0026-895X/92/010134-13$03.00/0

MOLECULAR PHARMACOLOGY, 41:134-146

High Affinity [^{3}H]Dextrorphan Binding in Rat Brain Is Localized to a Noncompetitive Antagonist Site of the Activated *N*-Methyl-D-aspartate Receptor-Cation Channel

PAUL H. FRANKLIN and THOMAS F. MURRAY
College of Pharmacy, Oregon State University, Corvallis, Oregon 97331
Received May 29, 1991; Accepted September 23, 1991

SUMMARY

[^{3}H]Dextrorphan recognition sites were characterized in rat brain membranes. The pharmacological profile and regional distribution of [^{3}H]dextrorphan binding sites appear to distinguish these sites from those labeled either by [^{3}H]dextromethorphan or by putative σ receptor radioligands. Data from thoroughly washed forebrain membranes suggest that [^{3}H]dextrorphan predominantly labels a high affinity site defined by the activated state of the *N*-methyl-D-aspartate (NMDA) receptor-channel complex. Regulation of [^{3}H]dextrorphan binding by specific modulators of NMDA receptor function suggests that [^{3}H]dextrorphan binding is predominantly localized to a domain of the receptor-channel complex also recognized by the prototypical noncompetitive antagonist radioligands (+)-[^{3}H]5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK-801) and [^{3}H]1-[1-(2-thienyl)cyclohexyl]piperidine (TCP). The critical relationship between [^{3}H]dextrorphan binding and activation of the NMDA receptor-complex is suggested by the profound dependence of [^{3}H]dextrorphan binding on glutamate in well washed membranes. Basal specific [^{3}H]dextrorphan binding is nearly totally suppressed by the specific competitive NMDA antagonist D(−)-2-amino-5-phosphonopentanoic acid (D-AP5), in a glutamate- but not glycine-surmountable manner. Glutamate and glycine each stimulate [^{3}H]dextrorphan binding in a concentration-dependent manner, effecting maximal increases from control of up to 30- and 14-fold, respectively. The NMDA receptor specificity of the modulation of [^{3}H]dextrorphan binding by glutamate and glycine is indicated by the sensitivity of their effects to competitive antagonism by D-AP5 and 3-amino-1-hydroxy-2-pyrrolidone (HA-966), respectively, and by the accordant rank orders of potency of glycine analogs as modulators of [^{3}H]dextrorphan binding and as ligands at the strychnine-insensitive glycine site. The divalent cations Mg^{2+} and Zn^{2+} and the polyamines spermine and spermidine regulate [^{3}H]dextrorphan binding in a manner consistent with radioligand interaction at the noncompetitive NMDA antagonist domain. Mg^{2+} and spermidine regulate [^{3}H]dextrorphan binding biphasically in well washed forebrain membranes, whereas Zn^{2+} monotonically inhibits [^{3}H]dextrorphan binding. Mg^{2+} and spermidine regulate [^{3}H]dextrorphan binding with qualitative similarity and in a contrasting fashion to their regulation of [^{3}H]MK-801 and [^{3}H]TCP binding. First, spermidine and Mg^{2+} are significantly more potent modulators of [^{3}H]dextrorphan binding than of [^{3}H]MK-801 and [^{3}H]TCP binding in well washed membranes; second, whereas the potencies of spermidine and Mg^{2+} as modulators of [^{3}H]MK-801 and [^{3}H]TCP binding are significantly increased by glutamate and glycine in well washed membranes, their potencies as regulators of [^{3}H]dextrorphan binding appear to be unaffected by glutamate and glycine. Furthermore, putrescine, which does not influence [^{3}H]MK-801 or [^{3}H]TCP binding, inhibits basal [^{3}H]dextrorphan binding in a manner dissimilar from that of spermidine- and spermine-mediated inhibition of binding. The kinetics of [^{3}H]dextrorphan binding in the presence of saturating concentrations of glutamate and glycine are complex and inadequately described by monoexponential association and dissociation processes. The differential distribution of [^{3}H]dextrorphan recognition sites in rat brain regions and the pharmacological profile specified by the rank order of potency of an extensive set of compounds as competitors for high affinity [^{3}H]dextrorphan binding unambiguously suggest that [^{3}H]dextrorphan binding in rat brain membranes corresponds to the site of the NMDA antagonist activity of dextrorphan *in vivo*. In contrast, the pharmacological signature and distribution of high affinity [^{3}H]dextrorphan binding sites in rat brain are incongruous with those of either the σ receptor or [^{3}H]dextromethorphan binding sites. Accordingly, the interaction of dextrorphan and dextromethorphan at sites labeled by [^{3}H]dextrorphan, but not at sites labeled by [^{3}H]dextromethorphan or by σ ligands, adequately accounts for the anticonvulsant and neuroprotective efficacies of these compounds *in vivo*.

Dextrorphan is the dextrorotatory form of the synthetic opioid 3-hydroxy-*N*-methylmorphinan. Unlike its enantiomer levorphanol, an analgesic nearly 5 times more potent than morphine, dextrorphan is essentially devoid of opioid receptor activity (1). A number of naturally occurring and synthetic opioids possess antitussive efficacy and, because this property is unrelated to classical opioid receptor activity, compounds

This work was supported by United States Public Health Service Grant DA 07218.

ABBREVIATIONS: PCP, 1-(1-phenylcyclohexyl)piperidine; D-AP5, D(−)-2-amino-5-phosphonopentanoic acid; DTG, 1,3-di-(*o*-tolyl)guanidine; HA-966, 3-amino-1-hydroxy-2-pyrrolidone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; NMDA, *N*-methyl-D-aspartate; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; SKF-10047, *N*-allylnormetazocine; TCP, 1-[1-(2-thienyl)cyclohexyl]piperidine.

such as dextrorphan and particularly its 3-methyl ether derivative, dextromethorphan, have been clinically valuable as cough suppressants. Although comparable to codeine in antitussive potency, the dextrorotatory morphinans have a wide margin of safety in humans and at therapeutic doses do not appreciably depress respiration (2); moreover, because they do not produce morphine-like subjective effects and fail to substitute for morphine in addicted individuals, these dextrorotatory morphinans were presumed to possess a minimum potential for abuse (3). Ironically, although the dextrorotatory morphinans are indeed devoid of morphine-like effects, they induce a striking PCP-like syndrome of effects at supraantitussive doses.

An early indication of a common pharmacological basis for the effects of the structurally dissimilar dextrorotatory morphinans and PCP-like drugs came from reports of dextromethorphan abuse (4, 5). After consumption of doses approximately 10 times higher than the antitussive dose of dextromethorphan, patients presented with signs of sedative-hypnotic effects, sensory distortion, pupillary dilation, slurred speech, and reported feelings of drunkenness and euphoria (4). Subsequently, a controlled study of the effects of dextromethorphan in humans revealed that dextromethorphan had prominent psychotomimetic effects at doses 6–10 times greater than the normal antitussive dose (6).

The discovery by Lodge (7) of the capacity for PCP-like drugs to antagonize specifically neuronal excitation mediated by *N*-methylaspartate provided a critical and unifying neuronal mechanism that reconciled the behavioral and subjective effects shared by this structurally diverse class of substances. Inhibition by PCP-like drugs is selective for the NMDA class of excitatory amino acid receptors and occurs in a manner that is not competitive with respect to agonist (7–9). The voltage- and use-dependence of PCP and ketamine in antagonism of NMDA-mediated neuronal excitation has suggested that noncompetitive antagonists may act as open channel blockers of the receptor-gated cation conductance (10, 11).

Church *et al.* (12) have shown dextrorphan to be a potent and selective antagonist of *N*-methylaspartate-mediated excitation in spinal neurons. Dextromethorphan and levorphanol also selectively antagonized *N*-methylaspartate excitation but with 5- and 7-fold lower potency, respectively, than dextrorphan (12). Levorphanol produces PCP-like discriminative effects when coadministered with the specific opioid receptor antagonist naltrexone (13, 14) and, like other compounds in this class, is an effective competitor for sites labeled by [^{3}H] PCP (15, 16).

Dextrorphan and dextromethorphan are effective antagonists of NMDA receptor-mediated neuropathology both *in vitro* and *in vivo*, although dextromethorphan is markedly less potent than dextrorphan in this regard. Presumably by virtue of their NMDA antagonist activity, these compounds are effective neuroprotective agents and reduce the magnitude and extent of neuronal damage after ischemic or traumatic brain insult *in vivo* (17, 18) or that resulting from hypoxic or hypoglycemic challenge *in vitro* (19, 20). Dextrorphan and dextromethorphan also possess significant antiepileptic activity *in vitro* (21–23) and anticonvulsant efficacy *in vivo* in several seizure models (24–27). Dextromethorphan, in addition, has been proposed to be prototypical of a unique class of anticonvulsants acting through a site labeled by [^{3}H]dextromethorphan (25). After systemic administration, however, dextromethorphan is rapidly metabolized to dextrorphan, suggesting that, to a large extent, the effects of dextromethorphan *in vivo* result from its conversion to dextrorphan (28, 29).

In a prior report, we identified high affinity [^{3}H]dextrorphan binding sites in rat forebrain membranes (30). We found that [^{3}H]dextrorphan binding, in a minimally washed membrane preparation, displayed a pharmacological profile consistent with the labeling of a noncompetitive NMDA antagonist domain. Further support for a possible association of [^{3}H]dextrorphan binding sites with an NMDA receptor channel domain was suggested by the regulation of [^{3}H]dextrorphan binding by glutamate and glycine.

In this report, we have further characterized the relationship between [^{3}H]dextrorphan binding and the functional state of the NMDA receptor complex, in the context of quantifying the response of [^{3}H]dextrorphan binding to the modulation of channel activity by pharmacologically specific regulators of activation of the individual NMDA receptor-ionophore functional domains. In addition we have extended the pharmacological characterization of [^{3}H]dextrorphan recognition sites through evaluation of the competition binding properties of a diverse set of compounds for sites labeled by [^{3}H]dextrorphan.

Experimental Procedures

Materials. [^{3}H]Dextrorphan (12.7 Ci/mmol) was provided by New England Nuclear (Boston MA); dextrorphan was obtained from Hoffman-LaRoche (Nutley, NJ); D(−)-AP5 and HA-966 were from Tocris Neuramin (Essex, England); MK-801, TCP, dextromethorphan, desipramine, DTG, (+)-3-PPP, and (−)-3-PPP were from Research Biochemicals Incorporated (Wayland, MA); levorphanol, (−)-cyclazocine, (+)-cyclazocine, (−)-ketamine, (+)-ketamine, (−)-SKF-10047, (+)-SKF-10047, and PCP were provided by the National Institute on Drug Abuse; and the remaining compounds were from Sigma Chemical Co. (St. Louis, MO).

Male Sprague-Dawley rats (150–200 g) were from Simonsen Labs (Gilroy, CA), and frozen rat brains were obtained from Pel-Freeze (Rogers, AK).

Care of animals. Rats were housed at no more than 4–6/cage in a professionally managed vivarium, maintained on a normal 12-hr dark-light cycle, and provided with rat chow and water *ad libitum*. Unanaesthetized rats were sedated by CO_2 narcosis before decapitation by guillotine.

