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Glycine and N-Methyl-D-Aspartate Receptors: Physiological Significance and Possible Therapeutic Applications

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I. Introduction . . . 598
II. N-Methyl-D-aspartate receptors . . . 600
A. Basic characteristics . . . 600
B. NR1 splice variants . . . 602
C. Distribution and ontogeny . . . 602
D. Glutamate and glycine binding sites . . . 603
III. Physiological role of glycine . . . 605
A. Glycine as a coagonist . . . 605
B. Desensitization . . . 606
C. Endogenous agonists: glycine and/or D-serine . . . 610
1. Distribution within the central nervous system . . . 610
2. Uptake . . . 612
3. Release . . . 613
4. Source and synthesis . . . 613
5. Metabolism . . . 614
6. N-Methyl-D-aspartate receptor-coupled recognition site and its distribution . . . 614
7. Are glycine$_B$ sites saturated in vivo? . . . 615
D. Kynurenic acid as an endogenous antagonist of the glycine$_B$ site . . . 617
IV. Exogenous ligands . . . 617
A. Agonists of the glycine$_B$ site . . . 617
B. Partial agonists of the glycine$_B$ site . . . 618
C. Antagonists . . . 620
1. Kynurenic acid derivatives . . . 620
2. 2-Carboxyindoles . . . 621
3. 2-Carboxytetrahydroquinolines . . . 621
4. 4-Hydroxy-2-quinolones . . . 622
5. Quinoxaline-2,3-diones . . . 622
6. 3-Hydroxy-1H-1-benzazepine-2,5-diones . . . 623
7. Tricyclic glycine$_B$ antagonists . . . 623
8. Prodrugs . . . 623
9. Modification of endogenous kynurenic acid metabolism . . . 624
D. Implications . . . 624
E. ^{3}H-Radiolabeled ligands for glycine$_B$ site . . . 624
F. Allosteric interactions involving the glycine$_B$ site . . . 625
V. N-Methyl-D-aspartate receptor subtypes: differences in glycine$_B$ recognition sites . . . 626
VI. Therapeutic aspects for agents acting at the glycine$_B$ site . . . 628
A. Pharmacokinetic aspects . . . 628
B. Side effects . . . 629
1. Drug discrimination . . . 629
2. Learning impairment . . . 630
3. Ataxia, myorelaxation, and sedation . . . 631

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4. Neurotoxicity in the retrosplenial/cingulate cortex . . . 632
5. Psychotomimetic side effects . . . 633
C. Anxiety . . . 633
D. Depression . . . 634
E. Schizophrenia . . . 635
F. Convulsions and epilepsy . . . 636
G. Drug dependence and tolerance . . . 638
1. Opioids . . . 638
2. Cocaine . . . 639
H. Pain . . . 640
I. Ethanol dependence and abuse . . . 641
J. Huntington's disease . . . 642
K. Alzheimer's disease and dementia . . . 642
L. Parkinson's disease . . . 643
M. Neuroprotection against acute insults . . . 645
1. Introduction . . . 645
2. Ischemia . . . 646
N. Other neurodegenerative diseases . . . 649
VII. First clinical experiences with glycine$_B$ antagonists . . . 650
VIII. Concluding remarks . . . 650
IX. Acknowledgments . . . 650
X. References . . . 650

I. Introduction

Since the finding by Johnson and Ascher (1987) demonstrating that glycine enhances electrophysiological responses mediated by N-methyl-D-aspartate (NMDA)[b]-sensitive glutamatergic receptors, considerable interest has been devoted to this topic (for reviews, see Dingledine *et al.*, 1990; Thomson, 1990; Huettner, 1991; Carter, 1992; Kemp and Leeson, 1993; Leeson, 1993; Wood, 1995). Although Johnson and Ascher (1987) were the first to connect the strychnine-insensitive glycine recognition site with the NMDA receptor, the strychnine-insensitive effects of glycine had been recognized earlier, indicating the diversity of presumed glycine receptors (table 1). Kishimoto *et al.* (1981) reported that the saturation isotherm of Na^+-independent [^{3}H]glycine binding in the medulla oblongata and spinal cord (but not cerebral cortex) showed biphasic features. In this assay, D-serine was surprisingly >40 times more potent as an inhibitor of [^{3}H]glycine binding in the cortex than was L-serine, and strychnine was inactive, indicating that these sites were not related to the inhibitory glycine receptors forming Cl^- channels (Young and Snyder, 1974). Later, it was reported that the distribution of [^{3}H]glycine and [^{3}H]strychnine binding is not colocalized, but rather complementary, being strongest in the forebrain and pons/spinal cord, respectively (Bristow *et al.*, 1986). Johnson and Ascher (1987) then published their observations, based on patch-clamp experiments in primary cultures of mouse cortical neurons, that the magnitude of NMDA responses was dependent on the speed of perfusion of agonist solution, i.e., slower flow resulted in larger responses. Subsequently, by using conditioned medium or adding exogenous glycine, it was clarified that the enhancement of NMDA responses was the result of glycine released from cultured cells. These authors reported similar augmentation when serine or alanine was used, although the differences between isomers were not established. Soon after, at the Society for Neuroscience meeting in 1987, it was reported that glycine enhanced 3′,5′-cyclic guanidine monophosphate (cGMP) levels in the cerebellum in vivo after intracisternal application (Danysz *et al.*, 1987, 1989c). This finding was soon confirmed in the same experimental model (Wood *et al.*, 1989), and both studies indicated that the glycine site of NMDA receptors is not always saturated

[b] Abbreviations: ACBC, 1-aminocyclobutanecarboxylic acid; ACPC, 1-aminocyclopropanecarboxylic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic; AP5, 2-amino-5-phosphonopentanoic acid; ASCT, neutral amino acid transporter; B_{max}, maximal binding; cGMP, 3′,5′-cyclic guanidine monophosphate; 7-Cl-KYN, 7-chlorokynurenic acid; CNS, central nervous system; CPP, 3-[(R)-2-carboxypiperazine-4-yl]propyl-1-phosphonic acid; CSF, cerebrospinal fluid; DCQX, 6,7-dichloroquinoxaline-2,3-dione; 5,7-diCl-KYN, 5,7-dichlorokynurenic acid; EC_{50}, 50% effective concentration; ECF, extracellular fluid; ED_{50}, 50% effective dose; EPSP, excitatory postsynaptic potential; GABA, γ-aminobutyric acid; GLYT, glycine transporter; HEK, human embryonic kidney; IC_{50}, 50% inhibitory concentration; i.c.v., intracerebroventricular(ly); ID_{50}, 50% inhibitory dose; i.p., intraperitoneal(ly); i.v., intravenous(ly); K_b, affinity calculated according to the Cheng-Prussoff relationship; LTP, long term potentiation; MCAo, middle cerebral artery occlusion; MES, maximal electroshock; MNQX, 5,7-dinitroquinoxaline-2,3-dione; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mRNA, messenger ribonucleic acid; NMDA, N-methyl-D-aspartate; PCP, phencyclidine; p.o., oral(ly); PTZ, pentylenetetrazol; SNc, substantia nigra pars compacta; TCP, thienylcyclohexylpiperidine; TI, therapeutic index; TM, transmembrane domain.

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TABLE 1
Chronology of milestones leading to the development of our current knowledge on the modulation of NMDA receptors through the strychnine-insensitive glycine site

Year	Observation	References
1974	Glycine enhancement of cGMP in the cerebellum is strychnine independent	Mao *et al.,* 1974
1978	[3H]Glycine binding is only partially strychnine sensitive	DeFeudis *et al.,* 1978
1978	HA-966 attenuates excitatory amino acid responses in isolated spinal cord of frogs and immature rats	Biscoe *et al.,* 1978
1979	Glycine enhances K^+-stimulated dopamine release in striatal prisms in a strychnine-insensitive manner	Martin and Mitchell, 1979
1981	Strychnine-insensitive binding sites for [3H]glycine are distributed mainly in forebrain areas and inhibited by D-amino acids	Kishimoto *et al.,* 1981
1986	Receptor autoradiography shows that strychnine-insensitive [3H]glycine binding predominates in the forebrain and cerebellum, whereas [3H]strychnine labels lower brain areas (e.g., medulla oblongata)	Bristow *et al.,* 1986
1987	Glycine enhances NMDA responses in patch-clamp studies in cortical neurons in a strychnine-insensitive manner	Johnson and Ascher, 1987
1987	Glycine enhances binding of NMDA channel blockers in a strychnine-insensitive manner	Bonhaus *et al.,* 1987; Reynolds *et al.,* 1987; Snell *et al.,* 1987; Wong *et al.,* 1987
1987	In vivo, glycine enhances NMDA-stimulated cGMP synthesis in mouse and rat cerebellum; its recognition site is not saturated	Danysz *et al.,* 1987, 1989c
1987	Glycine enhances NMDA receptor-mediated EPSPs in cultured neurons	Forsythe *et al.,* 1987, 1988
1987	Kynurenic acid is found to be the first glycine$_B$, site antagonist (not very selective)	Kessler *et al.,* 1987, 1989b
1988	CNQX[a] and DNQX are nonselective glycine site antagonists	Birch *et al.,* 1988a,b
1988	Glycine enhances the affinity of [3H]glutamate	Nguyen *et al.,* 1987; Fadda *et al.,* 1988
1988	HA-966 attenuates NMDA responses by acting at the glycine site	Fletcher and Lodge, 1988
1988	7-Cl-KYN is introduced as the first selective full antagonist of the glycine site; HA-966 is a partial agonist	Kemp *et al.,* 1988a,b
1988	Occupation of the glycine site is necessary for the activation of NMDA receptors (coagonist concept)	Kleckner and Dingledine, 1988
1988	In vivo, glycine potentiates strychnine-induced convulsions by acting through the NMDA receptor	Larson and Beitz, 1988
1989	Glycine inhibits desensitization of NMDA receptors	Mayer *et al.,* 1989a
1989	In vivo, D-serine excites some thalamic neurons in a strychnine-insensitive manner; its recognition site is not saturated	Salt, 1989
1989	In vivo, D-serine enhances NMDA-stimulated cGMP synthesis in mice and rat cerebellum; its recognition site is not saturated	Wood *et al.,* 1989
1989	D-Serine enhances Ca^{2+} influx induced by NMDA in cultures of cerebellar granule cells	Wroblewski *et al.,* 1989
1993	D-Serine is present in the CNS and might be an endogenous agonist at the strychnine-insensitive glycine site	Hashimoto *et al.,* 1993c
1994	The glycine recognition site is located on the NR1 subunit	Kuryatov *et al.,* 1994
1997	GLYT1 uptake can very efficiently regulate local glycine levels	Supplisson and Bergman, 1997

[a] CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione.

in vivo (see Section III.C.7. for a more detailed discussion). At the same Neuroscience Society Meeting, glycine was also shown to potentiate a slow excitatory postsynaptic potential (EPSP) between hippocampal neurons in culture (Forsythe *et al.*, 1987), and the first glycine$_B$ antagonist, kynurenic acid, was introduced (Kessler *et al.*, 1987, 1989b); this is of particular interest considering that kynurenic acid is an endogenous substance (Stone *et al.*, 1987).

Another important milestone was the report by Kleckner and Dingledine (1988) indicating that glycine is in fact probably obligatory for the activation of NMDA receptors. In turn, the term "coagonist" was proposed. This finding has very important implications, because it indicates that maximal inhibition of the glycine site should result in total inhibition of NMDA receptor activity, which in fact has been confirmed in several studies (see Section III.A.). Therefore, this opened new avenues for drug development targeted at the modulation of NMDA receptors.

One of the concerns regarding glycine have been its dual role in the central nervous system (CNS). It activates both inhibitory strychnine-sensitive receptors and the NMDA receptor-coupled site. Therefore, a selective endogenous ligand for the latter site has been sought. Hashimoto *et al.* (1993c) were the first to show that D-serine is present in the brain at levels that might affect the glycine$_B$ recognition site (for review, see Hashimoto and Oka, 1997). Since that report, at least two candidates have been proposed to be endogenous agonists for the glycine site of NMDA receptors.

One of the major problems in accepting the dynamic role of glycine or D-serine in the regulation of NMDA receptor function was the apparent lack of sufficiently robust regulatory mechanisms (uptake, release, and metabolism). Also, considering the micromolar concentrations of glycine present in the extracellular fluid (ECF) (Matsui *et al.*, 1995), some authors strongly suggested that these glycine sites must be saturated under physiological conditions. However, using coexpression of glycine transporter (GLYT)1 and NMDA receptors in oocytes, it was recently shown that very efficient buffering of local glycine concentration exists, leading to a >100-fold concentration gradient (Supplisson and Bergman, 1997). This strongly implies that the glycine concentra-

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tion might be well below its K_d value for the glycine site at NMDA receptors if this transporter is expressed sufficiently at the right location in vivo.

There is no uniformly accepted term for the "NMDA receptor-coupled, strychnine-insensitive, glycine modulatory site," which is both confusing and highly impractical. For purely pragmatic reasons, we abbreviate this as the "glycine$_B$ site," in contrast to the inhibitory, strychnine-sensitive, "glycine$_A$ site."

II. N-Methyl-D-Aspartate Receptors

A. Basic Characteristics

NMDA-sensitive ionotropic glutamate receptors probably consist of tetrameric, heteromeric, subunit assemblies that have different physiological and pharmacological properties and are differentially distributed throughout the CNS (Seeburg, 1993; Hollmann and Heinemann, 1994; McBain and Mayer, 1994; Danysz *et al.*, 1995a; Parsons *et al.*, 1998b). The exact subunit stoichiometry of these subunit assemblies is still a matter of debate. Although previous data were consistent with pentameric α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors (Wenthold *et al.*, 1992; Brose *et al.*, 1993, Wu and Chang, 1994; Ferrer Montiel and Montal, 1996; Sutcliffe *et al.*, 1996; Premkumar and Auerbach, 1997), recent findings indicate that both AMPA receptors (Rosenmund *et al.*, 1998) and NMDA receptors (Laube *et al.*, 1998) are in fact tetrameric assemblies. However, the debate is not yet over, because two studies using similar single-channel recording techniques came to very different conclusions regarding the number of NR1 subunits, with Behe *et al.* (1995) claiming two copies and Premkumar and Auerbach (1997) claiming three.

These receptors are positively modulated by glycine, which, as mentioned, binds to a specific, strychnine-insensitive, glycine$_B$ site (see Section II.D.), by polyamines (spermine and spermidine), by histamine, and, under some conditions, by cations (fig. 1). NMDA receptors are coupled to high conductance cationic channels permeable to K^+, Na^+, and Ca^{2+} (McBain and Mayer, 1994).

The NMDA channel is blocked in a use- and voltage-dependent manner by Mg^{2+} (fig. 1). This means that NMDA receptors are activated only after depolarization of the postsynaptic membrane by, for example, AMPA receptor activation, which relieves the voltage-

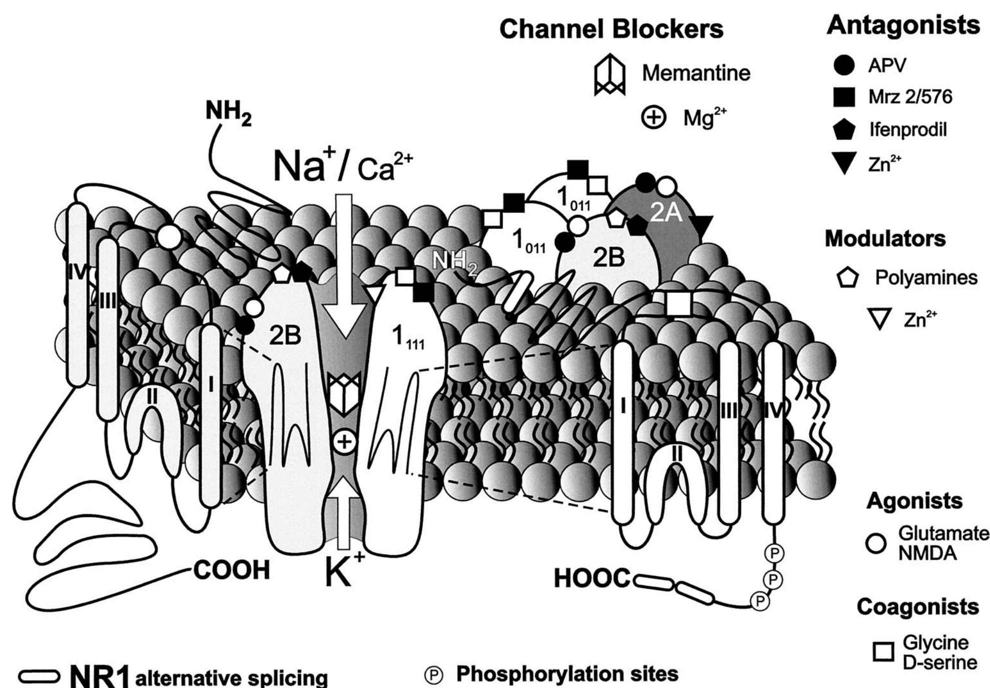

FIG. 1. Model showing that NMDA receptors are probably heteromeric assemblies of four subunits. Each subunit has four hydrophobic regions, although only three form membrane-spanning domains (TM1, TM2, and TM4). TM2 makes a hairpin bend within the membrane and forms the channel pore; the "TM" terminology is therefore inappropriate. Functional NMDA receptor complexes are formed by combinations of NR1 and NR2 subunits, which contain the glycine and glutamate recognition sites, respectively. Alternative splicing at three exons, one in the amino-terminal domain (N1) and two in the carboxyl-terminal domain (C1 and C2), generates eight isoforms for the NR1 subfamily. The NR2 subfamily consists of four individual subunits, NR2A to NR2D. Competitive antagonists such as APV probably bind to one site, which may be distinct from the agonist recognition sites but isosterically coupled in such a way as to allow competitive interactions. Glycine is a coagonist at the glycine$_B$ site and prevents Ca^{2+}-independent receptor desensitization. MRZ 2/576 is an example of a glycine$_B$ antagonist. Polyamines such as spermine and spermidine are positive modulators but also block the channel at higher concentrations. All heteromeric and homomeric NMDA receptor subtype complexes are permeant to Ca^{2+}, Na^+, and K^+. The open NMDA channel is blocked by Mg^{2+} and uncompetitive NMDA receptor antagonists, such as memantine and (+)MK-801, in a voltage-dependent manner, although the speed and voltage dependence of this effect depend on the antagonist affinity and the subunit composition. Ifenprodil is a selective antagonist for NR2B-containing receptors. Zn^{2+} is a potent, voltage-independent antagonist at NR2A-containing receptors. In addition, most NMDA receptors are influenced by Zn^{2+} ions in a voltage-dependent manner, as well as by oxidation/reduction and pH.

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dependent blockade by Mg^{2+}. This biophysical property and their high Ca^{2+} permeability render NMDA receptors inherently suitable for their role in mediating synaptic plasticity, such as that underlying learning processes and development (Collingridge and Singer, 1990; Danysz *et al.*, 1995b). Similar to Mg^{2+}, uncompetitive NMDA receptor antagonists such as ketamine, dextromethorphan, memantine, phencyclidine (PCP), and (+)MK-801 [(+)5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate] block the NMDA channel in the open state, although the blocking kinetics and voltage dependence of this effect depend on the antagonist (Rogawski, 1993; Parsons *et al.*, 1995).

To date, two major subunit families, designated NR1 and NR2, have been cloned. It is generally accepted that functional receptors in the mammalian CNS are only formed by combination of NR1 and NR2 subunits, which express the glycine and glutamate recognition sites, respectively (Kuryatov *et al.*, 1994; Grimwood *et al.*, 1995b; Wafford *et al.*, 1995; Hirai *et al.*, 1996; Williams *et al.*, 1996; Laube *et al.*, 1997; Anson *et al.*, 1998) (figs. 1 and 2).

The NR2 subfamily consists of four individual subunits, i.e., NR2A to NR2D (Nakanishi *et al.*, 1990, 1992; Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992; Hollmann and Heinemann, 1994; McBain and Mayer, 1994; Danysz *et al.*, 1995a; Parsons *et al.*, 1998b). Various heteromeric NMDA receptor channels formed by combinations of NR1 and NR2 subunits are known to differ in gating properties, magnesium sensitivity, and pharmacological profile (Sucher *et al.*, 1996; Parsons *et al.*, 1998b). The heteromeric assembly of NR1 and NR2C subunits, for instance, has much lower sensitivity to Mg^{2+} but increased sensitivity to glycine and very restricted distribution in the brain. In situ hybridization has revealed overlapping but different expression profiles for NR2 messenger ribonucleic acid (mRNA). For example, NR2A mRNA is distributed ubiquitously (like NR1), with the highest densities occuring in hippocampal regions, and NR2B is expressed predominantly in forebrain but not in cerebellum, where NR2C predominates; NR2D is localized mainly in the brainstem (Moriyoshi *et al.*, 1991; Monyer *et al.*, 1992; Nakanishi, 1992; McBain and Mayer, 1994). NMDA receptors cloned from murine CNS have a different terminology, compared with those from rats (Kutsuwada *et al.*, 1992); ζ1 remains the designation for the mouse equivalent of NR1, and ϵ1 to ϵ4 represent NR2A to -2D subunits, respectively.

In addition to NR1 and NR2, the NR3A subunit has recently been discovered. This receptor subunit (previously termed chi-1, or NMDAR-L) is a relatively recently

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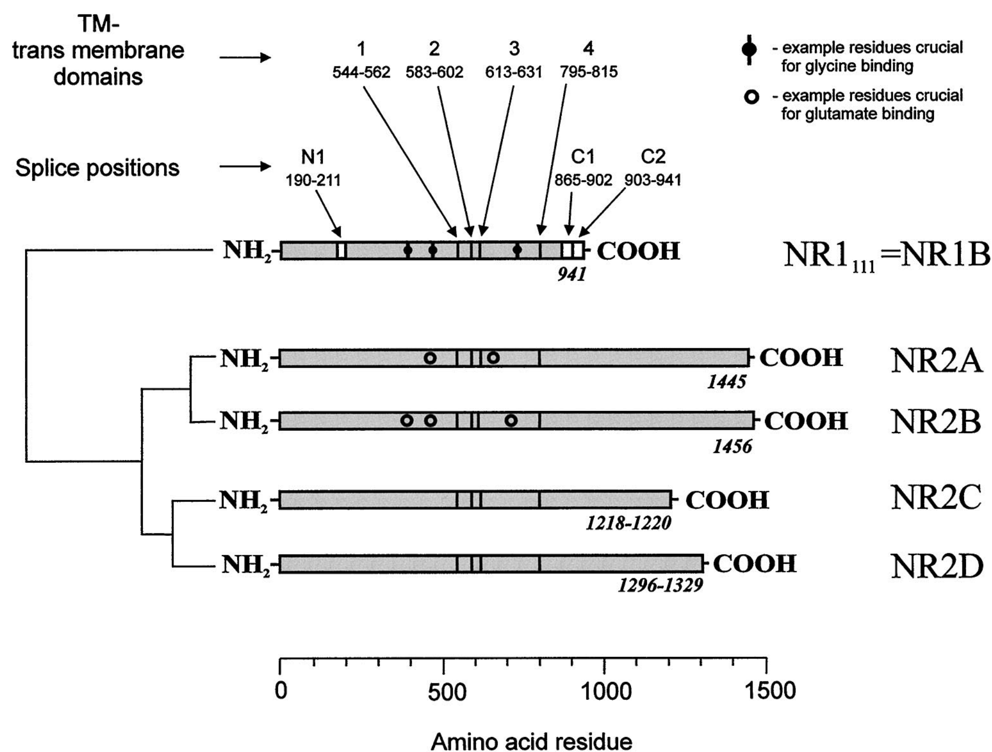

FIG. 2. Schematic presentation of the NMDA receptor subunit family. The tree indicates the degree of homology. For clarity, a NR1 receptor subunit splice variant having all alternatively spliced exons, i.e., N1 (exon 5, 21 amino acids), C1 (exon 21, 37 amino acids), and C2 (exon 22, 38 amino acids), is shown (Zukin and Bennett, 1995). Therefore, the TM and point mutation positions are numbered taking into account the presence of the N1 exon, i.e., positions are shifted by 21 amino acids, compared with original publications. Amino acid residues crucial for the binding of glutamate at NR2A or NR2B subunits and that of glycine at NR1 subunits are shown. The marks (closed and open circles) indicate residues at which point mutations resulted in at least 100-fold decreases in the affinity of glycine or glutamate. Glutamate binding sites also exist in the NR2C and NR2D subunits, but their exact locations have not been defined and are therefore not indicated in the scheme.

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identified member of a new class in the ionotropic glutamate receptor family that attenuates NMDA receptor currents when coexpressed with NR1/NR2 subunits in *Xenopus* oocytes but has no effect when tested with non-NMDA receptors or when expressed alone (Ciabarra *et al.*, 1995; Sucher *et al.*, 1995; Das *et al.*, 1998). This subunit has an open reading frame coding for a predicted polypeptide of 1115 amino acids (with a predicted mass of 110 kDa) and shares 23% homology with other NMDA subunits and 27% homology with non-NMDA subunits. Highest levels are present in the spinal cord, brainstem, hypothalamus, thalamus, CA1 field of the hippocampus, and amygdala, and this distribution remains the same throughout life. However, the absolute levels show a strong peak between postnatal days 7 and 14, which then declines to adulthood. This subunit is expressed as a glycosylated protein subunit (135 kDa) with a distribution that parallels that observed for its mRNA, as determined by in situ hybridization (Ciabarra and Sevarino, 1997). Genetic knockout of NR3A in mice results in enhanced NMDA responses and increased dendritic spines in early postnatal cortical neurons, suggesting that NR3A is involved in the development of synaptic elements by modulating NMDA receptor activity (Das *et al.*, 1998).

Before the molecular biological features of NMDA receptors were resolved, it was reported (Sekiguchi *et al.*, 1990) that NMDA receptors expressed from mRNA from guinea pig cerebellum (as opposed to other brain regions) do not show sensitivity to glycine. However, strychnine-insensitive glycine binding is present in the cerebellum (Bristow *et al.*, 1986; Danysz *et al.*, 1989c), and glycine potentiates electrophysiological responses to NMDA in the cerebellum in situ (Netzeband *et al.*, 1990). It should be stressed that in the cerebellum there are mainly NMDA receptors containing NR2C subunits (with NR1), which show strong sensitivity to glycine (Matsui *et al.*, 1995), and the failure of Sekiguchi *et al.* (1990) to observe further facilitation may have been the result of saturation of the glycine$_B$ site by background levels of glycine.

B. NR1 Splice Variants

Because of the location of the glycine recognition site on the NR1 subunit, this review focuses more on the molecular biological features and ontogeny of this subunit. For more information on the molecular biological characteristics of NR2 subunits, readers are referred to previous overviews (Seeburg, 1993; Hollmann and Heinemann, 1994; McBain and Mayer, 1994).

Alternative splicing generates eight isoforms for the NR1 subfamily (Nakanishi *et al.*, 1992; Durand *et al.*, 1993; Zukin and Bennett, 1995). The variants arise from splicing at three exons; one encodes a 21-amino acid insert in the amino-terminal domain (N1, exon 5), and two encode adjacent sequences of 37 and 38 amino acids in the carboxyl-terminal domain (C1, exon 21, and C2, exon 22, respectively) (figs. 1 and 2). NR1 variants are sometimes denoted by the presence or absence of these three alternatively spliced exons (N1, C1, and C2); $NR1_{111}$ has all three exons, $NR1_{000}$ has none, and $NR1_{100}$ has only the amino-terminal exon (Durand *et al.*, 1993; Zukin and Bennett, 1995). The variants from $NR1_{000}$ to $NR1_{111}$ are alternatively denoted as NR1E, -C, -D, -A, -G, -F, -"H", and -B or NMDAR1–4a, -2a, -3a, -1a, -4b, -2b, -3b, and -1b, respectively, but the more frequently used terminology uses noncapitalized suffixes for the most common splice variants, i.e., NR1a ($NR1_{011}$, NMDAR1–1a, or NR1A) and NR1b ($NR1_{100}$, NMDAR1–4b, or NR1G). This terminology is very confusing, and in our opinion the most logical and useful is that proposed by Zukin and Bennett (1995), i.e., $NR1_{xxx}$. The human NR1 gene is composed of 21 exons distributed over a total length of approximately 31 kilobases. Exons 4, 20, and 21 are identical in their amino acid sequences to N1, C1, and C2, respectively, in rats, suggesting that all eight NR1 isoforms found in rats would also be expressed in the human brain (Lebourdelles *et al.*, 1994; Zimmer *et al.*, 1995). In contrast, others reported that the human NR1 sequence diverges from the rodent and murine homologues near the carboxyl terminus (Planellscases *et al.*, 1993). Studies on the function of these splice variants in homomeric receptors expressed in *Xenopus* oocytes must be viewed with caution, because homomeric NR1 receptors (Durand *et al.*, 1993; Planellscases *et al.*, 1993; Rodriguez Paz *et al.*, 1995) are probably only functionally expressed because of the presence of an endogenous NR2-like protein (XenU1) in these cells (Barnard, 1997; Soloviev and Barnard, 1997).

C. Distribution and Ontogeny

The highest levels of NR1 mRNA in the adult rat and mouse CNS are in the olfactory bulb, and the lowest levels are expressed in the spinal cord. Intermediate levels were found in frontal cortex, hippocampus, cerebellum, and whole brain (Franklin *et al.*, 1993; Akazawa *et al.*, 1994). Similar findings have been reported with antibodies to NR1 subunits (Petralia *et al.*, 1994; Benke *et al.*, 1995). mRNA for double-splice variants in the C1/C2 regions, such as $NR1_{011}$ (NR1a), show an almost complementary pattern with respect to those lacking both of these inserts, such as $NR1_{100}$ (NR1b); the former are more concentrated in rostral structures such as cortex, caudate, and hippocampus, whereas the latter are principally found in more caudal regions such as thalamus, colliculi, locus coeruleus, and cerebellum (Laurie and Seeburg, 1994b; Luque *et al.*, 1995a; Paupard *et al.*, 1997). Others reported that the predominant splice variants in cortex and hippocampus were those without the N1 insert (also NR1a), whereas in the cerebellum the major variant was $NR1_{100}$ (NR1b), containing N1 (Zhong *et al.*, 1995). In other words, cell-specific patterns for NR1 mRNA lacking N1 inserts parallel those for mRNA containing C1/C2 inserts ($NR1_{011}$, NR1a) and

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vice versa ($NR1_{100}$, NR1b). In the hippocampus, NR1a mRNA shows high levels in all regions, whereas $NR1_{100}$ is expressed more intensely in CA3 pyramidal neurons (Paupard *et al.*, 1997). mRNA for $NR1_{001}$ and $NR1_{101}$ splice forms is found nearly homogeneously throughout the adult CNS, whereas mRNA with alternative splicing at C1 but not C2 ($NR1_{010}$ and $NR1_{110}$) is scarce, being detected only at very low levels in postnatal cortex and hippocampus (Laurie and Seeburg, 1994b; Paupard *et al.*, 1997). Important from a methodological perspective is the finding that the predominant splice variants in cultured cortical neurons are also those lacking the N1 insert, such as $NR1_{011}$ (NR1a) (Zhong *et al.*, 1994).

In developing rats, NR1 mRNA levels in cortex and hippocampus increased nearly three-fold from postnatal day 3 to day 15 and approximately doubled from day 15 to day 67 (Franklin *et al.*, 1993; Riva *et al.*, 1994; Nowicka and Kaczmarek, 1996). In contrast, cerebellum and brainstem showed no change in NMDAR1 mRNA levels between postnatal days 3 and 15 but levels also doubled from day 15 to day 67 (Franklin *et al.*, 1993). Similar results were reported by a different group, although levels in the hippocampus peaked at postnatal day 10 and declined thereafter (Pujic *et al.*, 1993). In the hippocampus, NR1 mRNAs lacking the N1 insert (such as $NR1_{011}$) dominate at birth and exhibit mature patterns of labeling, with high levels of expression in the CA1 and CA3 regions and the dentate gyrus. In contrast, mRNAs containing this insert (such as $NR1_{100}$) are initially expressed at lower uniform levels but levels increase more in the CA3 region than in the CA1 region or the dentate gyrus in the second and third postnatal weeks (Paupard *et al.*, 1997).

Antisera against the carboxyl- and amino-terminal domains of NR1 receptors revealed similar distributions, which increased strongly in most brain regions until postnatal day 21; the exception was that carboxyl-terminal domain staining decreased in the thalamus, tectum, and brainstem, possibly because of the emergence of carboxyl-terminal splice variants not recognized by the antiserum (Benke *et al.*, 1995; Luo *et al.*, 1996). Interestingly, the amino acid sequences contained within the seven-amino acid, carboxyl-terminal domain of C1 NR1 splice variants and all NR2 subunits may serve to localize NMDA receptors to synaptic domains by interactions with postsynaptic density protein-95 (Ehlers *et al.*, 1995; Kornau *et al.*, 1995). An important finding is that the absolute density of NR1 receptors detected with antisera is close to that found using [^{3}H]MK-801 binding, suggesting that most of the NR1 subunits expressed in the brain exist in an active form (Luo *et al.*, 1996).

NR1 subunit immunostaining in the rat visual cortex is associated with the plasma membrane at early stages of development, before innervation by axons, whereas clustering of receptors at junctions may be promoted by axonal contact (Aoki *et al.*, 1994; Aoki, 1997). At all ages, the prevalence of NR1-immunoreactive profiles was lamina 1 > laminae 4/5 > laminae 6/6B (Aoki *et al.*, 1994). In contrast, others reported that cat and ferret cortical neurons initially show high levels of immunostaining for NR1, which then decline gradually during development, with the notable exception of cortical layers 2/3, where levels of NMDAR1 immunostaining remain high into adulthood (Catalano *et al.*, 1997).

D. Glutamate and Glycine Binding Sites

In human embryonic kidney (HEK) 293 cells expressing homomeric NMDAR1 receptors, significant levels of specific binding of the $glycine_B$ antagonists ^{3}H-labeled L-689,560 [4-*trans*-2-carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline] (fig. 3) and MDL-105,519 [(E)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1H-indole-2-carboxylic acid] (fig. 4) but not of glutamate antagonists or [^{3}H]MK-801 were seen (Grimwood *et al.*, 1995b; Siegel *et al.*, 1996). Similarly, Lynch *et al.* (1994) reported, in HEK 293 cells transfected with the NR1 subunit, significant binding to the $glycine_B$ site [^{3}H-labeled 5,7-dichlorokynurenic acid (5,7-diCl-KYN)] (fig. 5) but not to the NMDA site [^{3}H-labeled CGP-37849 [DL-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid]] or the PCP site ([^{3}H]MK-801).

It should also be noted that NR2 subunits contribute to the affinity of glycine. With homomeric NR1 receptors the glycine K_i is in the range of 1 to 5 μM, but with some heteromeric receptors it is in the nanomolar range (Grimwood *et al.*, 1995b). Interestingly, glycine affinity with wild-type receptors is usually higher, i.e., 100 to 300 nM (Kishimoto *et al.*, 1981; Danysz *et al.*, 1990; Grimwood *et al.*, 1992).

Site-directed mutagenesis of the NR1 subunit at residues corresponding to positions forming the binding site of homologous, bacterial, amino acid-binding proteins indicates conservation of a common amino acid-binding

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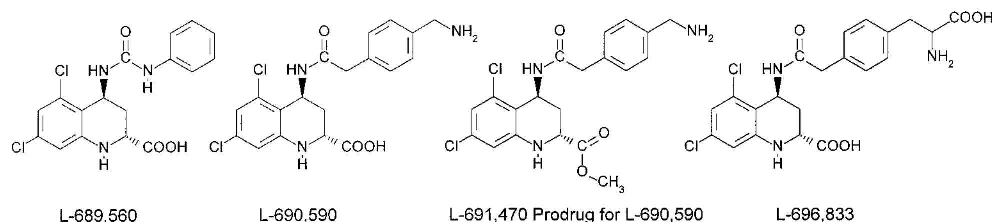

FIG. 3. 2-Carboxytetrahydroquinoline antagonists of the $glycine_B$ site.

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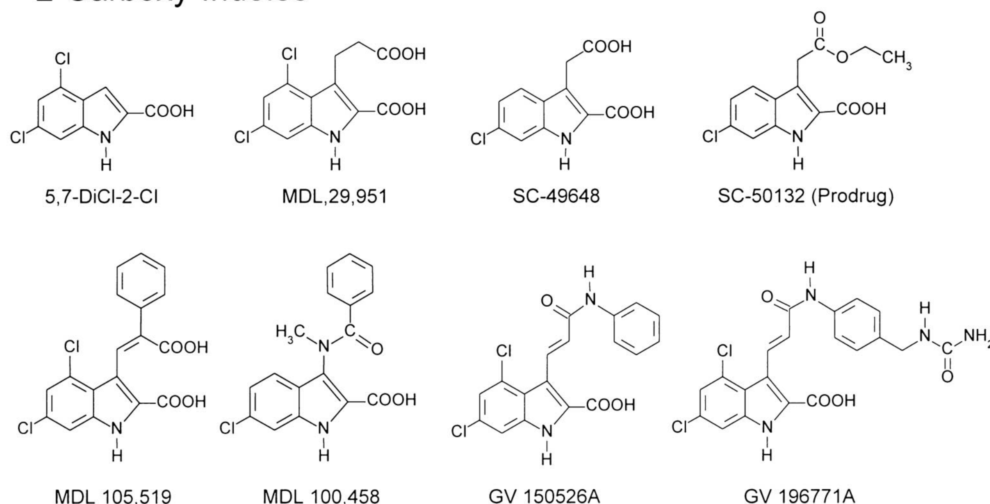

FIG. 4. 2-Carboxyindole (CI) antagonists of the glycine$_B$ site.

