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SITES FOR ANTAGONISM ON THE N-METHYL-D-ASPARTATE RECEPTOR CHANNEL COMPLEX

Erik H. F. Wong[1] *and John A. Kemp*

Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex, CM20 2QR, England

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INTRODUCTION

Interest in the physiology and pharmacology of the dicarboxylic amino acids L-glutamate and L-aspartate originally stemmed from identification of the powerful excitatory actions of these amino acids in the motor cortex by Hayashi in 1954 (1) and in the spinal cord by Curtis and colleagues in 1960 (2). After a fairly prolonged period of doubt, excitatory amino acids (EAAs) have gradually become accepted as the major transmitter of fast excitatory signals in the mammalian central nervous system (CNS). The receptors mediating these actions have been classified into three subtypes based on the selective agonists N-methyl-D-aspartate (NMDA), quisqualate, and kainate (3–6). The recent rapid advances in this field have primarily resulted from the identification and radiolabeling of potent and selective agonists and antagonists for elucidating the localization, density, ligand binding, and modulatory properties of these various subtypes of EAA receptors (7, 8). In particular, this approach has been very fruitful in the characterization of various sites associated with the NMDA receptor channel complex. This has complemented the powerful electrophysiological tech-

[1]Present address: Department of Neurosciences, Institute of Pharmacology, Syntex Research, 3401 Hillview Avenue, Palo Alto, California 94304

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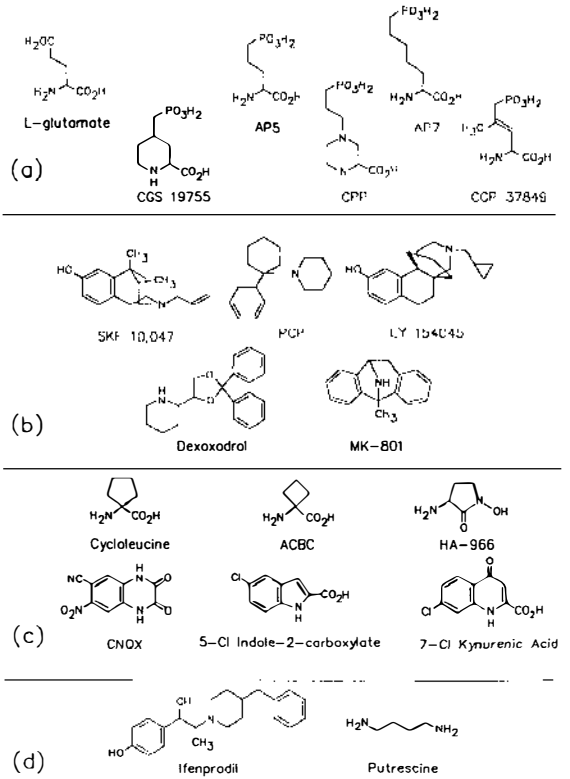

Figure 1 Structures of antagonists for the NMDA receptor channel complex at (*a*) the transmitter recognition site (*b*) the ion channel site (*c*) the glycine site, and (*d*) other sites.

niques such as voltage- and patch-clamp that have allowed the study of the conductance characteristics of the channels gated by these receptors, as well as the consequences of their blockade by competitive and noncompetitive antagonists (9).

The NMDA receptor has captured the attention of researchers because of its demonstrated involvement in various physiological roles (10). Functions of particular importance include: developmental plasticity (11–15); learning and memory (16, 17); sensory transmission (18–20); and the control of respiration (21) and blood pressure (22). Furthermore, NMDA receptors have also been implicated in such neurological disorders as ischemic brain damage (23–25), hypoglycemia (26), epilepsy (27, 28), anxiogenesis (29), motor-neurone diseases (30, 31) and olivopontocerebellar atropy (32). The possibility of providing a rational therapy for these conditions has prompted the explosion in the development of selective and potent antagonists for the NMDA receptor in recent years (6, 33).

In this review we concentrate on recent findings showing the existence of pharmacologically distinct sites on the NMDA receptor channel complex at which antagonists appear to act. These are (*a*) the transmitter recognition site, (*b*) the ion channel site, (*c*) the glycine site, (*d*) the zinc site, and (*e*) the other possible sites. The evidence indicating the coupling of these sites to form the NMDA receptor channel complex, as well as their physiological and pharmacological relevance, is discussed. The therapeutic significance of antagonism of the NMDA receptor has been extensively reviewed in other articles (23, 34), and is not discussed here.

THE TRANSMITTER RECOGNITION SITE

The first type of organic compounds to be developed as NMDA receptor antagonists were those that acted competitively with agonists at the transmitter recognition site. Almost all of these antagonists are D-isomers of longer chain analogues of glutamate and originated mainly from the work of Watkins and colleagues (3, 4, 6, 33; Figure 1). The initial selective and useful NMDA receptor antagonists were D-isomers of simple longer chain glutamate analogues, D-α-aminoadipate (D-α-AA) and D-α-aminosuberate (D-α-AS), and the diamino compound α-ϵ-diaminopimelic acid (DAP; 33). The optimum chain length for the monoamino series was 5 and 7 carbons (D-α-AA and D-α-AS, respectively), while for the diamino series a 6 carbon chain length (DAP) appeared most effective (6, 33). A rapid improvement in potency was achieved with replacement of ω-carboxylic acid of D-α-AA and D-α-AS with a phosphonic acid group to give D-2-amino-5-phosphovalerate (D-AP5) and D-2-amino-7-phosphonohepanoate (D-AP7), respectively (35–38; Figure 1). These antagonists, which have good selectivity and low micromolar affinity for the transmitter recognition site, have played an important part in defining many of the functional roles played by NMDA receptor in the mammalian CNS (39). However, the polar nature of these compounds results in poor CNS penetration, and this factor has limited their development as therapeutic agents.

