³HCGP 61594: the first photoaffinity ligand for a structural analysis and differentiation of the glycine antagonist site of NMDA receptor subtypes

Author(s):
Honer, Michael

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$[^3]H\text{CGP 61594}$: the first photoaffinity ligand for a structural analysis and differentiation of the glycine antagonist site of NMDA receptor subtypes

A dissertation submitted to the
Swiss Federal Institute of Technology Zurich
for the degree of
Doctor of Natural Sciences

presented by

Michael Honer
Dipl. Natw. ETH Zürich
born February 7th, 1968
citizen of Germany

accepted on the recommendation of

Prof. Dr. H. Möhler, examiner
Prof. Dr. A. Beck-Sickinger, co-examiner
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Summary

Glutamatergic neurotransmission via NMDA receptors is implicated in the pathophysiology of various CNS disorders (e.g. stroke, Parkinson’s disease, schizophrenia). For the treatment of these diseases the glycine binding site of NMDA receptors is of considerable therapeutic interest since glycine site antagonists offer advantages over other types of NMDA receptor antagonists in terms of their side effect profile. Further improvements are expected from the targeting of distinct NMDA receptor subtypes. As a tool for the investigation of the structural determinants of the glycine binding domain and for the identification of a putative subtype-selective interaction, a novel glycine antagonist and photoaffinity ligand was developed, termed $[^3H]CGP$ 61594.

The first part of the study provides an extensive characterization of $[^3H]CGP$ 61594 binding to crude brain membranes demonstrating that $[^3H]CGP$ 61594 interacts selectively and with high affinity with the glycine site of native NMDA receptors.

In the second part of the study, the glycine antagonist binding domain was directly targeted by a photolabeling approach for the first time. As a photoaffinity ligand $[^3H]CGP$ 61594 identifies the NR1 subunit of native and recombinant NMDA receptors as the main constituent of the glycine antagonist binding domain.

The third part of the study comprises a detailed analysis of the interaction of $[^3H]CGP$ 61594 with various native and recombinant NMDA receptor subtypes. High affinity binding sites for $[^3H]CGP$ 61594 are exclusively displayed by NR1/2B receptors whereas the NMDA receptor subtypes NR1/2A, NR1/2C and NR1/2D are clearly distinguished by reduced affinities. Thus, a heterogeneity of the glycine antagonist site among NMDA receptor subtypes is identified for the first time. This finding disproves the previous view that glycine antagonists interact with domains on the NR1 subunit that are insensitive to modulation by adjacent NR2 subunit variants. The subtype-specific interaction of $[^3H]CGP$ 61594 might be of great importance for the development of future glycine antagonists of the NMDA receptor since such agents are expected to be neuroprotective but less prone to side effects than presently available NMDA receptor antagonists.
Zusammenfassung


Im dritten Teil der Arbeit wurde die Interaktion dieses neuen Liganden mit verschiedenen Subtypen nativer und rekombinanter NMDA-Rezeptoren im Detail analysiert. Dabei wurde festgestellt, dass nur der Rezeptor-Subtyp NR1/2B eine hohe Affinität für CGP 61594 besitzt, während die Subtypen NR1/2A, NR1/2C und NR1/2D eine deutlich geringere Affinität aufweisen. Somit konnte zum ersten Mal eine Heterogenität der Bindungstelle für Glycin-Antagonisten in verschiedenen NMDA-Rezeptor-Subtypen identifiziert werden. Der Nachweis der selektiven Bindung eines Glycin-Antagonisten an einen NMDA-Rezeptor-Subtyp könnte für die zukünftige Medikamentenentwicklung
1. Introduction

1.1 Excitatory amino acid receptors

Glutamate is the most important excitatory neurotransmitter in the mammalian central nervous system (CNS). Nearly all excitatory neurons are glutamatergic, and it is estimated that over half of all brain synapses release this amino acid. Glutamate exerts its effects by binding to two different classes of glutamate receptors, metabotropic and ionotropic receptors. Metabotropic glutamate receptors are coupled to G-proteins and modulate intracellular second messenger systems (Pin and Duvoisin, 1995) whereas ionotropic glutamate receptors are the principle mediators of excitatory neurotransmission and represent ligand-gated cation channels permeable to Na⁺, K⁺ and Ca²⁺. The ionotropic receptor family is further subdivided into three major classes based upon pharmacological and electrophysiological data. They are named according to their selective agonists which structurally resemble glutamate but do not occur naturally within the brain: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) (Bettler and Mulle, 1995; Hollmann and Heinemann, 1994; Mori and Mishina, 1995).

AMPA receptors are assembled from four homologous subunits (GluR1-GluR4) in varying combinations to form functional receptor subtypes. The types of subunits present in receptor complexes determine their biophysical and pharmacological properties. AMPA receptors are found in most excitatory synapses and mediate the majority of fast excitatory neurotransmission. They show fast onset and offset kinetics, a rapid desensitization time course and a very low permeability for Ca²⁺ (Bettler and Mulle, 1995; Hollmann and Heinemann, 1994).

The role of kainate receptors in excitatory neurotransmission is less clear, although low and high affinity kainate receptors (GluR5-GluR7 and KA1/KA2, respectively) have been cloned and characterized. Kainate is thought to specifically activate both presynaptic and postsynaptic receptors. Presynaptically, kainate inhibits the release of the inhibitory neurotransmitter GABA (Clarke et al., 1997) whereas activation of postsynaptic kainate receptors leads to excitatory synaptic currents (Castillo et al., 1997; Vignes and Collingridge, 1997).
The NMDA receptors differ from both AMPA and kainate receptors in four fundamental ways: (1) They possess a considerably higher Ca\(^{2+}\) permeability (Burnashev et al., 1995; Schnegggenburger et al., 1993). (2) Their evoked excitatory postsynaptic currents have much slower onset and offset kinetics (McBain and Mayer, 1994; Monyer et al., 1992). (3) The NMDA receptor is the only member of the family of ligand-gated ion channels that requires the occupation of two distinct ligand recognition sites by a principal neurotransmitter (glutamate) and a 'co-agonist' (glycine) for receptor activation (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). (4) NMDA receptor function is voltage-dependent owing to a Mg\(^{2+}\) block of the channel pore at resting potential. This channel inhibition is only relieved when the postsynaptic membrane is sufficiently depolarized by AMPA/kainate receptors or other excitatory inputs to release the Mg\(^{2+}\) ion (Fig. 1) (Ascher and Nowak, 1988; Johnson and Ascher, 1990; Nowak et al., 1984). Consequently, NMDA receptor activation depends on the coincidence of presynaptic activity (neurotransmitter release) and postsynaptic activity (membrane depolarization). According to the Hebbian concept, synaptic connections are modified upon
simultaneous presynaptic and postsynaptic activity (Hebb, 1949) and the unique features of NMDA receptors make them coincidence detectors at Hebbian synapses (Seeburg et al., 1995).

1.2 Molecular biology of NMDA receptors

1.2.1 Cloning of NMDA receptor subunits

Molecular cloning revealed a striking structural heterogeneity of NMDA receptors by the identification of several subunit classes: NR1, NR2 (NR2A-NR2D) and NR3A (also termed NMDAR-L or \( \chi \)-1) (Ciabarra et al., 1995; Das et al., 1998; Hollmann and Heinemann, 1994; Ikeda et al., 1992; Ishii et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992; Moriyoshi et al., 1991; Sucher et al., 1995). The NR1 subunit shares 21–29% amino acid sequence identity with the four types of NR2 subunits (NR2A-NR2D), the NR3A subunit as well as with the AMPA/kainate receptor subunits.

Further molecular diversity of the NMDA receptor is generated by alternative splicing of the NR1 subunit mRNA (Anantheram et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Nakanishi, 1992; Sugihara et al., 1992). Differential splicing of three short exons gives rise to eight NR1 splice variants. The alternatively spliced exons encode a 21 amino acid sequence on the N-terminal region (termed N1) and adjacent sequences of 37 and 38 amino acids at the very C-terminus (termed C1 and C2, respectively) (Fig. 2) (Zükin and Bennett, 1995). Among the NR2 subunits, alternative mRNA splicing has been proposed to generate two different splice variants of the NR2D subunit (NR2D-1 and NR2D-2) (Ikeda et al., 1992). However, the NR2D-1 variant does not seem to be expressed in the brain (Wenzel et al., 1996).

1.2.2 Topology of NMDA receptors

The structural organization of glutamate receptor subunits is conserved throughout the NMDA, AMPA and kainate receptor classes. Features of this structure include a large extracellular N-terminal domain that contributes to ligand binding, four hydrophobic membrane domains (M1-M4) and a C-terminal regulatory domain (Fig. 2) (Hollmann, 1997; Wo and Oswald, 1995). The M2 domain is thought to be involved in the formation of the channel pore since a conserved asparagine within M2 of all NMDA receptor subunits (N598 in NR1) is critical for the Mg\(^{2+}\) block of the NMDA receptor channel (Burnashev et al., 1996).
The intracellular C-terminus of NMDA receptor subunits displays the highest degree of sequence diversity and is considerably larger for the NR2 subunits compared to the NR1 subunit. The current topology model of the NMDA receptor subunits implies three transmembrane segments (M1, M3 and M4), a re-entrant membrane loop (M2) and an extracellular localization of the loop connecting M3 and M4 (Fig. 2). This model is consistent with recent studies of the transmembrane topology of mammalian AMPA and kainate receptor subunits (Bennett and Dingledine, 1995; Hollmann and Heinemann, 1994; Kuusinen et al., 1995; Stern-Bach et al., 1994), but differs from the originally proposed topology with four transmembrane-spanning segments derived by analogy from the prototypical ligand-gated ion channel, the nicotinic acetylcholine receptor (Stroud et al., 1990). Definite evidence for the novel model with an extracellular M3-M4 loop was provided by epitope tagging (Hirai et al., 1996) as well as by introduction of a novel N-linked glycosylation site in this loop (Wood et al., 1997).

1.2.3 Stoichiometry of NMDA receptors

Experimental studies have clearly indicated that NMDA receptors exist as heteromeric complexes of NR1 and NR2 subunits (Buller et al., 1994; Chazot et al., 1994; Ishii et al., 1993; Laurie and Seeburg, 1994; Lynch et al., 1994; Priestley et al., 1995; Stern et al., 1992). The presence of two copies of NR1 subunits in functional NR1/2A receptor complexes was revealed by co-expression of wild-type (high conductance) and mutant (low conductance) NR1 subunits since an intermediate conductance state was produced (Béhé et al., 1995). In addition, two different types of NR1 splice variants were shown to be potentially part of the same NMDA receptor complex (Blahos and Wenthold, 1996). For the NR2 subunits, immunoprecipitation studies of NMDA receptors indicated that the NR1 subunit can be assembled with two different types of NR2 subunits (Chazot et al., 1994; Didier et al., 1995; Dunah et al., 1998; Luo et al., 1997; Sheng et al., 1994; Wafford et al., 1993). The exact stoichiometry of NMDA receptors is still a matter of debate and many reports produced contradictory data. A pentameric structure is supported by biochemical crosslinking experiments (Brose et al., 1993) as well as initial electrophysiological studies (Ferrermontiel and Montal, 1996; Premkumar and Auerbach, 1997), the latter pointing to the presence of three NR1 subunits in a receptor complex. In contrast, recent electrophysiological approaches provided
Fig. 2: Topology of NMDA receptor subunits.
The current topology model of the NMDA receptor subunits NR1 and NR2 implies a large extracellular N-terminal domain of approximately 500 amino acids, three transmembrane segments (M1, M3 and M4) and a re-entrant membrane loop (M2) as well as an extracellular localization of the 170 amino acid loop connecting M3 and M4. A conserved asparagine residue within M2 of all NMDA receptor subunits (N598 in NR1) is critical for the Mg\(^{2+}\) block of the NMDA receptor channel. The intracellular C-terminus of NMDA receptor subunits displays the highest degree of sequence diversity and is considerably larger for the NR2 subunits compared to the NR1 subunit. Three alternatively spliced exons of the NR1 subunit gene encode a 21 amino acid sequence on the N-terminal region (termed N1) and adjacent sequences of 37 and 38 amino acids at the very C-terminus (termed C1 and C2, respectively).
strong evidence for a tetrameric structure of recombinant NMDA receptors with a copy number of two for NR1 and NR2 subunits (Laube et al., 1998; Rosenmund et al., 1998). It remains yet to be determined whether this result can be extrapolated to native NMDA receptors.

1.2.4 Requirements for functional NMDA receptors

Fundamental properties of native NMDA receptors include a high Ca\(^{2+}\) permeability, a voltage-dependent channel blockade by Mg\(^{2+}\) and a cooperative channel gating by the primary neurotransmitter glutamate and the co-agonist glycine (Hollmann and Heinemann, 1994; Mori and Mishina, 1995; Seeburg et al., 1995; Sucher et al., 1996). Injection of NR1 subunit cRNA in Xenopus oocytes led to the formation of NMDA-gated channels which possess these basic features (Durand et al., 1993; Hollmann et al., 1993; Moriyoshi et al., 1991; Zheng et al., 1994). However, homomeric NR1 receptor channels formed with low efficiency and displayed only small agonist-induced responses. In addition, they exhibited unusual properties not detectable in native NMDA receptors, such as a potentiation by micromolar concentrations of Zn\(^{2+}\) (Hollmann et al., 1993) and a resistance to redox modulation (Sullivan et al., 1994). In contrast to Xenopus oocytes, expression studies in mammalian cells revealed that homomeric NR1 receptors fail to form functional channels (Chazot et al., 1992; Grimwood et al., 1995). Only the co-expression of the NR1 subunit with at least one type of NR2 subunit led to the expression of fully functional receptors in both expression systems and to efficient targeting of receptor complexes to the plasma membrane (Boeckman and Aizenman, 1994; Ishii et al., 1993; McIlhinney et al., 1996; Meguro et al., 1992; Monyer et al., 1992; Priestley et al., 1995). It was therefore suggested that endogenous NR2-like proteins, which might be present in Xenopus oocytes but not in mammalian cells, are responsible for the assembly of functional NMDA receptor channels in the Xenopus oocyte expression system (Sucher et al., 1996).

Recent ligand binding studies on recombinant homomeric NR1 and NR2 receptors pointed to a structural segregation of glutamate and glycine binding sites. Homomeric NR1 receptors contained high affinity binding sites for glycine agonists and antagonist (Grimwood et al., 1995; Laurie and Seeburg, 1994; Lynch et al., 1994; Moriyoshi et al., 1991; Siegel et al., 1996) whereas homomeric NR2A receptors displayed specific high affinity binding sites for glutamate and NMDA (Kendrick et al., 1996). Furthermore, extensive site-
directed mutagenesis analysis identified regions on the NR1 and NR2B subunits that are involved in the formation of the glycine and glutamate binding site, respectively (Kuryatov et al., 1994; Laube et al., 1997). Therefore, the glycine binding pocket appears to be formed primarily by the NR1 subunit and the glutamate binding pocket by the NR2 subunits (Laube et al., 1997) underlining the necessity of NR1 and NR2 subunits for the formation of functional NMDA receptors.

In contrast to the NR2 subunits, co-expression of the NR1 subunit with the NR3A subunit gives rise to unusual receptors with decreased NMDA-evoked currents. Receptors containing this subunit are suggested to play a role only during brain development (Das et al., 1998).

1.2.5 Heterogeneity of NMDA receptors
The combinatorial assembly of the NR1 subunit with at least one of the different types of NR2 subunits (NR2A-NR2D) gives rise to receptor subtypes with distinct pharmacological and electrophysiological properties (Buller et al., 1994; Chazot et al., 1994; Ishii et al., 1993; Laurie and Seeburg, 1994; Lynch et al., 1994; Priestley et al., 1995; Stern et al., 1992). For example, recombinant NR1/2B receptors show a higher affinity for glutamate and NMDA than NR1/2A receptors, but a lower affinity for glutamate antagonists (Buller et al., 1994; Kutsuwada et al., 1992; Laurie and Seeburg, 1994). Furthermore, the various recombinant NMDA receptor subtypes differ in their electrophysiological properties such as conductance levels, decay time constants and Mg$^{2+}$ sensitivity (Kuner and Schoepfer, 1996; Kutsuwada et al., 1992; Monyer et al., 1994; Monyer et al., 1992; Stern et al., 1992; Wagner and Leonard, 1996; Wyllie et al., 1996).

In general, these characteristics of individual recombinant NMDA receptor subtypes were found to resemble those observed in radioligand binding experiments or single channel analysis of distinct NMDA receptor subtypes in situ (Beaton et al., 1992; Buller et al., 1994; Farrant et al., 1994; Gibb and Colquhoun, 1992; Momiyama et al., 1996; Monaghan et al., 1988; Widdowson et al., 1995). Native NMDA receptor subtypes containing different NR2 subunits display characteristic expression profiles during development and in the adult brain (Benke et al., 1995; Monyer et al., 1994; Wenzel et al., 1997; Wenzel et al., 1995) and are thus thought to fulfil distinct physiological functions. For instance, only the NMDA receptor subtypes NR1/2B and NR1/2D are expressed in
embryonic and perinatal brain, whereas NR1/2A and NR1/2C subtypes are exclusively expressed postnatally, suggesting that the different subtypes are necessary to meet the specific requirements of the developing and mature neuronal circuitries, respectively. Likewise, in the adult brain, each NMDA receptor subtype shows a unique pattern of distribution. The NR1/2A subtype is expressed almost ubiquitously while the expression of the NR1/2B, NR1/2C and NR1/2D subtypes is much more restricted to specific brain regions where they are thought to contribute to distinct neuronal circuits and to subserve different functions. These findings imply that the observed regional, physiological and pharmacological heterogeneity of NMDA receptors in situ can be attributed to distinct receptor subtypes characterized by the differential inclusion of the four NR2 subunits.

1.3 Function of NMDA receptors and receptor subtypes

The NMDA receptor is involved in complex physiological functions based on its characteristic properties. NMDA receptor channels are activated slowly and remain open for a comparatively long time. This slow time course provides the synapse with the memory of its recent activation and allows the summation of responses to events tens of milliseconds apart. Moreover, the voltage-dependent channel blockade by extracellular Mg$^{2+}$ renders the channel inoperative when the postsynaptic membrane is in a resting state. Thus, activation only takes place when the membrane is sufficiently depolarized by AMPA/kainate receptors or other excitatory inputs to release the Mg$^{2+}$ block. Finally, NMDA receptors are highly permeable to Ca$^{2+}$ which provokes a massive influx of Ca$^{2+}$ into the postsynaptic cell upon receptor stimulation. The increase in intracellular Ca$^{2+}$ then triggers a complex series of molecular events, for example the activation of downstream signal transduction cascades such as the mitogen activating protein kinase (MAPK) pathway (Bading et al., 1993; English and Sweatt, 1997; Xia et al., 1996).