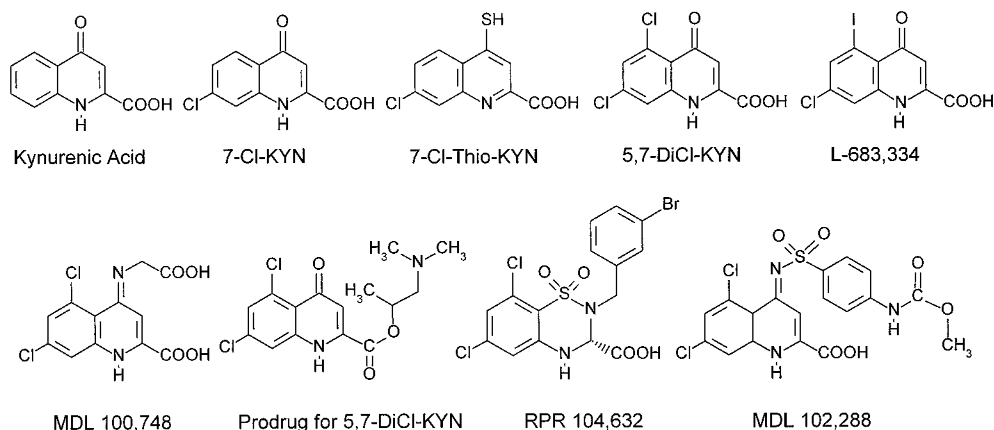

FIG. 5. Selected kynurenic acid derivatives that are antagonists of the glycine$_B$ site.

fold from prokaryotic periplasmic proteins to glutamate receptors in the mammalian brain (Kuryatov *et al.*, 1994). Glutamine substitutions at position 387 in NR1 subunits reduce glycine affinity (Kuryatov *et al.*, 1994). Similarly, replacement of cysteines 402 and 418 by alanine largely abolishes the potentiation of glutamate currents by glycine (Laube *et al.*, 1993) (fig. 2). These residues in the amino-terminal domain of NR1 subunits are extracellular before transmembrane domain (TM)1. It should be noted that these mutations in NR1 subunits had little effect on glutamate binding or the affinity of the glycine$_B$ antagonist 7-chlorokynurenic acid (7-Cl-KYN) (fig. 5). However, site-directed mutagenesis of the NR1 subunit expressed in *Xenopus* oocytes revealed that aromatic residues at positions 390, 392, and 466 are crucial determinants of both agonist and antagonist binding, as observed in patch-clamp experiments (Kuryatov *et al.*, 1994). Glutamate efficacy was little affected by these mutations, but inhibition by 7-Cl-KYN was also greatly reduced (Kuryatov *et al.*, 1994). Very similar findings were reported for human NR1 receptors, expressed in *Xenopus* oocytes, with mutation of residues 481 and 483 (Wafford *et al.*, 1995).

Valine and serine substitutions in NR1 subunits at positions 666 and 669, respectively (in the loop between TM1 and TM4) (figs. 1 and 2), were also found to reduce glycine efficacy (Kuryatov *et al.*, 1994). Others found that alternative mutation of D669 in NR1a to asparagine, alanine, or glutamate had little effect on the potency of glycine but abolished the "glycine-independent" form of spermine stimulation, indicating the importance of this residue for the binding of polyamines and/or the formation of part of the proton sensor (Kashiwagi *et al.*, 1996). Mutation of alanine at position 714 also greatly reduced the apparent affinity for glycine (Wood *et al.*, 1997), as did substitutions of the phenylalanine residues at positions 735 or 736 (Hirai *et al.*, 1996). Interestingly, these mutations did not alter the affinity of 7-Cl-KYN, indicating that this part of the extracellular domain contributes to glycine binding but not antagonist binding. Mutation of D732 to glutamate (D732E), asparagine (D732N), alanine (D732A), or glycine (D732G) not only

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dramatically reduced the potency of glycine but also changed the sensitivity to other glycine site agonists (and, in some cases, their efficacy) (Williams *et al.*, 1996) (fig. 2). For example, D-serine was a full coagonist at receptors containing NR1(D732N) and NR1(D732A), a partial agonist at receptors containing NR1(D732G), and a competitive antagonist at receptors containing NR1(D732E) (Williams *et al.*, 1996). All of these residues are found in the extracellular M3-M4 loop (figs. 1 and 2).

Homology-based molecular modeling of the glutamate and glycine binding domains indicates that the NR2 and NR1 subunits use similar residues to form their respective agonist binding sites. Therefore, similar mutations of residues within the amino-terminal domain (E387A and K459E) and the loop region between segments TM3 and TM4 (S664G) of NR2B subunits reduced the potency of glutamate >100-fold but had no effect on glycine affinity (Laube *et al.*, 1997). Mutations in NR2A subunits (T671A) dramatically reduced glutamate potency and produced faster deactivation kinetics, without changing channel gating or the affinity for glycine (Anson *et al.*, 1998). Similarly, NR2 subunits containing mutations at NR2A position D731 and NR2B position D732, which correspond to NR1 position D732, did not produce functional receptors when coexpressed with NR1 (Williams *et al.*, 1996). Taken together, these results indicate that the extracellular region before TM1 and the extracellular TM3-TM4 loop form a ligand-binding pocket for glutamate and glycine in NR2 and NR1 subunits, respectively; these findings provide the basis for a refined model for agonist and coagonist binding sites of the NMDA receptor (Hirai *et al.*, 1996).

The absolute stoichiometry of NMDA receptor subunits is not clear. It is widely accepted that NMDA receptors are activated only after the binding of glutamate to at least two NR2 subunits for each receptor (Mayer *et al.*, 1989b; Javitt *et al.*, 1990; Patneau and Mayer, 1990; Curras and Dingledine, 1992; Sather *et al.*, 1992; Wafford *et al.*, 1993; Hirai *et al.*, 1996; Laube *et al.*, 1997), and the same is true for glycine as a coagonist at NR1 subunits (Thedinga *et al.*, 1989; Benveniste *et al.*, 1990a,b; Benveniste and Mayer, 1991; Clements and Westbrook, 1991; Siegel *et al.*, 1996; Williams *et al.*, 1996). This assumption is normally made on the basis of Hill coefficients for steady-state responses, which are considerably greater than unity. Similarly, sigmoidal activation kinetics of NMDA channels in outside-out patches from cultured hippocampal neurons were best fitted by a kinetic model with two glutamate binding sites and two glycine sites, with agonist and coagonist binding being better described by an independent, rather than a sequential, model (Clements and Westbrook, 1991). Taken together, these data led to the hypothesis that the NMDA receptor is at least a tetramer containing four ligand-binding subunits, with a single binding site in each subunit (Clements and Westbrook, 1991). In contrast, others have reported Hill coefficients of less than unity for glycine (Lerma *et al.*, 1990), and detailed analysis of glycine concentration-response curves and kinetics indicated Hill coefficients for glycine of 1.1, with a corrected affinity of 130 nM, consistent with the idea that there is only one glycine binding site (Johnson and Ascher, 1992).

III. Physiological Role of Glycine

A. Glycine as a Coagonist

Kleckner and Dingledine (1988) were the first to report that glycine is essential for activation of the NMDA receptors. Using *Xenopus* oocytes injected with whole-brain mRNA, those authors observed that the response to NMDA vanished when the contamination with glycine was reduced to negligible levels. A similar conclusion regarding the necessity of glycine for the activation of NMDA receptors was later drawn on the basis of patch-clamp studies in neuronal cultures (Mayer *et al.*, 1989a; Henderson *et al.*, 1990; Huettner, 1990; Vornov and Coyle, 1991; Aoshima *et al.*, 1992; Chen *et al.*, 1997). Further evidence that the activation of the glycine$_B$ site is a prerequisite for NMDA receptor activation in vitro or in vivo was obtained by showing that selective antagonists of the glycine$_B$ site completely block the effects of NMDA receptor stimulation (see Section IV.C.). This suggests that residual responses in the nominal absence of glycine are the result of contamination with low background levels of glycine (Benveniste *et al.*, 1990a; Lerma *et al.*, 1990; Kemp and Priestley, 1991; Parsons *et al.*, 1993, 1997; Molnar and Erdo, 1996). Indeed, extrapolation of the lower linear part of glycine concentration-response curves indicates that approximately 20 to 40 nM glycine is found in the extracellular solution, a value that agrees very well with that measured by high pressure liquid chromatography (Benveniste *et al.*, 1990). This provides support for the notion that glycine is an essential coagonist at the NMDA receptor and that responses to NMDA cannot be obtained in the complete absence of glycine (Mayer *et al.*, 1989a,b; Vornov and Coyle, 1991; Aoshima *et al.*, 1992). However, it remains a remote possibility that blockade of the residual peak in the nominal absence of glycine by glycine$_B$ site full antagonists is the result of inverse agonistic effects and that a component of the peak response would still be present in the complete absence of glycine (Kemp *et al.*, 1988b; Mayer *et al.*, 1989a).

The binding of use-dependent NMDA receptor channel blockers, such as [^{3}H]MK-801, under nonequilibrium conditions can be used to investigate certain aspects of receptor function; levels of bound ligand are proportional to the degree of activation (Foster and Wong, 1987; Wong *et al.*, 1988). Using this method, in most cases it was only possible to demonstrate the essential role of glycine by blocking this site with an antagonist, probably because of widespread contamination with glycine. However, in extensively washed membranes it was

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shown that glutamate and glycine failed to enhance [^{3}H]MK-801 binding when used separately but did produce enhancement when applied together (Ratti *et al.*, 1990). On the other hand, spermine enhanced functional [^{3}H]MK-801 binding in the presence of the glycine$_B$ antagonist 7-Cl-KYN but not when the NMDA site was blocked by 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid (CPP) (Marvizon and Baudry, 1993). It was suggested by the authors of the latter study that glycine is not an absolute requirement for NMDA receptor activation. However, binding is probably not the optimal method to explore such questions, because, for example, [^{3}H]MK-801 has been reported to access the NMDA channel in a closed state via the so-called "lipophilic pathway" (Javitt and Zukin, 1989).

It is also noteworthy that, although glycine concentrations up to 3 mM do not activate NMDA receptors in vitro without NMDA agonists, there are indications that at concentrations above these levels glycine can actually damage neurons (Kleckner and Dingledine, 1988; McNamara and Dingledine, 1990; Wallis *et al.*, 1994; Newell *et al.*, 1997) and induce inward currents in cultured hippocampal neurons (Pace-Asciak *et al.*, 1992), via activation of both glycine$_B$ and NMDA sites. It has also been suggested that, under certain conditions, glycine might achieve such high levels and contribute to neurotoxicity in vivo (Newell *et al.*, 1997).

In vivo activation of glycinergic inhibitory interneurons in the spinal cord by stimulation of 1b afferents elicited a classical, short-latency, glycinergic inhibitory postsynaptic potential followed by an NMDA receptor-mediated EPSP. The EPSP was blocked by ketamine and R(+)HA-966 [R(+)-3-amino-1-hydroxypyrrolidin-2-one] and showed classical voltage dependence. The authors proposed that glycine released at inhibitory interneurons spills over to activate nonsaturated glycine$_B$ sites in vivo. This interpretation is controversial, because 1b afferents also activate excitatory interneurons and NMDA EPSPs are always delayed because of slower activation kinetics (Fern *et al.*, 1996).

B. Desensitization

Glycine greatly potentiates NMDA receptor-mediated responses by reducing desensitization both in native mammalian neurons and in *Xenopus* oocytes or HEK 293 cells expressing NMDA receptors (Mayer *et al.*, 1989a; Vornov and Coyle, 1991; Aoshima *et al.*, 1992; Chen *et al.*, 1997) (fig. 6). Glycine-sensitive desensitization is accompanied by a five- to seven-fold decrease in the affinity of the glycine$_B$ site in the presence of agonists for the NMDA site (Lerma *et al.*, 1990; Parsons *et al.*, 1993). With higher concentrations of glycine, the

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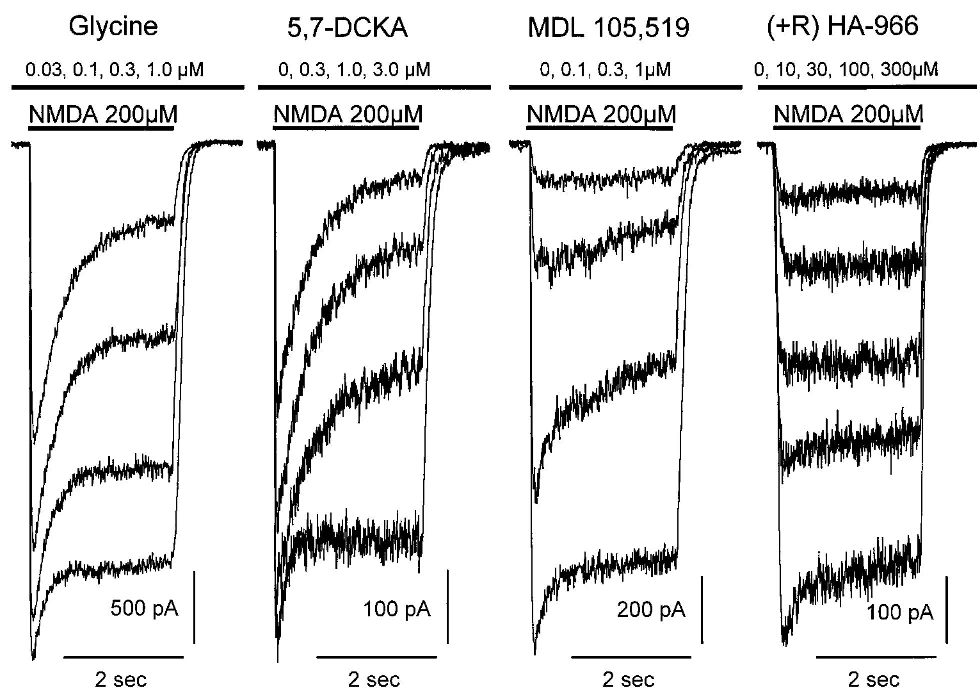

FIG. 6. Patch-clamp experiments with four different cultured hippocampal neurons, showing that glycine$_B$ antagonists have different effects on glycine-sensitive desensitization. NMDA (200 μM) was applied with a rapid concentration clamp (exchange times, ≤20 msec) for 2.5 sec every 30 sec, in the continuous presence of glycine (0.03 to 1 μM), with low Ca^{2+} levels (0.2 mM) and a constant membrane potential of −70 mV. Responses at equilibrium have been superimposed for each concentration of glycine or glycine$_B$ antagonist tested (continuously present for at least 2 min). Reduction of glycine concentrations reveals glycine-sensitive desensitization. 5,7-diCl-KYN (5,7-DCKA) (0.3 to 3 μM) concentration-dependently reveals glycine-sensitive desensitization in the continuous presence of glycine (1 μM). The higher affinity glycine$_B$ antagonist MDL-105,519 (0.1 to 1 μM) reveals less pronounced glycine-sensitive desensitization in the continuous presence of glycine (1 μM). The low intrinsic activity, glycine$_B$ site partial agonist R(+)HA-966 (10 to 300 μM) does not reveal glycine-sensitive desensitization in the continuous presence of glycine (1 μM). Note that the current scale bars are different for each antagonist tested.

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magnitude of this desensitization is decreased but the rate becomes faster. Furthermore, recovery from desensitization after step increases in the concentration of glycine or lower affinity glycine agonists in the continuous presence of NMDA reflects the association kinetics of the agonist concentrations used (Lerma *et al.*, 1990; Parsons *et al.*, 1993). Therefore, desensitization probably occurs rapidly upon binding of both glutamate and glycine, and the apparent rate reflects the balance between slow dissociation from and concentration-dependent reassociation of glycine with the altered receptor. In other words, at higher concentrations of glycine, the forward rate constant for rebinding greatly exceeds the rate of dissociation and the time course of desensitization appears to be faster. The affinities of agonist to induce desensitization are five-fold higher than their respective affinities as agonists at the peak of the response (Chizhmakov *et al.*, 1992).

The opposite has been reported in binding experiments, namely a mutually positive allosteric interaction, with glutamate increasing glycine affinity and glycine increasing glutamate affinity (table 2). Moreover, other electrophysiological studies concluded that desensitization involves structural changes in the channel-lining section of the protein, rather than the glycine or NMDA binding sites, because the induction of desensitization was dependent on channel opening (Zilberter *et al.*, 1991). This seems unlikely, because single-channel recordings and fluctuation analysis show an increase in opening frequency with no change in mean open time or conductance in the presence of glycine and the opposite in the presence of glycine$_B$ antagonists, suggesting that glycine regulates transitions to states that are intermediate between the binding of NMDA receptor agonists and ion-channel gating (Mayer *et al.*, 1989a; Vornov and Coyle, 1991; Parsons *et al.*, 1993). It has also been claimed that aspartate induces desensitization in the absence of glycine, but this interpretation is again complicated by the presence of background levels of glycine in all experiments (Chizhmakov *et al.*, 1992).

A similar form of desensitization is seen in the presence of some glycine$_B$ site full antagonists (Kemp and Priestley, 1991; Parsons *et al.*, 1993), but these vary in their ability to induce glycine-sensitive desensitization. L-689,560, L-701,324 [7-chloro-4-hydroxy-3-(3-phenoxy)phenyl-2(1H)-quinolinone], L-695,902 [7-chloro-4-hydroxy-3-methoxy-carbonyl-2(1H)-quinolinone] (fig. 7), and RPR-104,632 [2-(3-bromobenzyl)-6,8-dichloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-1,1-dioxide-3-carboxylic acid] (fig. 5) reveal little or no desensitization but other compounds, such as 7-Cl-KYN and 5,7-diCl-KYN, show 10-fold higher potencies against plateau responses (Molnar and Erdo, 1996; Karcz-Kubicha *et al.*, 1997). Other compounds, such as ACEA 1021 (5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione) (fig. 8), MRZ 2/571 [8-fluoro-4-hydroxy-1-oxo-1,2-dihydropyridazinol[4,5-b]quinoline-5-oxide (choline salt)], and MRZ 2/576 [8-chloro-4-hydroxy-1-oxo-1,2-dihydropyridazinol[4,5-b]quinoline-5-oxide (choline salt)] (fig. 9), induce moderate desensitization. The glycine$_B$ site partial agonist R(+)HA-966 (fig. 10) does not induce desensitization but produces a three-fold allosteric reduction in the affinity of agonists for the glutamate recognition site, which is reflected by slowing of the response onset rise time and acceleration of offset kinetics (Kemp and Priestley, 1991). Taken alone, this result could be considered indicative of a negative reciprocal interaction between the glycine and glutamate recognition sites. However, D-cycloserine and 1-aminocyclopropanecarboxylic acid (ACPC), which are partial agonists with higher intrinsic activity (fig. 10), are also unable to induce desensitization

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TABLE 2
Allosteric interactions within the NMDA receptor complex involving the glycine$_B$ recognition site (binding studies)

Unlabeled ligands	[^{3}H]Glycine	[^{3}H]5,7-diCl-KYN	[^{3}H]L-689,560	[^{3}H]Glutamate	[^{3}H]CPP	[^{3}H]CGS-19755	[^{3}H]CGP-39653
Polyamines	↑ [a] (1, 24, 25), 0 (9)	↓ (1, 26)	↓ (8)[b]	↓ (22)	↑ (2, 22, 23)		↑ (20)[c]
7-Cl-KYN				0 (8)	0 (8, 13)	0 (13)	↑ (20)[d]
L-701,324[e]				↓ (9)	↑ (9)	↑ (9)	
D-Cycloserine				0 (8)	0 (8)	↓ (8)	
Glycine, D-serine				↑ (4, 7, 8, 16)	0 (4, 13)	↓ (8, 13)	↓ (19)
ACPC				0 (3)	0 (3)		
ACBC				↓ (3), 0 (8)	↑ (3, 8, 10)	0 (8)	
HA-966				↓ (3, 8)	↑ (4, 8, 21, 23)	0 (8)	0 (21)
L-687,414				↓ (8)	↑ (8)	0 (8)	
CPP (NPC 12626, SC 46643)	0 (4, 8, 13)	↓ (1)	↓ (8)				
AP5, CGS-19755	↓ (6, 8, 11, 12, 14, 17, 18)[f]	↓ (1)	↓ (8)		0 (13)		
Glutamate	↑ (7, 13, 14, 16, 24)	↑ (1)					
CGS-19755	↓ (8, 11, 13)		↓ (8)				

[a] ↑, increase; ↓, decrease; 0, no change; references are given in parentheses. 1, Baron *et al.,* 1991; 2, Carter *et al.,* 1989; 3, Compton *et al.,* 1990; 4, Danysz *et al.,* 1989a; 5, Danysz *et al.,* 1990; 6, Danysz *et al.,* 1989b; 7, Fadda *et al.,* 1988; 8, Grimwood *et al.,* 1993; 9, Grimwood *et al.,* 1995a; 10, Hood *et al.,* 1989b; 11, Hood *et al.,* 1990; 12, Johnson *et al.,* 1988a; 13, Kaplita and Ferkany, 1990; 14, Kessler *et al.,* 1989b; 15, Keith *et al.,* 1989; 16, Monaghan *et al.,* 1988; 17, Monahan *et al.,* 1989a; 18, Monahan *et al.,* 1990a; 19, Mugnaini *et al.,* 1993; 20, Oblin and Schoemaker, 1994; 21, Pingping *et al.,* 1993; 22, Pullan and Powel, 1991; 23, Pullan *et al.,* 1990a; 24, Ransom and Deschenes, 1990; 25, Sacaan and Johnson, 1989; 26, Yoneda *et al.,* 1994.
[b] Inhibition by agonists.
[c] Only in the presence of 7-Cl-KYN.
[d] Only in the presence of spermine; not antagonized by ifenprodil.
[e] The same effect was observed with L-695,902, L-698,532; and L-703,717.
[f] This effect could be reversed by C7 antagonists and NMDA agonists.

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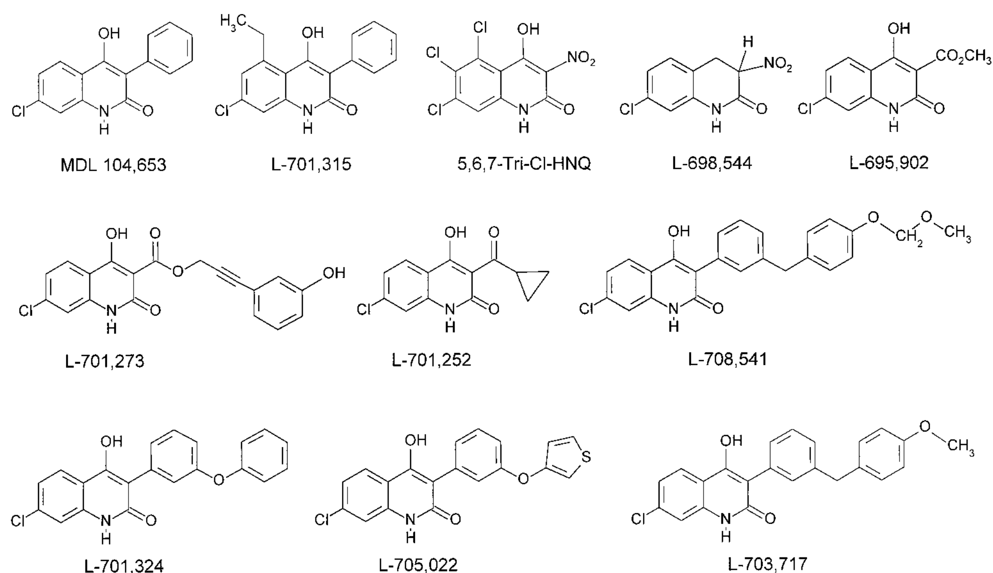

FIG. 7. 4-Hydroxyquinolone antagonists of the glycine$_B$ site. HNQ, 4-hydroxy-3-nitroquinolin-2(1H)-one.

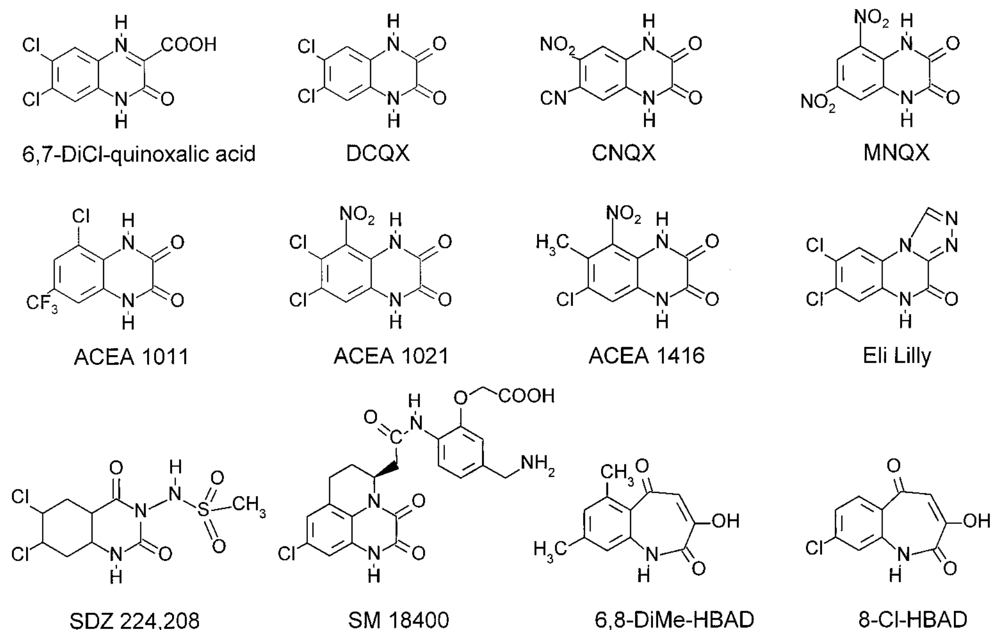

FIG. 8. Quinoxaline-diones and related compounds that are antagonists of the glycine$_B$ site. CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; HBAD, 3-hydroxy-1H-1-benzazepine-2,5-dione.

(Karcz-Kubicha *et al.*, 1997) but have intermediate effects on glutamate deactivation kinetics, in line with their relative intrinsic activities (Priestley and Kemp, 1994). Furthermore, the full antagonists L-701,324 and L-695,902 (fig. 7) were recently reported to decrease glutamate affinity in a manner similar to that of R(+)HA-966 (Priestley *et al.*, 1996). The same group reported a reciprocal three-fold interaction between partial agonists at the NMDA recognition site and glycine affinity, with the off-rate of glycine being fastest in the presence of a saturating concentration of the competitive NMDA receptor antagonist *cis*-2,3-piperidinedicarboxylic acid and progressively slower in the presence of quinolinate, NMDA, and L-glutamate (Priestley and Kemp, 1994). Taken together, these data were interpreted as being more in agreement with binding studies showing reciprocal positive interactions between full agonists at the NMDA and glycine recognition sites, with the extent of the modulation of one site by the other being related to the intrinsic activity of the agonist used, rather than its affinity.

However, it should be stressed that the comparisons in offset kinetics were all made at steady state and did not address the relative changes in apparent affinities at the peak and plateau of the response. The same is true for binding studies, which all assessed affinities at steady state under highly nonphysiological conditions. Alterna-

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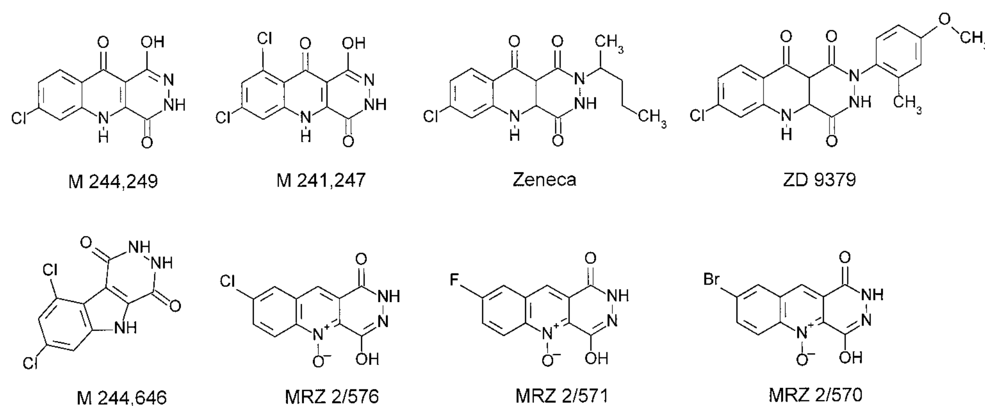
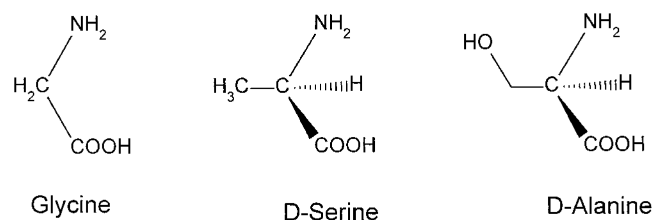


FIG. 9. Tricyclic antagonists of the glycine$_B$ site.



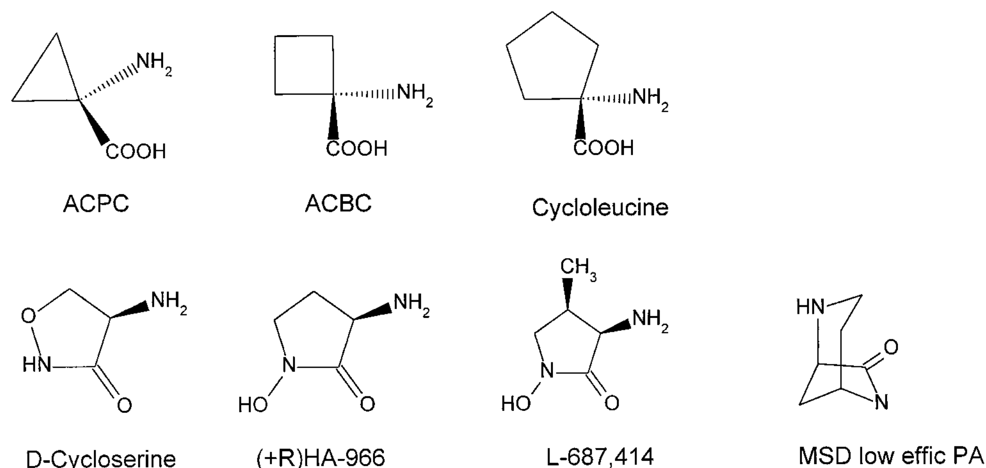


FIG. 10. Selected agonists and partial agonists of the glycine$_B$ site. MSD low effic PA, Merck Sharp and Dohme low efficiency partial agonist.

tively, the fact that R(+)HA-966 and D-cycloserine do not exhibit glycine-sensitive desensitization (fig. 6) could be the result of both their low potency and allosteric slowing in onset kinetics. It may be that an agonist-induced change in the affinity of the glycine site still occurs and would be seen as very fast desensitization, reflecting the rapid unbinding of this low affinity partial agonist, if it were not masked by the slower rise time of the response. This idea is supported by the fact that desensitization is seen with intermediate concentrations of the higher affinity partial agonist ACPC in the absence of glycine (Karcz-Kubicha *et al.*, 1997). A similar argument can be applied to the apparent lack of desensitization seen with some glycine$_B$ site full antagonists. We previously showed a trend for less desensitization with higher potency full antagonists (Parsons *et al.*, 1997) (fig. 6). In this case, the association of low concentrations of antagonist after agonist-induced dissociation of glycine is much slower than the forward rate constant for reassociation of glycine. Therefore, glycine-sensitive desensitization would be predicted to be revealed with a much slower time course.

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Several studies found that the kinetics of NMDA receptor currents in outside-out patches are dominated by a pronounced glycine-insensitive form of desensitization (Sather *et al.*, 1991, 1992; Lester *et al.*, 1993; Tong and Jahr, 1994). This is particularly evident after longer recording periods, indicating the importance of dialysis of intracellular factors in mediating this effect. However, the glycine and glutamate recognition sites are still allosterically coupled, as evidenced by changes in the offset kinetics of glycine in the presence of glutamate (Lester *et al.*, 1993). The appearance of glycine-insensitive desensitization is reduced by intracellular application of 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (EDTA) or preincubation of neurons with the Ca^{2+} release inhibitor dantrolene, suggesting that this form of desensitization is triggered by a transient increase in intracellular Ca^{2+} levels. The extent of glycine-insensitive desensitization is also reduced by intracellular application of adenosine-5′-*O*-(3-thio)-triphosphate, the phosphatase inhibitor microcystin, or a peptide inhibitor of calcineurin, implying regulation by the phosphorylation state of the receptor (Tong and Jahr, 1994). Moreover, this form of desensitization seems to be dependent on activation of G proteins (Turecek *et al.*, 1995). This may explain the relatively robust glycine-sensitive desensitization seen in the study of Parsons *et al.* (1993), because single-channel recordings were first made after 20 to 30 min of whole-cell recording with low extracellular Ca^{2+} levels and high intracellular ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, adenosine-5′-triphosphate, and 3′,5′-cyclic adenosine monophosphate levels, i.e., under conditions chosen to selectively investigate glycine-sensitive desensitization.

The improved therapeutic profiles of some systemically active, $glycine_B$ site full antagonists could be the result of their abilities to reveal glycine-sensitive desensitization (Parsons *et al.*, 1993). Receptor desensitization may represent a physiological process serving as an endogenous control mechanism to prevent long-term neurotoxic activation of glutamate receptors but allow their transient physiological activation. Interestingly, ischemia increases not only the concentration of extracellular glutamate but also that of glycine and, although this latter effect is less pronounced, it persists for much longer (Globus *et al.*, 1991b). Prolonged repetitive activation of NMDA receptors during ischemia would be effectively reduced at concentrations of $glycine_B$ site full antagonists having less effect on more transient activation during EPSPs, because the time course for glycine-sensitive desensitization (300 to 500 msec) (Mayer *et al.*, 1989a) is somewhat longer than that for NMDA receptor-mediated synaptic events (typically 100 to 200 msec) (Clements *et al.*, 1992). This property may also allow such compounds to differentiate between various forms of NMDA receptor-mediated synaptic plasticity, e.g., to block drug tolerance and dependence and chronic pain states at concentrations having less effect on learning and memory.

C. Endogenous Agonists: Glycine and/or D-Serine

Glycine has been traditionally regarded as an inhibitory transmitter in lower CNS regions, where it activates receptors forming chloride channels (Leu *et al.*, 1987). Only during the past 10 years has its function as a positive modulator acting at NMDA receptors been recognized (see Section I.). Meanwhile, high free D-serine (fig. 10) levels, i.e., concentrations of 2 to 300 μM, depending on the brain structure and experimental conditions, have been detected in the mammalian (including human) CNS (for review, see Hashimoto and Oka, 1997). In turn, D-serine has been suggested as a possible endogenous ligand for the $glycine_B$ site. Here the features crucial for dynamic regulation of the concentration of both ligands are discussed in parallel, according to the criteria given in table 3.

It is noteworthy that dynorphin(1–13) was also recently proposed as an endogenous agonist at the $glycine_B$ site, as evidenced by very pronounced increases in the amplitude of NMDA-activated currents in *Xenopus* oocytes in the presence of low extracellular glycine concentrations (Zhang *et al.*, 1997). Such an effect may exacerbate the well documented spinal toxicity seen with dynorphin peptides after intrathecal administration (Shukla and Lemaire, 1994), although the vasoconstrictive actions of this peptide are probably also important.

To determine the physiological importance of either glycine or D-serine, their extracellular CNS levels should be compared with their affinities. Affinity is often difficult to assess accurately, because of variable levels of contamination with glycine, which have been estimated to be between 20 and 130 nM in binding and patch-clamp studies (Benveniste *et al.*, 1990; Lerma *et al.*, 1990; Johnson and Ascher, 1992; Parsons *et al.*, 1993; Berger, 1995). In the majority of binding studies in brain homogenates, the affinity of glycine is slightly (two- to three-fold) higher than that of D-serine (Kishimoto *et al.*, 1981; Danysz *et al.*, 1990) (table 4). On the other hand, some authors report that, in experiments with recombinant NMDA receptors, D-serine seems to be slightly more potent as an agonist than glycine (Matsui *et al.*, 1995) (but see table 5).

1. Distribution within the central nervous system. In brain homogenates, glycine has been reported to be present at concentrations of 6 to 10 nmol/mg of protein

TABLE 3
Comparison of some of the criteria indicating dynamic regulation of modulator/transmitter concentrations within the synaptic cleft for glycine and D-serine

Criteria	Glycine	D-Serine
Heterogeneous distribution within CNS	Yes	Yes
Ca^{2+}-dependent synaptic release	No	No
Uptake	Yes	Yes
Synthesis in neurons	Yes	Yes
Metabolism	Yes	Yes
Receptor-coupled recognition site	Yes	Yes

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TABLE 4

Radioactive ligands used to label the glycine$_B$ site of the NMDA receptor (data for hippocampal/cortical or hemisphere membrane preparations)

Radiolabeled ligand	K_d (nM)	B_{max} (pmol/mg of protein)	References
[^{3}H]5,7-diCl-KYN	69	14.5	Baron *et al.,* 1991
[^{3}H]L-689,560	2.9	4.2	Grimwood *et al.,* 1992
[^{3}H]MDL 105,519	3.8	12.1	Baron *et al.,* 1996a
[^{3}H]ACPC	129 (H)[a], 7300 (L)	2.3 (H), 20 (L)	Popik *et al.,* 1995
D-[^{3}H]Serine (cortex)	355	5	Danysz *et al.,* 1990
[^{3}H]Glycine (cortex)	126	3.4	Kishimoto *et al.,* 1981

[a] H, high affinity site; L, low affinity site.

