Differentiation of Glycine Antagonist Sites of N-Methyl-D-aspartate Receptor Subtypes

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The binding site for the co-agonist glycine on N-methyl-D-aspartate (NMDA) receptors has been mapped to the NR1 subunit whereas binding of the principal agonist glutamate is mediated by the NR2 subunits. Using the novel glycine site antagonist and photoaffinity label CGP 61594, distinct contributions of the NR2 subunit variants to the glycine antagonist binding domains of NMDA receptor subtypes are demonstrated. High affinity sites for CGP 61594 were exclusively displayed by NR1/2B receptors, as shown by their co-distribution with the NR2B subunit, by subunit-selective immunoprecipitation and by functional analysis of NR1/2B receptors expressed in Xenopus oocytes (inhibitory potency, IC₅₀ = 45 ± 11 nM). Other NMDA receptor subtypes are clearly distinguished by reduced inhibitory potencies for CGP 61594, being low for NR1A2 and NR1D2 receptors (IC₅₀ = 430 ± 105 nM and 340 ± 61 nM, respectively) and intermediate for NR1C2 receptors (IC₅₀ = 164 ± 27 nM). Glycine antagonist sites with low and intermediate affinity for [³H]CGP 61594 were detected also in situ by radioligand binding in brain areas predominantly expressing the NR2A and NR2C subunits, respectively. Thus, [³H]CGP 61594 is the first antagonist radioligand that reliably distinguishes the glycine site of NMDA receptor subtypes. [³H]CGP 61594 is a promising tool to identify the NR2 subunit domains that contribute to differential glycine antagonist sites of NMDA receptor subtypes.

The NMDA³ subtype of glutamate receptors is a heterooligo-meric ligand-gated ion channel, which contributes to excitatory neurotransmission throughout the central nervous system. NMDA receptors are characterized by unique features such as a voltage-dependent Mg²⁺ block, slow activation and inactivation kinetics, and the requirement for glycine as a co-agonist in channel gating (1–6). They play a crucial role in brain development, synaptic plasticity and the pathophysiology of major neurological disorders (7–11). NMDA receptors are assembled from the NR1 subunit in combination with at least one type of NR2 subunit (NR2A-2D) (12–15), giving rise to receptor subtypes with distinct electrophysiological and pharmacological properties (2, 16–21).

Inhibition of NMDA receptors by glycine site antagonists is of major interest for the therapeutic intervention in pathological central nervous system states like stroke, head injury, and epilepsy. At doses evoking remarkable anticonvulsant and neuroprotective effects, glycine site antagonists are less prone to psychotomimetic actions and exhibit less severe motoric and cognitive side effects than glutamate site antagonists (22). Further improvements are expected from the targeting of the glycine site of NMDA receptor subtypes.

The NR1 subunit harbors main determinants of the glycine binding site in recombinant receptors as well as in situ. Recombinant homomic NR1 receptors bind glycine agonists and antagonists (19, 23, 24), and various amino acid residues involved in high affinity binding have been identified by mutational analysis of the NR1 subunit (25–28). Similarly, in native receptors, the novel glycine antagonist and photoaffinity ligand [³H]CGP 61594 labels exclusively the NR1 subunit of NMDA receptors in brain membranes.² In contrast, the glutamate binding pocket is formed by the NR2 subunits (29). Interestingly, the properties of the glycine site are influenced by the type of NR2 subunit that is assembled with the NR1 subunit. Glycine displays an up to 10-fold 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 L-serine, 1-aminoacyclohexaneacarbonylic acid, β-alanine, HA-966, and L-687,414 (16, 19, 21, 30–33). However, glycine site antagonists (e.g. L-701,324, L-689,560, L-695,902, CKA) did not clearly differentiate between recombinant NMDA receptor subtypes (23, 34). Mutational analysis of the NR1 subunit suggested structural differences in the binding domains of glycine site agonists and antagonists (25–28). Some of the mutated residues affected the affinity for

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CGP 61594 Differentiates Glycine Sites of NMDA Receptor Subtypes