The rise in the intracellular Ca$^{2+}$ concentration can finally result in long-term changes in synaptic strength. It is generally agreed that Ca$^{2+}$ flux through NMDA receptor channels during high frequency electrical stimulation enhances synaptic transmission and hence plays a crucial role in the induction of long-term potentiation (LTP), a putative mechanism of memory storage in the CA1 region of the hippocampus (Collingridge and Singer, 1990; Kullmann and...
Siegelbaum, 1995; Malenka, 1994; Malenka and Nicoll, 1993; Rison and Stanton, 1995). The degree of receptor activation is thought to determine the threshold for LTP induction (Malenka, 1991) and the amount of Ca\textsuperscript{2+} flow through the channel may regulate the magnitude and direction of changes in synaptic efficacy (Cummings et al., 1996; Perkel et al., 1993). Moreover, Ca\textsuperscript{2+} influx through NMDA receptors is considered to play a key role in the use-dependent strengthening and stabilization of synaptic connections during CNS development (Constantine-Paton et al., 1990; Hahm et al., 1991; Schnupp et al., 1995; Simon et al., 1992).

The complex physiological function of NMDA receptors is thought to be mediated by distinct receptor subtypes that are characterized by different NR2 subunits. However, the precise functional significance of individual NMDA receptor subtypes is poorly understood and has therefore been approached by gene-targeting techniques. The ablation of the NR1 subunit resulted in neonatal death, the failure to form the whisker-related neural pattern (barelettes) in the brainstem trigeminal complex and in an impairment in synaptic plasticity in the hippocampus (Forrest et al., 1994; Li et al., 1994). Perinatal lethality was also found for NR2B knockout mice (Kutsuwada et al., 1996) indicating that the NMDA receptor subtype NR1/2B is indispensable for postnatal development. Knockout mice devoid of other embryonically expressed subunits (NR2D or NR3A) were viable as were mice lacking the postnatally expressed NR2A and NR2C subunits. Mice with a targeted disruption of the NR2D gene exhibited reduced spontaneous behavioural activity (Ito et al., 1996). NR3A deficient mice were characterized by enhanced NMDA responses and an increase in dendritic spines in early development (Das et al., 1998). NR2A mutant mice showed a higher threshold in LTP and contextual learning (Kiyama et al., 1998; Sakimura et al., 1995) whereas NR2C mutant mice exhibited little overt deficits (Ebralidze et al., 1996; Sprengel et al., 1998). Further advances in the elucidation of the physiological role of different NMDA receptor subtypes are expected from the inducible and region-specific ablation of receptor subunit genes or from the development of subtype-selective receptor blockers.
1.4 Modulation of NMDA receptor function

The activity of NMDA receptor subtypes is modulated by a variety of endogenous ligands (polyamines, protons, Mg$^{2+}$, Zn$^{2+}$), by the phosphorylation state (regulated via kinases and phosphatases) as well as by the redox state (Fig. 3).

Fig. 3: Schematic representation of modulatory sites of the NMDA receptor.
NMDA receptor activation requires the binding of the principle agonist glutamate and the co-agonist glycine to distinct binding sites. A variety of endogenous ligands (polyamines, protons, Mg$^{2+}$, Zn$^{2+}$) and exogenous ligands (ifenprodil, channel blockers) as well as kinases/phosphatases and the redox state allow the modulation of NMDA receptor activity via interaction with specific binding sites. The localization of these binding sites is shown in simplified terms and does not represent the exact localization on a specific subunit.

1.4.1 Polyamine binding site
Polyamines such as spermine and spermidine are endogenous compounds in the brain with largely unknown function (Johnson, 1996). They exert various effects on NMDA receptors including a ‘glycine-dependent stimulation’ which is mediated by an increase in the affinity for glycine (Benveniste and Mayer, 1993;
Sacaan and Johnson, 1989) as well as a ‘glycine-independent stimulation’ that is observed in the presence of saturating glycine concentrations (Lynch et al., 1995; Williams et al., 1990). At higher concentration spermine was found to decrease the affinity for glutamate agonists and to inhibit the NMDA receptor in a voltage-dependent manner which becomes more pronounced at hyperpolarized potentials (Rock and MacDonald, 1992; Williams et al., 1995). It is therefore supposed that spermine interacts with different binding sites on the NMDA receptor. Interestingly, the effects of spermine are dependent on both the type of NR1 splice variant and the NR2 subunit composition of the receptor. For example, spermine exerts no effect on NMDA receptors containing the NR2C or NR2D subunit whereas NR2A-containing receptors exhibit only the ‘glycine-dependent stimulation’ and the voltage-dependent block (Kashiwagi et al., 1996; Williams et al., 1994). All effects are seen in recombinant receptors containing the NR2B subunit. However, only NR1/2B receptors which lack the splice cassette N1 in the N-terminus of the NR1 subunit exhibit a ‘glycine-independent potentiation’ by spermine (Durand et al., 1993; Gallagher et al., 1996).

1.4.2 Proton binding site
The ion current mediated by NMDA receptors is sensitive to protons in a subunit-specific manner. Like stimulation by polyamines, inhibition by protons depends on the absence or presence of the N1 cassette of the NR1 subunit. Splice variants containing the N1 cassette have an EC50 for proton inhibition of pH 7.3 whereas splice variants lacking this insert are characterized by a reduced proton sensitivity (EC50 = pH of 6.8) (Traynelis et al., 1995). Mutational analyses suggested that protons and polyamines share common determinants on NR2 subunits and that the mechanism of polyamine stimulation is linked to a relief of tonic receptor inhibition by protons (Gallagher et al., 1997; Traynelis et al., 1995) which is thought to occur at physiological pH (Traynelis and Cull-Candy, 1991; Traynelis and Cull-Candy, 1990). Furthermore, the extracellular acidity that accompanies brain hypoxia/ischaemia is known to reduce NMDA receptor-mediated currents (Tang et al., 1990) and neurotoxicity (Kaku et al., 1993). Thus, the proton sensitivity of the NMDA receptor may provide a negative feedback mechanism on receptor function (Tombaugh and Sapolsky, 1990; Traynelis and Cull-Candy, 1991; Traynelis and Cull-Candy, 1990).
1.4.3 Zn\textsuperscript{2+} binding site

Zn\textsuperscript{2+} is stored in synaptic vesicles in a number of glutamatergic terminals and is released during synaptic activity (Frederickson, 1989). Zn\textsuperscript{2+} inhibits NMDA receptor function through both voltage-dependent and voltage-independent mechanisms at two different binding sites (Christine and Choi, 1990; Legendre and Westbrook, 1990). The voltage-dependent effect of Zn\textsuperscript{2+} occurs probably via the same site as the voltage-dependent Mg\textsuperscript{2+} block within the channel pore (Paoletti et al., 1997). An additional high affinity site is located outside the channel pore and causes a voltage-independent inhibition. At this site, receptors containing the NR2A subunit show a much higher affinity for Zn\textsuperscript{2+} than other NR2 subunits (Chen et al., 1997; Paoletti et al., 1997; Traynelis et al., 1998; Williams, 1996). The IC\textsubscript{50}-value for Zn\textsuperscript{2+} at this high affinity site is in the range of the Zn\textsuperscript{2+} concentration present in the cerebrospinal fluid suggesting that NR1/2A receptors are tonically inhibited by extracellular Zn\textsuperscript{2+} (Paoletti et al., 1997, Zheng et al., 1998). Recently, amino acid residues influencing the Zn\textsuperscript{2+} sensitivity were identified on the NR1 subunit and the same residues were found to affect the proton sensitivity. Furthermore, concentrations of spermine which relieve the tonic proton inhibition also provoke a release of the tonic Zn\textsuperscript{2+} inhibition indicating that binding sites for protons, polyamines and Zn\textsuperscript{2+} are linked in some way (Traynelis et al., 1998).

1.4.4 Redox site

Modulation of NMDA receptor-mediated responses by oxidizing and reducing agents has been described in a variety of neuronal preparations (Aizenmann et al., 1989; Lazarewicz et al., 1989; Levy et al., 1990). The redox modulatory site is supposed to consist of two cysteine residues in the NR1 subunit (C744 and C798) which might form a disulfide bond under oxidizing conditions (Sullivan et al., 1994). Under reducing conditions, the opening frequency of NMDA receptors increases (Tang and Aizenman, 1993). Thus, in the presence of a reducing milieu, which occurs for example after stroke (Ginsberg et al., 1976), the extent of NMDA receptor-mediated neurotoxicity is increased (Levy et al., 1990).

1.4.5 Mg\textsuperscript{2+} binding site

NMDA receptor channels are blocked by extracellular Mg\textsuperscript{2+} in a voltage-dependent manner (Mayer et al., 1984; Nowak et al., 1984). There is evidence
for at least two Mg$^{2+}$ binding sites on the NMDA receptor (Burnashev et al., 1992; Premkumar and Auerbach, 1996; Shamma and Stevens, 1996). Mg$^{2+}$ can block the channel from both the outside and the inside, and the two Mg$^{2+}$ binding sites differ in their kinetics and voltage dependence (Johnson and Ascher, 1990). Mg$^{2+}$ additionally interacts with permeant monovalent ions such as Na$^+$ which might explain the paradoxically high degree of voltage dependence of the Mg$^{2+}$ block (Antonov et al., 1998). Several asparagine residues within the M2 segment of NR1 and NR2 subunits were reported to determine Mg$^{2+}$ binding (Burnashev et al., 1992; Kuner and Schoepfer, 1996; Wollmuth et al., 1996). At the narrow constriction of the NMDA receptor channel two adjacent asparagine residues N595 (N-site) and N596 (N+1 site) in the M2 domain of the NR2A subunit and the corresponding asparagine of the NR1 subunit (N598) form a critical binding site for extracellular Mg$^{2+}$ (Wollmuth et al., 1996).

In addition, Mg$^{2+}$ exhibits an allosteric, non-competitive interaction with the binding site for the atypical antagonist ifenprodil (see below) and mediates a 'glycine-independent potentiation' of NMDA-evoked currents, thus mimicking the effects of spermine. This finding supports the view that Mg$^{2+}$ might be the physiological ligand acting at the spermine site (Kew and Kemp, 1998; Paoletti et al., 1995; Traynelis et al., 1995).

1.4.6 Phosphorylation sites

The function of NMDA receptors is regulated by protein phosphorylation at serine or threonine residues by protein kinase C (PKC), protein kinase A (PKA) and calcium/calmodulin-dependent protein kinase II (CaMKII) (Moon et al., 1995; Raymond et al., 1993) as well as at tyrosine residues by the tyrosine kinases Src and Fyn (Köhr and Seeburg, 1996; Wang and Salter, 1994; Wang et al., 1996). Receptor phosphorylation upregulates NMDA receptor function (Kitamura et al., 1993; Lieberman and Mody, 1994; Wang and Salter, 1994) whereas protein phosphatases such as PP1, PP2A and calcineurin reduce NMDA-induced responses (Lieberman and Mody, 1994; Raman et al., 1996; Tong et al., 1995; Wang et al., 1994; Wang and Salter, 1994). PKA and PKC phosphorylate several serine residues at the C-termini of NR1, NR2A and NR2B subunits (Leonard and Hell, 1997; Tingley et al., 1997) while CaMKII phosphorylates a single residue (S1303) in the C-terminal tail of the NR2B subunit (Omkumar et al., 1996). Both NR2A and NR2B subunits are
phosphorylated on tyrosine residues (Lau and Huganir, 1995; Moon et al., 1994). The endogenous tyrosine kinase Src was shown to be associated with the NMDA receptor complex (Yu et al., 1997) and acts only on receptors assembled from NR2A subunits (Köhrl and Seeburg, 1996). Recent evidence suggests that Src-induced potentiation of NMDA receptor-mediated currents is due to the relief of Zn$^{2+}$ inhibition. Src is thought to interact with three tyrosine residues at the C-terminal tail of the NR2A subunit which induces a conformational change preventing the binding of Zn$^{2+}$ on the extracellular side of the receptor (Zheng et al., 1998).

1.5 NMDA receptor pathophysiology and drug development

Ca$^{2+}$ flux through NMDA receptors triggers a variety of downstream signal transduction cascades in the postsynaptic cell, thus representing an important intracellular messenger for neuronal plasticity. However, excessive Ca$^{2+}$ influx into the postsynaptic cell activates many enzymes such as kinases, lipases, proteases, phosphatases or endonucleases which contribute to a series of membrane, cytoplasmatic and nuclear events involved in neurotoxicity (Lipton and Rosenberg, 1994). Overstimulation of NMDA receptors is thought to be a major mechanism for Ca$^{2+}$ overload in neurons, and therefore excessive activation of NMDA receptors mediates neuronal injury and death (Choi, 1994; Dingledine et al., 1990; Olney and Farber, 1995). This condition is termed 'excitotoxicity' and might constitute a final common pathway of diseases with diverse pathophysiological processes (Choi, 1994; Choi, 1987). These diseases range from acute neurological disorders (stroke, head trauma, epilepsy, hypoglycaemic injury) to more chronic neurodegenerative conditions (Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, neuropathic pain and AIDS dementia) (Lipton and Rosenberg, 1994). Furthermore, an imbalance in NMDA receptor-mediated neurotransmission is thought to contribute to certain psychiatric states such as schizophrenia, depression or anxiety disorders (Olney and Farber, 1995; Skolnick et al., 1996; Trullas et al., 1991; Trullas and Skolnick, 1990). However, it is unlikely that a disturbance of glutamatergic neurotransmission via hypo- or hyperactivation of NMDA receptors is the major etiological factor of these diseases per se. NMDA receptors play rather a fundamental role in the execution of neuronal damage produced by other factors. For example, severe
energy deficits are thought to be implicated in the excitotoxic effects of glutamate in acute insults such as ischaemia or hypoxia whereas more mild but continuous malfunctioning of the glutamatergic system might be involved in many chronic neurodegenerative diseases or in psychiatric disorders (Danysz et al., 1995; Rothman and Olney, 1995).

There are several molecular sites for a pharmacological intervention to attenuate glutamate-mediated damage. In view of the universal role of NMDA receptors in both physiological and pathophysiological processes, it seems rational to consider the NMDA receptor as the main drug target for therapeutic agents to normalize glutamatergic function and to protect neurons from excitotoxicity. Major aims in drug development for the NMDA receptor are high affinity ligands with a large bioavailability in the CNS which act in a use-dependent mode to inhibit only the pathological activity of the receptor while sparing physiological neurotransmission. With regard to the profile of side effects of NMDA receptor antagonists, particular improvement is expected from the targeting of specific receptor subtypes since they are largely localized in different neuronal circuitries and probably fulfill distinct physiological functions. Thus, novel agents affecting only specific NMDA receptor subtypes in a use-dependent manner should open new avenues in the pharmacotherapy of a variety of pathological CNS states.

A multitude of synthetic ligands has been developed in the last decade to target the different binding sites of the NMDA receptor for pharmacological intervention such as the primary binding site for the neurotransmitter glutamate, the polyamine-related ifenprodil site, the open channel blocker site and the glycine co-agonist site.

1.5.1 Glutamate site antagonists

In animal models of cerebral ischaemia and epilepsy NMDA receptor blockade by competitive glutamate antagonists was shown to have neuroprotective and anticonvulsant effects (Danysz et al., 1995). Interestingly, glutamate site antagonists such as the archetypal D-AP5 (Davies et al., 1981), CPP (Harris et al., 1986), CGS 19755 (Murphy et al., 1988), CGP 39653 (Sills et al., 1991) and CGP 55802A (Marti et al., 1993) pointed to a heterogeneity of the glutamate recognition domain. For example, the brain distribution of the NMDA-sensitive binding sites for the agonist \(^3H\)glutamate differed from the pattern observed with the NMDA antagonists \(^3H\)CPP (Monaghan et al., 1988), \(^3H\)CGP 39653
(Jaarsma et al., 1993) and CGP 55802A (Marti et al., 1993), which resembled the distribution of the NR2A subunit. These results suggested that only NR1/2A receptors form high affinity binding sites for glutamate antagonists. Indeed, studies on recombinant receptors revealed that $[^3H]$CGP 39653 (Kendrick et al., 1996; Laurie and Seeburg, 1994) and $[^{125}I]$CGP 55802A (Marti et al., 1993) bind with high affinity only to NR1/2A receptors and with considerable lower affinity to other receptor subtypes. A major drawback of competitive glutamate site antagonists lies in the fact that therapeutic doses produced severe side effects such as neuronal vacuolization in the retrosplenial and cingulate cortex (Olney et al., 1991), psychotomimetic-type activity as well as dysfunctions of the glucose metabolism (Hargreaves et al., 1993; Hargreaves et al., 1993) and of the autonomic nervous system leading to elevated heart rate and blood pressure (for review see Muir and Lees, 1995). The preferential targeting of NR1/2A receptors by glutamate site antagonists suggests that this NMDA receptor subtype might be responsible for the severe side effect profile. Nevertheless, novel glutamate antagonists such as CGS 19755, CPPene and MDL-100,453 are in clinical trial for stroke and epilepsy, respectively (Parsons et al., 1998).

1.5.2 Open channel blockers

Various organic compounds of diverse chemical structure inhibit current flow through the NMDA receptor by blocking the channel in a non-competitive and use-dependent manner. These so-called open channel blockers include phencyclidine (PCP), dizocilpine (MK-801), ketamine, dextromethorphan, memantine and amantadine (Collingridge and Lester, 1989; Huettner and Bean, 1988; Yamakura et al., 1993). Photoaffinity labeling experiments using $[^3H]3$-azido-MK-801 labeled a 120 kDa protein which corresponds in size to the NR1 subunit (Sonders et al., 1990), suggesting that the NR1 subunit contains the main structural determinants for this binding site. However, the NR2 subunit variant present in the receptor complex also appears to contribute to the affinities of several channel blockers (Laurie and Seeburg, 1994; Monaghan and Larsen, 1997; Yamakura et al., 1993). Effective doses of open channel blockers were shown to ultimately evoke the same unwanted functional consequences as competitive glutamate site antagonist (Carter, 1994; Rogawski, 1993; Willetts et al., 1990). However, low affinity open channel blockers with fast unblocking kinetics offer a more
favourable therapeutic profile (Kornhuber and Weller, 1997). For example, memantine and amantadine are clinically used in the therapy of dementia and Parkinson's disease at doses having few side effects (Kornhuber et al., 1994; Parsons et al., 1998).