TABLE 5

Potency and intrinsic activity of ligands acting at the glycine$_B$ site of expressed NMDA receptor subtypes

Ligand	Potency (μM)				References; species, NR1 splice variant
	NR1/NR2A	NR1/NR2B	NR1/NR2C	NR1/NR2D	
Glycine		1.2		0.16	Buller and Monaghan, 1997; NR1a
Glycine	0.97	0.84	0.76	0.56	Matsui *et al.,* 1995; mouse
Glycine	1.9	1.4			Hess *et al.,* 1996; human, NR1a
Glycine	2.1	0.3	0.2		Kutsuwada *et al.,* 1992; mouse
Glycine	0.84	0.19	0.15	0.096	Woodward *et al.,* 1995a,b; rat, NR1a
Glycine	0.53 (100%)[a]	0.06 (100%)			Priestley *et al.,* 1995; human, NR1a
Glycine	3.7	2.1	0.36	2.3	Laurie and Seeburg, 1994a; binding
D-Serine	0.32 (88%)	0.26 (102%)	0.21 (118%)	0.17 (100%)	Matsui *et al.,* 1995; mouse
D-Serine	2.2	0.6			Hess *et al.,* 1996; human, NR1a
D-Serine	0.22 (99%)	0.15 (113%)			Priestley *et al.,* 1995; human, NR1a
D-Serine	0.79 (100%)	0.72 (100%)	1.24 (110%)	0.75 (100%)	Krueger *et al.,* 1997; human, NR1a
ACPC	0.6 (100%)	0.3 (100%)	3.2 (150%)	0.08 (110%)	Krueger *et al.,* 1997; mouse
D-Cycloserine	15.2 (20%)	8.4 (20%)	1.6 (120%)	2 (100%)	Krueger *et al.,* 1997; mouse
D-Cycloserine	3.2 (93%)	1.3 (88%)			Priestley *et al.,* 1995; human, NR1a
R(+)HA-966	121 (12%)	4.6 (14%)			Priestley *et al.,* 1995; human, NR1a
ACBC	45 (13%)	6.6 (33%)			Priestley *et al.,* 1995; human, NR1a
7-Cl-KYN	0.63 (0%)	0.2 (0%)			Priestley *et al.,* 1995; human, NR1a
5,7-diCl-KYN (K_d)	0.059 (0%)	0.097 (0%)	0.058 (0%)	0.113 (0%)	Laurie and Seeburg, 1994a; binding, rat
ACEA 1011 (K_b)	0.33	0.46	0.21	0.74	Woodward *et al.,* 1995a; rat, NR1a
ACEA 1021 (K_b)	0.0028	0.0059	0.0019	0.011	Woodward *et al.,* 1995a; rat, NR1a
GV 156526A	0.05 (0%)		0.07 (0%)		Bunnermann *et al.,* 1996; HEK cells
L-687,414	4.0 (5%)	1.1 (3%)			Priestley *et al.,* 1995; human, NR1a
L-701,324	0.005 (0%)	0.005 (0%)			Priestley *et al.,* 1995; human, NR1a

Data refer to electrophysiological studies unless stated otherwise. The species of mRNA and the type of NR1 splice variant are also shown.
[a] Values in parentheses are intrinsic activities (with respect to glycine).

in mice (Saransaari and Oja, 1994), 1 nmol/mg of wet tissue in rats (Katsura *et al.*, 1992), and 27 nmol/mg of protein in humans (Waziri *et al.*, 1993). In the same preparation in rats, D-serine concentrations range from 10 μM in the cerebellum to 400 μM in the cerebral cortex (Nagata *et al.*, 1994b). In adult human brain homogenates, D-serine is present at 66 to 130 μM (Hashimoto *et al.*, 1993c; Nagata *et al.*, 1994b; Hashimoto and Oka, 1997) or 2 to 15 nmol/mg of protein (Chouinard *et al.*, 1993; Waziri *et al.*, 1993). It should be kept in mind that homogenate concentrations indicate very little regarding free extracellular levels, because accumulation in intracellular compartments is likely (for example, see Danysz *et al.*, 1997). In human cerebrospinal fluid (CSF), free glycine levels of 7 to 10 μM have been detected (Ferraro and Hare, 1985).

Microdialysis studies have also shown that both D-serine and glycine are present at micromolar levels in the mammalian CNS. In rats, it was found that D-serine reaches concentrations of 6 and 0.5 to 1 μM (corrected for in vitro recovery) in the frontal cortex and cerebellum, respectively (Matsui *et al.*, 1995; compare with Westergren *et al.*, 1994; Hashimoto *et al.*, 1995a). In contrast, higher levels of glycine were detected in the cerebellum (18 to 28 μM) than in the frontal cortex (7 to 9 μM) (Matsui *et al.*, 1995; see also Fabricius *et al.*, 1993). It has therefore been suggested that in the cerebellum glycine might be a major ligand acting at the glycine$_B$ site, whereas in the forebrain D-serine is more likely to have this function (Matsui *et al.*, 1995; Schell *et al.*, 1997). In fact, it has been shown that the distribution of NMDA receptors labeled by [^{3}H]MK-801 binding corresponds better to the distribution of D-serine itself or D-[^{3}H]serine binding than to glycine levels or [^{3}H]glycine binding (Danysz *et al.*, 1990; Hashimoto *et al.*, 1993c; Schell *et al.*, 1995).

In accordance with these data, immunohistochemical studies also revealed a discrete, complementary distribution of glycine and D-serine in the brain (Schell *et al.*, 1997). Glycine immunoreactivity predominates in the cerebellum, hypothalamus, and hindbrain, whereas D-serine preponderates in the gray matter of the cerebral cortex, hippocampus, olfactory bulbs, striatum, and amygdala (Schell *et al.*, 1995, 1997). Interestingly, in the majority of cases the levels of D-serine (but not those of glycine) correlate both spatially and ontogenetically

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with the expression of NMDAR2A and -2B subunits (Schell *et al.*, 1997). Based on in situ hybridization studies, it seems that D-serine distribution corresponds best to NMDAR2B receptor expression, whereas glycine and NMDAR2A receptor distributions are both more widespread (Watanabe *et al.*, 1993a; Monyer *et al.*, 1994; Hashimoto *et al.*, 1995a; Schell *et al.*, 1997). There are, however, some unexplained exceptions to this pattern. In the substantia nigra reticulata, there are high levels of both NMDAR2A/B mRNA and glycine immunoreactivity but no D-serine was detected (Schell *et al.*, 1997). In the adult cerebellum, where D-serine levels are low, D-serine does not colocalize with the glycine$_B$ sites; D-serine binding is seen in the granule cell layer, whereas D-serine immunoreactivity is localized in the molecular layer (Schell *et al.*, 1995).

It is noteworthy that the CNS concentration of D-serine seems to be inversely correlated with the distribution of its metabolizing (deaminating) enzyme, i.e., D-amino acid oxidase (Nagata *et al.*, 1994b; Wood *et al.*, 1996). This discrete distribution appears at 3 weeks after birth in rats and is likely to be a consequence of brain structure-specific increases in D-amino acid oxidase expression (Weimar and Neims, 1977; Hashimoto *et al.*, 1993c; Hashimoto *et al.*, 1995a). At the cellular level, D-serine immunoreactivity was detected only in glia (astrocytes) (Schell *et al.*, 1995), whereas glycine was also found in neurons, probably participating in this case in inhibitory transmission (Hagan *et al.*, 1987).

D-Serine concentrations show substantial variations during ontogenesis. In the human prefrontal cortex, D-serine levels peak at gestational week 14 and then decline rapidly, suggesting involvement in the regulation of NMDA receptors during development (Hashimoto *et al.*, 1993a). Similarly, in the periphery D-serine levels are high on the day of birth but shortly thereafter fall to very low levels (Hashimoto *et al.*, 1995a).

2. Uptake. Early studies demonstrated the presence, in the cerebral cortex (Hagan *et al.*, 1988), cerebellum (Wilson *et al.*, 1976; Wilkin *et al.*, 1981), and hippocampus (Toth and Lajtha, 1986), of a sodium-dependent glycine uptake system that did not seem to transport D-serine. In the hippocampus, both low affinity (minor Na^+ dependence) and high affinity (strong Na^+ dependence) carriers were detected (Fedele and Foster, 1992). Based on lesion studies, it was concluded that this carrier is localized on both neurons and glia (mainly astroglia) (Magnuson *et al.*, 1988). A similar conclusion was derived from studies on cultured cerebellar granule cells and astrocytes (Eberhard and Holtz, 1988) and autoradiographic experiments with [^{3}H]glycine in the cerebellum (Wilkin *et al.*, 1981).

Cloning studies revealed the existence of GLYT1a–c (Nicholls, 1989; Guastella *et al.*, 1992; Smith *et al.*, 1992) and GLYT2 (Liu *et al.*, 1992) families of transporters (table 6). GLYT1 carriers are encoded by the same gene, and a, b, and c subtypes are produced via different promoters or splicing of small fragments in the amino-terminal region (Nicholls, 1989; Guastella *et al.*, 1992; Smith *et al.*, 1992). Both transporters are Na^+ and Cl^- dependent (transporting 2 Na^+ and 1 Cl^- ions) (Aragon *et al.*, 1987; Nicholls, 1989; Guastella *et al.*, 1992; Smith *et al.*, 1992).

GLYT1, apart from being an element of inhibitory glycine transmission, is found in some regions with low levels of glycine$_A$ receptors, such as the cerebellum (molecular layer), thalamus, hypothalamus, olfactory bulb, cortex, and hippocampus (Zafra *et al.*, 1995b). This pattern probably reflects the distribution of the GLYT1b subtype, which is suggested to be colocalized with NMDA receptors (table 6) (Smith *et al.*, 1992; Fedele *et al.*, 1993). GLYT1 mRNA has been detected in glia and neurons, but immunohistochemical analysis failed to confirm the presence of the respective protein in neurons (Zafra *et al.*, 1995a). Hence, only glial localization of this transporter is proven at present. Interestingly, in cultures, glial cells express GLYT1 only under conditions of direct contact with neurons, i.e., "on demand," supporting its role in neurotransmission (Zafra *et al.*, 1997).

The GLYT1 uptake system has a K_m in the range of 20 to 40 μM; however, maximal velocity in the cerebral cortex is 10 times less than that in the spinal cord (Fedele and Foster, 1992), which could indicate lower buffering capacity in the former region. However, GLYT1b transporters expressed in *Xenopus* oocytes can buffer glycine concentrations fairly efficiently, decreas-

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TABLE 6
Classification of glycine transporters based on recent cloning data

Name	Cellular localization	Distribution	Colocalizes with	References
GLYT1a	Glia	Gray matter, olfactory bulb, cerebellum, hypothalamus, mesencephalon, brainstem, spinal cord, thalamus, liver, lung, stomach	Glycine$_A$ receptors	Guastella *et al.,* 1992; Liu *et al.,* 1992; Adams *et al.,* 1995; Zafra *et al.,* 1995a,b
GLYT1b	Glia	White matter, olfactory bulb, corpus collosum, cerebellum, hypothalamus, mesencephalon, brainstem, spinal cord, thalamus, pineal gland, hippocampus	NMDA receptors	Liu *et al.,* 1992; Fedele *et al.,* 1993; Luque *et al.,* 1995b; Zafra *et al.,* 1995a,b
GLYT1c	Glia	Brain, human (?)	?	Kim *et al.,* 1994
GLYT2	Neuronal (presynaptic?)	Spinal cord, brainstem (CNS only)	Glycine$_A$ receptors	Liu *et al.,* 1993a; Luque *et al.,* 1995b; Zafra *et al.,* 1995a,b

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ing levels within the vicinity of NMDA receptors 10- to 100-fold (Supplisson and Bergman, 1997) (see Section III.C.7. for more details). In contrast to GLYT1, the distribution of GLYT2 mRNA (and proteins) is seen mainly in the pons, cerebellum, and spinal cord, which corresponds very well to localization of glycine$_A$ receptors, as revealed by immunocytochemical analysis (Van den Pol and Gorcs, 1988) and autoradiography of [^{3}H]strychnine binding (Zarbin *et al.*, 1981; Bristow *et al.*, 1986).

Glycine uptake can be inhibited by glycyldodecylamide and sarcosine (Javitt and Frusciante, 1997). The former is systemically active but nonselective, whereas sarcosine is a preferential blocker of the GLYT1 family of transporters (Liu *et al.*, 1993).

Much less is known regarding D-serine uptake. Recently, novel Na^+-dependent neutral amino acid transporters (ASCT1 and -2) were cloned and characterized, and D-serine was shown to inhibit ASCT2 but not ASCT1; although, ASCT2 mRNA has not been detected in the brain thus far (Utsunomiya-Tate *et al.*, 1996). The D-serine uptake system is probably located on astrocytes (Schell *et al.*, 1995). In line with this suggestion, D-serine injected intracerebroventricularly (i.c.v.) has been found to accumulate in glial cells, as evidenced by immunohistochemical analysis (Wako *et al.*, 1995).

3. Release. In cerebellar cultures, veratridine and high potassium levels induced release of preloaded [^{3}H]glycine from both astrocytes and granule cells, but only the latter was partially Ca^{2+} dependent (Halopainen and Konto, 1989). In primary cultures of striatal neurons, KCl-induced depolarization produced a two-fold increase in glycine release that was partially (75%) Ca^{2+} dependent (Weiss *et al.*, 1989). Also in synaptosomes from human cortex, Ca^{2+}-dependent release of preloaded [^{3}H]glycine was observed upon exposure to high potassium levels (15 mM) (Wullner *et al.*, 1993). On the other hand, kainate-evoked release in striatal neurons was apparently not dependent on extracellular Ca^{2+} (Weiss *et al.*, 1989). In hippocampal synaptosomes preloaded with 0.5 μM [^{3}H]glycine, there was also significant enhancement of radioactivity efflux by veratridine and high potassium levels, which was not affected by Ca^{2+} removal (Galli *et al.*, 1993). Because replacement of Na^+ with Li^+ attenuated [^{3}H]glycine efflux, it can be concluded that efflux was governed by a glycine carrier working in a reverse mode (Galli *et al.*, 1993). In HEK 293 cells stably expressing GLYT1 and preloaded with [^{3}H]glycine, release was observed that was evoked by, for example, sarcosine, extracellular glycine, and replacement of Na^+ with Li^+ (Sakata *et al.*, 1997). The latter finding indicates again that a decrease in the sodium gradient reverses the glycine carrier. Similarly, in hippocampal slices preloaded with radioactive [^{3}H]glycine, potassium-evoked efflux of glycine was not calcium dependent, as concluded by the authors (Saransaari and Oja, 1994), although fig. 5 of that report clearly indicates an inhibitory effect of Ca^{2+} removal in old animals.

In vivo studies using brain microdialysis indicate that glycine can be released by high potassium levels and veratridine, but in both cases it is Ca^{2+} independent, in contrast to the release of aspartate and glutamate (Semba *et al.*, 1995). Using the same method, Hashimoto *et al.* (1995b) reported that Ca^{2+} removal increased glycine and to a lesser extent D-serine levels, whereas tetrodotoxin increased both equally. These findings suggest that release from the cytoplasmic pool by reversal of the uptake system is a major contributor. Interestingly, it has also been reported that veratridine increases glycine and decreases D-serine concentrations in the microdialysate (Hashimoto *et al.*, 1995b). This finding is difficult to interpret and requires clarification. It has been shown that D-serine can be released from astrocytes after activation of glutamate receptors of the AMPA/kainate type (Schell *et al.*, 1995); in this context, it has been suggested that synaptically released glutamate might stimulate glia to release D-serine, which in turn enhances NMDA responses.

These studies present quite confusing findings, but it seems that, in the vicinity of glutamatergic synapses, glial release of glycine is present and is usually not Ca^{2+} dependent. There are indications that reversal of glycine and D-serine uptake might play a major role in increasing the concentration of these amino acids near the synaptic cleft.

4. Source and synthesis. Glycine in the CNS is probably derived from general metabolic pathways (for example, it can be synthesized from L-serine by serine hydroxymethyl transferase) (Pycock and Kerwin, 1981), whereas the precise source of D-serine in the brain is not certain. It could theoretically be derived from (*a*) ingested food, (*b*) intestinal bacteria, (*c*) metabolically stable proteins containing this isomer, or (*d*) L-serine (Hashimoto and Oka, 1997). It has been suggested that ontogenic changes (e.g., distinct peak periods in various organs) argue against the first three possibilities, although the possibility that the differences observed result not from different rates of synthesis or selective target tissue uptake but from different rates of metabolism (i.e., the activity of D-amino acid oxidase) must be considered (Hashimoto and Oka, 1997). Also arguing against an external source are the findings that serum levels of D-serine are very low, compared with some organs (Hashimoto and Oka, 1997), and the transport of D-amino acids through the blood-brain barrier is rather poor (Olendorf, 1973; Sato *et al.*, 1991). Moreover, it has been suggested that D-serine in the brain does not originate from microorganisms (which contain some D-amino acids) (Man and Bada, 1987), because germ-free and pathogen-free mice have similar brain contents of D-serine (Nagata *et al.*, 1994b) and the distribution of D-serine within the brain is heterogeneous. The third possibility is also unlikely, as argued by Hashimoto and

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Oka (1997), because D-serine levels are high in the human cortex at embryonic stages, but the contribution of D-amino acid residues to metabolically stable proteins actually increases with age. Another option, namely that D-serine might be synthesized from L-serine, has recently been pursued. It was shown that D-serine is present in brain synaptosomes (Hashimoto *et al.*, 1993c), where it is formed from L-phosphoserine by the actions of phosphoserine phosphatase followed by racemase (Wood *et al.*, 1996). In fact, it was reported that injection of huge doses of L-serine into infant rats produced an increase of D-serine levels (two-fold) that lasted >24 h (Takahashi *et al.*, 1997). The peak of L-serine was higher (four-fold) but of shorter duration (10 h), in accord with the precursor concept. D-Serine injection also produced an increase in L-serine content (but not that of other amino acids, including glycine), suggesting a bidirectional activity of the racemase, which, unfortunately, has not been detected in the brain thus far (Takahashi *et al.*, 1997).

Another possibility is that D-serine is synthesized from glycine by the glycine cleavage system, assuming that it is not strictly chiral (Daly *et al.*, 1976), or by reverse action of D-amino acid oxidase, which can also use glycine as a substrate (DeMarchi and Johnston, 1969). In accord with this, it has been reported that glycine injection produces an increase in both L- and D-serine levels (Takahashi *et al.*, 1997).

5. Metabolism. Metabolic inactivation of glycine is most probably accomplished through the glycine cleavage system, which is localized in the inner membrane of the mitochondria of astroglial cells but is absent in neurons (Daly *et al.*, 1976; Sato *et al.*, 1991). The reaction of the system is as follows: 2 glycine + H_2O + NAD^+ $\rightarrow$ serine + CO_2 + NH_2 + NADH + H^+. The distribution of the glycine cleavage system corresponds to some extent to the distribution of NMDA receptors and is also somewhat inversely related to local glycine levels in the brain (Daly *et al.*, 1976; Sato *et al.*, 1991), indicating its importance in maintaining certain spatially determined basal levels of this amino acid. For example, astrocytes in the telencephalon and cerebellum are strongly labeled with antibody toward this enzyme, and those in spinal cord and medulla oblongata are much more weakly labeled (Daly *et al.*, 1976; Sato *et al.*, 1991).

D-Serine can be metabolized by the stereoselective, glial enzyme D-amino acid oxidase, as follows: D-serine + H_2O + O_2 $\rightarrow$ hydroxypyruvate + H_2O_2 + NH_3. This enzyme is present at high levels in the kidney but is less abundant in the CNS (Hamilton, 1985), where it is concentrated in microperoxisomes of Bergmann glial cells and other astrocytes but not in oligodendrocytes or neurons (Horiike *et al.*, 1987; Nagata *et al.*, 1992; for review, see Hashimoto and Oka, 1997). In addition to D-serine, this enzyme can use other D-amino acids as substrates (D′Aniello *et al.*, 1993). Also, as already mentioned, the CNS concentration of D-serine seems to be inversely correlated with the distribution of D-amino acid oxidase and positively correlated with the distribution of NMDA receptors (DeMarchi and Johnston, 1969; Nagata *et al.*, 1994b; Schell *et al.*, 1995: Wood *et al.*, 1996).

It is noteworthy that, in transgenic mice with a deficit in D-amino acid oxidase, a substantial increase in the content of D-serine is observed in the periphery and in the cerebellum, which in normal animals has a very high activity of this enzyme (Nagata *et al.*, 1992; Hashimoto *et al.*, 1993b). A very modest change was observed in forebrain regions, indicating that this enzyme probably has a minor role in the maintenance of D-serine levels in this region (Nagata *et al.*, 1992; Hashimoto *et al.*, 1993b). In conclusion, during postnatal development a rapid decrease in the D-serine concentrations in the CNS parallels an increase in D-amino acid oxidase activity (Hashimoto and Oka, 1997), indicating that this enzyme is indeed a key factor in the regulation of D-serine levels.

6. N-Methyl-D-aspartate receptor-coupled recognition site and its distribution. In Na^+-free medium, at 4°C (to avoid binding to carriers), [^{3}H]glycine labels two types of recognition sites in the CNS. The first type is the the inhibitory glycine$_A$ receptor (chloride channel), which is also labeled by [^{3}H]strychnine (Young and Snyder, 1974; Aprison and Daly, 1978; Danysz *et al.*, 1990) and is abundant mainly in the spinal cord and pons. The second type is the strychnine-insensitive glycine$_B$ site coupled to NMDA receptors (Kishimoto *et al.*, 1981; Galli *et al.*, 1988). This site predominates in the striata oriens and radiatum of the hippocampal CA1 area, the CA3 region, the dentate gyrus, the superficial layer of the cerebral cortex, the striatum, the dorsolateral septum, and the amygdala.

Both sites show complementary rather than similar distributions (Kishimoto *et al.*, 1981; Galli *et al.*, 1988; DeFeudis *et al.*, 1978; Bristow *et al.*, 1986; Bowery, 1987). The distribution of strychnine-insensitive sites labeled by [^{3}H]glycine corresponds, as expected, to the distribution of other ligands labeling NMDA receptors, e.g., [^{3}H]glutamate, ^{3}H-labeled 2-amino-5-phosphonopentanoic acid (AP5), and [^{3}H]CPP labeling NMDA receptor recognition sites (Greenamyre *et al.*, 1985; Monaghan *et al.*, 1985; Olverman *et al.*, 1986; Jarvis *et al.*, 1987) and [^{3}H]MK-801 and ^{3}H-labeled thienylcyclohexylpiperidine (TCP) labeling the PCP site within the ionic channel (Bowery and Hudson, 1986; Gundlach *et al.*, 1986; Jansen *et al.*, 1989). Glycine$_B$ sites can be labeled with D-[^{3}H]serine (Danysz *et al.*, 1990) (see Section IV.E.) or with an antagonist such as [^{3}H]MDL-105,519 (Chmielewski *et al.*, 1996) with greater selectivity, because these ligands do not label inhibitory sites in, for example, the spinal cord. The distribution is similar to that seen with [^{3}H]glycine in the presence of strychnine (Schell *et al.*, 1995).

More direct evidence for a functional connection between glycine$_B$ sites and NMDA receptors was obtained in studies using radiolabeled ligands acting at the PCP

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site. The initial finding that glycine enhances [^{3}H]TCP and [^{3}H]MK-801 binding (Fagg, 1987; Wong *et al.*, 1987; Benavides *et al.*, 1988; Thomas *et al.*, 1988) was attributed to an allosteric interaction expressed as a change in affinity and maximal binding (B_{max}). However, most subsequent studies performed under equilibrium conditions failed to detect any influence of glycine on the affinity or B_{max} of channel blockers (Bonhaus and McNamara, 1988; Johnson *et al.*, 1988b; Kloog *et al.*, 1988). At present, it is clear that binding experiments using these ligands have features of a functional assay only when performed under nonequilibrium conditions, i.e., short incubation times with low concentrations of glycine and glutamate. Under these conditions, the degree of ligand bound is proportional to the affinity, concentration, and intrinsic activity of the added agents acting at the glycine and glutamate recognition sites. Hence, binding at the NMDA channel is an expression of site accessibility; it reflects the use-dependent nature of channel blockade, as clearly demonstrated in electrophysiological studies (Huettner and Bean, 1988).

In the rat forebrain, strychnine-insensitive [^{3}H]glycine binding is already present at prenatal stages and increases to the adult level by postnatal day 10. The change observed involved B_{max} (number of recognition sites) but not affinity (Shinohara *et al.*, 1989). Using the functional [^{3}H]MK-801 assay, it was reported that stimulation by glycine was approximately similar in postnatal and adult rats (Boje and Skolnick, 1992). In aged animals, there are indications of a decrease in [^{3}H]glycine binding primarily in forebrain structures (hippocampus, cortex, striatum, and thalamus), as studied by receptor autoradiography (Miyoshi *et al.*, 1990; Araki *et al.*, 1996); this is not apparently the consequence of general NMDA receptor loss, because the binding of [^{3}H]MK-801 was not changed (Araki *et al.*, 1996).

It has long been recognized that, in addition to the CNS, NMDA receptors are present in the periphery (Erdo, 1991), e.g., in the myenteric plexus (Moroni *et al.*, 1989), and these receptors are also sensitive to positive modulation by glycine (Galli *et al.*, 1990; Campbell *et al.*, 1991). Glycine-sensitive NMDA receptors are not unique to mammals but are also seen in invertebrates such as molluscs and insects (Darlison, 1992; Moroz *et al.*, 1993).

7. Are glycine$_B$ sites saturated in vivo? It has been often claimed that the glycine$_B$ site is saturated under physiological conditions (e.g., see Obrenovitch *et al.*, 1997). However, this conclusion is mainly based on in vitro experiments using brain slices (Fletcher and Lodge, 1988; Kemp *et al.*, 1988a; Taylor *et al.*, 1988; Crawford and Roberts, 1989; Ransom and Deschenes, 1989) and on the observation that the free glycine concentration in ECF in vivo is much higher than the K_d of glycine for the receptor site (see Section III.C.1.). However, the following arguments argue against the assumption that glycine$_B$ sites are saturated.

1. It should be kept in mind that the traumatic preparation of slices and/or the application of the NMDA agonists leads to release of glutamate and glycine; in other words, slice preparations are not ideally suited to address this question. However, even under such conditions some authors have reported that additional glycine or D-serine enhances NMDA receptor-mediated events (Thomson *et al.*, 1988; Minota *et al.*, 1989).
2. Estimates of the ECF concentration of any molecule do not necessarily reflect concentrations in the synaptic cleft. If this were the case, NMDA receptors would be continuously saturated by glutamate, because the "resting" ECF concentration of glutamate could be as high as 345 μM, based on in vivo microdialysis (5 μl/min flow) results corrected for in vitro recovery (5%) and a diffusion factor (Benveniste and Hansen, 1991), i.e., 1000 times greater than the K_i of glutamate for NMDA receptors (Watkins *et al.*, 1990). It is, however, likely that the basal level of glutamate was overestimated by Benveniste and Hansen (1991). Using the zero-net flux and mass-transfer methods of in vivo recovery, lower values were obtained by other investigators (Jacobson *et al.*, 1985; Miele *et al.*, 1996), i.e., 3.0 and 4.3 μM, respectively. Even these conservative estimates of resting glutamate levels would be sufficient to strongly activate NMDA receptors. Fortunately, NMDA receptors are clearly not saturated in vivo, because of efficient local buffering mechanisms.
3. The glycine$_B$ antagonist kynurenic acid is endogenous to the brain and under certain conditions may rise to concentrations sufficient to block the glycine$_B$ recognition site, according to some authors (Moroni *et al.*, 1988a; Wu *et al.*, 1992). It should be noted that kynurenic acid is not selective for the glycine$_B$ site.
4. Zinc has been shown to decrease the affinity of [^{3}H]glycine binding (Yen *et al.*, 1990). Hence, the affinity of glycine in vitro may be overestimated, compared with in vivo conditions in brain regions where zinc is present (Aniksztejn *et al.*, 1987). However, it should be stressed that Ca^{2+} and Mg^{2+} have been reported to increase glycine affinity (Gu and Huang, 1994; Wang and Macdonald, 1995; McBain and Mayer, 1994).

Referring to the second point, uptake mechanisms probably also buffer the local glycine concentration and thereby maintain a diffusion gradient between the ECF and the synaptic cleft. In fact, the feasibility of this prediction was clearly demonstrated by very elegant electrophysiological experiments in which NMDAR1/2A or NMDAR1/2B receptors were coexpressed with GLYT1b in *Xenopus* oocytes (Supplisson and Bergman, 1997). By studying responses to NMDA, it was demon-

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strated that this glycine transporter can buffer glycine concentrations by up to 95%, i.e., 10 μM glycine would be buffered within 1 sec to 0.5 μM near the NMDA receptor (Supplisson and Bergman, 1997). D-Serine was not affected because it is not a substrate for this transporter (Supplisson and Bergman, 1997). This could have very important consequences for glutamatergic transmission, because this transporter is colocalized with NMDA receptors in the brain (Smith *et al.*, 1992; Adams *et al.*, 1995; Luque and Richards, 1995; Zafra *et al.*, 1995b). It is of course not clear whether the local density of this carrier in vivo is as high as that expressed in oocytes. Moreover, one could argue that depolarization during synaptic transmission would weaken this glycine uptake considerably (Supplisson and Bergman, 1997).

The most convincing evidence that the glycine$_B$ site is not saturated in vivo is the fact that there are >40 in vivo studies demonstrating clear-cut effects of glycine agonists or partial agonists stimulating NMDA receptors. These have been summarized in table 7. In some cases in which glycine was used as an agonist, the participation of strychnine-sensitive receptors cannot be ruled out. For example, the enhancement of dopamine release in the striatum, as studied by microdialysis experiments, might be mediated by glycine$_A$ receptors, because it was sensitive to strychnine (Yadid *et al.*,

TABLE 7
Compilation of in vivo studies showing effects of glycine$_B$ site agonists or partial agonists, indicating that the glycine$_B$ sites are not saturated

Effect	Agents used (route of administration)	References
Analgesia after unilateral ligation of sciatic nerve	Glycine (intrathecal)	Simpson *et al.,* 1997
Enhancement of tail flick response	D-Serine (intrathecal)	Kolhekar *et al.,* 1994
Anxiogenic response	D-Serine, glycine (PAG)[a]	Schmitt *et al.,* 1995
Pressor responses	Glycine, D-serine (caudal ventrolateral medulla)	Kapoor *et al.,* 1996
Enhancement of NMDA-induced hypertension	Glycine (PAG)	Berrino *et al.,* 1992
Enhancement of NMDA receptor-dependent cGMP stimulation in the cerebellum	Glycine (systemic, cisterna magna)	Danysz *et al.,* 1987, 1989c
Enhancement of cGMP production in the cerebellum	D-Cycloserine (i.p.) (at high doses, antagonism), D-serine (i.p.)	Wood *et al.,* 1989; Emmett *et al.,* 1991
Enhancement of NMDA-induced elevation of cGMP in the cerebellum	D-Serine (local, microdialysis)	Vallebuona and Raiteri, 1995; Fedele *et al.,* 1997
Enhancement of cGMP production in the hippocampus	D-Serine (local, microdialysis)	Uckele *et al.,* 1989
Enhancement of NMDA-induced convulsions in rats at postnatal day 7	D-Serine (i.p.)	Peeters and Vanderheyden, 1992
Enhancement of NMDA-induced convulsions	D-Serine (i.c.v.)	Singh *et al.,* 1990c, 1991
Potentiation of strychnine-induced convulsions	Glycine; AP5-sensitive (intrathecal)	Larson and Beitz, 1988
Depression of positive waves in the hippocampus; electrophoresis	Glycine (local)	Erdemli *et al.,* 1990
Enhancement of NMDA responses in the spinal cord and dorsal horn; electrophoresis	Glycine (local)	Budai *et al.,* 1992
Enhancement of NMDA responses in the cerebellum; electrophoresis (dependent on the firing rate)	Glycine (local)	Netzeband *et al.,* 1990
Enhancement of NMDA responses in the visual cortex; electrophoresis	D-Serine (local)	Czepita *et al.,* 1996
Enhancement of NMDA responses in the red nucleus; electrophoresis	Glycine, D-serine (local)	Goldstein *et al.,* 1989
Enhancement of NMDA responses; electrophoresis	Glycine, D-serine (red nucleus)	Goldstein *et al.,* 1989
Enhancement of field potential in hippocampus (DG)	D-Cycloserine (local)	Pitkanen *et al.,* 1994
Enhancement of field potential in DG	D-Cycloserine (hippocampus)	
Enhancement of NMDA-induced negative field potential in the hippocampus (CA1); electrophoresis	Glycine (local)	Dalkara *et al.,* 1992
Enhancement of synaptic responses in the red nucleus; electrophoresis	D-Serine (local), glycine produced inhibition	Davies *et al.,* 1994
Enhancement of eye blink response learning	D-Cycloserine, monoclonal antibody to glycine site	Thompson *et al.,* 1992
Enhancement of LTP in CA1	D-Serine (hippocampus)	Thiels *et al.,* 1992
Enhancement of spatial learning	D-Cycloserine (i.p.)	Monahan *et al.,* 1989b; Sirvio *et al.,* 1992; Baxter *et al.,* 1994; Zajaczkowski and Danysz, 1997
Improvement in word recall test in humans	D-Cycloserine (subcutaneous)	Wesnes *et al.,* 1991
Enhancement of LTP in the hippocampus (CA1) in microcephalic rats	D-Serine (local)	Ramakers *et al.,* 1993
Attenuation of PCP-induced hyperlocomotion	D-Serine, D-alanine (i.c.v.)	Tanii *et al.,* 1991, 1994
Attenuation of (+)MK-801 and PCP stereotypy	D-Serine, glycine (i.c.v.)	Contreras, 1990
Attenuation of PCP-induced hyperlocomotion	Glycine (i.p.)	Toth and Lajtha, 1986
Enhancement of noradrenaline release in the red nucleus of stria terminalis	D-Serine (local)	Aliaga *et al.,* 1995
Enhancement of NMDA-stimulated dopamine release in the striatum	Glycine (local), microdialysis	Krebs *et al.,* 1989; Martinez-Fong *et al.,* 1992
Enhancement of NMDA-stimulated glutamate release in the striatum	D-Serine (local), microdialysis	Bustos *et al.,* 1992
Enhancement of ischemia-induced damage	Glycine, serine (i.c.v.)	Dalkara *et al.,* 1990

[a] PAG, periaqueductal gray; DG, dorsal ganglia.

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1993). Although no glycine$_A$ site mRNA has been detected in this structure, it is possible that the receptor protein is transported from the substantia nigra pars compacta (SNc), the origin of dopaminergic terminals (Yadid *et al.*, 1993). In some cases, different effects of glycine and D-serine have been observed, such as enhancement of synaptic responses in the red nucleus (Davies *et al.*, 1994). This is probably because only the latter ligand has sufficient selectivity for glycine$_B$ sites. Moreover, the existence of inhibitory glycine receptors that are insensitive to strychnine has been suggested, based on noradrenaline release studies in hypothalamus slices (Johnson *et al.*, 1994).

The degree of saturation of glycine$_B$ sites also depends on the conditions and brain structure. D-Serine enhanced NMDA-induced cGMP production, as detected by microdialysis in the cerebellum, but was ineffective in the hippocampus (Vallebuona and Raiteri, 1995). Similarly, Fletcher and Lodge (1988) found that glycine failed to enhance NMDA responses in the spinal cord, but this result contrasts with some other studies in the same region (Budai *et al.*, 1992). Saturation of glycine$_B$ sites in vivo is implied by experiments demonstrating that mice lacking D-amino acid oxidase show no signs of overstimulation of NMDA receptors, despite having higher levels of D-serine in the cerebellum and serum (Hashimoto *et al.*, 1993b; Nagata *et al.*, 1992).

To summarize, there is still considerable debate regarding whether the glycine$_B$ site is saturated in vivo (Peeters and Vanderheyden, 1992; Matsui *et al.*, 1995; Wood, 1995; Fedele *et al.*, 1997; Obrenovitch *et al.*, 1997); we think that no general answer can be given, because the degree of occupation depends on numerous factors, such as regional differences in the expression of receptor subtypes (with different affinities for glycine), the type and density of glycine carriers present, cation and kynurenic acid concentrations, and local glycine and/or D-serine levels (Czepita *et al.*, 1996). However, on the basis of the evidence discussed above, it seems clear that fluctuations of glycine or D-serine concentrations may be an important factor determining the magnitude of NMDA receptor activation during both physiological and pathological processes (Dalkara *et al.*, 1990) (also see Section VI.M.).

D. Kynurenic Acid as an Endogenous Antagonist of the Glycine$_B$ Site

Kynurenic acid is an endogenous molecule found in both rodent and human brain, at concentrations of 5.8 to 36.3 and 50 pmol/g, respectively (Moroni *et al.*, 1988b; Turski *et al.*, 1988, 1989b). It is most likely produced by astroglia (Speciale *et al.*, 1989; Eastman *et al.*, 1994). It is a product of the kynurenine pathway after tryptophan degradation, and its direct precursor is kynurenine (Moroni *et al.*, 1988b; Turski *et al.*, 1988, 1989b). Kynurenic acid is also potentially an endogenous glycine$_B$ antagonist, with a 50% inhibitory concentration (IC_{50}) of 43 μM ([^{3}H]glycine binding) (Danysz *et al.*, 1989b; see also Kessler *et al.*, 1989b). When exogenously applied, it clearly attenuates activation of NMDA receptors (Birch *et al.*, 1988b,c; Velisek *et al.*, 1995). Questions arise regarding whether the brain levels of endogenous kynurenic acid are sufficient to block NMDA receptors under certain conditions and, if they are, why nature would create both agonists and an antagonist for the same site. The kynurenic acid concentration has been reported to be high in prenatal rodents (300 fmol/mg of protein), but it decreases after birth (Beal *et al.*, 1992). Increased levels have also been reported in aged animals (Moroni *et al.*, 1988a; Gramsbergen *et al.*, 1992), associated with various brain insults/diseases, such as septicemia (Heyes and Lackner, 1990), excitotoxic brain lesions (Wu *et al.*, 1992), and kindling (Loscher *et al.*, 1996), and in dystonic hamsters (Richter *et al.*, 1996). Enhanced levels of kynurenic acid in human brain have also been reported in Huntington's disease (Connick *et al.*, 1989; but see Beal *et al.*, 1990) and in Down's syndrome (Baran *et al.*, 1996). Brain levels of kynurenic acid can be efficiently raised to concentrations that interact with NMDA receptors by several pharmacological interventions, for example, by administering the kynurenic acid precursor 4-chlorokynurenine, inhibiting brain efflux with probenecid, or inhibiting kynurenic acid metabolism (see Section IV.C.9.).

IV. Exogenous Ligands

Numerous high affinity, full antagonists for the glycine$_B$ site were developed shortly after detection of this recognition site, but this was then recognized as being the easy step. It soon became clear that the real challenge was to develop compounds that penetrate the CNS well. Only with such compounds can the suggested usefulness of the glycine$_B$ site for drug targeting be properly verified in animal models. Both the central administration of systemically inactive, full antagonists and the use of partial agonists (which show better blood-brain barrier penetration) often produce confusing results. Although glycine$_B$ site partial agonists and antagonists influence the same site (by definition), in our opinion they should be considered separately. First, the agonist and antagonist glycine$_B$ sites of the NMDA receptor are overlapping but probably not exactly the same, as evidenced by mutation analysis of the receptor protein (see Section II.D.) and by comparison of the structures of these two groups of agents. Second, the outcome of treatment with glycine$_B$ site partial agonists is not predictable on the basis of the intrinsic activity determined in vitro (Karcz-Kubicha *et al.*, 1997).

