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Effect of 1-aminocyclopropanecarboxylic acid on N-methyl-D-aspartate-stimulated [^{3}H]-noradrenaline release in rat hippocampal synaptosomes

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1 The effect of 1-aminocyclopropanecarboxylic acid (ACPC), a partial agonist at the glycine site of the N-methyl-D-aspartate (NMDA) receptor complex that exhibits neuroprotective, anxiolytic and antidepressant-like actions, was investigated in a functional assay for presynaptic NMDA receptors.

2 NMDA (100 μM) produced a 36% increase of tritium efflux above basal efflux in rat hippocampal synaptosomes preincubated with [^{3}H]-noradrenaline ([^{3}H]-NA), reflecting a release of tritiated noradrenaline. This effect was prevented by 10 μM 7-chlorokynurenic acid, an antagonist of the glycine site of the NMDA receptor.

3 Glycine enhanced the effect of NMDA with E_{max} and EC_{50} values of $84 \pm 11\%$ and 1.82 ± 0.04 μM, respectively. ACPC potentiated the effect of NMDA on tritium overflow with a lower EC_{50} (43 ± 6 nM) and a lower maximal effect ($E_{max} = 40 \pm 9\%$) than glycine. Furthermore, ACPC (0.1 μM) shifted the EC_{50} of glycine from 1.82 μM to $\geqslant 3$ mM.

4 These results show that ACPC can reduce the potentiation by glycine of NMDA-evoked [^{3}H]-NA release and hence, may act as an antagonist at the glycine site of presynaptic hippocampal NMDA receptors when the concentration of glycine is high.

Keywords: ACPC; NMDA receptor; glycine; hippocampus; noradrenaline release

Introduction

The N-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptors is a ligand-gated ion channel that contains discrete but interdependent functional domains, each with separate ligand binding sites (recently reviewed in McBain & Mayer, 1994). Among others there is a transmitter recognition site for acidic amino acids such as L-glutamate; a cation channel with a unique voltage-dependent regulation by magnesium; a polyamine site; and a recognition site for neutral animo acids such as glycine. The binding of glycine to its recognition site on the NMDA receptor is strychnine-insensitive and was initially shown to augment the electrophysiological actions of NMDA in cultured neurones (Johnson & Ascher, 1987). Subsequent studies in both *Xenopus* oocytes expressing NMDA-gated cation channels (Kleckner & Dingledine, 1988; Lerma *et al.*, 1990) and primary cultures of the rat visual cortex (Huettner, 1989) have demonstrated that occupation of the glycine site by an agonist is an absolute requirement for NMDA receptor activation and that glycine acts as a co-agonist at this receptor (Kleckner & Dingledine, 1988).

1-Aminocyclopropanecarboxylic acid (ACPC) is a specific high affinity ligand ($K_i \sim 32$ nM) at strychnine-insensitive glycine sites (Marvizon *et al.*, 1989) that has been reported to exhibit partial agonist activity in both neurochemical (Marvizon *et al.*, 1989) and electrophysiological (Watson & Lanthorn, 1990; Priestley & Kemp, 1994) studies. It has been hypothesized that if the glycine co-agonist site needs to be occupied for the activation of the NMDA receptor complex and this is also an absolute requirement *in vivo*, then a partial agonist such as ACPC could function as an NMDA antagonist *in vivo* (Skolnick *et al.*, 1989; Trullas *et al.*, 1989). In agreement with this hypothesis, and similarly to both competitive NMDA antagonists and use-dependent channel blockers, ACPC exhibited anticonvulsant (Skolnick *et al.*, 1989), neuroprotective (Von Lubitz *et al.*, 1992), anxiolytic (Trullas *et al.*, 1989), and antidepressant (Trullas & Skolnick, 1990) actions in animal models. To investigate the mechanisms involved in the functional NMDA receptor antagonist properties of ACPC, the present study was designed to evaluate the effects of this compound on NMDA receptor function using an *in vitro* functional assay for NMDA receptors (Pittaluga & Raiteri, 1990).