Preparation of rat brain membranes. Rat forebrains, dissected rat brain regions, or frozen rat forebrains were homogenized in 40 volumes of 5 mM HEPES, 10 mM EDTA buffer, pH 7.4, by Polytron (Brinkmann Instruments), for 30 sec at setting 7.5. The homogenate was then centrifuged for 20 min at 48,000 × *g*. The pellet resulting from this step was then washed by resuspension in 140 volumes of HEPES/EDTA buffer and subsequent centrifugation, as in the previous step. The pellet was then washed a second time by repeating the resuspension/centrifugation process, this time in the absence of EDTA. The final pellet was then frozen at −70° for a period of at least 1 and no longer than 14 days. At the time of experiment the pellet was resuspended in a volume of 5 mM HEPES, pH 7.4, sufficient to yield a concentration of 0.6–0.8 mg of protein/ml.

For preparation of membranes from rat brain regions, tissue corresponding to the desired regions was dissected and pooled from six animals for each experiment.

In order to evaluate the role of endogenous regulators of [^{3}H] dextrorphan binding, well washed membranes were prepared by subjecting the −70° frozen pellet from the penultimate step of the preparation described above to four additional cycles of resuspension-centrifugation in 140 volumes of 5 mM Na-HEPES, pH 7.4, followed by an additional freeze-thaw step before final resuspension at the time of experiment.

Membrane protein was measured by the method of Lowry *et al.* (31), using bovine serum albumin as standard.

Radioligand binding. [^{3}H]Dextrorphan binding was routinely determined in a reaction mixture buffered to pH 7.4 at 20° in 5 mM HEPES and containing 200–300 μg of membrane protein, 100 μM glycine, and 100 μM glutamate, in a final volume of 1 ml and at a final [^{3}H]dextrorphan concentration of 10 nM. Nonspecific binding is defined as that which occurs in the presence of 10 μM MK-801. Under these assay conditions at 20°, the [^{3}H]dextrorphan-recognition site association reaction progresses to >99% of completion by 4 hr. After a routine incubation of 4 hr at 20°, the reaction was terminated by rapid filtration over Whatman GF/B glass fiber filters, in a Brandel M24-R cell harvester. Before filtration, filter strips were soaked in 0.5% polyethyleneimine for at least 2 hr, to prevent the binding of ligand to filter. Filter disks were allowed to elute for at least 9 hr in Biocount liquid scintillation cocktail (Research Products International, Mount Prospect IL) and then counted in a Beckman LS6800 scintillation counter.

Data analysis. Saturation isotherms were parameterized through a nonlinear regression analysis-based fitting of a hyperbolic equation to the data, using the Public Procedure FITSAT of the NIH PROPHET computer resource, which uses an iterative process to minimize residual least squares. Competition analysis was performed, similarly, by nonlinear regression analysis using the PROPHET FITCOMP Public Procedure to fit a logistic equation to the data. The parameters of [^{3}H]dextrorphan binding reaction association and dissociation kinetics were determined by use of KINETIC (Biosoft, Cambridge, U.K.) or by the PROPHET Public Procedure FITFUN, fitting the following exponential equations:

$$RB_t = \sum_{i=1}^{n} [RB_0] \cdot e^{-k_{-1_i} \cdot t}$$

$$RB_t = \sum_{i=1}^{n} [RB_{eq_i}] \cdot (1 - e^{-k_{obs_i} \cdot t})$$

where, for site i of n total sites, RB_t is the amount of radioligand bound at time t, RB_0 is the amount of radioligand bound at time $t = 0$, RB_{eq} is the amount of radioligand bound at equilibrium, k_{-1} is a dissociation rate constant, and k_{obs} is an apparent association rate constant.

EC_{50} values, similarly, were determined by fitting a logistic equation to the data, using the PROPHET Public Procedure FITFUN:

$$E = \frac{E_{max}}{1 + (K/[X])^n}$$

where E and E_{max} are the response and maximal response, respectively, K is the ED_{50}, X is the dose, and n is a slope factor.

The criterion for asserting a multiple-site model in these equilibrium and kinetic ligand binding analyses is the F statistic, defined as follows:

$$F = \frac{[SS_1 - SS_2]/[df_1 - df_2]}{[SS_2/df_2]}$$

where SS_1 and SS_2 are the sum of squares of the residuals for the one- and two-site fits, respectively, and df_1 and df_2 are the degrees of freedom for one- and two-site fits.

Results

Regulation of [^{3}H]dextrorphan binding by glycine and glutamate. In our initial report (30), we observed that [^{3}H]dextrorphan equilibrium binding in rat forebrain is increased by glycine and glutamate in a manner consonant with their coactivation of the NMDA receptor-operated ion channel, when binding is assayed under low ionic strength in a divalent cation-depleted washed membrane preparation, conditions that have been used to amplify the binding of other noncompetitive NMDA antagonists such as [^{3}H]MK-801 and [^{3}H]TCP (32–35).

If the stimulation of [^{3}H]dextrorphan binding effected by glutamate or glycine results from activation of the NMDA receptor agonist or co-agonist sites, respectively, then these effects should display pharmacological sensitivities consonant with the known pharmacology of these NMDA receptor domains. To address this issue, the concentration-response relationships describing the stimulation of [^{3}H]dextrorphan binding by glycine and by glutamate were determined in the presence and absence of 1) the specific competitive NMDA antagonist D-AP5 and 2) the strychnine-insensitive glycine site antagonist HA-966, in thoroughly washed rat forebrain membranes (Fig. 1). The data of Fig. 1B show that added glutamate stimulates [^{3}H]dextrorphan binding in a concentration-dependent manner in thoroughly washed rat forebrain membranes and that the stimulatory effects of glutamate are antagonized by D-AP5 (0.3 mM) in an apparently competitive manner. D-AP5 markedly shifts the control glutamate concentration-response curve to the right but does not reduce the efficacy of glutamate to stimulate [^{3}H]dextrorphan binding. Glycine also mediates a significant and concentration-depend-

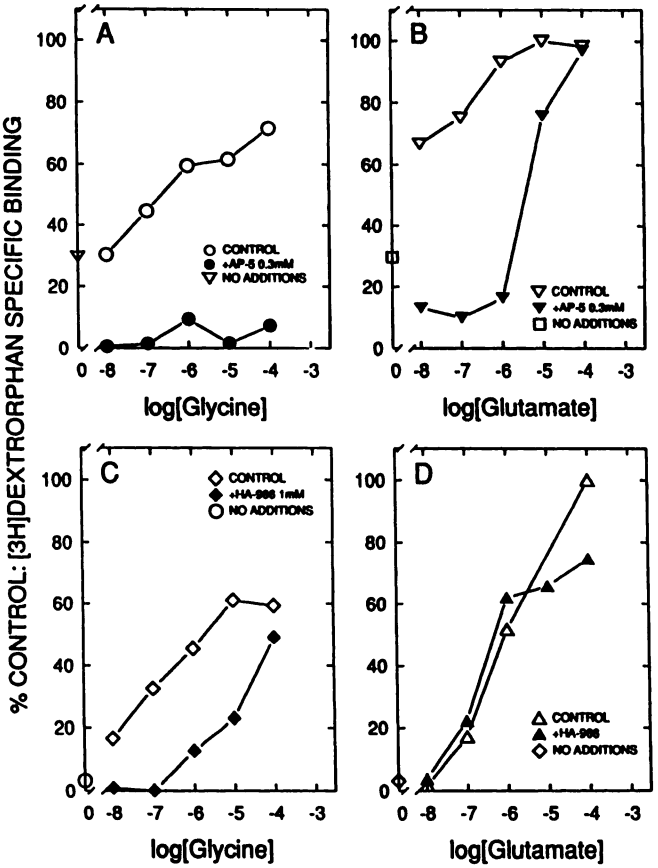

Fig. 1. Stimulation of [^{3}H]dextrorphan binding by glycine and glutamate in repetitively washed rat forebrain membranes: concentration-response relationships in the presence and absence of the specific NMDA antagonist D-AP5 and the strychnine-insensitive glycine site antagonist HA-966. [^{3}H]Dextrorphan binding was performed as described in Experimental Procedures. Values depict the results of representative experiments performed in parallel and repeated at least three times with equivalent results; values are expressed as percentages of maximum stimulation by either glycine or glutamate, as indicated. A and B, Glycine (A) and glutamate (B) were present at the indicated concentrations in the presence and absence (control) of D-AP5 (0.3 mM). C and D, Glycine (C) and glutamate (D) were present at the indicated concentrations in the presence and absence (control) of HA-966 (1 mM). Nonspecific binding was defined by 10 μM MK-801.

ent stimulation of [³H]dextrorphan binding in thoroughly washed forebrain membranes (Fig. 1A) but, in contrast to glutamate, the effects of glycine are inhibited completely and insurmountably by D-AP5 (0.3 mM). Moreover, specific [³H]dextrorphan binding is reduced essentially to zero by D-AP5, despite the presence of saturating concentrations of glycine in the absence of added glutamate.

The specificity of the glycine-mediated stimulation of [³H]dextrorphan binding is addressed in the complementary data of Fig. 1, C and D. HA-966 (1 mM) competitively inhibits glycine- but not glutamate-mediated stimulation of [³H]dextrorphan binding in thoroughly washed forebrain membranes; whereas the glycine concentration-response curve (Fig. 1C) is shifted to the right by HA-966, the potency of glutamate to increase [³H]dextrorphan binding (Fig. 1D) is not affected by HA-966 at this concentration.

Despite the extensive washing procedures used in this membrane preparation, the presence of residual glutamate, and presumably glycine as well, is suggested by the capacity of D-AP5 to reduce specific [³H]dextrorphan binding below the control, i.e., basal, level seen in the absence of added glycine and glutamate (Fig. 1, A and B). The washing procedure was apparently more effective in the experiment of Fig. 1, C and D; specific binding in the absence of added glycine and glutamate approaches the level of nonspecific binding. These data suggest that specific [³H]dextrorphan binding has a profound requirement for glutamate.

These data suggest that stimulation of [³H]dextrorphan binding by glutamate and glycine derives from activation of the NMDA receptor complex agonist and co-agonist sites, respectively, and that the access of [³H]dextrorphan to its recognition sites in rat forebrain membranes appears to be sustained by NMDA receptor activation.

Agonist activity at the NMDA receptor-associated, strychnine-insensitive glycine site is defined by a well established set of structural requirements (36, 37). Further evidence that glycine regulates dextrorphan binding through influence on the NMDA receptor is provided by the data of Fig. 2, which depict [³H]dextrorphan binding concentration-response curves for a series of glycine analogs, evaluated in the absence of added glutamate in well washed membranes. These results are consistent with the pharmacological profile of these compounds as modulators of NMDA receptor activation. Both serine stereoisomers effectively increase [³H]dextrorphan binding, with the potency of D-serine exceeding that of its L-isomer; D-valine, consonantly, has virtually no effect on [³H]dextrorphan binding.

Regulation of [³H]dextrorphan binding by Mg^{2+} and Zn^{2+}. The divalent cations Mg^{2+} and Zn^{2+} prototypically define at least two pharmacologically and electrophysiologically distinct functional sites associated with the NMDA-operated cation channel.

The binding of [³H]dextrorphan, like that of [³H]MK-801 (32, 34) and [³H]TCP (38–40), is regulated by Mg^{2+} and Zn^{2+} (Fig. 3). As has been reported for [³H]MK-801 and [³H]TCP binding, intrinsic differences in the mechanisms subserving Zn^{2+} and Mg^{2+} regulation of [³H]dextrorphan binding are suggested by the incongruence of the [³H]dextrorphan concentration-response curves for the two cations determined in the presence and absence of saturating glycine and glutamate in thoroughly washed membranes.