A. Agonists of the Glycine$_B$ Site

Of a multitude of amino acids tested in the pioneering study of Johnson and Ascher (1987) with cultured mouse cortical neurons, only alanine and serine were also active, and they were not as effective as glycine. In the

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same year, glycine and D-serine were reported to have similar potencies in enhancing [^{3}H]TCP and [^{3}H]MK-801 binding in the presence of glutamate, whereas D-alanine was somewhat less potent (Bonhaus *et al.*, 1987; Reynolds *et al.*, 1987; Snell *et al.*, 1987) (fig. 10). Interestingly, the L-isomers of alanine and serine were considerably less potent (Kessler *et al.*, 1987; Reynolds *et al.*, 1987), which was confirmed in electrophysiological studies in *Xenopus* oocytes (Kleckner and Dingledine, 1988). There are numerous binding and electrophysiological studies supporting the actions of these amino acids at the glycine$_B$ site. Most are listed within this review and confirm that the relative potencies at native receptors show the following rank order: glycine > D-serine > D-alanine ≫ L-serine > L-alanine (fig. 10). Much higher (millimolar) concentrations of L-serine, L-cysteine, L-alanine, L-proline, and glycine also evoke inward current responses in cultured hippocampal neurons by acting as agonists at the NMDA recognition site (Pace-Asciak *et al.*, 1992).

B. Partial Agonists of the Glycine$_B$ Site

One of the first glycine$_B$ site partial agonists described, ACPC (fig. 10), has an intrinsic activity of 80 to 92% (table 5) and a potency of approximately 0.09 to 0.4 μM (Nadler *et al.*, 1988; Marvizon *et al.*, 1989; Monahan *et al.*, 1989a; Watson and Lanthorn, 1990; Karcz-Kubicha *et al.*, 1997). Interestingly, the concentration-response curve for ACPC in patch-clamp experiments shows a biphasic nature, indicating spillover onto other recognition sites at higher concentrations (Karcz-Kubicha *et al.*, 1997). ACPC shows antidepressive, anxiolytic, and neuroprotective activity (see Section VI.) (table 8). These "antagonistic" effects at NMDA receptors are difficult to reconcile with its high intrinsic activity. Interestingly, in cultured neurons subjected to sustained exposure to ACPC, the neuroprotective effect vanishes after 24 h, which has been associated with an increase in the expression of the NR2C subunit, at which it has 150% intrinsic activity (relative to glycine) (Fossom *et al.*, 1995a; Krueger *et al.*, 1997). In contrast, repeated treatment in vivo resulted in diminution of NMDA-induced convulsions and antidepressive activity in the forced-swim test, but anxiolytic and neuroprotective effects remained unchanged (Skolnick *et al.*, 1992; Von Lubitz *et al.*, 1992). These diverse effects strongly suggest that there might be alterations in NMDA receptor composition, as found in vitro.

ACPC seems to penetrate well to the CNS. Initially it was reported that this agent has a brain half-life of <5 min (Rao *et al.*, 1990), which would make interpretation of any in vivo effects extending beyond this time difficult. However, subsequent studies in monkeys and rodents showed that its half-life in blood is in fact 2.5 h and that in the CNS is 6 h; in humans, it is even longer (Cherkofsky, 1995; Maccecchini, 1997). ACPC is currently being developed by Symphony Pharmaceuticals for treatment of stroke and depression (table 8).

Among this group of cyclic analogues, an increase in size decreases affinity and intrinsic activity. In *Xenopus* oocytes injected with mRNA from rat brain, ACPC was a potent partial agonist with high intrinsic activity at the glycine$_B$ site, 1-aminocyclobutanecarboxylic acid (ACBC) (fig. 10) was a weak partial agonist with low

TABLE 8
Glycine$_B$ site partial agonists and antagonists under development

Agent	Company	Application	Phase[a]	References
ACEA 1021 (Licostinel)	CoCensys	Stroke	III	Woodward *et al.*, 1995a; Lutfy and Weber, 1996
		Head injury	I	
		Pain	P	
D-Cycloserine	Searle Monsanto	Dementia	II, D	Wesnes *et al.*, 1991; Lanthorn, 1994; Mohr *et al.*, 1995; van Berckel, 1997
GV 150526	Glaxo Wellcome	Stroke	III	Bordi *et al.*, 1996; Di Fabio *et al.*, 1997
ZD9379	Zeneca	Stroke	II	Qiu *et al.*, 1997; Shors *et al.*, 1997
		Pain	II	
GV 196771A	Glaxo Wellcome	Chronic pain	I	Quartaroli *et al.*, 1997
ACPC	Symphony	Stroke, depression	I	Maccecchini, 1997
4-Chlorokynurenine (prodrug)	University of Maryland	Huntington's disease	P	Wu *et al.*, 1997
7-Cl-thio-KYN	Fidia	Neuroprotection	P	Chen *et al.*, 1993; Pellegrini-Giampietro *et al.*, 1994
L-701,324	Merck & Co	Antipsychotic, pain, neuroprotection	P, D	Bristow *et al.*, 1995, 1996a,b; Priestley *et al.*, 1996
M244249	Zeneca	Stroke	P, D	Patel *et al.*, 1993
M241247	Zeneca	Stroke	P, D	Patel *et al.*, 1993
MDL-29,951	Hoechst Marion Roussel	Neuroprotection	P	Baron *et al.*, 1992
MDL-100,748	Hoechst Marion Roussel	Epilepsy	P	Baron *et al.*, 1992
MRZ 2/570	Merz	Drug dependence, chronic pain, neuroprotection	P	Parsons *et al.*, 1997
MRZ 2/571	Merz			Parsons *et al.*, 1997
MRZ 2/576	Merz			Parsons *et al.*, 1997
SM-18400	Sumitomo	Stroke, epilepsy	P	Yasuda *et al.*, 1995

[a] I–III, clinical phases I to III; P, preclinical phase; D, discontinued; 7-Cl-thio-KYN, 7-chlorothiokynurenic acid.

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intrinsic activity but also interacted with other sites on the NMDA receptor, and cycloleucine was a very weak, $glycine_B$ site full antagonist (Watson and Lanthorn, 1990; Wong and Kemp, 1991). ACBC has a bigger ring than ACPC, lower intrinsic activity (approximately 16%), and much lower affinity [50% effective concentration (EC_{50}) = 17 to 19 μM], as shown by electrophysiological and binding experiments (Hood *et al.*, 1989b; Watson *et al.*, 1989). Because of its low potency, this compound has not been studied intensively, and it has not been proven that this compound actually penetrates to the CNS. Cycloleucine (fig. 10) has the biggest ring and is a very weak, full antagonist, inhibiting glycine responses at 1 mM concentrations to 36%, with an IC_{50} of approximately 500 to 600 μM (Snell and Johnson, 1988; Watson and Lanthorn, 1990). However, patch-clamp studies with cultured hippocampal neurons showed higher potency (IC_{50} = 24 μM with 1 μM glycine), although it should be noted that a component of blockade could not be overcome by higher glycine concentrations, indicating interactions with other sites on the NMDA receptor (Hershkowitz and Rogawski, 1989).

D-Cycloserine (fig. 10) has long been used at high doses as an antibiotic for the treatment of tuberculosis (Mandell and Petri, 1996); more recently, it was discovered to have partial agonistic properties at the $glycine_B$ site, with an intrinsic activity of 40 to 86%, depending on the experimental paradigm (Hood *et al.*, 1989a; Watson *et al.*, 1990; Priestley and Kemp, 1994; Karcz-Kubicha *et al.*, 1997). It has been shown that at lower doses (<20 mg/kg) D-cycloserine shows agonistic effects in vivo but at higher doses an antagonistic action, such as anticonvulsive activity, predominates (Emmett *et al.*, 1991; Peterson and Schwade, 1993; Lanthorn, 1994). Such bell-shaped dose-effect relationships are sometimes falsely interpreted to be "typical" for partial agonists, i.e., agonism at low doses and antagonism at high doses. However, partial agonism actually means that an agent reaches a limited nonmaximal effect at higher doses (intrinsic activity), i.e., antagonizes receptor activation by high concentrations of a full agonist but facilitates low concentrations of a full agonist (Henderson *et al.*, 1990; Karcz-Kubicha *et al.*, 1997). Recent data indicate that the consistent biphasic effects of D-cycloserine observed in vivo may, rather, be related to different affinities and intrinsic activities at NMDA receptor subtypes (see Section V. and table 5). Therefore, D-cycloserine is a partial agonist for the murine equivalents of NR1/NR2A and NR1/NR2B heteromers (38% and 56% intrinsic activity, respectively, compared with 10 μM glycine) but is more effective than glycine at NR1/NR2C receptors (130%) (O'Connor *et al.*, 1996). This effect is accompanied by higher affinity at NR1/NR2C receptors, i.e., NR1/NR2C > NR1/NR2D ≫ NR1/NR2B > NR1/NR2A (Krueger *et al.*, 1997). Therefore, it is likely that the biphasic effects seen in vivo are the result of agonistic actions at NR1/NR2C receptors at lower doses and inhibition of NR1/NR2A- and NR1/NR2B-containing receptors at higher doses. This does not explain, however, why complete inhibition of NMDA effects on cGMP production is observed in the cerebellum, where NR1/NR2C receptors predominate (Emmett *et al.*, 1991). The receptor subtype selectivity of D-cycloserine and its differential intrinsic activity could well underlie its promising preclinical profile in some animals models. Enhancement of NMDA receptor-mediated events by D-cycloserine in human and nonhuman subjects is lost after chronic administration (Lanthorn, 1994; Quartermain *et al.*, 1994; Randolph *et al.*, 1994). D-Cycloserine was developed by Searle-Monsanto as a cognitive enhancer for dementia but has been abandoned (table 8).

Racemic HA-966 (fig. 10) was originally developed by Organon in the late 1960s (Bonta *et al.*, 1971), but its antagonistic action at the $glycine_B$ site was recognized years later. In 1978, HA-966 was reported to produce Mg^{2+}-like selective antagonism of excitatory amino acid-induced responses in isolated spinal cords of frogs and immature rats in vitro (Evans *et al.*, 1978) and to antagonize amino acid-induced and synaptic excitation of mammalian spinal neurons in vivo (Biscoe *et al.*, 1978). HA-966 antagonism of NMDA-induced responses in rat cortical slices and cultured rat cortical neurons was then found to be sensitive to reversal by glycine (Fletcher and Lodge, 1988; Drejer *et al.*, 1989; Foster and Kemp, 1989; Keith *et al.*, 1989). Systemic administration of HA-966 was then reported to selectively reduce NMDA-induced lesions of the striatum in perinatal rats (Uckele *et al.*, 1989). The first indication that HA-966 might be a partial agonist was the finding that inhibition of [^{3}H]MK-801 binding, NMDA-induced Ca^{2+} influx, and NMDA-induced release of ^{3}H-labeled γ-aminobutyric acid (GABA) was not complete (Reynolds *et al.*, 1989). Conclusive evidence that both HA-966 and D-cycloserine are partial agonists, with low and high intrinsic activities, respectively, was first provided by patch- and concentration-clamp experiments (Henderson *et al.*, 1990). The in vivo and in vitro activity of HA-966 at the $glycine_B$ site was subsequently found to reside in the R(+)-stereoisomer (Pullan *et al.*, 1990b), whereas the (−)-isomer was reported to be a potent γ-butyrolactone-like sedative (Singh *et al.*, 1990a).

Hence, all old studies using racemic HA-966 should be interpreted with caution, and only R(+)HA-966 (fig. 10) should be used to test effects at the $glycine_B$ site. R(+)HA-966 inhibits NMDA receptors with a potency of 5.6 (K_i) or 28 (IC_{50}) μM and has an intrinsic activity of 10 to 40% (Watson and Lanthorn, 1990; Priestley and Kemp, 1994; Karcz-Kubicha *et al.*, 1997). In contrast to the accepted partial agonistic properties of R(+)HA-966, it was reported that, in the periphery, HA-966 inhibited NMDA responses completely (Campbell *et al.*, 1991). It is not clear whether this was attributable to different properties of NMDA receptors in vivo or the fact that the nonselective racemic mixture was used.

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Attempts to improve the potency of partial agonists by 4-substitutions of R(+)HA-966 led to L-687,414 [R-(+)*cis*-β-methyl-3-amino-1-hydroxypyrrolid-2-one] (a *cis*-methyl derivative) (fig. 10) and *cis*-hydroxy derivatives with increased potency, but there was a rapid loss of activity with longer or *trans* substitutions (Leeson *et al.*, 1990, 1993b). It was proposed that the axial conformation is preferred, and Merck Sharp and Dohme attempted to develop bicyclic compounds to test this hypothesis. The only compound to result from these attempts was a [3.2.1]bicyclic derivative. Both this compound and L-687,414 are partial agonists with very low intrinsic activities and would be predicted to have effects similar to those of full antagonists. Indeed, L-687,414 is active in DBA/2 mice, with a 50% inhibitory dose (ID_{50}) of 5.2 mg/kg [administered intraperitoneally (i.p.)], but its potency against maximal electroshock (MES)-induced convulsions leaves room for improvement [26.1 mg/kg, intravenously (i.v.)] (Foster *et al.*, 1992b; Leeson *et al.*, 1993b). The very strict structure-activity requirements for these partial agonists may be related to differences in allosteric interactions, compared with full antagonists (Kemp and Priestley, 1991). R(+)HA-966 and L-687,414 were under development by Merck Sharp and Dohme but have been abandoned (table 8) (Smith and Meldrum, 1992; Gill *et al.*, 1995).

Although none of these systemically active partial agonists induces receptor desensitization (Henderson *et al.*, 1990; Kemp and Priestley, 1991; Karcz-Kubicha *et al.*, 1997), they have favorable therapeutic profiles in some in vivo models and, in the case of D-cycloserine, also in humans (Lanthorn, 1994; van Berckel *et al.*, 1997; Witkin *et al.*, 1997). This may be, in part, the result of their own intrinsic activities as agonists at the $glycine_B$ site, which would serve to preserve a certain level of NMDA receptor function even at very high concentrations (Priestley and Kemp, 1994; Fossom *et al.*, 1995a; Krueger *et al.*, 1997).

C. Antagonists

1. Kynurenic acid derivatives. Although kynurenic acid has been known for years, Kessler *et al.* (1987, 1989b) were the first to recognize (using receptor binding assays) that it actually interacts with $glycine_B$ sites. Electrophysiological studies subsequently confirmed these $glycine_B$ antagonistic effects against NMDA responses in infant rat hemisected spinal cord (Birch *et al.*, 1988b; Watson *et al.*, 1988). However, kynurenic acid is very weak and is not selective, because it is also a competitive antagonist at NMDA and AMPA/kainate receptors (Birch *et al.*, 1988c; Stone, 1991).

Kemp *et al.* (1988a) then found that the halogen-substituted derivative 7-Cl-KYN (fig. 5) is also a $glycine_B$ site antagonist, with 70-fold higher affinity in patch-clamp experiments (IC_{50} = 0.3 μM). NMDA receptor antagonism in vivo was subsequently confirmed by anticonvulsant and neuroprotective activity after i.c.v. administration (Foster and Kemp, 1989; Singh *et al.*, 1990c). These findings were followed by intensive research on the structural requirements for actions at the $glycine_B$ site. Lipophilic and size-limited substitutions at the 5- and 7-positions increase activity at the $glycine_B$ site, whereas compounds with 6-substitutions show more activity as competitive AMPA receptor antagonists (McNamara *et al.*, 1990; Baron *et al.*, 1991; Leeson *et al.*, 1991a; Foster *et al.*, 1992a; Leeson and Iversen, 1994). The effects of these substitutions are additive, in terms of both potency and selectivity. Chloro, bromo, and methyl groups are best at the 7-position, whereas bulkier groups such as iodo and ethyl moieties are better at the 5-position. The most potent in vitro antagonist of this series is 5-iodo-7-chlorokynurenic acid (L-683,344), although 5,7-diCl-KYN (fig. 5) has become the standard potent and selective $glycine_B$ antagonist for in vitro studies. 4-Thiol substitutions of kynurenic acid derivatives retain activity at the $glycine_B$ site, despite existing as thiol tautomers in solution (Moroni *et al.*, 1991), and seem to exhibit better penetration to the CNS (Chen *et al.*, 1993). In contrast, 4-carboxymethylamino derivatives such as MDL-100,748 [4-carboxymethylamino-5,7-dichloroquinoline-2-carboxylic acid] (fig. 5) exist as 4-keto tautomers in solution, and this configuration was proposed to increase their potency at the $glycine_B$ site (Harrison *et al.*, 1990).

MDL-102,288 [5,7-dichloro-1,4-dihydro-4-[[[4-[(methoxycarbonyl)amino]phenyl]sulfonyl]imino]-2-quinolinecarboxylic acid] (fig. 5) is a moderately potent $glycine_B$ antagonist ([^{3}H]glycine K_i = 170 nM), and it blocked NMDA-stimulated cGMP accumulation in rat cerebellar slices with an IC_{50} of 7.8 μM (Kehne *et al.*, 1995). Although MDL-102,288 was without effect in DBA/2 mice [50% effective dose (ED_{50}) > 300 mg/kg, i.p.], it did reduce separation-induced ultrasonic vocalizations in rat pups (ED_{50} = 6.3 mg/kg, i.p.). This anxiolytic effect of MDL-102,288 was selective, in that it occured at doses that did not produce motor disruption, as measured using an inclined-plane test [ED_{50} > 160 mg/kg; therapeutic index (TI) > 25.4]. However, the possibility cannot be excluded that this in vivo activity was the result of the poorly developed blood-brain barrier in newborn animals.

Benzothiadiazine-1,1-dioxides were developed by Rhône Poulenc Rorer and have structural requirements similar to those of kynurenic acid derivatives (Mignani *et al.*, 1995; Boireau *et al.*, 1996). RPR 104632 (fig. 5) antagonized [^{3}H]5,7-diCl-KYN binding with a K_i of 4.9 nM and functional [^{3}H]MK-801 binding with an IC_{50} of 55 nM. This (−)-isomer was 500-fold more potent than the (+)-isomer. RPR 104632 was less potent in inhibiting NMDA-evoked increases in cGMP levels in neonatal rat cerebellar slices than in inhibiting [^{3}H]5,7-diCl-KYN binding but reduced NMDA-induced neurotoxicity in rat hippocampal slices and in cortical primary cell cultures at 1 to 10 μM (Boireau *et al.*, 1996).

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2. 2-Carboxyindoles. 2-Carboxyindoles such as 2-carboxybenzimidazole (fig. 4) were reported to be active as glycine$_B$ antagonists at the same time as kynurenic acid (Huettner, 1989; Smith *et al.*, 1993) and quinoxaline-2,3-dione derivatives, which led to the assumption that kynurenic acid derivatives exist as the 4-keto tautomer in solution (Leeson *et al.*, 1991a). The 3-substituent seems to have a structure-activity relationship similar to that for the 4-substituent in 2-carboxytetrahydroquinolines and is similarly responsible for hydrogen bonding (Huettner, 1989; Salituro *et al.*, 1990; Gray *et al.*, 1991; Baron *et al.*, 1992; Hood *et al.*, 1992; Rowley *et al.*, 1992; Salituro *et al.*, 1992; Vazquez *et al.*, 1992; Nichols and Yielding, 1993; Rao *et al.*, 1993). Compounds such as SC-49648 [3-(6-chloro-2-carboxyindol-3-yl)ethanoic acid] and MDL-29,951 [3-(4,6-dichloro-2-carboxyindol-3-yl)propionic acid] (fig. 4) are only active after i.c.v. administration (Baron *et al.*, 1992). SC-50132 [3-(6-chloro-2-carboxyindol-3-yl)ethanoic acid ethyl ester] is the 3-ethyl ester analogue of SC-49648 and was found to be a weak, systemically active prodrug for SC-49648 (Rao *et al.*, 1993).

MDL-100,458 [3-(benzoylmethylamino)-6-chloro-1H-indole-2-carboxylic acid] (fig. 4) selectively inhibits [^{3}H]glycine binding, with a K_i of 130 nM (Salituro *et al.*, 1991), and completely antagonizes NMDA-stimulated cGMP accumulation in rat cerebellar slices. MDL-100,458 antagonized audiogenic seizures in DBA/2 mice with an ED_{50} of 21 mg/kg, i.p. (Kehne *et al.*, 1995).

Another compound from this group, MDL-105,519 (fig. 4), selectively displaces [^{3}H]glycine binding, with an IC_{50} of 10 nM; as a tritiated radioligand, it labels glycine$_B$ sites with a K_d of 2 to 10 nM (Baron *et al.*, 1996, 1997; Siegel *et al.*, 1996; Parsons *et al.*, 1997). However, low-micromolar concentrations are required to block functional NMDA-induced Ca^{2+} influx and inward currents in cultured neurons. MDL-105,519 shows some activity in vivo, with ED_{50} values of 13, 29, and 36 mg/kg in juvenile DBA/2 mice and against MES- and quinolinic acid-induced convulsions, respectively (Baron *et al.*, 1996, 1997; Siegel *et al.*, 1996; Parsons *et al.*, 1997). However, it should be noted that the activity of MDL-100,458 and MDL-105,519 in juvenile DBA/2 mice may be attributable to the poorly developed blood-brain barrier and the weaker activity in adult rats was apparent even after i.v. administration (Baron *et al.*, 1997).

GV 150526A [4,6-dichloro-3-[2-[(phenylamino)carbonyl]ethenyl]-1H-indole-2-carboxylic acid] and GV 196771A [E-4,6-dichloro-3-(2-oxo-1-phenylpyrrolidin-3-ylidenemethyl)-1H-indole-2-carboxylic acid] (fig. 4) belong to a series of 2-carboxyindole derivatives in which chain substitutions at position 3 increased affinity at the glycine$_B$ site. The most active compounds had in vitro affinities in the low-nanomolar range and were active against convulsions in mice after i.v. and oral (p.o.) administration at low-milligram per kilogram doses (Di Fabio *et al.*, 1997). Potency increased with the lipophilicity and steric bulk of these substituents (Di Fabio *et al.*, 1997; see model).

GV 150526A blocks NMDA-induced inward currents in cultured hippocampal and spinal cord neurons with IC_{50} values of 0.10 and 0.14 μM, respectively (Bunnermann *et al.*, 1996). The effects are reversed by glycine, yielding affinity (K_b) values of 0.08 and 0.11 μM, respectively [with $K_b = IC_{50}/(1 + \{[\text{glycine}]/\text{glycine } EC_{50}\})$]. It showed no subtype selectivity, i.e., 1A/2B and 1A/2C receptors expressed in HEK 293 cells were inhibited with IC_{50} values of 0.05 and 0.07 μM, respectively. ^{3}H-labeled GV 150526A bound to cortical membranes with a K_d of 9.0 nM and a B_{max} of 3.4 pmol/mg of protein. Similar affinities were obtained using [^{3}H]glycine binding and functional [^{3}H]TCP binding assays. GV 150526A has no effect on other recognition sites of NMDA receptors or AMPA/kainate receptors. It has a half-life in rats of approximately 6 h and in humans of approximately 20 h. This compound is claimed to produce no side effects such as ataxia, learning impairment, or psychotomimetic-like activity (Bunnermann *et al.*, 1996). GV 150526A is currently under development by Glaxo Wellcome-Verona for the treatment of stroke; the related agent GV 196771A is being developed for the treatment of chronic pain (table 8).

3. 2-Carboxytetrahydroquinolines. The first compounds belonging to this group (fig. 3) were produced by Merck Sharp and Dohme and were developed to investigate the three-dimensional requirements for antagonists at the glycine$_B$ site (Carling *et al.*, 1992; Leeson *et al.*, 1992). The decreased potency of conformationally flexible 2,3-dihydrokynurenic acid derivatives was proposed to be the result of the fact that the pseudoaxial conformation is preferred in solution but the pseudoequatorial conformation is required for potency at the glycine$_B$ site. Introduction of 4-carboxyl groups introduced a second position for stereoisomerization, with *trans* substitutions being 100-fold more potent than *cis*. These *trans* substitutions take on a pseudoaxial conformation in solution and hold the 2-carboxyl group in the optimal pseudoequatorial position. The absolute stereochemistry is 2R/4S, showing similarities to that of agonists and partial agonists at the 2-position.

Amide derivatives of the 2,4-dicarboxytetrahydroquinolines were the starting point for very potent glycine$_B$ antagonists such as L-689,560 (Carling *et al.*, 1992; Leeson *et al.*, 1992). They have the same stereoselectivity, i.e., 2-equatorial and 4-axial. There is considerable bulk tolerance at the 4-position. Activity is optimal when there is a single phenyl group and a single atom spacer between this phenyl group and the amide carbonyl. L-689,560 (fig. 3) is one of the most potent glycine$_B$ antagonists developed to date. This compound is a phenyl urea, and the greatly improved potency may be the result of enhanced hydrogen bonding. Compounds with additional substitutions of the amide moiety, resulting in zwitterionic molecules, retained potency and

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showed some degree of in vivo activity (L-690,590) (Carling *et al.*, 1993). This study also confirmed that the poor in vivo activity was the result of low concentrations being reached in the CSF. The methyl ester prodrug L-691,470 [4-*trans*-2-carboxymethyl-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline] shows improved water solubility but no change in potency after systemic administration. L-696,833 was designed in an attempt to use the amino acid transport system but was not notably more active in vivo.

4. 4-Hydroxy-2-quinolones. Acidity is an important factor for selective high affinity at the glycine$_B$ site. 2-Oxy plus 4-hydroxy substitutions of quinoxaline-diones (fig. 7) yield compounds with the desired pK_a of approximately 5 (Leeson *et al.*, 1993a). MDL-104,653 [3-phenyl-4-hydroxy-7-chloro-2(1H)-quinolinone] (fig. 7) is relatively weak as a glycine$_B$ antagonist (K_i = 170 nM against [^{3}H]glycine) but protects against sound-induced clonic seizures in DBA/2 mice after i.p. (ED$_{50}$ = 2 to 5 mg/kg) or p.o. (ED$_{50}$ = 6.2 mg/kg) administration. L-698,544 [7-chloro-3-nitro-2(1,3,4H)-quinolinone] is also a relatively weak, nonselective, AMPA/NMDA antagonist in vitro (10 μM) but is systemically active in DBA/2 mice (ED$_{50}$ = 13 mg/kg, i.p.). These compounds may therefore prove to be a basis for developing good neuroprotective agents (Carling *et al.*, 1993).

4-Hydroxy-3-nitroquinolin-2(1H)-ones have structural requirements and potencies similar to those of quinoxaline-diones but show a greater degree of selectivity (Cai *et al.*, 1996a). For example, 5,6,7-trichloro-4-hydroxy-3-nitro-2(1H)-quinolinone (fig. 7) has a potency similar to that of 6,7-dichloroquinoxaline-2,3-dione (DCQX) at the glycine$_B$ site (220 nM) but is 240-fold selective, compared with AMPA receptors. Substitutions at the 8-position cause a large decrease in affinity.

The ester L-695,902 and the cyclopropylketone L-701,252 [7-chloro-3-(cyclopropylcarbonyl)-4-hydroxy-2(1H)-quinolinone] (fig. 7) were the starting compounds for attempts to develop compounds with even better systemic activity (McQuaid *et al.*, 1992; Rowley *et al.*, 1993; Kulagowski *et al.*, 1994). Phenyl substitutions at the 3-position and halogen or small alkyl substitutions at the 6-position increased potency further and led to compounds such as L-701,315 [3-phenyl-4-hydroxy-5-ethyl-7-chloro-2(1H)-quinolinone]. Those authors proposed that the phenyl group at position 3 interacts in an electrostatic manner with the receptor via its π-system. This line of thinking led to the development of L-701,324, L-703,717 [7-chloro-4-hydroxy-3-[3-(4-methoxybenzyl)phenyl]-2(1H)-quinolinone], L-705,022 [7-chloro-4-hydroxy-3-(3-thiophenoxy)phenyl-2(1H)-quinolinone], and L-708,541 [7-chloro-4-hydroxy-3-[3-(4-O-ethyloxomethylbenzyl)phenyl]-2(1H)-quinolinone] (fig. 7), which are very potent glycine$_B$ antagonists in vitro. These compounds are also active against audiogenic seizures in DBA/2 mice at $<$1 mg/kg, and the first two are active after p.o. administration (Kulagowski *et al.*, 1994). This purported improved bioavailability was ascribed to the β-dicarbonyl system delocalizing negative charges across five atoms. However, it is unclear whether this improved potency in vivo really reflects improved bioavailability, because these compounds are 100-fold more potent than MDL-104,653 or L-695,544 in vitro but only 10- to 20-fold more potent in vivo.

Interestingly, 4-amino-3-phenylquinolin-2(1H)-dione was recently reported to exhibit a 40-fold lower binding affinity but to be only 4-fold weaker as an anticonvulsant, compared with the acidic 4-hydroxy compound MDL-104,653 (Carling *et al.*, 1997). Methylation or ethylation at the 4-position plus attachment of neutral, hydrogen bond-accepting groups to these groups produced compounds with in vitro and in vivo activities comparable to those of the acidic 4-hydroxy compound. This indicates that anionic functionality is not an absolute requirement for good affinity at the glycine/NMDA site (Carling *et al.*, 1997).

5. Quinoxaline-2,3-diones. The first quinoxaline-2,3-dione (fig. 8) derivatives synthesized were structurally related to the kynurenic acid derivatives and were claimed to be selective AMPA receptor antagonists (Drejer and Honore, 1988; Honore *et al.*, 1989). However, it soon became clear that most were not very selective and also blocked NMDA receptors by interacting with the glycine$_B$ site (Harris and Miller, 1989; Kessler *et al.*, 1989a; Sheardown *et al.*, 1989; Verdoorn *et al.*, 1989; Yamada *et al.*, 1989; Patel *et al.*, 1990). They showed different structure-activity requirements for aromatic ring substitution (Leeson *et al.*, 1991b). 6,7-Dinitroquinoxaline-2,3-dione was most potent as an AMPA antagonist, DCQX was nonselective, 5,7-dinitroquinoxaline-2,3-dione (MNQX) was preferential for the glycine$_B$ site, and 6-cyano-7-nitroquinoxaline-2,3-dione was approximately 10-fold selective for the glycine$_B$ site (Kessler *et al.*, 1989a; Sheardown *et al.*, 1989; Verdoorn *et al.*, 1989; Yamada *et al.*, 1989; Watkins *et al.*, 1990).

Heterocyclic derivatives such as 7,8-dichloro-1,2,4-triazolo[4,3-a]quinoxalin-4(5H)-one and 7,8-dichloroimidazo-[1,2-a]quinoxalin-4(5H)-one had affinities for the glycine site of 0.63 and 1.26 μM, respectively. Although the structure-activity relationship for the heterocyclic compounds did not directly parallel that for known quinoxalinediones, there was little improvement in selectivity, with five-fold higher concentrations also blocking [^{3}H]AMPA binding (McQuaid *et al.*, 1992). 6,7-Dichloroquinoxalic acid was also active in vitro but was not selective (Carling *et al.*, 1993).

Trisubstituted compounds such as ACEA 1011 (5-chloro-7-trifluoromethyl-1,4-dihydro-2,3-quinoxalinedione) and ACEA 1021 (fig. 8) have improved selectivity for the glycine$_B$ site (Epperson *et al.*, 1993; Keana *et al.*, 1995; Woodward *et al.*, 1995a) and are active in vivo in various models of glutamate-induced pathogenesis (Warner *et al.*, 1995; Lutfy *et al.*, 1996a,b, 1997; Lutfy and Weber, 1996; Takaoka *et al.*, 1997a). In general, a methyl group is a good replacement for chloro or bromo in the 6-position, and

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alkoxy-substituted quinoxaline-diones have lower potencies than alkyl- or halogen-substituted compounds. An ethyl group is not as good as a methyl group, especially in the β-position of 5,6,7-trisubstituted quinoxaline-diones (Cai *et al.*, 1997). ACEA 1416 (7-chloro-6-methyl-5-nitroquinoxaline-dione) (K_b = 8 to 11 nM) (fig. 8) and 7-bromo-6-methyl-5-nitroquinoxaline-dione (IC_{50} = 9 nM) are comparable to ACEA 1021 in potency as glycine site antagonists and have ED_{50} values of 1.2 and 2.0 mg/kg (i.v.), respectively, in the mouse MES assay (Ilyin *et al.*, 1996). Surprisingly, compounds with water/octanol partition coefficients of 0.5 are more active in vivo. 7-Chloro-6-methyl-5-nitroquinoxaline-dione, with 440-fold selectivity for NMDA versus AMPA receptors, was claimed to have the best combination of in vitro and in vivo potency of all compounds tested in studies of the quinoxaline-dione series (Cai *et al.*, 1997).

1,2,3,4-Tetrahydroquinoline-2,3,4-trione-3-oximes have structural requirements similar to those of quinoxaline-diones but show 10-fold higher potencies (Cai *et al.*, 1996a,b). For example, 5,6,7-trichloro-1,2,3,4-tetrahydroquinoline-2,3,4-trione-3-oxime binds to the glycine_B site with 12 nM affinity. SDZ 224-208 (fig. 8) is a mixed, orally active, glycine_B/AMPA receptor antagonist. It inhibits responses to AMPA in cortical wedge preparations with an affinity (based on Schild plots) of 5.2 (PA_2 negative common logarithm of the concentration producing an agonist shift to the right of 2 units) (equivalent to an IC_{50} of approximately 10 μM). Field EPSPs in the CA1 region of the hippocampus are blocked in vitro and in vivo with IC_{50} values of 6.2 μM and 31 mg/kg (i.p.), respectively. This compound also inhibits handling-induced seizures in strychnine-treated rats, kindled seizures in rats, and MES-induced convulsions in mice, with ID_{50} values of approximately 10 to 15 mg/kg (i.p. and p.o.). SDZ 224-208 has a long duration of action (4 to 10 h) and good solubility and shows no kidney toxicity. Brain levels are not known. This compound was claimed to have no effect on rotarod performance at 15 mg/kg (Meier *et al.*, 1996).

6. 3-Hydroxy-1H-1-benzazepine-2,5-diones. A β-dicarbonyl system is also present in 3-hydroxy-1H-1-benzazepine-2,5-diones such as 8-chloro-3-hydroxy-1H-1-benzazepine-2,5-dione (Kulagowski, 1996), some of which are also active in vivo (Guzikowski *et al.*, 1995, 1996; Jackson *et al.*, 1995). The in vivo potency of these compounds does not depend just on the in vitro affinity at the glycine_B site, as demonstrated by the 7,8- and 6,8-dimethyl derivatives (fig. 8), which both have high-nanomolar affinity in vitro but are effective in the DBA/2 model at 4 to 6 mg/kg (i.p.) (Guzikowski *et al.*, 1996). These compounds contain elements of both the quinoxaline and kynurenic acid structures, but some may act at multiple sites of the NMDA receptor and also at non-NMDA receptors (Swartz *et al.*, 1992). The 7,8-dichloro-6-methyl and 7,8-dichloro-6-ethyl analogues were the most potent 3-hydroxy-1H-1-benzazepine-2,5-diones, with 4.1 and 2.8 nM affinity at NMDA receptors, respectively, and were 100- to 200-fold less potent at AMPA receptors (Guzikowski *et al.*, 1997).

7. Tricyclic glycine_B site antagonists. 2-Aryl-1H-pyrazolo[3,4-c]quinoline-1,4(2H)-diones also have high affinity at the glycine_B receptor. In particular, structure-activity studies identified 7-chloro-3,5-dihydro-2-(4-methoxyphenyl)-1H-pyrazolo[3,4-c]quinoline-1,4(2H)-dione as the most potent of a series of analogues, with an IC_{50} of 3.3 nM. The fact that these compounds have acidity equivalent to that of carboxylic acids (pK_a typically of 4.0) probably underlies the fact that they are not systemically active (Macleod *et al.*, 1995). However, some tricyclic quinoline-diones, such as SM-18400 [(S)-9-chloro-5-[*p*-aminomethyl-*o*-(carboxymethoxy)phenylcarbamoylmethyl]-6,7-dihydro-1H,5H-pyrido[1,2,3-de]quinoxaline-2,3-dione] (fig. 9), are also active in vivo, despite having a much higher pK_a than classical glycine_B antagonists (Nagata *et al.*, 1994a, 1995; Tanaka *et al.*, 1995; Yasuda *et al.*, 1995; Maruoka *et al.*, 1998). The pyridazino[4,5-b]quinoline-diones M244249 and M241247 (fig. 9) and the cyclic diacyl hydrazide M244,646 were reported to be active against global ischemia in gerbils (Patel *et al.*, 1993), although similar compounds were not effective at up to 100 mg/kg (i.p.) in the MES test in our own laboratories.

Merz is developing a series of novel tricyclic pyridophthalazine-diones (fig. 9), which are moderately potent glycine_B antagonists in vitro but show good penetration to the brain in vivo (Parsons *et al.*, 1997; Danysz *et al.*, 1996). They are potent selective NMDA receptor antagonists against responses of single neurons in the rat spinal cord to microelectrophoretic application of NMDA (ID_{50} = 1.2 to 4.7 mg/kg, i.v.), and they also inhibit MES- and pentylenetetrazol (PTZ)-induced convulsions in mice (ID_{50} = 7 to 16 mg/kg, i.p.). The duration of anticonvulsive action in mice is rather short but is prolonged by the organic acid transport inhibitor probenecid, indicating the importance of transport out of the brain, via the choroid plexus, in governing the pharmacokinetics of these compounds. Microdialysis studies after i.p. administration of a high dose of 30 mg/kg to rats indicate that these compounds reach peak extracellular concentrations in the brain that are approximately 5 to 10 times greater than their in vitro potencies at the glycine_B site. The half-lives in rat brain reflect those seen for anticonvulsive activity and can also be prolonged by probenecid (200 mg/kg, i.p.) (Hesselink *et al.*, 1997; Parsons *et al.*, 1997).

8. Prodrugs. Many of the compounds described above were essentially inactive in vivo, indicating very poor access to the CNS. Several strategies were followed to develop prodrugs with improved solubility and reduced polarity. Prodrug esters (L-691,470) (fig. 3) and amides of tetrahydroquinoline and kynurenic acid derivatives (fig. 5) seemed to show somewhat increased in vivo potencies, although it should be stressed that even these prodrugs were active only at 30 to 60 mg/kg in the DBA/2 mouse audiogenic seizure model, which is exquis-

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itely sensitive to NMDA receptor antagonists (Carling *et al.*, 1993; Moore *et al.*, 1993a).