EXPERIMENTAL PROCEDURES

Reversible Binding and Photolabeling with [3H]CGP 61594 ([2]-trans-4-(2/4-Acylphenylacetylamino)-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-carboxylic Acid)—Membranes were prepared from male Sprague-Dawley rat brains, frozen, thawed, and washed twice with 5 mM Tris acetate, pH 7.0, and once with 50 mM Tris/HCl, pH 7.4. Aliquots of the membranes (100 μg of protein) were incubated with 25 nM [3H]CGP 61594 (29 Ci/mmol, custom synthesized in tritiated form by Amersham Pharmacia Biotech) or, for saturation binding experiments, with concentrations ranging from 2 to 200 nM for 2 h on ice in 50 mM Tris/HCl, pH 7.4, in a final volume of 200 μl. Nonspecific binding was determined in the presence of 1 μM glycine and amounted to 20–30% of total binding at 25 nM [3H]CGP 61594. At high radioligand concentrations (180 nM), nonspecific binding amounted to about 80% of total binding in cerebellum and to 60–70% of total binding in hippocampus/cortex. After filtration through Whatman GF/C filters, the radioactivity was quantified by liquid scintillation counting. Scatchard analysis of the saturation binding data was performed using the “Ligand” program (35).

For irreversible labeling, membranes containing 60 μg of protein were incubated with 25 nM [3H]CGP 61594 in 50 mM Tris/HCl, pH 7.4, for 2 h on ice, followed by irradiation with UV light in a Stratalinker (1 J at 254 nm; distance: 10 cm). Nonspecific binding was determined in the presence of 1 mM glycine. After centrifugation, the membranes were dissolved in SDS-PAGE sample buffer, heated at 60 °C for 30 min and subjected to SDS-PAGE using 5% mini gels. After staining for proteins with Coomassie Brilliant Blue R-250, the gel was incubated in Amplify (Amersham Pharmacia Biotech) for 30 min, dried, and exposed to an x-ray film (Fuji RX) for at least 2 weeks.

Immunopurification of [3H]CGP 61594-photolabeled Receptors—Whole brain membranes were photolabeled with 25 nM [3H]CGP 61594 as described above, washed with 50 mM Tris/HCl, pH 7.4, and solubilized in 50 mM Tris/HCl, pH 9.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS (RIPA) (36) for 1 h on ice, followed by centrifugation at 100,000 × g for 1 h. The supernatant was carefully removed, and aliquots (500 μl) were incubated with NR1, NR2A, NR2B, NR2C, or NR2D antiserum (37–40) overnight at 4 °C in the absence or presence of the respective peptide antigen (10 μg/ml). Antibody-antigen complexes were precipitated by incubation with 125 μl of Pansorbin (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 of 50 mM Tris/HCl, pH 7.4, and the radioactivity was quantified by liquid scintillation counting. The ability of the different antisera to immunoprecipitate the respective receptor subtype was verified by Western blot analysis of the NR1, NR2A, NR2B, NR2C, and NR2D subunits.

Receptor Autoradiography—The regional distribution of [3H]CGP 61594 was analyzed on unfixed parasagittal cryostat-cut sections (12 μm) of brains of adult male Sprague-Dawley rats (200–250 g). Slidemounted sections were thawed and washed three times for 10 min at 4 °C in 50 mM Tris/HCl, pH 7.5. For [3H]CGP 61594 binding, sections were incubated for 2 h at 4 °C with 10 nM [3H]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 read and exposed to Hyperfilm-12H (Amersham Pharmacia Biotech) for 15 days. Nonspecific [3H]CGP 61594 binding was assessed in parallel in the presence of 1 μM glycine.

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 μg total) using the calcium phosphate precipitation method of Chen and Okayama (41). In co-transfection cultures, either 5 μM MK-801 and 10 μM DCKA or 500 μM ketamine were added. After overnight incubation (35 °C, 5% CO2), the medium was renewed and the cells incubated for further 24–48 h (37 °C, 5% CO2). The plates were then rinsed with phosphate-buffered saline (37 °C, pH 7.5), and the cells were harvested into the same buffer containing 50 mM KCl. The cell suspension was centrifuged (10,000 × g, 15 min, 4 °C), and the resultant pellet homogenized (Polytron) in ice-cold 0.32 M sucrose, 10 mM EDTA, 1 mM glycine, and centrifuged (1000 × g, 15 min, 4 °C). After rehomogenization of the pellet and recentrifugation, the supernatants were combined and centrifuged at 30,000 × g for 30 min at 4 °C. The membrane pellet was washed once in 10 mM Tris acetate, pH 7.5, 10 mM EDTA, and frozen to −70 °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.

