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# Effects of RPR 118723, a novel antagonist at the glycine site of the NMDA receptor, in vitro

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**Abstract**

RPR 118723 ((8-chloro-5-methyl-2,3-dioxo-1,4-dihydro-5*H*-indeno[1,2-b]pyrazin-5-yl) acetic acid) was previously reported to exhibit potent affinity for the glycine site of the *N*-methyl-D-aspartate (NMDA) receptor–channel complex in the nanomolar range ($K_i = 3.1 \pm 0.8$ nM). We now report on the effects of RPR 118723 in two functional tests reflecting the interaction between the glycine site and the NMDA receptor. First, RPR 118723 potently inhibited [$^3$H]*N*-[1-(2-thienyl)cyclohexyl]-3,4-piperidine ([$^3$H]TCP) binding in the presence of NMDA ($IC_{50} = 3.5 \pm 0.4$ nM). Second, RPR 118723 antagonized the NMDA-induced increase in [$^3$H]dopamine release in mouse striatal slices ($IC_{50} = 8.0 \pm 1.1$ nM). In both experimental models, an excess of glycine reversed the effect of RPR 118723. These results show that RPR 118723 interferes functionally in the nanomolar range with the glycine site coupled to the NMDA receptor in vitro. The blockade of the glycine site with RPR 118723 may be useful for the therapy of the disorders linked to excessive NMDA stimulation.
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*Keywords:* RPR 118723; Glycine antagonist; Glutamate; *N*-methyl-D-aspartate (NMDA) receptor–channel complex; TCP binding; Dopamine release

## 1. Introduction

A strychnine-insensitive glycine modulatory site on NMDA receptors was first reported by Johnson and Ascher (1987). Since then, various compounds with affinity and specificity for the glycine site have been reported (see Carter, 1992; Bigge, 1993; Kemp and Leeson, 1993; Ornstein et al., 1994; Grimwood et al., 1995; Mignani et al., 1995; Ilyin et al., 1996; Kehne et al., 1995; Kulagowski and Leeson, 1995; Boireau et al., 1996; Wood and Hawkinson, 1997).

A new chemical series led to the optically active RPR 118723 (Fig. 1) ((8-chloro-5-methyl-2,3-dioxo-1,4-dihydro-5*H*-indeno[1,2-b]pyrazin-5-yl) acetic acid), one of the very few water-soluble glycine/NMDA antagonists of nanomolar potency range ([$^3$H]5,7-dichlorokynurenate binding: $K_i = 3.1 \pm 0.8$ nM), which displays in vivo activity at low doses in animal model of convulsions and in electrophysiological studies (Jimonet et al., 2000). In the present work, we investigated the in vitro functional activity of RPR 118723 in two models in which the response to NMDA is modulated by glycine. First, we tested the effect of RPR 118723 on [$^3$H]*N*-[1-(2-thienyl)cyclohexyl]-3,4-piperidine ([$^3$H]TCP) binding in the presence of NMDA (see Kloog et al., 1990; Hori et al., 1991). Second, as the release of dopamine (DA) is modulated by NMDA receptors (Werling et al., 1990; Hanbauer et al., 1992; Krebs et al., 1991; Nankai et al., 1995), we tested the effects of RPR 118723 in the mouse striatal slices on the evoked release of [$^3$H]dopamine induced by NMDA. We used 5,7-dichlorokynurenic acid as a reference compound; some data obtained with this glycine site antagonist were previously reported (Boireau et al., 1996).

## 2. Materials and methods

### 2.1. [$^3$H]TCP binding

#### 2.1.1. Membrane preparation

Rats (IcO:OFA-SD 200–300 g) were decapitated and their cerebral cortices removed on ice and frozen at −80°C for at least 24 h. The tissue was rapidly thawed, homogenised with a Polytron® in 20 volumes of cold (4°C) sucrose (0.32 M) and centrifuged at $1000 \times g$ for 20 min.

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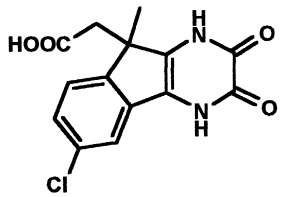


Fig. 1. Structure of RPR 118723.

The supernatant was recovered and recentrifuged at 17,500 $\times g$ for 20 min. The resulting pellet was resuspended in 50 volumes of ice-cold distilled water, incubated for 30 min at 37°C and centrifuged at 32,000 $\times g$ for 20 min. This procedure was repeated twice. The pellet was resuspended in 50 volumes of HEPES–NaOH 10 mM, pH 7.5 and centrifuged at 32,000 $\times g$ for 20 min. The resulting pellet was resuspended in 30 volumes of HEPES buffer and frozen at −80°C until use. On the day of the binding assay, the membranes were thawed and centrifuged at 32,000 $\times g$ for 20 min. This procedure was repeated twice. The final pellet was resuspended in the appropriate buffer for use in the binding assay.