Kynurenic acid is a putative endogenous NMDA glycine$_B$ site antagonist in the CNS. Its concentration under normal conditions is definitely too low to affect NMDA receptors. An interesting approach is to produce kynurenine derivatives that are metabolized to higher affinity antagonists in vivo (Salituro *et al.*, 1994). In fact, Schwarcz and colleagues (Wu *et al.*, 1997) showed that central injection of 4-chlorokynurenine results in the production of its metabolite 7-Cl-KYN in quantities sufficient to block NMDA receptors; 7-Cl-KYN has 50 times higher affinity for the glycine$_B$ site (K_d = 0.3 μM) than does kynurenic acid (K_d = 15 μM) (Henderson *et al.*, 1990). After systemic administration of high doses, 7-Cl-KYN concentrations sufficient to block NMDA receptors were achieved in the brain (Schwarcz R, personal communication).

9. Modification of endogenous kynurenic acid metabolism. It has been suggested that inhibition of the synthesis of quinolinic acid by kynurenine 3-hydroxylase decreases levels of this NMDA receptor agonist and increases levels of endogenous kynurenic acid by facilitating alternative pathways. This can be accomplished with compounds like FCE 28833A [(R,S)-3,4-dichlorobenzoylalanine; Upjohn-Pharmacia], which, when administered systemically at 400 mg/kg, increases kynurenic acid levels in the brain, as shown in microdialysis experiments (Speciale *et al.*, 1996). However, this treatment produced estimated maximal brain concentrations of only 120 nM (assuming 20% recovery), i.e., much less than the K_i of kynurenic acid for the glycine$_B$ site of the NMDA receptor (15 μM) (Kessler *et al.*, 1989a). Nevertheless, this treatment has been reported to provide a neuroprotective effect against global ischemia in gerbils (Speciale *et al.*, 1996).

D. Implications

A model accounting for glycine receptor binding of *trans*-4-amido-2-carboxytetrahydroquinolines was proposed, comprising (*a*) size-limited, hydrophobic binding of substituents on the benzene ring, (*b*) hydrogen bond acceptance by the 4-substituent, (*c*) hydrogen bond donation by the 1-amino group, (*d*) Coulombic attraction of the 2-carboxylate, and (*e*) a large hydrophobic bulk tolerance region approximately 5 Å above the plane of the tetrahydroquinoline ring system (Carling *et al.*, 1992). The model can also account for the binding of quinoxaline-2,3-diones, quinoxalic acids, 4-hydroxy-2-quinolones, and 2-carboxybenzimidazoles. The model accounts for differences in the structural requirements for substitutions on the benzene ring, with 7-substitutions with halogens having similar effects but substitutions at the 5-position being different for various classes of antagonists, as exemplified for the 4-hydroxy-2-quinolones by L-701,315.

However, the fact that most very high affinity glycine$_B$ antagonists developed to date are essentially inactive after systemic administration indicates that attempts to improve in vivo activity solely by increasing the in vitro potency of glycine$_B$ antagonists with poor pharmacodynamic properties may be the wrong approach. Moreover, the ability of some full antagonists of the glycine$_B$ site to unmask glycine-sensitive NMDA receptor desensitization may underlie their promising therapeutic profiles (see Section VI.) and seems, in part, to be inversely related to affinity.

Binding of 4-hydroxyquinolones and other glycine$_B$ antagonists to plasma protein was recently proposed to limit the brain penetration of higher affinity compounds (Rowley *et al.*, 1997). Thus, whereas affinity at the glycine$_B$ site increases with lipophilicity, up to a maximum at a water/octanol partition coefficient of approximately 3, binding to plasma protein also correlates with the water/octanol partition coefficient. This was supported by the findings that warfarin increased in vivo potency and that brain penetration in an in situ brain perfusion model in rats was good in the absence of plasma protein. This has important implications for the design of novel compounds and suggests that it is necessary to maintain the water/octanol partition coefficient below 2.4 or keep the pK_a high, to obtain good CNS activity (Rowley *et al.*, 1997).

E. ^{3}H-Radiolabeled Ligands for the Glycine$_B$ Site

[^{3}H]Glycine (table 4) has long been used as a ligand to label strychnine-sensitive glycine receptors (Young and Snyder, 1974); more recently, investigators observed that, under certain conditions, this compound labels glycine$_B$ sites (Kishimoto *et al.*, 1981). This ligand has been used in several studies, but centrifugation had to be used for bound ligand separation, because of the low ligand affinity. It is also possible to use the filtration method if Mg^{2+} is added to increase glycine affinity (Canton *et al.*, 1992). [^{3}H]Glycine has also been used for receptor autoradiography (Bristow *et al.*, 1986).

D-[^{3}H]Serine (table 4) was suggested to be a more useful ligand because, in contrast to radiolabeled glycine, it clearly allows exclusion of binding to the inhibitory glycine receptor (Danysz *et al.*, 1990). Originally it was used at 20 nM, and incubations were performed in 50 mM Tris acetate, at pH 7.4, for 20 min at 4°C. Specific binding was determined using 1 mM D-serine (approximately 70%). The major problems involved rather high levels of nonspecific binding and low affinity, again necessitating the use of centrifugation methods to terminate incubations. D-Serine has also been used for receptor autoradiography (Schell *et al.*, 1995). It was recently suggested that D-[^{3}H]serine does not label a single population of sites, because 5,7-diCl-KYN failed to completely inhibit binding (Matoba *et al.*, 1997). The profile of the 5,7-diCl-KYN-insensitive component differed from those of all other defined recognition sites. This compo-

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nent was detected at high levels in the cerebral cortex and in the cerebellum (Matoba *et al.*, 1997). These mysterious sites could be important for the in vivo effects of D-serine.

The partial agonist [^{3}H]ACPC (table 4) was used in two investigations to study glycine$_B$ sites, and it seems to label two sites, with different affinities and densities (Monahan *et al.*, 1990b; Popik *et al.*, 1995). [^{3}H]ACPC was used at 10 to 30 nM, and incubations were performed in Tris N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (10 mM) for 1 h at 25°C, using 1 mM glycine to determine specific binding. Both the filtration and centrifugation termination methods were used. To our knowledge, [^{3}H]ACPC is not commercially available.

[^{3}H]5,7-diCl-KYN (table 4) was introduced by Baron *et al.* (1991). They used a 10 nM concentration of this radioligand, in 50 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) KOH, and continued the incubation for 15 min at 4°C at pH 8. D-Serine (100 μM) was used to assess specific binding, which was >70%. This assay was originally based on separation by centrifugation (which was not optimal) but was later adapted to filtration methods (Canton *et al.*, 1992; our own experience) but, in this case, inhibition curves are very shallow and results are quite variable. Standard glycine$_B$ antagonists displaced binding to levels below nonspecific levels determined with unlabeled glycine (Parsons *et al.*, 1997; see also Baron *et al.*, 1991; Yoneda *et al.*, 1993). Moreover, there are some indications that this ligand might be unstable, i.e., the equilibrium binding began to decline spontaneously after 90 min of incubation (Wamsley *et al.*, 1994a). [^{3}H]5,7-diCl-KYN can also be used for autoradiography, but high levels of nonspecific binding (40 to 70%) are an obstacle (Wamsley *et al.*, 1994a).

[^{3}H]L-689,560 (fig. 4), with very high affinity, represents an important advancement because it allows incubation termination by filtration (Grimwood *et al.*, 1992). A 1 nM concentration of ligand was used and the incubation was performed in 50 mM Tris acetate buffer at pH 7 for 120 min at 4°C. Glycine was used at 1 mM to determine specific binding, which was >90%. Unfortunately, this ligand is not commercially available.

Of all ligands used to date, [^{3}H]MDL-105,519 (table 4) has the highest affinity and is available from NEN Life Science Products. It is therefore the ligand of choice to label glycine$_B$ sites (Baron *et al.*, 1996; Hofner and Wanner, 1997). It is routinely used in our laboratory for screening purposes and shows very good reliability and ease of handling. In our studies, membranes were suspended and incubated in 50 mM Tris-HCl, pH 8.0, for 45 min at 4°C, with a fixed [^{3}H]MDL-105,519 concentration of 2 nM. Nonspecific binding was defined by the addition of unlabeled glycine at 1 mM (Parsons *et al.*, 1997). This compound has also been used for autoradiography, which showed that B_{max} and K_d values were heterogeneous in different structures (Chmielewski *et al.*, 1996). B_{max} was highest in the cortex and hippocampus (20 to 25 fmol/mm^2) and lowest in the cerebellum (6 fmol/mm^2). The K_D ranged from 7 nM in the cortex to 21 nM in the caudate putamen. For autoradiography, 50 mM Tris acetate at 22°C and pH 7.4 was used for incubation (10 nM ligands, 30-min incubation), and specific activity was determined with 1 mM glycine (Chmielewski *et al.*, 1996).

In an analysis of the data presented in table 4, one of the striking inconsistencies is that different B_{max} values were obtained by using different radioligands for the glycine$_B$ site. In general, the compounds seem to fall into two categories, i.e., those having B_{max} values of approximately 3 to 5 or 12 to 15 pmol/mg of protein. This could indicate that [^{3}H]5,7-diCl-KYN and [^{3}H]MDL-105,519 label additional sites.

F. Allosteric Interactions Involving the Glycine$_B$ Site

There are several binding studies indicating complex allosteric interactions among various recognition sites of the NMDA receptor. Initially, after the discovery of the glycine$_B$ site, such experiments gave valid support for the existence of several recognition sites on the same molecular entity, i.e., the NMDA receptor complex. The physiological importance of some of these findings is unclear, because similar allosteric effects are not always seen in electrophysiological studies or in vivo.

Glutamate has been reported to enhance [^{3}H]glycine binding in many experiments (e.g., Nguyen *et al.*, 1987) (table 2). In patch-clamp experiments, the off-rate of glycine (which is often slower with higher affinity) is fastest in the presence of partial agonists with low intrinsic activity at the NMDA site and progressively slower in the presence of quinolinate, NMDA (partial agonists), and L-glutamate (full agonists) (Priestley and Kemp, 1994). In contrast, most patch-clamp studies indicate that the more physiologically relevant effect of glutamate involves a decrease in glycine affinity, and this phenomenon finds expression as desensitization (Benveniste *et al.*, 1990a,b).

These interactions in binding experiments are reciprocal, i.e., glycine and D-serine enhance both glutamate affinity and glutamate efficacy to increase [^{3}H]TCP binding (Fadda *et al.*, 1988; Monaghan *et al.*, 1988; McKernan *et al.*, 1989; Stirling *et al.*, 1989). In line with this, electrophoresis experiments in the hemisected spinal cord showed that D-serine increases the affinity of NMDA (Siarey *et al.*, 1990). Again, this effect may be related to intrinsic activity at the glycine$_B$ site, because partial agonists with low intrinsic activity, such as R(+)HA-966, ACBC, and L-687,414 (table 2), actually inhibit NMDA agonist binding. Similar effects on response kinetics were seen in patch-clamp experiments (Priestley and Kemp, 1994). Moreover, the opposite has been observed in binding experiments using radiolabeled C7 competitive NMDA receptor antagonists such as [^{3}H]CPP (Danysz *et al.*, 1989a; Compton *et al.*, 1990;

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Hood *et al.*, 1990; Kaplita and Ferkany, 1990; Grimwood *et al.*, 1993). In that case, enhancement was associated with lower intrinsic activity (Grimwood *et al.*, 1993). In contrast, the binding of competitive antagonists of the C5 type [e.g., ^{3}H-labeled CGS-19755 [*cis*-4-(phosphonomethyl)-2-piperidinecarboxylic acid]] is decreased by agonists at the glycine$_B$ site and partial agonists with high intrinsic activity, such as D-cycloserine, but not by partial agonists with low intrinsic activity (table 2).

Glycine$_B$ site full antagonists have been thought not to allosterically affect the NMDA recognition site (table 2). This hypothesis has been confused by the finding that glycine$_B$ site full antagonists such as L-701,324 and L-695,902 also enhance the binding of [^{3}H]CPP and [^{3}H]CGS-19755 (Grimwood *et al.*, 1995a) and decrease glutamate affinity in a manner similar to that of R(+)HA-966 (Priestley *et al.*, 1996). Hence, this allosteric interaction is not directly related to intrinsic activity at the glycine$_B$ recognition site but rather involves other features of the interacting molecules (Grimwood *et al.*, 1995a). In addition, other ligands show interactions with the glycine$_B$ site; this refers particularly to polyamines such as spermine, which have been shown to enhance glycine but not 7-Cl-KYN potency in the functional [^{3}H]MK-801 assay (Sacaan and Johnson, 1989; Ransom and Deschenes, 1990; Benveniste and Mayer, 1993; Marvizon and Baudry, 1994).

Similarly, glycine affinity is modified by divalent cations in a manner not related to direct actions on channel conductance (Peters *et al.*, 1987; Forsythe *et al.*, 1988). As previously mentioned, Zn^{2+} has been shown to decrease the affinity of glycine (Reynolds and Miller, 1988; Yen *et al.*, 1990). This effect could possibly increase the threshold for saturation of the glycine$_B$ site under physiological conditions, because zinc is present in the brain and in some regions, e.g., dentate gyrus of the hippocampus, can be released in an activity-dependent manner (Aniksztejn *et al.*, 1987).

The endogenous polyamines spermine and spermidine have multiple effects on the activity of NMDA receptors. These include an increase in the magnitude of NMDA-induced whole-cell currents seen in the presence of saturating concentrations of glycine, an increase in glycine affinity, a decrease in glutamate affinity, and voltage-dependent inhibition at higher concentrations (Johnson, 1996; Williams, 1994a,b, 1997; Williams *et al.*, 1994).

Glycine-independent stimulation by polyamines requires the presence of NR1 variants that lack an amino-terminal insert, such as NR1$_{011}$ (NR1a). The stimulatory effect is also controlled by NR2 subunits in heteromeric complexes; it is observed at heteromeric NR1a/NR2B receptors but not at heteromeric NR1$_{011}$/NR2A or NR1$_{011}$/NR2C receptors (Williams *et al.*, 1994; Kashiwagi *et al.*, 1997). Glycine-dependent stimulation is mediated via a three-fold increase in glycine affinity (Williams, 1994a; Williams *et al.*, 1994) accompanied by a similar decrease in the rate of development of glycine-sensitive desensitization and a decrease in the rate of dissociation of glycine (Benveniste and Mayer, 1993). This effect probably involves a second binding site, because it is also seen at NR1$_{011}$/NR2A receptors. Argiotoxin 636 produced a similar potentiation of NMDA responses at positive potentials, accompanied by a slowing in the rate of current desensitization and an increase in the affinity for glycine (Donevan and Rogawski, 1996).

Protons inhibit NMDA receptor function through interactions with the NR1 subunit, and both polyamines and the N1 (exon 5) insert potentiate receptor function through relief of the tonic proton inhibition present at physiological pH. A single amino acid (lysine 211) mediates the effects of N1 in the rat brain. This effect, together with the structural similarities between polyamines and the surface loop encoded by N1, suggest that exon 5 may act as a tethered, pH-sensitive, constitutive modulator of NMDA receptor function (Traynelis *et al.*, 1995).

Mg^{2+} also induces a shift of the pH sensitivity of NMDA receptors at the same site as polyamines (Paoletti *et al.*, 1995), and relatively high concentrations (EC_{50} = 3 mM) potentiate NMDA receptor-mediated responses at positive potentials by increasing the potency of glycine. This effect is associated with a reduction in the magnitude of desensitization and slower desensitization and glycine offset kinetics. Similar effects on kinetics were seen with Ca^{2+}, indicating that both cations may enhance NMDA responses at positive potentials via interactions with the glycine site (Gu and Huang, 1994; Wang and Macdonald, 1995). Ketamine has also been reported to increase NMDA receptor-mediated currents in cultured mouse hippocampal neurons and HEK 293 cells expressing NMDA $\xi 1/\epsilon 2$ receptors by increasing the affinity of the glycine$_B$ site (Zhang *et al.*, 1994; Wang and Macdonald, 1995), and we have made similar observations with memantine and some novel uncompetitive NMDA receptor antagonists (Parsons *et al.*, 1998a).

Nondesensitizing responses of NR1/NR2B receptors expressed in *Xenopus* oocytes elicited in the presence of subsaturating concentrations of glycine were converted into desensitizing responses by the addition of ethanol, an effect that was reversed with increasing glycine concentrations. The ability of ethanol to promote glycine-sensitive desensitization further suggests an interaction between glycine and ethanol inhibition of the NMDA receptor (Buller *et al.*, 1995).

V. N-Methyl-D-Aspartate Receptor Subtypes: Differences in Glycine$_B$ Recognition Sites

Heterogeneity of glycine$_B$ sites was first suggested by Monaghan *et al.* (1988), who showed that NMDA receptors in various brain regions differed in the stimulatory effects of glycine on [^{3}H]glutamate binding, as assessed by autoradiography, i.e., the effect was stronger in the thalamus and cortex than in the striatum, septum, and

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cerebellum. Similarly, D-cycloserine was approximately 6 times more potent in inhibiting [^{3}H]glycine binding in cortical membranes than in the cerebellum; a similar phenomenon was observed for glycine, but the difference was much smaller (2.5-fold) (O'Shea *et al.*, 1991). In contrast, there was no difference in the potencies of D-serine and 7-Cl-KYN (O'Shea *et al.*, 1991). Also, in comparisons of [^{3}H]glycine and D-[^{3}H]serine binding in the spinal cord and cortex, it was observed that 7-Cl-KYN was a very weak inhibitor in the former preparation, whereas HA-966 was equally active in the two preparations (Danysz *et al.*, 1990). In the slice preparation, glycine was potent in reversing the desensitization-related reduction of NMDA-stimulated dopamine release but failed to reverse the diminution of acetylcholine release, possibly indicating the involvement of different NMDA receptor subtypes (Cai *et al.*, 1991).

In fact, it was confirmed that NMDA receptor subtypes do indeed show different pharmacological characteristics of the glycine$_B$ site. This aspect is very important for drug development, because targeting of specific NMDA receptor subtypes might allow better separation of therapeutic and side effects (Watanabe *et al.*, 1993b; Wenzel *et al.*, 1995). Studies in *Xenopus* oocytes and cell lines expressing NMDA receptor subtypes show substantial differences in the sensitivity to glycine and other ligands at the glycine$_B$ site (table 5). The most consistent feature is the observation that glycine is more potent at NMR2D-containing receptors. Although one binding study indicates relative affinities of NR1/NR2C $\gg$ NR1 (homomeric) = NR1/NR2B = NR1/NR2D > NR1/NR2A (Laurie and Seeburg, 1994a), most electrophysiological studies indicate a rank order of NR1a/NR2D > NR1a/NR2C > NR1a/NR2B > NR1a/NR2A (Kutsuwada *et al.*, 1992; Wafford *et al.*, 1993; Matsui *et al.*, 1995; Priestley *et al.*, 1995; Woodward *et al.*, 1995a; Buller and Monaghan, 1997) (table 5). This difference is reflected in very much slower deactivation kinetics of receptors with higher affinity for glycine (Monyer *et al.*, 1992) and glutamate (P. Spielmanns, unpublished data). Interestingly, D-serine shows far less selectivity (Matsui *et al.*, 1995; Priestley *et al.*, 1995; Krueger *et al.*, 1997). The partial agonists R(+)HA-966 and L-687,414 were three-fold more potent at NR1a/NR2B receptors than at NR1a/NR2A receptors, but their intrinsic activities were similar (Priestley *et al.*, 1995). Differences in the potencies and intrinsic activities of other partial agonists were discussed in Section IV.B. (see also table 5).

The molecular basis for this selectivity has not been fully elucidated but is probably related to allosteric interactions between NR2 subunits and the glycine recognition site on NR1 subunits (Hirai *et al.*, 1996; Laube *et al.*, 1997; Wood *et al.*, 1997). It is also likely that affinity is directly dependent on splice variants of NR1 subunits, but this is difficult to test conclusively because homomeric NR1 receptors do not normally form functional receptors when expressed in mammalian cell lines (Grimwood *et al.*, 1995b; Ishmael *et al.*, 1996). Nonetheless, when expressed as homomeric receptors in *Xenopus* oocytes, splice variants containing the N1 insert, such as NR1$_{100}$, showed lower affinity for glutamate, NMDA, and 2-amino-5-phosphonovaleric acid (AP5), no glycine-independent potentiation by spermine, histamine, or Mg^{2+}, greater potentiation by protein kinase C, and stronger inhibition by (+)MK-801 and ketamine, compared with NR1a receptors (Durand *et al.*, 1992, 1993; Williams, 1994b; Paoletti *et al.*, 1995; Rodriguez Paz *et al.*, 1995; Albrecht *et al.*, 1996). It should be noted that others have attributed apparent differences in sensitivity to channel blockers to different blocking kinetics (Monaghan and Larsen, 1997). Levels of inhibition by Zn^{2+} were similar, but potentiation at lower concentrations was seen only with receptors lacking the amino-terminal insert (Hollmann *et al.*, 1993). Variants differing only in the carboxyl-terminal domain showed little change in agonist affinity or spermine potentiation (Durand *et al.*, 1992, 1993).

More relevant for the present review is the fact that NR1$_{011}$ and NR1$_{100}$ receptors showed nearly identical affinities for glycine and Mg^{2+} (Durand *et al.*, 1992). It is truly unfortunate that other studies have not directly addressed this point of paramount importance, because the glycine recognition site is located on NR1 receptors. This is probably because the splice variants of NR1 receptors could be predicted to have a minor influence on the putative binding pocket for glycine, and hence glycine affinity. However, confirmation of this concept would be helpful. Coexpression studies with common NR2 subunits such as NR2A would overcome problems in the interpretation of such experiments in *Xenopus* oocytes.

There are surprisingly few data on the NMDA receptor subtype selectivity of glycine$_B$ antagonists for all four NR2 subunits. The exception is the thorough electrophysiological studies by CoCensys, which showed that the IC$_{50}$ values of ACEA 1011 against NR1A/NR2A, NR1A/NR2B, NR1A/NR2C, and NR1A/NR2D subunit combinations expressed in *Xenopus* oocytes ranged from 0.4 to 7.8 μM in the presence of 1 μM glycine. This 20-fold variation in sensitivity was the result of a combination of subunit-dependent differences in glycine and antagonist affinities; EC$_{50}$ values for glycine ranged from 0.09 to 0.7 μM, and K_b values for ACEA 1011 ranged from 0.2 to 0.7 μM (Woodward *et al.*, 1995b). The same group reported similar data for ACEA 1021 (Woodward *et al.*, 1995a).

[^{3}H]5,7-diCl-KYN was reported to bind with similar affinities to all recombinant receptors ($K_d \sim 50$ to 100 nM) (Laurie and Seeburg, 1994a). However, it should be noted that the rank orders of potency of both glutamate and glycine in that study diverged strongly from those observed in electrophysiological studies. [^{3}H]L-689,560 was also reported to bind to both NR1$_{011}$- and NR1$_{000}$-

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transfected HEK 293 cells with high affinity. The affinities of glycine$_B$ antagonists to inhibit [^{3}H]L-689,560 binding to NR1$_{000}$-transfected cells were similar to those observed with rat brain membranes, whereas affinities at NR1$_{011}$ receptors were two-fold lower. Affinity values for agonists and partial agonists were 4- to 16-fold lower, indicating that the glycine site of homomeric NR1 receptors is in an antagonist-preferring state (Grimwood *et al.*, 1995b).

Receptor subtype selectivity would allow glycine$_B$ site full antagonists to block NMDA receptor function in a manner similar to that of partial agonists in cells expressing heterologous populations of NMDA receptors. For example, highly selective antagonists for receptors containing NR2A subunits would block responses to a maximum of 50% in cells expressing mostly NR1/NR2A and NR1/NR2B receptors at equal levels. Although some glycine$_B$ antagonists have already been reported to show true NMDA receptor subtype selectivity, perhaps more important for in vivo therapeutic profiles is functional subtype selectivity, which could also be related to differences in the affinity of glycine and/or regional variations in endogenous glycine concentrations. Such effects may underlie the improved TIs of some glycine$_B$ site antagonists. For example, MDL-100,458 and MDL-102,288 are equipotent as glycine$_B$ antagonists in vitro but exhibit strikingly different in vivo profiles for audiogenic seizures in DBA/2 mice and for separation-induced ultrasonic vocalizations in rat pups (a model of anxiolytic activity) (Kehne *et al.*, 1995).

It should be stressed that the selectivity of most of the existing glycine ligands for certain subtypes of NMDA receptors is probably far too small for determination of pharmacological characteristics in vivo. However, our intention was to emphasize, by giving selected examples, potential pharmacological targeting of specific subtypes.

VI. Therapeutic Aspects for Agents Acting at the Glycine$_B$ Site

Although some studies using central injection of glycine$_B$ antagonists are discussed in the present section, they are of limited therapeutic importance. In our opinion, only experiments based on systemic administration yield findings regarding possible therapeutic applications, as exemplified by the study showing paradoxically anticonvulsive effects of the GABA$_A$ receptor antagonist bicuculline after central microinjections into the striatum (Turski *et al.*, 1989a). Exemption from this rule should include therapeutic indications where local administration is desirable, e.g., intrathecal infusion to obtain antinociceptive effects.

The general potential usefulness of NMDA receptor antagonists as therapeutic agents has been discussed elsewhere (Meldrum, 1985; Rogawski, 1993; Leeson and Iversen, 1994; Danysz *et al.*, 1995a; Besnard *et al.*, 1996; Ishimaru and Toru, 1997; Parsons *et al.*, 1998b). In the present review, we therefore decided to focus on specific aspects of glycine or its recognition site on NMDA receptors. It is necessary to stress that the common opinion that NMDA receptors are involved only in learning and pathological changes, because of their voltage-dependent blockade by Mg^{2+}, must be put into context. If the situation were so simple, then the development of NMDA receptor antagonists (including glycine$_B$ antagonists) for any application except neuroprotection would seem pointless. It is becoming clear that NMDA receptors also play crucial roles in other forms of plasticity, such as drug dependence and addiction, chronic pain, and CNS development, as well as in normal or disturbed synaptic transmission in some areas of the CNS. In view of the basic aspects discussed above, it is clear that activation of NMDA receptors depends not only on the level of synaptic activity but also on other factors, such as agonist affinity, gating kinetics, and Mg^{2+} sensitivity. In turn, the role of NMDA receptors in various processes depends on the subtype composition and area of the CNS involved.

The crucial questions that have been addressed are as follows:

1. Is there specific dysfunction in glycine/D-serine modulation of NMDA receptors in various disease states or models thereof?
2. Are glycine$_B$ antagonists different from or better than agents acting at other recognition sites of the NMDA receptor?
3. Is initial clinical experience with glycine$_B$ antagonists promising?

There is a rational theoretical basis for a "better" therapeutic potential of glycine$_B$ antagonists, particularly when neuroprotection is considered. Moderate concentrations of glycine$_B$ antagonists would not "switch off" responses completely but, rather, would negatively modulate NMDA receptors through an increase in receptor desensitization, i.e., by potentiation of a built-in protective mechanism against prolonged overactivation.

A. Pharmacokinetic Aspects

In our opinion, there have been two major negative trends in the development of new glycine$_B$ antagonists, which have slowed progress in this area. First, a great deal of effort was put into the development of high affinity agents without much initial concern regarding brain availability; this point has already been discussed. Second, very sensitive convulsion tests are often selected for detection of in vivo activity in the CNS; this favors a positive effect. For example, observation of audiogenic seizures in DBA/2 mice seems to be an exquisitely sensitive model for NMDA receptor antagonists, compared with either MES- or NMDA-induced convulsions (Chapman, 1991; Tricklebank *et al.*, 1994; Bristow *et al.*, 1996b). In fact, on the basis of activity in this model, several glycine$_B$ antagonists were claimed to be system-

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ically active, but they were obviously not sufficiently active for further development (Russi *et al.*, 1992; Moore *et al.*, 1993a; Rowley *et al.*, 1993; see Parsons *et al.*, 1997).

Another major problem with some glycine$_B$ antagonists (e.g., SC-49648, ACEA 1021, and MRZ 2/576) is not brain penetration, which is quite good and fast (1 to 5 min), but rapid transport back out of the brain. This is clearly reflected in brain pharmacokinetics or short duration of functional effects in the CNS, such as anticonvulsive actions or inhibition of cGMP stimulation in the cerebellum (Robinson *et al.*, 1993; Baron *et al.*, 1997; Hesselink *et al.*, 1997; Parsons *et al.*, 1997). This efflux from the brain is most likely associated with the activity of the organic acid transporter in the choroid plexus, because the addition of probenecid considerably prolongs half-lives (Hesselink *et al.*, 1997; Parsons *et al.*, 1997). This may be a less important problem for other compounds such as MDL-105,519 and L-701,324, which have longer half-lives of approximately 3 and 1 h (or >15 h for the β-phase), respectively (Bristow *et al.*, 1996b; Baron *et al.*, 1997); in human subjects, half-lives may be even longer (see Section VII.).

Because glycine$_B$ antagonists are generally intended to be CNS-active agents, the brain concentrations reached must compare with in vitro activity at the glycine$_B$ site, to relate therapeutic effects to the proposed mechanism of action and permit adjustment of dosages accordingly. One of the most spectacular examples (from another area of investigation) in which this rule was ignored involves the claim that aniracetam acts as a cognitive enhancer through inhibition of AMPA receptor desensitization, which is seen at 2 mM concentrations in vitro (Ito *et al.*, 1990). In contrast, brain levels reached at cognition-enhancing doses are in the low-nanomolar range (Ichihara *et al.*, 1986).

The best method to study brain concentrations of drugs is microdialysis with in vivo recovery (Benveniste and Huttemeier, 1990). By comparisons of brain and serum levels, ratios can be established, which, in turn, are very helpful for adjusting doses in clinical studies.

B. Side Effects

Based on studies in animals, there are indications that glycine$_B$ antagonists do not produce many of the side effects often observed with competitive NMDA receptor antagonists and high affinity channel blockers. Early studies suggested better TIs for glycine$_B$ antagonists, but it should be stressed that agents with very poor brain penetration (such as 7-Cl-KYN) were used (Rao *et al.*, 1993). It was reported that 7-Cl-KYN at up to 500 mg/kg did not affect locomotion and was devoid of myorelaxant activity in mice (screen test) (Ginski and Witkin, 1994). The same agent, however, did produce ataxia at low doses (20 nmol) when administered i.c.v. to rats (Danysz and Wroblewski, 1989). It has even been claimed that agents acting at the glycine site are completely devoid of certain side effects (Chiamulera *et al.*, 1990). This is difficult to reconcile with the necessity of glycine for NMDA receptor activation (Kleckner and Dingledine, 1988) and the fact that higher concentrations of glycine$_B$ antagonists produce complete blockade of channel activity. These apparent contradictions can possibly be explained as follows.

1. The differences between glycine$_B$ antagonists and competitive antagonists or channel blockers are not qualitative but rather are quantitative, resulting in better TIs.
2. Compared with competitive or uncompetitive antagonists, some glycine$_B$ antagonists may show better selectivity for NMDA receptor subtypes of "therapeutic" interest versus those mediating side effects.
3. Subtype-selective glycine$_B$ antagonists could produce functional partial antagonism in cells expressing heterologous populations of NMDA receptors.
4. The induction of desensitization by glycine$_B$ antagonists could differentiate among different forms of NMDA receptor activation.

1. Drug discrimination. Drug discrimination is often used to test whether different agents share similar subjective cues, e.g., compared with commonly abused agents such as cocaine or PCP. In the case of NMDA receptor antagonists, this paradigm has been used to address the question of whether agents acting at different sites might have similar side effects and abuse potential.

In rats, R(+)HA-966 (at 30 mg/kg), but not its S(−) isomer, has been found to produce discriminative behavior in the two-lever operant responding paradigm (Singh *et al.*, 1990b). Using the T-maze with mice to study interceptive cues, it was found that glycine$_B$ site partial agonists such as ACPC (ED_{50} = 74 mg/kg) and D-cycloserine (ED_{50} = 60 mg/kg) substituted for the R(+)HA-966 cue (150 mg/kg), indicating similar subjective effects of these partial agonists (Witkin *et al.*, 1995). In contrast, 7-Cl-KYN was ineffective up to a huge dose of 1 g/kg (Witkin *et al.*, 1995). Glycine blocked the R(+)HA-966 cue, indicating that antagonistic (not agonistic) properties play a major role in the discriminative cue of this partial agonist (Witkin *et al.*, 1995). In accordance with this, D-cycloserine substituted for R(+)HA-966 only at a high dose of 100 mg/kg (Witkin *et al.*, 1995), which is regarded as antagonistic in vivo (Emmett *et al.*, 1991; Peterson and Schwade, 1993; Lanthorn, 1994).

The glycine$_B$ site partial agonist ACPC (50 to 400 mg/kg) did not produce place preference in rats, suggesting a lack of abuse potential (Papp *et al.*, 1996). Also, the glycine$_B$ site full antagonist MRZ 2/570 [8-bromo-4-hydroxy-1-oxo-1,2-dihydropyridazinol[4,5-b]quinoline-5-oxide (choline salt)] failed to induce place preference in rats at a dose of 5 mg/kg, which did attenuate a morphine cue (Popik *et al.*, 1998). These studies could indi-

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cate that glycine$_B$ site partial agonists, but probably not full antagonists (only one study known), produce a characteristic interceptive cue.

In pigeons, R(+)HA-966 produced fairly good generalization (three of four pigeons) to a PCP cue (Baron and Woods, 1995). On the other hand, in rats, R(+)HA-966 at up to 50 mg/kg failed to generalize to a PCP cue, and vice versa, indicating substantially different subjective effects of the two agents in this species (Singh *et al.*, 1990b). R(+)HA-966 (200 mg/kg) also failed to produce substitution in mice trained with (+)MK-801 in the T-maze paradigm (Witkin *et al.*, 1995). Balster *et al.* (1995) showed that glycine$_B$ site full antagonists that penetrate to the brain (ACEA 1011 and ACEA 1021) neither substitute for nor attenuate a PCP cue in rats (up to 25 mg/kg, i.p.) and rhesus monkeys (10 mg/kg, i.v.). In rats, both agents failed to act as discriminative stimuli by themselves (Balster *et al.*, 1995).

The differences among NMDA antagonists seen in drug discrimination studies are somewhat reminiscent of results of ex vivo 2-deoxyglucose utilization experiments. In simplified form, it can be stated that after systemic administration uncompetitive antagonists increase glucose utilization in most brain regions, whereas competitive antagonists (Nehls *et al.*, 1988; Kurumaji *et al.*, 1989; Sharkey *et al.*, 1994), glycine$_B$ site antagonists (Hargreaves *et al.*, 1993a), and the polyamine antagonist eliprodyl (Cudennec *et al.*, 1994) are ineffective or much less effective (even a decrease has been reported).

In conclusion, although the number of studies with glycine$_B$ site full antagonists is very limited, there is consensus that glycine$_B$ site full antagonists do not share a discriminative cue with NMDA channel blockers. Therefore, a different behavioral profile can be expected for these agents.

2. Learning impairment. In animals, most NMDA receptor antagonists produce impairment of learning when given at sufficiently high doses before the association phase but not when administered after this phase or during retrieval (Danysz *et al.*, 1995b). Although this statement is an oversimplification, it is the intention of the present review not to discuss the specificity of such effects but, rather, to assess the risk of learning impairment by glycine$_B$ site antagonists. This is of therapeutic relevance because other types of NMDA receptor antagonists, e.g., ketamine (Malhotra *et al.*, 1996), PCP (Luby *et al.*, 1959), and D-3-(2-carboxypiperazine-4-yl)-1-propenyl-1-phosphonic acid (Rockstroh *et al.*, 1996; Herrling *et al.*, 1997), produce clear learning deficits in humans.

Long term potentiation (LTP) is often used to study basic mechanisms of memory formation (Collingridge and Bliss, 1987). As expected for agents inhibiting NMDA receptor function, LTP in the CA1 region in vitro is completely blocked by high concentrations of 5,7-diCl-KYN (Baron *et al.*, 1990; Izumi *et al.*, 1990; Oliver *et al.*, 1990; Bashir *et al.*, 1991; Watanabe *et al.*, 1992). Similar effects were observed in rats in vivo after central administration of this agent (Mizutani *et al.*, 1991). In contrast, GV 150526A did not affect LTP in vivo [at 60 mg/kg, p.o., or 3 mg/kg, i.v., which is the protective dose in the middle cerebral artery occlusion (MCAo) ischemia model], whereas (+)MK-801 (1 mg/kg) produced impairment (Bordi *et al.*, 1996).

Studies on the effects of partial agonists on learning processes yielded highly variable results, probably reflecting the different intrinsic activities and receptor selectivities of the agents used. HA-966 (200 nmol, i.c.v.) was shown to impair passive avoidance learning in rats (Danysz and Wroblewski, 1989), but the racemate was used in that study, making interpretation difficult. In fact, no effect was reported using R(+)HA-966 (up to 80 mg/kg) in the same test in rats (Dunn *et al.*, 1992). In contrast, in rats responding with lever-pressing for food delivery, R(+)HA-966 but not D-cycloserine impaired accuracy in a task involving eight responses on the same lever, which should then be switched to the alternate one (Willmore *et al.*, 1997). To our knowledge, there are no data indicating negative effects of either ACPC or D-cycloserine on learning. In fact, positive effects have been found with D-cycloserine, promoting the use of this agent in human dementia (see Section VI.K. and Lanthorn, 1994).

Initial studies of the effects of glycine$_B$ site full antagonists on learning used central injections. Intracerebroventricular administration of 7-Cl-KYN (1.1 μg) was found to produce learning impairment in the passive avoidance test in rats below anticonvulsive doses, whereas AP5 was relatively more potent as an anticonvulsant (Danysz and Wroblewski, 1989). Similarly, Murata and Kawasaki (1993) found 7-Cl-KYN to be amnesic at a minimal effective dose of 4.5 μg (administered i.c.v. to rats). In a three-panel runway, working-memory task, 7-Cl-KYN at a dose of 3.2 μg/side (injected into the dorsal hippocampus) significantly increased the number of errors in a multiple three-choice runway in rats (Ohno *et al.*, 1994). Also, using a different learning paradigm (the Morris water maze), it was demonstrated that 7-Cl-KYN (10 nmol, i.c.v.) impaired acquisition of spatial learning in rats (Watanabe *et al.*, 1992). In contrast, Chiamulera *et al.* (1990) reported that this agent (also administered i.c.v.) showed very good separation between amnesic and anticonvulsive effects (TI > 30; no impairment at 6.7 μg), whereas (+)MK-801 had a TI of <1. This obvious contradiction can possibly be explained by the different species used (mice in the latter case). The aforementioned studies indicate that glycine$_B$ antagonists probably bear a risk of amnesic action when given centrally, but the effects after systemic administration remain to be determined.