1.5.3 Ifenprodil and related compounds

Ifenprodil is a neuroprotective NMDA receptor antagonist which was originally thought to interact in a competitive manner at the polyamine site (Carter et al., 1990). However, several studies suggested that this atypical antagonist binds to a site distinct from that of polyamines (Carter et al., 1990; Reynolds and Miller, 1989; Tamura et al., 1993). Site-directed mutagenesis identified an amino acid residue on the NR2B subunit that is absolutely required for high affinity interaction with ifenprodil but not with spermine (Gallagher et al., 1996). Furthermore, a largely kinetic based approach by Kew and Kemp (1998) showed that spermine binding to the NMDA receptor leads to a reduction in receptor affinity for ifenprodil and vice versa. This finding suggests a non-competitive, allosteric interaction between polyamine site ligands and ifenprodil. Interestingly, ifenprodil has a 140-fold selectivity for the NR1/2B subtype over the NR1/2A subtype (Williams, 1993). A major drawback of ifenprodil, however, lies in the fact that it cross-reacts at higher concentrations with σ-receptors (Karbon et al., 1990), high voltage-activated Ca\(^{2+}\) channels (Bath et al., 1996), \(\alpha_1\)-adrenergic receptors (Chenard et al., 1991) and the 5HT\(_3\) receptor (McCool and Lovinger, 1995).

Meanwhile, several ifenprodil-like antagonists have been developed such as Ro 25-6981 (Fischer et al., 1997), CP 101-606 (Chenard et al., 1995) and Ro 8-4304 (Kew et al., 1998) which also show a selectivity for the NR1/2B subtype. Members of this class of compounds are neuroprotective both in in vitro and in vivo models of ischaemia but notably appear to lack many of the side effects associated with non-selective NMDA receptor antagonists in vivo (Menniti et al., 1997). Ifenprodil, Ro 25-6981 and Ro 8-4304 act via a novel state-dependent mechanism of action which together with their subunit-selectivity seems likely to underlie the desirable neuropharmacological profile of this class of drug (Fischer et al., 1997; Kew et al., 1996; Kew et al., 1998).
1.5.4 The glycine binding site of the NMDA receptor

Activation of NMDA receptors requires the presence of glycine as co-agonist which binds to a site that is allosterically linked to the glutamate binding site (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). The mechanism of glycine potentiation of glutamate-evoked currents is not understood but may involve the allosteric modulation of agonist binding affinities (Fadda et al., 1988; Kessler et al., 1989). For instance, glycine enhances \[^{3}H\]glutamate binding and reduces the binding of antagonists of the glutamate recognition site (Benveniste et al., 1990; Kessler et al., 1989; Monahan et al., 1990). Conversely, glutamate increases the affinity of \[^{3}H\]glycine (Monaghan et al., 1988).

1.5.4.1 Physiological role

The functional significance of the glycine binding site of NMDA receptor is still a matter of debate. Initially, measurements of glycine in extracellular and cerebrospinal fluids suggested that glycine occurs at micromolar concentrations which would be sufficient to saturate the glycine site, arguing against a modulatory role of glycine under physiological conditions (Obrenovitch et al., 1997; Westergren et al., 1994). However, several reports indicated that glycine may be present at subsaturating concentrations in the synaptic cleft (Ascher, 1990; Kew et al., 1998; Wood, 1995). Externally applied glycine potentiated NMDA-dependent LTP (Thiels et al., 1992) as well as neuronal activity in rat spinal cord and thalamus in vivo (Budai et al., 1992). Furthermore, the active uptake of glycine by high affinity transporters is thought to be sufficient to reduce the glycine concentration in the synaptic cleft below saturation (Attwell et al., 1993; Supplisson and Bergman, 1997), thereby allowing the variations in the local glycine concentration to become a signal acting on NMDA receptors. This was also recently suggested by the generation of two mouse lines carrying a point mutation in the putative glycine binding domain (Kew et al., 1998). Mice having a glycine site with a five-fold lower affinity for glycine showed deficits in LTP and spatial memory. Introduction of a point mutation resulting in a 80-fold reduction in glycine affinity led to a lethal phenotype. These findings provide strong evidence for the modulation of NMDA receptor activity by glycine under physiological conditions and a glycine level in the synaptic cleft well below saturation.
1.5.4.2 Structural domains contributing to the glycine site of recombinant NMDA receptors

Site-directed mutagenesis and ligand binding studies on recombinant NMDA receptors identified the NR1 subunit to contain all structural domains required for high affinity binding of glycine agonists and antagonists (Grimwood et al., 1995; Hirai et al., 1996; Kuryatov et al., 1994; Laurie and Seeburg, 1994; Moriyoshi et al., 1991; Wafford et al., 1995; Williams et al., 1996). Based on this data, sequence alignments of the NR1 subunit with the soluble bacterial amino acid binding proteins LAOBP (lysine/arginine/ornithine binding protein) and QBP (glutamine binding protein) gained first insights into the putative structural organization of the glycine binding site of recombinant NMDA receptors (O'Hara et al., 1993). The identified mutated residues on NR1 were shown to correspond to positions in the bacterial proteins which are known to interact with the ligand (Kuryatov et al., 1994; O'Hara et al., 1993; Oh et al., 1993). Several mutations of the NR1 subunit in the large extracellular N-terminal domain preceding the first membrane spanning segment M1 were found to strongly increase the EC\textsubscript{50} values for glycine agonists (Hirai et al., 1996; Kuryatov et al., 1994). This N-terminal domain corresponds to the lobe S1 in the bacterial proteins whereas further mutations identified in the loop connecting the transmembrane segments M3 and M4 correspond to the lobe S2 in the homologous bacterial proteins (Stern-Bach et al., 1994). Based on structural information available for the bacterial amino acid binding protein LAOBP (Oh et al., 1993) a bilobate S1-S2 structure of the glycine binding site was proposed, with a deep cleft between the two lobes accommodating the binding site for glycine (Kuryatov et al., 1994; Paas, 1998).

Interestingly, mutational analysis of the NR1 subunit pointed to structural differences between the binding domains for glycine site agonists and antagonists (Hirai et al., 1996; Kuryatov et al., 1994; Wafford et al., 1995; Williams et al., 1996) since the mutated residues in the loop connecting M3 and M4 affected the affinity for glycine site agonists (D-serine, D-cycloserine, ACBC) but not or only moderately for glycine site antagonists (DCKA, L-689,560, L-701,324). This finding suggested that the determinants for the binding site of glycine antagonists might be predominantly located on the putative S1 lobe region of the NR1 subunit.

Electrophysiological and radioligand binding studies on recombinant NMDA receptor subtypes pointed to a heterogeneity of the glycine agonist but not the
glycine antagonist binding site. Glycine site agonists (D-serine, ACBC, Dalanine, HA-966 and L-687,414) displayed an up to tenfold lower potency at NR1/2A receptors than at NR1/2B, NR1/2C or NR1/2D receptors (Buller et al., 1994; Grimwood et al., 1996; Ikeda et al., 1992; Kutsuwada et al., 1992; Laurie and Seeburg, 1994; Priestley et al., 1995; Wafford et al., 1993) whereas glycine site antagonists (L-701,324, L-689,560, L-695,902, CKA) did not significantly differentiate between recombinant NMDA receptor subtypes (Grimwood et al., 1995; Priestley et al., 1996). Thus, glycine antagonists are thought to interact with a domain on the NR1 subunit that is insensitive to modulation by adjacent NR2 subunit variants (Priestley et al., 1995), which would consequently rule out the development of subtype-specific glycine site antagonists for the targeting of individual NMDA receptor subtypes.

1.5.4.3 The glycine site as a target in drug development

Inhibition of NMDA receptors by glycine site antagonists is of major interest for pharmacological intervention in a variety of pathological CNS states. Glycine site antagonists show considerable therapeutic promise as neuroprotective, anticonvulsant, antipsychotic, anxiolytic, antinociceptive and nootropic agents (Kulagowski and Leeson, 1995). They may offer advantages over other types of NMDA receptor antagonists in terms of their side effect profile, especially in the long-term therapy of chronic degenerative diseases but also in the treatment of serious acute disorders such as status epilepticus or stroke. At doses evoking remarkable neuroprotective and anticonvulsant effects, glycine site antagonists are less prone to psychotomimetic actions and exhibit less severe motoric and cognitive side effects than glutamate site antagonists or open channel blockers (Dannhardt and Kohl, 1998; Kemp and Leeson, 1993; Leeson and Iversen, 1994). A number of glycine site antagonists (e.g. ACEA 1021, ACPC, GV-150526, GV-196771A, ZD9379) undergo currently clinical trials for stroke, depression and chronic pain, respectively (Parsons et al., 1998). A specific glycine antagonist with selectivity for a distinct NMDA receptor subtype has not yet been reported but is expected to further improve the therapeutic value of this class of drug.
2. Aim of the study

A major part of the excitatory neurotransmission in the brain is mediated by glutamate receptors of the NMDA-type. These receptors have been implicated in a variety of neurological and mental disorders. The activation of NMDA receptors requires the binding of the co-agonist glycine to a site that is allosterically linked to the binding site of the primary neurotransmitter glutamate. The glycine site of the NMDA receptor is an attractive target in drug development since glycine antagonists are expected to be beneficial in various CNS disorders. The spatial organization of the glycine binding domain in situ is thus of topical interest. So far, tools for a structural analysis of the glycine site of native NMDA receptors were not available. Therefore, an attempt was made to develop a radioligand ([3H]CGP 61594) which can be irreversibly linked to the glycine binding domain upon photoactivation. This photoaffinity ligand was used for a direct identification of structural constituents of the glycine binding site of native NMDA receptors.

Further advances in the design and development of novel glycine site antagonists are expected from the targeting of specific NMDA receptor subtypes. Previous experiments suggested that glycine antagonists do not permit a distinction of NMDA receptor subtypes. These findings appeared to preclude the development of subtype-specific glycine site antagonists. Since [3H]CGP 61594 represents a structurally unique glycine site antagonist it was expected to interact differentially with NMDA receptor subtypes. [3H]CGP 61594 was synthesized as a derivative of the high affinity glycine site antagonist L-689,560, but differs from its lead compound and other glycine antagonist by the presence of an azide group and by the orientation of the phenyl ring containing this group. To test the hypothesis of a subtype-selectivity of [3H]CGP 61594, the interaction of [3H]CGP 61594 with various native and recombinant NMDA receptor subtypes was analyzed biochemically and electrophysiologically. The search for a subtype-specificity might have an important impact on future drug development. Subtype-specific glycine antagonists are expected to display selective therapeutic effects with a reduced side effect profile compared to non-selective NMDA receptor antagonists.
3. Materials and Methods

3.1 Synthesis of CGP 61594 ((±)-trans-4-[2-(4-azidophenyl)-acetyl-amino]-5,7-dichloro-1,2,3,4-tetrahydro-quinoline-2-carboxylic acid).

The non-radioactive photoaffinity ligand CGP 61594 (Fig. 4A, 2a) was synthesized by acylation of the hydrobromide of trans-methyl-4-amino-5,7-dichloro-1,2,3,4-tetrahydro-quinoline-2-carboxylate (Leeson et al., 1992) with 4-azidophenylacetic acid (Cremo and Schimerlik, 1984) in 70% yield after alkaline hydrolysis with LiOH (Fig. 4).

For the preparation of the corresponding tritiated compound (Fig. 4B, 2b) the protocol of Boehm (1991) was adapted to 4-amino-3,5-diiodophenylacetic acid (Fig. 4B, 4). The latter was synthesized by iodination of the known monoiodo derivative (Fig. 4B, 3) with 1.5 equivalents of chloramine-T and sodium iodide, thus obviating the use of the iodine-monochloride. The diiodo compound (Fig. 4B, 4) was then catalytically tritiated (custom synthesis at Amersham Pharmacia Biotech) and converted to the 3,5-ditritiated 4-azidophenylacetic acid (Fig. 4B, 6) via the procedure of Cremo and Schimerlik (1984). Coupling as described for 4-azidophenyl acetic acid and alkaline hydrolysis resulted in the radioactive photoaffinity label (Fig. 4A, 2b).

3.2. Preparation of brain membranes

Male Sprague-Dawley rats (200-250 g) were decapitated, their brains or selected brain regions were rapidly removed and homogenized in 10 volumes of sucrose buffer (5 mM Tris/HCl pH 7.4, 0.32 M sucrose) using a Polytron (level 3-4, 2 min). The homogenate was centrifuged at 1000 x g for 15 min and the resulting supernatant was stored on ice while the pellet was washed in 5 volumes of sucrose buffer by resuspension and centrifugation. To obtain the crude membrane fraction the combined supernatants were centrifuged at 17,000 x g for 30 min. Endogenous ligands possibly interfering with the binding assay were removed by a combination of hypoosmotic lysis, freezing and detergent treatment of the membranes. The membranes were homogenized in 20 volumes of 5 mM Tris/acetate pH 8.0, stirred on ice for 1 h followed by centrifugation for 30 min at 17,000 x g. The resulting pellet was frozen at -30°C for at least 12 h, then resuspended in 5 mM Tris/acetate pH 7.0 containing 0.1% saponine and centrifuged for 15 min at 30,000 x g. After washing the pellet
twice with 5 mM Tris/acetate pH 7.0 and once with 50 mM Tris/HCl pH 7.4, the membranes were resuspended in 50 mM Tris/HCl pH 7.4 to give a protein concentration of about 10 mg/ml and were stored at -30°C.

(A) Synthesis of unlabeled CGP 61594 (2a) and tritiated CGP 61594 (2b).
(B) Synthesis of the 3,5-ditritiated 4-azidophenyl acetic acid (6); trans-methyl-4-amino-5,7-dichloro-1,2,3,4-tetrahydro-quinoline-2-carboxylate (1); 4-amino-3-iodophenyl acetic acid (3); 4-amino-3,5-diodophenyl acetic acid (4); 3,5-ditritiated 4-amino-phenyl acetic acid (5); EDC, N-ethyl-N‘-(3-dimethyl-amo-propyl)-carbodiimide hydrochloride.
3.3 Reversible radioligand binding assay

Prior to the binding experiment, aliquots of the frozen membranes were thawed, washed twice with 5 mM Tris/acetate pH 7.0 and once with 50 mM Tris/HCl pH 7.4. Binding assays were carried out with 100 µg membrane protein and 25 nM [³H]CGP 61594 (29 Ci/mmol) in a final volume of 200 µl 50 mM Tris/HCl pH 7.4 for 120 min on ice. The incubation was stopped by vacuum filtration through Whatman GF/C filters followed by washing the filters with 3 x 4 ml cold 50 mM Tris/HCl pH 7.4. Retained radioactivity was quantified by liquid scintillation counting. The high affinity and the relatively slow dissociation kinetics of [³H]CGP 61594 (see Results) justified the use of a filtration assay.

For saturation binding experiments, membranes were incubated either with increasing concentrations of [³H]CGP 61594 (2-200 nM) or with increasing concentrations of cold CGP 61594 (1 nM-10 µM) at a fixed radioligand concentration (180 nM). Drug displacement studies were performed by incubating membranes with serial dilutions (1 nM-10 µM) of various drugs at a fixed concentration of [³H]CGP 61594 (25 nM). All binding data were analyzed using the 'LIGAND' program (Biosoft, Cambridge, UK).

3.4 Photoaffinity labeling

Washed membranes (60 µg protein) were incubated with 25 nM [³H]CGP 61594 for 120 min on ice in 50 mM Tris/HCl pH 7.4, followed by UV-irradiation on ice (UV-Stratalinker 1800, 254 nm at a distance of 10 cm). The total irradiation energy was 1 J corresponding to an irradiation time of 6 min. Non-specific photolabeling was determined in the presence of 1 mM glycine. Following the photolabeling reaction, the membranes were pelleted by centrifugation, resuspended in 125 mM Tris/HCl pH 6.8, 20% glycerol, 0.002% bromphenol blue, 10% β-mercaptoethanol, 4% SDS and incubated for 30 min at 60°C. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5% mini-gels (Mini Protean II, BioRad) and proteins were stained with Coomassie brilliant blue R-250. For visualization of radioactively labeled proteins by fluorography, the gel was soaked for 30 min in Amplify (Amersham Pharmacia Biotech), dried and exposed to an X-ray film (Fuji RX) for at least two weeks.
For quantitative analysis of the incorporated radioactivity, the gels were sliced and individual gel pieces were dissolved in 30% H$_2$O$_2$ (6 h at 60°C) followed by determination of the incorporated radioactivity by liquid scintillation counting.

3.5 Purification of antibodies

The antisera used were raised in rabbits (NR1, NR2A, NR2C and NR2D) and guinea pigs (NR2B) against the following amino acid sequences: NR1, amino acids 901-938; NR2A, 219-319; NR2B, 27-297; NR2C, 1043-1218; NR2D, 1307-1323 (Benke et al., 1995; Wenzel et al., 1995).

For affinity purification of the antisera, synthetic peptide antigens (NR1, NR2D) were coupled via an additional cysteine residue to Thiopropyl-Sepharose 6B (Pharmacia), whereas 6His-tagged recombinant antigens (NR2B, NR2C) were coupled to Affi-Gel 10 or 15 (BioRad) depending on their isoelectric point. Since the NR2A antigen generated in bacterial expression systems was not soluble under conditions needed for coupling to the various matrices, it was subjected to 10% SDS-PAGE, blotted onto nitrocellulose membranes and used as an affinity matrix for the isolation of the respective antibodies.

Binding of the antisera (diluted 1:2 with 10 mM Tris/HCl pH 8, 150 mM NaCl) to the respective affinity matrices was performed overnight by recirculating the antiserum at 6 ml/h through the column. After extensive washing with 10 mM Tris/HCl pH 8, 150 mM NaCl, bound antibodies were eluted with 3.5 M MgCl$_2$.

Protein containing fractions (1 ml) were pooled, supplemented with BSA to a final concentration of 1 mg/ml, dialyzed against 10 mM Tris/HCl pH 8, 150 mM NaCl containing 0.05% NaN$_3$ and finally concentrated by ultrafiltration (Amicon). Purified antisera were stored at 4°C until use.

3.6. Enzyme-linked immunosorbent assay (ELISA)

The reactivity of antisera and the efficiency of purification were assessed by ELISA. The wells of immunoplates (Nunc, Maxisorb) were pre-treated with 100 µl glutaraldehyde (0.025%) in 50 mM Na$_2$CO$_3$ pH 9.5 for 10 min at room temperature, washed several times with H$_2$O and finally coated with antigen (0.1 µg per well) at 4°C overnight. Following extensive washing with PBS (50 mM phosphate buffer pH 7.4, 150 mM NaCl) protein binding sites on the plates were blocked with 2% BSA in PBS for 1 h at room temperature. After washing (2x H$_2$O, 1x buffer), the antisera were applied in serial dilutions ranging from 1:50 to
1:100'000 in PBS containing 2% BSA and incubated at 4°C overnight. Following extensive washing, horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-guinea pig IgG (Jackson Immunoresearch) was applied (diluted 1:5'000 in PBS/1% BSA) for 2 h at room temperature. After extensive washing (2x H$_2$O, 1x PBS, repeated twice) 100 μl of the HRP substrate solution was added (20 mM 3.3’5.5’ tetramethylbenzidine in 0.2 M sodium citrate pH 3.95, 0.006% H$_2$O$_2$). After colour development the staining reaction was terminated by addition of 100 μl 1 M H$_2$SO$_4$ and the optical density was recorded at 450 nm (reference filter: 620 nm) in a microplate reader (Anthos reader 2001).

