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Inorganic iron complexes derived from the nitric oxide donor nitroprusside: competitive *N*-methyl-D-aspartate receptor antagonists with nanomolar affinity

Hans Neijt, Manuel Koller*, Stephan Urwyler

Novartis Pharma AG, Therapeutic Area Nervous System, CH-4002 Basel, Switzerland

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Abstract

Aquopentacyanoferrate(II), $[Fe^{II}H_2O(CN)_5]^{3-}$, is one of the photodegradation products of the vasodilator and nitric oxide donor nitroprusside. Earlier observations concerning the light dependence of *N*-methyl-D-aspartate (NMDA) receptor blockade by nitroprusside prompted us to examine the effects of this iron complex on the NMDA receptor. $[Fe^{II}H_2O(CN)_5]^{3-}$ and two other related species, aminopentacyanoferrate(II) and aminopentacyanoferrate(III), were found to be highly potent, competitive, and selective NMDA receptor antagonists. In a binding assay for the transmitter recognition site on the NMDA receptor, these iron complexes displaced the radioligand [^{3}H]CGP 39653 with nanomolar affinities. They did not displace radioligands labeling the channel ([^{3}H]MK-801) or the glycine co-agonist ([^{3}H]glycine) sites of the NMDA receptor, nor did they have any relevant affinities for a number of other neurotransmitter (α-adrenergic, 5-hydroxytryptamine, dopamine, opiate) receptors. The iron complexes blocked NMDA-induced depolarizations in rat cortical slices at submicromolar concentrations, whereas responses to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate were not affected. In another functional receptor assay (potentiation of [^{3}H]MK-801 binding by glutamate under non-equilibrium conditions), Schild analysis demonstrated the competitive nature of the NMDA receptor antagonism. The pA_2 values obtained from these experiments were similar to the pK_i values derived from radioligand ([^{3}H]CGP 39653) binding assays. To explain the high affinity and selectivity of these compounds for the NMDA receptor, a novel mechanism of antagonist–receptor interaction is proposed, involving a ligand exchange process in which a loosely bound species (here H_2O or NH_3) in the coordination sphere of the iron complex is replaced by a functional group of an amino acid side chain placed at the glutamate recognition site of the NMDA receptor, thereby hindering agonist binding.

Keywords: NMDA receptor; Inorganic metal complexes; Iron complexes; Nitroprusside

1. Introduction

The role of NO as a messenger molecule in the central nervous system has been well established. Glutamate, by activating NMDA receptors, causes a Ca^{2+} influx and consequently the release of NO in cerebellar cells [1]. NO, in turn, has been suggested to block agonist binding to the NMDA receptor [2] and to inhibit NMDA-evoked intracellular Ca^{2+} responses [3], thereby exerting a feedback inhibition on the NMDA receptor [4]. NO has also been proposed to have, depending on its redox state (NO^+ or $NO^{\cdot}$), either neuroprotective or neurotoxic effects via an interaction with the redox modulatory site of the NMDA receptor [5]. Because a direct application of gaseous NO in biological assays is usually not feasible (due to immediate and strong acidification of the solution and difficulty of accurate dosage), the effects of NO were frequently studied using one of several NO-donors, such as nitroglycerine, nitrosocysteine, SIN-1 (3-morpholino-sydnonimine), SNAP (*S*-nitroso-*N*-acetyl-D,L-penicillamine), or the iron complex nitroprusside ($[Fe^{II}NO(CN)_5]^{2-}$). However, concerning the latter, several studies raised doubts about NO being at the origin of its inhibitory effects at the NMDA receptor. For

* Corresponding author. Tel.: +41 61 324 66 08; fax: +41 61 324 67 60.

E-mail address: manuel.koller@pharma.novartis.com (M. Koller).

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; D-AP5, D-2-amino-5-phosphonopentanoic acid; 8OH-DPAT, 8-hydroxy-2-dipropylaminotetralin; [^{125}I]GTI, 5-hydroxytryptamine-5-*O*-carboxymethyl-glycyl[^{125}I]tyrosinamide; 5-HT, 5-hydroxytryptamine (serotonin); NMDA, *N*-methyl-D-asparate; NO, nitric oxide; and PCP, 1-(1-phenylcyclohexyl)piperidine (phencyclidine).