Previous investigations have shown that NMDA-sensitive receptors are present on noradrenergic axon terminals of rat cerebral cortex and hippocampus (Pittaluga & Raiteri, 1990; Fink *et al.*, 1990). Activation of these presynaptic receptors results in a Ca^{2+}-dependent [^{3}H]-noradrenaline ([^{3}H]-NA) release and their pharmacological characterization indicates that they are indistinguishable from the NMDA receptors described in electrophysiological studies (Pittaluga & Raiteri, 1990; 1992; Göthert & Fink, 1991; Raiteri *et al.*, 1992; Wang *et al.*, 1992). The NMDA-stimulated [^{3}H]-NA release is potentiated by glycine and is blocked by Mg^{2+} and selective NMDA antagonists (Pittaluga & Raieri, 1990; 1992; Göthert & Fink, 1991; Wang *et al.*, 1992).

Using this functional assay for presynaptic NMDA receptors, we now show that ACPC reduces the facilitatory effect of glycine on NMDA-evoked [^{3}H]-NA release. This result provides further evidence to support the hypothesis that ACPC may act as a functional NMDA receptor antagonist.

Methods

Preparation of synaptosomes

Male Sprague-Dawley rats (3-4 months) were housed 3 per cage and maintained on a 12 h light/dark cycle with food and water freely available. Animals were killed by decapitation and the brains were quickly removed and kept on ice during dissection. Crude hippocampal synaptosomes were prepared according to Gray & Whittaker (1962). Briefly, the hippocampus

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was rapidly dissected on ice and homogenized with a Teflon-glass Potter homogenizer in 40 volumes of 0.32 M sucrose buffered at pH 7.4 with sodium phosphate (0.01 M). Homogenates were centrifuged at 1000 *g* for 5 min, the pellet was discarded and the supernatant was again centrifuged at 12,000 *g* for 20 min. The crude synaptosomal pellet was then resuspended in a standard medium having the following composition (in mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 22, NaH_2PO_4 1, glucose 10, pH 7.2 to 7.4 and continuously aerated with 95% O_2 and 5% CO_2 at 37°C.

Neurotransmitter release

The release of [^{3}H]-NA from synaptosomes was measured in a continuous superfusion system as described by Raiteri *et al.* (1974). Briefly, after incubation of synaptosomes (15 min, 37°C) in the presence of [^{3}H]-NA (0.04 μM, 39 Ci mmol^{-1}) aliquots of the synaptosomal suspension (0.24 mg protein/chamber) were distributed on 0.65 μm Millipore filters and placed at the bottom of a set of parallel superfusion chambers. After 20 min of superfusion the standard medium was replaced with a medium without $MgSO_4$. Two-minute fractions were collected starting at $t=37.5$ min ($t=0$ being the beginning of superfusion). The amount of radioactivity released into each fraction was calculated as the percentage of the total synaptosomal tritium at the start of the fraction collected. Tritium release in each fraction was expressed as a ratio over the first fraction collected (Fx/F1). Preliminary studies indicated that the maximal effects of drugs on tritium release were reached in the third fraction (results not shown). Thus, the effects of drugs on tritium release were evaluated by calculating the ratio of percentage tritium release in fraction 3 (F3), corresponding to the maximal effect, and the tritium release in the first fraction collected (F1, in which no drugs were present). This F3/F1 ratio was compared with the corresponding F3/F1 ratio obtained for the respective control groups. NMDA, glycine and ACPC were added at the end of the first fraction. In experiments evaluating the effects of ACPC on the potentiation by glycine of NMDA-evoked tritium release, ACPC was added 10 min before NMDA and glycine. Estimates of E_{max} (maximal effect) and EC_{50} (molar concentration of drug producing 50% of its maximal effect) were calculated for glycine and ACPC from concentration-response experiments by non-linear regression analysis using the SigmaPlot computer programme (Jandel, San Rafael, CA, U.S.A.).

Statistical analysis

Results represent mean ± s.e.mean. Statistical differences between groups were analyzed with Student's paired *t* test; $P<0.05$ was considered to be significant.

Drugs

The following drugs were used: (−)-[7,8-^{3}H]-noradrenaline (39 Ci mmol^{-1}) was obtained from Amersham International, N-methyl-D-aspartic acid and glycine from Sigma Chemical (St Louis, MO, U.S.A.), 7-chlorokynurenic acid (7Kyn) from RBI (Natick, MA, U.S.A.); 1-aminocyclopropanecarboxylic acid (ACPC) from Tocris-Cookson (Bristol, U.K.).