Indole-2-carboxylic acid (up to 50 mg/kg) failed to affect rat performance in the Morris water maze (Smith *et al.*, 1993). In our own studies (not published) with a new generation of glycine$_B$ antagonists, initial experiments indicated that both MRZ 2/570 and L-701,324 (adminis-

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tered systemically at 5 to 10 mg/kg) impaired passive avoidance learning in rats when given before the training. Further studies, however, revealed that at amnesic doses these compounds inhibited vocalization/startle reactions to foot shock (shock titration), suggesting that the learning impairment might have been related to diminished shock sensitivity. To further explore this aspect, both agents were studied in the radial maze test in rats (four of eight arms reinforced); neither affected working memory (at 5 to 10 mg/kg), whereas reference memory was impaired by L-701,324 only (at 5 and 10 mg/kg). The cause of this unexpected difference is unclear and is under investigation. In the same test (radial maze), ACEA 1021 (5 to 20 mg/kg) had no effect on either working or reference memory errors in rats but increased the time to complete the task, which most probably reflects alterations in motor coordination (Kretschmer *et al.*, 1997). Similarly, MDL-104,653 did not disrupt acquisition and performance of food-reinforced instrumental tasks, up to doses that suppressed responding in the same species (up to 32 mg/kg) (Baron and Moerschbaecher, 1996). Rats given GV 150526A at doses of 10 or 60 mg/kg (p.o.) showed normal learning in the water maze test. GV 150526 (up to 30 mg/kg, i.v.) also did not impair passive avoidance performance in mice (Bordi *et al.*, 1996).

These data indicate that systemically administered, glycine$_B$ site antagonists show a low risk of memory impairment, compared with, for example, some high affinity uncompetitive NMDA receptor antagonists. The cause of this favorable profile is not clear but might be related to selectivity for certain NMDA receptor subtypes and/or different modes of interactions with NMDA receptors (see Section V.). For example, our own unpublished data with 5,7-diCl-KYN indicate that the IC_{50} against LTP in the CA1 region of hippocampal slices is 2.5 μM. This value agrees very well with the IC_{50} against peak currents in response to exogenous NMDA in cultured hippocampal neurons (IC_{50} = 2.1 μM with 1 μM glycine) but is 10-fold higher than that against steady-state currents (IC_{50} = 0.24 μM), because of the induction of desensitization. Because steady-state currents are more relevant for the pathological activation of NMDA receptors, it might be predicted that 10-fold lower doses are needed against mild pathological versus physiological activation of NMDA receptors. Obviously, this situation may be different in more severe pathological states, as the concentration of glycine increases.

3. Ataxia, myorelaxation, and sedation. Ataxia and myorelaxation seem to be very common side effects observed with NMDA receptor antagonists (Koek and Colpaert, 1990; Murata and Kawasaki, 1993; Carter, 1994). These side effects (measured using rotarod or inclined mesh tests, for example) have often been used in comparison with anticonvulsive potencies to provide TI values. It should be stressed that the absolute TI values are almost meaningless, because they depend on the type of seizures, the strength/dose of convulsive treatment, rotarod speed, etc.; modification of these parameters may result in a wide range of TI values. This also implies that often no sensible comparison of results from different investigations is possible. Instead, reference agents should always be used to draw any conclusions regarding the relative risk of side effects.

Koek and Colpaert (1990) did not observe ataxia in mice after i.c.v. injections of either HA-966 or 7-Cl-KYN. This contrasts with other reports showing clear ataxia in rats after i.c.v. injections of 7-Cl-KYN (Danysz *et al.*, 1990; Murata and Kawasaki, 1993; De Sarro *et al.*, 1996a). After intrathecal injection, DCQX (fig. 2) and 7-Cl-KYN produced consistent deficits in grasping reflex and righting reflex, indicating myorelaxation and/or ataxia (Coderre and Van Empel, 1994a,b). After central injections of 5,7-diCl-KYN and 7-Cl-KYN in DBA/2 mice, the TI of 0.8 to 2 was similar to that of systemically administered (+)MK-801 (TI = 1.8) but was lower than that observed after systemic injection of competitive antagonists (TI = 4.0 to 20) (Chapman, 1991).

Intravenous injection of R(+)HA-966 was reported to be only slightly more potent in producing inhibition of low intensity electroshock convulsions in mice (ED_{50} = 106 mg/kg) than in evoking ataxia/myorelaxation, as measured by the inclined screen test (ED_{50} = 184 mg/kg) (Vartanian and Taylor, 1991). Similarly, systemic injection of the partial agonist L-687,414 impaired rotarod performance in mice with an ED_{50} of 97 mg/kg, which is very close to the potency of this compound as an anticonvulsant against NMDA responses (Carter, 1994). In contrast, when audiogenic seizures in DBA/2 mice were studied, a TI of 5 was reported for this agent (Saywell *et al.*, 1990); in baboons, no evident side effects were reported at anticonvulsive doses of up to 45 mg/kg (Smith *et al.*, 1992). In another study, L-687,414 failed to produce sedation in rats but did induce ataxia starting at 50 mg/kg, which was >2 times higher than the anticonvulsive ED_{50} (Tortella and Hill, 1996). The TI was greater when neuroprotective activity against intrastriatal injection of NMDA was considered (Vartanian and Taylor, 1991). However, a serious deficiency of that study is that neuroprotective activity was tested in 7-day-old mice, whereas other tests were made in adult animals. Skolnick *et al.* (1989) reported that ACPC did not change rotarod or hanging wire performance at up to 2 g/kg in mice, whereas the anticonvulsive effect exhibited an ED_{50} of 234 mg/kg. However, an inverted U-shaped dose-response curve was obtained (i.e., no dose produced 100% protection), making the interpretation difficult.

The glycine$_B$ site full antagonist indole-2-carboxylic acid evoked ataxia when injected i.v. into rats at 20 to 50 mg/kg; at higher doses, loss of consciousness and respiratory depression were observed (Smith *et al.*, 1993). MDL-104,653 administered systemically to DBA/2 mice provided a TI of 4, which is acceptable but lower than values for competitive antagonists (Chapman *et al.*,

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1995). Our own experience with L-701,324 and MRZ 2/576 indicates that, after systemic application, the TI values for these compounds are not evidently greater than that for (+)MK-801, as measured using the MES-induced convulsion model (Parsons *et al.*, 1997). Similarly, others found ataxic effects of L-701,324 at anticonvulsive doses (approximately 3 mg/kg) (Bristow *et al.*, 1996b; Parsons *et al.*, 1997). Quite strong myorelaxant/ataxic effects were seen with MDL-105,519, i.e., the TI calculated as a ratio between anticonvulsive (against quinolinic acid) or anxiolytic (isolation-induced vocalization in pups) potency and motor impairment was within a range of 1 to 2 (Baron *et al.*, 1997). For ACEA 1021, the TI was even lower (Baron *et al.*, 1997).

Therefore, it seems that ataxia/myorelaxation is the major side effect observed after administration of glycine$_B$ site full antagonists in most cases. Surprisingly, the exception seems to be GV 150526, which was suggested to have a large therapeutic window; no side effects were observed at doses effective in stroke models (Bordi *et al.*, 1996, 1997). However, these claims require verification by an independent laboratory.

It is possible that subtype-selective antagonists would be devoid of such side effects. Several studies have shown that NR2B-selective agents such as ifenprodil, eliprodil, and Ro 25-6981 [R-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol] reduce seizures and are effective neuroprotectants against focal and global ischemia and trauma at doses that do not cause ataxia or impair learning (for review, see Parsons *et al.*, 1998b). Moreover, no signs of motor deficits were observed in knockout mice lacking either NR2A or NR2C subunits, but when both subunits were missing a deficit was seen in mice subjected to demanding tests such as narrow beam walking and quickly rotating rotarod tests (Kadotani *et al.*, 1996). Hence, glycine$_B$ antagonists acting selectively at one NMDA receptor subtype might be devoid of negative effects on motor coordination. Sedation is another side effect described for glycine$_B$ antagonists but, according to most authors, this effect seems to be less pronounced than with competitive antagonists (Chen *et al.*, 1993; Pellegrini-Giampietro *et al.*, 1994; Karcz-Kubicha *et al.*, 1997).

4. Neurotoxicity in the retrosplenial/cingulate cortex. Olney *et al.* (1989, 1991) observed that high doses of competitive or uncompetitive NMDA receptor antagonists produce neuronal vacuolization in the cingulate/retrosplenial cortex in rodents. Some of the neurons containing vacuoles (emerging from mitochondria) may eventually die through necrosis and possibly programmed cell death (Fix *et al.*, 1994). This feature is seen with all known and properly tested uncompetitive and competitive NMDA receptor antagonists.

The glycine$_B$ site partial agonist L-687,414 failed to increase glucose utilization (used as an indication of possible insult in this region) or to induce neuronal vacuolization in cingulate/retrosplenial cortex in rats, at a dose shown to be neuroprotective in ischemia (17.5 mg/kg plus 24 mg/kg/h infusion) (Hargreaves *et al.*, 1993a,b; Gill *et al.*, 1995). Similarly, D-cycloserine seems to be devoid of this action (Haggerty *et al.*, 1993). The full antagonist 7-chlorothiokynurenic acid also did not produce such cortical toxicity up to 50 mg/kg (Chen *et al.*, 1993). Similarly, no pathological changes have been observed with MRZ 2/576 (administered i.p.) at the maximal tested (anesthetic) dose of 100 mg/kg (A. Schwaier, unpublished observation) or with ACEA 1021 at up to 50 mg/kg (i.v.) (Hawkinson *et al.*, 1997). In the case of the latter agent, a detailed study revealed some alterations in the cingulate/retrosplenial cortex and rare vacuoles (four in the entire study) that were apparently not followed by noticeable necrosis (Auer, 1997). Using induction of a 70-kDa heat shock protein as a marker for neurodegenerative changes, no effects were detected with ACPC (1.8 g/kg), D-cycloserine (200 mg/kg), or R(+)HA-966 (180 mg/kg) (Berger *et al.*, 1994). These findings indicate that glycine$_B$ antagonists seem not to produce neurotoxicity in the cingulate/retrosplenial cortex, which favors their therapeutic use, although why they do not remains unknown.

Regarding the safety concerns in relation to pathological alterations in the retrosplenial/cingulate cortex, there are two aspects that should be kept in mind. First, this phenomenon has, until now, seemed to be specific for rats and mice, because it has never been reproduced in primates. A study with memantine in baboons revealed no neuronal toxicity at high doses causing obvious behavioral signs of intoxication (A. Schwaier, unpublished observations). Similarly, Auer *et al.* (1996) failed to detect any changes in the cingulate/retrosplenial cortex of squirrel monkeys after a high dose of (+)MK-801 (1 mg/kg), although this finding should be treated with caution because of several factors that could lead to false-negative findings (see the discussion in that report). Another group also did not observe changes in monkeys with the competitive NMDA receptor antagonist CGS-19755 (up to 20 mg/kg) (Huber *et al.*, 1997). Interestingly, in a recent study in guinea pigs, even after a very high dose of (+)MK-801 (12 mg/kg), vacuoles were seen only occasionally in the cingulate/retrosplenial cortex, although neocortical areas were affected (Raboisson *et al.*, 1997). This raises questions regarding the relevance of using rats or mice to assess the safety of NMDA receptor antagonists for use in humans, with respect to pathological changes in the cingulate/retrosplenial cortex. Second, experiments on vacuolization and necrosis in the cingulate/retrosplenial cortex have been performed, in the great majority of cases, with female animals, because this gender shows much higher (up to 10-fold) susceptibility (Fix *et al.*, 1995). However, most pharmacological profiling studies in animal models are performed with male animals, leading to a false, low therapeutic window.

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5. Psychotomimetic side effects. Psychotomimetic effects are often seen in human subjects treated with high doses of either uncompetitive or competitive NMDA receptor antagonists (Luby *et al.*, 1959; Albers *et al.*, 1991; Kristensen *et al.*, 1992; Muir *et al.*, 1994). In rodents, indications of such activity are (among others) locomotor activation, backpedaling, and stereotypic sniffing, circling, and head weaving. Indeed, in rats most uncompetitive NMDA receptor antagonists produce these effects when given at sufficiently high doses (Contreras *et al.*, 1988; Koek and Colpaert, 1990; Hoffman, 1992; Kretschmer *et al.*, 1992; Murata and Kawasaki, 1993; Schmidt and Kretschmer, 1997). However, it has been shown that competitive antagonists also cause locomotor activation when the test is performed after a sufficient time delay or high doses are given (Löscher and Honack, 1993; Furuya and Ogura, 1997).

The glycine$_B$ site partial agonists R(+)HA-966 (30 mg/kg) and D-cycloserine (30 mg/kg) do not increase locomotion in rats and mice when given systemically (Herberg and Rose, 1990; Danysz *et al.*, 1994; Karcz-Kubicha *et al.*, 1998b). Systemically administered glycine$_B$ site full antagonists such as L-701,324 (1 to 10 mg/kg) (Bristow *et al.*, 1993, 1996a) or MRZ 2/576 (1 to 10 mg/kg) (Karcz-Kubicha *et al.*, 1998b) do not increase locomotor activity in rats and, if anything, produce sedation. Similar observations were made recently using ACEA 1021 (up to 20 mg/kg) in the same species (Kretschmer *et al.*, 1997).

Behaviors in rodents that are more often interpreted as being indicative of psychotomimetic potential are stereotyped sniffing, circling, and head weaving. In contrast to uncompetitive NMDA receptor antagonists, a negligible amount of head weaving was observed in mice only with high doses of R(+)HA-966, 7-Cl-KYN, and D-cycloserine (Tricklebank and Saywell, 1990), with modest effects with L-687,414 (minimal effective dose of 27 mg/kg, i.p., in mice and 20 mg/kg, i.v., in rats) (Tricklebank *et al.*, 1994). Circling was reported after central injection of 7-Cl-KYN, although the effect was of lower magnitude than with PCP or (+)MK-801 (Murata and Kawasaki, 1993). In rats, ACEA 1021 (7.5 mg/kg) produced a moderate, not clearly dose-dependent, increase in stereotyped sniffing that was antagonized by D-cycloserine (Kretschmer *et al.*, 1997). Another glycine$_B$ antagonist, L-701,324, produced stereotyped behavior in mice (head weaving) but again with 3 times lower intensity than with (+)MK-801 (Bristow *et al.*, 1996a).

A better method for detecting psychotomimetic potential seems to be prepulse inhibition of the acoustic startle response; this is a model of sensory gating that is disrupted in schizophrenic subjects and in normal individuals treated with PCP (Swerdllow *et al.*, 1994; Johansson *et al.*, 1995; Alamin and Schwarzkopf, 1996). In animals, it is also impaired by NMDA channel blockers (Mansbach and Balster, 1991; Alamin and Schwarzkopf, 1996; Furuya and Ogura, 1997) but, according to some authors, not by competitive NMDA receptor antagonists (Mansbach and Balster, 1991; Wedzony *et al.*, 1994). However, according to others, competitive antagonists do attenuate prepulse inhibition when injected 120 min but not 30 min before the test (Furuya and Ogura, 1997).

After i.c.v. administration of 5,7-diCl-KYN, a mild (50%) decrease in prepulse inhibition was observed (Furuya and Ogura, 1997). Similarly, 7-Cl-KYN injected into the nucleus accumbens produced impairment (Kretschmer and Koch, 1997). However, all published studies with glycine$_B$ antagonists administered systemically indicated no effect. After L-701,324 (5 mg/kg) administration to rats, no impairment of prepulse inhibition was observed; rather, there was improvement of deficits resulting from isolation (Bristow *et al.*, 1995). Similarly, we did not observe any effects of the glycine$_B$ antagonists MRZ 2/576 (1 to 10 mg/kg) and L-701,324 (2.5 to 5 mg/kg), administered systemically, on prepulse inhibition in rats (Karcz-Kubicha *et al.*, 1998b). MDL-105,519 also failed to affect prepulse inhibition at up to 200 mg/kg, i.e., 6 times higher than the anticonvulsive dose (Baron *et al.*, 1997). Finally, neither ACEA 1011 nor ACEA 1021 (up to 30 mg/kg) disrupted prepulse inhibition of the acoustic startle reflex in rats (Balster *et al.*, 1995; Kretschmer *et al.*, 1997). Therefore, the present data indicate that, after systemic administration of glycine$_B$ antagonists, the risk of psychotomimetic effects is negligible or minor.

C. Anxiety

In 1986 it was reported that a high dose of the competitive NMDA receptor antagonist 2-amino-7-phosphonoheptanoic acid produced anxiolytic activity (Bennett and Amrick, 1986; Stephens *et al.*, 1986). These findings were followed by several publications confirming this action with competitive and uncompetitive NMDA receptor antagonists in the conflict test (Corbett and Dunn, 1993; Plaznik *et al.*, 1994), the social interaction test (Dunn *et al.*, 1989; Corbett and Dunn, 1993), the elevated plus-maze test (Dunn *et al.*, 1989; Corbett and Dunn, 1993), and the test of separation-induced vocalization in rat pups (Kehne *et al.*, 1991), as well as by blockade of the fear-potentiated startle response (Anthony and Nevins, 1993). Similarly, glycine$_B$ antagonists and partial agonists have been suggested to show an encouraging therapeutic anxiolytic profile (Trullas *et al.*, 1989; Corbett and Dunn, 1991, 1993; Kehne *et al.*, 1991, 1995; Anthony and Nevins, 1993; Plaznik *et al.*, 1994).

The partial agonist D-cycloserine (30 mg/kg) exerts anxiolytic activity in the fear-potentiated startle response test in rats (Anthony and Nevins, 1993). Similarly, we recently observed a clear anxiolytic effect of this agent in the elevated plus-maze test in rats (starting at 10 mg/kg) (Karcz-Kubicha *et al.*, 1997). In the same model, ACPC at 300 to 400 mg/kg (but not at 500 mg/kg) was shown by Trullas *et al.* (1989, 1991) to in-

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crease both the percentage of time spent in open arms and the percentage of entries into open arms for mice, which was interpreted as a decrease in anxiety. However, the efficacy of ACPC was significantly lower than that of chlordiazepoxide (Trullas *et al.*, 1989). Anxiolytic properties of ACPC have also been shown in rats in the fear-potentiated startle response test (starting at 200 mg/kg, with complete blockade at 500 mg/kg) (Anthony and Nevins, 1993) and in the conflict test (100 and 200 mg/kg) (Przegalinski *et al.*, 1996). Similarly, positive effects of this agent (12.5 to 200 mg/kg) in the model of separation-induced ultrasonic vocalization in rat pups have been reported (Winslow and Insel, 1991). In mice trained in a dark avoidance task, the step-down latencies measured within 24 h, but not 1 h, were prolonged by posttraining administration of ACPC (400 mg/kg), 7-Cl-KYN (30 mg/kg), or D-cycloserine (1 mg/kg), indicating anxiolytic activity (Faiman *et al.*, 1994). Pretreatment with glycine abolished this effect, confirming the involvement of the glycine$_B$ site (Faiman *et al.*, 1994). In contrast, we failed to observe anxiolytic effects of ACPC (up to 600 mg/kg) in rats using the plus-maze test (Karcz-Kubicha *et al.*, 1997).

Corbett and Dunn (1991) demonstrated anxiolytic-like activity of racemic HA-966 (1 to 3 mg/kg) in the conflict, social interaction, and plus-maze tests in rats. The selective R(+)-enantiomer has also been shown to decrease fear-potentiated startle responses in the same species at doses of 10 and 30 mg/kg (Anthony and Nevins, 1993). In accordance with this result, R(+)HA-966 injected into dorsal periaqueductal gray matter increased both the percentage of time spent in open arms and the percentage of entries into open arms in the elevated plus-maze test in rats (Matheus *et al.*, 1994). In our studies, R(+)HA-966 also produced anxiolytic effects in this model (starting at a dose of 1 mg/kg), but the magnitude of this action was very modest, in comparison with those of diazepam and D-cycloserine (Karcz-Kubicha *et al.*, 1997).

The glycine$_B$ site full antagonist 7-Cl-KYN, given i.p. at a dose of 25 mg/kg or administered directly into the dorsal periaqueductal gray matter, decreased anxiety in the elevated plus-maze test (Trullas *et al.*, 1989; Matheus *et al.*, 1994). Another glycine site antagonist, 5,7-diCl-KYN, when injected i.p. significantly increased social interaction behavior (doses of 30 and 100 mg/kg), open-arm exploration time (100 mg/kg), and conflict responding in rats (Corbett and Dunn, 1993). This compound was also tested, with positive effects for anxiety, in the model of separation-induced ultrasonic vocalization in rat pups (Kehne *et al.*, 1991) and in the Vogel conflict test (5 μg, i.c.v.) (Plaznik *et al.*, 1994). Another glycine$_B$ antagonist, MDL-105,519, has been shown to inhibit ultrasonic vocalization in rat pups (Baron *et al.*, 1997), with an ED_{50} of 40 mg/kg, which is close to its ED_{50} for myorelaxant effects (73 mg/kg). In our studies, the specific and high affinity glycine$_B$ antagonist L-701,324 (Bristow *et al.*, 1996b; Priestley *et al.*, 1996) had very weak anxiolytic effects in the elevated plus-maze test (3 to 10 mg/kg) and was ineffective in the Vogel conflict test (0.1 to 10 mg/kg) (Karcz-Kubicha *et al.*, 1997). Similarly, no effect of MRZ 2/576 (or related agents) was seen in rats in the elevated plus-maze test or in the Vogel conflict test, at doses that result in brain levels sufficient to inhibit NMDA receptors (i.e., up to 10 mg/kg) (Karcz-Kubicha *et al.*, 1997; Parsons *et al.*, 1997). Somewhat in line with these findings, Wiley *et al.* (1995) failed to show consistent anxiolytic activity of ACEA 1021 in the elevated plus-maze test in rats; a significant but modest increase was seen only in open-arm entries at the highest dose of 30 mg/kg. In contrast, anxiolytic activity of L-701,324 (at 2.5 and 5 mg/kg) in the plus-maze test and in the punished drinking test was recently reported (Kotlinska and Liljequist, 1998).

An intriguing question is whether anxiolytic activity is an inverse function of the intrinsic activity of glycine$_B$ site antagonists/partial agonists, i.e., whether activity is simply a consequence of NMDA receptor inhibition. By analyzing the data described above and by directly comparing several agents with different levels of intrinsic activity (from 0 to 92%), we obtained a negative answer to this question (Karcz-Kubicha *et al.*, 1997).

These somewhat inconsistent results could indicate that the antagonists studied have preferences for NMDA receptor subtypes differently involved in fear regulation. In fact, studies with MDL-100,458 and MDL-102,288 indicated that the former was 100 times more potent as an anticonvulsant in DBA/2 mice, whereas the latter was 13 times more potent as an anxiolytic in a separation-induced vocalization model (Kehne *et al.*, 1995). One consistent finding was that, in the majority of cases, the anxiolytic efficacy of glycine$_B$ antagonists is far weaker than that of benzodiazepines. Therefore, although they are scientifically interesting, this class of agents cannot currently be considered to be promising for the development of new anxiolytic agents that are superior to existing treatments.

D. Depression

The cause of depression is largely unknown, despite decades of intensive research. Therefore, any new hypothesis in this field is refreshing, including a recent one involving NMDA receptors (see below this section).

Many antidepressants seem to have NMDA channel-blocking properties, but their modest affinities in this regard do not allow conclusions to be drawn regarding the relevance of these properties to the therapeutic efficacy of these drugs (Reynolds and Miller, 1988; Leander, 1989; Sills and Loo, 1989; Kitamura *et al.*, 1991). However, there are indications that dysfunctions of glycine$_B$ site regulation could occur in depression. In patients with major depression, decreases in serum glycine concentrations and increases in the serine/glycine ratio were observed (Altamura *et al.*, 1995); another report

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indicated an increase in plasma serine levels (Maes *et al.*, 1995).

In the frontal and parietal cortex of suicide victims, no difference in the glycine stimulation of [^{3}H]MK-801 binding was observed (Palmer *et al.*, 1994a), but Nowak *et al.* (1995a) reported that the proportion of high affinity, glycine-displaceable binding of ^{3}H-labeled CGP-39653 [DL-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid] was reduced in the frontal cortex. However, other parameters, such as the potency or efficacy of glycine to interact with [^{3}H]5,7-diCl-KYN, [^{3}H]CGP-39653, or [^{3}H]MK-801 binding, were not altered (Nowak *et al.*, 1995a).

In animal models of depression, such as the forced-swim test and stress-induced anhedonia test, NMDA receptor antagonists exert positive effects in most cases (Maj, 1992; Moryl *et al.*, 1993; Papp and Moryl, 1994; Przegalinski *et al.*, 1997). A positive effect of ACPC (400 mg/kg) in the forced-swim test in rats was probably the first indication that glycine$_B$ ligands might have antidepressive potential (Trullas and Skolnick, 1990). An antagonistic effect of ACPC at the glycine$_B$ site was involved in these findings, because the effect was reversed by glycine (Trullas and Skolnick, 1990). This original study was recently replicated, i.e., ACPC (200 to 400 mg/kg) produced a reduction of immobility time in the forced-swim test in rats (Przegalinski *et al.*, 1997). ACPC and D-cycloserine were also studied in a chronic mild stress model of depression (Papp and Moryl, 1996), where a decrease in the consumption of sucrose is used as an index of anhedonia and is sensitive to antagonism by classical antidepressant drugs. A positive effect was seen after chronic (5-week) treatment with ACPC (starting at 100 mg/kg), whereas less consistent (non-dose-dependent) effects were seen with D-cycloserine (significant effect at 10 mg/kg) (Papp and Moryl, 1996).

It is noteworthy that chronic treatment of mice with (+)MK-801 or ACPC produces β-adrenoceptor down-regulation (measured as [^{3}H]dihydroalprenolol binding) in cortical membranes (Paul *et al.*, 1992); this effect was previously reported to be the most common change after various antidepressive therapies (Vetulani and Sulser, 1975). Antidepressants also change NMDA receptor function. Chronic antidepressant treatment (or electroconvulsive shock treatment) has been reported to cause a reduction in the potency of glycine to displace [^{3}H]5,7-diCl-KYN binding and a reduction in the proportion of high affinity, glycine-displaceable binding of the competitive NMDA receptor antagonist [^{3}H]CGP-39653 (Nowak *et al.*, 1993; Paul *et al.*, 1993, 1994). It is interesting to note that various treatments, such as classical antidepressants (amitriptyline and desipramine), atypical antidepressants having different mechanisms of action (mianserine and citalopram), and electroconvulsive shocks, all produce the same reduction in glycine potency to inhibit [^{3}H]5,7-diCl-KYN binding. On the other hand, chronic stress (which produces motivational deficits in rats) had the opposite effect, i.e., an increase in the potency of glycine to inhibit [^{3}H]5,7-diCl-KYN binding (Nowak *et al.*, 1995b).

Recently, chronic treatment with huge doses of glycine (500 mg/kg) was shown to attenuate the increase in activity after bulbectomy (another model used to study antidepressive agents) and to attenuate the activity change in response to PCP in this model, resembling results obtained with antidepressants (Redmond *et al.*, 1996). This is somehow in contrast to the antidepressive-like effects of NMDA receptor inhibition described above.

In summary, there are data suggesting that chronic treatment with antidepressants produces a change in glycine interactions with the NMDA receptor (possibly resulting from alterations in the subunit composition), which requires further investigation. The impact of this change on the function of NMDA receptors and the pathomechanism of depression is also unclear. Concerning the therapeutic potential of glycine$_B$ antagonists as antidepressants, only ACPC is being developed in this direction (table 8); ongoing clinical trials of treatments for depression should validate the therapeutic utility of glycine$_B$ modulators in depression. In our opinion, even if ACPC proves to be effective, this finding should not be generalized to other glycine$_B$ antagonists or partial agonists, which all seem to have very different pharmacological profiles.

E. Schizophrenia

Because high affinity NMDA channel blockers, such as PCP, mimic both positive and negative symptoms of schizophrenia in humans, it has been suggested that hypofunction of the glutamatergic system might occur in this disease (Carlsson and Carlsson, 1990; Javitt and Zukin, 1991; Halberstadt, 1995; Ishimaru and Toru, 1997). In fact, there are some indications of glutamatergic hypofunction in schizophrenic patients, such as decreased NMDA receptor-stimulated glutamate release in synaptosomal preparations (Sherman *et al.*, 1991; Ishimaru and Toru, 1997). It has been suggested that enhancement of glutamatergic transmission could potentially have beneficial effects (Carlsson and Carlsson, 1990). It would be difficult to consider use of direct NMDA receptor agonists, because of their neurotoxic and convulsive potential, but positive stimulation of the glycine site might be a favorable approach. The latter idea is supported by the findings that some schizophrenic patients exhibit deficiencies in a glycine-synthesizing enzyme (serine hydroxymethyl transferase) and may respond positively to glycine administration (Waziri, 1988). In contrast, other authors did not find differences in glycine concentrations for schizophrenic patients after loading with high doses of L-serine (the direct precursor of glycine), arguing against a deficit in serine hydroxymethyl transferase (Lucca *et al.*, 1993; but see Ishimaru *et al.*, 1994). Nev-

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ertheless, in schizophrenia there might be dysfunctions of the glycine/serine balance, because increased glycine levels in the orbitofrontal cortex and reduced serine levels in the putamen have been shown (Kurumaji *et al.*, 1996). The number of strychnine-insensitive [^{3}H]glycine binding sites (B_{max}) was found to be increased in 6 of 16 cortical areas studied in postmortem brain samples from chronic schizophrenics (Kurumaji *et al.*, 1996). The authors suggested that the increase could possibly be ascribed to compensation for impaired glutamatergic neurotransmission.

In rats, chronic treatment with neuroleptic agents (haloperidol, pimozide, clozapine, and risperidone) resulted in decreases in glycine stimulation of [^{3}H]MK-801 binding (McCoy and Richfield, 1996). The effect was specific, because stimulation by NMDA or spermidine was not affected. Interestingly, based on glycine-sensitive inhibition of NMDA responses in voltage-clamped hippocampal neurons (IC_{50} = 1.9 μM), it has been suggested that haloperidol is a partial agonist at the glycine$_B$ site (Fletcher and Macdonald, 1993).

In 1986, systemic p.o. treatment with high doses of glycine (approximately 370 mg/kg) was shown to decrease PCP-induced hyperactivity in mice (Toth and Lajtha, 1986), indicating antipsychotic potential. Also, D-serine administered i.c.v. attenuated stereotypic behavior produced by the NMDA channel blockers PCP and (+)MK-801 in rats (Contreras, 1990). Similarly, in this species D-serine and D-alanine injected i.c.v. inhibited stereotypy, hyperactivity, and ataxia induced by PCP (Tanii *et al.*, 1994) but, surprisingly, the former was less potent. D-Cycloserine (3 mg/kg) was also effective in counteracting hypermotility after (+)MK-801 administration (Dall'Olio and Gandolfi, 1993). A systemically active glycine uptake inhibitor, glycyldodecylamide, was found to be very potent in reversing PCP-induced hyperactivity in rodents (Javitt and Frusciante, 1997). The fact that glycine$_B$ site activation attenuates the behavioral effects of NMDA channel blockers is in contrast to in vitro experiments, where the opposite effect is seen as an expression of use dependence (Huettner and Bean, 1988; Reynolds and Miller, 1988). One explanation might be that the glycine$_B$ site is far from saturated in vivo and the apparent competitive reversal of MK-801 effects is the result of recruitment of more receptors by stimulation of the glycine$_B$ site with D-cycloserine. Stimulation of the glycine$_B$ site might also increase the efficacy of neuroleptic agents, e.g., D-cycloserine enhanced (−)-sulpiride potency to attenuate apomorphine-induced stereotypy (Dall'Olio and Gandolfi, 1993).

As already mentioned, in humans, glycine has been shown by some authors to be beneficial in the alleviation of negative symptoms or the enhancement of the efficacy of neuroleptic agents (Waziri, 1988; Rosse *et al.*, 1989; Javitt *et al.*, 1994; Herescolevy *et al.*, 1996; Leiderman *et al.*, 1996). In contrast, D-cycloserine occasionally produced psychotic symptoms at high doses (250 to 500 mg) in patients with tuberculosis (Mandell and Petri, 1996) and aggravated positive symptoms in schizophrenic patients (Cascella *et al.*, 1994). Others found that D-cycloserine did not improve symptoms in patients when used as an adjunct to clozapine therapy (Goff *et al.*, 1996). In a study showing that D-cycloserine attenuated negative symptoms, this finding was possibly the result of arousal effects (Goff *et al.*, 1995). In another study, D-cycloserine produced an improvement at low doses (50 mg) but caused worsening at higher doses (250 mg), which probably reflects actions at different NMDA receptor subtypes at which D-cycloserine has different intrinsic activities (Cascella *et al.*, 1994; Goff *et al.*, 1995) (table 5).

In recent years, data opposed to the "classical" hypothesis of glutamatergic deficits in schizophrenia have appeared. It was reported that L-701,324 (at 5 mg/kg) antagonized amphetamine-induced locomotor activation and increases in dopamine turnover in the nucleus accumbens and also attenuated the deficit in the prepulse inhibition of the acoustic startle response induced by isolation in rats (Bristow *et al.*, 1995, 1996a). Interestingly, it did not inhibit apomorphine-induced stereotyped behavior (sniffing and licking/biting), which is believed to result from action in the striatum and is sensitive to the typical neuroleptic haloperidol (Bristow *et al.*, 1996a). On the basis of these findings, L-701,324 was suggested to have features like those of the atypical neuroleptics, without extrapyramidal side effects. In our studies, although this agent did attenuate PCP and amphetamine locomotor stimulation in an apparently specific manner at 10 mg/kg (significant interaction factor in an analysis of variance), it failed to affect the PCP-induced deficit of prepulse inhibition, in contrast to atypical neuroleptics, in rats (Karcz-Kubicha *et al.*, 1998b). Similar results were obtained with MRZ 2/576 at 10 mg/kg (Karcz-Kubicha *et al.*, 1998b) and R(+)HA-966 (Furuya *et al.*, 1998). In contrast, another glycine$_B$ antagonist, MDL-103,371 [(E)-3-[2-(3-aminophenyl)-2-carboxyethanol]-4,6-dichloroindol-2-carboxylic acid], had no effect on prepulse inhibition or dopamine levels by itself but antagonized the (+)MK-801-induced increase in dopamine levels, implicating antipsychotic-like activity (Schmidt *et al.*, 1996).

These clinical and preclinical data indicate that glycine and D-cycloserine might find some use in the alleviation of negative symptoms in schizophrenia. In the case of the latter agent, an important obstacle is the narrow range of effective doses. In our opinion, the concept of the use of glycine$_B$ antagonists as "atypical neuroleptics" is interesting but is not substantiated by sufficient data from animal models using different types of compounds.

F. Convulsions and Epilepsy

Of all epileptic patients (approximately 20 to 50 million people), 75% experience partial seizures; of these

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patients, approximately 85% suffer from complex partial seizures. Therefore, this type of epilepsy should be a major target for drug development (Rogawski and Porter, 1990; Löscher, 1993).

Although one of the first suggested therapeutic applications of NMDA receptor antagonists was in epilepsy (Czuczwar and Meldrum, 1982; Meldrum, 1985), only a few such agents reached clinical testing for this indication; these agents failed to show sufficient benefits and produced serious side effects (Troupin *et al.*, 1986; Leppik *et al.*, 1988; Sveinbjornsdottir *et al.*, 1993). It should be pointed out that the efficacy of the agents under investigation has often been tested in animal models that are more relevant for global seizures (NMDA, MES, and sound models), whereas clinical testing usually involves patients with complex partial seizures, which are better modeled in animals by kindling experiments (Löscher and Schmidt, 1988). Using that model, Löscher and Hönack (1991) demonstrated the lack of evident anticonvulsive efficacy of NMDA receptor antagonists in the expression of symptoms, accompanied by an exaggeration of side effects indicative of psychotomimetic activity. This observation later found confirmation in a clinical study of D-3-(2-carboxypiperazine-4-yl)-1-propenyl-1-phosphonic acid in patients with complex partial seizures, which was terminated because of several CNS-related side effects (Sveinbjornsdottir *et al.*, 1993). Of course, these failures should not discourage testing of glycine$_B$ antagonists, which might show quite different profiles.

Are there any indications for the specific involvement of glycine, or the glycine$_B$ site, in these seizures? In rats, during tonic seizures evoked by PTZ administration, there was a rapid increase (approximately 40%) in glycine levels measured in the cisterna magna (Halonen *et al.*, 1992). This effect was not specific, however, because other amino acid levels were also increased. Tissue samples collected from epileptogenic loci of 35 epileptic patients showed marked increases in glycine levels (Perry and Hansen, 1981). In addition, increases in glycine concentrations were detected in biopsy brain samples (Hamberger *et al.*, 1991). Analysis of surgically removed vascular malformations (cavernous angiomas causing neurodegeneration and epileptoform activity) revealed high levels of serine and glycine (Von Essen *et al.*, 1996), which were related to overactivation of NMDA receptors. Similarly, using brain microdialysis in humans, it was reported that during seizures glycine levels in epileptic loci rose (eight-fold) more than did levels of glutamate (seven-fold) and serine (four-fold); the basal levels were 0.4, 1.2, and 2.0 μM, respectively (Ronneengstrom *et al.*, 1992; see also Carlson *et al.*, 1992). If nonsaturating glycine levels are assumed before seizure initiation (see discussion above), this could indicate a contribution of glycine to this phenomenon. However, considering the poor temporal resolution of the microdialysis method, it is not clear whether amino acid changes are a causal factor or a direct or indirect consequence of the seizures. Even if the latter is the case, the use of glycine$_B$ antagonists might be useful in preventing the brain damage seen in epilepsy, because this damage is probably related to overactivation of NMDA receptors (Meldrum, 1991).