Expression in Xenopus Oocytes—Linearized plasmid DNAs were used for the in vitro synthesis of cRNA (mCAP mRNA capping kit, Stratagene). cRNAs of NR1 and NR2A-2D subunits were synthesized using T7 or T3 RNA polymerase, respectively. cRNA concentrations were determined by both measuring optical densities at 280 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/μl, 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 (42).

RESULTS

[3H]CGP 61594 is a novel glycine site antagonist. Upon photolabeling it is selectively cross-linked to the NR1 subunit of NMDA receptors. By analyzing the interaction of [3H]CGP 61594 with various NMDA receptor subtypes, the contribution of the NR2 subunit variants to the glycine antagonist domain was investigated.

Regional and Ontogenetic Distribution of [3H]CGP 61594 Binding Sites—The distribution of [3H]CGP 61594 binding sites was mapped autoradiographically on parasagittal cryostat sections of adult brains. High affinity [3H]CGP 61594 binding was mainly restricted to forebrain regions with highest densities in the hippocampal formation, followed by cerebral cortex, striatum, olfactory bulb, and thalamus (Fig. 1). In mid- and hindbrain areas only marginal labeling of receptors over background was observed (Fig. 1). In contrast, strychnine-insensitive [3H]glycine binding, which recognizes the entire population of NMDA receptors, is detected in virtually all brain
observed autoradiographically. Affinity labeling closely corresponded to the labeling pattern of low affinity sites (see below). The rank order of regional high correspondence to that of the protein band varied in different regions with a rank order NR1 subunit (115 kDa) was specifically photolabeled in all visualized by fluorography. A single protein with the size of the 61594, photolabeled proteins were resolved by SDS-PAGE and lacked high affinity binding sites for [3H]CGP 61594 (Fig. 1, NR2B subunit such as the adult cerebellum (38, 40, 46, 47). Furthermore, brain regions that lack expression of the previously visualized at the level of the mRNA and protein (38, 40, 46, 48) also was observed in the hippocampal formation, followed by the cerebral cortex and striatum. Lower \( B_{\text{max}} \) values were apparent in olfactory bulb and thalamus, while very low binding was found in brainstem regions (mesencephalon, medulla/pons). In cerebellar membranes high affinity binding sites were not detected, although specific binding detected at high nanomolar concentrations (150–200 nM) pointed to the potential presence of low affinity sites (see below). The rank order of regional high affinity labeling closely corresponded to the labeling pattern observed autoradiographically.

As a photoaffinity ligand, \(^{3}H\)CGP 61594 is known to interact with the NR1 subunit. It was therefore tested whether the molecular target specificity was retained in brain regions containing high affinity \(^{3}H\)CGP 61594 sites. Following UV irradiation of brain membranes in the presence of 25 nM \(^{3}H\)CGP 61594, photolabeled proteins were resolved by SDS-PAGE and visualized by fluorography. A single protein with the size of the NR1 subunit (115 kDa) was specifically photolabeled in all brain areas analyzed as shown by its displacement with glycine (Fig. 2). The relative radiolabeling intensity of the 115-kDa protein band varied in different regions with a rank order corresponding to that of the \( B_{\text{max}} \) values in reversible binding in the respective brain regions. Thus, the molecular specificity of \(^{3}H\)CGP 61594 photolabeling was retained, and the regional intensity of photolabeling was paralleled by the level of reversibly bound \(^{3}H\)CGP 61594.

The regional distribution of \(^{3}H\)CGP 61594 binding was strikingly similar to the distribution of the NR2B subunit previously visualized at the level of the mRNA and protein (38, 40, 46, 47). Furthermore, brain regions that lack expression of the NR2B subunit such as the adult cerebellum (38, 40, 46, 48) also lacked high affinity binding sites for \(^{3}H\)CGP 61594 (Fig. 1, Table 1). Thus, at low nanomolar concentrations \(^{3}H\)CGP 61594 appears to be selective for NMDA receptors containing the NR2B subunit.

In keeping with this notion, high levels of \(^{3}H\)CGP 61594 binding sites were expected to occur in neonatal brain. At this developmental stage, the subunits NR1 and NR2B are strongly expressed, while the NR2D subunit represents only a minor subunit population and the NR2A and NR2C subunits are hardly detectable or absent at birth (39, 40, 46, 49). Indeed, high affinity \(^{3}H\)CGP 61594 binding sites were found to be present in neonatal brain at levels close to 50% of that found in adult brain (P 0 (postnatal day): \( B_{\text{max}} \) = 1.5 ± 0.6 pmol/mg protein, \( K_D \) = 21 ± 3 nM; adult: \( B_{\text{max}} \) = 3.2 ± 0.7 pmol/mg, \( K_D \) = 21 ± 3 nM). In addition, in photolabeling experiments only a 115-kDa protein, corresponding to the NR1 subunit, was specifically photolabeled by \(^{3}H\)CGP 61594 in neonatal brain (not shown). This further supports the notion that \(^{3}H\)CGP 61594 preferentially recognizes NMDA receptors containing the subunits NR1 and NR2B.