Results

The spontaneous basal tritium efflux from synaptosomes prepared from rat hippocampus and pre-incubated with [^{3}H]-NA in the first fraction collected (F1) was 1.29 ± 0.04% (per 2 min) of the total remaining radioactivity. In the absence of added drugs, the ratio of % tritium efflux of the third over the first fraction (F3/F1) was 0.87 ± 0.02. NMDA (100 μM) stimulated tritium release in the third fraction, raising the F3/F1 ratio to 1.18 ± 0.04 which represents a 36% increase in F3/F1 ratio when compared to unstimulated basal levels. Addition of glycine (10 μM) potentiated the effects of NMDA on tritium release and significantly increased the F3/F1 ratio by 27% to 1.50 ± 0.07 (Figure 1). Similarly, the addition of ACPC (0.1 μM) to the superfusion medium enhanced the NMDA-evoked tritium release in the third fraction and increased the F3/F1 ratio by 26% to 1.49 ± 0.04. 7Kyn, an antagonist at the glycine site of the NMDA receptor, at a concentration of 10 μM significantly antagonized the effects of both glycine and ACPC. Furthermore, 7Kyn completely blocked the effect of NMDA (100 μM) in the absence of added glycine, indicating the presence of glycine either in the synaptosomal samples or in the assay buffers (Figure 1).

Glycine produced a concentration-related increase in the NMDA-evoked overflow of tritium, with an EC_{50} of 1.82 ± 0.04 μM and an E_{max} of 84 ± 11% when compared to effect of NMDA in the absence of added glycine. ACPC also potentiated the effect of NMDA but with a significantly lower EC_{50} (43 ± 6 nM) and a significantly lower maximal effect ($E_{max}=40\pm9\%$) when compared to glycine (Figure 2).

The concentration-response curve of glycine was also assessed in the presence of fixed concentrations (0.1 nM and 0.1 μM) of ACPC to determine the partial agonist activity of ACPC at the glycine modulatory site. The EC_{50} of glycine, which was 1.82 ± 0.04 μM in the absence of ACPC, was increased to 51 ± 3 μM and ⩾3 mM in the presence of 0.1 nM and 0.1 μM concentrations of ACPC respectively (Figure 3).

Discussion

NMDA increased tritium efflux in superfused rat hippocampal synaptosomes. This increase reflects [^{3}H]-NA release and was potentiated by glycine and ACPC. The facilitatory effect of both glycine and ACPC on NMDA-evoked [^{3}H]-NA release was prevented by the selective antagonist at the glycine site 7Kyn, indicating that it is mediated by the strychnine-insensitive glycine site of the NMDA receptor complex.

Receptor binding experiments have shown that ACPC is a competitive inhibitor of strychnine-insensitive [^{3}H]-glycine binding to the NMDA receptor in rat forebrain membranes with an apparent affinity ($K_i=0.03$ μM) that is approximately three times higher than that of glycine ($K_D=0.1$ μM) (Marvi-

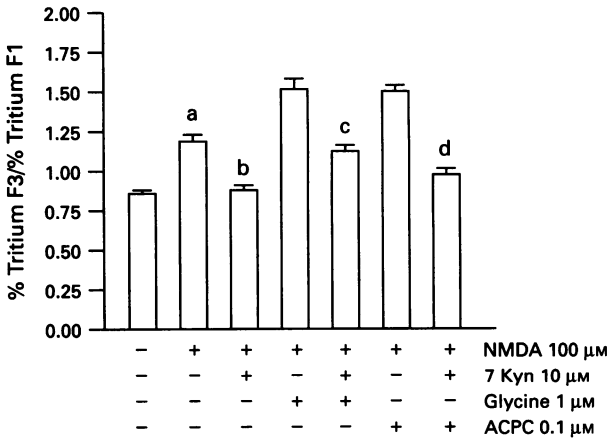

Figure 1 Effects of ligands at the glycine site of the NMDA receptor complex on the ratio of % ^{3}H efflux in fraction 3 over % ^{3}H efflux in fraction 1 induced by 100 μM NMDA in rat superfused hippocampal synaptosomes. For the control group (no NMDA, no glycine), the ratio of % tritium efflux of the third fraction over the first was 0.87 ± 0.02, corresponding to 1.13 ± 0.03% tritium efflux in the third fraction (0.207 ± 0.03 nCi). Values refer to 2-min fractions and represent mean ± s.e.mean of at least four experiments run in triplicate. [a]$P<0.001$ versus control group; [b]$P<0.001$ versus NMDA; [c]$P<0.001$ versus NMDA + glycine; [d]$P<0.001$ versus NMDA + ACPC.

