glycine. At present, there is no direct evidence that kynurenate or any other compound actually functions as an inverse agonist, although this cannot yet be ruled out. On the other hand, when contamination by glycine is minimized, HA-966 does cause enhancement of current gated by NMDA [77], suggesting that it is not a simple antagonist but, instead, a weak partial agonist. Cycloserine (Fig. 1), which has a structure similar to HA-966 and a similar affinity for the glycine site, appears to be a more effective partial agonist, producing about 60% of the maximal response to glycine [57].

A number of the compounds that have been found to inhibit potentiation by glycine, including kynurenic acid, are also antagonists at kainate and quisqualate receptors [23, 78–85] and in some cases at the NMDA binding site as well [23, 79, 80]. In evaluating these antagonists it is clearly important to consider the structural features that may determine selectivity among the various amino acid binding sites in addition to the properties that enhance affinity for any particular site. Most of the antagonists that are active at the glycine potentiation site are comprised of derivatives of four bicyclic parent compounds: kynurenic acid, indole-2-carboxylic acid (I2CA), quinoxaline-2,3-dione, and 3 hydroxy-2-quinoxaline carboxylic acid (HQC) (Fig. 2).

Kynurenic acid is often represented as 4-hydroxyquinoline-2-carboxylic acid; however, the tautomer shown in Fig. 2, a and b, is likely to predominate in aqueous solution [86–88]. Although it is not known what form of the molecule binds to the glycine site or to the other receptors, the charged canonical form (Fig. 2b) bears the closest structural similarity to fully ionized amino acids (cf. Fig. 1). The other three parent compounds have the potential to adopt a similar configuration. The two quinoxalines exhibit tautomers with charged canonical forms (Fig. 2, c, d, e, and f) and I2CA can be considered a hybrid of structures including its conventional representation (Fig. 2i) and six forms, in which nitrogen donates electrons to the ring system. Two of these forms are shown in Fig. 2, j and k. Thus, the common features expected for these compounds include a partial positive charge on nitrogen, the potential for a hydrogen to be bound to nitrogen, and nearby negative oxygens, either from a 2-carboxyl group or, in the case of quinoxalinedione, oxygens bound directly to the ring. An additional property of these molecules is that they are almost perfectly flat. It is not yet clear how important each of these common features actually is for antagonism; however, for a number of compounds that lack activity at the glycine site [43, 45], such as quinaldic acid, quinoxaline-2-carboxylic acid and indoline-2-carboxylic acid, these configurations are absent or less favorable.

In the case of all four parent compounds, the addition of electronegative substituents to the benzene ring greatly increases antagonist potency and can influence the selectivity for the glycine site relative to other amino acid receptors [23, 45, 78]. Addition of F or Cl at the 5 position of I2CA enhances the potency at the glycine site by a factor of 1.7 and 2.4 respectively [45]. The 5-methyl derivative is slightly less potent than I2CA, and the 5-OCH_3 and 5-OH derivatives have a much lower affinity. Kemp and colleagues [23] found 7-chlorokynurenic acid to have approximately 70-fold higher affinity for the glycine site than its parent compound; affinity for other sites was enhanced much less. In physiological experiments, 7-chlorokynurenic acid is about 40-fold more potent as a glycine site antagonist than as an inhibitor of responses to kainate [78], while blockade by kynurenic acid shows 8- to 10-fold selectivity for the glycine site relative to the receptor activated by kainate [23, 41]. Similarly, Cl substitution at the 6 and 7 positions of HQC increases the affinity for the glycine site 45-fold ($K_i = 0.3$ vs 15 μM) and for the kainate site 22-fold ($K_i = 3$ vs 67 μM) [78, see also 80, 81].

The widest range of derivatives has been tested for quinoxalinedione [43, 78, 79, 83–85]. All substitutions of electronegative groups in the 6 and 7 position enhance the potency against both kainate and glycine, but Cl derivatives show a preference for the glycine site while derivatives with NO_2 or CN groups favor antagonism of kainate [78]. It is tempting to speculate that this difference results from the tendency for NO_2 and CN to withdraw electrons by both induction and resonance, in contrast to halogens which release electrons by resonance and withdraw them by induction. Another possibility is that direct interactions of the substituent groups with the receptor play the major role in determining antagonist affinity. In this regard, compounds bearing halogens would be much more hydrophobic than derivatives with NO_2 or CN groups.