Zn^{2+} inhibits [³H]dextrorphan binding (Fig. 3B) monotonically and with equal potency in the presence (IC_{50} = 18.5 ± 0.6 μM) or absence (IC_{50} = 18.5 ± 1.3 μM) of added glycine and glutamate. Mg^{2+}, similarly, is an essentially equipotent inhibitor of [³H]dextrorphan binding in the presence (IC_{50} = 12.3 ± 0.7 μM) or absence (IC_{50} = 14.3 ± 7.5 μM) of glycine and glutamate. In contrast to Zn^{2+}, however, Mg^{2+}, in the presence of glycine and glutamate, modulates [³H]dextrorphan binding

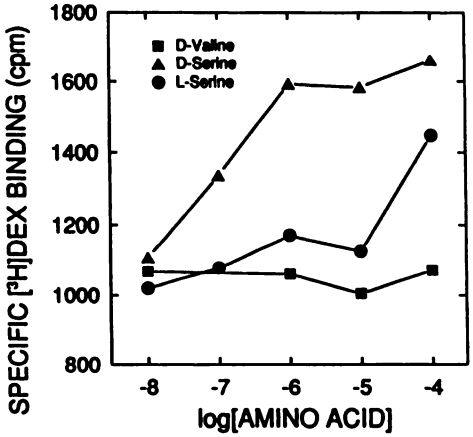

Fig. 2. Stimulation of [³H]dextrorphan binding by glycine analogs in repetitively washed rat forebrain membranes: concentration-response. D-Valine and D- and L-serine were evaluated at the indicated concentrations in the absence of added glutamate. [³H]Dextrorphan binding was performed as described in Experimental Procedures. Values are from a representative experiment. Nonspecific binding was defined by 10 μM MK-801.

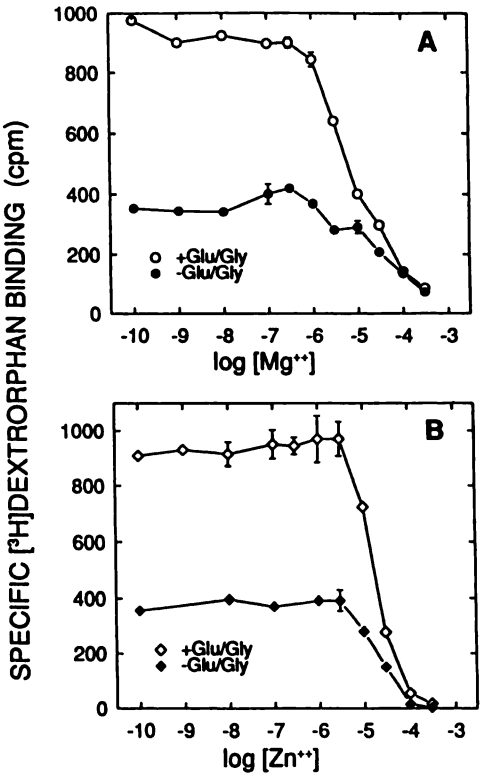

Fig. 3. Regulation of [³H]dextrorphan binding in thoroughly washed rat forebrain membranes by magnesium (A) and zinc (B) at the indicated concentrations in the presence (+) and absence (−) of glycine (*Gly*) and glutamate (*Glu*), each at a concentration of 100 μM, as indicated in the figure. [³H]Dextrorphan binding was performed as described in Experimental Procedures. Values are the means ± standard errors. Nonspecific binding was defined by 10 μM MK-801.

biphasically, effecting a modest (19%; $p < 0.001$, Student's t test) but reliable stimulation of binding with a peak effect at [Mg^{2+}] of ≤1 μM in this preparation; the stimulatory phase is then succeeded by a significant inhibitory phase at higher [Mg^{2+}]. This biphasic pattern is characteristic of the regulation of both [^{3}H]MK-801 and [^{3}H]TCP binding by Mg^{2+} in exhaustively washed membranes (32, 34, 38, 41).

[^{3}H]Dextrorphan binding, however, is significantly more sensitive to the inhibitory effects of Mg^{2+} than is the binding of either [^{3}H]MK-801 or [^{3}H]TCP, whether in the presence or in the absence of added glycine and glutamate. In well washed membranes, in the absence of glycine and glutamate, Mg^{2+} (Fig. 3A) is 200–700 times more potent an inhibitor of [^{3}H]dextrorphan binding than of [^{3}H]TCP or [^{3}H]MK-801 binding (IC_{50} = 3–10 mM); in the presence of maximally activating levels of added glycine and glutamate, Mg^{2+} is 12–27 times more potent an inhibitor of [^{3}H]dextrorphan binding than of either [^{3}H]MK-801 or [^{3}H]TCP binding (IC_{50} = 150–330 μM) (32, 34, 38, 41).

Regulation of [^{3}H]dextrorphan binding by polyamines. The data of Fig. 4 show that the polyamines spermidine (Fig. 4A) and spermine (Fig. 4B), but not putrescine (Fig. 4C), increase [^{3}H]dextrorphan binding in a concentration-dependent manner in well washed membranes in the absence of added glycine and glutamate; under these nominally "closed channel" conditions, [^{3}H]dextrorphan binding is elevated 144 ± 3% by spermidine (EC_{50} = 4.8 ± 0.6 μM) and 127 ± 5% by spermine (EC_{50} = 3.9 ± 2.2 μM). In the presence of saturating levels of glycine and glutamate, however, only spermidine increases [^{3}H]dextrorphan binding appreciably (33 ± 2%; EC_{50} = 3.3 ± 1.2 μM). Spermidine stimulates [^{3}H]dextrorphan binding with 3–6 times greater potency than that for [^{3}H]MK-801 (42, 43) and [^{3}H]TCP (41) binding in well washed membranes and, unlike its regulation of [^{3}H]MK-801 and [^{3}H]TCP binding, spermidine is equipotent in the presence and in the absence of added glutamate and glycine. A steep inhibitory phase is characteristic of the spermine and spermidine concentration-response curves at polyamine concentrations of >10 μM; this inhibition occurs in the presence or in the absence of glycine and glutamate and is of a larger magnitude than the stimulatory effects of these polyamines (Fig. 4) (additional data not shown). Putrescine, unlike spermine and spermidine, does not increase specific [^{3}H]dextrorphan binding. Instead, putrescine effects a relatively shallow concentration-dependent inhibition of [^{3}H]dextrorphan binding, which reaches a maximum of 43 ± 5% and 38 ± 6% of control binding in the presence and absence of added glycine and glutamate, respectively (Fig. 4C). The capacity of putrescine to inhibit [^{3}H]dextrorphan binding in the absence of added stimulatory polyamines distinguishes [^{3}H]dextrorphan binding from that of either [^{3}H]MK-801 or [^{3}H]TCP (42, 44, 45).

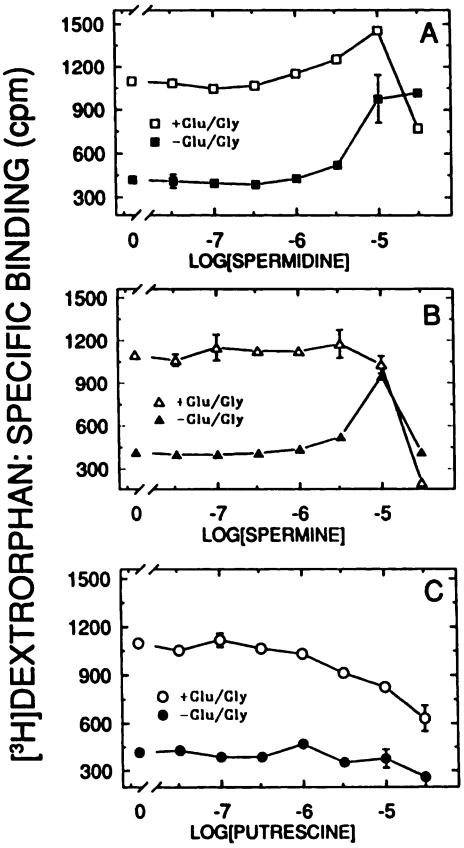

Fig. 4. Regulation of [^{3}H]dextrorphan binding in thoroughly washed rat forebrain membranes by the polyamines spermidine (A), spermine (B), and putrescine (C) in the presence (+) and absence (−) of glutamate (*Glu*) and glycine (*Gly*), each at a concentration of 100 μM, as indicated in the figure. [^{3}H]Dextrorphan binding was performed as described in Experimental Procedures. Values are the means ± standard errors of duplicate experiments. Nonspecific binding was defined by 10 μM MK-801.

Kinetics of [^{3}H]dextrorphan binding. The association of [^{3}H]dextrorphan with its recognition sites progresses in the presence of saturating concentrations of glycine and glutamate at 20° and at a radioligand concentration of 10 nM with a $t_{0.5}$ of 5.42 min; equilibrium, however, is not attained under these conditions until $t \geq 180$ min (Fig. 5, *upper*). Nonlinear regression analysis of the kinetics of [^{3}H]dextrorphan association indicates that a simple bimolecular reaction model cannot adequately account for the observed binding complexity. A significant improvement in fit ($F = 11.91$, $p < 0.001$) results from acceptance of a more complex biexponential binding model and derives from the resolution of two kinetic components, $k_{obs_1} = 0.33 \pm 0.06$ min^{-1} and $k_{obs_2} = 0.016 \pm 0.009$ min^{-1}, with 55% of the total in the fast phase and 45% in the slow. The kinetics of [^{3}H]dextrorphan dissociation, initiated by addition of 10 μM MK-801, in the presence of saturating glycine and glutamate at 20° are also complex; $t_{0.5}$ for the overall dissociation process is 46.2 min (Fig. 5, *lower*). Under these conditions nonlinear regression analysis indicates that a significant improvement in goodness of fit is achieved if a biexponential process is fit to the data, compared with a simple unimolecular dissociation model ($F = 31.91$; $p < 0.001$). Estimates of the dissociation rate constants of the two components from nonlinear regression analysis are as follows: $k_{-1_1} = 0.031 \pm 0.009$ min^{-1} and $k_{-1_2} = 0.005 \pm 0.001$ min^{-1}, with 53% in the fast component and 47% in the slow phase.