**Immunoprecipitation of NMDA Receptors Irreversibly Labeled by \(^{3}H\)CGP 61594**—To test the hypothesis of a preferential interaction of \(^{3}H\)CGP 61594 with receptors containing the NR2B subunit in situ, the type of photolabeled receptor was identified. Since the NR1 subunit is selectively photolabeled, immunoprecipitation of labeled receptors with NR2 subunit-selective antisera was expected to unravel the subunit combination displaying high affinity \(^{3}H\)CGP 61594 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 (36). NMDA receptor subtypes were immunoprecipitated with antisera selective for the subunits NR1, NR2A, NR2B, NR2C, or NR2D, and the radioactivity of the immunoprecipitate was measured by liquid scintillation counting. Nonspecifically precipitated radioactivity was determined by co-incubating the antiserum with an excess of the respective peptide antigen. Photolabeled proteins were specifically immunoprecipitated only with the NR1 or NR2B antisera (Fig. 3A). The amount of radioactivity immunoprecipitated was much higher with the NR1 antisera than with the NR2B antisera. This difference may be due to either an excess of free NR1 subunits in brain tissue (50, 51) or to differential reactivities of the NR1 and NR2B antisera. The NR2A, NR2C, or NR2D antibodies did not result in specific immunoprecipitation of radioactivity (Fig. 3A), although the respective NMDA receptor subtypes were immunoprecipitated as verified by the detection of the NR1 subunit by Western blotting (Fig. 3B). These findings support the view that \(^{3}H\)CGP 61594 interacts with high affinity with NMDA receptors containing the NR1 and NR2B subunits.

**Glycine Antagonist Sites with Low Affinity for \(^{3}H\)CGP 61594 in Native NMDA Receptors**—The heterogeneity of the glycine sites of NMDA receptors in situ was further analyzed by investigating the potential presence of low affinity binding sites for \(^{3}H\)CGP 61594 in NMDA receptors other than those containing the NR2B subunit. A competition binding assay was performed in the adult cerebellum, a region that contains almost exclusively the NR2A and NR2C subunits but virtually

### Table I

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

**FIG. 2.** Photolabeling of membranes from selected brain regions with \(^{3}H\)CGP 61594. Membranes from the indicated brain regions were incubated with \(^{3}H\)CGP 61594 (2–200 nM) for 2 h at 4 °C, 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 115 kDa, faint nonspecific labeling was detected in cerebral cortex at about 120 kDa that was not displaced by 1 mM glycine or 10 μM CGP 61594.
therefore tested whether low affinity \([3H]\)CGP 61594 sites were attributable to NR1/2C or NR1/2A receptors or both. It was

Momeric NR1 receptors and, surprisingly, all heteromeric subunits containing the NR2A subunit display low affinity sites for \([3H]\)CGP 61594. These results suggest that receptors containing the NR2A subunit display low affinity sites for \([3H]\)CGP 61594. Thus, the major NMDA receptor subtypes in situ are differentiated by distinct inhibitory potencies of \([3H]\)CGP 61594 at the respective glycine sites.

\([3H]\)CGP 61594 Binding to Recombinant Receptors—The interaction of \([3H]\)CGP 61594 with different heteromeric NMDA receptors was further examined on recombinant receptors with defined subunit composition. Homomeric NR1 and heteromeric NR1/2A, NR1/2B, NR1/2C, and NR1/2D receptors were expressed in HEK 293 cells by transient transfection with the corresponding cDNAs. In reversible binding experiments, homomeric NR1 receptors and, surprisingly, all heteromeric subunit combinations exhibited specific high affinity \([3H]\)CGP 61594 binding (not shown). This appeared not to be due to a nonspecific interaction, since photolabeling of the transfected cells with \([3H]\)CGP 61594 resulted in the labeling of the 115-kDa NR1 subunit in homomeric NR1 receptors as well as in all double subunit combinations (Fig. 4A). Thus, the reversible labeling was most likely due to homomeric NR1 receptors present in HEK 293 cells, even when the NR1 subunit is coexpressed with an NR2 subunit. The presence of NR1 homomeric receptors has previously been described in HEK 293 cells cotransfected with the NR2A subunit cDNA (30). Therefore, \([3H]\)CGP 61594 binding was analyzed in a test that is operative only in heteromeric receptors. Glutamate antagonists, such as AP5, interact with heteromeric receptors but virtually lack affinity for homomeric NR1 receptors, inhibition of \([3H]\)CGP 61594 by AP5 is expected to occur only at heteromeric NR1/NR2 receptors. The subtype selectivity of \([3H]\)CGP 61594 for the NR1/2B subunit combination is demonstrated. The data shown are the mean ± S.D. of three independent experiments.