In the search for possible therapeutic agents, several conformationally restricted analogues of AP5 and AP7 have subsequently been synthesized that incorporate a ring system into the molecule. The development of a rigid piperazine analogue of AP7 gave rise to 3-(2-carboxy-piperazin-4-yl)propyl-1-phosphonic acid (CPP, 40, 41; Figure 1). When a similar strategy was applied to AP5 cis-4-(phosphonomethyl)-2-piperidine-carboxylic acid was synthesized (CGS 19755; 42). The incorporation of a cyclohexyl ring into AP7 produced 2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid (NPC 12626; 43). In these cases selectivity for the NMDA recognition site was maintained while potency and activity following systemic administration were significantly improved over the parent structures (Figure 1). Another

simple modification of AP5, which has produced the most potent competitive NMDA receptor antagonist to date, has been the introduction of a double bond and methyl group into the carbon chain to give D,L-(E)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 37849; 44; Figure 1). An interesting feature of this compound and its carboxyethylester; CGP 39551, is that they are the first competitive antagonists to show significant anticonvulsant activity after oral administration (45) and thus may represent novel candidates for human antiepileptic and anti-ischemic therapy.

Radiolabeling some of the above recognition-site specific ligands have produced D-[^{3}H]AP5 (46–48), [^{3}H]CPP (49, 50) and [^{3}H]CGS 19755 (51). These ligands have allowed detailed characterization of the NMDA receptor recognition site in vitro and in general exhibit features comparable to NMDA-sensitive L-[^{3}H] glutamate binding in terms of anatomical distribution (49, 52–55), pharmacological specificity, and subcellular localization (48, 55–60). However, detailed analysis of the binding properties of L-[^{3}H]-glutamate and [^{3}H]-CPP has uncovered regionally distinct agonist- and antagonist-preferring binding sites (55). It is still unclear whether this is a reflection of two classes of NMDA recognition sites or two states of the same site. Nevertheless, it is of interest that measurement of the target/molecule sites of the NMDA-sensitive L-[^{3}H] glutamate binding site and [^{3}H] CPP binding sites by radiation inactivation technique have produced different values, i.e. 121000 and 209000, respectively (61, 62). This has been interpreted in terms of an allosteric coupling between distinct agonist and antagonist binding sites. Alternatively, high affinity CPP binding might require an additional macromolecule that is associated with, but not part of, the agonist recognition site. The identification of novel competitive antagonists such as CGS 19755, NPC 12626, and CGP 37849, with possibly different ligand binding requirements from CPP, may prove useful in determining whether the above observations are specific to CPP or general to all competitive antagonists.

THE ION-CHANNEL SITE

A substantial body of information now suggests that a diverse group of substances can act as blockers of the NMDA receptor gated ion channel. The first to be identified was magnesium (Mg^{2+}), which was initially shown to be a selective depressant of depolarizing responses to NMDA rather than those to quisqualate or kainate (63). It has subsequently been shown that Mg^{2+} ions produce a voltage-dependent block of the NMDA ion channel by binding to a site deep within the ionophore (64). When examined at the single channel level using patch-clamp techniques, low concentrations of Mg^{2+} produce a flickering block of the ion channel (64) characteristic of fast open channel block (65), although this may not fully explain all of the actions of Mg^{2+}.

Several other divalent cations also act to block NMDA responses in the same manner as Mg^{2+}. However, the important feature of the block produced by Mg^{2+} is that it occurs at concentrations well below those normally present in extracellular fluid. This confers a unique voltage-, as well as agonist-, dependency on the NMDA receptor-mediated response. Thus, at normal resting membrane potentials very little NMDA receptor-mediated response is evoked by synaptic activation at low frequencies. Only when the membrane is depolarized (above −50 to −30mV) for more prolonged periods (e.g. during repetitive, high-frequency synaptic activation) do NMDA receptor-mediated responses become apparent, due to the reduction in the block produced by Mg^{2+}. This important feature of NMDA receptor activation enables it to fulfill its specialized physiological roles, e.g. induction of LTP and generation of patterned pacemakerlike activity (16, 66).