Distribution of [^{3}H]dextrorphan binding sites in rat brain. The regional distribution of [^{3}H]dextrorphan binding sites in the rat central nervous system was assessed by comparing the equilibrium binding of a fixed concentration of radioligand (10 nM) in homogenates prepared from rat cortex, hippocampus, striatum, hypothalamus, thalamus, superior and inferior colliculi, cerebellum, and cervical spinal cord (Fig. 6). These data suggest that [^{3}H]dextrorphan binding is distributed nonuniformly in the rat central nervous system. Among the regions evaluated, the highest levels of binding are found in

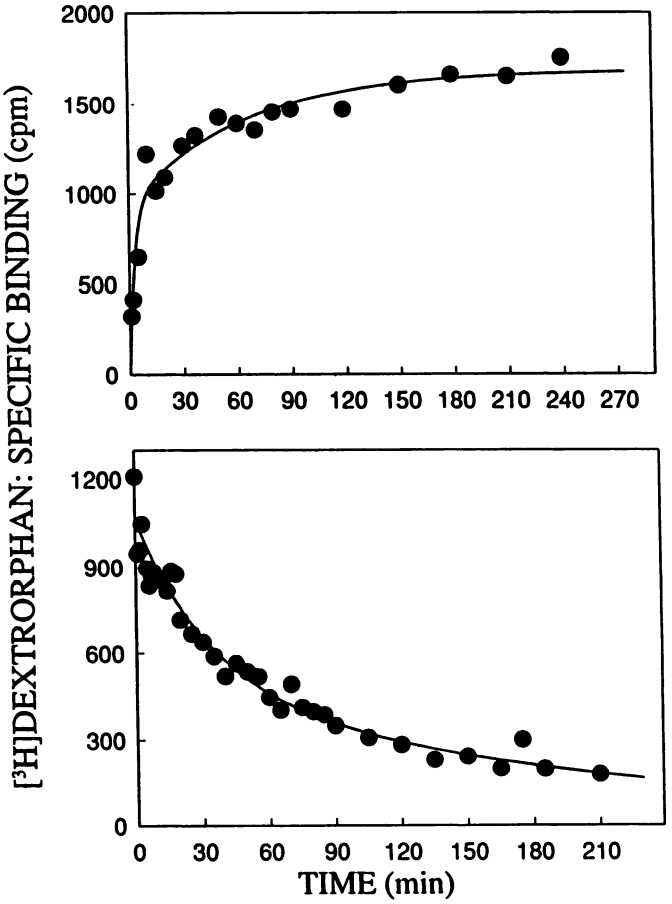

Fig. 5. Kinetics of [³H]dextrorphan binding at 20°. [³H]Dextrorphan at a concentration of 10 nM was incubated with washed rat forebrain membranes in the presence of glutamate and glycine, each at a concentration of 100 μM, for the indicated times, as described in Experimental Procedures. The [³H]dextrorphan association reaction (*upper*) and dissociation (*lower*) were inadequately described by monoexponential processes (see Results), and the curves were generated from the biexponential parameter estimates derived by nonlinear regression analysis (see Experimental Procedures). Dissociation was initiated by addition of MK-801 at a final assay concentration of 10 μM. Values are the results of representative experiments. Nonspecific binding was defined at each time point by 10 μM MK-801.

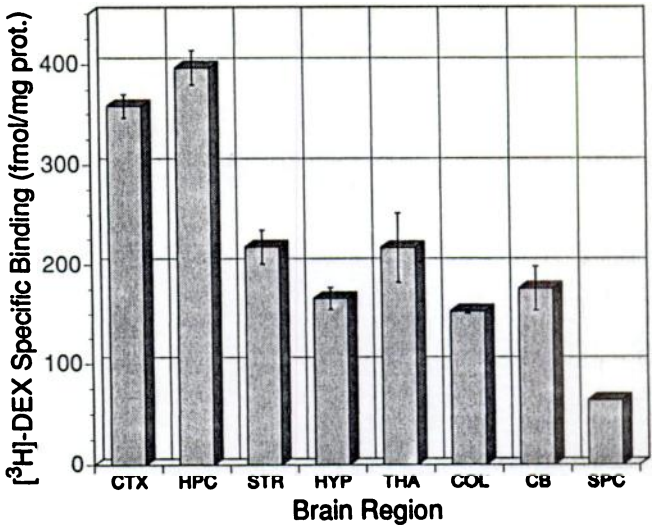

Fig. 6. Regional distribution of [³H]dextrorphan binding in rat brain. [³H]Dextrorphan binding assay was performed at a fixed radioligand concentration of 10 nM in the presence of glutamate and glycine, each at a concentration of 100 μM, as described in Experimental Procedures, in washed rat brain membranes prepared from the indicated regions. *CTX*, cortex; *HPC*, hippocampus; *STR*, striatum; *HYP*, hypothalamus; *THA*, thalamus; *COL*, colliculi; *CB*, cerebellum; *SPC*, cervical spinal cord. Values are the means ± standard errors of duplicate experiments. Nonspecific binding was defined by 10 μM MK-801.

hippocampus (397 ± 17 fmol/mg of protein) and cortex, intermediate levels are present in the thalamus and striatum, and cervical spinal cord contains the lowest level of [³H]dextrorphan binding (64 ± 1 fmol/mg of protein).

Equilibrium saturation binding isotherms from membranes prepared from hippocampus, cortex, and cerebellum (Fig. 7) indicate that the differential distribution of [³H]dextrorphan binding sites suggested by the data of Fig. 6 is consonant, at least for these three brain regions, with differences in their relative densities of [³H]dextrorphan binding sites. Nonlinear regression analysis of these saturation isotherms indicates that [³H]dextrorphan labels a population of binding sites in cortex with an apparent K_D of 76 ± 7 nM and B_{max} of 2.72 ± 0.11 pmol/mg of protein; similarly, in hippocampus [³H]dextrorphan labels a population with an apparent K_D of 58 ± 5 nM and a B_{max} of 3.03 ± 0.13 pmol/mg of protein. In cerebellum, saturation analysis suggests that [³H]dextrorphan labels a population of similar apparent affinity but substantially lower apparent density than those of either cortex or hippocampus (K_D = 74 ± 15 nM, B_{max} = 1.67 ± 0.18 pmol/mg of protein).

Pharmacological characterization of [³H]dextrorphan binding: competition analysis. To establish the pharmacological profile of [³H]dextrorphan binding, we evaluated the concentration-response relationships for a diverse series of compounds as competitors for [³H]dextrorphan binding sites in rat forebrain. Table 1 compiles the best-fit parameter estimates describing logistic curves derived for each competition data set by nonlinear, least squares, regression analysis. The *F* test, as described in Experimental Procedures, was used as the criterion for acceptance of binding models of increasing complexity; $p < 0.001$ was set as the level of acceptance. The competition curves expanded from the logistic equation using these parameter estimates are plotted, along with the data, in Fig. 8.

Several generalizations can be made from the data of Table 1 regarding [³H]dextrorphan binding in rat forebrain. First, for the majority of compounds evaluated, competition for [³H]dextrorphan binding sites cannot be adequately explained by a simple one-site model. Second, if the relative apportionment in Table 1 of sites into high and low affinity states is considered, two nonoverlapping populations of compounds can be discriminated; 1) if all compounds with >60% of their sites in the high affinity state are considered, then the set will include all the compounds evaluated that have established efficacy as NMDA antagonists, and 2) if all compounds with >70% apportionment of sites in the low affinity state are considered, then this set exclusively and exhaustively includes compounds considered to be selective σ receptor ligands. Thus, the most potent compound tested, MK-801, recognizes high and low affinity sites labeled by [³H]dextrorphan in forebrain that are characterized by IC_{50} values of 0.62 ± 0.14 nM and 606 ± 417 nM, respectively; these high and low affinity sites represent 69 ± 5 and 31 ± 4% of the total bound, respectively. Compounds such as (−)-cyclazocine, TCP, PCP, (+)-cyclazocine, dextrorphan, and (+)-SKF-10047 compete for the high affinity [³H]dextrorphan binding site with relatively high potency (IC_{50} = 4.4–42.7 nM) and yet are from

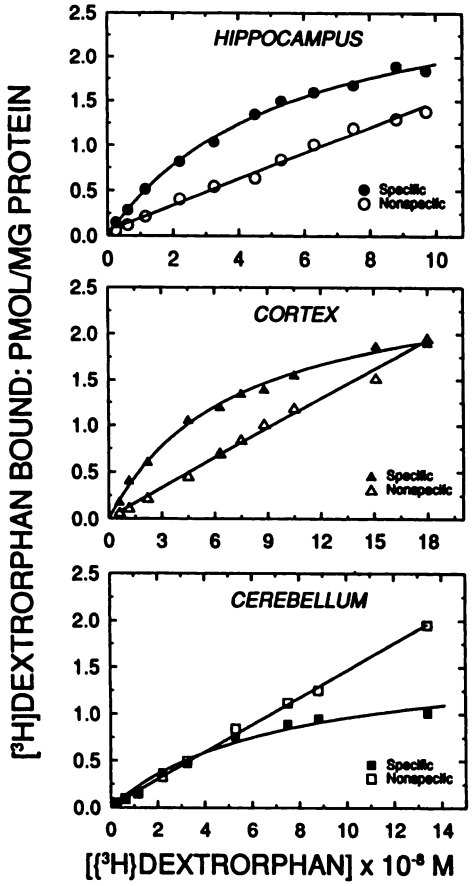

Fig. 7. [³H]Dextrorphan equilibrium saturation binding isotherms from rat brain regions. [³H]Dextrorphan at the indicated concentrations was incubated in the presence of glutamate and glycine, each at a concentration of 100 μM, with washed rat brain membranes prepared from hippocampus, cortex, and cerebellum. Values are from representative experiments. Nonspecific binding was defined at the indicated [³H]dextrorphan concentrations by 10 μM MK-801.

8 to 70 times less potent than MK-801. This group of compounds also preferentially recognizes the high affinity [³H]dextrorphan binding site in forebrain, which accounts for 61–82% of the total recognized by each compound.

The σ receptor ligands DTG, (+)-3-PPP, and haloperidol also compete with [³H]dextrorphan binding in a complex manner in forebrain, consistent with their recognition of two binding sites. These substances, however, uniquely among the compounds tested, interact predominantly with the low affinity component ($IC_{50} > 1$ μM), which accounts for 79–83% of their binding. Although the high affinity [³H]dextrorphan site recognized by these compounds represents a commensurately small fraction of the domain of their binding (18–22%), these compounds interact with this site with relatively high affinity (IC_{50} = 1.2–8.5 nM).

Support for a relationship between forebrain [³H]dextrorphan binding sites and the NMDA-operated cation channel is provided by the strong correlation ($r = 0.97, p < 0.001$) between the IC_{50} values of MK-801, (−)-cyclazocine, TCP, PCP, (+)-SKF-10047, (+)-cyclazocine, and (−)-SKF-10047 at the high affinity [³H]dextrorphan binding site and the respective IC_{50} values of these compounds against [³H]MK-801 binding in human cortex reported by Quarum *et al.* (46) (Fig. 10A).

In contrast, from the data of Wong *et al.* (32), no correlation exists ($r = 0.03, p < 0.5$) between the IC_{50} values of MK-801, TCP, PCP, dextrorphan, ketamine, and (+)-SKF-10047 at the high affinity [³H]dextrorphan binding site and the IC_{50} values of these compounds against [³H]SKF-10047 labeling of the σ receptor in rat pons-medulla (Fig. 10D). Furthermore, no significant correlation exists between the high affinity sites labeled by [³H]dextrorphan and those labeled by [³H]dextromethorphan ($r = -0.53, p < 0.20$), based on the IC_{50} values of those compounds evaluated both in the present study and by Craviso and Musacchio (47) in guinea pig brain, i.e., (+)- and (−)-cyclazocine, dextrorphan, levorphanol, and dextromethorphan (Fig. 10C).