3.7 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE was performed according to Laemmli (1970) using 5%, 7.5% and 10% mini gels (90 x 60 x 1.5 mm) with up to 40 μg protein per lane. The following proteins served as molecular weight marker (Pharmacia): myosin, 212 kDa; α$_2$-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa. After SDS-PAGE, proteins were transferred to nitrocellulose membranes (0.45 μm, BioRad) in a semi-dry electro-blotting apparatus (BioRad) for 60 min at 15 V (transfer buffer: 39 mM Tris, 48 mM glycine, 1.3 mM SDS, 20% methanol). For immunodetection, the blots were blocked for 2 h at room temperature in TBST (10 mM Tris/HCl pH 8, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk (BioRad). Incubation with the different antisera (NR1, NR2A-NR2D) was carried out overnight at 4°C in the same solution, followed by extensive washing of the blots with RIPEA (20 mM Tris/HCl pH 7.5, 60 mM NaCl, 2 mM EDTA, 0.4% SDS, 0.4% Triton X-100, 0.4% deoxycholate) and two times with TBST. After washing the blots were incubated with HRP-conjugated goat anti-rabbit IgG (Promega Corp.) diluted 1:5'000 or with HRP-conjugated goat anti-guinea pig IgG (Jackson Immunoresearch) diluted 1:5'000 in TBST/5% blocker for 1 h at room temperature. Following extensive washing with RIPEA and TBST, the immunoreaction was visualized using the enhanced chemoluminescence method (ECL, Amersham Pharmacia Biotech). Chemoluminescence was detected on Fuji X-ray films exposed for 3 s to 20 min depending on the signal intensity. The specificity of the immunoreaction was assessed by preincubating
the antisera with 10 μg/ml of the respective antigen in TBST/5% blocker for 1 h at room temperature.

3.8 Histoblotting

Parasagittal cryostat sections of adult rat brain (16 μm) were collected on dry nitrocellulose membranes (0.45 μm, BioRad) and stored at -20°C until use. For protein blotting the nitrocellulose membranes were moistened with transfer buffer (39 mM glycine, 48 mM Tris, 20% methanol, 2% SDS) for 15 min at room temperature. Following incubation for 2 h in TBST/5% blocker, the blots were washed with TBST and incubated overnight in 0.1 M Tris pH 7.5, 2% SDS, 0.1 M β-mercaptoethanol at room temperature to remove the tissue. Following extensive washing in TBST, the blots were blocked again with TBST/5% blocker and processed for immunostaining as described above for Western blots.

3.9 Immunoaffinity chromatography

For the construction of an NR1-specific immunoaffinity column, 8 mg of the affinity-purified NR1 antibody was incubated with 2 ml of protein A-agarose (Pierce) for 1 h at room temperature to couple the antibody in an oriented manner via its Fc domain. Unbound antibodies were removed by washing with 20 volumes of PBS and 10 volumes 0.2 M triethylamine pH 8.3. The bound antibodies were irreversibly linked to protein A-agarose by incubation with two volumes of 20 mM dimethyl pimelimidate (in 0.2 M triethylamine pH 8.3) for 30 min at room temperature under constant gentle agitation. The reaction was terminated by incubation with an equal volume of 0.2 M ethanolamine for 5 min, followed by extensive washing with PBS.

For immunoaffinity chromatography, whole brain membranes were photolabeled with 25 nM [3H]CGP 61594 (see above), washed with 50 mM Tris/HCl pH 7.4 and solubilized in RIPA (50 mM Tris/HCl pH 9.0, 1 mM EDTA, 1% NP40, 0.5% deoxycholate, 0.1% SDS) for 1 h on ice. Following centrifugation at 100,000 x g for 1 h, the solubilized fraction of photolabeled receptors, which contained about 30% of the totally photolabeled 116 kDa protein, was diluted fourfold with Tris buffer and circulated overnight at 4°C through the immunoaffinity column. After extensive washing with 10 mM Tris/HCl pH 9.0, 150 mM KCl, 1 mM EDTA, 0.05% deoxycholate, 0.05% NaN₃,
proteins were eluted from the immunoaffinity column by changing the pH-value to 11.5 (0.1 M Na₂HPO₄, pH 11.5, 150 mM KCl, 1 mM EDTA, 0.05% deoxycholate). The eluate was immediately neutralized with 1 M NaH₂PO₄, concentrated by ultrafiltration, followed by SDS-PAGE and fluorography. About 80-100% of the solubilized photolabeled 116 kDa protein bound to the NR1 immunoaffinity column as analyzed by determination of the amount of radioactivity in gel slices containing the 116 kDa protein before and after subjection of the detergent extract to the immunoaffinity column. 50-60% of the radioactivity that had been retained on the column was eluted.

3.10 Immunoprecipitation

Whole brain membranes were photolabeled with 25 nM [³H]CGP 61594, washed with 50 mM Tris/HCl pH 7.4 and solubilized in RIPA as described above. Aliquots of the detergent extract (500 μl) were incubated with NR1, NR2A, NR2B, NR2C or NR2D antiserum overnight at 4°C in the absence or presence of the respective peptide antigen (10 μg/ml). Antigen-antibody complexes were precipitated by incubation with 125 μl Pansorbin (suspension of 10% Staphylococcus aureus, Calbiochem) for 1 h at room temperature. After washing the pellets three times in 50 mM Tris/HCl pH 7.4, 0.2% Triton X-100 the precipitates were resuspended in 200 μl 50 mM Tris/HCl pH 7.4, and the radioactivity was determined by liquid scintillation counting. The ability of the different antisera to immunoprecipitate the respective receptor subtype was verified by Western blot analysis.

3.11 Electrophysiological slice recordings

Electrophysiological studies on neocortical slices were essentially done as described previously (Brugger et al., 1990). Male rats (Tif:RAI f(SPF), 150-180 g) were killed by decapitation under slight halothane anaesthesia. The brains were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF, 124 mM NaCl, 2.5 mM KCl, 1.25 mM KH₂PO₄, 2.5 mM CaCl₂, 2 mM MgSO₄, 26 mM NaHCO₃, 10 mM glucose; gassed with 95% O₂, 5% CO₂). Coronal neocortical slices (500 μm) were placed in a recording chamber, perfused with Mg²⁺-free ACSF and recordings were carried out as described previously (Brugger et al., 1990).
3.12 Receptor autoradiography

The regional distribution of \[^{3}H\]CGP 61594 binding sites was analyzed on unfixed parasagittal cryostat-cut sections (12 \(\mu\)m) of brains of adult male Sprague-Dawley rats (200-250 g). Slide-mounted sections were thawed and washed three times for 10 min at 4\(^\circ\)C in 50 mM Tris/HCl pH 7.5. For \[^{3}H\]CGP 61594 binding, sections were incubated for 2 h at 4\(^\circ\)C with 10 nM \[^{3}H\]CGP 61594 in 50 mM Tris/HCl pH 7.5, followed by washing five times for 2 min in buffer. After washing in buffer and a quick dip in distilled water, the sections were dried and exposed to Hyperfilm-\(^{3}\)H (Amersham Pharmacia Biotech) for 15 days. Non-specific \[^{3}H\]CGP 61594 binding was assessed in parallel in the presence of 1 mM glycine.

3.13 Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown to 50-60% confluence and transfected with the NR1 cDNA alone or in combination with the cDNA encoding the NR2A, NR2B, NR2C, or NR2D subunit (25 \(\mu\)g total) using the calcium phosphate precipitation method of Chen and Okayama (1987). In co-transfection cultures, either 5 \(\mu\)M MK-801 and 10 \(\mu\)M DCKA or 500 \(\mu\)M ketamine were added. After overnight incubation (35\(^\circ\)C, 3% \(CO_2\)), the medium was renewed and the cells were incubated for further 24-48 h (37\(^\circ\)C, 5% \(CO_2\)). The plates were then rinsed with PBS (37\(^\circ\)C, pH 7.5) and the cells were harvested into the same buffer containing 50 mM KCl. The cells were pelleted by centrifugation (10'000 \(x\) g, 15 min, 4\(^\circ\)C), homogenized (Polytron) in ice-cold 0.32 M sucrose, 10 mM EDTA, 1 \(\mu\)g/ml PMSF, followed by preparation of the crude membrane fraction as described above. The membrane pellet was washed once in 10 mM Tris/acetate pH 7.5, 10 mM EDTA and frozen to -70\(^\circ\)C at least overnight. Before the binding experiment, the thawed membrane suspension was washed twice with 5 mM Tris/acetate pH 7.0 and once with 50 mM Tris/HCl pH 7.4.

3.14 Expression and recordings in Xenopus oocytes

Linearized plasmid DNAs were used for in vitro synthesis of cRNA (mCAP mRNA Capping Kit, Stratagene). cRNAs of NR1 and NR2A-NR2D subunits were synthesized using T7 or T3 RNA polymerase, respectively. cRNA concentrations were determined by both measuring optical densities at 260 nm
and comparing methylene blue staining intensities after gel electrophoresis. For oocyte injection, the concentration of the cRNA samples was adjusted to 200-500 ng/ml, and diluted aliquots of the NR1 and NR2 cRNAs were mixed at the RNA ratio of 1:1. Microinjection of about 50 nl of cRNA into *Xenopus laevis* oocytes and voltage-clamp recordings of agonist responses in Mg²⁺-free frog Ringer's solution at a holding potential of -70 mV were performed as described (Schmieden et al., 1989).
4. Results

Activation of NMDA receptors requires the presence of glycine as a co-agonist which binds to a site that is allosterically linked to the binding site of the primary neurotransmitter glutamate. Since the glycine site of the NMDA receptor is a promising target in drug development, its structural organization is of topical interest. The photoaffinity ligand and glycine site antagonist \([3\text{H}]\text{CGP 61594}\) was developed as a novel tool for a direct identification and analysis of the glycine binding site of native NMDA receptors. Moreover, by investigating the interaction of \([3\text{H}]\text{CGP 61594}\) with various native and recombinant NMDA receptor subtypes a putative heterogeneity of the glycine binding domain was examined.

4.1. Synthesis of CGP 61594

For the development of a photoaffinity label for the glycine binding site of the NMDA receptor, L-689,560 was derivatized which was the antagonist with the highest affinity for this binding site available at that time (Fig. 5) (Grimwood et al., 1992). The novel compound CGP 61594, which contains a phenylacetic acid side chain with a photoreactive azide group, was synthesized based on the tetrahydroquinoline structure of L-689,560. In preliminary experiments CGP 61594 showed high displacement potency in binding assays using the high affinity glycine site antagonist \([3\text{H}]\text{DCKA}\) (IC\(_{50}\) = 5 \(\pm\) 2 nM, \(n = 3\)). Therefore, CGP 61594 was tritiated (see Materials and Methods, Fig. 4) to serve as a radioactive label for a structural analysis of the glycine site of NMDA receptors.

![Chemical structure of the glycine site antagonists \([3\text{H}]\text{L-689,560}\) and the novel photoaffinity ligand \([3\text{H}]\text{CGP 61594}\). The glycine-like structure of the molecules is marked by a box.](image-url)
4.2 Antagonism of NMDA-evoked currents by CGP 61594

To test whether CGP 61594 interacts as an antagonist at the glycine site, NMDA receptors were analyzed electrophysiologically in neocortical slices (Fig. 6). NMDA-evoked responses decreased to 41 ± 7% of controls (n = 12) in the presence of 10 μM unlabeled CGP 61594. Co-application of the glycine site agonist D-serine (200 μM) together with CGP 61594 reversed the antagonistic effect of CGP 61594 by restoring the current response to 88 ± 4% (n = 12) of control values (100%). Consequently, the inhibition of NMDA-evoked responses by CGP 61594 in a D-serine reversible manner characterizes CGP 61594 as a glycine site antagonist.

Fig. 6: Antagonism of NMDA-evoked currents by CGP 61594 in brain slices. Electrophysiological recordings were made from coronal neocortical slices (500 μm) in Mg²⁺-free ACFS. NMDA receptors were activated by 10 μM NMDA. Inhibition of NMDA-evoked currents by CGP 61594 (10 μM) was reversed by co-application of D-serine (200 μM).
4.3 Characterization of reversible $[^3]H$CGP 61594 binding

Preliminary reversible binding assays with $[^3]H$CGP 61594 were performed using membranes prepared from rat brain regions with a high NMDA receptor density (cerebral cortex and hippocampus). Membranes were incubated with 25 nM $[^3]H$CGP 61594 in the absence and presence of 1 mM glycine and subsequently vacuum-filtrated to separate bound from free radioactivity. Specific $[^3]H$CGP 61594 binding amounted to 70-80% of total binding. The specific binding increased linearly with membrane protein concentration up to 500 μg/ml. Consequently, a maximum of 100 μg protein in a total volume of 0.2 ml was used in the reversible binding assays.

4.3.1 Kinetics of $[^3]H$CGP 61594 binding

Kinetic analysis of $[^3]H$CGP 61594 binding at a concentration of 25 nM resulted in an association equilibrium of $[^3]H$CGP 61594 that was reached by 1 h. The isotherm was best fitted to a double exponential function with $t_{1/2}$-values of 5 ± 1 min and 21 ± 6 min, with the fast component constituting 54 ± 11% of the specific binding (Tab. 1). Likewise, two components were found for the dissociation kinetics and gave $t_{1/2}$-values of 10 ± 1 min and 46 ± 9 min (Tab. 1). The component with fast dissociation amounted to 52 ± 7% of specific binding. Similar binding kinetics had previously been identified for the lead compound of $[^3]H$CGP 61594, the glycine site antagonist $[^3]H$L-689,560 (Grimwood et al., 1992).

4.3.2 High affinity $[^3]H$CGP 61594 binding

Saturation binding assays were performed in a concentration range of 2-200 nM of $[^3]H$CGP 61594. Since the non-specific binding of $[^3]H$CGP 61594 strongly increased with high ligand concentration, 200 nM was the upper limit of radioligand concentration which could be used in a saturation analysis. Scatchard analysis of $[^3]H$CGP 61594 saturation binding data resulted in a single high affinity binding site of $[^3]H$CGP 61594 with a dissociation constant ($K_D$) of 23 ± 2 nM and a maximum number of binding sites ($B_{max}$) of 5.84 ± 0.42 pmol/mg protein (Fig. 7, Tab. 2). In the presence of 0.25 mM glycine an increase of the $K_D$-value for $[^3]H$CGP 61594 was observed with no change in the $B_{max}$-value, indicative of a competitive interaction with glycine (Fig. 7, Tab. 2). In contrast, co-incubation with an excess of the polyamine site agonist spermine
(100 µM) or the glutamate site antagonist AP5 (100 µM) reduced the maximum number of binding sites without affecting the affinity, consistent with a non-competitive and allosteric interaction (Tab. 2).

<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$: M$^{-1}$min$^{-1}$</th>
<th>$k_{off}$: min$^{-1}$</th>
<th>sites (%)</th>
<th>$t_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{on1}$</td>
<td>$4.2 \pm 0.3 \times 10^6$</td>
<td>54 ± 11</td>
<td></td>
<td>5 ± 1</td>
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<tr>
<td>$k_{on2}$</td>
<td>$1.1 \pm 0.5 \times 10^6$</td>
<td>46 ± 11</td>
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<td>21 ± 6</td>
</tr>
<tr>
<td>$k_{off1}$</td>
<td>$7.0 \pm 0.6 \times 10^{-2}$</td>
<td>52 ± 7</td>
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<td>10 ± 1</td>
</tr>
<tr>
<td>$k_{off2}$</td>
<td>$1.5 \pm 0.3 \times 10^{-2}$</td>
<td>48 ± 7</td>
<td></td>
<td>46 ± 9</td>
</tr>
</tbody>
</table>

Tab. 1: Kinetics of [$^3$H]CGP 61594 binding
Association kinetics were determined by incubating [$^3$H]CGP 61594 (25 nM) with cortex/hippocampus membranes on ice for different times (1-180 min), followed by determination of bound radioactivity. For dissociation kinetics, [$^3$H]CGP 61594 (25 nM) was incubated with cortex/hippocampus membranes for 90 min on ice, followed by a 10-fold dilution with ice-cold buffer containing 1 mM glycine. Radioactivity bound to the membranes was determined at various time intervals (1-90 min) after dilution. Non-specific binding was determined by co-incubation with 1 mM glycine. The data are the mean ± S.D. of four independent experiments.

Fig. 7: Scatchard analysis of [$^3$H]CGP 61594 saturation binding data under reversible conditions. Cortex/hippocampus membranes were incubated with various concentrations of [$^3$H]CGP 61594 (2-200 nM) in the absence or presence of 0.25 mM glycine. Non-specific [$^3$H]CGP 61594 binding was determined in the presence of 1 mM glycine. The data shown are from a representative experiment. The mean $K_p$ and $B_{max}$ values of four independent experiments are given in Table 2.
KD (nM)  Bmax (pmol/mg Protein)

<table>
<thead>
<tr>
<th>Reversible binding</th>
<th>K_D (nM)</th>
<th>B_max (pmol/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without drug)</td>
<td>23 ± 2</td>
<td>5.839 ± 0.416</td>
</tr>
<tr>
<td>+ 0.25 μM Glycine</td>
<td>44 ± 7</td>
<td>4.981 ± 1.035</td>
</tr>
<tr>
<td>+ 100 μM Spermine</td>
<td>23 ± 2</td>
<td>2.912 ± 1.617</td>
</tr>
<tr>
<td>+ 100 μM AP5</td>
<td>23 ± 4</td>
<td>2.753 ± 0.143</td>
</tr>
<tr>
<td>Photolabeling</td>
<td>27 ± 3</td>
<td>0.435 ± 0.111</td>
</tr>
</tbody>
</table>

Tab. 2: Scatchard analysis of [3H]CGP 61594 binding
Cortex/hippocampus membranes were incubated with increasing concentrations of [3H]CGP 61594 (2-200 nM) in the absence (control) or presence of 0.25 mM glycine, 100 mM spermine or 100 mM AP5. For the determination of a 'pseudo KD' under irreversible conditions, the incubation was followed by irradiation with UV-light (254 nM, 1 J). After SDS-PAGE, gel slices containing the photolabeled 116 kDa protein band were dissolved in H2O2 and radioactivity was determined by liquid scintillation counting. Non-specific binding or photolabeling was determined in the presence of 1 mM glycine. The data shown are the mean ± S.D. of four independent experiments.

