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Antagonist properties of the stereoisomers of ifenprodil at NR1A/NR2A and NR1A/NR2B subtypes of the NMDA receptor expressed in *Xenopus* oocytes

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Abstract

The NMDA receptor antagonist ifenprodil contains two asymmetric centres which give rise to four stereoisomeric forms of this molecule. The inhibitory effects of each of these stereoisomers on recombinant NMDA receptors expressed from NR1A/NR2A and NR1A/NR2B subunit combinations were studied in *Xenopus* oocytes by voltage-clamp recording. All four ifenprodil stereoisomers were potent antagonists at NR1A/NR2B ($IC_{50} < 0.8$ μM), but weak antagonists at NR1A/NR2A receptors ($IC_{50} > 100$ μM). In heteromeric NR1A/NR2B receptors, (+) *erythro*- and (−) *threo*-ifenprodil (IC_{50} 0.21 and 0.22 μM, respectively) were about 4 times more potent than (−) *erythro*- and (+) *threo*-ifenprodil (IC_{50} 0.81 and 0.76, respectively). These results show that the stereoisomers of ifenprodil exhibit a weak though significant stereoselectivity at the NR1A/NR2B NMDA receptor subtype.

Keywords: Recombinant NMDA receptor; *Xenopus* oocyte; Ifenprodil stereoisomer; Voltage clamp

1. Introduction

Ifenprodil is a non-competitive atypical NMDA receptor antagonist which has been shown to block the polyamine modulatory site of the NMDA receptor (for review see Carter et al., 1991; Schoemaker et al., 1994). In recent studies, using recombinant NMDA receptors expressed in *Xenopus* oocytes, ifenprodil was found to potently antagonize NMDA-induced currents in oocytes expressing the NR1A/NR2B but not in those expressing the NR1A/NR2A subunit combination (Williams, 1993). In addition to blocking the NMDA receptor, ifenprodil also antagonizes voltage-dependent N-, P- and L-type Ca^{2+} channels (Church et al., 1994). Furthermore, ifenprodil exhibits a high affinity for sigma (σ) opiate binding sites (Schoemaker et al., 1994; Hashimoto and London, 1995). These diverse properties of ifenprodil may contribute to its potent neuroprotective properties demonstrated in animal models of stroke (Gotti et al., 1988).

Ifenprodil contains two asymmetric centres which give rise to two diastereoisomeric forms of this molecule, namely the *erythro* form and the *threo* form. In the *erythro* form, the relative stereochemistry of the asymmetric centres is (R^*-S^*) and that of the *threo* form is (R^*-R^*). Enantiomers of the *erythro* and *threo* diastereoisomers exist, leading to a total of four stereoisomeric forms of the ifenprodil molecule, namely (+) and (−) *erythro* enantiomers and (+) and (−) *threo* enantiomers. The synthesis of ifenprodil is diastereoselective and the drug substance is composed entirely of the racemic *erythro* diastereoisomer i.e. (±) *erythro*-ifenprodil.

The stereoselectivity of ifenprodil at NMDA receptors has previously been investigated in an in vitro glutamate neurotoxicity test. In this study (±) *threo*-ifenprodil appeared as a more potent (5 times) neuroprotective agent than (±) *erythro*-ifenprodil and among the individual enantiomers, (−) *threo*-ifenprodil had the highest potency (Chenard et al., 1991). The expres-

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sion in heterologous systems of recombinant NMDA receptors is now possible and should allow a more direct characterization of the stereoselectivity of ifenprodil at these receptors. We used this strategy in the present study where we compared, using the double-electrode voltage clamp technique, the effects of the four stereoisomers of ifenprodil at two recombinant heteromeric NMDA receptor subtypes, namely NR1A/NR2A and NR1A/NR2B subunit combinations, that were transiently expressed in *Xenopus* oocytes.