In addition to these correlations derived from ligand binding data, activity at the high affinity [³H]dextrorphan binding site in rat forebrain membranes is highly correlated with NMDA receptor antagonism *in vivo*; the IC_{50} values of MK-801, PCP, dextrorphan, SKF-10047, dextromethorphan, and ketamine are highly correlated ($r = 0.97, p < 0.002$) with the EC_{50} values of these compounds in protecting against sound-induced seizures in mice (26). Taken together, this pharmacological profile indicates that [³H]dextrorphan binding labels a site physically near and functionally associated with the NMDA-operated cation channel in rat forebrain. In contrast, no evidence can be found to support a relationship between high affinity [³H]dextrorphan binding and σ receptor activity in the rat forebrain. Moreover, this profile unambiguously distinguishes [³H]dextrorphan binding sites from sites labeled by [³H]dextromethorphan, which has been suggested to selectively label σ receptors with high affinity (48).

Although the equilibrium saturation binding data (Fig. 7) from the three brain regions evaluated, cortex, hippocampus, and cerebellum, suggest that the differential distribution of [³H]dextrorphan binding sites in rat brain seen in Fig. 6 can, to a large extent, be regressed to differences in absolute receptor densities among these regions, qualitative differences in the pharmacological character of [³H]dextrorphan binding domains among these regions cannot be ruled out as a source of the observed variance.

In order to evaluate the possibility of a regional heterogeneity in the pharmacological signature of [³H]dextrorphan binding sites in rat brain, an additional series of [³H]dextrorphan competition binding experiments were performed in rat cerebellum. The best-fit parameter estimates describing logistic curves fit to the cerebellum competition data by nonlinear, least squares regression are presented in Table 2; the competition curves expanded from these parameterizations of the logistic equation, as well as the experimental data points, are plotted in Fig. 9. These data suggest that [³H]dextrorphan labels populations of sites in the cerebellum that may differ either intrinsically or stoichiometrically from those of the forebrain.

As in forebrain, MK-801 is the most potent ligand competing for sites labeled by [³H]dextrorphan in the rat cerebellum; similarly, MK-801 binds to sites labeled by [³H]dextrorphan in cerebellum in a manner best explained by its interaction with high and low affinity sites. MK-801, however, is nearly 19-fold less potent at the high affinity site of cerebellum (IC_{50} = 11.5 ± 2.8 nM) than at the high affinity forebrain site; furthermore, in contrast to forebrain, the majority of sites recognized by MK-801 (62 ± 2%) in cerebellar membranes are of the low affinity form (IC_{50} = 2.0 ± 0.3 μM). TCP, similarly, recognizes two sites in the rat cerebellum and is >4-fold less potent at the cerebellar than the forebrain high affinity site; like MK-801, a

TABLE 1

Potencies of competitors for [^{3}H]dextrorphan binding to rat forebrain membranes

The [^{3}H]dextrorphan binding assay was performed as described in Experimental Procedures, using washed forebrain membranes in the presence of glutamate and glycine, each at a concentration of 100 μM. Data were fit to a logistic equation by nonlinear regression analysis, using the PROPHET Public Procedure FITCOMP, as described in Experimental Procedures. *R*, Receptor population; *H* and *L*, high and low affinity binding species, respectively.

Compound[a]	IC_{50_H}	R_H	IC_{50_L}	R_L
	nM	%	nM	%
MK-801	0.62 ± 0.14	69 ± 5	606 ± 417	31 ± 4
(−)-Cyclazocine	4.4 ± 0.9	74 ± 4	365 ± 184	26 ± 4
TCP	5.9 ± 0.7	71 ± 2	662 ± 183	29 ± 2
PCP	23.1 ± 2.1	81 ± 2	4,001 ± 1,628	19 ± 2
(+)-Cyclazocine	25.0 ± 8.8	61 ± 12	427 ± 223	39 ± 12
Dextrorphan	39.5 ± 5.1	82 ± 5	860 ± 486	18 ± 5
(+)-SKF-10047	42.7 ± 5.5	78 ± 3	2,920 ± 1,393	22 ± 3
(−)-SKF-10047	76.5 ± 9.9	100		
(+)-Ketamine	96.2 ± 13.3	72 ± 4	4,749 ± 1,944	28 ± 3
Levorphanol	120 ± 12.5	76 ± 3	6,956 ± 2,925	24 ± 3
Dextromethorphan	321 ± 17.9	100		
(−)-Ketamine	761 ± 86	100		
Desipramine	838 ± 111	100		
Haloperidol	1.25 ± 0.6	22 ± 4	526,300 ± 123,984	79 ± 1
DTG	2.39 ± 1.3	18 ± 3	2,189 ± 242	83 ± 2
(+)-3-PPP	8.47 ± 6.1	21 ± 3	49,771 ± 7,732	79 ± 2
(−)-3-PPP	21.9 ± 9.3	18 ± 2	16,248 ± 1,243	82 ± 2

[a] Compounds with IC_{50} values of ≥100 μM included verapamil, nifedipine, naloxone, and dynorphin(1–13)NH_2.

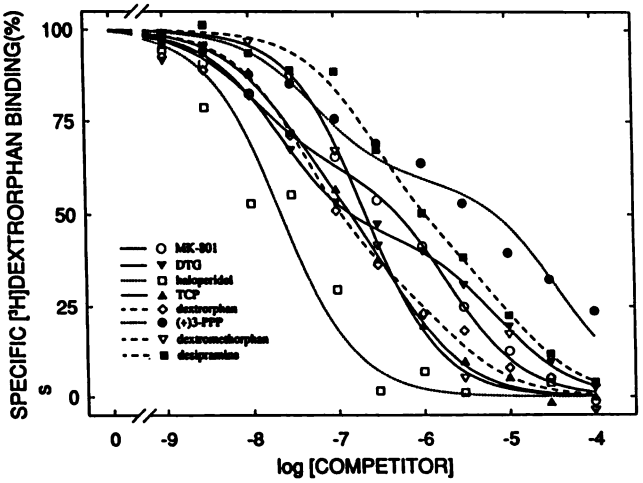

Fig. 8. [^{3}H]Dextrorphan competition binding in rat forebrain membranes. Assay was performed as described in Experimental Procedures, using washed membranes in the presence of glycine and glutamate, each at a concentration of 100 μM. Binding of the indicated compounds is depicted by plots of the data points and their respective theoretical curves generated from expansion of the logistic equation by the parameter estimates fit to the data sets by nonlinear regression analysis (see Experimental Procedures). Values are the means of data from three experiments. Nonspecific binding was defined by 10 μM MK-801.

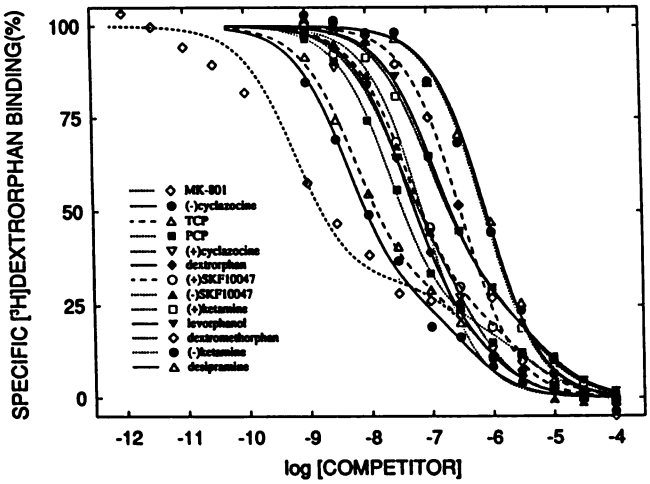

Fig. 9. [^{3}H]Dextrorphan competition binding in washed membranes prepared from rat cerebellum. Assay was performed as described in Experimental Procedures, in the presence of glycine and glutamate, each at a concentration of 100 μM. Binding of the indicated compounds is depicted by plots of the data points and their respective theoretical curves generated from expansion of the logistic equation by the parameter estimates fit to the data sets by nonlinear regression analysis (see Experimental Procedures). Values are the means of data from three experiments. Nonspecific binding was defined by 10 μM MK-801.

majority of sites recognized by TCP in cerebellar membranes are of the low affinity form (56 ± 4%). The predominance of low affinity site binding by MK-801 and TCP in the cerebellum is not characteristic of the binding of dextrorphan or dextromethorphan in this brain region. The percentage of dextrorphan binding to the high affinity site decreases from 81 ± 2% in forebrain to 65 ± 5% in the cerebellum but, unlike MK-801 and TCP, dextrorphan is essentially equipotent in self-competition in rat cerebellum and forebrain.

Among the compounds evaluated, dextromethorphan competes for [^{3}H]dextrorphan binding in a manner that appears to distinguish it from other compounds with significant NMDA antagonist efficacy *in vivo*. Although competition data show dextromethorphan to be of relatively low potency as a competitor for [^{3}H]dextrorphan binding sites in both rat forebrain (IC_{50} = 321 ± 18 nM) and cerebellum (IC_{50} = 220 ± 53 nM), in contrast to other NMDA antagonists dextromethorphan is 1.5-fold more potent in the cerebellum than in forebrain. The only other compound that proved to be more potent in cerebellum than in forebrain was the tricyclic antidepressant desipramine, which among all compounds evaluated in both brain areas was the least potent competitor for high affinity [^{3}H]dextrorphan binding.

Discussion

A central finding of this report is that high affinity [^{3}H] dextrorphan binding in rat forebrain membranes appears to be

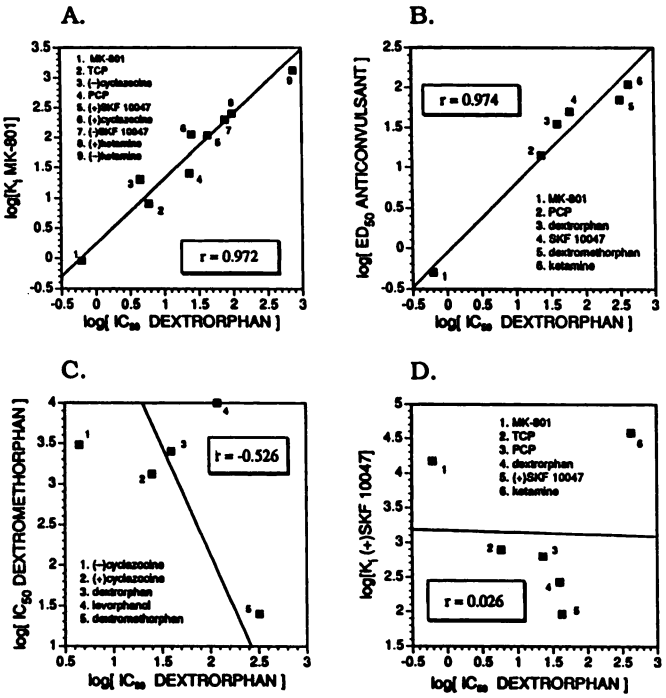

Fig. 10. Correlation between the potencies of compounds to compete for high affinity [^{3}H]dextrorphan binding in rat forebrain membranes and their reported potencies to compete for sites labeled by [^{3}H]MK-801 in human cortex ($r = 0.97$, $p < 0.001$), from the data of Quarum *et al.* (46) (A), to block sound-induced seizures in mice ($r = 0.97$, $p < 0.002$), from the data of Chapman and Meldrum (26) (B), to compete for sites labeled by [^{3}H]dextromethorphan in guinea pig brain ($r = -0.53$, $p < 0.20$), reported by Craviso and Musacchio (47) (C), and to compete for putative σ receptors labeled by [^{3}H]SKF-10047 in rat brain ($r = 0.03$, $p < 0.5$), reported by Wong *et al.* (32) (D). For each correlation, the data represent the potencies of all possible combinations of compounds evaluated both as competitors for [^{3}H]dextrorphan binding in the present study and in the indicated measures as reported in the literature. The significance of the correlations was evaluated by *t* test of the probability of $r = 0$ for each of the comparisons.

predominantly localized to sites within an "open channel" domain of the NMDA receptor-operated cation channel. This relationship is clearly seen in data from thoroughly washed rat forebrain membranes (Figs. 1–4), which demonstrate a striking degree of reciprocity between the magnitude of [^{3}H]dextrorphan binding and the implied level of activation of the NMDA-operated channel resulting from pharmacological modulation of the known regulatory sites of this receptor-channel complex. A consensus of electrophysiological and ligand binding data currently suggest that activation of the NMDA receptor-ionophore complex is regulated through the cooperative interaction of at least five pharmacologically distinct modulatory domains, including 1) the NMDA agonist recognition site, 2) a strychnine-insensitive glycine coagonist site, 3) a divalent cation site prototypically defined by Mg^{2+}, 4) a second divalent cation site defined by Zn^{2+}, and 5) a polyamine site.