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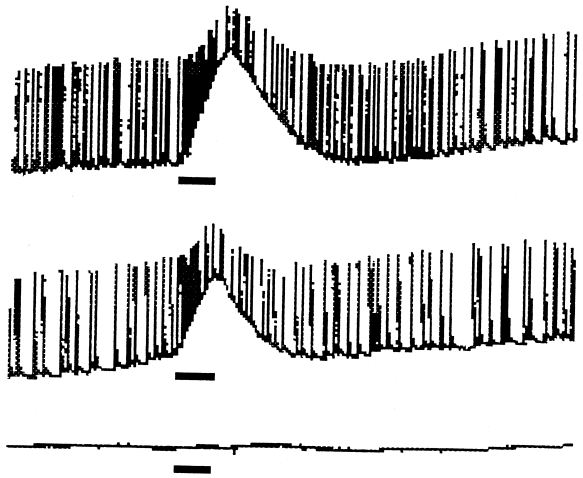

Fig. 1. Light dependence of NMDA receptor antagonism by sodium nitroprusside. Responses to NMDA (20 μM, applied for 1 min as indicated by the horizontal bars) and spontaneous epileptiform activity (mediated via activation of NMDA receptors by the endogenous agonist [29]) were recorded using the rat neocortical slice preparation. Treatment with a fresh solution containing 1 mM sodium nitroprusside (prepared and subsequently applied to the slice for at least one hour in the dark) only slightly decreased the frequency of spontaneous activity and the amplitude of NMDA-induced responses (middle trace) when compared to its own pretreatment control (top trace). In other slices continuously perfused with an irradiated solution (exposed for at least 30 min to an ordinary light source [halogen, 150 W]) containing a 10-fold lower concentration of sodium nitroprusside, both spontaneous activity and NMDA-induced responses were completely abolished (bottom trace). Results were similar when the fresh nitroprusside solution was exposed to direct sunlight (data not shown).

example, solutions of nitroprusside that had been extensively degassed with O_2/CO_2 to expel any NO formed were still able to block NMDA responses [6]. Furthermore, whereas the effects of nitroprusside on NMDA receptors were mimicked by the NO-free iron complex hexacyanoferrate(II) ($[Fe^{II}(CN)_6]^{4-}$) [6,7], they were, in different assay systems, not shared by some of the non-iron NO donors mentioned above [8–10].

We observed earlier [6] that the potencies of nitroprusside as well as hexacyanoferrate(II) ($[Fe^{II}(CN)_6]^{4-}$) and, to a lesser extent, hexacyanoferrate(III) ($[Fe^{III}(CN)_6]^{3-}$) to block NMDA responses in rat cortical slices were markedly enhanced by irradiation with light (Fig. 1). This prompted us to search for a common, NMDA receptor-blocking, NO-free photochemical transformation product of these iron complexes. A survey of the literature concerned with the photolytic pathways of cyanoferrates revealed a complex situation. Different factors seem to influence the photodegradation of nitroprusside, such as the wavelength and intensity of the light, pH of the solution, and the presence or absence of oxygen. Overall, however, the photodegradation of nitroprusside to aquopentacyanoferrate(II) and aquopentacyanoferrate(III) ($[Fe^{II}H_2O(CN)_5]^{3-}$ and $[Fe^{III}H_2O(CN)_5]^{2-}$, respectively) is well established [11–13]. Regarding the hexacyanoferrates, the photolytic formation of $[Fe^{II}H_2O(CN)_5]^{3-}$ from $[Fe^{II}(CN)_6]^{4-}$ and of $[Fe^{III}H_2O(CN)_5]^{2-}$ from $[Fe^{III}(CN)_6]^{3-}$

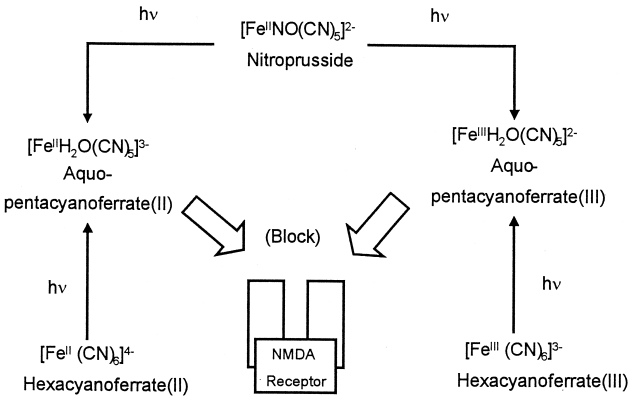

Fig. 2. Schematic representation of known interconversions of iron complexes by light, leading to the species $[Fe^{II}H_2O(CN)_5]^{3-}$ and $[Fe^{III}H_2O(CN)_5]^{2-}$ which, we suggest, block the NMDA receptor. See the text for further details.