4.3.3 Low affinity [3H]CGP 61594 binding
To identify putative additional low affinity binding sites of [3H]CGP 61594, competition binding assays with a high radioligand concentration were performed. Membranes prepared from the cerebral cortex and the hippocampus were incubated with 180 nM [3H]CGP 61594 and radioligand binding was competed with an increasing concentration of unlabeled CGP 61594 (1 nM-10 μM). Non-specific binding was determined by co-incubation with 1 mM glycine and amounted to 60-70% of total binding. Under these conditions, a low affinity binding component for CGP 61594 with an IC50 of 371 ± 85 nM was identified in hippocampus/cortex membranes, in addition to the high affinity binding site revealed by Scatchard analysis. Furthermore, [3H]CGP 61594 binding was analyzed in membranes prepared from the cerebellum, a brain region which is known to differ from hippocampus/cortex in its NMDA receptor pharmacology and modulation (Widdowson et al., 1995). Surprisingly, the high affinity binding component for
[³H]CGP 61594 present in hippocampus/cortex membranes was not detectable in this brain region. Therefore, NMDA receptors in the cerebellum may contain a [³H]CGP 61594 binding site which is strikingly different from the one present in hippocampus and cerebral cortex. Incubation of cerebellar membranes with a high [³H]CGP 61594 concentration (180 nM) in the absence and presence of 1 mM glycine yielded little specific [³H]CGP 61594 binding which amounted to about 15% of total binding. The affinity of these specific binding sites in the cerebellum was determined by competition experiments with cold CGP 61594 (1 nM-10 µM). An IC₅₀-value of 231 ± 56 nM for CGP 61594 was found in this brain region which is 10 times higher compared to the high affinity binding site identified in hippocampus/cortex membranes but is clearly lower than the low affinity binding component detected in membranes prepared from hippocampus/cortex (IC₅₀ = 371 ± 85 nM). These different affinities of the [³H]CGP 61594 binding site expressed in hippocampus/cortex and cerebellum point to a regional heterogeneity of the glycine antagonist binding site.

4.3.4 Drug displacement of [³H]CGP 61594 binding
Drug displacement studies were performed to investigate whether [³H]CGP 61594 interacts specifically with the glycine site of the NMDA receptor. The displacing potencies of various glycine agonists (glycine, D-serine), partial agonists (ACPC, HA-966) and antagonists (CKA, DCKA, MDL 100,458, MDL 102,288) were analyzed and found to be in a nanomolar range except for HA-966 (Tab. 3, left column), similar to those reported previously for other radioligand binding assays with glycine site ligands (Danysz et al., 1990; Grimwood et al., 1992; Monahan et al., 1989; Monahan et al., 1990; Yoneda et al., 1994). [³H]CGP 61594 did neither interact with the glycine site of the glycine-gated chloride receptor channel nor with the AMPA and kainate subtypes of glutamate receptors since the respective selective ligands (strychnine, AMPA and kainate) lacked displacing potency in [³H]CGP 61594 binding (Tab. 3, left column). These results support the specificity of [³H]CGP 61594 as a glycine site ligand of the NMDA receptor.
### Tab. 3: Inhibition of reversible binding and photoaffinity labeling with [³H]CGP 61594

The potencies of various agents in inhibiting reversible binding of [³H]CGP 61594 were determined by incubating cortex/hippocampus membranes with [³H]CGP 61594 (25 nM) in the presence of various concentrations of the respective compounds. To determine the displacing potencies under photolabeling conditions, the incubation was followed by irradiation with UV-light (254 nm, 1 J). After SDS-PAGE, gel slices containing the photolabeled 116 kDa protein band were dissolved in H₂O₂ and radioactivity was determined by liquid scintillation counting. Non-specific binding or photolabeling was determined in the presence of 1 mM glycine. The data are the mean ± S.D. of four independent experiments.
4.3.5 Allosteric modulation of $[^3H]CGP\ 61594$ binding

The ability of ligands for the glutamate and polyamine sites of the NMDA receptor to allosterically modulate $[^3H]CGP\ 61594$ binding was analyzed in reversible binding experiments (Tab. 4, left column). Glutamate site agonists (glutamate and NMDA) slightly enhanced $[^3H]CGP\ 61594$ binding, whereas glutamate site antagonists (AP5, D-CPP, CGP 37849 and CGP 40116) decreased $[^3H]CGP\ 61594$ binding to a maximal extent of 25-45% depending on the ligand. Furthermore, allosteric modulation of $[^3H]CGP\ 61594$ binding showed stereoselectivity. The pharmacologically active stereoisomers D-CPP and CGP 40116 inhibited $[^3H]CGP\ 61594$ binding to $23 \pm 8\%$ (1 $\mu$M) and $21 \pm 4\%$ (1 $\mu$M), respectively, whereas the inactive stereoisomers L-CPP (Aebischer et al., 1995) and CGP 40117 (Fagg et al., 1990) did not influence $[^3H]CGP\ 61594$ binding at this concentration. Moreover, polyamine site agonists inhibited $[^3H]CGP\ 61594$ binding. Spermine was more potent than spermidine, whereas the polyamine site antagonist eliprodil was inactive in modulating $[^3H]CGP\ 61594$ binding (Tab. 4, left column).

Scatchard analysis of $[^3H]CGP\ 61594$ binding performed in the presence and absence of the glutamate site antagonist AP5 or the polyamine site agonist spermine verified their allosteric manner of inhibition of $[^3H]CGP\ 61594$ binding. Both AP5 and spermine exerted their inhibitory action by reducing the maximum number of binding sites while leaving the affinity constant $K_D$ unchanged, consistent with a non-competitive interaction with $[^3H]CGP\ 61594$ (Tab. 2).
<table>
<thead>
<tr>
<th>Drug</th>
<th>% Effect</th>
<th>reversible</th>
<th>irreversible</th>
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<tbody>
<tr>
<td><strong>Glutamate site agonists:</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Glutamate (100 μM)</td>
<td>+ 4 ± 1</td>
<td>+ 13 ± 14</td>
<td></td>
</tr>
<tr>
<td>NMDA (100 μM)</td>
<td>+ 22 ± 5</td>
<td>+ 5 ± 7</td>
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</tr>
<tr>
<td><strong>Glutamate site antagonists:</strong></td>
<td></td>
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<tr>
<td>AP5 (100 μM)</td>
<td>- 45 ± 8</td>
<td>- 39 ± 5</td>
<td></td>
</tr>
<tr>
<td>D-CPP (1 μM)</td>
<td>- 21 ± 8</td>
<td>- 25 ± 5</td>
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</tr>
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<td>L-CPP (1 μM)</td>
<td>0 ± 3</td>
<td>3 ± 9</td>
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<tr>
<td>CGP 37849 (100 μM)</td>
<td>- 25 ± 3</td>
<td>- 26 ± 5</td>
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<td>CGP 40116 (1 μM)</td>
<td>- 21 ± 4</td>
<td>- 21 ± 8</td>
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<td>CGP 40117 (1 μM)</td>
<td>0 ± 5</td>
<td>0 ± 11</td>
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<tr>
<td><strong>Polyamine site ligands:</strong></td>
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<tr>
<td>Spermine (100 μM)</td>
<td>- 48 ± 7</td>
<td>- 50 ± 10</td>
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<tr>
<td>Spermidine (100 μM)</td>
<td>- 22 ± 1</td>
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<tr>
<td>Eliprodil (100 μM)</td>
<td>- 6 ± 4</td>
<td>n.d.</td>
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</tr>
</tbody>
</table>

Tab. 4: Allosteric modulation of reversible binding and photoaffinity labeling with [3H]CGP 61594.

[3H]CGP 61594 (25 nM) was incubated with cortex/hippocampus membranes in the absence or presence of glutamate site and polyamine site ligands followed by determination of bound radioactivity. For an analysis of the modulatory drug effects under photolabeling conditions, the incubation was followed by irradiation with UV-light (254 nm, 1 J). After SDS-PAGE, gel slices containing the photolabeled 116 kDa protein were dissolved in H2O2 and radioactivity was determined by liquid scintillation counting. CGP 40116 and CGP 40117 are the pharmacologically active and inactive enantiomers of the racemate CGP 37849. Non-specific binding and photolabeling was determined in the presence of 1 mM glycine. The data are the mean ± S.D. of four independent experiments (n.d. not determined).
4.4 Photoaffinity labeling of the glycine binding domain

[³H]CGP 61594 was developed as a glycine site antagonist that can be used as a photoaffinity label. A photosensitive azide group was introduced into the molecule at position 4 of the phenyl ring of [³H]CGP 61594. Upon UV-illumination, this azide group generates a highly reactive, short-lived nitrene species by concomitant release of gaseous nitrogen (Bouchet and Goeldner, 1997). [³H]CGP 61594 was thus expected to be covalently crosslinked to an amino acid within the glycine binding domain. This would allow [³H]CGP 61594 to serve as a covalent marker of the protein constituents of this binding site.

4.4.1 [³H]CGP 61594 photolabeling of native NMDA receptors

To test the optimal conditions for photolabeling, membrane preparations from adult rat brain were incubated with 25 nM [³H]CGP 61594 for 120 min at 4°C and irradiated with UV-light in a Stratalinker at 254 nm. The photolabeled proteins were then separated on a SDS-PAGE and the amount of incorporated radioactivity was either visualized by fluorography or quantified by measuring the radioactivity in individual gel slices by liquid scintillation counting. Non-specifically photolabeled proteins were determined by incubation with [³H]CGP 61594 in the presence of 1 mM glycine and separation of proteins in parallel lanes of the gel. Quantification of specifically incorporated radioactivity amounted to 66 ± 9% (n = 3) and revealed a prominent peak at 116 kDa (Fig. 8A). Maximum incorporation of [³H]CGP 61594 was observed at an irradiation energy of 1 J, which corresponded to an irradiation time of 6 min (Fig. 8C). Consequently, 1 J was used in the photocrosslinking experiments. To test whether UV-irradiation per se induced alterations in the glycine binding site membrane preparations were irradiated prior to incubation with [³H]CGP 61594, but this procedure did not change the capacity of the membranes for [³H]CGP 61594 binding or photolabeling.
Fig. 8: Photolabeling with [³H]CGP 61594: Molecular size of the photolabeled protein (A, B), labeling efficiency at different levels of irradiation energy (C) and immunological identity of the photolabeled protein (D).

(A) Cortex/hippocampus membranes (60 μg protein) were photolabeled with 25 nM [³H]CGP 61594 in the absence or presence of 1 mM glycine. After SDS-PAGE, the gel was cut in pieces (1 mm) in the range from 70-220 kDa. Slices were dissolved in H₂O₂ and radioactivity was determined by liquid scintillation counting.

(B) Cortex/hippocampus membranes (60 μg protein) were photolabeled with 25 nM [³H]CGP 61594 in the absence or presence of 1 mM glycine (+Gly),
10 μM unlabeled CGP 61594 (+CGP) or 10 μM DCKA (+DCKA) followed by SDS-PAGE and fluorography. Specific incorporation of radioactivity was apparent at 116 kDa. Very faint non-specific photolabeling was detected at 94 kDa. Cortex/hippocampus membranes (200 μg protein) were incubated with 25 nM [3H]CGP 61594 for 120 min at 4°C followed by irradiation with UV-light (254 nm) of increasing energy (0.25-2 J, corresponding to an irradiation time of 2.5-12 min). Following SDS-PAGE, gel slices containing the photolabeled 116 kDa protein were dissolved in H2O2 and radioactivity was determined by liquid scintillation counting. Non-specific incorporation of radioactivity was determined in the presence of 1 mM glycine. Data are expressed as percent of maximum incorporated radioactivity and represent the mean ± S.D. of three experiments. The range of radioactivity incorporated was: 200-800 dpm (for 0.25 J), 800-2000 dpm (for 0.5 J), 1200-3200 dpm (for 1 J), 1100-3000 dpm (for 2 J). Cortex/hippocampus membranes were photolabeled with 25 nM [3H]CGP 61594, solubilized and subjected to immunoaffinity chromatography using NR1 subunit-specific antibodies immobilized on protein A-agarose. Analysis of the eluate by SDS-PAGE and fluorography revealed radioactivity in the 116 kDa band, indicating that the 116 kDa photolabeled protein had been retained on the NR1 immunoaffinity column.

4.4.1.1 Specificity of [3H]CGP 61594 photolabeling
Scatchard analysis of irreversible [3H]CGP 61594 binding data (2-200 nM) revealed a half-maximal incorporation of [3H]CGP 61594 at a concentration of 27 ± 3 nM. This value corresponds to the dissociation constant in reversible binding and suggests that the affinity of the [3H]CGP 61594 binding site is unchanged under irreversible labeling conditions. A maximum of 0.435 ± 0.111 pmol binding sites per mg protein was covalently labeled (Fig. 9B). Thus, compared to the Bmax-value under reversible conditions, 7.5% of specifically bound [3H]CGP 61594 was irreversibly incorporated into the 116 kDa protein (Tab. 2).

Photolabeling of the 116 kDa band was inhibited by glycine site agonists as well as antagonists with potencies corresponding to those observed in the reversible binding experiments (Tab. 3, right column). Only for HA-966 a three times lower affinity was determined in the irreversible binding assay. These data demonstrate that the specificity of [3H]CGP 61594 is unaltered under crosslinking conditions.

Furthermore, it was tested whether the allosteric modulation of [3H]CGP 61594 binding can be also observed under irreversible conditions. Indeed, photolabeling of the 116 kDa band was modulated by ligands of the glutamate and polyamine sites with a stereoselectivity and in an extent expected from the reversible ligand binding experiments (Tab. 4, right column). Thus, [3H]CGP
61594 binding is also accessible to allosteric modulation under crosslinking conditions.

Fig. 9: Comparison of Scatchard analysis of [3H]CGP 61594 saturation binding data under reversible (A) and irreversible (B) conditions. (A) Cortex/hippocampus membranes were incubated with various concentrations of [3H]CGP 61594 (2-200 nM). (B) Under irreversible conditions incubation was followed by irradiation with UV-light (254 nM, 1 J). After SDS-PAGE, gel slices containing the photolabeled 116 kDa protein band were dissolved in H2O2 and radioactivity was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 1 mM glycine. The data shown are from a representative experiment. The mean KD- and Bmax-values of four independent experiments are given in Table 2.

4.4.1.2 Identity of the photolabeled 116 kDa protein
Analysis of the irreversibly labeled proteins by fluorography revealed a single specifically labeled protein band of 116 kDa, which was completely blocked by co-incubation with 1 mM glycine, 10 μM unlabeled CGP 61594 or 10 μM DCKA (Fig. 8B). In addition to the 116 kDa protein, a faintly radioactive band was observed at 94 kDa, which was insensitive to the presence of glycine, CGP 61594 or DCKA and thus represented non-specific labeling.

The 116 kDa protein band that was specifically photolabeled by [3H]CGP 61594 in brain membrane preparations corresponded in size to the NR1 subunit of the NMDA receptors (Benke et al., 1995; Brose et al., 1993). The identity of this 116 kDa protein as the NR1 subunit was analyzed immunobiochemically. Brain
membranes were subjected to photolabeling with [³H]CGP 61594 (25 nM) and solubilized with the detergent mixture RIPA (Sheng et al., 1994). The solubilized fraction, which contained about 30% of the photolabeled 116 kDa protein, was diluted with Tris buffer and subjected to NR1 subunit-specific immunoaffinity chromatography. About 80-100% of the solubilized photolabeled 116 kDa protein bound to the NR1 immunoaffinity column as analyzed by determination of the amount of radioactivity in gel slices containing the 116 kDa protein before and after subjection of the solubilisate to the immunoaffinity column. 50-60% of the photolabeled proteins retained on the immunoaffinity column could be eluted by changing the pH-value to 11.5 and were analyzed by SDS-PAGE and fluorography. A single photolabeled protein band with a size of 116 kDa was observed in the eluate (Fig. 8D), indicating that the protein labeled in membrane preparations by [³H]CGP 61594 is the NR1 subunit.

These results suggest that the NR1 subunit of native NMDA receptors represents the main constituent of the glycine binding domain. In contrast, the NR2 subunits (140-180 kDa) are not major targets for [³H]CGP 61594 photolabeling since labeled proteins of higher molecular weight were observed neither in crude membrane preparations (Fig. 8B) nor in immunoaffinity-purified NMDA receptors (Fig. 8D).

4.4.2 [³H]CGP 61594 photolabeling of recombinant NMDA receptors

Photolabeling of rat brain membranes with [³H]CGP 61594 attributed the glycine site of native NMDA receptors to the NR1 subunit (Fig. 8B, D). To identify whether the same target specificity would be found on recombinant NMDA receptors, HEK 293 cells were transfected with NR1 cDNA alone or together with NR2B cDNA. In reversible binding experiments, [³H]CGP 61594 bound specifically and with high affinity to homogenates prepared from cells expressing homomeric NR1 receptors or heteromeric NR1/2B receptors (Fig. 10A). Likewise, in photolabeling experiments the 116 kDa band was detected in cells transfected with the NR1 subunit alone or together with NR2B (Fig. 10B). Again, the NR2 subunit was not labeled in heteromeric receptors, confirming that the NR1 subunit is the main target of the interaction with [³H]CGP 61594 also in recombinant receptors.
4.4.3 [³H]CGP 61594 binding to mutated recombinant NMDA receptors

To identify a structural element contributing to the [³H]CGP 61594 site, binding of [³H]CGP 61594 to point mutated NMDA receptors was analyzed. A mutated NR1 subunit in which phenylalanine 466 was replaced by a histidine was used for reversible and irreversible binding assays. This mutation had been shown to increase the EC₅₀ for glycine 2150-fold and that for the glycine site antagonist DCKA 160-fold (Kuryatov et al., 1994) suggesting that F466 is a crucial residue shaping the binding pocket for glycine site agonists and antagonists. Both, homomeric and heteromeric receptors containing the mutated NR1 subunit (NR1⁴⁶⁶H) displayed no specific [³H]CGP 61594 binding, not even at high radioligand concentrations (200 nM) (Fig. 10A). Correspondingly, in photocrosslinking experiments labeling of the 116 kDa band was not detected in cells transfected with the mutated NR1 subunit cDNA (Fig. 10B). These experiments suggest that F466 on the NR1 subunit is an essential amino acid for the constitution of the glycine antagonist binding pocket of homomeric NR1 as well as heteromeric NR1/2B receptors. Moreover, these results clearly demonstrate that the NR1 subunit is necessary and sufficient for the formation of a high affinity binding site for the glycine antagonist [³H]CGP 61594.
**Fig. 10:** [$^3$H]CGP 61594 binding to wild-type and point mutated recombinant NMDA receptors

(A) HEK 293 cells expressing wild-type NR1 or mutant NR1$_{F466H}$ subunits alone or in combination with NR2B were incubated with 200 nM [$^3$H]CGP 61594 in the presence or absence of 1 mM glycine. The amount of specifically bound radioactivity was not the same for the homomeric and heteromeric NR1/2B receptors, most likely owing to different transfection efficiencies. The data shown are the mean ± S.D. of three independent experiments. (B) HEK 293 cells expressing wild-type NR1 or mutant NR1$_{F466H}$ subunits alone or in combination with NR2B were incubated with 25 nM [$^3$H]CGP 61594, irradiated with UV-light and subjected to SDS-PAGE and fluorography. The fluorogram shows total labeling (-) and non-specific labeling (+) in the presence of 1 mM glycine.
4.5 Heterogeneity of [$^3$H]CGP 61594 binding sites among NMDA receptor subtypes

The heteromeric assembly of the NR1 subunit with different NR2 subunit variants gives rise to receptor subtypes with distinct pharmacological properties (Buller et al., 1994; Chazot et al., 1994; Ishii et al., 1993; Laurie and Seeburg, 1994; Priestley et al., 1995). Previous experiments suggested that the glycine agonist binding domain but not the glycine antagonist binding domain on the NR1 subunit is sensitive to modulation by the NR2 subunit variant present in the receptor complex (Grimwood et al., 1995; Priestley et al., 1996). However, the lack of high affinity binding sites of [$^3$H]CGP 61594 in the cerebellum and the presence of distinct low affinity [$^3$H]CGP 61594 binding sites in hippocampus/cortex and cerebellum (see above) pointed to a heterogeneity of the glycine antagonist site as well. Therefore, a putative differentiation of [$^3$H]CGP 61594 binding sites among native and recombinant NMDA receptor subtypes was investigated in detail.