The magnitude and direction of the response of [^{3}H]dextrorphan binding to modulation of NMDA receptor-channel activity through its functional domains parallels the response of both [^{3}H]MK-801 and [^{3}H]TCP binding to these manipulations. Like [^{3}H]MK-801 (32, 49, 50) and [^{3}H]TCP (33, 35, 38, 39) binding, [^{3}H]dextrorphan binding is enhanced by activators of NMDA-operated cation channel activity and inhibited by pharmacological antagonists or physiological inhibitors of channel activity. Moreover, the competition binding of a broad range of compounds for high affinity [^{3}H]dextrorphan binding sites unambiguously specifies a noncompetitive NMDA antagonist profile in rat forebrain.

Although these data clearly suggest that the high affinity site labeled by [^{3}H]dextrorphan lies within the domain of the NMDA-operated cation channel, they also suggest that this site may be neither identical nor coincident to those labeled by [^{3}H]TCP or [^{3}H]MK-801. Furthermore, heterogeneity in [^{3}H] dextrorphan binding sites in rat brain is suggested by competition binding data from rat cerebellum, which indicate that [^{3}H]dextrorphan labels, in addition to the NMDA receptor-regulated site characterized in forebrain, a site of unknown function that appears to recognize "σ" receptor ligands with high affinity.

A critical issue in asserting an NMDA receptor-open-channel site for [^{3}H]dextrorphan binding is the fidelity of its dependence upon inferred open-channel conditions, as effected by activators of the NMDA receptor complex. The primary mediators of this activation are, presumably, glutamate and glycine, endogenous agonists at the NMDA and strychnine-insensitive glycine coagonist sites, respectively. We have shown previously that glycine and glutamate stimulate [^{3}H]dextrorphan binding moderately, but with concentration dependency, in a minimally washed rat forebrain membrane preparation; [^{3}H]dextrorphan binding in this preparation was increased maximally 56% by glycine and 30% by glutamate (30). In repetitively washed membranes, however, glycine and glutamate stimulate [^{3}H]dextrorphan binding in a fashion qualitatively similar to that in the minimally washed preparation, but to a significantly greater maximal extent. In well washed mem-

TABLE 2

Potencies of competitors for [^{3}H]dextrorphan binding to membranes prepared from rat cerebellum

The [^{3}H]dextrorphan binding assay was performed as described in Experimental Procedures using washed membranes prepared from cerebellum, in the presence of glutamate and glycine, each at a concentration of 100 μM. Data were fit to a logistic equation by nonlinear regression analysis, using the PROPHET Public Procedure FITCOMP, as described in Experimental Procedures. *R*, Receptor population; *H* and *L*, high and low affinity binding species, respectively.

Compound	IC_{50_H}	R_H	IC_{50_L}	R_L
	nM	%	nM	%
MK-801	11.5 ± 2.8	38 ± 2	2,001 ± 296	62 ± 2
TCP	25.5 ± 12.0	44 ± 11	519 ± 192	56 ± 11
Dextrorphan	41.5 ± 8.7	65 ± 5	1,744 ± 657	35 ± 3
Dextromethorphan	220 ± 53	100		
Desipramine	265 ± 141	57 ± 13	10,192 ± 9,852	43 ± 12
DTG	21.3 ± 6.7	56 ± 4	7,213 ± 2,915	44 ± 4
Haloperidol	22.5 ± 9.5	100		
(+)-3-PPP	57.4 ± 35	41 ± 6	38,521 ± 14,451	59 ± 5

branes, glycine and glutamate effect maximal increases in [^{3}H] dextrorphan binding over control of 2–19-fold and 3–34-fold, respectively. The magnitude with which glutamate and glycine maximally elevate [^{3}H]dextrorphan binding then, as has been reported for both [^{3}H]TCP (33, 35) and [^{3}H]MK-801 (49, 50) binding, appears to depend on the degree to which endogenous glycine and glutamate have been depleted by repetitive washing of the membranes. Basal specific [^{3}H]dextrorphan can approach zero in this preparation (Fig. 1, C and D) but when present is either significantly reduced or eliminated by D-AP5 (Fig. 1, A and B). The profound dependence of [^{3}H]dextrorphan binding on glutamate and the NMDA receptor specificity implied by its sensitivity to D-AP5 suggest that [^{3}H]dextrorphan binding under these assay conditions can be related nearly quantitatively to an activated NMDA-receptor complex domain.

The NMDA agonist-appropriate regulation of [^{3}H]dextrorphan binding by glutamate, together with the accordance of the NMDA coagonist-appropriate rank order of agonist potency of the glycine-mediated stimulation of binding (Fig. 2) (36, 37) and its sensitivity to antagonism by HA-966 (Fig. 1), consistently argue that glutamate and glycine regulate [^{3}H]dextrorphan binding through activation of their respective agonist and coagonist domains at the NMDA receptor-channel complex.

The antecedent requirement of NMDA receptor activation/channel opening for blockade by noncompetitive antagonists *in vitro* (11, 51, 52) is likely to contribute significantly to the observed kinetic complexity with which these ligands bind (34, 53–57). An NMDA receptor activation-promoted binding mechanism at a channel recognition site could account for the apparent kinetic complexity of [^{3}H]dextrorphan binding, as well as its regulation by glycine and glutamate (Fig. 5). Fast and slow phases of binding are characteristic of both [^{3}H] dextrorphan association and dissociation in rat forebrain membranes, despite the presence of saturating concentrations of glutamate and glycine. Similarly, both [^{3}H]MK-801 (53) and [^{3}H]TCP (57, 58) binding have been reported to progress by apparently biexponential association and dissociation processes in rat brain membranes.

The mechanisms subserving the regulation of noncompetitive antagonist binding by NMDA agonists and co-agonists have not been completely elucidated. One possibility is that NMDA receptor activation could increase the density of noncompetitive antagonist binding sites without increasing their ligand affinity (59). A second possibility, which has found greater experimental support, is that channel opening may be coupled with an increase in binding affinity without an increase in B_{max} (38, 49, 50, 56). A third construct, which does not necessarily exclude the second, suggests that NMDA receptor activation by glycine and glutamate increases the rates of both ligand-receptor association and dissociation to an equal extent, thereby having no net effect on the parameters of equilibrium binding (54, 55, 57).

A consensus finding, however, is that a primary determinant of noncompetitive antagonist binding is access to the channel binding site, which in turn is determined by channel opening frequency. As a consequence, the time to equilibrium for [^{3}H] MK-801 and [^{3}H]TCP binding appears to be extremely long in thoroughly washed membrane preparations in the absence of supplementary glutamate and glycine. The explicit dependence of [^{3}H]dextrorphan binding on glutamate and glycine and the apparent kinetic complexity of [^{3}H]dextrorphan binding in the presence of saturating concentrations of these NMDA channel activators suggest that in their absence [^{3}H]dextrorphan binding may not be at equilibrium in well washed membranes even after a 4-hr incubation (Figs. 1–4, control conditions). [^{3}H]TCP binding, however, has been found to reach equilibrium after 4 hr when evaluated under similar assay conditions and in an even more extensively washed rat brain membrane preparation (39). Species differences, as well as differences in assay conditions and membrane preparation, may play a large role in this regard. Specific binding of low nanomolar concentrations of [^{3}H]MK-801, under similar assay conditions, in minimally washed brain membranes has been reported to reach equilibrium after 20 min in rat brain (32) but requires 6 hr in membranes prepared from human cortex (46). In another report using thoroughly washed membranes supplemented by glutamate and glycine, [^{3}H]MK-801 binding in human frontal cortex failed to reach equilibrium until nearly 20 hr of incubation (60).

The qualitative similarity among the responses of [^{3}H]dextrorphan, [^{3}H]MK-801, and [^{3}H]TCP binding to pharmacological modulation of the NMDA agonist and coagonist sites suggests that these radioligands label either a common site or closely related sites in rat forebrain. The regulation of [^{3}H] dextrorphan binding by Mg^{2+} and polyamines, in contrast, appears to distinguish [^{3}H]dextrorphan binding from both [^{3}H] MK-801 and [^{3}H]TCP binding.

Mg^{2+} appears to regulate [^{3}H]dextrorphan binding in well washed membranes in a biphasic manner, which parallels its regulation of [^{3}H]MK-801 and [^{3}H]TCP binding; Mg^{2+}, however, is a much more potent inhibitor of [^{3}H]dextrorphan binding than of either [^{3}H]MK-801 (32, 34) or [^{3}H]TCP (38, 41) binding. In addition, a differential sensitivity of the inhibitory effects of Mg^{2+} to glutamate and glycine contrasts [^{3}H] dextrorphan binding with that of [^{3}H]MK-801 and [^{3}H]TCP. In well washed membranes, Mg^{2+} appears to be significantly more potent an inhibitor of both [^{3}H]MK-801 (34) and [^{3}H] TCP (39) binding in the presence of glutamate and glycine than in their absence. In contrast, Mg^{2+} appears to be an equipotent inhibitor of [^{3}H]dextrorphan binding in well washed membranes in the presence or in the absence of supplementary glutamate and glycine.

Recent evidence has suggested that both [^{3}H]MK-801 (42, 61) and [^{3}H]TCP (41, 45) binding are regulated by Mg^{2+}-like divalent cations, and by polyamine agonists such as spermidine, through modulation of a common cation-sensitive site at the NMDA-receptor complex. It is interesting, in this context, that the distinguishing features of the Mg^{2+}-mediated regulation of [^{3}H]dextrorphan binding are paralleled in the regulation of [^{3}H]dextrorphan binding by spermidine and, accordingly, distinguish [^{3}H]dextrorphan binding from that of [^{3}H]MK-801 and [^{3}H]TCP. Thus, relative to the spermidine regulation of [^{3}H]MK-801 (41, 45) and [^{3}H]TCP (41, 45) binding, the spermidine-[^{3}H]dextrorphan binding concentration-response curve is shifted to the left 3–6-fold in well washed membranes and, furthermore, is insensitive to the presence of glutamate and glycine, which, in contrast, increase the potency of spermidine to stimulate [^{3}H]MK-801 and [^{3}H]TCP binding by a minimum of 3–5-fold.