has been clearly demonstrated [14,15]. Based on these reactions, outlined in Fig. 2, we reasoned that the two iron aquo complexes, $[Fe^{II}H_2O(CN)_5]^{3-}$ and $[Fe^{III}H_2O(CN)_5]^{2-}$, are the likely candidates to explain the NMDA receptor antagonism produced by nitroprusside or by $[Fe^{II}(CN)_6]^{4-}$ and $[Fe^{III}(CN)_6]^{3-}$. To confirm this hypothesis, we first tested $[Fe^{II}H_2O(CN)_5]^{3-}$ as a candidate to explain the NMDA receptor-blocking properties of nitroprusside, whereas later on, to compare the effects of Fe^{II} and Fe^{III} complexes, we extended our investigation to the related aminopentacyanoferrates $[Fe^{II}NH_3(CN)_5]^{3-}$ and $[Fe^{III}NH_3(CN)_5]^{2-}$ (the latter was more easily accessible than the corresponding aquo complex).

2. Materials and methods

2.1. Binding assays for different recognition sites on the NMDA receptor complex

Well-washed membranes for the [^{3}H]CGP 39653, [^{3}H]glycine, and [^{3}H]MK-801 binding assays were prepared from cortex and hippocampus of male adult Sprague–Dawley rats as described previously [16]. The methods used for measuring [^{3}H]CGP 39653 binding to the NMDA recognition site and the binding of [^{3}H]glycine to the strychnine-insensitive co-agonist site were also described earlier [16–18]. To determine the affinities of test compounds for the MK-801/PCP binding site within the NMDA receptor-operated ion channel, [^{3}H]MK-801 binding was measured *under equilibrium conditions* also according to a previously described method [16].

2.2. Radioligand binding assays for other neurotransmitter receptors

Binding assays for α_1- and α_2-adrenergic, dopamine D1 and D2, serotonin (5-HT1A, 1C, 1D and 5-HT2), and opiate receptors were also performed using well-established methods and radioligands [19–25].

Table 1
Affinities and potencies of iron complexes in different NMDA receptor assays

Compound	[^{3}H]CGP 39653 binding (pK_i)	NMDA-induced depolarizations (pIC_{50})	MK-801 potentiation (pA_2)
$[Fe^{II}H_2O(CN)_5]^{3-}$	7.8 ± 0.06	6.3 ± 0.1	7.4 ± 0.18
$[Fe^{II}NH_3(CN)_5]^{3-}$	7.7 ± 0.03	6.8 ± 0.1	8.0 ± 0.13
$[Fe^{III}NH_3(CN)_5]^{2-}$	7.9 ± 0.09	§	7.2 ± 0.10
$FeSO_4$	NT	<4	<5

The numbers (−log [M]) are means ± SEM from at least 3 independent experiments or from the fit of pooled data in the case of cortical slice experiments (NMDA-induced depolarizations). The Hill coefficients in the [^{3}H]CGP 39653 binding and electrophysiological experiments and the slopes of the Schild plots of the MK-801 potentiation assays were not significantly different from unity. §: full block of NMDA responses at 10 μM; NT: not tested.

2.3. Data analysis

In the receptor binding assays, IC_{50} values were derived from the curves describing the inhibition of specific [^{3}H]ligand binding by the test compounds and converted into K_is using the Cheng–Prusoff equation [26]. Prism software (GraphPad Software) was used for non-linear curve fitting. Results are expressed in logarithmic form, i.e. as pK_i values.

2.4. Functional NMDA receptor tests

2.4.1. MK-801 potentiation assay

The method for measuring the stimulation of [^{3}H]MK-801 binding *under non-equilibrium conditions* by glutamate has been described earlier [16]. Antagonist affinities were obtained from the shift of glutamate stimulation curves towards higher concentrations in the presence of varying antagonist concentrations, and calculated as pA_2 values from the corresponding Schild plots [27].