As expected for agents inhibiting NMDA receptor function, glycine$_B$ antagonists (after i.c.v. administration) inhibit convulsions induced by sound, MES, PTZ, or NMDA (Danysz and Wroblewski, 1989; Chiamulera *et al.*, 1990; Koek and Colpaert, 1990; Singh *et al.*, 1991; Baron *et al.*, 1992; Rowley *et al.*, 1993; De Sarro *et al.*, 1996b; Ilyin *et al.*, 1996). However, because systemically active compounds are obviously more relevant as possible therapeutic agents, primarily these are discussed further. For example, an anticonvulsant effect of MNQX was one of the first reported CNS effects of selective and systemically active glycine$_B$ site full antagonists (Sheardown *et al.*, 1989). To our knowledge, no confirmation of this finding has ever been published (see Bisaga *et al.*, 1993).

In general, the effects of glycine$_B$ site partial agonists are quite unpredictable on the basis of their degree of NMDA receptor inhibition. For example, the partial agonists D-cycloserine and ACPC have high intrinsic activities but are quite effective anticonvulsants, whereas conflicting results have been obtained with R(+)HA-966, despite its lower intrinsic activity (Skolnick *et al.*, 1989; Vartanian and Taylor, 1991; Peterson *et al.*, 1992; Bisaga *et al.*, 1993; Carter, 1994). It should be stated that there are other studies challenging the concept that there is a simple inverse relationship between the intrinsic activity of partial agonists and anticonvulsive effects (Peterson, 1991a,b, 1992; Peterson and Schwade, 1993). Using the MES model, Peterson (1991) reported that in rats glycine (4 g/kg, p.o.) and D-serine (2 g/kg, p.o.) enhanced the anticonvulsive activities of traditional anticonvulsive drugs, apparently via agonistic effects at the glycine$_B$ site, because this action was antagonized by 7-Cl-KYN, which was ineffective when administered alone. Also, D-cycloserine attenuated MES-induced tonic convulsions with an ED_{50} of 153 mg/kg, acting at the same site (Peterson and Schwade, 1993). Further complicating the situation, it has been shown that glycine potentiates convulsions produced by strychnine (Larson and Beitz, 1988).

Although the low intrinsic activity, partial agonist L-687,414 (fig. 10) did inhibit MES-induced convulsions in rats starting at 10 mg/kg, the dose-response curve was very flat (12-fold dose difference between 20 and 80% protection), and 100% protection was not obtained (Tortella and Hill, 1996). L-687,414 has also been shown to inhibit photically induced myoclonic seizures in primates (baboons) without producing any adverse effects (Smith and Meldrum, 1992).

The glycine$_B$ site full antagonist MDL-104,653 (fig. 7) protected DBA/2 mice against sound-induced clonic sei-

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zures, with an ED_{50} of 1.7 mg/kg (i.p., 45 min) (Chapman *et al.*, 1995), whereas MDL-100,458 (fig. 4) exhibited an ED_{50} of 20.8 mg/kg (Kehne *et al.*, 1995). Also, with L-701,324 (fig. 7) a potent anticonvulsive effect was seen against NMDA-, PTZ-, MES-, and sound-induced convulsions in mice (ED_{50} values of 0.96 to 3.4, i.v., and 1.9 to 3.4, p.o.) (Bristow *et al.*, 1996b; see also Parsons *et al.*, 1997). Similarly, several phthalazine-diones (MRZ 2/570, MRZ 2/571, and MRZ 2/576) (fig. 9) were quite effective inhibitors of MES-induced tonic convulsions in mice, in the range of 7 to 15 mg/kg (Parsons *et al.*, 1997).

As stated above, the kindling model is probably much more relevant for screening of potential therapies, compared with simple audiogenic or chemically induced seizure models (Löscher, 1993). Croucher and Bradford (1990, 1991) were the first to describe a positive effect of $glycine_B$ antagonists, using 7-Cl-KYN and R(+)HA-966 administered directly into the amygdala, against kindled seizures. This finding was not confirmed in another study after the i.c.v. route of administration, although a significant effect on seizure development was observed (Namba *et al.*, 1993). Rundfeldt *et al.* (1994) tested the $glycine_B$ antagonist 7-Cl-KYN and the partial agonists R(+)HA-966 and D-cycloserine, also using i.c.v. administration. All three drugs increased the focal seizure threshold, and none of them induced adverse behavioral effects or motor impairment (measured in the rotarod test) at anticonvulsant doses, indicating the superiority of $glycine_B$ site antagonists and partial agonists, compared with competitive or uncompetitive NMDA receptor antagonists. In this model, i.c.v. injection of D-serine or i.p. administration of D-cycloserine (160 to 320 mg/kg), but not R(+)HA-966 (10 to 40 mg/kg), increased the afterdischarge threshold in amygdala-kindled rats (Löscher *et al.*, 1994; see also Wlaz *et al.*, 1994a).

It was recently reported that L-701,324 alone (up to 10 mg/kg) failed to alter the afterdischarge threshold or seizure severity in kindled rats, despite apparent ataxia in the rotarod test (Ebert *et al.*, 1997). However, it is noteworthy that combination with ifenprodil resulted in anticonvulsive effects without further exaggeration of negative motor effects (Ebert *et al.*, 1997). Similarly, MRZ 2/576 was not effective in this model, up to doses that impair motor behavior (10 mg/kg) (Wlaz and Löscher, 1998). In contrast, systemic administration of another $glycine_B$ antagonist, MDL-104,653 (20 mg/kg), inhibited both the development and expression of kindled seizures in rats (Chapman *et al.*, 1995).

Felbamate was shown to possess clear anticonvulsive activity that was weakened by coadministration of glycine or D-serine and was thus claimed to involve $glycine_B$ site antagonistic effects (McCabe *et al.*, 1993; White *et al.*, 1995). However, felbamate inhibits [^{3}H]5,7-diCl-KYN binding with an IC_{50} of only 306 μM (Wamsley *et al.*, 1994b), which is probably far above concentrations reached in the brain during therapy. It showed a promising therapeutic profile for the treatment of epilepsy and was introduced to the American market but was withdrawn shortly thereafter because of reported cases of aplastic anemia (McCabe *et al.*, 1993; Burdette and Sackellares, 1994). Also, its mechanism of action is more complicated than initially anticipated; recent electrophysiological studies showed that felbamate is more likely to act as a low affinity NMDA receptor channel blocker than it is to act at the $glycine_B$ site (Subramaniam *et al.*, 1995), and the compound also has Na^+ channel-blocking properties (Srinivasan *et al.*, 1996).

In summary, at present there is no indication that $glycine_B$ site full antagonists could be useful in monotherapy for the treatment of epilepsy. Still open as a possibility is the combination of these agents with traditional treatments (Czuczwar *et al.*, 1996; Wlaz *et al.*, 1996) or agents acting at other recognition sites of the NMDA receptor (Norris *et al.*, 1992; Wlaz *et al.*, 1994b; Ebert *et al.*, 1997). Moreover, paradoxically, the best preclinical profiles in animal models recorded to date have been observed with high intrinsic activity, partial agonists such as D-cycloserine.

G. Drug Dependence and Tolerance

1. Opioids. The tolerance seen after repeated treatment with opioids (mainly morphine) is an important therapeutic obstacle because it develops with respect to antinociceptive (therapeutic) effects but not to side effects related to gastrointestinal motility or respiratory depression (Ling *et al.*, 1989). It has been demonstrated that NMDA receptor antagonists block the development of tolerance to opiates without increasing the acute effects of morphine (Marek *et al.*, 1991; Trujillo and Akil, 1991; for reviews, see Herman *et al.*, 1995; Trujillo and Akil, 1995; for conflicting data, see Grass *et al.*, 1996; Bhargava, 1997). Interestingly, NMDA receptor antagonists [LY 274614 [3SR,4aRS,6SR,8aRS-6-(phosphonomethyl)decahydroisoquinoline-3-carboxylic acid], dextromethorphan, and memantine] not only prevent but also reverse (when given repetitively) tolerance to morphine after it has been established (Tiseo and Inturrisi, 1993; Elliott *et al.*, 1994; Popik and Skolnick, 1996; Shimoyama *et al.*, 1996), indicating that NMDA receptors are involved not only in development but also in maintenance of morphine dependence. Similar effects can possibly be expected with $glycine_B$ site antagonists.

The $glycine_B$ site partial agonist ACPC (50 or 150 mg/kg) prevented tolerance to morphine and the δ ligand [D-Pen^2,D-Pen^5]enkephalin in rats but did not influence tolerance to the κ-opioid U50,488H [*trans*-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide] (Kolesnikov *et al.*, 1994). In this study, acute effects of morphine were not changed by either acute or repetitive treatment with ACPC, indicating a specific effect on adaptive changes. ACPC also reverses morphine tolerance after it has been established (Kolesnikov *et al.*, 1994).

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The glycine$_B$ site full antagonist ACEA 1328 (20 mg/kg) completely blocked tolerance to morphine-induced antinociception in the tail flick test in CD-1 mice, without affecting the basal nociceptive response or potentiating morphine-induced antinociceptive effects in the tail flick test (Lutfy *et al.*, 1995). Much lower doses (5 mg/kg) were sufficient to block both early and late phases of formalin-induced hyperalgesia but also impaired rotarod performance and decreased locomotion (Lutfy *et al.*, 1996b). To inhibit morphine tolerance in the formalin test, doses as high as 40 mg/kg were needed (Lutfy *et al.*, 1996b). Recently, Bespalov *et al.* (1998) observed an inhibitory effect of MRZ 2/576 on the development of tolerance to the analgesic action of morphine, but only when this glycine$_B$ antagonist was combined with probenecid to prolong its half-life.

Therefore, this is still a largely unexplored area, but the data generated so far are encouraging. Apart from tolerance, opioid abuse is another important therapeutic challenge associated with the chronic use of these compounds. Although the contribution of physical dependence to sustained drug use is not clear (Robinson and Berridge, 1993), it is widely accepted that the optimal treatment should inhibit both withdrawal signs and motivational aspects of abuse. D-Cycloserine (3 and 10 mg/kg) attenuated naloxone-precipitated withdrawal signs in rats, but R(+)HA-966 (3 and 10 mg/kg) actually worsened morphine withdrawal signs (Kosten *et al.*, 1995). These results may not be surprising because, as discussed above, at the doses used D-cycloserine might have an agonistic character, whereas R(+)HA-966 should behave as an antagonist. In contrast, in a recent study in rats, R(+)HA-966 (30 and 70 mg/kg) did inhibit the naloxone-precipitated withdrawal syndrome (wet dog shakes, ejaculations, mouth movements, ptosis, irritability to touch, and diarrhea) (Bristow *et al.*, 1997).

Recently, Popik *et al.* (1998) demonstrated that the glycine$_B$ antagonists L-701,324 (3 mg/kg) and MRZ 2/570 (5 mg/kg) inhibited the expression of the naloxone-precipitated morphine withdrawal syndrome in mice (jumping). It has often been stated that nonspecific effects, such as sedation, ataxia, and myorelaxation, could produce false-positive results in this test (Herman *et al.*, 1995). This, however, was clearly not the case, because the dose-response curves for both agents, but not for a NMDA channel blocker tested in parallel, had a bell-shaped character, i.e., higher and lower doses were ineffective (Popik *et al.*, 1998). In the same study, extinction (detoxification) of morphine dependence was also observed, i.e., when glycine$_B$ antagonists were administered after termination of morphine treatment, the decline of the withdrawal syndrome was faster in treated animals.

Most relevant for drug abuse are studies focusing on motivational aspects. The place preference test used to study the reinforcing aspects of drug dependence is usually performed with a two-chamber apparatus; morphine injection is followed by restriction to one compartment and on alternating days saline injection is associated with the other chamber, leading to gradual increases in preference for the morphine-associated chamber. It has been demonstrated that NMDA receptor antagonists [e.g., memantine and (+)MK-801] inhibit both the development and the expression of morphine place preference in rats (Popik and Danysz, 1997; Tzschentke and Schmidt, 1997). Recently, it was shown that L-701,324 (3 and 10 mg/kg) and MRZ 2/570 (2.5 to 10 mg/kg) also inhibit both the development and the expression of morphine place preference in rats (Popik *et al.*, 1998). The mechanisms of this effect are not clear but could involve actions through inhibition of the reinforcing effects of morphine, inhibition of morphine recognition, or general learning impairment. The latter possibility is a rather unlikely explanation for the inhibition of place preference expression, because NMDA receptor antagonists do not inhibit retrieval of previously learned responses (Danysz *et al.*, 1995b).

An increase in self-stimulation (electrical stimulation of the brain pleasure center by, for example, lever pressing) produced in rats by morphine was attenuated by the nonselective glutamate receptor antagonist kynurenic acid (Bespalov *et al.*, 1994). However, it should be stressed that kynurenic acid, apart from actions at the glycine site, also acts as a competitive NMDA and AMPA/kainate receptor antagonist at slightly higher concentrations (Stone *et al.*, 1987; Birch *et al.*, 1988b; Stone, 1991). These limited data suggest that glycine$_B$ antagonists might be a promising co-treatment to decrease tolerance to the analgesic effects of morphine, as well as possibly a valuable treatment for opioid abuse.

2. *Cocaine.* NMDA receptor antagonists [e.g., (+)MK-801] inhibit sensitization (locomotor response) to repetitive administration of cocaine (Karler *et al.*, 1989; Carey *et al.*, 1995). In the place preference test, NMDA receptor antagonists block the acquisition of cocaine-induced place preference but not its expression (Cervo and Samanin, 1995). Also, established self-administration of cocaine, and its development in rats, is decreased by dextromethorphan (Schenk *et al.*, 1993; Pulvirenti *et al.*, 1997). However, another report actually indicates that (+)MK-801 increased enhancement of electric self-stimulation by cocaine (Ranaldi *et al.*, 1997).

R(+)HA-966 (15 to 100 mg/kg) does not affect the acute stimulatory response to cocaine in rats (Witkin, 1993; Morrow *et al.*, 1995) but inhibits behavioral sensitization (increase in locomotion) after repetitive administration (Morrow *et al.*, 1995; Ramirez *et al.*, 1997). In contrast, with i.c.v. administration 5 min before each cocaine administration, this partial agonist failed to prevent sensitization but inhibited cocaine self-administration (Shoaib *et al.*, 1995).

Much less is known regarding the effects of full antagonists of the glycine$_B$ site on cocaine activity. There is only one study showing that ACEA 1021, ACEA 1328,

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and ACEA 1022 (up to 60 mg/kg) attenuated cocaine toxicity and the development and expression of behavioral sensitization in rats (locomotion) (Ramirez *et al.*, 1997).

Because there is only one positive study with glycine$_B$ site full antagonists, the data are not sufficient to yield any conclusions regarding the potential use of these compounds in cocaine abuse. More agents should be studied and more attention should be paid to the motivational aspects of cocaine use, i.e., self-administration and place preference tests.

H. Pain

It is now well established that NMDA receptors play a key role in chronic pain states and hyperalgesia (Dickenson, 1990; Coderre, 1993b), and agents such as ketamine (Eide *et al.*, 1995a) and dextromethorphan (Price *et al.*, 1994; Ilkjaer *et al.*, 1997; Vierck *et al.*, 1997) are finding increasing use for this indication. These agents are unfortunately not without side effects. The ability of glycine$_B$ antagonists to induce receptor desensitization may allow such compounds to differentiate among various forms of NMDA receptor-mediated synaptic plasticity. It is therefore possible that glycine$_B$ antagonists might block the development of chronic pain states at doses causing few or no side effects.

The first evidence for actions of glycine$_B$ antagonists in the modulation of chronic pain was the observation that intrathecal injection of kynurenic acid blocked intrathecal NMDA-induced biting behavior in mice (Raigorodsky and Urca, 1990). Dickenson and Aydar (1991) were quick to follow and showed that intrathecal administration of the selective glycine$_B$ antagonist 7-Cl-KYN blocked the induction of wind-up, an electrophysiological model of the induction of chronic pain states in rats. Similarly, both pre- and postadministration of intrathecally applied 7-Cl-KYN (10 nmol) selectively blocked the second phase of the response of convergent dorsal horn neurons to peripheral injection of formalin, whereas 6-cyano-7-nitroquinoxaline-2,3-dione had nonselective effects (Chapman and Dickenson, 1995). Moreover, electrophoretic administration of glycine plus strychnine enhanced the responses of dorsal horn neurons to concomitant administration of NMDA or peripheral stimulation, indicating that the glycine$_B$ site is not saturated in the spinal cord in vivo (Budai *et al.*, 1992). Intrathecally administered 7-Cl-KYN and (±)HA-966 were also effective in behavioral models in mice, such as the mouse hot-plate (ED$_{50}$ = 13.6 and 114 mg/kg, respectively) and formalin (14.8 and 65 mg/kg, respectively) tests, but it should be noted that high doses also impaired motor function (Nasstrom *et al.*, 1992) and that the first two models reflect acute pain, which is generally accepted to be insensitive to NMDA receptor antagonists. Such aspects are of particular importance for preclinical models of pain, because antinociceptive effects are most commonly assessed on polysynaptic nociceptive reflexes, necessitating verification of the specificity of the effects observed.

In contrast, others reported that intrathecal 7-Cl-KYN (90 nmol) failed to produce any analgesic effects in either the early or late phase of the formalin test (Coderre, 1993a), and the antinociceptive activity of intrathecal AP5 was actually reduced by combination with 7-Cl-KYN, as was glycine-induced enhancement of antinociception (Coderre and Van Empel, 1994b). Moreover, D-serine (100 μg, i.c.v.) actually potentiated antinociception produced by morphine (0.3 to 10 mg/kg, subcutaneously) in both the acute and tonic phases of the formalin response (Hunter *et al.*, 1994).

More relevant are studies with systemically active agents. Administration of ACEA 1011, before formalin injection but not after, reduced pain-like behavior in the late tonic phase (Vaccarino *et al.*, 1993). These findings suggest that the development of the late phase of formalin-induced pain is the result of NMDA-mediated activity during the early phase. Similarly, in mice, R(+)HA-966, L 687,414, D-cycloserine (fig. 10), and MDL-29,951 (fig. 4) dose-dependently (IC$_{50}$ = 3.4, 1.7, 22, and 1 mg/kg, respectively) and selectively attenuated the tonic nociceptive phase of the formalin test at doses exerting little motor disruption in the rotarod test (Hunter and Singh, 1994; Millan and Seguin, 1994; Seguin *et al.*, 1995). These agents had a more favorable profile than did competitive or uncompetitive NMDA receptor antagonists, which nonselectively blocked both the early and late phases of the formalin response and induced ataxia at comparable doses (Hunter and Singh, 1994; Millan and Seguin, 1994; Seguin *et al.*, 1995).

Glycine$_B$ antagonists are also effective in other models of chronic pain. L-687,414 (fig. 10) and L-701,324 (fig. 7) produced a selective, dose-dependent reversal of mechanical hyperalgesia in rats with carrageenan-induced paw inflammation, with minimal effective doses of 100 and 3 mg/kg, respectively (Laird *et al.*, 1996). GV 196771A (fig. 4) was effective in blocking the development of hyperalgesia after chronic sciatic nerve ligation in rats when given at 3 mg/kg, p.o., twice daily for 10 days. GV 196771A also dose-dependently reversed established hyperalgesia for up to 8 h (ID$_{50}$ =3 mg/kg). The therapeutic profile was promising, inasmuch as the second phase of formalin-induced hyperalgesia was antagonized with an ID$_{50}$ of 0.6 mg/kg, p.o., whereas 10 mg/kg had no effect on the first phase (Quartaroli *et al.*, 1997). There is also evidence for synergistic antinociceptive interactions between nonsteroidal anti-inflammatory drugs and glycine$_B$ antagonists. Coadministration of low doses of niflumic acid with R(+)HA-966 (1 mg/kg, i.v., and 2.5 mg/kg, subcutaneously, respectively) significantly reduced carrageenan-evoked spinal c-Fos expression without influencing peripheral carrageenan-evoked edema. Neither compound was effective alone (Chapman *et al.*, 1996).

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Recent data indicate that peripheral NMDA receptors may also contribute to the development of chronic pain states (Carlton and Hargett, 1995; Zhou *et al.*, 1996; Davidson *et al.*, 1997). Indeed, local injection of ketamine has been reported to have long term effects in preventing the development of burn-induced primary and secondary hyperalgesia in healthy volunteers (Warncke *et al.*, 1997). Ketamine was also reported to profoundly enhance the anesthetic and analgesic actions of locally administered buvivacaine after herniorrhaphy, via a peripheral mechanism of action (Tverskoy *et al.*, 1996). Although very encouraging, these results should be verified, because antinociceptive effects in these studies were assessed after local administration of systemically active antagonists, which does not allow conclusions to be drawn regarding whether peripheral receptors were involved. However, if these findings can be substantiated, then they would indicate a novel approach to minimize the side effects of NMDA receptor antagonists. There are many glycine$_B$ antagonists that have been abandoned essentially because of their poor penetration to the brain. Systemic administration of such compounds might effectively block peripheral NMDA receptors without producing any centrally mediated side effects. Indeed, a very recent report (Zhou and Carlton, 1997) showed that i.p. administration of the glycine$_B$ antagonist 5,7-diCl-KYN blocked the second phase of formalin-induced nociception in rats with an ID_{50} of 10 mg/kg, and this effect was reversed by systemically administered glycine (120 mg/kg). Unfortunately, it is not clear whether this truly reflects antinociceptive activity, because the early phases were also affected to a lesser degree; this could indicate nonselective effects.

There is also evidence that NMDA receptors are involved in visceral nociception even under acute conditions. Nociceptive responses of multireceptive spinal neurons to colorectal distension are potentiated by intrathecally administered D-serine and are antagonized by 7-Cl-KYN (Kolhekar and Gebhart, 1996). These findings demonstrate the involvement of spinal NMDA receptors in mediating the hyperexcitability of spinal neurons in response to visceral nociceptive input, and they also indicate that the glycine$_B$ site is not saturated in the spinal cord in vivo (Kolhekar and Gebhart, 1996). Ketamine (1 to 10 mg/kg) also reduces acute components of visceral nociceptive responses (Laird *et al.*, 1995; Olivar *et al.*, 1997). Recent data show that, although noxious stimulation of viscera evokes increases in receptive fields, little or no wind-up is seen, in contrast to the powerful wind-up and reflex facilitation seen with noxious stimulation of somatic tissues (Laird *et al.*, 1995). Very recent observations with the systemically active glycine$_B$ antagonist MRZ 2/576 indicate that it is 10 times more potent against uretal distension-induced nociception in rats after i.v. administration (ID_{50} = 0.2 mg/kg) (Olivar *et al.*, 1997), compared with its previously determined activity in the CNS as an anticonvulsant in the MES test (Parsons *et al.*, 1997). It is also possible that the NMDA receptors involved in acute visceral nociception are not spinal. The effects seen with systemic administration of NMDA receptor antagonists (Laird *et al.*, 1995) were more pronounced than those observed after local intrathecal administration (Kolhekar and Gebhart, 1996).

It is thought that phenomena such as sensitization, tolerance, and drug dependence might also involve synaptic plasticity, and numerous studies indicate that NMDA receptor antagonists block tolerance and dependence to opioids in animal models (see above). Systemically active glycine$_B$ antagonists attenuate both physical dependence on morphine and the development of tolerance to the antinociceptive effects of opioids after repeated administration. ACEA 1328 and ACEA 1021 (20 mg/kg) completely blocked tolerance to morphine-induced antinociception in the tail flick test in CD-1 mice, without affecting the basal nociceptive response or potentiating morphine-induced antinociceptive effects (Lutfy *et al.*, 1995, 1996b). Similar effects were reported by others, although tolerance to κ-opioids was not changed (Pasternak and Inturrisi, 1995). When given alone, ACPC (50 and 150 mg/kg) had no analgesic actions in the tail flick assay and did not change the potency of morphine in naive mice (Kolesnikov *et al.*, 1994). However, chronic administration of this "functional" NMDA receptor antagonist both prevented and reversed morphine tolerance (Kolesnikov *et al.*, 1994).

Taken together with the aforementioned effects of some glycine$_B$ antagonists in models of chronic pain, these data indicate the utility of the combined use of therapeutically safe glycine$_B$ antagonists with opioids in the treatment of chronic pain (Eide *et al.*, 1995b; Elliott *et al.*, 1995). The antinociceptive effects could be predicted to be synergistic, and the presence of a glycine$_B$ antagonist should both block the development of chronic pain states and inhibit tolerance to the analgesic effects of morphine.

I. Ethanol Dependence and Abuse

Ethanol inhibits NMDA receptors with affinity in the low-millimolar range, and these concentrations are actually seen in the brains of alcohol abusers (Dildy and Leslie, 1989; Danysz *et al.*, 1992; Hoffman, 1995). It seems that ethanol preferentially interacts with NMDA receptors containing the NR2B subunit (Lovinger and Zieglgansberger, 1996; Yang *et al.*, 1996). Moreover, it has been shown that, upon withdrawal from ethanol in dependent rats, an increase in glutamate release is seen in the striatum and corresponds to the duration of the withdrawal syndrome (hyperactivity, treading, shakes, jerks, and twitches) (Rossetti and Carboni, 1995). (+)MK-801 normalized both biochemical and behavioral changes, whereas diazepam affected only the latter aspects.

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Several authors showed that in vitro glycine can reverse the inhibitory effects of ethanol on NMDA receptors, implying a competitive interaction (Rabe and Tabakoff, 1990; Woodward and Gonzales, 1990; Buller *et al.*, 1995), but these findings have not been confirmed by others (Peoples and Weight, 1992).

Consistent with the role of NMDA receptors in alcohol tolerance is the finding that D-cycloserine (10 to 30 mg/kg) given before (but not after) ethanol administration enhances tolerance to the motor-impairing effect in rats (Khanna *et al.*, 1995). This effect of D-cycloserine is probably attributable to agonism at the glycine$_B$ site, because it was antagonized by ketamine (Khanna *et al.*, 1995).

Glycine$_B$ antagonists also attenuate the expression of the withdrawal syndrome. Audiogenic seizures associated with alcohol withdrawal after 7-day treatment in rats are inhibited by L-701,324 at 5 but not 2.5 mg/kg (Kotlinska and Liljequist, 1996), similarly to NMDA receptor antagonists acting at other recognition sites (Grant *et al.*, 1990; Danysz *et al.*, 1992).

There are several possibilities regarding how glycine$_B$ antagonists exert this effect. One is that they produce alcohol-like effects, as suggested by drug discrimination studies (Bienkowski *et al.*, 1997; Kotlinska and Liljequist, 1997). Bienkowski *et al.* (1997) reported significant substitution for an ethanol cue by L-701,324 (80% at 3 mg/kg) and MRZ 2/576 (50% at 5 mg/kg) in rats, at doses that did not affect responding rates. Similarly, others (Kotlinska and Liljequist, 1997) found up to 100% substitution with L-701,324 at 7.5 mg/kg, without impairment of performance. In this respect, L-701,324 seem to be like uncompetitive antagonists, which usually substitute fully for the alcohol cue (Sanger, 1993). On the other hand, ACEA 1021 (20 mg/kg) failed to substitute for the ethanol cue in an operant drug discrimination paradigm (Balster *et al.*, 1995), again indicating substantial differences in the actions of various glycine$_B$ antagonists.

These limited data suggest that glycine$_B$ antagonists behave like NMDA receptor antagonists acting at other recognition sites in tests assessing tolerance/withdrawal-related changes. However, to confirm the validity of alcohol abuse treatment with glycine$_B$ antagonists, more elaborate models assessing the motivational aspects of alcohol use should be applied. Studies with some NMDA receptor antagonists (such as memantine) in a chronic model of alcohol craving in rats are promising (Holter *et al.*, 1996), and glycine$_B$ antagonists will definitely be tested soon.

J. Huntington's Disease

The involvement of NMDA receptor overactivation in neurodegeneration in Huntington's disease has often been suggested (Young *et al.*, 1988; Beal *et al.*, 1991; Beal, 1992). In patients with Huntington's disease, glycine concentrations are significantly increased (25%) in platelets but not in plasma; it has been argued that this probably reflects an analogous increase in glycine levels in the CNS, because this disease is caused by a dominant gene and should affect metabolism similarly in all tissues (Reilmann *et al.*, 1997). In accordance with this suggestion is the finding that glycine levels are increased by 30% in the CSF of patients with Huntington's disease (Nicoli *et al.*, 1993). It has been suggested that this change in glycine concentrations might contribute to the progression of the disease (Reilmann *et al.*, 1997). In general, if the suitability of glycine$_B$ antagonists as neuroprotective agents in chronic neurodegenerative disease is accepted, then Huntington's disease will probably be among the first to be tested clinically. This is because this disease shows fast progression, which would allow the demonstration of neuroprotection within a relatively short study period. The important issue is whether symptomatological improvement or worsening can be expected.

K. Alzheimer's Disease and Dementia

According to the hypothesis proposed originally by Greenamyre *et al.* (1988), and subsequent publications, glutamate may play an important role in the neuropathomechanism and symptoms of dementia (Greenamyre and Young, 1989; Palmer and Gershon, 1990; Francis *et al.*, 1993). Both in old rats with learning deficits and in patients with Alzheimer's dementia, a decrease in [^{3}H]glycine binding and/or glycine potency to enhance functional [^{3}H]MK-801 binding has been reported by some authors (Procter *et al.*, 1989; Steele *et al.*, 1989; Miyoshi *et al.*, 1990; Tamaru *et al.*, 1991). In patients with Alzheimer's disease, a decrease of [^{3}H]glycine binding was observed in area 18 (layers I and II) of the cortex (Carlson *et al.*, 1993). This could indicate a deficit in NMDA receptor function. In contrast, others (Palmer and Burns, 1994) failed to detect any change in glycine potency to modulate [^{3}H]MK-801 binding in the brains of patients with Alzheimer's disease, but aging itself was associated with lower B_{max} values for [^{3}H]MK-801 binding at saturating concentrations of glycine and glutamate (see also Delbel and Slater, 1991).

As described above, there are conflicting data on the function of NMDA receptors in patients with Alzheimer's disease, but according to some authors (Ulas *et al.*, 1992) the problem could result from pooling of data, which might obscure case-related differences. In other words, in patients with dementia an increase in variability is usually observed, which is only expressed as a consistent deficit in a subset of patients.

In aged rats (24 months), an increase in glycine uptake and a decrease in glycine release was observed in hippocampal synaptosomes (Pittaluga *et al.*, 1993) and, surprisingly, the EC_{50} value of glycine to stimulate [^{3}H]MK-801 binding was decreased four-fold. This increase in glycine potency might have been an artifact resulting from lower levels of glycine in the brain ho-

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mogenates used for binding studies. However, Kumashiro *et al.* (1995) failed to detect any differences in the postmortem concentrations of glycine and D-serine in brains of patients with Alzheimer's disease (prefrontal cortex). Another study reported that the concentration of total D-amino acids was higher (approximately 50%) in both lumbar and ventricular CSF in patients with Alzheimer's disease (Fisher *et al.*, 1994); unfortunately, those authors did not resolve various amino acids, but it can be assumed that the D-serine contribution was significant (Hashimoto and Oka, 1997).

Because inhibition of LTP and learning disruption have been observed after glycine$_B$ site blockade (Danysz and Wroblewski, 1989; Chiamulera *et al.*, 1990; Oliver *et al.*, 1990a; Danysz *et al.*, 1995b), it has been suggested that it might be possible to obtain cognitive enhancement by stimulation of the glycine$_B$ site. In fact, in a simultaneous brightness-discrimination task, high doses of glycine (750 mg/kg) were reported to reverse learning deficits induced by entorhinal cortex lesions (Myhrer *et al.*, 1993). Glycine enhanced LTP in the hippocampus (Abe *et al.*, 1990), and D-cycloserine (10 mg/kg) increased the excitability of hippocampal dentate granule cells (Pitkanen *et al.*, 1994). In fact, several studies showed positive effects of D-cycloserine (0.3 to 30 mg/kg) in rodents in a variety of learning tasks, such as passive avoidance (Monahan *et al.*, 1989b), T-maze (Monahan *et al.*, 1989b; Flood *et al.*, 1992), Morris water maze (Sirvio *et al.*, 1992; Baxter *et al.*, 1994), and radial maze (Schuster and Schmidt, 1992) tests. Eyeblink classical conditioning in rabbits was also enhanced by D-cycloserine (Thompson *et al.*, 1992). D-Cycloserine at low doses (0.3 to 1.0 mg/kg) also attenuated the learning induced by scopolamine in rats (Sirvio *et al.*, 1992; Zajaczkowski and Danysz, 1997). Spontaneous alterations in the Y-maze and passive avoidance learning in mice are both impaired by i.c.v. pretreatment with amyloid β_{25-35} 7 or 14 days earlier (Maurice *et al.*, 1996). In such animals, D-cycloserine attenuated this deficit with a bell-shaped dose-response curve; the best effect was obtained with 10 mg/kg D-cycloserine (Maurice *et al.*, 1996). An elaborate study (Quartermain *et al.*, 1994) using the two-unit linear maze test in mice showed a clear improvement with pretraining administration of D-cycloserine (starting at 3 mg/kg) but no effect when the compound was administered after training and before testing (24 h after training). However, the positive effects of D-cycloserine on learning disappeared after repeated administration for 15 days (Quartermain *et al.*, 1994), an effect that parallels clinical observations (see below). In accordance with this study, repeated treatment with D-cycloserine (6 mg/kg) failed to antagonize a radial maze learning deficit, induced by lesions of the entorhinal cortex, in rats (Zajaczkowski *et al.*, 1996). Similarly, in a study in primates, D-cycloserine (4 to 14 mg/kg, intramuscularly) failed to consistently attenuate scopolamine- or PCP-induced spatial delayed-response deficits (Rupniak *et al.*, 1992).

Although clinical studies in healthy volunteers treated with scopolamine (Mohr *et al.*, 1995) revealed some positive cognitive effects of D-cycloserine, further development has been abandoned because in patients with Alzheimer's disease D-cycloserine failed to demonstrate consistent beneficial effects (Mohr *et al.*, 1995). This could be related to its narrow therapeutic window and/or tolerance to its positive cognitive effects developing after chronic administration (Lanthorn, 1994).

Milacemide is a glycine prodrug that shows positive effects in some learning tasks, e.g., Y-maze (Handelmann *et al.*, 1988, 1989) and Morris water maze (Finkelstein *et al.*, 1994) tests. Its positive effects on learning are blocked by inhibition of monoamine oxidase-B, the activity of which is necessary for the metabolism of milacemide to glycineamide, which is then further transformed to glycine (Handelmann *et al.*, 1989). Hence, the action of milacemide is thought to involve glycine, which activates both NMDA receptor-coupled and -uncoupled (inhibitory) glycine recognition sites, but could also be related to monoamine oxidase inhibition (Obrien *et al.*, 1994).

Recently, an interesting observation was made by Cowburn *et al.* (1997), indicating a role for the glycine$_B$ site in the neurotoxic effects of β-amyloid. They found that β-amyloid(25–35) inhibited [^{3}H]glycine binding and stimulated [^{3}H]MK-801 binding but only when glycine concentrations were low. This suggests that this β-amyloid fragment might enhance NMDA receptor function by acting as a glycine$_B$ site agonist or partial agonist. To our knowledge, this is the only report directly connecting the glycine$_B$ site, or glycine itself, with the pathological changes of Alzheimer's disease, but there are many indications that glutamate and/or NMDA receptors could play a role (see Parsons *et al.*, 1998b); therefore, a neuroprotective potential of glycine$_B$ antagonists should be taken into account.

L. Parkinson's Disease

There are indications of overactivity of the glutamatergic system in Parkinson's disease and animal models thereof, probably involving descending corticostriatal, corticosubthalamic, and subthalamic-pallidal/nigral glutamatergic projections (for reviews, see Carlsson and Carlsson, 1990; Greenamyre and O'Brien, 1991; Schmidt *et al.*, 1992). Based on these observations, NMDA antagonists have been proposed as possible symptomatological treatments for this disorder (Carlsson and Carlsson, 1990; Greenamyre and O'Brien, 1991; Schmidt *et al.*, 1992; Parsons *et al.*, 1998b).

No change in D-serine content was observed in the brains of patients with Parkinson's disease (Chouinard *et al.*, 1993), but plasma levels of aspartate, glutamate, and glycine were found to be increased (Iwasaki *et al.*, 1992). The latter finding was interpreted by the authors

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as suggesting ongoing excitotoxic processes. Also, a [^{3}H]MK-801 functional assay performed in striatal membranes from patients with Parkinson's disease suggested an increase in the glycine sensitivity of NMDA receptors containing the NR2B subunit, which might contribute to increased glutamatergic tone (Nash *et al.*, 1997). The importance of these findings in the pathomechanism of Parkinson's disease is unknown.

Both competitive and uncompetitive NMDA receptor antagonists have been reported to possess antiparkinsonian-like activity in animal models, i.e., they reduce neuroleptic agent-induced catalepsy, restore locomotor activity, and reduce muscular rigidity in reserpine-treated animals, as well as potentiate the effects of L-3,4-dihydroxyphenylalanine in the latter model (Schmidt *et al.*, 1991; Moore *et al.*, 1993b; Skuza *et al.*, 1994; Kaur and Starr, 1995; Ossowska, 1994; McAllister, 1996; Schmidt and Kretschmer, 1997). In this regard, uncompetitive NMDA receptor antagonists have much better efficacy than competitive antagonists in animal models (Schmidt *et al.*, 1991; Moore *et al.*, 1993b; Skuza *et al.*, 1994; Kaur and Starr, 1995; Ossowska, 1994; McAllister, 1996; Schmidt and Kretschmer, 1997). However, less is known regarding the therapeutic potential of glycine$_B$ antagonists for this indication. Kretschmer *et al.* (1994) showed that 7-Cl-KYN (administered i.c.v.) reduced haloperidol-induced catalepsy. Also, the Merz glycine$_B$ antagonists MRZ 2/570, MRZ 2/571, and MRZ 2/576 dose-dependently antagonized haloperidol-induced catalepsy in rats (10 to 30 mg/kg), but L-701,324 (up to 5 mg/kg) tested in the same study was ineffective (Karcz-Kubicha *et al.*, 1998a). Similarly, others (Kretschmer *et al.*, 1997) recently reported very weak effects of ACEA 1021 (up to 20 mg/kg) in this model.