FIG. 3. Immunoprecipitation of NMDA receptors irreversibly labeled with \([3H]\)CGP 61594 by NR1 and NR2B subunit-specific antisera. A, total brain membranes were photolabeled with 25 nM \([3H]\)CGP 61594, solubilized, and immunoprecipitated with antiserum directed against the NMDA receptor subunits NR1, NR2A, NR2B, NR2C, or NR2D. The radioactivity in the immunoprecipitates was determined by liquid scintillation counting. Nonspecific precipitation was determined in parallel assays in presence of the respective peptide antigen (10 μg/ml). The columns show the mean of two to three 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. Nonspecific immunoreactivity (right lane) was determined in presence of the respective peptide antigen (10 μg/ml).

FIG. 4. Photolabeling and allosteric modulation of recombinant NMDA receptor subtypes. A, photolabeling of NMDA receptor subtypes by \([3H]\)CGP 61594. 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 followed by SDS-PAGE and fluorography. Total and nonspecific photolabeling are shown in adjacent lanes. A 115-kDa protein, corresponding to the NR1 subunit, was ubiquitously labeled presumably due to the presence of homomeric NR1 receptors that coexist with heteromeric NR1/NR2 receptors. B, allosteric modulation of high affinity \([3H]\)CGP 61594 binding by AP5. 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. Nonspecific binding was determined in the 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 subtype selectivity of \([3H]\)CGP 61594 for the NR1/2B subunit combination is demonstrated. The data shown are the mean ± S.D. of three independent experiments.
absence of 10 μM AP5 in HEK 293 cells transfected with cDNAs of the NR1 subunit or the subunit combinations NR1/2A, NR1/2B, NR1/2C, and NR1/2D. Only HEK 293 cells transfected with the NR1/2B combination displayed inhibition of [3H]CGP 61594 binding by AP5, whereas [3H]CGP 61594 binding to cells transfected with the cDNA for NR1 alone or in combination with the cDNAs of NR2A, NR2C, or NR2D remained unaffected (Fig. 4B). Thus, only the NR1/2B subunit combination displayed an allosteric modulation of [3H]CGP 61594 binding.

Electrophysiological Analysis of CGP 61594 on Recombinant Receptors—The differential interaction of CGP 61594 with NMDA receptor subtypes was functionally analyzed in Xenopus oocytes expressing the NR1 subunit in combination with the NR2 subunit variants NR2A, NR2B, NR2C, and NR2D. In this test system, homomeric NR1 receptors were not expected to appreciably interfere with the analysis, since they generate only very small currents compared with heteromeric receptors (32, 55, 56). Glutamate-evoked responses were measured in the presence of glycine concentrations eliciting a half-maximal response (26, 29). The glutamate-evoked currents were inhibited by CGP 61594 with high potency only when the NR1 and NR2B subunits were co-expressed (IC$_{50}$ = 45 ± 11 nM) (Fig. 5). The other heteromeric receptors were less sensitive to inhibition by CGP 61594. The IC$_{50}$ value determined for the NR1/2C receptor was four times higher (IC$_{50}$ = 164 ± 27 nM) as compared with NR1/2B. An even lower inhibitory potency of CGP 61594 was found for the NR1/2A receptor (IC$_{50}$ = 430 ± 105 nM) and the NR1/2D receptor (IC$_{50}$ = 340 ± 61 nM) (Fig. 5). Thus, 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.

DISCUSSION

This report shows that the glycine antagonist [3H]CGP 61594 constitutes a novel tool to differentiate the glycine sites of the major NMDA receptor subtypes. CGP 61594 binds with high affinity to NR1/2B receptors, intermediate affinity to NR1/2C receptors, and low affinity to NR1/2A and NR1/2D receptors.