4.5.1 Regional distribution of high affinity [$^3$H]CGP 61594 binding sites

The NR1 subunit, which is the main constituent of the [$^3$H]CGP 61594 binding site, is ubiquitously expressed throughout the CNS whereas the NR2 subunits show a characteristic and more restricted pattern of distribution (Benke et al., 1995; Wenzel et al., 1995). To analyze whether [$^3$H]CGP 61594 recognizes the entire population of NMDA receptors, the distribution of high affinity [$^3$H]CGP 61594 binding sites was visualized autoradiographically on parasagittal cryostat sections of adult rat brain. Surprisingly, high affinity [$^3$H]CGP 61594 binding was mainly restricted to forebrain regions with highest densities in the CA1 region and the dentate gyrus of the hippocampal formation, followed by cerebral cortex, striatum, olfactory bulb and thalamus (Fig. 11A). In mid- and hindbrain areas only marginal labeling of receptors over background was seen. The distribution of [$^3$H]CGP 61594 binding sites clearly contrasts to that of the NR1 subunit which was detected in virtually all brain areas including cerebellum and brain stem, as shown by the histoblot technique using an NR1 subunit-selective antiserum (Fig. 11C). Thus, only a subpopulation of NMDA receptors, which is almost exclusively present in the forebrain, displayed high affinity [$^3$H]CGP 61594 binding sites. Interestingly, this pattern of [$^3$H]CGP 61594 binding sites strikingly corresponded to the distribution of the NR2B
Fig. 11: Regional distribution of $[^3\text{H}]$CGP 61594 binding sites and of NR1 and NR2B subunits in adult rat brain.

(A) Parasagittal cryostat sections (12 μm) were incubated with 10 nM $[^3\text{H}]$CGP 61594 and exposed to Hyperfilm-$^3\text{H}$ for 15 days. Colour-coding of the section ranges from black for background to blue, orange and yellow for maximal staining intensity. (B, C) Parasagittal cryostat sections of adult rat brain (16 μm) were blotted onto nitrocellulose membranes and stained with affinity-purified antisera recognizing the subunits NR1 (B) and NR2B (C).
subunit in histoblots (Fig. 11B). In line with this finding, high affinity sites for 
[^3]H]CGP 61594 were absent in the cerebellum which lack expression of the 
NR2B subunit. These results suggest that the restricted distribution pattern of 
[^3]H]CGP 61594 may be due to a preferential interaction with the NR1 subunit of 
NMDA receptors containing the NR2B subunit.

The autoradiographic determination of the expression pattern and level of high 
affinity[^3]H]CGP 61594 binding sites was performed with a radioligand 
concentration (10 nM) well below the K_D-value (23 nM) in order to minimize 
non-specific binding. Thus, minor changes in the affinity for[^3]H]CGP 61594 in 
different brain regions would considerably affect the signal intensity. To 
exclude that the regional variation in the density of high affinity[^3]H]CGP 61594 
binding sites is due to a shift in the affinity of[^3]H]CGP 61594, the K_D-values of 
[^3]H]CGP 61594 binding sites were determined in different brain regions. 
Scatchard analysis of saturation binding experiments revealed that the affinity 
of[^3]H]CGP 61594 within the range of 2-200 nM was unchanged among the 
different brain regions tested (Tab. 5). The highest maximum number of binding 
sites (B_max-values) was observed in the hippocampal formation, followed by the 
cerebral cortex and striatum. Lower B_max-values were apparent in olfactory bulb 
and thalamus, while very low binding was found in brainstem regions 
(mesencephalon, medulla/pons). This rank order of B_max-values closely 
corresponded to the regional labeling intensities in brain slices (Fig. 11A).

As[^3]H]CGP 61594 specifically photolabels the NR1 subunit of NMDA receptors 
in membranes prepared from hippocampus/cortex, it was tested whether this 
molecular target specificity was retained in other brain regions containing high 
nM[^3]H]CGP 61594), photolabeled proteins were resolved by SDS-PAGE and 
visualized by fluorography. In all brain areas analyzed, a single protein of 116 
kDa was specifically photolabeled, corresponding in size to the NR1 subunit 
(Fig. 12). The relative radiolabeling intensity of the 116 kDa protein band varied 
in different regions with a rank order corresponding to that of the B_max-values in 
reversible binding in the respective brain regions (Tab. 5). Thus, the molecular 
specificity of[^3]H]CGP 61594 photolabeling was unchanged in different brain 
regions. Furthermore, photolabeling of cerebellar membranes which lack high 
affinity[^3]H]CGP 61594 binding sites in reversible experiments did not yield a 
photoaffinity labeled protein.
The NR2B-like distribution of the high affinity \( ^{3}H \)CGP 61594 binding sites detected in the autoradiography points to a selective interaction of \( ^{3}H \)CGP 61594 with the NMDA receptor subtype NR1/2B. The hypothesis of a high affinity interaction with a distinct NMDA receptor subtype is further supported by the finding that the affinity (within a range of 2-200 nM) as well as the target specificity of \( ^{3}H \)CGP 61594 were unchanged within the various brain regions.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>( K_D ) (nM)</th>
<th>( B_{\text{max}} ) (pmol/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampal formation</td>
<td>22 ± 1</td>
<td>6.43 ± 1.07</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>25 ± 3</td>
<td>5.47 ± 0.62</td>
</tr>
<tr>
<td>Striatum</td>
<td>22 ± 1</td>
<td>4.51 ± 0.53</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>25 ± 1</td>
<td>2.75 ± 0.24</td>
</tr>
<tr>
<td>Thalamus / Hypothalamus</td>
<td>21 ± 1</td>
<td>2.46 ± 0.61</td>
</tr>
<tr>
<td>Mesencephalon</td>
<td>23 ± 2</td>
<td>1.54 ± 0.08</td>
</tr>
<tr>
<td>Medulla / Pons</td>
<td>23 ± 2</td>
<td>0.92 ± 0.07</td>
</tr>
</tbody>
</table>

Tab. 5: Affinity and abundance of \( ^{3}H \)CGP 61594 binding sites in different brain regions
Membranes from the indicated brain regions were incubated with \( ^{3}H \)CGP 61594 (2-200 nM). \( K_D \) and \( B_{\text{max}} \) values were determined by Scatchard transformation of reversible binding data. Non-specific \( ^{3}H \)CGP 61594 binding was determined in the presence of 1 mM glycine. The data represent the mean ± S.D. of three independent experiments.

4.5.2 Developmental expression of \( ^{3}H \)CGP 61594 binding sites
Each NMDA receptor subunit displays a characteristic tempo-spatial expression pattern during embryonic and postnatal development (Benke et al., 1995; Monyer et al., 1992; Watanabe et al., 1992; Wenzel et al., 1997). For instance, the NMDA receptor subunits NR1, NR2B and NR2D are already well expressed in embryonic and neonatal CNS structures, whereas NR2A and NR2C subunits emerge later in postnatal development. Thus, considerable amounts of high affinity \( ^{3}H \)CGP 61594 binding sites present in neonatal brain would point to the
Photolabeling of membranes from selected brain regions with [³H]CGP 61594

Membranes from the indicated brain regions (60 μg protein) were incubated with 25 nM [³H]CGP 61594, irradiated with UV-light and subjected to SDS-PAGE and fluorography. The fluorogram shows total labeling (left lane) and non-specific labeling (right lane) in the presence of 1 mM glycine for each brain region (Hi, hippocampus; Cx, cerebral cortex; St, striatum; Cb, cerebellum). In addition to the specifically incorporated activity at 116 kDa faint non-specific labeling was detected in cerebral cortex at about 120 kDa that was not displaced by 1 mM glycine or 10 μM CGP 61594.

Labeling of NR1/2B receptors, since the NR1/2D subtype represents only a minor receptor population and the NR1/2A and NR1/2C subtypes are hardly detectable or absent at birth, respectively. Indeed, Scatchard analysis of reversible binding data using membranes prepared from whole rat brains of various postnatal stages demonstrated large amounts of high affinity [³H]CGP 61594 binding sites already in neonatal brain (Bₘₐₓ = 1.54 ± 0.57 pmol/mg protein). The number of [³H]CGP 61594 binding sites increased after birth and reached a peak in expression three weeks after birth, decreasing slightly thereafter to adult levels (Fig. 13A, Tab. 6). Hence, the expression of [³H]CGP 61594 binding sites parallels the developmental expression levels of the NR1 and NR2B proteins (Benke et al., 1995; Wenzel et al., 1997). Moreover, at all developmental stages tested, the affinity of [³H]CGP 61594 remained unchanged over a concentration range of 2-200 nM (Tab. 5), and irreversible labeling of membranes prepared from different developmental stages revealed a selective labeling of the NR1 subunit (Fig. 13B). The invariability of the photoaffinity target specificity and of the high affinity Kᵦ-value of [³H]CGP 61594
suggests that its binding site does not undergo a developmental maturation. Furthermore, the developmental course of the expression of high affinity [³H]CGP 61594 binding site further supports the view of a preferential interaction of [³H]CGP 61594 with NR1/2B receptors.

<table>
<thead>
<tr>
<th>Age</th>
<th>K_D (nM)</th>
<th>B_max (pmol/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>21 ± 3</td>
<td>1.54 ± 0.57</td>
</tr>
<tr>
<td>P5</td>
<td>22 ± 3</td>
<td>2.14 ± 0.41</td>
</tr>
<tr>
<td>P10</td>
<td>23 ± 5</td>
<td>2.64 ± 0.43</td>
</tr>
<tr>
<td>P21</td>
<td>22 ± 2</td>
<td>4.38 ± 0.24</td>
</tr>
<tr>
<td>Adult</td>
<td>21 ± 2</td>
<td>3.23 ± 0.71</td>
</tr>
</tbody>
</table>

Tab. 6: Affinity and abundance of [³H]CGP 61594 binding sites during postnatal development
Membranes from the indicated developmental stages were incubated with [³H]CGP 61594 (2-200 nM). K_D- and B_max-values were determined by Scatchard transformation of reversible binding data. Non-specific [³H]CGP 61594 binding was determined in the presence of 1 mM glycine. The data represent the mean ± S.D. of three independent experiments.
Fig. 13: Reversible (A) and irreversible (B) binding of [³H]CGP 61594 to brain membranes from different developmental stages

(A) Membranes from the indicated developmental stages (postnatal day (P) 0, P5, P10, P21 and adult) were incubated with [³H]CGP 61594 (2-200 nM). Bmax-values were obtained by Scatchard transformation of reversible binding data. Non-specific [³H]CGP 61594 binding was determined in the presence of 1 mM glycine. The data shown represent the mean ± S.D. of three independent experiments. Bmax- and Kd-values are given in Tab. 6. (B) Membranes prepared from brains of the indicated developmental stage were incubated with 25 nM [³H]CGP 61594, irradiated with UV light and subjected to SDS-PAGE and fluorography. The fluorogram shows total labeling (left lane) and non-specific labeling (right lane) in the presence of 1 mM glycine for each developmental stage (P0, P5, P10, P21, adult).
4.5.3 Immunoprecipitation of photolabeled NMDA receptor subtypes
The hypothesis of a preferential interaction of $[^3]$H]CGP 61594 with native NMDA receptors containing the NR2B subunit was tested by immunoprecipitation experiments. Since the NR1 subunit is selectively targeted by $[^3]$H]CGP 61594, immunoprecipitation of photolabeled receptors with NR2 subunit-selective antisera was expected to identify the NMDA receptor subtype constituting high affinity $[^3]$H]CGP 61594 binding sites. Membranes prepared from whole adult brain were photoaffinity-labeled in the presence of 25 nM $[^3]$H]CGP 61594 and solubilized with the detergent mixture RIPA (Sheng et al., 1994). NMDA receptors were immunoprecipitated with antisera selective for the subunits NR1, NR2A, NR2B, NR2C or NR2D and the radioactivity incorporated in the immunoprecipitates was measured by liquid scintillation counting. Non-specifically precipitated radioactivity was determined by co-incubating the antiserum with an excess of the respective peptide antigen. Photolabeled proteins were specifically immunoprecipitated only by antisera directed against the NR1 or NR2B subunits (Fig. 14A). In contrast, the NR2A, NR2C or NR2D antibodies did not yield specific immunoprecipitation of photolabeled receptors (Fig. 14A), although the respective NMDA receptor subtypes could be immunoprecipitated by the antisera as verified by Western blotting (Fig. 14B). The amount of radioactivity immunoprecipitated was higher with the NR1 antiserum than with the NR2B antiserum, possibly due to differences in the reactivity of the NR1 and NR2B antisera. These findings provide strong evidence for the view that $[^3]$H]CGP 61594 interacts with high affinity selectively with native NMDA receptors containing the NR1 and NR2B subunits.
Fig. 14: Immunoprecipitation of NMDA receptors irreversibly labeled with \[^{3}H\]CGP 61594 by subunit-specific antisera.

(A) Total brain membranes were photolabeled with 25 nM \[^{3}H\]CGP 61594, solubilized and immunoprecipitated with antisera directed against the NMDA receptor subunits NR1, NR2A, NR2B, NR2C or NR2D. The radioactivity in the immunoprecipitates was determined by liquid scintillation counting. Non-specific precipitation was determined in parallel assays in presence of the respective peptide antigen (10 \(\mu\)g/ml). The columns show the mean of 2-3 independent experiments and the maximum and minimum value. (B) The ability of the different antisera to immunoprecipitate the respective receptor subtype was verified by the detection of the NR1 subunits in Western blots of each immunoprecipitate. Non-specific immunoreactivity (right lane) was determined in presence of the respective peptide antigen (10 \(\mu\)g/ml).
4.5.4 $[^{3}H]$CGP 61594 binding to recombinant NMDA receptor subtypes

The putative preferential interaction of $[^{3}H]$CGP 61594 with NR1/2B receptors was further examined on recombinant receptors with a defined subunit composition. Homomeric NR1 and heteromeric NR1/2A, NR1/2B, NR1/2C and NR1/2D receptors were transiently expressed in HEK 293 cells and their interaction with $[^{3}H]$CGP 61594 was measured in the absence and presence of 1 mM glycine to determine total and non-specific binding. Homomeric NR1 receptors and, surprisingly, all heteromeric subunit combinations exhibited specific high affinity $[^{3}H]$CGP 61594 binding under reversible and photolabeling conditions (Fig. 15A, B). This result clearly contradicts the data of the distribution of $[^{3}H]$CGP 61594 binding sites in situ as well as the immunoprecipitation of photolabeled native NMDA receptor subtypes. However, recently it was shown that homomeric NR1 receptors are present in HEK 293 cells co-transfected with the NR2A subunit cDNA (Grimwood et al., 1996). Therefore, the observed specific labeling of the recombinant subtypes NR1/2A, NR1/2C and NR1/2D is most likely due to additional homomeric NR1 receptors present in HEK 293 cells, even when the NR1 subunit was co-expressed together with an NR2 subunit.

This hypothesis was investigated in a binding assay which only detects heteromeric NR1/NR2 receptors. Glutamate antagonists, such as AP5, bind to a site mainly formed by domains on the NR2 subunits and, thus, interact only with heteromeric receptors but virtually lack affinity for homomeric NR1 receptors (Kendrick et al., 1996; Laurie and Seeburg, 1994; Lynch et al., 1995). As a consequence, glutamate antagonists do not modulate glycine antagonist binding to NR1 homomeric receptors as shown for $[^{3}H]$-689,560 and $[^{3}H]$MDL 105,519 binding (Grimwood et al., 1995; Siegel et al., 1996). An allosteric modulation of the glycine binding site by AP5 was therefore expected to occur only at heteromeric receptors. High affinity $[^{3}H]$CGP 61594 binding was performed in the presence and absence of 10 μM AP5 to HEK 293 cells transfected with cDNAs of the NR1 subunit or the subunit combinations NR1/2A, NR1/2B, NR1/2C and NR1/2D. As expected, $[^{3}H]$CGP 61594 binding to homomeric NR1 receptors was not modulated by AP5. Among the heteromeric combinations tested, HEK 293 cells transfected with the NR1/2B combination displayed inhibition of $[^{3}H]$CGP 61594 binding by AP5 (Fig. 15C). The extent of allosteric inhibition of $[^{3}H]$CGP 61594 was comparable to the one observed in modulation experiments using membranes from hippocampus/cortex (Tab. 4).
In contrast, [3H]CGP 61594 binding to cells transfected with the cDNA for NR1 in combination with the cDNAs of NR2A, NR2C or NR2D remained unaffected (Fig. 15C).

![Graph A]

![Graph B]

![Graph C]

Fig. 15: Reversible binding (A), photolabeling (B) and allosteric modulation (C) of recombinant NMDA receptor subtypes.