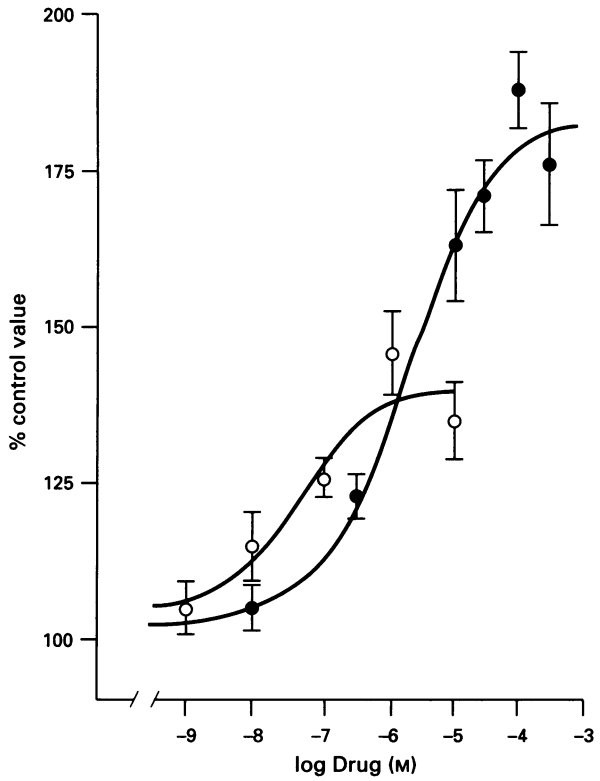

Figure 2 Concentration-response curves for the effects of glycine (●) and ACPC (○) on NMDA-induced tritium release (F3/F1 ratio). Modulation of NMDA-stimulated tritium release by glycine and ACPC was assessed in superfused synaptosomes from rat hippocampus. Values are the percentage of the tritium overflow elicited by 100 μM NMDA in the absence of added glycine and ACPC and represent mean ± s.e.mean of at least four experiments run in triplicate.

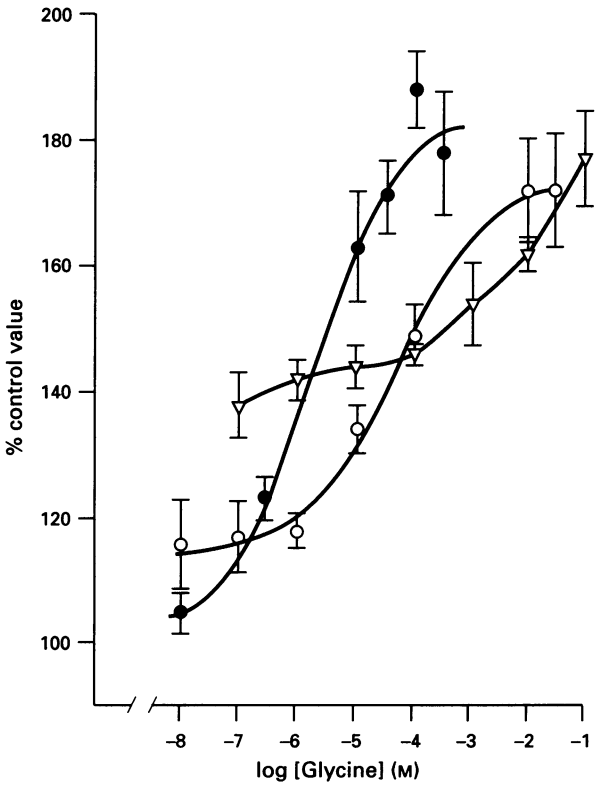

Figure 3 Concentration-response curves for the effect of glycine on NMDA-induced tritium release in the presence of fixed concentrations of ACPC. Modulation of NMDA-stimulated tritium release by glycine was assessed in superfused synaptosomes from rat hippocampus exposed to NMDA (100 μM) and various concentrations of glycine in the absence (●) and in the presence of 0.1 nM (○) and 0.1 μM (▽) ACPC. ACPC was added to the superfusion medium 10 min before NMDA and glycine. Values are the percentage of the tritium overflow elicited by 100 μM NMDA in the absence of added glycine and ACPC and represent mean ± s.e.mean of at least four experiments run in triplicate. In the presence of 0.1 μM ACPC, the concentration-response curve for glycine did not level off at high concentrations. In this condition, the EC_{50} of glycine was estimated to be $\geqslant$ 3 mM. This estimate was calculated by using the effect observed with 100 mM glycine, the highest concentration that could be reached in these experiments, as the maximal effect.