The partial agonists ACPC (up to 600 mg/kg) and D-cycloserine (up to 300 mg/kg) also failed to antagonize haloperidol-induced catalepsy (Karcz-Kubicha *et al.*, 1998a). In fact, D-cycloserine at low agonistic doses (12 mg/kg) attenuated the anticataleptic effects of CGP-37849 but not those of (+)MK-801 (Kretschmer *et al.*, 1992; Maj *et al.*, 1993). In contrast, the low intrinsic activity, glycine$_B$ site partial agonist R(+)HA-966 (6 to 30 mg/kg) dose-dependently reduced the cataleptic effects of haloperidol (Kretschmer *et al.*, 1992). However, the highest dose (30 mg/kg) produced myorelaxation, akinesia, and sedation.

In the context of antiparkinsonian-like effects of glycine$_B$ antagonists, a very interesting observation was made by Kretschmer and Schmidt (1996). They showed that the glycine$_B$ site antagonist 7-Cl-KYN, when administered directly into the striatum, abolished catalepsy induced by D2 dopamine receptor antagonists (e.g., haloperidol) but was without effect on catalepsy induced by D1 dopamine receptor antagonists [e.g., SCH 23390 [R-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol]]. This is in contrast to observations with competitive and uncompetitive NMDA receptor antagonists, which can block catalepsy induced by both types of dopamine antagonists (Elliot *et al.*, 1990; Schmidt *et al.*, 1991; Kretschmer *et al.*, 1992; Moore *et al.*, 1993b), and indicates that motor behavior mediated by the striatopallidal and striatonigral output pathways is regulated by NMDA receptors containing glycine$_B$ sites with different pharmacological characteristics.

In monoamine-depleted mice, the glycine$_B$ site antagonists 5,7-diCl-KYN, 7-Cl-KYN, M247258, and M244646 (figs. 5 and 9), as well as the partial agonist R(+)HA-966, significantly increased locomotion when injected i.c.v. (Slusher *et al.*, 1994; Carroll *et al.*, 1995). R(+)HA-966 produced a similar effect after i.p. administration at 10 to 100 mg/kg (after double reserpine treatment), but at 60 to 100 mg/kg muscle relaxation was observed (Carroll *et al.*, 1995). In our study (Karcz-Kubicha *et al.*, 1998a), none of the glycine$_B$ site antagonists or partial agonists tested (MRZ 2/570, MRZ 2/571, MRZ 2/576, L-701,324, ACPC, and D-cycloserine at up to 30, 30, 30, 10, 600, and 300 mg/kg, respectively) increased locomotor activity in reserpine-treated rats. MRZ 2/571 and MRZ 2/576 at 30 mg/kg actually potentiated the sedative effect of reserpine, most likely as a result of ataxia.

Using rats with unilateral 6-hydroxydopamine lesions of the nigrostriatal system, it was observed that R(+)HA-966 injected ipsilaterally into the striatum produced contralateral rotations, suggesting potential antiparkinsonian activity of this drug involving mechanisms postsynaptic to dopaminergic terminals (Carroll *et al.*, 1995). On the other hand, we (Karcz-Kubicha *et al.*, 1998a) failed to observe effects of glycine$_B$ antagonists (MRZ 2/571 to 30 mg/kg, MRZ 2/576 to 30 mg/kg, and L-701,324 to 10 mg/kg) or partial agonists [ACPC at 600 mg/kg and R(+)HA-966 to 10 mg/kg] in this rotation model after systemic administration, whereas D-cycloserine (100 mg/kg) produced modest ipsilateral rotations. None of the glycine$_B$ antagonists tested in this model was able to increase contralateral rotations after L-3,4-dihydroxyphenylalanine treatment (Karcz-Kubicha *et al.*, 1998a). In animals with bilateral 6-hydroxydopamine lesions, R(+)HA-966 injected directly into the striatum (but not systemically) attenuated the parkinsonian syndrome (bradykinesia, decreased spontaneous locomotion, and hunched posture) (Mitchell *et al.*, 1995).

In accordance with the concept of glutamatergic hyperfunction in Parkinson's disease, the glycine prodrug milacemide increased the severity of parkinsonian symptoms in a double-blind, placebo-controlled study (Giuffra *et al.*, 1993), but no clinical data on glycine$_B$ antagonists are currently available. In general, it seems that glycine$_B$ antagonists produce antiparkinsonian-like effects when injected directly into brain structures but not when administered systemically. It is possible that these compounds interact with multiple sites in the brain, producing mutually opposing effects. Also, it is

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likely that the differences observed with various agents are a reflection of different affinities and/or intrinsic activities of partial agonists at different NMDA receptor subtypes and/or differences in local glycine concentrations (Priestley *et al.*, 1995; Krueger *et al.*, 1997). There is no evidence supporting an antiparkinsonian potential of glycine$_B$ antagonists, regarding treatment of symptoms.

It has been suggested that in Parkinson's disease neurodegeneration of dopaminergic neurons in the SNc involves excitotoxicity. In rats, NMDA receptor antagonists protect against damage to dopaminergic neurons induced by the dopaminomimetic methamphetamine (Sonsalla *et al.*, 1989). In monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a metabolite of which (1-methyl-4-phenylpyridinium) is a mitochondrial complex I inhibitor, protective effects have been also demonstrated with NMDA receptor antagonists (Kupsch *et al.*, 1992; Lange *et al.*, 1993).

Methamphetamine toxicity to SNc neurons in mice was attenuated by i.p. injection of (+)MK-801 but not by R(+)HA-966 (50 mg/kg), 7-Cl-KYN (50 mg/kg), or ACPC (200 mg/kg) (Layer *et al.*, 1993). In the case of ACPC, a chronic treatment regimen of 200 mg/kg, which was previously shown to be effective in the treatment of global ischemia (Von Lubitz *et al.*, 1992), failed to provide protection (Layer *et al.*, 1993). In contrast, in another study, R(+)HA-966 (10 and 30 mg/kg) protected C57 black mice from MPTP toxicity, as assessed by attenuation of dopamine and dihydroxyphenylethanoic acid decreases and the number of tyrosine hydroxylase-positive cells in the SNc (Kanthasamy *et al.*, 1997).

Conclusions regarding the utility of glycine$_B$ antagonists as possible treatments to inhibit disease progression can only be made when agents with optimal brain penetration and long half-lives have been tested. The latter is obligatory because both methamphetamine and MPTP initiate an ongoing insult that persists long after administration.

M. Neuroprotection Against Acute Insults

1. Introduction. There are several indications that glycine$_B$ antagonists are at least as good as other NMDA receptor antagonists in providing neuroprotection against acute insults (Moroni *et al.*, 1992; Newell *et al.*, 1995; Yenari *et al.*, 1997). Initial suggestions resulted from in vitro studies showing neuroprotective effects of 7-Cl-KYN and 5,7-diCl-KYN against NMDA- or glutamate-induced insults in cell cultures (Shalaby *et al.*, 1989; McNamara *et al.*, 1990; Patel *et al.*, 1990). 7-Cl-KYN is also neuroprotective against hypoxia-induced damage in cultured primary cortical neurons, with 1000-fold lower potency than (+)MK-801 but similar efficacy (Priestley *et al.*, 1990). Interestingly, R(+)HA-966 showed similar efficacy (but lower potency), indicating that the residual intrinsic activity of this agent (10 to 40%) is not sufficient to allow excitotoxic activation of NMDA receptors.

There is also evidence of favorable neuroprotective activity of glycine$_B$ antagonists in a more elaborate model, i.e., hippocampal slices in which damage to the CA1 region induced by 35 min of hypoxia/ischemia is prevented by preapplication of 100 μM 7-Cl-KYN (Newell *et al.*, 1995). A more systematic approach was recently used by Frankiewicz and Parsons (manuscript in preparation), who compared the IC$_{50}$ values of several NMDA receptor antagonists acting at different recognition sites in hippocampal slices with ischemia/hypoxia-induced functional deficits and the induction of LTP. They came to the conclusion that the in vitro TI obtained with the glycine$_B$ antagonist 5,7-diCl-KYN (TI = 0.77) was 3 times better than that seen with (+)MK-801 (TI = 0.25). Although even with 5,7-diCl-KYN somewhat higher concentrations were required to block these pathological changes (IC$_{50}$ = 3.3 μM), compared with LTP (IC$_{50}$ = 2.53 μM), it should be noted that the 7-min complete ischemia/hypoxia treatment used was severe. It is predicted that the absolute potencies of both agents would be greater against more mild pathological activation but the relative difference in their TIs would remain the same or might even increase.

Because of the previous lack of systemically active glycine$_B$ antagonists, initial studies on neuroprotection in vivo used central injections. In young rats (postnatal day 7), the damage produced by a direct striatal injection of NMDA was attenuated when 7-Cl-KYN was coadministered by the same route (Uckele *et al.*, 1989). In this model, peripheral injection of HA-966 also provided significant protection (McDonald *et al.*, 1989), but the interpretation of these findings is complicated by the lack of selectivity of racemic HA-966, which was used in this study (see Section IV.B.).

Recently, Wenk *et al.* (1998) reported dose-dependent neuroprotective effects of MRZ 2/570, MRZ 2/571, and MRZ 2/576 (starting at 10 mg/kg) administered twice, 15 min before and 15 min after a direct unilateral injection of NMDA into the nucleus basalis of Meynert (magnocellularis in rats). Neuroprotection was scored as the difference between left and right frontal cortex choline acetyltransferase activities measured 7 days after the insult.

Widdowson *et al.* (1996) found no effect of R(+)HA-966 (2 × 10 mg/kg), D-cycloserine (2 × 50 mg/kg) (fig. 10), MDL-29,951 (2 × 10 mg/kg) (fig. 4), MNQX (2 × 10 mg/kg) (fig. 8), or L-701,252 (5 mg/kg) (fig. 7) against L-2-chloropropionic acid-induced neurotoxicity in the cerebellum. CPP, CGP-40116 [D-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid], and (+)MK-801 were effective. According to the authors, this indicates a lack of glycine$_B$ site involvement in cerebellar damage. This is difficult to reconcile with the high levels of expression of NR1C subunits, which are very sensitive to glycine (table 5). Instead, the lack of effects could result from either

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poor brain penetration of the glycine$_B$ antagonists used and/or the intrinsic activities of the partial agonists used.

2. Ischemia. Before the question of whether glycine$_B$ antagonists are effective in clinically relevant models of ischemia is addressed, it would be valid to analyze whether NMDA receptor antagonists in general are active under these conditions. Unfortunately, it is not always obvious what "clinically relevant" actually means. In our opinion, the following points are important: the treatment should be administered after a relatively long delay (minimum, 1 to 2 h) after initiation of the insult, verification of damage and functional deficits should be performed not less than 1 to 2 weeks later, transient and preferably focal ischemia should be used, and the brain temperature should be maintained at the same level in control and treated animals (see Parsons *et al.*, 1998b). In reviewing all published data, we were unable to find convincing and consistent evidence for positive effects of NMDA receptor antagonists under these conditions. Nonetheless, it has been suggested that glycine$_B$ antagonists might be particularly useful for the treatment of stroke, based on the following findings.

1. As studied by brain microdialysis, the rise in glycine (or serine) concentrations under ischemic (or traumatic) conditions is in most cases smaller than that for glutamate but might persist much longer (Benveniste *et al.*, 1984; Baker *et al.*, 1991; Globus *et al.*, 1991a; Cantor *et al.*, 1992; Gemba *et al.*, 1992). In one study comparing brain structures, a persistent elevation of glycine concentrations was seen in the hippocampus, which was most severely affected, but the elevation was less pronounced in brain regions showing more moderate damage, such as the thalamus and cortex (Globus *et al.*, 1991a). Also, in newborn rats subjected to ischemia/hypoxia, enhanced CSF levels of glycine are sustained much longer than those of glutamate (Andine *et al.*, 1991). This increase is probably associated with a decrease of glycine transporter efficiency upon depolarization (Supplisson and Bergman, 1997). Therefore, glycine$_B$ antagonists would not need to be present at very high concentrations to compete with endogenous agonists but should be present for a longer time. Better TIs might be anticipated, and prolonged intervention with glycine$_B$ antagonists might be sensible. It should be noted that microdialysis data on the relationship between amino acid levels and ischemia-induced damage should be interpreted with caution. Food deprivation can protect the rat striatum against hypoxia/ischemia but results in higher glutamate levels in the dialysate (Dijk *et al.*, 1994). In contrast to in vivo models, in slices subjected to hypoxia in vitro the increase in serine and glycine levels was greater than that of glutamate, suggesting the importance of these amino acids for neuronal insults (Hirai and Okada, 1993). However, others came to the conclusion that the alterations of glycine levels were not of crucial significance in a similar model (Jones and Szatkowski, 1995).
2. Transient cerebral ischemia causes severe reduction in [^{3}H]glycine binding throughout the brain, and this reduction precedes the neuronal damage in selectively vulnerable brain regions (Araki *et al.*, 1995).
3. In the four-vessel occlusion ischemia model in rats, neuroprotective hypothermia did not change glutamate, GABA, or aspartate cortical levels (as determined by brain microdialysis) but did significantly decrease the glycine concentration, suggesting a causal relationship between glycine levels and brain damage and indicating the glycine$_B$ site as an attractive target for neuroprotective agents (Simpson *et al.*, 1991). Similar findings were obtained in a global ischemia model in rabbits (Baker *et al.*, 1991).
4. Using brain microdialysis (hippocampus) it was found that a neuroprotective adenosine agonist failed to change the glutamate concentration after transient global ischemia in rabbits (neck tourniquet inflation) but significantly lowered glycine levels in this structure (Cantor *et al.*, 1992).
5. Areas known to be susceptible to ischemia-induced brain damage show low levels of expression of mRNA for GLYT transporters, indicating weak buffering capacities (Goebel, 1996).
6. In striatal slices taken ex vivo from dogs subjected to a model of cardiac arrest, there was an increase in glycine potency to augment NMDA-induced dopamine release (Werling *et al.*, 1994).
7. The glycine (and glutamate) concentrations in plasma and serum are higher in patients with progressing ischemic stroke, compared with stable stroke subjects (Castillo *et al.*, 1997). Similarly, in patients with closed-head injuries there is a substantial (up to eight-fold) and long-lasting (70 h) increase in glycine concentrations in the CSF (Palmer *et al.*, 1994b).
8. Globus and co-workers (Globus *et al.*, 1991a; Lin *et al.*, 1992) suggested that glycine or glutamate levels alone are not a predictive measure of neuronal damage but that the ratio of (glutamate $\times$ glycine)/GABA (the so-called excitotoxic index) is more relevant.

The first studies with glycine$_B$ antagonists in brain ischemia used agents with no or very poor brain penetration, such as 7-Cl-KYN and 5,7-diCl-KYN, necessitating i.c.v. injection. In transient global ischemia in rats (common carotid artery occlusion plus hypotension), 7-Cl-KYN administered i.c.v. shortly before initiation of the insult prevented CA1 cell damage and discrimina-

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tion learning impairment (Wood *et al.*, 1992, 1993) (table 9). Similar observations were made by Verrecchia *et al.* (1995) (table 9) in a permanent MCAo model. The neuroprotective effect was reversed by coadministration of D-cycloserine, suggesting involvement of the glycine$_B$ site. Systemic (i.p.) pretreatment with either 7-Cl-KYN (20 mg/kg) or HA-966 (10 mg/kg) has also been shown to be effective in neuroprotection in global ischemia in gerbils (Patel *et al.*, 1989) (table 9). In the case of the former agent, ischemia-induced opening of the blood-brain barrier could play a role in its increased brain accessibility.

In a MCAo model of permanent ischemia, the glycine$_B$ site partial agonist L-687,414, given immediately after occlusion as a 14 mg/kg i.v. bolus, followed by infusion at 14 mg/kg/h, reduced cortical but not striatal damage (Gill *et al.*, 1995). At a higher dose (table 9) apparently weaker protection was obtained, which could be explained by a slight fall in blood pressure (Gill *et al.*, 1995). These treatments resulted in L-687,414 plasma levels of 25 and 60.8 μg/ml, respectively. Bolus injection only (17.6 mg/kg) was ineffective, indicating the importance of secondary excitotoxic mechanisms extending far beyond the direct insult (Gill *et al.*, 1995) (table 9). L-687,414 did not change glucose utilization in the brain or produce pathological alterations in the retrosplenial/cingulate cortex at these neuroprotective doses (Hargreaves *et al.*, 1993a) (see Section VI.B.).

The glycine$_B$ site partial agonist ACPC, at doses of 300 and 600 mg/kg (but not 150 mg/kg) given 5 min (but not 30 min) after initiation of global ischemia in gerbils, increased 7-day survival rates and produced significant preservation of neurons in the CA1 hippocampal region (Fossom *et al.*, 1995b). These effects are rather unexpected, considering the very high intrinsic activity of this agent (Marvizon *et al.*, 1989; Karcz-Kubicha *et al.*, 1997; Maccecchini, 1997). Also, chronic administration of ACPC (300 mg/kg) for 1 week before the production of global ischemia in gerbils resulted in an improvement of survival rates (three-fold), an improvement in neurological scores, and decreased cell loss in the CA1 hippocampal region (Von Lubitz *et al.*, 1992) (table 9). No negative behavioral alterations by this compound have been reported at doses up to 1 g/kg (Maccecchini, 1997). The positive effect in the chronic study is of particular interest because neuroprotective effects were present at a time point (24 h after the last dose) when no significant plasma/brain levels of this agent are expected (Cherkofsky, 1995). This indicates that ACPC leads to long-lasting changes in NMDA receptors. However, paradoxically, exposure of cultured neurons to ACPC in vitro decreases its neuroprotective potency, possibly as a result of changes in NMDA receptor subunit expression, namely an increase of NR2C subunits (the subunit at which ACPC has very high intrinsic activity, i.e., 150% that of glycine) (Boje *et al.*, 1993; Fossom *et al.*, 1995a; Krueger *et al.*, 1997) (table 5). ACPC also showed neuroprotection (attenuated by glycine) at 100 or 200 mg/kg (but not 50 mg/kg) when injected 30 min before ischemia produced by subarachnoid injection of dynorphin-A; improvement of neurological scores and attenuation of spinal cell loss were observed (Long and Skolnick, 1994).

A slight modification in the structure of 7-Cl-KYN, namely introduction of a thiol group, seems to increase brain penetration. In the rat permanent MCAo model, 7-chlorothiokynurenic acid provided neuroprotective effects (mainly in the cortex) when given as an i.v. bolus 5 min before or 15 min after occlusion, but not 60 min later (Chen *et al.*, 1993) (table 9). This effect was apparently the result of antagonism at the glycine$_B$ site and not scavenging properties of this agent, because an analogue having the latter properties only (5-chlorothiokynurenic acid) was ineffective (Chen *et al.*, 1993). The same compound was also effective in global ischemia in gerbils when treatment was initiated at the time of reperfusion (Pellegrini-Giampietro *et al.*, 1994) (table 9). It should be added that the neuroprotective effect (prevention of CA1 damage) was halved when normal body temperature was maintained (Pellegrini-Giampietro *et al.*, 1994).

7-Chloroindole-2-carboxylate ethyl ester, given 30 min before global ischemia in gerbils, attenuates damage in the CA1 region (Hood *et al.*, 1992) (table 9). The related compound indole-2-carboxylic acid (20 or 50 mg/kg) attenuates cognitive and neurological deficits, as well as edema and ionic alterations produced by fluid percussion trauma, when given 15 min after the insult (Smith *et al.*, 1993).

In the global forebrain ischemia model in gerbils, Lazarewicz *et al.* (1997) showed approximately 40% neuroprotection (as determined by cell counts in the CA1 hippocampal region) in animals treated with MRZ 2/576 or MRZ 2/571, given at a dose of 3 × 30 mg/kg, starting 15 min after initiation of ischemia (table 9). However, this treatment was accompanied by significant hypothermia (approximately 3°C) lasting 30 to 180 min after reperfusion (Lazarewicz *et al.*, 1997). The glycine$_B$ antagonist SM-18400, given as a bolus injection followed by infusion (i.v.) starting 1 h after initiation of transient (2-h) MCAo, provided remarkable protection in the cortex and minor protection in the striatum (Tanaka *et al.*, 1995) (table 9).

In a rat model of cardiac arrest, ACEA 1011 and ACEA 1021 significantly attenuated the appearance of myoclonus (Matsumoto *et al.*, 1995). In a MCAo model, both ACEA 1021 and ACEA 1031 (5-nitro-6,7-dibromo-1,4-dihydro-2,3-quinoxalinedione) (but not ACEA 1011) injected i.p. reduced cerebral infarction volumes and the incidence of hemiparesis (Warner *et al.*, 1995) (table 9). In the same model (75-min occlusion), ACEA 1021 administered shortly (10 min) after occlusion, followed by infusion for 24 h (table 9), provided approximately 40% protection under conditions where normal body temperature was maintained during ischemia and for 6 h thereafter (Takaoka *et al.*, 1997a). The neuroprotection was evident in the cortex and was less pronounced in the

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TABLE 9
Compilation of studies in animal models of ischemia with glycine$_B$ site antagonists or partial agonists

Compound studied	Ischemia model[a]	Animals	Analysis period	Dose	Route	Time of administration[b]	Infarction volume	References
7-Cl-KYN	per. MCAo	Mice	24 h	0.44 μg	i.c.v.	−5 min	38% redn.	Verrecchia *et al.*, 1995
7-Cl-KYN	per. MCAo	Mice	24 h	0.88 μg	i.c.v.	−5 min	27% redn.	Verrecchia *et al.*, 1995
7-Cl-KYN	? min CCAo + hypot.	Rats?	7 days	0.22 μg	i.c.v.	−5 min	50% redn.	Wood *et al.*, 1992, 1993
7-Cl-KYN	10-min CCAo	Gerbils	?	20 mg/kg	?	?	?	Patel *et al.*, 1989
7-Cl-thio-KYN	per. MCAo	SD rats	24 h?	10 mg/kg	i.v.	−5 min	No protection	Chen *et al.*, 1993
7-Cl-thio-KYN	per. MCAo	SD rats	24 h?	20 mg/kg	i.v.	−5 min	40% redn.	Chen *et al.*, 1993
7-Cl-thio-KYN	per. MCAo	SD rats	24 h?	20 mg/kg	i.v.	+15 min	40% redn.	Chen *et al.*, 1993
7-Cl-thio-KYN	per. MCAo	SD rats	24 h?	20 mg/kg	i.v.	+60 min	No protection	Chen *et al.*, 1993
7-Cl-thio-KYN	per. MCAo	SD rats	24 h?	30 mg/kg	i.v.	−5 min	40% redn.	Chen *et al.*, 1993
7-Cl-thio-KYN	5-min CCAo	Gerbils	7 days	4 × 15, 25, 50, or 100 mg/kg	i.p.	+5, +35, +95, and +365 min	No protection	Pellegrini-Giampietro *et al.*, 1994
7-Cl-thio-KYN	5-min CCAo	Gerbils	7 days	4 × 15, 25, or 50 mg/kg	i.p.	+5, +35, +95, and +365 min	No protection	Pellegrini-Giampietro *et al.*, 1994
7-Cl-thio-KYN	5-min CCAo	Gerbils	7 days	4 × 100 mg/kg	i.p.	+5, +35, +95, and +365 min	90% protection; 46% in normothermic animals	Pellegrini-Giampietro *et al.*, 1994
ACEA 1011	90-min MCAo	Wistar rats	96 h	3 × 3 mg/kg	i.p.	−55, +90, and +180 min	No protection	Warner *et al.*, 1995
ACEA 1021	per. MCAo + 2-h ipsilateral CCAo	Rats	22 h	10 mg/kg bolus followed by 7 mg/kg/h 22-h infusion	i.v.	0	89% redn. in cortex, 42% subcortical	Marek *et al.*, 1994
ACEA 1021	per. MCAo	SD rats	24 h	?	i.v.	Before ischemia	50% redn.	Pietra *et al.*, 1996
ACEA 1021	per. MCAo	SD rats	24 h	?	i.v.	+1 and +3 h	No protection	Pietra *et al.*, 1996
ACEA 1021	per. MCAo	Rats	28 days	40 mg/kg i.v. bolus and 40 mg/kg i.p.	i.v./i.p.	0, and +2 h	47% redn.	Sauer *et al.*, 1995
ACEA 1021	per. MCAo	Rats	28 days	40 mg/kg	i.v.	0 and +2 h	47% redn.	Sauer *et al.*, 1995
ACEA 1021	per. MCAo	Rats	28 days	40 mg/kg	i.v.	0	No protection	Sauer *et al.*, 1995
ACEA 1021	75-min MCAo	Wistar rats	7 days	5 mg/kg bolus and 3.5 mg/kg/h infusion for 24 h	i.v.	+10 min	40% redn.	Takaoka *et al.*, 1997b
ACEA 1021	75-min MCAo	Wistar rats	7 days	10 mg/kg bolus and 7 mg/kg/h infusion for 24 h	i.v.	+10 min	40% redn.	Takaoka *et al.*, 1997b
ACEA 1021	90-min MCAo	Wistar rats	96 h	3 × 10 mg/kg	i.p.	−55, +90, and +180 min	50% redn.	Warner *et al.*, 1995
ACEA 1021	90-min MCAo	Wistar rats	96 h	3 × 30 mg/kg	i.p.	−55, +90, and +180 min	75% redn.	Warner *et al.*, 1995
ACEA 1021	5-min CCAo + hypot.	SD rats	5 days	10 mg/kg	i.p.	−55, +90, and +180 min	No protection	Warner *et al.*, 1995
ACEA 1021	5-min CCAo + hypot.	SD rats	5 days	30 mg/kg	i.p.	−55, +90, and +180 min	No protection	Warner *et al.*, 1995
ACEA 1031	90-min MCAo	Wistar rats	96 h	3 × 30 mg/kg	i.p.	−55, +90, and +180 min	59% redn.	Warner *et al.*, 1995
ACEA 1031	90-min MCAo	Wistar rats	96 h	3 × 30 mg/kg	i.p.	−55, +90, and +180 min	61% redn.	Warner *et al.*, 1995
ACEA 1031	5-min CCAo + hypot.	SD rats	5 days	10 mg/kg	i.p.	−55, +90, and +180 min	No protection	Warner *et al.*, 1995
ACEA 1031	5-min CCAo + hypot.	SD rats	5 days	30 mg/kg	i.p.	−55, +90, and +180 min	No protection	Warner *et al.*, 1995
ACPC	20-min CCAo	Gerbils	7 days	300 mg/kg	i.p.	+5 min	90% redn.	Fossom *et al.*, 1995b
ACPC	20-min CCAo	Gerbils	7 days	150 mg/kg	i.p.	+5 min	?	Fossom *et al.*, 1995b
ACPC	20-min CCAo	Gerbils	7 days	600 mg/kg	i.p.	+5 min	?	Fossom *et al.*, 1995b
ACPC	20-min CCAo	Gerbils	7 days	300 mg/kg	i.p.	+30 min	No protection	Fossom *et al.*, 1995b
ACPC	20-min CCAo	Gerbils	7 days	300 mg/kg	i.p.	−7, −6, −5, −4, −3, and −2 days	Significant protection?	Von Lubitz *et al.*, 1992
ACPC	20-min CCAo	Gerbils	7 days	300 mg/kg	i.p.	−7, −6, −5, −4, −3, and −2 days and −30 min	Significant protection?	Von Lubitz *et al.*, 1992
GV 150526A	per. MCAo	SD rats	24 h	0.3 mg/kg bolus	i.v.	+1 h	Trend to reduce but NS	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	1 mg/kg bolus	i.v.	+1 h	Trend to reduce but NS	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	3 mg/kg bolus	i.v.	+1 h	81% redn.	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	0.3 mg/kg bolus	i.v.	+3 h	Trend to reduce but NS	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	1 mg/kg bolus	i.v.	+3 h	Trend to reduce but NS	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	3 mg/kg bolus	i.v.	+3 h	65% redn.	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	1 mg/kg bolus	i.v.	+6 h	Trend to reduce but NS	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	3 mg/kg bolus	i.v.	+6 h	42% redn.	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	10 mg/kg bolus	i.v.	+6 h	68% redn.	Bordi *et al.*, 1997
GV 150526A	per. MCAo	SD rats	24 h	3 and 10 mg/kg	i.v.	+1 and +3 h	>50% redn.	Pietra *et al.*, 1996
GV 150526A	per. MCAo	SD rats	24 h	3 and 10 mg/kg	i.v.	6 h	>50% redn.	Pietra *et al.*, 1996
HA-966	10-min CCAo	Gerbils	?	10 mg/kg	?	?	?	Patel *et al.*, 1989
HA-966	per. MCAo	Mice	24 h	20 μg	i.c.v.	−5 min	20% redn.	Verrecchia *et al.*, 1995
HA-966	per. MCAo	Mice	24 h	40 μg	i.c.v.	−5 min	40% redn.	Verrecchia *et al.*, 1995
I-2-CA	5-min CCAo	Gerbils	7 days	60 mg/kg	i.p.	−30 min	No protection	Hood *et al.*, 1992
I-2-CA	5-min CCAo	Gerbils	7 days	300 mg/kg	i.p.	−30 min	>2-fold increase in surviving neurons	Hood *et al.*, 1992
L-687,414	per. MCAo	SD rats	4 h	17.6 mg/kg bolus	i.v.	0	No protection	Gill *et al.*, 1995
L-687,414	per. MCAo	SD rats	4 h	7 mg/kg bolus followed by 7 mg/kg/h 4-h infusion	i.v.	0	No protection	Gill *et al.*, 1995

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TABLE 9
Continued.

Compound studied	Ischemia model[a]	Animals	Analysis period	Dose	Route	Time of administration[b]	Infarction volume	References
L-687,414	per. MCAo	SD rats	4 h	14 mg/kg bolus followed by 14 mg/kg/h 4-h infusion	i.v.	0	34% redn.	Gill *et al.*, 1995
L-687,414	per. MCAo	SD rats	4 h	30 mg/kg bolus followed by 30 mg/kg/h 4-h infusion	i.v.	0	20% redn.	Gill *et al.*, 1995
MRZ 2/570	3-min CCAo	Gerbils	14 days	3 × 30 mg/kg	i.p.	+15, +30, and +45 min	40% redn.	Lazarewicz *et al.*, 1997
MRZ 2/576	3-min CCAo	Gerbils	14 days	3 × 30 mg/kg	i.p.	+15, +30, and +45 min	40% redn.	Lazarewicz *et al.*, 1997
SM-18400	per. MCAo	Rats	24 h	15 mg/kg bolus followed by 3 mg/kg/h 22-h infusion	i.v.	+2 h	72% redn. in cortex and 27% redn. in striatum	Tanaka *et al.*, 1995
SM-18400	per. MCAo	Rats	24 h	7.5 mg/kg bolus followed by 1.5 mg/kg/h 22-h infusion	i.v.	+2 h	42% redn. in cortex	Tanaka *et al.*, 1995
SM-18400	per. CCAo	SHR rats	3 h	?	i.v.	0 and +2 h	Edema redn.	Yasuda *et al.*, 1995
SM-18400	? min MCAo + per. ipsilateral CCAo	SD rats	?	? mg/kg bolus followed by ? mg/kg/h 25-h infusion	i.v.	+1 h	?	Yasuda *et al.*, 1995
ZD9379	per. MCAo	Rats	?	1 mg/kg bolus and infusion of 1 mg/kg/h for ?	i.v.	+30 min	43% redn.	Takano *et al.*, 1997
ZD9379	per. MCAo	Rats	?	5 mg/kg bolus and infusion of 5 mg/kg/h for ?	i.v.	+30 min	51% redn.	Takano *et al.*, 1997
ZD9379	MCAo + ipsilateral CCAo	Rats	6 h	10 mg/kg bolus and infusion of 2.5 mg/kg/h for 4 h	i.v.	0	40% redn., magnetic resonance microscopy	Qiu *et al.*, 1997
ZD9379	per. MCAo	Rats	?	10 mg/kg bolus and infusion of 10 mg/kg/h for ?	i.v.	+30 min	44% redn.	Takano *et al.*, 1997

[a] per., permanent; hypot., hypothermia; CCAo, common cerebral artery occlusion; SD, Sprague Dawley; redn., reduction; ?, not known/not stated in the cited publication; 7-Cl-thio-KYN, 7-chlorothiokynurenic acid; I-2-CA, indole-2-carboxylic acid.
[b] The time of treatment is given in relation to occlusion initiation (i.e., −, before; +, after).

striatum; it was also observed in neurological testing (Takaoka *et al.*, 1997a). This neuroprotective effect was not accompanied by a direct current potential shift (unfiltered change in field potential) (Takaoka *et al.*, 1997b). These studies indicate that stimulation of the glycine site could play a role in the brain damage observed in the MCAo model of ischemia, although from the therapeutic perspective a 10-min treatment delay is probably much too short.

In contrast, in a forebrain ischemia model in rats, ACEA 1021 had no effect on delayed neuronal necrosis in the hippocampal CA1 region, neocortex, or caudate putamen (Warner *et al.*, 1995). At the highest dose (30 mg/kg) of ACEA 1021, an increase in anesthesia duration was observed; with ACEA 1031, sedation, ataxia, and an increase in mortality rates were seen (Warner *et al.*, 1995).

Both pre- and postinjury treatment with ACEA 1021 significantly reduced hemispheric ischemic damage produced by subdural hematoma (26 to 39% reduction in infarction size) (Tsuchida and Bullock, 1995) (table 9). It is noteworthy that 15 mg/kg (administered i.v.) produced strong hypotension and death (Tsuchida and Bullock, 1995). ACEA 1021 was recently reported to block both the glycine$_B$ site and AMPA receptors in vivo, and this combined action was claimed to contribute to its neuroprotective activity (Woodward *et al.*, 1995a).

In a permanent MCAo model, ZD9379 [7-chloro-2,3-dihydro-2-(4-methoxy-2-methylphenyl)pyridazino[4,5b]quinoline-1,4,10(5H)-trione] injected as an initial bolus 30 min after occlusion, followed by infusion for 4 h, produced significant protection (43 to 51%) (Takano *et al.*, 1997) (table 9). ZD9379 was also effective against the development of stroke volume, as assessed by three-dimensional, diffusion-weighted, magnetic resonance microscopy after 2.5 and 6 h of MCAo (Qiu *et al.*, 1997).

In a permanent distal MCAo model of focal ischemia, the glycine$_B$ site full antagonist GV 150526A (3 mg/kg) showed neuroprotection even when administered as late as 6 h after ischemia (Bordi *et al.*, 1997). This dose was more effective when given at shorter intervals after ischemia (table 9). No changes in blood parameters or body temperature were observed (Bordi *et al.*, 1997).

In conclusion, although there are only limited data on the effects of systemically active glycine$_B$ antagonists in animal models of ischemia and trauma, recent data obtained with compounds such as GV 150526A seem very promising. Taken together with in vitro data and the predicted superiority of this class of agents, it seems likely that glycine$_B$ antagonists will find therapeutic use as neuroprotective agents in the near future.

N. Other Neurodegenerative Diseases

In amyotrophic lateral sclerosis, a deficit in glutamate transporter 1 has been reported (Rothstein, 1995), implicating increased glutamate levels as a causative factor in this disorder. In addition, mRNA for the glycine

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carrier GLYT1 (which may be colocalized with NMDA receptors) seems to be decreased in the ventral spinal region of patients with amyotrophic lateral sclerosis (Virgo and Debelleroche, 1995), supporting the involvement of excitotoxicity in this disease.

The glycine$_B$ antagonist 7-Cl-KYN also protects cultured neurons against injury produced by the human immunodeficiency virus envelope protein gp120 (Lipton, 1992). Indeed, there are indications that gp120 might actually stimulate NMDA receptors via the glycine$_B$ site. In cultured hippocampal neurons, gp120 overcame the inhibition of NMDA-induced GABA release by the glycine$_B$ antagonist 5,7-diCl-KYN (Fontana *et al.*, 1997).

VII. First Clinical Experiences with Glycine$_B$ Antagonists

The partial agonist ACPC (Symphony Pharmaceuticals) (tables 8 and 9) reached phase I of clinical trials and is being developed for treatment of stroke and depression. In the former case, a single i.v. dose of 10 or 20 mg/kg was used (Maccecchini, 1997). Pharmacokinetic studies revealed that this compound is readily distributed in the body, has a half-life of 5.9 h, and is not metabolized (Maccecchini, 1997). No adverse effects were reported at the doses tested.

Another antagonist, ACEA 1021, has a half-life of 6 h in humans. This is a very interesting observation because in rodents the half-life was much shorter (approximately 30 min) (Baron *et al.*, 1997), pointing out that unsatisfactory pharmacokinetic features in animal models are not always predictive of the parameters in humans. Recently, codevelopment with Novartis was terminated because of drug crystals appearing in the urine of patients in a clinical study (Hughes, 1997), indicating the possibility of renal toxicity. For further studies, CoCensys is planning to test a combination of ACEA 1021 with probenecid, which (through inhibition of the renal organic acid transporter) would slow renal excretion and possibly reduce nephrotoxicity. Apart from this, in phase I studies ACEA 1021 administered i.v. produced no serious side effects at up to 2 mg/kg/15 min; only sedation, dizziness, and nausea were noted (Yenari *et al.*, 1997). ACEA 1021 (without probenecid) is presently in phase III clinical trials for treatment of stroke and in phase I trials for treatment of head injuries (table 8).

ZD9379 is under development by Zeneca as a treatment for stroke and pain (table 8), but no details of the ongoing clinical trials are available. GV 150526 (Glaxo Wellcome) was recently tested in patients after strokes (phase III trials) (table 8), and the results are expected soon. Its analogue GV 196771A is currently being developed for treatment of chronic pain (phase I) (table 8).

The reports discussed above indicate that there are several interesting glycine$_B$ antagonists under development as neuroprotective agents (table 8). There is a strong mechanistic basis for thinking that this indication (neuroprotection) is probably the most promising for this group of compounds.

VIII. Concluding Remarks

1. The term "glycine$_B$" is proposed as an abbreviation for the strychnine-insensitive glycine site of the NMDA receptor complex, as opposed to the strychnine-sensitive glycine$_A$ site that is coupled with chloride channels.
2. The glycine$_B$ site is necessary for NMDA receptor activation; hence, the term coagonist seems appropriate.
3. The evidence presented indicates that glycine$_B$ sites of NMDA receptors are not always saturated in vivo. This suggests the glycine$_B$ site as an attractive target for drug development, where both full antagonists and partial agonists might find therapeutic applications.
4. Glycine$_B$ ligands show differences in affinities and intrinsic activities with various subtypes of NMDA receptors.
5. Preclinical evidence suggests that glycine$_B$ antagonists are most likely to find clinical use in the treatment of chronic pain, drug abuse, and tolerance and as neuroprotectants.

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