2. Materials and methods

2.1. Plasmid constructions

The cDNAs coding for the NMDA receptor subunits NR1A, NR2A and NR2B were a gift from Prof. S. Nakanishi (Kyoto University, Kyoto, Japan). The NR1A subunit was subcloned from pN60 into pcDNA3 expression vector (Invitrogen, San Diego, CA) opened at the HindIII and EcoRV sites. The NR1A cDNA containing 265 of 5′ and 379 of 3′ untranslated base pairs (bp), respectively, was cloned in correct orientation to be driven by the CMV promoter. To subclone the NR2 subunits, we first constructed the pCISMCS vector by inserting between the ClaI and XhoI sites of the pCIS expression vector (provided by Dr. P.H. Seeburg, University of Heidelberg, Germany) a multicloning site made with complementary oligonucleotides CGATTCTAGA TTCGAACTCG AGGTTAACGG GCCCGCTAGC GATATCGCGG CCGCG and TCGACGCGGC CGCGATATCG CTAGCGGGCC CGTTAACCTC GAGTTCGAAT CTAGAAT. The NR2A cDNA was then subcloned from pNR2A into pCISMCS between the HpaI and NheI sites. The 5′ and 3′ untranslated regions of the subcloned NR2A cDNA contained 216 and 770 bp, respectively. Similarly, the NR2B cDNA from pNR2BF was inserted into the HpaI site of pCISMCS. The 5′ and 3′ untranslated regions of this subcloned cDNA contained 149 and 67 bp, respectively. For oocyte injection, plasmids were purified by ion exchange chromatography (Qiagen).

2.2. Oocyte injection and recording

Oocytes, stage V-VI were dissected from the ovary of anesthetized (in ice-cold water) *Xenopus laevi* (CNRS, Montpellier, France). Defolliculation of oocytes was performed by treatment, under agitation, with collagenase (Gibco, Grand Island, NY), 7.5 mg/ml for 30 min at room temperature in a modified Ringer solution (see composition below). Oocytes were then stored for 24 h at 18°C in Barth medium. cDNAs (2 ng of NR1A plus 10 ng of NR2A or NR2B, total volume 20 nl) were pressure-injected directly in the oocyte nuclei using a calibrated injection device (inject + matic, Geneva). Oocytes were then incubated for 2–5 days at 18°C. For recording, oocytes were transferred in a Perspex chamber superfused at 5 ml/min with a high Ba^{2+} Ringer solution (see composition below). Drugs were applied in the superfusing solution. Oocytes were voltage-clamped to negative voltages (−60 to −80 mV) via two 3 M KCl filled electrodes (resistance 0.5 MΩ) connected to a GeneClamp 500 amplifier (Axon Instruments, Foster City, California). Currents were digitized on-line at 25 Hz using the Axotape (Axon Instruments) software and the data stored on an optical disk. For analysis and presentation, data were transferred in ASCII format to Fig. P (Biosoft, Cambridge, UK). For IC_{50} determinations, the least square fitting routine of the same software was used. Fitting was performed using all individual data points and fitting parameters are given with a 95% confidence interval. Values are expressed as means ± standard error of the number (n) of oocytes tested.