Lodge and colleagues made the first important finding that the dissociative anesthetics phencyclidine (PCP) and ketamine were NMDA antagonists when they showed that these compounds selectively blocked NMDA-induced excitations of rat and cat spinal neurones (67). Since that original observation a large number of organic molecules have been shown to act in a similar manner as NMDA antagonists, an effect thought to be mediated by an action at the level of the ion channel (68). PCP, ketamine, and the sigma opiate SKF10,047 (N-allylnormetazocine, NAMN; Figure 1) had been in the forefront of psychopharmacology research because of their stimulatory effects on behavior in animals (69–71), and the psychotomimetic effects and abuse potential of PCP and opiates of the benzomorphan series in humans (72). Interest was further strengthened by the demonstration of high affinity binding sites for PCP and NAMN in rat brain (73–76). The apparent correlation between the dissociative anesthetics and sigma compounds as displacers of [^{3}H] PCP binding and their ability to induce PCP-like behavior (77, 78) prompted the speculation that PCP produced its behavioral/psychotomimetic effects by an action at this PCP binding site in the brain. However, such a deduction was complicated by the lack of pharmacological specificity of PCP and NAMN, which have high affinity for sigma sites in brain, in addition to the NMDA ion channel (79). The mechanisms by which PCP produces its behavioral effects remain unclear, although several cellular actions have been proposed (80).

Early electrophysiological studies carried out in vitro suggested that this group of compounds did not share the same site of action as those of competitive NMDA antagonists (81, 82). The identification that they act as blockers of the NMDA receptor operated ion-channel came about largely from the work of MacDonald and coworkers (83, 84) and from the discovery that MK-801 (dizocilpine) was the most potent NMDA antagonist within this class of compounds (85). MacDonald and colleagues showed that the block by ketamine and PCP of NMDA-induced currents in voltage-

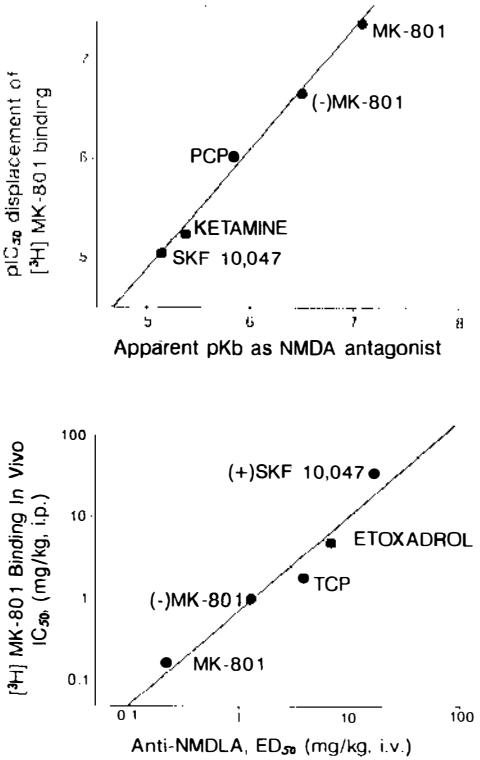

Figure 2 Top: Correlation between the potency of noncompetitive NMDA receptor antagonists as blockers of NMDA-induced depolarizations of rat cortical tissue and inhibitors of [^{3}H]-MK-801 binding to rat cortical membranes. Bottom: Correlation between the potency of noncompetitive NMDA antagonists as inhibitors of [^{3}H]-MK-801 binding in vivo and their potency as blockers of NMDLA-induced convulsions.

clamped neurones was both use- and voltage-dependent (83, 84), indicative of a channel-blocking mechanism of action. Also in the mid-1980s, it was discovered that dizocilpine bound with high affinity, in a stereoselective manner, to a single population of sites within rat brain membranes, which were regionally specific and heat labile (85, 86). Electrophysiological studies on rat cortical slices demonstrated that dizocilpine was a potent and selective blocker of NMDA receptor-mediated responses (85, 87). Furthermore, the block produced by dizocilpine was markedly agonist-dependent, requiring repeated applications of NMDA to fully develop the block (68, 87–90). A clear correlation between the ability of dizocilpine, ketamine, PCP, and other noncompetitive antagonists to displace [^{3}H]dizocilpine binding and to block NMDA receptor responses (Figure 2a) suggested that the high affinity di-

zocilpine binding site corresponded to the site responsible for mediating the NMDA antagonism of these compounds (85, 89) and presumably their anticonvulsant actions (91; Figure 2b). Subsequent patch-clamp studies on cultured neurones (90) demonstrated that the unblocking rate for dizocilpine was highly voltage-dependent, similar to the results obtained with ketamine and PCP (83). This, combined with the marked use-dependency of dizocilpine (which is probably due to its extremely slow dissociation rate (90)), provided strong evidence that this group of chemically diverse compounds block NMDA receptor responses by an open-channel blocking mechanism of action. Furthermore, the agonist-dependency of recovery from their blocking action suggests that they can become "trapped" by the ion channel closing around them (83, 84).