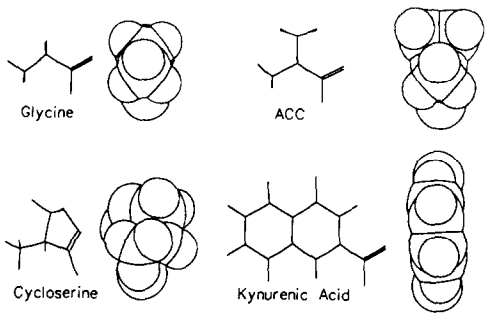

Fig. 3. Four ligands of the glycine site. Each compound is shown in two orientations: on the left is the sigma backbone and on the right is a space-filling model that has been rotated 90°.

As mentioned above, ACBC [62] and cycloleucine [63, 64] also serve as glycine site antagonists. In contrast to the bicyclic antagonists these compounds are not planar, but have the ring oriented perpendicular to the plane of the α-carbon and the NH_3^+ and COO^- termini. Affinity for the glycine site decreases as the ring is enlarged. The cyclopropane agonist, ACC, has the highest affinity [57, 60, 61], whereas ACBC [62] and cycloleucine [63, 64] bind with roughly 40- and 200-fold lower affinity, respectively, and do not cause potentiation.

Taken together, these considerations suggest that the glycine binding site lies within a narrow groove oriented along the axis defined by the NH_3^+ and COO^- termini. The receptor accepts relatively large compounds, such as the bicyclic antagonists, provided that they are nearly flat molecules. Affinity for the site appears to be reduced progressively by substituent groups that extend out from the major plane of the molecule containing the NH_3^+–COO^- axis. Figure 3 shows space-filling models of four ligands of the glycine potentiation site viewed down this axis. The smaller straight or branched chain glycine derivaives may be able to adopt a relatively planar configuration but, unless such a conformation is energetically favored, only a fraction of the molecules that impinge on the binding site will be configured appropriately, and so activity will be low [cf. 57]. An additinal factor that may enhance binding affinity is the ability of β-carbon substituents to accept a hydrogen bond [57].

Further work on glycine site antagonists, at least initially, will probably involve additional derivatives of the bicyclic parent compounds considered above. Detailed comparison of selected compounds may shed greater light on the structural requirements for effecive binding. For example, it would be particularly interesting to compare the activity of 6-chloro,7-nitro-HQC with 6-nitro,7-chloro-HQC and with the 6- and 7-monosubstituted derivatives, due to the difference in resonance expected for these

compounds. In searching for further lead compounds, it would appear prudent to seek molecules that are constrained to be planar.

Glycine modulation

Several authors [89–91] have raised the possibility that the degree of potentiation by glycine may be regulated by an endogenous antagonist of the glycine site, rather than by rapid control of the extracellular glycine concentration. Kynurenic acid, which is an intermediate in tryptophan metabolism, could play such a role [92–94], although its affinity and selectivity for the glycine site are relatively low [23, 78]. The speed with which glycine antagonists inhibit current through the NMDA channel appears to be governed by the relatively slow rate of glycine dissociation (approximately 1–3 sec^{-1}) [41, 42]. Therefore, control of the level of potentiation on the millisecond time scale seems fairly unlikely.

Initial results suggest that antagonists of the glycine potentiation site can be neuroprotective in excitotoxicity models. Recent studies [95–98] on neurons in culture have shown that HA-966 and 7-chlorokynurenic acid block neuronal death induced by NMDA. Much additional work will be needed to assess the potential therapeutic value of competitive blockers of the glycine site, but in theory they offer several possible advantages over other types of NMDA antagonists. Both the onset of inhibition and recovery from block should be much faster for glycine site antagonists than for the use-dependent channel blocking drugs such as MK-801 [35–38] (ignoring the difficult problem of access through the blood–brain barrier). In addition, a partial blockade of the glycine site, or saturation with a partial agonist, could prevent excessive channel activation while still allowing for a low level of transmitter-dependent activity, which may be necessary to maintain synaptic stability [9, 10].

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