The preferential interaction of CGP 61594 with NR1/2B receptors is based on several lines of evidence. 1) In contrast to the ubiquitous labeling of glycine sites by [3H]glycine and [3H]DCKA (19, 43, 45), [3H]CGP 61594 binding is restricted to the forebrain with a regional distribution that strikingly corresponds to the localization of NR2B mRNA and protein in adult rodent brain (38, 40, 46, 47, 57). The presence of low numbers of [3H]CGP 61594 sites in medulla/pons is consistent with the expression of NR2B mRNA in a variety of brain stem nuclei, including superior and inferior colliculus, substantia nigra pars compacta, ventral tegmental area, locus coeruleus, pontine nucleus, and inferior olive (58). Furthermore, the prominent expression of [3H]CGP 61594 binding sites in neonatal brain corroborates their identity with receptors containing the NR2B subunit, since the NR2A and NR2C subunits are virtually absent at birth and the NR2D subunit is expressed only at low levels (37, 39, 40, 46, 58).

2) NMDA receptors photolabeled with [3H]CGP 61594 in adult brain could be immunoprecipitated with antisera directed against the NR1 and NR2B subunit only, but not with NR2A, NR2C, or NR2D antisera. The lack of the NR2A antisera to immunoprecipitate the photolabeled NMDA receptors is of particular interest in view of the presence of NMDA receptors containing both NR2B and NR2A subunits in situ (36, 51, 59). Thus, it appears that the NR2A, NR2C, or NR2D subunits are not prominent constituents of the photolabeled receptors.

3) Among the recombinant receptors tested, only the NR1/2B subtype displayed high affinity [3H]CGP 61594 binding sites as shown by its allosteric modulation with the glutamate antagonist AP5. Furthermore, in an electrophysiological analysis, high affinity CGP 61594 antagonism was seen with NR1/2B receptors. The potency of CGP 61594 in inhibiting glutamate-induced currents (IC$_{50}$ = 45 ± 11 nM) was similar to the affinity of [3H]CGP 61594 binding determined in forebrain regions (K$_D$ = 23 ± 2 nM). Thus, at low ligand concentrations CGP 61594 is a selective ligand for NR1/2B receptors not only in situ but also with recombinant receptors.

In contrast to the NR2B subunit, the NR2A subunit generates a glycine binding domain that binds CGP 61594 with lower potency. This is shown electrophysiologically for recombinant NR1/2A receptors (IC$_{50}$ = 430 ± 105 nM) and in situ by radioligand binding in cerebral cortex/hippocampus, where, in addition to the NR1/2B receptors, the NR1/2A receptors are predominant (IC$_{50}$ = 371 ± 85 nM). A similar low affinity CGP 61594 binding site is formed upon co-expression of the NR2D subunit (IC$_{50}$ = 340 ± 61 nM). Notably, the assembly of the NR1 and the NR2C subunits results in an antagonist domain with intermediate affinity for CGP 61594, most clearly demonstrated by the inhibitory potency of CGP 61594 for NR1/2C receptors (IC$_{50}$ = 164 ± 27 nM). A site with comparable affinity is found in situ in the cerebellum (IC$_{50}$ = 231 ± 56 nM in radioligand binding), where the NR2C subunit is prevalent (38–40, 46, 48), although NR2A containing receptors may also contribute to this IC$_{50}$ value. Thus, using CGP 61594 as a tool, a comparative analysis of NR2 subunit sequences should help
to define the NR2 domains that differentially modulate the glycine site of the major NMDA receptors. New insights into the subunit configurations of NMDA receptor subtypes may be gained.

[3H]CGP 61594 provides the first radioligand that reliably discriminates the glycine site of different NMDA receptor subtypes. This is of particular interest since the glycine site of NR1/2B receptors is of therapeutic relevance as a drug target for the treatment of diseases such as stroke or epilepsy. Similar to CGP 61594, the neuroprotective agents ifenprodil and eliprodil, acting at the polyamine site of NMDA receptors, interact preferentially with NR1/2B receptors (60, 61). They are characterized by a favorable side effect profile at neuroprotective doses compared with agents that inhibit NMDA receptors non-selectively (62–64). Thus, the glycine antagonist CGP 61594 may serve as a valuable tool for developing potential therapeutic agents targeted to the NR1/2B receptor.

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