*2.1.2. [$^3$H]TCP binding assay*

The extent of [$^3$H]TCP binding to the dissociative anaesthetic binding site was determined by the method described by Hori et al. (1991). Membranes (0.2 mg protein/ml) suspended in 10 mM HEPES buffer (pH 7.5) were incubated for 45 min at 25°C with 2.5 nM [$^3$H]TCP and the studied compound, plus NMDA 100 μM. Dizocilpine (MK801; 10 μM) was added to some of the aliquots for determination of non-specific binding. The binding interaction was terminated by filtration through Whatman GF/B glass fibre filters with a Skatron® cell harvester, and filters were immediately rinsed with 3 × 4 ml of cold buffer. Each determination was performed in duplicate. The radioactivity remaining on the filters was measured by liquid scintillometry in Ready Solv HP scintillant.

*2.2. [$^3$H]dopamine release in mouse striatal slices*

Male mice (Charle Rivers, France) weighing 20–35 g were housed 10 to a cage in a controlled environment with a 12-h light–dark cycle. Food and water were freely available. The in vitro release of dopamine was studied as previously described (Boireau et al., 1993) with slight modifications. Briefly, mouse striata were sliced into ribbons (0.3 × 0.3 mm) with a McIlwain tissue chopper and incubated for 15 min at 37°C in an oxygenated (95% $O_2$, 5% $CO_2$) physiological medium composed of (mM): NaCl 118, KCl 5, $NaHCO_3$ 25, $NaH_2PO_4$ 1, $MgSO_4$ 1.2, $CaCl_2$ 1.9, glucose 11.1, ascorbic acid 0.1, pargyline 10 μM and 0.05 μM [$^3$H]dopamine (1140 GBq/mmol, New England Nuclear). The tissue was rinsed and 1-ml aliquots containing approximately 10 mg of tissue were transferred to superfusion chambers consisting of Millipore filters (Millex HA; 0.45 μm). After 30 min of superfusion at 0.4 ml/min in the absence of $Mg^{2+}$, 2-min fractions were collected directly into vials and the amount of radioactivity was determined by liquid scintillation spectrometry. NMDA was added in fractions 4–5 and RPR 118723 (or 5,7-dichlorokynurenic acid) were added 4 min before NMDA and maintained throughout the superfusion. Overall, nine fractions of superfusion were collected. The radioactivity remaining in the filter at the end of the superfusion was measured. Radioactivity was expressed as a percentage of the total radioactivity present in the slices at the beginning of each fraction. The results are given in terms of percent release for each fraction. The percent release for the fifth fraction was generally the fraction of maximal response. The concentration inhibiting by 50% the effects of NMDA on the overflow from the fifth fraction ($IC_{50}$ value) was calculated by computer-assisted iterative non-linear regression analysis using a GraphPad PRISM™ software package. Data from the fifth fraction were analyzed by Student's *t* test or one-way analysis of variance followed by a Student–Newman–Keuls' test. Significance was defined as $P < 0.05$. NMDA was purchased from Sigma (La Verpillière, France). Our chemical department synthetized the 5,7-dichlorokynurenic acid.

## 3. Results

*3.1. Effect of RPR 118723 on [$^3$H]TCP binding*

NMDA (100 μM) enhanced basal [$^3$H]TCP binding (fmol/mg protein: basal = 26 ± 3, NMDA present = 158

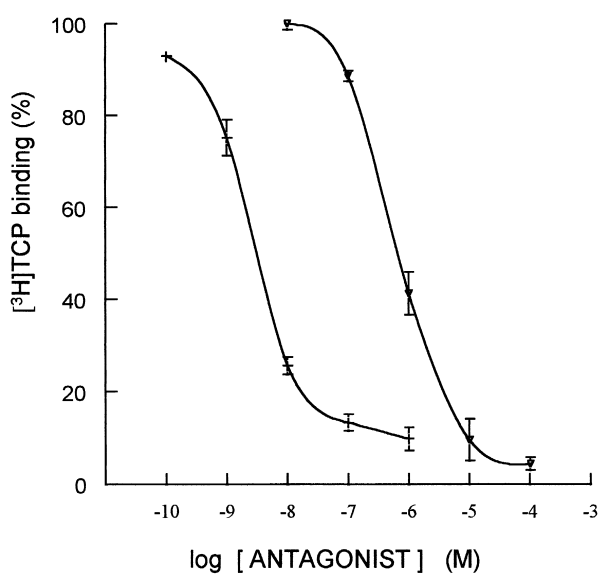


Fig. 2. Effects of RPR 118723 (+) and 5,7-dichlorokynurenic acid: DCKA (▽) on the specific binding of [$^3$H]TCP. Specific binding of 2.5 nM [$^3$H]TCP was determined in the presence of 100 μM NMDA. The data shown are the mean ± S.E.M. of data obtained from at least three independent experiments.