2.4.2. Antagonism of excitatory amino acid-induced depolarizations in rat cortical slices

The effects of iron complexes on spontaneous epileptiform discharges and slow depolarizations evoked by bath applications of NMDA, AMPA, and kainate in a rat cortical slice preparation placed in a chamber consisting of two compartments separated by a grease barrier were measured as described earlier [28,29].

2.5. Chemicals

The iron complex $Na_2[Fe^{III}NH_3(CN)_5]$ was prepared from $Na_2NH_4[Fe^{II}NH_3(CN)_5]$ by oxidation with sodium nitrite in cold water [30]. The crude product was dissolved three times in water at 0° and precipitated with ethanol. Calculated values of the element composition (with 0.5 H_2O) in %: C 23.3, H 1.6, Fe 21.7, N 32.6, Na 17.8, O 3.1; composition found by microanalysis: C 23.3, H 1.9, Fe 20.8, N 32.2, Na 18.3, O 2.8. The compounds $Na_2NH_4[Fe^{II}NH_3(CN)_5]$ and $FeSO_4$ (as the heptahydrate) were purchased from Fluka AG. The iron complex $Na_3[Fe^{II}H_2O(CN)_5]$ was synthesized from sodium nitroprusside by reduction with hydroxylamine following the procedure of Hofmann [31]. Calculated values in %: C 22.0, H 0.7, Fe 20.5, N 25.7, Na 25.3, O 5.9; found: C 22.5, H 0.5, Fe 20.2, N 26.0, Na 24.7, O 5.6. The radioligands used in this study, in particular [^{3}H]CGP 39653, [^{3}H]glycine, and [^{3}H]MK-801, were purchased either from Amersham Pharmacia Biotech or from NEN Life Science Products.

3. Results

The iron complex $[Fe^{II}H_2O(CN)_5]^{3-}$ potently inhibited the binding of the radioligand [^{3}H]CGP 39653 to the transmitter recognition site of the NMDA receptor in rat cortical membranes (pK_i = 7.8). Similarly high affinities were found for the amino complexes $[Fe^{II}NH_3(CN)_5]^{3-}$ and $[Fe^{III}NH_3(CN)_5]^{2-}$ (Table 1 and Fig. 3). The corresponding

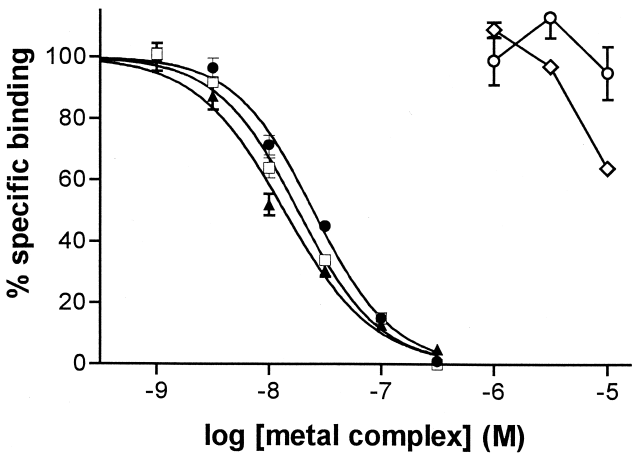

Fig. 3. Displacement of [^{3}H]CGP 39653 from the glutamate recognition site on the NMDA receptor by the iron complexes $[Fe^{III}NH_3(CN)_5]^{2-}$ (▲), $[Fe^{II}H_2O(CN)_5]^{3-}$ (□), and $[Fe^{II}NH_3(CN)_5]^{3-}$ (●). For $[Fe^{II}H_2O(CN)_5]^{3-}$, its weak or lacking activity as a displacer of [^{3}H]glycine (○) and [^{3}H]MK-801 (◇) from the glycine co-agonist and NMDA receptor channel sites, respectively, is also illustrated. The data points shown are means ± SEM from triplicate determinations of a typical experiment; the mean pK_i values derived from several such experiments are given in Table 1.