zon *et al.*, 1989). The glycine and NMDA recognition sites of the NMDA receptor complex are allosterically coupled. Glycine facilitates the opening of the NMDA receptor channel and, as a result, enhances the binding of compounds such as [^{3}H]-MK801 that bind to a site located within the NMDA cation channel. Since ACPC has a lower E_{max} (40%) than glycine to enhance [^{3}H]-MK801 binding to rat forebrain membranes, it was suggested that ACPC is a partial agonist at the glycine site of the NMDA receptor (Marvizon *et al.*, 1989). Electrophysiological studies performed in several laboratories confirm this hypothesis but suggest that the estimation of the intrinsic activity of ACPC at the strychnine-insensitive glycine site may depend on the experimental assay system and the type of functional NMDA receptor activity measured. Thus, electrophysiological studies report intrinsic activity values for ACPC between 77 and 95% compared to glycine, significantly higher than the reported values in radioligand binding experiments (McBain *et al.*, 1989; Watson & Lanthorn, 1990; Bashir *et al.*, 1990; Priestley & Kemp, 1994).

In the present experiments, the E_{max} of ACPC for potentiation of the effects of NMDA on synaptosomal [^{3}H]-NA release was 48% of the E_{max} of glycine (Figure 2), a result that it is more consistent with the intrinsic activity shown by ACPC in enhancing [^{3}H]-MK801 binding in receptor binding assays. In our superfused hippocampal synaptosome preparation, the potency of glycine in enhancing the effects of NMDA on [^{3}H]-NA release was found to be somewhat lower (EC_{50} = 1.82 μM, Figure 2) but within the range of the affinities reported in electrophysiological experiments (EC_{50} = 0.7 ± 0.1 μM, Kleckner & Dingledine, 1988). However, ACPC showed an almost thirty fold higher potency than glycine. These findings suggest that ACPC exhibits lower maximal effect and higher potency in presynaptic NMDA receptor assays than in assay preparations that contain either postsynaptic or mixed populations of NMDA receptor subtypes. In support of this notion, the apparent affinities of glycine site ligands have been shown to differ remarkably among brain regions (Monaghan & Anderson, 1991) and the ligand binding affinities of NMDA receptors have been shown to depend on subunit composition (Laurie & Seeburg, 1994).

Glycine is thought to be present continuously in the extracellular space (McBain & Mayer, 1994). However, the physiological contribution of the modulatory action of glycine to the activity of the NMDA receptor complex *in vivo* remains controversial. If occupation by glycine of the glycine site is essential for activation of NMDA receptors, then a partial agonist like ACPC may function as a glycine antagonist at high glycine concentrations. In support of this interpretation, the results reported in the present studies, showing that ACPC (0.1 μM) shifts the EC_{50} of glycine to enhance NMDA-evoked [^{3}H]-NA release from 1.82 μM to $\geqslant$ 3 mM, indicate that ACPC may act as an antagonist at the glycine site of hippocampal NMDA receptors.

In summary, the present results show that ACPC can reduce the potentiation by glycine of NMDA-evoked [^{3}H]-NA release in a functional assay for presynaptic NMDA receptor activity and suggest that a partial agonist like ACPC may act as an antagonist at the glycine site of the NMDA receptor at high glycine concentrations.

References

BASHIR, Z.I., TAM, B. & COLLINGRIDGE, G.L. (1990). Activation of the glycine site in the NMDA receptor is necessary for the induction of LTP. *Neurosci. Lett.*, **108,** 261–266.

FINK, K., BÖNISCH, H. & GÖTHERT, M. (1990). Presynaptic NMDA receptors stimulated noradrenaline release in the cerebral cortex. *Eur. J. Pharmacol.*, **185,** 115–117.

GÖTHERT, M. & FINK, K. (1991). Stimulation of Noradrenaline Release in the Cerebral Cortex via Presynaptic N-Methyl-D-Aspartate (NMDA) Receptors and Their Pharmacological Characterization. *J. Neural Trans.*, Suppl., **34,** 121–127.

GRAY, E.G. & WHITTAKER, V.P. (1962). The isolation of nerve endings from brain: An electron-microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.*, **96,** 79–87.