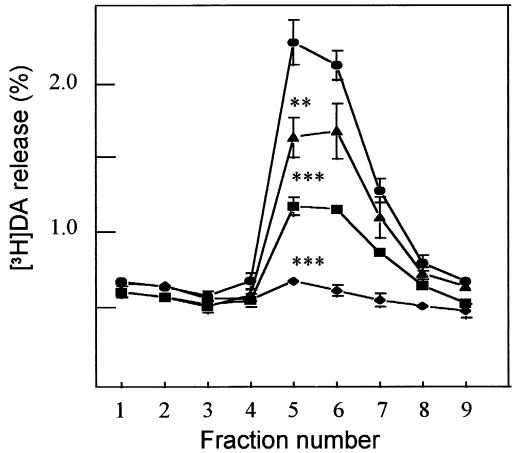


Fig. 3. Effect of RPR 118723 on the NMDA-induced release of [$^3$H]dopamine from striatal slices. NMDA ([●]: 100 μM) was added in fractions 4–5 and RPR 118723 ([▲]: 0.5 nM; [■]: 5 nM; [♦]: 50 nM) was added 4 min before NMDA and maintained throughout the superfusion. $^{**}$: $P < 0.01$; $^{***}$: $P < 0.001$, significantly different from controls. Each point represents the mean ± S.E.M. of three individual determinations.

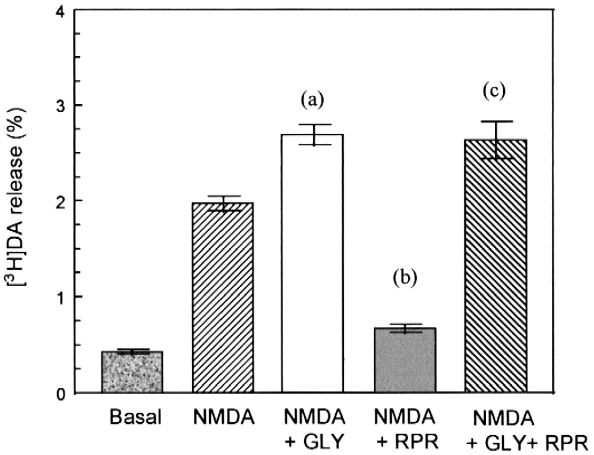


Fig. 5. Modulation of [$^3$H]dopamine ([$^3$H]DA) release by 100 μM NMDA and 50 nM RPR 118723 (RPR) in the absence or in the presence of added glycine (GLY). Values given are the mean of three individual determinations of the radioactivity released in the fifth fraction. (a): $P < 0.01$ vs. NMDA alone. (b): $P < 0.001$ vs. NMDA alone. (c): $P < 0.001$ vs. NMDA + RPR.

± 16; mean of seven experiments in duplicate). As shown in Fig. 2, RPR 118723 potently inhibited ($IC_{50} = 3.5 \pm 0.4$ nM) the increase in [$^3$H]TCP binding brought about by NMDA. This result is in good agreement with our previous data showing that RPR 118723 is a potent inhibitor of [$^3$H]5,7-dichlorokynurenic acid binding at the glycine site of the NMDA receptor–channel complex (Jimonet et al., 2000). In the presence of NMDA and 1 mM glycine, RPR 118723 was more than 7000 times less potent in displacing [$^3$H]TCP from its binding sites ($IC_{50}$ close to 25 μM) suggesting it to be an antagonist at the glycine site. Interestingly, 5,7-dichlorokynurenic acid also inhibited the binding of [$^3$H]TCP in the presence of NMDA ($IC_{50} = 685$ ± 105 nM), an effect totally reversed by glycine ($IC_{50} >$ 100 μM).