displacement curves (Fig. 3) all had Hill coefficients of one. To verify the specificity of this effect, we tested the binding affinities of $[Fe^{II}H_2O(CN)_5]^{3-}$ for a variety of other binding sites using the appropriate, well-established radioligands. The compound was inactive at the strychnine-insensitive glycine co-agonist site associated with the NMDA receptor (labeled by [^{3}H]glycine) and displayed only a weak affinity for the NMDA channel site (labeled by [^{3}H]MK-801) (Fig. 3). Furthermore, $[Fe^{II}H_2O(CN)_5]^{3-}$ turned out to be inactive in binding assays for serotonin 5-HT1A (radioligand: [^{3}H]8OH-DPAT), 1C ([^{3}H]mesulergine), 1D ([^{125}I]GTI), noradrenergic α_1 ([^{3}H]prazosin), dopamine D1 ([^{3}H]SCH23390) and D2 ([^{3}H]spiperone) as well as opiate ([^{3}H]naloxone) receptors, and was only weakly bound by 5-HT2 ([^{3}H]ketanserin) and α_2 ([^{3}H]clonidine) receptors with pK_i values of 5.6 in both cases (data not shown).

To demonstrate functional antagonism, responses to NMDA, AMPA, and kainate in rat cortical slices were measured in the absence and presence of 10 μM $[Fe^{II}H_2O(CN)_5]^{3-}$ (Fig. 4, top panel). Responses to NMDA (20 μM) were completely blocked, and spontaneous "epileptiform" activity (which is also NMDA receptor-mediated [29]) was also abolished. The inhibitory effect on NMDA-induced responses was concentration-dependent with an IC_{50} of about 0.5 μM (pIC_{50} = 6.3) and in several slices was found to be surmountable by increased concentrations of NMDA (data not shown). In contrast, responses induced with AMPA or kainate (15 μM) were not affected by $[Fe^{II}H_2O(CN)_5]^{3-}$. Exposure to light has been previously shown [6] to dramatically enhance the inhibition of NMDA-induced responses by nitroprusside (Fig. 1). Light dependence could, however, not be observed with $[Fe^{II}H_2O(CN)_5]^{3-}$ (data not shown). The amino complexes $[Fe^{II}NH_3(CN)_5]^{3-}$ and $[Fe^{III}NH_3(CN)_5]^{2-}$ prevented NMDA responses in the cortical slice preparation with potencies similar to that of the aquo complex (Table 1). On the other hand, no inhibition was observed with $FeSO_4$ up to 100 μM.

The rate of association of the "use-dependent" NMDA antagonist MK-801 to its binding site inside the receptor-operated ion channel depends on the degree of channel opening which, in turn, is a function of the activation of the glutamate recognition and the glycine co-agonist sites. Therefore, when measured under the appropriate *non-equilibrium conditions*, the stimulation of the binding of [^{3}H]MK-801 by glutamate (in the presence of a saturating concentration of glycine) can be taken as an index of agonism at the NMDA recognition site [32]. As shown in Fig. 5, the aquo complex $[Fe^{II}H_2O(CN)_5]^{3-}$ antagonized the activation of the NMDA receptor in a competitive fashion. At all antagonist concentrations, the same maximal effect could be achieved with saturating concentrations of glutamate. The pA_2 value derived from the corresponding Schild plot (Fig. 5, inset) correlated well with the NMDA receptor affinity determined from the [^{3}H]CGP 39653 binding assay