The apparently unique features of the regulation of [^{3}H] dextrorphan binding by Mg^{2+} and spermidine are more readily explained by the existence of a [^{3}H]dextrorphan binding site that to some extent is noncoincident with the sites of [^{3}H]MK-801 and [^{3}H]TCP binding than by the unique regulation of [^{3}H]

dextrorphan binding by Mg^{2+} and spermidine at a common noncompetitive antagonist binding site. The significant glycine- and glutamate-mediated shift of the potencies of Mg^{2+} and spermidine as regulators of [^{3}H]MK-801 and [^{3}H]TCP binding suggests that their recognition sites may be localized to a conformationally sensitive channel domain not occupied by [^{3}H]dextrorphan. It is possible, given the apparent localization of noncompetitive NMDA antagonist binding to sites within a membrane-spanning channel domain, that the relatively greater potencies of Mg^{2+} and spermidine as regulators of [^{3}H]dextrorphan binding *in vitro* could result from a relatively greater accessibility of [^{3}H]dextrorphan binding to the effects of these exogenously applied modulators, as a consequence of its occupation of a channel binding site that is relatively external to the sites of [^{3}H]MK-801 or [^{3}H]TCP binding. Consistent with this possibility, it would be plausible for the channel binding sites of ligands such as [^{3}H]MK-801 and [^{3}H]TCP, which appear relatively insulated, under closed channel conditions, from the modulatory effects of an exogenous channel-permeant cation such as Mg^{2+}, to occupy a relatively deeper channel location than ligands such as [^{3}H]dextrorphan, which appear relatively less sensitive to channel opening and closing.

Although it would be premature to attempt to assign functional or mechanistic significance to the features of the ligand behavior of [^{3}H]dextrorphan that distinguish it from [^{3}H]MK-801 and [^{3}H]TCP binding, a direct comparison of functional antagonism of NMDA-operated channel activity by dextrorphan and MK-801 *in vitro* suggests that the two act through nonidentical mechanisms (22). In contrast to MK-801, which was found to act in a strictly use-dependent manner, dextrorphan showed no evidence of use dependency in mediating a potent, selective, and dose-dependent noncompetitive inhibition of NMDA receptor-mediated depolarizations in the hippocampal slice (22). These results are not inconsistent with the possibility of dextrorphan acting at a site relatively exterior to that of MK-801 in the channel domain. Because the probability of opening of the NMDA-operated channel appears to be very low ($p < 0.002$) (51), a more superficially liganded antagonist such as dextrorphan would presumably encounter, during a prolonged closed channel interval, less of an impediment to reaching its site of action than would a compound acting at a site deeper in the channel domain, such as MK-801. In support of this possibility, the onset of dextrorphan blockade of channel activity has been shown to progress slowly but completely in the hippocampal slice in the absence of exogenous agonist or evoked excitation; in contrast, onset of MK-801 blockade fails to progress under these closed channel conditions *in vitro* (22).

The regulation of [^{3}H]dextrorphan binding by pharmacological modulators of NMDA receptor-channel complex activity provides strong indirect evidence for the association of [^{3}H]dextrorphan binding in rat forebrain with the noncompetitive antagonist domain of the NMDA receptor complex. The competition binding data of Table 1 (Fig. 8) provide a direct assessment of the nature of the sites labeled by [^{3}H]dextrorphan in rat forebrain. The pharmacological signature of [^{3}H]dextrorphan binding provided by these data suggests unambiguously that the site that is labeled primarily by [^{3}H]dextrorphan in rat forebrain and that occurs in rat cerebellum at much lower abundance is that which mediates the well documented noncompetitive NMDA antagonist activity of dextrorphan observed *in vivo* and *in vitro* (12, 21–24, 27). Furthermore, the rank order of potency of ligands as competitors for [^{3}H]dextrorphan binding in forebrain is not consistent with interaction at either σ receptors (32) or dextromethorphan binding sites (47) (Fig. 10).

Further evidence of the pharmacological congruence between the forebrain [^{3}H]dextrorphan binding domain and the NMDA receptor-ionophore complex comes from the relative potencies of the cyclazocine ketamine and SKF-10047 stereoisomers as competitors for [^{3}H]dextrorphan binding. As can be seen from the data of Table 1 (Fig. 8), the levorotatory form of cyclazocine, in contrast to the SKF-10047 and ketamine stereoisomers, is more potent than its dextrorotatory isomer in competition for [^{3}H]dextrorphan binding (Table 1; Fig. 8), whereas (+)-SKF-10047 and (+)-ketamine are more potent than their (−)-forms in this regard. An identical stereoselectivity is characteristic of these compounds at the NMDA receptor-ionophore complex, with (−)-cyclazocine being a more potent antagonist than (+)-cyclazocine of *N*-methylaspartate-mediated neuronal excitation (62) and (−)-cyclazocine and the (+)-forms of SKF-10047 and ketamine being more potent than their optical antipodes in competition for [^{3}H]PCP (15) or [^{3}H]MK-801 (46) binding.

The direct labeling of [^{3}H]dextrorphan recognition sites provides unique insight into the mechanisms of action of dextromethorphan and dextrorphan. Both compounds have been shown to compete effectively for high affinity [^{3}H]PCP binding sites in rat brain, but dextrorphan exhibits nearly 4-fold greater potency than dextromethorphan (15). In contrast, dextrorphan is >400-fold less potent than dextromethorphan at [^{3}H]dextromethorphan binding sites; moreover, neither PCP nor ketamine appears to compete for [^{3}H]dextromethorphan binding sites with appreciable efficacy (47).

It is significant, in the context of the behavioral effects of these compounds, that dextromethorphan binds with 8-fold lower potency than dextrorphan to the high affinity [^{3}H]dextrorphan site in rat forebrain (Table 1; Fig. 8). Although the rapid metabolic conversion of dextromethorphan to dextrorphan following its peripheral administration (28, 29) prevents a clear resolution of its effects from those of dextrorphan *in vivo*, Craviso and Musacchio (63) found no evidence of metabolism of dextromethorphan in brain microsomes *in vitro*. The ligand-binding properties of dextromethorphan *in vitro*, therefore, are not confounded by the presence of dextrorphan. The relative potency and complete efficacy with which dextromethorphan competes for sites labeled by [^{3}H]dextrorphan in rat forebrain appear to account adequately for its efficacy as an anticonvulsant and neuroprotective agent.

Although dextromethorphan and dextrorphan have NMDA antagonist efficacy both *in vitro* and *in vivo*, dextromethorphan is clearly less potent than dextrorphan in most experimental models of NMDA antagonism or in antagonism of NMDA-mediated pathology, including the attenuation of hypoxic damage in neuronal culture (19), blockade of low Mg^{2+}-induced epileptiform activity in slices from neocortex (21) and hippocampus (22), protection against sound-induced seizures (26), protection against maximal electroshock-induced seizures (24), and protection against NMDA-induced lethality (64). The consistently greater potency of dextrorphan over dextromethorphan is antithetical to the reported pharmacological profile of [^{3}H]dextromethorphan binding sites (47) but is consistent with that of the [^{3}H]dextrorphan profile.

It is, then, by virtue of both its intrinsic activity as a noncompetitive NMDA antagonist and its rapid conversion to

dextrorphan that dextromethorphan at high doses produces psychotomimetic effects and effects a PCP-like behavioral syndrome in humans (6). Because of its PCP-like effects, over-the-counter availability, oral efficacy, and relatively wide margin of safety, dextromethorphan has enjoyed popularity as a drug of abuse in many parts of the world (5).

Because a preponderance of the behavioral and ligand-binding effects of dextromethorphan can be regressed to its NMDA antagonist activity, the possible functional role of [³H]dextromethorphan binding sites remains unclear. Musacchio and co-workers (48, 65) have suggested recently, based on ligand-binding studies in guinea pig brain, that dextromethorphan and σ receptor ligands share a common high affinity binding domain. No clear relationship appears to exist, however, between the potencies of compounds as σ receptor ligands and their efficacies as anticonvulsant or neuroprotective agents.

The domain of [³H]dextrorphan binding in rat cerebellum (Table 2; Fig. 9), however, appears to differ qualitatively from that of rat forebrain. The σ receptor ligands DTG, haloperidol, and (+)-3-PPP compete for sites labeled by [³H]dextrorphan in rat cerebellar membranes with approximately 10-fold lower potency than in forebrain, yet the high affinity [³H]dextrorphan binding component recognized by these compounds accounts for a significantly larger fraction of the total specific binding in this brain region than in forebrain. The prototypical non-competitive NMDA antagonist ligands MK-801 and TCP are 19- and 4-fold less potent, respectively, at the high affinity site labeled by [³H]dextrorphan in cerebellum than at the high affinity forebrain [³H]dextrorphan site. Furthermore, the high affinity site recognized by MK-801 and TCP is, respectively, 44% and 38% less abundant in cerebellum than in forebrain.

Other evidence of relative disparity in the character of the NMDA receptor-channel complex in cerebellum has been reported. Monaghan *et al.* (66), using quantitative autoradiography, have shown that NMDA-sensitive L-[³H]glutamate binding is significantly less sensitive to inhibition by competitive NMDA antagonists in the cerebellum than in other brain regions. [³H]MK-801 has been reported to label a high affinity site in human cerebellum with significantly lower affinity than its counterpart in cortex (46). Johnson and co-workers (67) have reported a virtual insensitivity of cerebellar [³H]TCP binding to modulation by glutamate, yet they found the sensitivity of NMDA-mediated [³H]norepinephrine release to inhibition by PCP in cerebellum to be consonant with that elsewhere in brain.

In summary, we have provided evidence suggesting that [³H]dextrorphan binding is predominantly associated with the NMDA receptor-channel complex and most likely resides in a channel-binding domain approximating the site or sites of binding of other specific noncompetitive NMDA antagonists. The data from competition radioligand binding experiments confirm that the high affinity [³H]dextrorphan recognition sites pharmacologically correspond to a noncompetitive antagonist domain of the NMDA receptor and that they are unrelated to those labeled by [³H]dextromethorphan.

The therapeutic potential of noncompetitive NMDA antagonists has yet to be realized, primarily because of the overlapping dose ranges of these compounds in producing therapeutic and untoward effects. Dextrorotatory morphinans such as dextrorphan could provide an alternative to arylcycloalkylamines or methyldibenzo[*a*,*d*]cycloheptenimines, such as MK-801, as points of departure in the design of novel, therapeutically useful, noncompetitive NMDA antagonists. In addition to its NMDA antagonist efficacy *in vivo*, dextrorphan has established a record of clinical safety and low toxicity by virtue of the widespread use of its *de facto* prodrug, dextromethorphan (68). Thus, dextrorphan-like compounds could provide a unique approach to addressing the great therapeutic potential of non-competitive NMDA antagonists.

Future studies will address the nature of the apparent complexity of [³H]dextrorphan binding, as well as the apparent regional heterogeneity of binding in rat brain, and will seek to identify the basis for and possible functional significance of the unique regulation of [³H]dextrorphan binding by polyamines and Mg^{2+}.

Acknowledgments

We thank Ms. Janice Woelfl and Mr. Tom Jacobsen for their expert technical assistance and Dr. Mark Leid for helpful discussions.