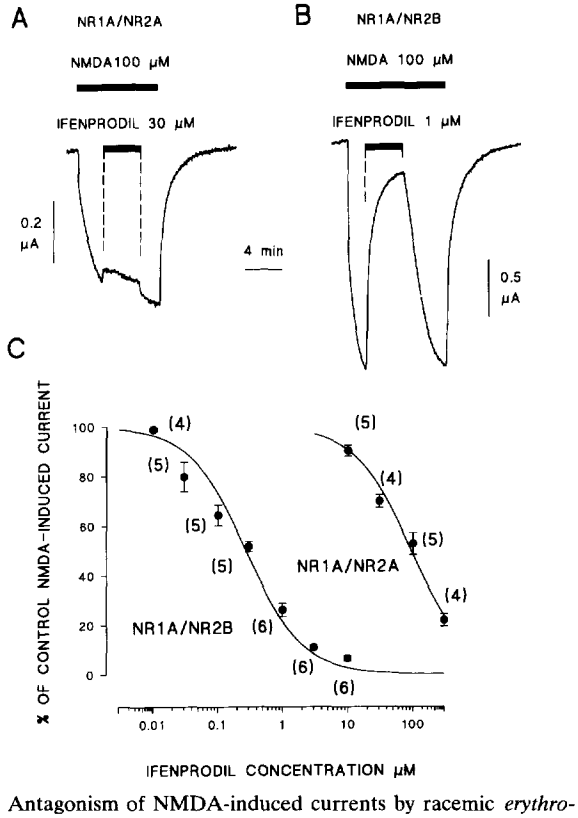

Fig. 1. Antagonism of NMDA-induced currents by racemic *erythro*-ifenprodil. (A,B) (±) *erythro*-Ifenprodil was applied at the concentration indicated during the current response obtained with 100 μM NMDA, at a voltage clamped at −80 mV, on oocytes expressing the NR1A/NR2A (A) and NR1A/NR2B (B) subunits. (C) Concentration-response curves obtained with oocytes expressing the two NMDA receptor subtypes using the protocol illustrated in (A) and (B). The data were fitted with the equation $100-(100*C/(C+IC_{50}))$ were C is the racemic ifenprodil concentration. Corresponding IC_{50} values are given in Table 1.

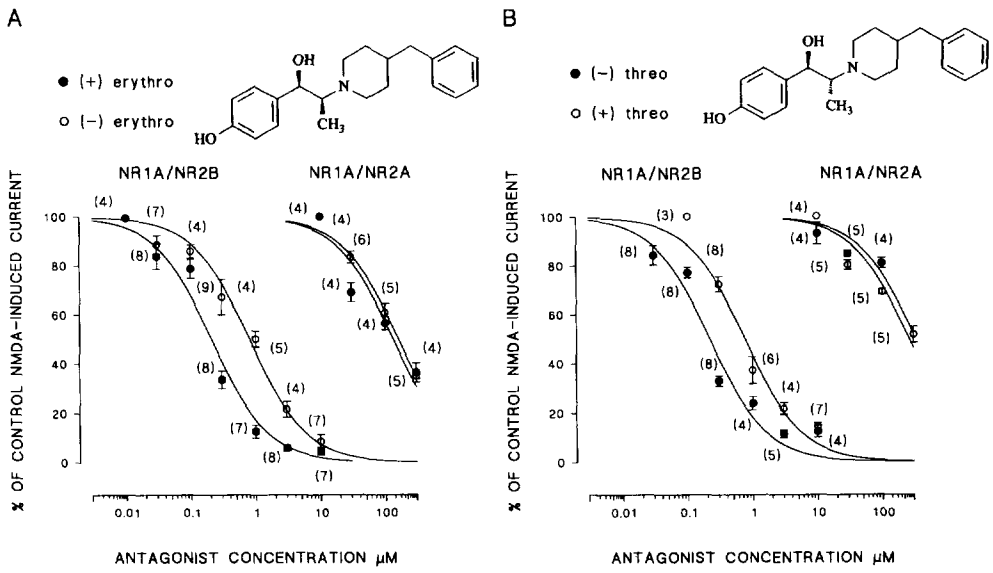

Fig. 2. Antagonism of NMDA-induced currents by the stereoisomers of ifenprodil. The protocol used was identical to that described in Fig. 1. (A) Antagonism of the NMDA response obtained with the enantiomers of *erythro*-ifenprodil in oocytes expressing NR1A/NR2B and NR1A/NR2A receptors. (B) Antagonism of the NMDA response obtained with the enantiomers of *threo*-ifenprodil in oocytes expressing NR1A/NR2B and NR1A/NR2A receptors. The data were fitted with the equation $100-(100*C/(C+IC_{50}))$ were C is the antagonist concentration. IC_{50} values are given in Table 1.