The agonist-dependent blocking action of dizocilpine and PCP is paralleled in studies of the binding of [^{3}H]-dizocilpine and [^{3}H]-TCP to brain membranes. These studies showed that selective binding of [^{3}H]-dizocilpine (92–94) or [^{3}H]-TCP (95–97) is markedly enhanced by NMDA receptor agonists and inhibited by competitive NMDA receptor antagonists. The enhancement appears to be due to an increase in the apparent on-rate of binding by removal of a steric barrier (presumably opening of the channel) (95). The specificity of this effect is indicated by the blockade of the glutamate stimulation of [^{3}H]dizocilpine binding by D-AP5 in a competitive manner (Figure 3a; 92), while the mixed antagonist kynurenic acid, which has actions at both the agonist recognition and the glycine site (see next section), reverses the glutamate effect by a clearly noncompetitive action (Figure 3b). These studies highlight the possibility of utilizing the binding of labeled uncompetitive antagonists that only bind to the activated state of the receptor (dizocilpine-sensitive [^{3}H]PCP binding exhibits a similar use-dependence profile; B. Ebert & E. H. F. Wong, unpublished data) as an assay for activation and blockade of the NMDA receptor, and support the concept of measuring affinity and efficacy by radioligand binding studies as first observed for the GABA/benzodiazepine/Cl channel complex (98, 99). The sensitivity of the binding of these open channel blockers to receptor activation and conformation change has allowed the detection and characterization of other novel modulatory sites on the NMDA receptor channel complex for compounds such as glycine, divalent cations, desipramine, ifenprodil, polyamines, all of which are covered in the subsequent sections.

THE GLYCINE SITE

The exciting finding that the classical inhibitory neurotransmitter, glycine, markedly potentiates the action of agonists at the NMDA receptor has generated an enormous amount of interest since it was first reported (100). In this

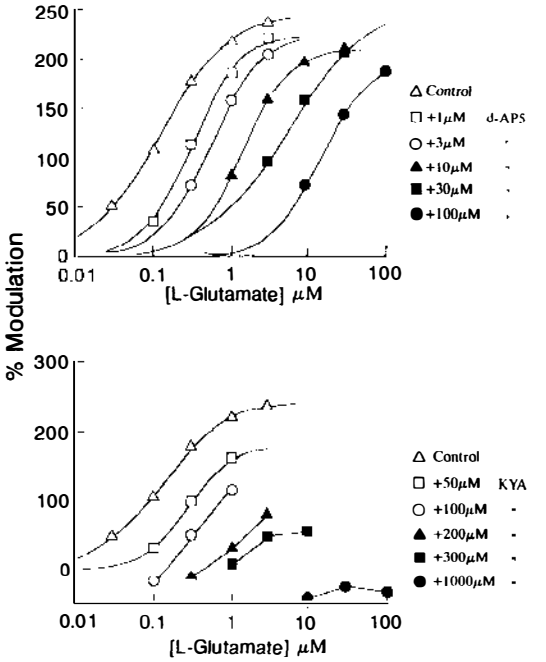

Figure 3 Antagonism of L-glutamate stimulation of [^{3}H]-MK-801 binding by D-AP5 (top) and kynurenic acid (bottom).

period insights have rapidly been made into the site and mechanism of action of glycine at the NMDA receptor complex.

The discovery of the glycine modulatory site was prompted by the observation that the magnitude of the whole cell current evoked by NMDA-receptor agonists on cultured neurones depended upon the speed and the mode of perfusion of NMDA-containing solutions (100, 101). One of the considered explanations of this result was that a substance augmenting the response to NMDA was tonically released into the environment of the cultured cell and that this was washed away when a fast perfusion was used. This theory also explained the large "off" response seen when NMDA was perfused using the U-tube technique (101, 102). Further investigations corroborated this explanation and revealed that a low molecular weight compound contained in the culture medium greatly augmented the response to NMDA. Screening of the common amino acids revealed that glycine mimicked the effects of the conditioned culture medium, producing a potent and marked increase in the size of the NMDA response (100).

Only very low concentrations of glycine are required for this effect (EC_{50}: 100–300nM), about tenfold lower than those required to activate the in-

hibitory, strychnine-sensitive receptor. A high affinity, strychnine-insensitive [^{3}H]-glysine binding site in rat brain membranes had been described by Kishimoto et al (103) and Bristow et al (104). After the discovery of the glycine potentiating effect on NMDA receptors, it was immediately noted (105) that there was a near identical neuroanatomical distribution between these [^{3}H]-glycine binding sites and those of NMDA-sensitive [^{3}H]-glutamate binding sites (54). These earlier studies had also shown that the D-isomers of serine and alanine had higher affinities for this glycine binding site than the L-isomers, with D-serine being only slightly less active than glycine itself. This agreed with the emerging pharmacology of this glycine site where D-serine was found to be a potent agonist (106, 107). Because of its lack of affinity for glycine uptake, D-serine became a useful pharmacological tool (108) for this NMDA receptor related site.

Patch-clamp recordings in outside-out patches were used to show that glycine facilitated the NMDA response by an increase in channel-opening probability (100). This observation also ruled out the possibility that this effect was mediated by a second-messenger system and it was suggested that glycine may act at an allosteric site within the NMDA-receptor/ion-channel complex. Several other observations have supported this suggestion: (*a*) The close association between the distribution of the NMDA receptor and glycine sites outlined above; (*b*) Interactions between the agonist recognition site, the ion channel and the glycine site can be seen in brain membranes using radioligand binding studies (92, 93, 107, 109–112); (*c*) These are also observed in a soluble receptor preparation, indicating that the glycine site constitutes part of the NMDA receptor protein (113); (*d*) The NMDA receptor expressed in Xenopus oocytes following injection of rat brain mRNA is also facilitated by glycine (114).