(A) HEK 293 cells, transfected with the NR1 subunit cDNA alone or in combination with the HEK
293 cells, transfected with the NR1 subunit cDNA alone or in combination with the indicated NR2 subunit cDNA, were incubated with 25 nM [³H]CGP 61594 in the presence or absence of 1 mM glycine. The columns represent the percentage of specific binding. The amount of specifically bound radioactivity was not the same for the homomeric and the various heteromeric receptors, most likely owing to different transfection efficiencies. The data shown are the mean ± S.D. of three independent experiments. (B) HEK 293 cells, transfected with the NR1 subunit cDNA alone or in combination with the NR2 subunit cDNA indicated, were incubated with 25 nM [³H]CGP 61594 in the presence or absence of 1 mM glycine followed by SDS-PAGE and fluorography. Total and non-specific photolabeling are shown in adjacent lanes. A 116 kDa protein, corresponding to the NR1 subunit, was ubiquitously labeled presumably due to the presence of homomeric NR1 receptors which coexist with heteromeric NR1/NR2 receptors. (C) HEK 293 cells, transfected with the NR1 subunit cDNA alone or in combination with one type of NR2 subunit cDNA, were incubated with 25 nM [³H]CGP 61594 in absence or presence of 10 µM AP5. Non-specific binding was determined in presence of 1 mM glycine. The columns represent the percentage of specific binding which is inhibited by AP5. Since AP5 virtually lacks affinity for homomeric NR1 receptors, inhibition of [³H]CGP 61594 by AP5 is expected to occur only at heteromeric NR1/NR2 receptors. The data shown are the mean ± S.D. of three independent experiments.

4.5.5 Electrophysiological analysis on recombinant NMDA receptors

The preferential interaction of [³H]CGP 61594 with NR1/2B receptors was finally proven functionally in an electrophysiological analysis using the Xenopus oocyte expression system. This test system was chosen since homomeric NR1 receptors, which might be co-expressed together with heteromeric receptors, do not interfere with the analysis, because they generate only very small currents compared to heteromeric receptors (Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992). Glutamate-evoked responses were measured in the presence of glycine concentrations eliciting a half-maximal response (Kuryatov et al., 1994; Laube et al., 1997). As expected, the glutamate-evoked currents were inhibited by CGP 61594 with high potency only when the NR1 and NR2B subunits were co-expressed (IC⁵₀ = 45 ± 11 nM, Fig. 16). However, there was no absolute selectivity for the NR1/2B subtype since the other heteromeric receptors were also sensitive to inhibition by CGP 61594 at higher concentrations (Fig. 16). The IC⁵₀-value determined for the NR1/2C receptor was four times higher (IC⁵₀ = 164 ± 27 nM) as compared to the NR1/2B combination. An even lower inhibitory potency of CGP 61594 was found for the NR1/2A receptor (IC⁵₀ = 430 ± 105 nM) and the NR1/2D receptor (IC⁵₀ = 340 ± 61 nM). Hence, the major NR2 variants contribute to distinct glycine antagonist
sites as shown by an up to 10-fold difference in their sensitivity to inhibition by CGP 61594.

![Graph showing inhibition of glutamate-evoked currents by CGP 61594 of different NMDA receptor subtypes expressed in Xenopus oocytes.](image)

**Fig. 16**: Inhibition of glutamate-evoked currents by CGP 61594 of different heteromeric NMDA receptor subtypes expressed in *Xenopus* oocytes. Defolliculated *Xenopus* oocytes were co-injected with cRNAs of the NR1 subunit and one type of NR2 subunit. The inhibition curves of CGP 61594 for the subunit combinations NR1/2A, NR1/2B, NR1/2C and NR1/2D are given. The inhibitory constants (IC₅₀), the Hill coefficients (nₗ) and the number of experiments (n) were: NR1/2A, IC₅₀ = 430 ± 105 nM, nₗ = 1.46, n = 4; NR1/2B, IC₅₀ = 45 ± 11 nM, nₗ = 1.4, n = 7; NR1/2C, IC₅₀ = 164 ± 27 nM, nₗ = 1.6, n = 7; NR1/2D, IC₅₀ = 340 ± 61 nM, nₗ = 1.59, n = 4.

### 4.5.6 Overview of CGP 61594 binding sites

The various CGP 61594 binding sites formed by different recombinant heteromeric receptor assemblies can be correlated with CGP 61594 binding sites of native NMDA receptors identified in rat brain membranes prepared from different brain regions (Tab. 7). In hippocampus/cortex membranes, a high affinity binding site for [³H]CGP 61594 was identified (Kᵦ = 23 ± 2 nM) which can be attributed to the high affinity binding site in recombinant NR1/2B receptors (IC₅₀ = 45 ± 11 nM). In the same brain regions, a second binding site for [³H]CGP 61594 with a 10-fold lower affinity was detected (IC₅₀ = 371 ± 85 nM) which resembled that of recombinant NR1/2A receptors (IC₅₀ = 430 ± 105 nM).
Since the hippocampus and cerebral cortex preferentially express NR2A and NR2B subunits and largely lack the NR2C and NR2D subunits (Watanabe et al., 1993; Wenzel et al., 1995), this low affinity binding site most likely represents NR1/2A receptors. A contribution of NR1/2B receptors to this low affinity binding site could be ruled out because a low affinity CGP 61594 binding component was not detected in NR1/2B receptors in the electrophysiological analysis (Fig. 16). A CGP 61594 binding site with an intermediate affinity was identified in cerebellar membranes (IC$_{50}$ = 231 ± 56 nM) which corresponds well to the affinity observed for recombinant NR1/2C receptors (IC$_{50}$ = 164 ± 27 nM). The adult cerebellum contains almost exclusively NR2A and NR2C subunits but virtually lacks NR2B and NR2D subunits (Monyer et al., 1994; Watanabe et al., 1994; Wenzel et al., 1997; Wenzel et al., 1996). Therefore, the $[^3]$HCGP 61594 binding site with an intermediate affinity present in the cerebellum can be attributed to NMDA receptor subtypes containing the NR2C. However, a contribution of NR1/2A receptors to this IC$_{50}$ is also likely.

In conclusion, native as well as recombinant NMDA receptor subtypes can be clearly distinguished by different affinities of $[^3]$HCGP 61594. Therefore, apart from serving as a covalent marker for the glycine site of NMDA receptors, $[^3]$HCGP 61594 represents the first radioligand that reveals a heterogeneity of the glycine antagonist binding site of NMDA receptors and may thus be used in the future as a tool for the differentiation of glycine sites.

<table>
<thead>
<tr>
<th>Table 7: Correlation of CGP 61594 binding sites identified in recombinant and native NMDA receptor subtypes.</th>
<th>High affinity</th>
<th>Intermediate affinity</th>
<th>Low affinity</th>
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<tr>
<td><strong>Recombinant</strong></td>
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<tr>
<td>Affinity</td>
<td>IC$_{50}$ = 45 ± 11 nM</td>
<td>IC$_{50}$ = 164 ± 27 nM</td>
<td>IC$_{50}$ = 430 ± 105 nM</td>
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<tr>
<td>Subtype</td>
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<td>NR1/2C</td>
<td>NR1/2A</td>
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<td><strong>Native</strong></td>
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<tr>
<td>Affinity</td>
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<td>IC$_{50}$ = 231 ± 56 nM</td>
<td>IC$_{50}$ = 371 ± 85 nM</td>
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5. Discussion

The novel glycine site antagonist and photoaffinity ligand [3H]CGP 61594 was developed as a tool for the structural characterization of the glycine binding domain and for the identification of a putative selective interaction with distinct NMDA receptor subtypes. Using this photolabel, the present study provides an extensive analysis of the glycine antagonist site of NMDA receptors and yields three main results:

- [3H]CGP 61594 binds selectively and with high affinity to the glycine site of native NMDA receptors.
- As a photoaffinity ligand [3H]CGP 61594 serves as the first covalent marker for the glycine binding site which is a prerequisite for a structural characterization of this site in native NMDA receptors. By means of the photolabeling approach [3H]CGP 61594 identifies the NR1 subunit as the main constituent of the glycine binding domain.
- A heterogeneity of glycine antagonist sites among NMDA receptor subtypes is demonstrated for the first time. High affinity binding sites for [3H]CGP 61594 are exclusively displayed by NR1/2B receptors whereas the other NMDA receptor subtypes are clearly distinguished by reduced affinities.

5.1 Specific and high affinity interaction of [3H]CGP 61594 with the glycine site of NMDA receptors

[3H]CGP 61594 was synthesized as a derivative of the specific and high affinity glycine site antagonist L-689,560 (Grimwood et al., 1992), carrying a photoreactive azide group for a covalent crosslinking of the molecule to its binding domain. Several lines of evidence demonstrate that [3H]CGP 61594 specifically interacts with the glycine site of NMDA receptors:

1. Scatchard analysis of reversible binding data revealed a high affinity interaction of [3H]CGP 61594 with a single binding site within a concentration range of 2-200 nM (Fig. 7). The maximum number of binding sites corresponded to the number determined in radioligand binding studies using the specific glycine site ligands [3H]DCKA and [3H]L-689,560 (Grimwood et al., 1992; Widdowson et al., 1995; Yoneda et al., 1994). Moreover, [3H]CGP 61594 binding was inhibited by glycine in a competitive manner as shown by an increase in the $K_D$-value without alteration of the $B_{max}$-value and displayed a
non-competitive interaction with the polyamine agonist site and glutamate antagonist site (Tab. 2). (2) The displacing potencies of various glycine site agonists, partial agonists and antagonists corresponded to those reported for previously available glycine site radioligands (Danysz et al., 1990; Grimwood et al., 1992; Monahan et al., 1989; Monahan et al., 1990; Yoneda et al., 1994). Moreover, [³H]CGP 61594 did neither interact with the strychnine-sensitive glycine receptor (GlyR) nor with the AMPA and kainate receptors since [³H]CGP 61594 binding was not influenced by the respective ligands (Tab. 3). (3) Binding of [³H]CGP 61594 was allosterically modulated by ligands of the glutamate and polyamine sites in accordance with previous reports on [³H]L-689,560 and [³H]DCKA binding (Grimwood et al., 1993; Yoneda et al., 1994). Modulation of binding occurred in a stereoselective manner as shown for D- and L-CPP as well as the enantiomers CGP 40116 and CGP 40117 (Tab. 4). (4) The antagonistic action of CGP 61594 to block NMDA-evoked currents in brain slices could be reversed by application of D-serine, a potent and specific glycine site agonist of the NMDA receptor (Fig. 6). (5) Binding of [³H]CGP 61594 to recombinant NMDA receptors carrying a point mutation in the putative glycine binding domain was completely abolished (Fig. 10).

In conclusion, [³H]CGP 61594 represents a novel photoaffinity radioligand that interacts with high affinity and selectivity with the glycine site of the NMDA receptor and can thus be used as a tool for the structural analysis of this site.

5.2 Targeting the glycine binding domain by photoaffinity labeling

The structural characterization of the glycine binding site of native NMDA receptors has not been achieved so far since specific radioligands for the covalent labeling of this binding site have not been available. This experimental problem was approached by the development of the photoaffinity ligand [³H]CGP 61594 which is irreversibly crosslinked to the glycine binding domain upon photoactivation. Photolabeling with [³H]CGP 61594 allowed a direct identification of the protein that is involved in the formation of the glycine binding site of NMDA receptors in situ.

5.2.1 Efficiency and specificity of [³H]CGP 61594 photolabeling

Photoaffinity labeling with [³H]CGP 61594 was carried out at a wave length of 254 nm because irradiation at a larger wave length (366 nM) yielded extremely
low labeling efficiencies. Although a wave length of 254 nM represents harsh conditions for protein irradiation, photolabeling did not induce alterations in the glycine binding site since pre-irradiation of membranes prior to incubation with \( ^3\text{H} \text{CGP 61594} \) did not change the capacity of the membranes for reversible and irreversible \( ^3\text{H} \text{CGP 61594} \) binding. Interestingly, pre-irradiation of \( ^3\text{H} \text{CGP 61594} \) before the binding experiment did not result in a loss in specific reversible binding activity, indicating that UV-illumination does not provoke a complete decomposition of the molecule. As expected, the ability of \( ^3\text{H} \text{CGP 61594} \) to be irreversibly crosslinked was abolished by pre-irradiation of the photoaffinity ligand.

7.5% of reversibly bound \( ^3\text{H} \text{CGP 61594} \) was covalently linked to the protein constituent (Tab. 2), a value that is lower compared to previous reports for other photoaffinity ligands (Dellouve-Courillon et al., 1989; Foucaud et al., 1995; McIntosh et al., 1996) but the reason for this modest efficiency is unknown. The displacement and allosteric modulation of \( ^3\text{H} \text{CGP 61594} \) photolabeling occurred in a degree and with a stereoselectivity corresponding to that seen in reversible binding experiments (Tab. 3, 4) confirming that UV-illumination at 254 nM does not alter the specificity of \( ^3\text{H} \text{CGP 61594} \) for the glycine site of the NMDA receptor.

5.2.2 Photolabeling of the \( ^3\text{H} \text{CGP 61594} \) binding domain

The photoaffinity ligand \( ^3\text{H} \text{CGP 61594} \) allowed a direct identification of the protein constituent that is involved in the formation of the glycine binding site of native and recombinant NMDA receptors. Photocrosslinking of \( ^3\text{H} \text{CGP 61594} \) to its binding domain resulted in the irreversible labeling of a protein band with a size of 116 kDa (Fig. 8B). The structural component of the photolabeled 116 kDa protein represents the NR1 subunit of the NMDA receptor as demonstrated by the following findings:

1. The 116 kDa photolabeled protein in membrane preparations corresponded in size to the NR1 subunit of native NMDA receptors (Benke et al., 1995; Brose et al., 1993).
2. In HEK 293 cells transiently transfected with the NR1 cDNA, but not in mock transfected cells, a 116 kDa protein was specifically photolabeled (Fig. 10B).
3. Introduction of a point mutation in the putative glycine binding domain on the NR1 subunit abolished photolabeling of the NR1 subunit in transfected HEK 293 cells (Fig. 10B).
4. The photolabeled 116 kDa
protein in brain membranes was specifically retained on an NR1 subunit-specific immunoaffinity column (Fig. 8D).

Thus, the main protein constituent of the glycine binding site of native and recombinant NMDA receptors is formed by the NR1 subunit and not by the NR2 subunits which have larger molecular weights (140-175 kDa). Furthermore, these results corroborate earlier findings that the structure of the glycine recognition site for agonists and antagonists is fully represented by recombinant homomeric NR1 receptors (Grimwood et al., 1995) and suggest that the glycine site is similarly formed in native and recombinant NMDA receptors. In contrast, the binding domain for glutamate antagonists clearly differed in native and recombinant NMDA receptors as revealed by the pattern of photoreactive proteins of the glutamate site antagonist and photoaffinity ligand $^{[125]}$I$^{[125]}$CGP 55802A (Marti et al., 1993). Moreover, the glutamate antagonist site was shown to undergo a developmental maturation (Marti et al., 1993) suggesting that this binding site of native NMDA receptors is influenced by other factors such as an additional subunit or post-translational modification. The present study, however, demonstrates that the $[^3H]$CGP 61594 photolabeling pattern is unchanged (1) among various brain regions (Fig. 12), (2) during brain ontogeny (Fig. 13), (3) in native and recombinant NMDA receptors (Fig. 8, 10) and (4) in recombinant homomeric and heteromeric NMDA receptors (Fig. 10).

5.2.3 Structure of the glycine binding domain

Four distinct domains on the NR1 subunit were previously suggested to be involved in the formation of the glycine binding site (Hirai et al., 1996; Kuryatov et al., 1994; Wafford et al., 1995; Williams et al., 1996). Interestingly, different determinants appeared to be crucial for agonist and antagonist binding. Several residues within all four domains were reported to affect glycine potency whereas for high affinity binding of glycine antagonists only the aromatic side chains of F390, Y392 and F466 in the first and second domain were found to be essential (Kuryatov et al., 1994). Replacement of F466 by a non-aromatic residue (histidine) decreased the EC$_{50}$ for glycine more than 1000-fold and the IC$_{50}$ for the glycine antagonist DCKA 160-fold (Kuryatov et al., 1994). It remained yet unknown whether this mutation affected the ligand binding domain directly or rather played a role in signal transduction. The photoaffinity label $[^3H]$CGP 61594 now provides the first tool which permits the direct identification of a structural determinant of the glycine antagonist domain by its
covalent attachment to amino acid(s) within the glycine binding pocket. Recombinant receptors containing the F466H point mutation on the NR1 subunit lacked specific [³H]CGP 61594 binding both under reversible and photolabeling conditions (Fig. 10), thus demonstrating the importance of the hydrophobic amino acid residue F466 for the formation of the glycine antagonist binding domain. By identifying the direct site(s) of irreversible labeling by [³H]CGP 61594, this photoaffinity ligand is expected to be a valuable tool for identifying additional amino acids within the glycine binding site on the NR1 subunit and to shed some light on the spatial organization of the binding pocket.

Sequence alignments of the NR1 and NR2 subunits with the soluble bacterial amino acid binding protein LAOBP gave first insights into the putative three-dimensional structural organization of the glycine site (Hirai et al., 1996; Kuryatov et al., 1994; Laube et al., 1997) as well as the glutamate site (Laube et al., 1997) of recombinant NMDA receptors. Thus, the same protein homology has been used to make structural predictions about the glycine as well as the glutamate binding site. However, the homologous binding domain of the LAOBP protein accommodates only a single amino acid (O'Hara et al., 1993; Oh et al., 1993) while in case of the NMDA receptor two different amino acids bind to two distinct binding sites. It seems therefore unlikely that models based on bacterial protein homology can predict the three-dimensional structure of both sites simultaneously (Wood et al., 1997). Although the recognized homology between NMDA receptor binding sites and bacterial amino acid binding proteins has significantly contributed to the current model of glycine and glutamate binding sites, it may not fully serve to predict the complex structure of the NMDA receptor and requires further investigations of the spatial organization of glycine and glutamate binding sites by photoaffinity labeling.
5.3 Heterogeneity of glycine antagonist sites among NMDA receptor subtypes

The NR1 subunit harbours main determinants of the glycine binding site in recombinant receptors as well as *in situ* (Fig. 8, 10). However, previous studies using glycine agonists indicated that the properties of the glycine site are also influenced by the type of NR2 subunit that is assembled with the NR1 subunit. The endogenous agonist glycine displayed an up to tenfold lower potency at NR1/2A receptors than at NR1/2B, NR1/2C or NR1/2D receptors, and a similar difference in affinity has also been observed for the glycine site agonists D-serine, ACBC, D-alanine, HA-966 and L-687,414 (Buller et al., 1994; Grimwood et al., 1996; Ikeda et al., 1992; Kutsuwada et al., 1992; Laurie and Seeburg, 1994; Priestley et al., 1995; Wafford et al., 1993). In contrast to glycine agonists, a clear differentiation between recombinant NMDA receptor subtypes was not found for glycine site antagonists (e.g. L-701,324, L-689,560, L-695,902, CKA) (Grimwood et al., 1995; Priestley et al., 1996). It was therefore concluded that glycine antagonists interact with NR1 subunit domains that are virtually insensitive to modulation by the NR2 subunit variants (Priestley et al., 1995) which would preclude the development of subtype-specific glycine site antagonists for the differentiation of NMDA receptor subtypes. However, by investigating the interaction of \[^{3}H\]CGP 61594 with various NMDA receptor subtypes, a heterogeneity of the glycine antagonist site due to a differential contribution of the NR2 subunit variants could be identified for the first time.