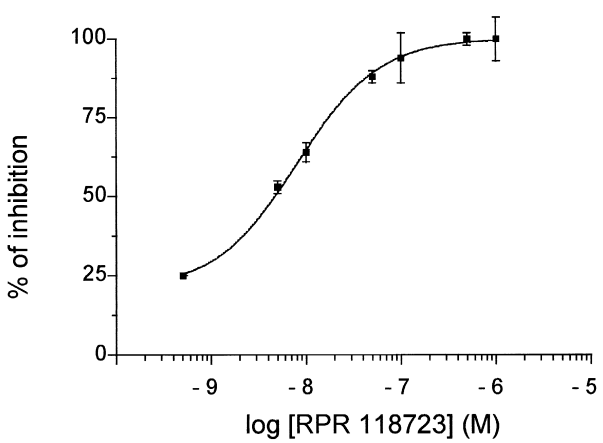


Fig. 4. Dose–response curve of the effect of RPR 118723 on NMDA-induced [$^3$H]dopamine ([$^3$H]DA) release from striatal slices. RPR 118723 (0.5 to 1000 nM) was added 4 min before NMDA (100 μM) and maintained throughout the superfusion. Values given are means for triplicate determinations in each of four different experiments.

### 3.2. Effect of RPR 118723 on [$^3$H]dopamine release

As shown in Fig. 3, 100 μM NMDA markedly increased the basal release of [$^3$H]dopamine, with a maximal effect observed generally in the fifth fraction of perfusion. As shown in Fig. 4, RPR 118723 dose-dependently antagonized this increase ($IC_{50} = 8.0 \pm 1.1$ nM). In the presence of 1 mM glycine, the effect of NMDA was slightly increased (Fig. 5), a result which suggests that under these experimental conditions, glycine sites were not totally saturated. Interestingly, the antagonist effect of 50 nM RPR 118723 was totally reversed in the presence of glycine (Fig. 5). 5,7-dichlorokynurenic acid also, dose-dependently antagonized the releasing effect of NMDA ($IC_{50}$ close to 1 μM; data not shown).

## 4. Discussion

This study reports that RPR 118723, a new indeno[1,2-b]pyrazin-2,3-dione derivative, interferes with the glycine site coupled to the NMDA receptor channel complex. In a previous study (Jimonet et al., 2000), we showed that RPR 118723 is a potent inhibitor of [$^3$H]5,7-dichlorokynurenic acid binding and interferes only at high (micromolar) concentrations with the NMDA and the dissociative anaesthetic sites of the NMDA receptor–channel complex, as well as the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor (Jimonet et al., 2000). In the present article, we report that RPR 118723 exhibits a

potent activity in two tests in which NMDA receptor stimulation is regulated by glycine. As discussed by Kloog et al. (1990), the binding of [$^3$H]TCP provides a means to assess the specificity of a given ligand for the glycine site, (and also for the NMDA site or the dissociative anaesthetic site located inside the channel). In the presence of NMDA and the absence of added glycine, glycine antagonists potently inhibit the binding of [$^3$H]TCP. Under our experimental conditions, [$^3$H]TCP binding was increased by NMDA and this effect was antagonized by RPR 118723. In the presence of glycine, the efficacy of RPR 118723 was greatly reduced (about 7000-fold). From these results, it can be concluded that RPR 118723 interferes specifically with the glycine site of the NMDA receptor–channel complex, which responds functionally to stimulation with both co-agonists. 5,7-dichlorokynurenic acid less potently than RPR 118723 antagonized the binding of [$^3$H]TCP and this effect was totally reversed by glycine.

The effects of RPR 118723 were also studied in another functional model in which the release of [$^3$H]dopamine from the mouse striatal slices was increased in the presence of NMDA. In this model, RPR 118723 caused dose-dependent antagonism of the effect of NMDA. In the presence of added glycine, the releasing effect of NMDA was slightly increased, suggesting that under these experimental conditions the glycine sites are not totally saturated. Furthermore, under these experimental conditions, the activity of RPR 118723 was totally abolished. Interestingly, 5,7-dichlorokynurenic acid also antagonized the releasing effect of NMDA, though being less potent than RPR 118723. In consequence, from the data obtained with this second model, we may propose that RPR 118723 interacts specifically with NMDA receptors at the glycine site.

In conclusion, RPR 118723 represents a new and extremely potent glycine antagonist which modulates functionally the activity of the NMDA receptor–channel complex at nanomolar concentrations. Excessive NMDA stimulation has been suggested to play a role in pathological events such as cerebral ischemia, stroke, trauma, epilepsy and transmission of pain. The acetic acid-type entity in the 5-position leads to a water-soluble compound (Jimonet et al., 2000). Thus, RPR 118723 represents one of the very few water-soluble glycine/NMDA antagonists of nanomolar potency, which furthermore display in vivo activity in animal models of convulsions at low doses (Jimonet et al., 2000). The blockade of the glycine site with RPR 118723 may be useful for the therapy of the disorders linked to excessive NMDA stimulation.

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