This latter study also showed that when glycine was removed from the receptor environment virtually no NMDA response could be elicited, implying that glycine acts as a coagonist at the NMDA receptor complex, i.e. agonist binding at both the glutamate recognition site and the glycine site is required for channel activation. This possibility had also been suggested by experiments with selective antagonists for the glycine site. Kynurenic acid, a well-known "broad spectrum" excitatory amino acid receptor antagonist, has a noncompetitive component to its NMDA antagonist activity (115, 116). It was found that kynurenic acid competitively inhibited strychnine-insensitive [^{3}H]-glycine binding to rat brain membranes (112) and part of its NMDA blocking effect in tissue slices could be reversed by addition of glycine, indicating an antagonist action at the glycine site (117). 7-chloro kynurenic (7-Cl KYNA) acid was shown to have a selective 70-fold increase in affinity for the glycine modulatory site (108). This compound markedly flattened NMDA responses on rat cortical tissue, an effect that could be reversed by

glycine and D-serine, and produced a complete block of NMDA responses on voltage-clamped cortical neurones in tissue culture, even those elicited in the absence of any added glycine (108). This effect suggested that 7-Cl KYNA was either a simple antagonist at the glycine site and that activation of this site was an absolute requirement for NMDA receptor activation or that it possessed negative modulatory (inverse-agonist) effects, in analogy with compounds acting at the benzodiazepine receptor on the $GABA_A$ receptor complex. The picture was further complicated by the finding that HA-966, one of the first NMDA antagonists discovered, also acted selectively at the glycine site by blocking glycine potentiation of NMDA responses in cultured neurones (118, 119) but had little effect in the absence of added glycine (119, 120). The possibilities here were that HA-966 was either a pure antagonist and blocked the glycine potentiation of NMDA responses but had no inhibitory effect of its own, or that it was a low-efficacy partial agonist and that its efficacy was similar to the level of response produced by contaminating amounts of glycine in the extracellular medium. In light of the results from Xenopus oocytes (114) and more recent observations on cultured neurones, it appears that the latter possibility is correct and that HA-966 is a partial agonist with approximately 10% of the efficacy of glycine itself (J. A. Kemp & T. Priestley, in preparation), whereas 7 Cl-kynurenic acid is an antagonist.

This low level of efficacy has important implications for the NMDA antagonist effect of HA-966. In intact adult cortical tissue, HA-966 produced a maximum threefold shift to the right of the NMDA concentration-response curve (119, 120), unlike the complete flattening produced by full antagonists (108, 120). In cortex and hippocampus, in the presence of a maximum blocking concentration of HA-966, NMDA is still able to evoke a full depolarization of these neurones. This indicates that in these brain regions, which are high in NMDA receptor density, there is a very large receptor reserve and that even 10% receptor activation can produce enough inward current to depolarize neuroncs completely.

Studies with these antagonists were also the first to demonstrate that the glycine site is functional in intact adult tissue. The glycine modulation of NMDA responses was originally shown on immature cells in tissue culture (100, 101). Most initial electrophysiological studies in vivo and in slices of adult tissue failed to show any consistent potentiating effect of glycine on NMDA responses (108, 117, 118, 121). The ability of selective glycine-site antagonists to produce a noncompetitive depression of NMDA responses, in a glycine or D-serine reversible manner, confirmed the presence of the glycine modulatory site on NMDA receptors in normal adult tissue (108, 117, 118, 121). These results also indicated that the glycine site was normally already maximally activated by the extracellular concentration of glycine present in these preparations. However, another possible explanation of these findings is

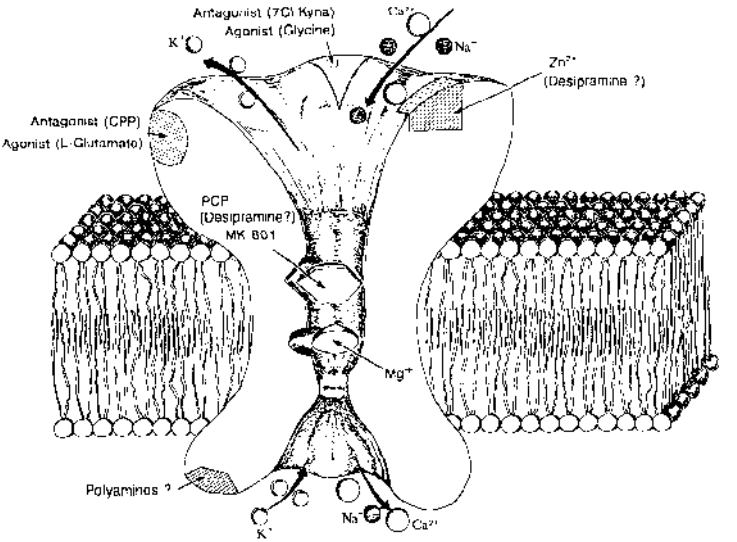

Figure 4 Schematic representation of the NMDA receptor ion channel complex illustrating sites of antagonist action (Drawn by Andy Butler).

that NMDA evokes the release of glycine from neurones or glia within the slice to levels that saturate the glycine facilitatory site. Therefore, when examined in this way, the glycine site will appear always maximally activated by glycine. Indeed, L-glutamate and L-aspartate have been shown to cause the release of glycine from retina (122) and hippocampal cultures (123).