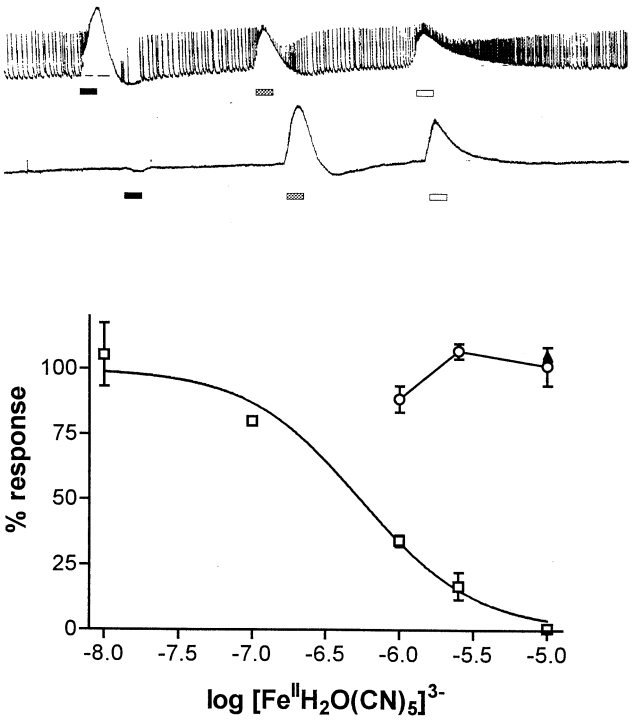

Fig. 4. Selective NMDA receptor antagonism by $[Fe^{II}H_2O(CN)_5]^{3-}$ in the cortical slice preparation. Top panel: Using a grease seal barrier technique, responses to NMDA (20 μM, black bars), AMPA (15 μM, gray bars), and kainate (15 μM, open bars) were recorded from rat neocortical slices while these were being exposed to magnesium-free buffer (top trace) and subsequently, after 30-min preincubation, to buffer containing 10 μM $[Fe^{II}H_2O(CN)_5]^{3-}$ (bottom trace). Note that spontaneous discharges (compound action potentials) were also inhibited. The horizontal bars represent a period of 1 min. Bottom panel: Inhibition curves constructed with the peak amplitudes of NMDA-induced responses (□) in percent of the control responses prior to antagonist exposure revealed a pIC_{50} = 6.3 ± 0.1. Inhibitory effects on AMPA- (○) or kainate- (▲) induced responses were not found. A single dose per slice was tested, and a total of 17 slices were used to construct the NMDA curve. The symbols represent mean values with their standard errors.

(Table 1). Similar observations were made with the two aminopentacyanoferrates, but not with $FeSO_4$ (Table 1).

4. Discussion

Aquopentacyanoferrate(II), $[Fe^{II}H_2O(CN)_5]^{3-}$, and the two related aminopentacyanoferrates $[Fe^{II}NH_3(CN)_5]^{3-}$ and $[Fe^{III}NH_3(CN)_5]^{2-}$ were shown to be competitive NMDA receptor antagonists with high affinity and selectivity in functional and radioligand binding assays. These surprising effects of inorganic species at the NMDA receptor allow several mechanistic interpretations. As transition metals are inherent redox systems, an electron transfer might occur between the iron complexes and the redox modulatory site of the NMDA receptor. However, the fact that similarly high potencies were found for both iron oxidation states ($[Fe^{II}NH_3(CN)_5]^{3-}$ and $[Fe^{III}NH_3(CN)_5]^{2-}$ (Table 1) indicates that a direct redox process at this site as

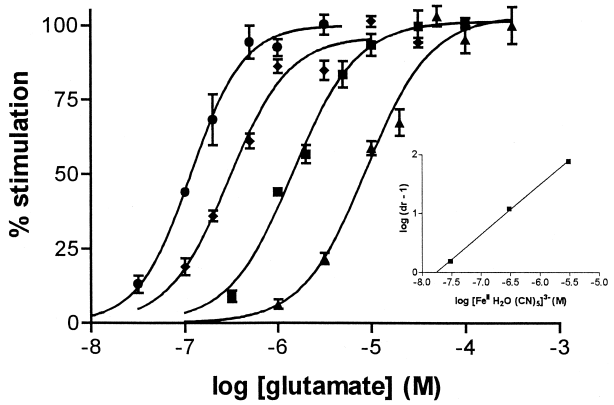

Fig. 5. Competitive antagonism of the glutamate-induced potentiation of [^{3}H]MK-801 binding by the iron complex $[Fe^{II}H_2O(CN)_5]^{3-}$. [^{3}H]MK-801 binding was measured under non-equilibrium conditions (in contrast to the experiments shown in Fig. 3) as described in the Methods section, i.e. in the absence (●) and the presence of 0.03 (◆), 0.3 (■), and 3 μM (▲) $[Fe^{II}H_2O(CN)_5]^{3-}$. The maximal stimulation obtained with glutamate under control conditions (i.e. in the absence of antagonist) was set to 100%. The data points shown are from a typical experiment and represent means ± SEM from triplicate determinations. The inset shows the corresponding Schild plot. The pA_2 values derived from several such experiments for the different iron complexes are given in Table 1.