HUETTNER, J.E. (1989). Indole-2-carboxylic acid: a competitive antagonist of potentiation by glycine at the NMDA receptor. *Science*, **243,** 1611–1613.

JOHNSON, J.W. & ASCHER, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature*, **325,** 529–531.

KLECKNER, N.W. & DINGLEDINE, R. (1988). Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science*, **241,** 835–837.

LAURIE, D.J. & SEEBURG, P.H. (1994). Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. *Eur. J. Pharmacol-Mol. Pharmacol.*, **268,** 335–345.

LERMA, J., ZUKIN, R.S. & BENNETT, M.V.L. (1990). Glycine decreases desensitization of N-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus* oocytes and is required for NMDA responses. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 2354–2358.

MARVIZON, J.C.G., LEWIN, A. & SKOLNICK, P. (1989). 1-Aminocyclopropanecarboxylic acid: A potent and selective ligand for the glycine modulatory site of the N-methyl-D-aspartate receptor complex. *J. Neurochem.*, **52,** 992–994.

MCBAIN, C.J., KLECKNER, N.W., WYRICK, S. & DINGLEDINE, R. (1989). Structural requirements for activation of the glycine coagonist site of N-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. *Mol. Pharmacol.*, **36,** 556–565.

MCBAIN, C.J. & MAYER, M.L. (1994). N-methyl-D-aspartic acid receptor structure and function. *Physiol. Rev.*, **74,** 723–760.

MONAGHAN, D.T. & ANDERSON, K.J. (1991). Heterogeneity and organization of excitatory amino acid receptors and transporters. In *Excitatory Amino Acids and Synaptic Transmission.* ed. Wheal, H. & Thomson, A. pp. 33–54. London: Academic Press Ltd.

PITTALUGA, A. & RAITERI, M. (1990). Release-enhancing glycine-dependent presynaptic NMDA receptors exist on noradrenergic terminals of hippocampus. *Eur. J. Pharmacol.*, **191,** 231–234.

PITTALUGA, A. & RAITERI, M. (1992). N-Methyl-D-Aspartic Acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. I. Location on axon terminals and pharmacological characterization. *J. Pharmacol. Exp. Ther.*, **260,** 232–237.

PRIESTLEY, T. & KEMP, J.A. (1994). Kinetic study of the interactions between the glutamate and glycine recognition sites on the N-methyl-D-aspartic acid receptor complex. *Mol. Pharmacol.*, **46,** 1191–1196.

RAITERI, M., ANGELINI, F. & LEVI, G. (1974). A simple apparatus for studying the release of neurotransmitters from synaptosomes. *Eur. J. Pharmacol.*, **25,** 411–414.

RAITERI, M., GARRONE, B. & PITTALUGA, A. (1992). N-Methyl-D-Aspartic Acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. II. Evidence for functional cooperation and for coexistence on the same axon terminal. *J. Pharmacol. Exp. Ther.*, **260,** 238–260.

SKOLNICK, P., MARVIZON, J.C., JACKSON, B.W., MONN, J.M., RICE, K.C. & LEWIN, A.H. (1989). Blockade of N-methyl-D-aspartate induced convulsions by 1-aminocyclopropanecarboxylates. *Life Sci.*, **45,** 1647–1655.

TRULLAS, R., JACKSON, B. & SKOLNICK, P. (1989). Anxiolytic properties of 1-aminocyclopropanecarboxylic acid, a ligand at strychnine-insensitive glycine receptors. *Pharmacol. Biochem. Behav.*, **34,** 313–316.

TRULLAS, R. & SKOLNICK, P. (1990). Functional antagonists at the NMDA receptor complex exhibit antidepressant actions. *Eur. J. Pharmacol.*, **185,** 1–10.

VON LUBITZ, D.K.J.E., LIN, R.C., MCKENZIE, R.J., DEVLIN, T.M., MCCABE, R.T. & SKOLNICK, P. (1992). A novel treatment of global cerebral ischemia with a glycine partial agonist. *Eur. J. Pharmacol.*, **219,** 153–158.

WANG, J.K.T., ANDREWS, H. & THUKRAL, V. (1992). Presynaptic glutamate receptors regulate noradrenaline release from isolated nerve terminals. *J. Neurochem.*, **58,** 204–211.

WATSON, G. & LANTHORN, T. (1990). Pharmacological characteristics of cyclic homologues of glycine at the N-methyl-D-aspartate receptor associated glycine site. *Neuropharmacology*, **29,** 727–730.

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