2.3. Solutions and reagents

The Barth medium (pH 7.6) contained (in mM): NaCl (88), KCl (1), $Ca(NO_3)_2$ (0.33), $CaCl_2$ (0.41), $MgSO_4$ (0.82), $NaHCO_3$ (2.4), Hepes/Tris-OH (10). Gentamicin (Sigma) was added at 1 ml/l. For defolliculation of oocytes, the simplified Ringer medium (pH 7.6) contained (in mM): NaCl (85), $MgCl_2$ (1), Hepes/KOH (5). The high Ba^{2+} Ringer solution used in recording conditions (pH 7.2) contained (in mM): NaCl (115), KCl (0.25), $BaCl_2$ (5.8), Hepes/Tris-OH (10).

Ifenprodil and its stereoisomers were prepared in the laboratory following procedures previously described (see Chenard et al., 1991 and references cited therein). (±) *erythro*-Ifenprodil was a tartrate salt; (+) *erythro*- and (−) *erythro*-ifenprodil were used as benzoate salts and (+) *threo*- and (−) *threo*-ifenprodil were used as free bases. All compounds were diluted in dimethyl sulfoxide (DMSO), the constant final concentration of which was 0.08% except for the 100 and 300 μM compound concentrations where the DMSO concentration was 0.2 and 0.8%, respectively. No difference was observed in preliminary experiments between the effects of (±) *erythro*-ifenprodil tartrate and (±) *erythro*-ifenprodil benzoate, indicating no effect of the accompanying ion.

Table 1
Antagonism by ifenprodil and its stereoisomers of NMDA-induced currents at NR1A/NR2B and NR1A/NR2A receptors

	IC_{50} (μM)			
	NR1A/NR2B		NR1A/NR2A	
(±) *erythro*-Ifenprodil	0.27[a]	[0.22,0.32]	92.5[c]	[74.5,110.5]
(+) *erythro*-Ifenprodil	0.21[a]	[0.17,0.25]	132.7[c]	[96.1,169.3]
(−) *erythro*-Ifenprodil	0.81[b]	[0.62,1.00]	160.0[c]	[134.3,185.7]
(+) *threo*-Ifenprodil	0.76[b]	[0.60,0.92]	256.2[d]	[202.6,309.8]
(−) *threo*-Ifenprodil	0.22[a]	[0.18,0.26]	324.8[d]	[192.4,457.2]

IC_{50} values were obtained by least square fitting of the concentration-response relationships and are given with a 95% confidence interval. The non-overlap of the confidence intervals indicates that a ≠ b ≠ c ≠ d.

3. Results

More than 80% of the injected oocytes responded to application of NMDA in the presence of 10 μM glycine by large inward currents when the oocytes were voltage-clamped at negative potentials (Fig. 1). EC_{50} values for NMDA were 36.4 [30.0,42.8] μM and 24.0 [14.3,33.7] μM for the NR1/NR2A and the NR1A/NR2B receptors, respectively, and currents recorded in response to 100 μM NMDA (at −80 mV holding voltage) ranged from 0.03 to 5 μA (mean 0.78 ± 0.11 μA, $n = 77$) and from 0.012 to 3.86 μA (mean 1.01 ± 0.08 μA, $n = 133$), respectively. When (±) *erythro*-ifenprodil, the drug substance currently used in most studies, was applied in the presence of NMDA, the current was concentration dependently reduced (Fig. 1). The blockade of the current was complete at 10 μM with an IC_{50} value of 0.27 [0.22,0.32] μM for oocytes injected with the NR1A/NR2B NMDA subunit combination and partial (80%), at the highest

concentration tested of 300 μM, for oocytes injected with the NR1A/NR2A assembly. These results confirm the selectivity of ifenprodil for the NR1A/NR2B receptors reported previously (Williams, 1993).

This selectivity was also found when each of the four stereoisomers were studied separately (Fig. 2A,B). *erythro*-Ifenprodil enantiomers, and *threo*-ifenprodil enantiomers antagonized the NMDA-induced current in NR1A/NR2A receptors with a low potency (IC_{50} > 100 μM) regardless of the optical isomer employed (Fig. 2A,B; Table 1). In contrast, all stereoisomers antagonized the NMDA response potently (IC_{50} < 0.8 μM) at NR1A/NR2B receptors: (+) *erythro*-ifenprodil exhibited a 4 times higher potency when compared to the (−) enantiomer while (−) *threo*-ifenprodil was 4 times more potent than the (+) enantiomer (Table 1).