an explanation of the effects of both iron species is unlikely. Furthermore, the antagonism produced by the iron complexes was found to be competitive (Fig. 5), whereas it is believed that the redox modulatory site of the NMDA receptor is distinct from its glutamate binding site [33]. The most likely mechanism, therefore, seems to be a ligand exchange between a functional group of the receptor binding site and the ligand sphere of the metal complex, a nucleophilic (thiol-, amino-, hydroxy-, or carboxyl) group at the receptor site displacing one of the ligands in the coordination shell of the iron complex. The high kinetic lability assigned to the readily exchangeable non-cyano ligand, i.e. the aquo or ammonia molecule loosely bound to the iron atom, is an important chemical feature of the three iron complexes found to be active in our assays. In fact, ligand exchange processes of aquo- and aminopentacyanoferrates in solution are well-documented reactions [34,35], and it is conceivable that they take place at receptor sites containing suitable functional groups. There is evidence [36] from mutagenesis and molecular modeling work that the amino acid residues glutamate[387], histidine[460], lysine[463], serine[486], threonine[488], asparagine[490], and arginine[493] on the NR2B subunit, all containing side chain functionalities adequate for ligand exchange at an iron complex, play an important role in the binding of agonists (e.g. glutamate) as well as competitive antagonists (e.g. D-AP5). Although it seems that somewhat different amino acid residues are important for glutamate binding to the NR2A subtype [37], some have also been identified which would be suitable for participation in an iron complex, e.g. histidine[466] (equivalent to histidine[460] in NR2B), threonine[665], or threonine[671].

In the light of the above-mentioned chemical lability of these metal complexes, the high selectivity observed with $[Fe^{II}H_2O(CN)_5]^{3-}$ may appear surprising. Most protein molecules contain functional groups capable of replacing a labile ligand at a metal center, but of the ones tested, only NMDA receptors were able to attract and bind $[Fe^{II}H_2O(CN)_5]^{3-}$ with high affinity. As a possible explanation, the negative overall charge of the complex ion comes to mind. Numerous other ligands with high affinity for the NMDA receptor, such as amino acid analogues bearing a terminal phosphono acid group (e.g. D-AP5 and derivatives thereof) [38] underline the preference of this receptor for negatively charged species. In line with this interpretation is the finding that aqueous solutions of $FeSO_4$, in which the iron ions are fully hydrated and form positively charged hexa–aquo complexes, were inactive in the concentration range tested (Table 1).

The similar IC_{50} values of the aquo- and aminopentacyanoferrates in both the binding experiments and the functional tests (Table 1) suggest that the amino complex dissolved in the aqueous medium might first be hydrolyzed to the aquo complex, being the species acting at the receptor. This hypothesis is strongly supported by the observation [39] that in an unbuffered aqueous solution at pH 6–8.5 and a temperature of 10–30°, the ammonia ligand is lost from the aminopentacyanoferrate(II) complex with a half-life of only 40 sec. Whether this might also be the case for the aminopentacyanoferrate(III) is not clear but seems unlikely, as this complex has been purified by repeatedly dissolving it in cold water followed by precipitation with ethanol.

Hexacyanoferrate(II), $[Fe^{II}(CN)_6]^{4-}$, has been reported by several authors to be a moderately active antagonist of NMDA receptor function, whereas hexacyanoferrate(III), $[Fe^{III}(CN)_6]^{3-}$, is weaker or even inactive [6–8,40]. However, according to the photolytic processes outlined in Fig. 2 and in analogy to nitroprusside, it seems likely that the antagonism of these hexacyanoferrates is mediated essentially by their more labile primary decay products, the aquopentacyanoferrates, formed slowly under the influence of daylight. The finding that the hexacyanoferrates are only weak antagonists would thus be attributed to their greater kinetic stability compared to their pentacyano counterparts, into which they would need to be transformed before binding at the NMDA receptor can occur.

To our knowledge, the type of ligand exchange mechanism which we propose here for an antagonist to interact with a hormone or neurotransmitter receptor has no precedent in the literature and represents a unique feature of these inorganic NMDA receptor antagonists. We are not aware of any description of a receptor expelling a labile atomic group from the ligand shell of a metal complex ion, resulting in a highly selective and competitive receptor–complex binding of nanomolar affinity.

In conclusion, the NMDA antagonistic effects of nitroprusside and presumably also of the hexacyanoferrates are attributed to their photodegradation products, the aquopen-

tacyanoferrates. Together with the related aminopentacyanoferrates, they represent a new class of selective and competitive antagonists with high affinity for the NMDA recognition site. A novel type of a receptor–ligand interaction is proposed, consisting of the participation of a functional group at the receptor site to the coordination sphere of an iron complex ion.

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