Some support for the view that the glycine site is not always fully saturated comes from experiments in slices where NMDA has been locally applied, rather than bath-perfused. Under these conditions potentiation of NMDA receptor-mediated events by glycine have been observed (124, 125). Evidence from experiments performed in vivo, demonstrating that injections of D-serine or glycine potentiate NMDA receptor-mediated responses (126–128), also suggests that the interstitial glycine concentration can be controlled to below supersaturating levels.

THE ZINC SITE

In addition to the well-defined, voltage-dependent, antagonistic action of Mg^{2+}, increasing evidence indicates that the divalent transition metal ion Zn^{2+} also produces a selective blocking action of NMDA receptor responses (102, 129–133). At concentrations of 1 to 100 μM (Kd= 13 μM), Zn^{2+} dose-dependently attentuates the activation of NMDA receptors in mouse dissociated neuronal cell cultures (133). This action is rapid in onset and

reversible, and in contrast to Mg^{2+}, does not exhibit voltage-dependency (130, 132, 133). The difference between the mode of action of Mg^{2+} and Zn^{2+} was further highlighted by the observation that whereas Mg^{2+} increased the dissociation rate and the binding of [^{3}H]dizocilpine and [^{3}H]TCP to extensively washed frozen and thawed rat cortical membranes, Zn^{2+} has the opposite effects (113, 134–137). These results argue for a distinct site of action for these two divalent cations. Cadmium, another group IIB metal cation, also acts in a similar manner to Zn^{2+} but is roughly four times less potent. Thus, it has been proposed that at least two binding sites for divalent cations on the NMDA receptor exist: one deep within the pore of the channel (Mg^{2+} site) and a second near the extracellular surface of the receptor (Zn^{2+} site) (Figure 4). The physiological relevance of Zn^{2+} remains unknown, although Zn^{2+} has been shown to be localized in distinct brain regions (138, 139), released upon stimulation (140, 141), and may be involved in synaptic transmission of the mossy fibre axons in the hippocampus (142). It has been calculated that high K^+-induced depolarization in hippocampal slices can release sufficient Zn^{2+} to achieve a uniform extracellular concentration of 300 μM (140). Corelease of Zn^{2+} together with endogenous glutamate would therefore be expected to reduce the resultant NMDA receptor component of excitatory amino acid transmission (129, 131, 132, 143). The demonstration that binding of channel blockers such as dizocilpine (90, 92–94, 107, 144) and TCP (95–97, 134) is dependent on glycine and NMDA activation of the receptor-gated ion channel has facilitated the investigations of the mechanism of action of Zn^{2+}. Zn^{2+} has no effect on NMDA-sensitive glutamate binding (58, 137), but is able to reduce channel ligand binding (134) by a decrease in association and dissociation rates (135–137). The fact that Zn^{2+} inhibits glycine binding and glycine stimulation of [^{3}H]TCP binding has led Yeh and coworkers (137) to propose that synaptically released zinc (140–142) could reduce the tonic excitatory influence of glycine (108–114). The ability of Zn^{2+} to reduce NMDA-mediated neurotoxicity (143) might constitute an important negative feedback mechanism to control excessive synaptic excitation during epileptic seizures and other disease states involving prolonged exposure to glutamate (24).

Some tricyclic antidepressants such as desipramine and imipramine resemble Zn^{2+} in terms of their ability to modulate the kinetics (147) of [^{3}H]dizocilpine binding (113, 134, 146), and reduce NMDA receptor-mediated Ca^{2+} influx into cultured rat cortical neurons. In vivo, desipramine and other tricyclic antidepressants prevent lethality in mice induced by large doses of NMDA, with a specificity corresponding to their ability to reduce [^{3}H]dizocilpine binding (147a). While it is tempting to ascribe the NMDA receptor blocking action of these tricyclic antidepressants to the Zn^{2+} site, the voltage-sensitivity of their blockade (148) argues against an action at the same

site and suggests that they act as open channel blockers in a manner analogous to that of MK-801 and PCP.