5.3.1 Regional heterogeneity of \[^{3}H\]CGP 61594 binding sites

Autoradiographical analysis revealed that high affinity \[^{3}H\]CGP 61594 binding sites were almost restricted to the forebrain, clearly demonstrating that \[^{3}H\]CGP 61594 does not label the NR1 subunit of all NMDA receptors in the brain with high affinity. In contrast, strychnine-insensitive \[^{3}H\]glycine binding as well as binding with the prototypical glycine site antagonist \[^{3}H\]DCKA recognized the entire population of NMDA receptors and was detected in virtually all brain areas including the cerebellum and brain stem (Laurie and Seeburg, 1994; Laurie and Seeburg, 1994; McDonald et al., 1990; Yoneda et al., 1994). Most interestingly, the restricted \[^{3}H\]CGP 61594 distribution pattern strikingly corresponded to the localization of NR2B mRNA and protein in adult rodent brain (Monyer et al., 1994; Standaert et al., 1994; Watanabe et al., 1993;
Wenzel et al., 1997; Wenzel et al., 1995). The presence of low numbers of [³H]CGP 61594 sites in medulla/pons revealed by Scatchard analysis is in line with the detection of NR2B mRNA in a variety of brain stem nuclei, including superior and inferior colliculus, substantia nigra pars compacta, pontine nucleus, ventral tegmental area, locus coeruleus and inferior olive (Watanabe, 1994). Furthermore, the complete absence of high affinity [³H]CGP 61594 binding sites in the cerebellum suggests that NR1/2A and NR1/2C receptors, which are virtually the only NMDA receptor subtypes in the cerebellum (Ishii et al., 1993; Wenzel et al., 1997), are not targeted by [³H]CGP 61594 with high affinity.

5.3.2 Subtype-specific immunoprecipitation of photolabeled receptors
Supporting the view that [³H]CGP 61594 preferentially interacts with the native NMDA receptor subtype NR1/2B, photolabeled receptors were immunoprecipitated with antisera directed against the NR1 and NR2B subunit only, but not with NR2A-, NR2C- or NR2D-specific antisera (Fig. 14). The amount of photolabeled immunoprecipitated receptors was much higher with the NR1 antiserum than with the NR2B antiserum which may be due to an excess of free NR1 subunits in brain tissue (Blahos and Wenthold, 1996; Chazot and Stephenson, 1997) or might result from distinct reactivities of the NR1 and NR2B antisera. The lack of the NR2A antiserum to immunoprecipitate the photolabeled NMDA receptors is of particular interest in face of the presence of ternary NMDA receptor complexes containing both NR2B and NR2A subunits in situ (Blahos and Wenthold, 1996; Luo et al., 1997; Sheng et al., 1994). Thus, NR1/2A/2B receptors may not exhibit high affinity binding sites for [³H]CGP 61594 or are expressed in very low amounts (Blahos and Wenthold, 1996). Moreover, it is important to note that immunoprecipitation experiments do not distinguish functional and non-functional receptors and that the detergent mixture RIPA might preferentially solubilize non-synaptic NMDA receptors.

5.3.3 Interaction of [³H]CGP 61594 with recombinant NMDA receptor subtypes
The preferential interaction of [³H]CGP 61594 identified for native NR1/2B receptors was also detected for recombinant NR1/2B receptors. However, [³H]CGP 61594 binding experiments with heteromeric NMDA receptors were interfered by the expression of homomeric NR1 receptors in the cells co-
transfected with NR1 and NR2 cDNAs since specific reversible and irreversible 
$[^3H]CGP$ 61594 labeling was detected in all double combinations (Fig. 15A, B).
This finding confirmed previous data showing that homomeric and heteromeric NMDA receptors coexist in transfected cells and that this may have implications when interpreting data from pharmacological analyses of heteromeric receptors (Grimwood et al., 1996). For an exclusive detection of $[^3H]CGP$ 61594 binding to heteromeric receptor subtypes, the allosteric interaction between $[^3H]CGP$ 61594 and the glutamate site antagonist AP5 was analyzed. This binding test is only operative in heteromeric receptors since AP5 virtually lacks affinity for homomeric NR1 receptors (Kendrick et al., 1996; Laurie and Seeburg, 1994; Lynch et al., 1995). Among all recombinant receptor combinations tested, only the NR1/2B subtype displayed high affinity $[^3H]CGP$ 61594 binding sites as shown by the clear allosteric inhibition of $[^3H]CGP$ 61594 binding by AP5. Thus, as observed for native NR1/2B receptors, at low nanomolar concentrations $[^3H]CGP$ 61594 binds selectively to the recombinant NMDA receptor subtype NR1/2B.

The preferential interaction of CGP 61594 with recombinant NR1/2B receptors was finally proven in a functional test using the Xenopus oocyte expression system. In this test system, homomeric NR1 receptors are not detectable upon co-expression of NR1 and NR2 subunits (Ikeda et al., 1992; Meguro et al., 1992; Monyer et al., 1992) since homomeric receptors generate only negligible currents. Solely the glutamate-evoked currents of NR1/2B receptors were inhibited with high potency by CGP 61594 with an IC$_{50}$-value (45 ± 11 nM) similar of the K$_{d}$-value (23 ± 2 nM) determined in reversible binding experiments. Likewise, in previous analyses with glycine site ligands, a good agreement between the electrophysiologically determined IC$_{50}$- and EC$_{50}$-values and affinities obtained from radioligand binding studies was observed. Especially the values for the glycine site antagonists L-689,560 and DCKA were similar in electrophysiological and binding assays (Grimwood et al., 1996; Hess et al., 1996; Lynch et al., 1994; Wafford et al., 1995). In comparison to NR1/2B receptors, the recombinant heteromeric receptor subtypes containing the NR2C, NR2A and NR2D subunits form glycine antagonist binding sites that interact with CGP 61594 with a four- to tenfold lower potency. In most of the reversible and irreversible binding experiments described above a radioligand concentration of 10-25 nM $[^3H]CGP$ 61594 was used, and according to the inhibition curves in Fig. 16 only NR1/2B receptors were detected at this
concentration. Thus, at low nanomolar concentrations [3H]CGP 61594 is a selective ligand for the glycine antagonist binding site of the NMDA receptor subtype NR1/2B as clearly shown by the autoradiographical distribution of [3H]CGP 61594 binding sites (Fig. 11A), the immunoprecipitation of photolabeled receptors (Fig. 14) and the allosteric modulation of [3H]CGP 61594 binding in recombinant NMDA receptor subtypes (Fig. 15C).

5.3.4 Binding sites of CGP 61594 in native and recombinant NMDA receptor subtypes

The inhibitory constant for recombinant NR1/2B receptors (IC$_{50}$ = 45 ± 11 nM) could be attributed to the high affinity binding site of [3H]CGP 61594 (K$_{D}$ = 23 ± 2 nM) which was detected in membranes prepared from various brain regions (Tab. 5, 7). In contrast, the NR2C subunit induces a glycine antagonist domain with an intermediate sensitivity for CGP 61594 as shown by its potency in inhibiting the current response (IC$_{50}$ = 164 ± 27 nM). A corresponding intermediate displacing potency for [3H]CGP 61594 could be demonstrated in situ in the cerebellum (IC$_{50}$ = 231 ± 56 nM), most likely reflecting the interaction of CGP 61594 with receptors containing the NR2C subunit. A contribution of the NR2A subunit to this IC$_{50}$ can not be excluded, whereas the NR2B subunit is virtually absent in the cerebellum and the NR2D subunit is only marginally expressed (Monyer et al., 1994; Watanabe et al., 1994; Wenzel et al., 1997; Wenzel et al., 1995; Wenzel et al., 1996). Furthermore, the NR2A and NR2D subunits in the functional analysis were found to define a glycine antagonist site which displays an even lower potency for CGP 61594 in inhibiting the current responses (IC$_{50}$ = 430 ± 105 nM and 340 ± 61 nM, respectively). This characteristic seems to be retained in situ, at least for NR1/2A receptors, as shown by low affinity binding sites for CGP 61594 in brain areas in which - in addition to NR1/2B receptors - the NR1/2A receptors predominate (IC$_{50}$ = 371 ± nM in cerebral cortex/hippocampus). NR1/2B receptors present in these brain regions are not expected to contribute to this IC$_{50}$ because a low affinity binding component was not observed for recombinant NR1/2B receptors.

5.3.5 Structural requirements for the subtype selectivity of [3H]CGP 61594

CGP 61594 is to our knowledge the first ligand which clearly discriminates the glycine antagonist sites of NMDA receptor subtypes. Interestingly, the parent compound of [3H]CGP 61594, the high affinity glycine site antagonist L-689,560
(Grimwood et al., 1992) and other bulky glycine site antagonists such as L-701,324 (Priestley, 1994) did not differentiate between NMDA receptor subtypes in electrophysiological and radioligand binding studies (Grimwood et al., 1996; Priestley et al., 1995). The structure of the tetrahydroquinoline phenylurea L-689,560 revealed the presence of a hydrophobic bulk tolerance region in the glycine antagonist binding pocket which can accommodate the phenyl ring of L-689,560 (Grimwood et al., 1992; Kemp and Leeson, 1993). Therefore, [³H]CGP 61594 was synthesized as a structurally unique derivative of L-689,560 with a different orientation of this phenyl ring domain achieved by the replacement of a urea-like linker by an amide structure. A second difference between the two molecules is provided by the introduction of the azide group as a 4-substituent in the phenyl ring of CGP 61594. These structural changes in CGP 61594 are sufficient to result in a subtype-selective interaction of CGP 61594 suggesting that the hydrophobic bulk tolerance domain which accommodates the 4-azido-phenyl moiety is sensitive to modulation by adjacent NR2 subunit variants. Thus, as it was already found for the pharmacology of the glutamate, polyamine and open channel blocker sites, the NR2 subunits apparently confer distinct, pharmacological properties to the glycine site as well (Buller et al., 1994; Laurie and Seeburg, 1994; Monaghan and Larsen, 1997; Avenet et al., 1997; Williams, 1993). This ligand constitutes a novel tool to identify and compare NR2 subunit domains that contribute - directly or allosterically - to the differential affinity of the glycine antagonist binding site on the NR1 subunit. Since glycine antagonist sites can be formed by homomeric NR1 receptors with similar high affinity compared to native NMDA receptors (Grimwood et al., 1995; Laurie and Seeburg, 1994; Moriyoshi et al., 1991), the influence of the NR2 subunit on the glycine antagonist binding site is most likely due to an allosteric modulation of the binding domain. However, a direct steric influence on the glycine binding site at the interface of NR1 and NR2 subunits may be envisaged as well.

5.3.6 Implications for drug development
The detection of a subtype-specific pharmacology of the glycine antagonist site is expected to have an important impact on the future development of NMDA receptor antagonists. Glycine site antagonists which are structurally related to [³H]CGP 61594 offer the possibility to target drugs to the NR1/2B receptor subtype. Ifenprodil and eliprodil, NMDA receptor modulators which bind to a
site distinct from the glycine binding domain, possess a selectivity for the NMDA receptor subtype NR1/2B (Nicolas and Carter, 1994; Williams, 1993). They are characterized by a favourable side effect profile which is likely to be, at least in part, due to the NR1/2B subtype selectivity of these drugs (Cudennec et al., 1994; Duval et al., 1992; Kew and Kemp, 1998; Patat et al., 1994). Therefore, glycine site antagonists targeted to NR1/2B receptors are also expected to be less prone to side effects than non-selective NMDA receptor antagonists.
6. Outlook

The development of \[^{3}H\]CGP 61594 provides the first photoaffinity label which permits an analysis of the structural determinants of the glycine binding domain of NMDA receptors *in situ*. Knowledge of the spatial organization of the glycine site is of major relevance for the design of glycine antagonists as therapeutic agents. As a photocrosslinker, \[^{3}H\]CGP 61594 is ideally suited for a further, detailed structural analysis of the glycine binding domain on the NR1 subunit, for example by identifying a photolabeled peptide fragment or even the labeled amino acid(s). In addition, a comparative analysis of NR2 subunit domains would be of major interest to identify amino acids on the NR2 subunits which differentially determine the affinity for \[^{3}H\]CGP 61594. In the future, the novel tool \[^{3}H\]CGP 61594 is anticipated to permit new insights into the protein configurations of the glycine site of native and recombinant NMDA receptor subtypes.

The discovery of a heterogeneity of the glycine antagonist site among NMDA receptor subtypes should encourage to search for further subtype-selective glycine site antagonists. CGP 61594 is expected to serve as a structural lead for the development of novel NR1/2B-selective glycine site antagonists. Such agents might be therapeutically useful for the treatment of a variety of neurological and mental disorders and are expected to display less side effects than the presently available NMDA receptor antagonists.
7. References


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8. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ACBC</td>
<td>1-aminocyclobutanecarboxylic acid</td>
</tr>
<tr>
<td>ACEA 1021</td>
<td>6,7-dichloro-5-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>ACPC</td>
<td>1-aminocyclopropanecarboxylic acid</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)-propionic acid</td>
</tr>
<tr>
<td>AP5</td>
<td>2-amino-5-phosphono-pentanoic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium calmodulin protein kinase type II</td>
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<tr>
<td>Cb</td>
<td>cerebellum</td>
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<tr>
<td>CGP 37849</td>
<td>D,L-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid</td>
</tr>
<tr>
<td>CGP 39653</td>
<td>D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid</td>
</tr>
<tr>
<td>CGP 40116</td>
<td>pharmacologically active R-enantiomers of the racemic CGP 37849</td>
</tr>
<tr>
<td>CGP 40117</td>
<td>pharmacologically inactive S-enantiomer of the racemic CGP 37849</td>
</tr>
<tr>
<td>CGP 55802A</td>
<td>(E)-2-amino-10-[(4-azido-2-hydroxy-3-iodobenzoyl)amino]-4-phosphonomethyl-dec-3-enolic acid</td>
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<tr>
<td>CGP 61594</td>
<td>(±)-trans-4-[2-(4-azidophenyl)-acetylamino]-5,7-dichloro-1,2,3,4-tetrahydro-quinoline-2-carboxylic acid</td>
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<tr>
<td>CGS 19755</td>
<td>(±)-(cis-4-phosphono-methyl-2-piperidine carboxylic acid</td>
</tr>
<tr>
<td>Ci</td>
<td>curie</td>
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<tr>
<td>CKA</td>
<td>7-chlorokynurenic acid</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CPP</td>
<td>(±)-4-(3-phosphonopropyl)-piperazine-2-carboxylic acid</td>
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<tr>
<td>CPPene</td>
<td>(±)-4-(3-phosphonopropenyl)-piperazine-2-carboxylic acid</td>
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<tr>
<td>Cx</td>
<td>cerebral cortex</td>
</tr>
<tr>
<td>DCKA</td>
<td>5,7-dichlorokynurenic acid</td>
</tr>
<tr>
<td>dpm</td>
<td>disintegrations per minute</td>
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<tr>
<td>ECL</td>
<td>enhanced chemo-luminescence</td>
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<tr>
<td>EDC</td>
<td>N-ethyl-N'-(3-dimethyl-amino-propyl)-carbodiimide hydrochloride</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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</table>
GluR AMPA or kainate receptor subunit
GlyR glycine receptor
HA-966 (±)-3-amino-1-hydroxy-pyrrolidin-2-one
HEK human embryonic kidney
Hi hippocampus
HRP horseradish peroxidase
IgG immunoglobuline G
KA kainate
L-687,414 (+)-cis-4-methyl-HA-966
L-689,560 (±)-4-trans-5,7-dichloro-4-phenylamino-carbonylamino-1,2,3,4-tetrahydroquinoline-2-carboxylic acid
L-695,902 7-chloro-4-hydroxy-2(1H)-quinolone-3-carboxylate
L-701,324 7-chloro-4-hydroxy-3-(3-phenoxyphenyl)-2(1H)quinolinone
LAOBP lysine/arginine/ornithine binding protein
LTP long-term potentiation
MAPK mitogen activating protein kinase
MDL 100,458 3-(benzoyl-methyl-amino)-6-chloro-1H-indole-2-carboxylic acid
MDL 102,288 5,7-dichloro-4-(4-methoxycarbonylaminobenzenesulfonylimino) -1,4-dihydro-quinoline-2-carboxylic acid monohydrate
MK-801 (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate ( = dizocilpine)
NMDA N-methyl-D-aspartate
NR N-methyl-D-aspartate receptor subunit
P postnatal day
PAGE polyacrylamide gel electrophoresis
PBS phosphate-buffered saline
PCP 1-[1-phenylcyclohexyl]piperidine ( = phencyclidine)
PKA protein kinase A
PKC protein kinase C
PMSF phenylmethanesulfonyl fluoride
PP1 protein phosphatase 1
PP2A protein phosphatase 2A
QBP glutamine binding protein
S.D. standard deviation
SDS sodium dodecyl sulfate
SL 82,0715  \((\pm)-\alpha-(4\text{-chlorophenyl})-4\text{-}([4\text{-fluorophenyl}]\text{methyl})\text{-}1\text{-piperidine}\) ethanol (= eliprodil)

Tris  tris(hydroxymethyl) aminomethane
TrK  tyrosine kinase
wt  wild-type
9. Curriculum Vitae

Personal data
Name: Michael Honer
Date of birth: February 7, 1968
Birthplace: Spaichingen, Germany
Nationality: German

School education
1974-1978 Primary school in Spaichingen
1978-1987 Secondary school in Spaichingen
1987 Abitur (school-leaving exam)
08/87-03/89 Community service at the Hospital of Spaichingen, Germany

University education
1989-1991 University of Tübingen, Germany
Student of Biochemistry
1991 Vordiplom (Bachelor of Science)
1991-1994 ETH Zürich, Switzerland
Student of Biochemistry
10/93-4/94 Diploma thesis at the Institute of Pharmacology:
'Immunobiochemical and pharmacological characterization of
GABA\textsubscript{A} receptor subtypes differing in the \gamma-subunit variant'
05/94 Graduation as Diplom Naturwissenschafter (Master of Science)
11/94-2/99 ETH and University of Zürich, Switzerland
Ph.D. thesis at the Institute of Pharmacology:
'[\textsuperscript{3}H]CGP 61594: the first photoaffinity ligand for a structural
analysis and differentiation of the glycine antagonist site of
NMDA receptor subtypes']
10. Publications arising from this Ph.D. thesis

Full papers


Abstracts


Honer, M., Benke, D., Kuhse, J., Laube, B., Heckendorn, R., Allgeier, H., Betz, H. and Mohler, H.: [3H]CGP 61594: A selective photoaffinity ligand for the glycine site of the NMDA receptor subtype NR1/2B.

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