4. Discussion

The results of the present study show that all stereoisomers of ifenprodil have a 500–1000-fold selectivity for the NR1A/NR2B versus NR1A/NR2A NMDA receptor. The two *erythro*-enantiomers were slightly more potent than the two *threo*-isomers at blocking NMDA-induced currents in NR1A/NR2A receptors (Table 1). In addition, essentially no difference was found when each of the enantiomers of *erythro*- and of *threo*-ifenprodil were compared at the NR1A/NR2A receptor combination. However, at the NR1A/NR2B receptor subtype, a 4-fold higher affinity was found for one of the enantiomers of each of the *erythro* ((+) enantiomer) and *threo* ((−) enantiomer) pairs. In the case of the *erythro* pair, for which we studied the racemate (the drug compound) and both enantiomers individually, the IC_{50} value of the racemate was intermediate between the IC_{50}'s obtained for each enantiomers and closer to the IC_{50} of the more potent enantiomer ((+) enantiomer) as would be expected from a competitive interaction of both enantiomers with a single receptor site.

Our results are only partially in accordance with those previously obtained with the different ifenprodil stereoisomers on glutamate-induced toxicity of cultured hippocampal neurones, a test taken as an index of NMDA receptor antagonism (Chenard et al., 1991). We found, like these authors, a higher potency of the (−) versus (+) *threo* isomer. However, calculations based on the activities of each individual enantiomer suggest no difference in potency between (±) *erythro*-ifenprodil and (±) *threo*-ifenprodil at NR1A/NR2A and NR1A/NR2B receptors, whereas in the latter study (±) *erythro*-ifenprodil was found about 5 times less potent than (±) *threo*-ifenprodil at preventing glutamate-induced hippocampal neuronal death. The discrepancy between our results and the in vitro glutamate neurotoxicity study suggests that blockade by this drug of the NMDA receptor may not be the sole mechanism involved in its neuroprotective effects. Indeed, ifenprodil was shown recently to possess voltage-operated Ca^{2+} channel antagonist properties in cultured hippocampal neurons (Church et al., 1994). A similar neuronal Ca^{2+} channel antagonism has been also demonstrated for other neuroprotective compounds acting at the NMDA receptor, such as dextromethorphan (Netzer et al., 1993) or eliprodil, an ifenprodil derivative (Avenet et al., 1994; Biton et al., 1994). The stereoselectivity of ifenprodil at these channels is not known and may completely differ from that observed at NMDA receptors.

A 4-fold decrease in the affinity for σ-1 sites has been reported when the relative stereochemistry of ifenprodil was changed from (±) *erythro* to (±) *threo*, whereas no difference in affinity was observed at σ-2 sites (Hashimoto and London, 1995). Since our results suggest no difference in the potency of (±) *erythro*- and (±) *threo*-ifenprodil at blocking NR1A/NR2B or NR1A/NR2A receptors, it is likely that stereochemical requirements for the σ-1 site and for the above NMDA receptor subtypes differ. This lends support to the current view that the NMDA receptor and the σ-1 site are separate molecular entities.

In conclusion, all four stereoisomers of ifenprodil display a high selectivity for the NR1A/NR2B versus NR1A/NR2A subtype of the NMDA receptor. At the NR1/NR2B receptor a small but nonetheless significant difference in potency exists between the enantiomers of each *erythro* and *threo* pairs. However, because of the similar potencies of (+) *erythro*- and (−) *threo*-ifenprodil, on one hand, and the similar potencies of (−) *erythro*- and (+) *threo*-ifenprodil, on the other hand, essentially no difference in the potencies of the racemates (±) *erythro*- and (±) *threo*-ifenprodil exists at this receptor subtype.

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