OTHER POTENTIAL AGONIST SITES ON THE NMDA RECEPTOR CHANNEL COMPLEX

Apart from the sites discussed above, there is increasing evidence for other sites associated with the NMDA receptor channel complex. In particular, the anti-ischemic actions of the di-phenylpiperidineethanol compounds ifenprodil and its analogue SL 82.0715 (149; Figure 1d) have been ascribed more recently to their interaction with a distinct site on the NMDA receptor complex. These two compounds antagonize the NMDA-induced elevation of cGMP levels in immature rat cerebellar slices in a noncompetitive manner with moderate potency (IC_{50}s of 0.4 and 10 μM, respectively), and inhibit [^{3}H]-CPP binding to the agonist recognition site and [^{3}H]TCP binding to the channel site in a noncompetitive manner, leading to a reduced B_{max} value (150). In contrast to other channel site ligands that inhibit [^{3}H]dizocilpine binding by a mass action profile, without having any influence on the dissociation kinetics, ifenprodil gave an apparent displacement of [^{3}H]dizocilpine binding with an extremely flat curve (nH = 0.3) spanning concentrations from 10^{-9}M to 10^{-4}M, by virtue of a drop in dissociation rate (151). The inability of glycine to alter the potencies of ifenprodil in reducing [^{3}H]dizocilpine binding argues against an action of ifenprodil at the glycine site. The noncompetitive effect of ifenprodil at the agonist recognition sites is indicated by incomplete antagonism of the L-glutamate-stimulated [^{3}H]TCP binding. Functionally, ifenprodil and SL 82.0715 exert a dose-dependent, but incomplete, inhibition of NMDA-induced depolarizations of the immature rat hemisected spinal cord, and in mouse cultured spinal cord neurones (150). When given systematically, the two compounds antagonized the effect of intrastriatally administered NMDA on dopamine release in rat with ED_{50}s of 0.9 and 0.3 mg/kg (i.p), respectively. The complicated interaction between ifenprodil and the NMDA receptor complex is also indicated by the biphasic displacement curve of ifenprodil in a [^{3}H]dizocilpine binding assay (Ki_{high} = 35nM; Ki_{low} = 13 μM). Detailed analysis of the effect of ifenprodil on the dissociation of [^{3}H]dizocilpine has suggested that the low affinity component of its action may be mediated by the Zn^{2+} site (151). SL 82.0715 also resembles ifenprodil in displaying biphasic displacement of [^{3}H]dizocilpine binding. Interestingly, SL 82.0715 was less potent at the high affinity ifenprodil site but more potent at the low affinity site. In view of the reported rank order of neuroprotective activity of these two compounds (ifenprodil > SL 82.0715) when injected intraventricularly, the neuroprotective action of ifenprodil has been ascribed to its action at the high affinity site (151).

Nevertheless, it is not possible to ignore the contribution of the low affinity ifenprodil site since ifenprodil was demonstrated to inhibit NMDA/glycine-induced elevation of intracellular Ca^{2+} levels in cultured neurones from rat cortex with a potency of 10 μM (151). Recent studies with radiolabeled [^{3}H]-ifenprodil have shown that under appropriate conditions, it binds with high affinity to a site that may be associated with the NMDA receptor complex (152). This binding is partially inhibited by some competitive NMDA receptor antagonists and completely by the polyamines spermine and spermidine (see below). However, the ability of ifenprodil to interact with α-adrenergic receptors (153), voltage-dependent Ca^{2+} channels (154), and the sigma site (155) argues for a cautious approach, particularly with regard to its anti-ischemic mechanism of action.

Elegant studies by Ransom & Stec (156) have identified a site on the NMDA receptor channel complex that is sensitive to endogenous polyamines, spermine, and spermidine (157). While these polyamines to some extent resemble glutamate and glycine in their ability to enhance [^{3}H]dizocilpine (156, 158–160) and [^{3}H]TCP (161, 162) binding, the inability of spermidine or spermine to inhibit [^{3}H]glycine or [^{3}H]CPP binding argues for a distinct site of action. The allosteric coupling of these sites can be observed in terms of shifts in the response curves of glutamate and glycine for stimulation of [^{3}H]dizocilpine binding by spermine, and the reciprocal shift on the spermidine response curve on [^{3}H]dizocilpine binding by glutamate or glycine. Spermidine and spermine, but not putrescine, also directly enhance [^{3}H]glycine binding in rat cortical membranes (163). However, Sacaan & Johnson (162) only observed enhancement of [^{3}H]glycine binding with spermine and not spermidine. A coupling has been suggested between the NMDA receptor and ornithine decarboxylase (ODC; 166, 167), the rate-limiting enzyme in polyamine synthesis. The ODC inhibitor, α-difluoromethylornithine (DFMO), has been reported to have a protective action against NMDA-induced neurotoxicity (168, 169), leading to the suggestion that activation of ornithine decarboxylase may be involved in the generation of NMDA receptor-mediated neurotoxicity and that inhibitors that penetrate the brain may represent novel neuroprotective agents. However, the concentrations of DFMO required to produce these effects are very large (5mM) and the exact mechanism by which DFMO exerts its protective effect remains to be determined.

Ifenprodil and SL 82.0715 have been proposed to act at the polyamine site (169a). However, while this study clearly demonstrated a dose-dependent antagonism of the potentiating effects of spermidine on [^{3}H]TCP binding by ifenprodil and SL 82.0715, the evidence was not convincing that this was a competitive interaction. Indeed, when the interaction between ifenprodil and spermine or spermidine was investigated in the [^{3}H]dizocilpine binding assay

in membranes (151) or a solubilized preparation (146) Schild analysis of the shift in the polyamine curves failed to indicate a competitive interaction. However, initial studies of [^{3}H]ifenprodil binding have shown inhibition by polyamines (152) compatible with a competitive interaction. The precise nature of this interaction remains to be determined. While the site of action for ifenprodil remains obscure, the above studies on the action of polyamines on the NMDA receptor at concentrations that are found in the brain (157) suggest a possible mechanism for endogenous modulation of this receptor system. If the polyamine production following activation of ODC (170) has anything to do with the modulation of NMDA receptors, it seems logical that polyamines will work on a site associated with the NMDA receptor channel complex but facing the interior of the neurone. Patch-clamping studies using internal and external application of polyamines (and ifenprodil) on NMDA receptor-mediated responses should help to clarify the location of action of these modulators. Preliminary published data using whole cell patch-clamp recordings on striatal (164) and hippocampal (165) cultured neurones suggest a rapid polyamine action, presumably reflecting an effect on the extracellular face. However, in our own investigations we have been unable to show any potentiating effect of extracellularly applied spermine or spermidine on NMDA responses in cortical slices or cultured cortical neurones (J. A. Kemp, T. Priestley & G. Marshall, unpublished observations).

NMDA RECEPTOR CHANNEL COMPLEX

Information concerning the molecular composition of the NMDA receptor channel complex has been sought by solubilization studies. The conventional objective of these studies has been to serve as a first step towards the purification of this receptor protein. This will provide sequence information and ultimately allow cloning and expression of possible subtypes of NMDA receptor. However, one should not disregard the value of utilizing the solubilized receptor preparation as a tool for pharmacological investigation. Channel ligands such as [^{3}H]dizocilpine and [^{3}H]TCP have served well for this purpose because their binding affinity displays a marked agonist-dependency (92, 97), presumably a reflection of the agonist-induced conformational change that allows access of these ligands to their binding site within the channel. It follows that other modulatory sites associated with the NMDA receptor channel complex will also express themselves in the form of modulation of channel ligand binding (109–113, 134–137, 151, 156, 158–163). Hence it is of interest that on solubilization of the NMDA receptor the channel site not only maintains high affinity binding for [^{3}H]dizocilpine (113, 146, 171, 172) and [^{3}H]TCP (173), but retains the modulatory activities previously observed in membrane-binding studies. These include stimulation of binding

by NMDA agonists in a manner that is blockable by competitive antagonists, and modulation by sites sensitive to glycine, Mg^{2+} and Zn^{2+} (113). While the sites of action of ifenprodil and polyamines remain unclear, recent studies on [^{3}H]dizocilpine binding in solubilized receptor preparation indicated that ifenprodil retains the ability to reduce glutamate-stimulated [^{3}H]dizocilpine binding in a biphasic manner (146, 151). In addition, spermine and spermidine can dose-dependently enhance [^{3}H]dizocilpine binding, while putrescine, cadaverine, and ifenprodil failed to directly enhance binding but were able to antagonize the effect of spermine in a noncompetitive manner (146). These results strongly suggest that all the modulatory sites so far identified by binding and functional assays maintained their coupling to the NMDA receptor and could be integral parts of the receptor protein complex.

An alternative approach to the above studies on the molecular composition of the NMDA receptor channel complex has been to obtain molecular size information by radiation inactivation techniques (174). The result has been the identification of a molecular size of around 120,000 daltons for sites/complex labeled by [^{3}H]L-glutamate (NMDA-sensitive component), [^{3}H]glycine, [^{3}H]TCP, and [^{3}H]dizocilpine (62, 175) and this coincides with the molecular weight of the NMDA receptor labeled by [^{3}H]azido-dizocilpine (176). Taken together, these studies have suggested a molecular complex of consistent size that apparently possesses all the modulatory sites of interest. Whether this proves to be the case will await successful cloning studies on the NMDA receptor channel complex.

CONCLUSIONS

It is hoped that the identification of novel sites on the NMDA receptor complex described above will aid the development of effective anticonvulsant and neuroprotection agents with fewer untoward side-effects than currently available NMDA receptor antagonists. A promising result in this direction has been the discovery that the glycine site partial agonist, (+)-HA-966, is an anticonvulsant and yet fails to produce PCP-like locomotor behavior (177), even at very high doses. It is also of interest to note the lack of effect of ifenprodil in PCP drug-discrimination tests (178). It remains to be determined whether this apparently desirable profile of action reflects a possible heterogeneity of NMDA receptors or is the result of an incomplete switching-off of NMDA receptor-mediated events within the CNS.

It is now clear that apart from the conventional glutamate recognition site agonists, which directly activate the NMDA receptor, there are other possible avenues for promoting the activation of this receptor complex, for example, by way of the strychnine-insensitive glycine site and the polyamine modulatory site. These sites may also provide targets for the potential discovery of

novel anticonvulsants, anxiolytics, muscle relaxants, analgesics, cognitive enhancers, and neuroprotective agents (23, 25, 34, 45, 128, 178–182). Antagonists that act at the glycine or polyamine site may provide a more subtle way of damping down NMDA receptor-mediated excitability (108, 159, 182) compared to competitive antagonists or channel–blocking drugs.

The identification of arylcyclohexylamines, such as PCP, benzomorphans, such as N-allylnormetazocine, and dibenzocycloheptinimines, such as dizocilpine, as uncompetitive antagonists has made a significant contribution to our knowledge of the pharmacological complexity of the NMDA receptor complex. The discovery of the agonist-dependent nature of the binding of these ligands has greatly facilitated the identification of different modulatory sites on the NMDA receptor channel complex, analogous to the utilization of the cage convulsant TBPS for the characterization of various modulatory sites on the $GABA_A$ receptor complex (98, 183). It is anticipated that the discovery of potent, selective and CNS-penetrating antagonists for the sites discussed in this chapter will have a major impact on the therapy of CNS disorders.

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