References

1. Jaffe, J. H., and W. R. Martin. Opioid analgesics and antagonists, in *The Pharmacological Basis of Therapeutics* (A. G. Gilman, T. W. Rall, A. S. Nies and P. Taylor, eds.). Pergamon Press, New York, 485–521 (1990).
2. Benson, W. M., P. L. Stefko, and L. O. Randall. Comparative pharmacology of levorphan, racemorphan, dextrorphan and related methyl ethers. *J. Pharmacol. Exp. Ther.* **109**:189–200 (1953).
3. Isbell, H. I., and H. F. Fraser. Actions and addiction liabilities of dromoran derivatives in man. *J. Pharmacol. Exp. Ther.* **107**:524–530 (1953).
4. Degkwitz, R. Dextromethorphan (Romilar) as an intoxicant. *Nervenarzt* **35**:412–414 (1964).
5. McCarthy, J. P. Some less familiar drugs of abuse. *Med. J. Aust.* **2**:1078–1081 (1971).
6. Jasinski, D. R., W. R. Martin, and P. A. Mansky. Progress report on the assessment of the antagonists nalbuphine and GPA-2087 for abuse potential and studies of the effects of dextromethorphan in man, in *Report of the 33rd Meeting, Committee on Problems of Drug Dependence*, (National Research Council) National Academy of Sciences, Washington DC. 143–178 (1971).
7. Lodge, D., and N. A. Anis. Effects of phencyclidine on excitatory amino acid activation of spinal interneurons in the cat. *Eur. J. Pharmacol.* **77**:203–204 (1982).
8. Anis, N. A., S. C. Berry, N. R. Burton, and D. Lodge. The dissociative anaesthetics, ketamine and phencyclidine, selectively decrease excitation of central neurons by *N*-methyl-aspartate. *Br. J. Pharmacol.* **79**:565–575 (1983).
9. Berry, S. C., S. L. Dawkins, and D. Lodge. Comparison of σ- and κ-opiate ligands as excitatory amino acid antagonists. *Br. J. Pharmacol.* **83**:179–185 (1984).
10. Honey, C. R., Z. Miljkovic, and J. F. MacDonald. Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid. *Neurosci. Lett.* **61**:135–139 (1985).
11. Mayer, M. L., G. L. Westbrook, and L. Vyklicky. Sites of antagonist action on *N*-methyl-D-aspartic acid receptors studied using fluctuation analysis and a rapid perfusion technique. *J. Neurophysiol.* **60**:645–663 (1988).
12. Church, J., D. Lodge, and S. C. Berry. Differential effects of dextrorphan and levorphanol on the excitation of rat spinal neurons by amino acids. *Eur. J. Pharmacol.* **111**:185–190 (1985).
13. Holtzman, S. G. Phencyclidine-like discriminative stimulus properties of opioids in the squirrel monkey. *Psychopharmacology* **77**:295–300 (1982).
14. Herling, S., R. E. Solomon, and J. H. Woods. Discriminative stimulus effects of dextrorphan in pigeons. *J. Pharmacol. Exp. Ther.* **227**:723–731 (1983).
15. Murray, T. F., and M. E. Leid. Interaction of dextrorotatory opioids with phencyclidine recognition sites in rat brain membranes. *Life Sci.* **34**:1899–1911 (1984).
16. Hampton, R. Y., F. Medzihradsky, J. H. Woods, and P. J. Dahlstrom. Stereospecific binding of [³H]phencyclidine to brain membranes. *Life Sci.* **30**:2147–2154 (1982).
17. Steinberg, G. K., J. Saleh, and D. Kunis. Delayed treatment with dextromethorphan and dextrorphan reduces cerebral damage after transient focal ischemia. *Neurosci. Lett.* **89**:193–197 (1988).
18. Faden, A. I., P. Demediuk, S. S. Panter, and R. Vink. The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science (Washington D. C.)* **244**:798–800 (1989).
19. Goldberg, M. P., P. C. Pham, and D. W. Choi. Dextrorphan and dextromethorphan attenuate hypoxic injury in neuronal culture. *Neurosci. Lett.* **80**:11–15 (1987).
20. Monyer, H., and D. W. Choi. Morphinans attenuate cortical neuronal injury induced by glucose deprivation *in vitro*. *Brain Res.* **446**:144–148 (1988).
21. Wong, B. Y., D. A. Coulter, D. W. Choi, and D. A. Prince. Dextrorphan and dextromethorphan, common antitussives, are antiepileptic and antagonize *N*-methyl-D-aspartate in brain slices. *Neurosci. Lett.* **85**:261–266 (1988).
22. Cole, A. E., C. U. Eccles, J. J. Aryanpur, and R. S. Fisher. Selective depression of *N*-methyl-D-aspartate-mediated responses by dextrorphan in the hippocampal slice in rat. *Neuropharmacology* **28**:249–254 (1989).

23. Aram, J. A., D. Martin, M. Tomczyk, S. Zeman, J. Millar, G. Pohler, and D. Lodge. Neocortical epileptogenesis *in vitro*: studies with *N*-methyl-D-aspartate, phencyclidine, *sigma* and dextromethorphan receptor ligands. *J. Pharmacol. Exp. Ther.* **248**:320–328 (1989).
24. Leander, J. D., R. C. Rathbun, and D. M. Zimmerman. Anticonvulsant effects of phencyclidine-like drugs: relation to *N*-methyl-D-aspartic acid antagonism. *Brain Res.* **454**:368–372 (1988).
25. Tortella, F. C., J. W. Ferkany, and M. J. Pontecorvo. Anticonvulsant effects of dextrorphan in rats: possible involvement in dextromethorphan-induced seizure protection. *Life Sci.* **42**:2509–2514 (1988).
26. Chapman, A. G., and B. S. Meldrum. Non-competitive *N*-methyl-D-aspartate antagonists protect against sound-induced seizures in DBA/2 mice. *Eur. J. Pharmacol.* **166**:201–211 (1989).
27. Ferkany, J. W., S. A. Borosky, D. B. Clissold, and M. J. Pontecorvo. Dextromethorphan inhibits NMDA-induced convulsions. *Eur. J. Pharmacol.* **151**:151–154 (1988).
28. Ramachander, G., F. D. Williams, and J. F. Emele. Determination of dextrorphan in plasma and evaluation of bioavailability of dextromethorphan hydrobromide in humans. *J. Pharm. Sci.* **66**:1047–1048 (1977).
29. Barnart, J. W. The urinary excretion of dextromethorphan and three metabolites in dogs and humans. *Toxicol. Appl. Pharmacol.* **55**:43–48 (1980).
30. Franklin, P. H., and T. F. Murray. Identification and initial characterization of high-affinity [^{3}H]dextrorphan binding sites in rat brain. *Eur. J. Pharmacol.* **189**:89–93 (1990).
31. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
32. Wong, E. H., A. R. Knight, and G. N. Woodruff. [^{3}H]MK-801 labels a site on the *N*-methyl-D-aspartate receptor channel complex in rat brain membranes. *J. Neurochem.* **50**:274–281 (1988).
33. Snell, L. D., R. S. Morter, and K. M. Johnson. Glycine potentiates *N*-methyl-D-aspartate-induced [^{3}H]TCP binding to rat cortical membranes. *Neurosci. Lett.* **83**:313–317 (1987).
34. Reynolds, I. J., and R. J. Miller. [^{3}H]MK801 binding to the NMDA receptor/ionophore complex is regulated by divalent cations: evidence for multiple regulatory sites. *Eur. J. Pharmacol.* **151**:103–112 (1988).
35. Loo, P., A. Braunwalder, J. Lehmann, and M. Williams. Radioligand binding to central phencyclidine recognition sites is dependent on excitatory amino acid receptor agonists. *Eur. J. Pharmacol.* **123**:467–468 (1986).
36. Snell, L. D., R. S. Morter, and K. M. Johnson. Structural requirements for activation of the glycine receptor that modulates the *N*-methyl-D-aspartate operated ion channel. *Eur. J. Pharmacol.* **156**:105–110 (1988).
37. McBain, C. J., N. W. Kleckner, S. Wyrick, and R. Dingledine. Structural requirements for activation of the glycine coagonist site of *N*-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. *Mol. Pharmacol.* **36**:556–565 (1989).
38. Loo, P. S., A. F. Braunwalder, J. Lehmann, M. Williams, and M. A. Sills. Interaction of L-glutamate and magnesium with phencyclidine recognition sites in rat brain: evidence for multiple affinity states of the phencyclidine/*N*-methyl-D-aspartate receptor complex. *Mol. Pharmacol.* **32**:820–830 (1987).
39. Johnson, K. M., L. D. Snell, A. I. Sacaan, and S. M. Jones. Pharmacological regulation of the phencyclidine-binding site associated with the *N*-methyl-D-aspartate receptor-operated ion channel. *Drug Dev. Res.* **17**:281–297 (1989).
40. Yeh, G.-C., D. W. Bonhaus, and J. O. McNamara. Evidence that zinc inhibits *N*-methyl-D-aspartate receptor-gated ion channel activation by noncompetitive antagonism of glycine binding. *Mol. Pharmacol.* **38**:14–19 (1990).
41. Sacaan, A. I., and K. M. Johnson. Competitive inhibition of magnesium-induced [^{3}H]*N*-(1-[thienyl]cyclohexyl)piperidine binding by arcaine: evidence for a shared spermidine-magnesium binding site. *Mol. Pharmacol.* **38**:705–710 (1990).
42. Reynolds, I. J., and R. J. Miller. Ifenprodil is a novel type of *N*-methyl-D-aspartate receptor antagonist: interaction with polyamines. *Mol. Pharmacol.* **36**:758–765 (1989).
43. Ransom, R. W., and N. L. Stec. Cooperative modulation of [^{3}H]MK-801 binding to the *N*-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. *J. Neurochem.* **51**:830–836 (1988).
44. Williams, K., C. Romano, and P. B. Molinoff. Effects of polyamines on the binding of [^{3}H]MK-801 to the *N*-methyl-D-aspartate receptor: pharmacological evidence for the existence of a polyamine recognition site. *Mol. Pharmacol.* **36**:575–581 (1989).
45. Sacaan, A. I., and K. M. Johnson. Characterization of the stimulatory and inhibitory effects of polyamines on [^{3}H]*N*-(1-[thienyl]cyclohexyl)piperidine binding to the *N*-methyl-D-aspartate receptor ionophore complex. *Mol. Pharmacol.* **37**:572–577 (1990).
46. Quarum, M. L., J. D. Parker, J. F. Keana, and E. Weber. (+)-[^{3}H]MK-801 binding sites in postmortem human brain. *J. Neurochem.* **54**:1163–1168 (1990).
47. Craviso, G. L., and J. M. Musacchio. High-affinity dextromethorphan binding sites in guinea pig brain. II. Competition experiments. *Mol. Pharmacol.* **23**:629–640 (1983).
48. Musacchio, J. M., M. Klein, and J. J. Paturzo. Effects of dextromethorphan site ligands and allosteric modifiers on the binding of (+)-[^{3}H]3-(-3-hydroxyphenyl)-*N*-(1-propyl)piperidine. *Mol. Pharmacol.* **35**:1–5 (1989).
49. Foster, A. C., and E. H. Wong. The novel anticonvulsant MK-801 binds to the activated state of the *N*-methyl-D-aspartate receptor in rat brain. *Br. J. Pharmacol.* **91**:403–409 (1987).
50. Reynolds, I. J., S. N. Murphy, and R. J. Miller. ^{3}H-labeled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. *Proc. Natl. Acad. Sci. USA* **84**:7744–7748 (1987).
51. Huettner, J. E., and B. P. Bean. Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc. Natl. Acad. Sci. USA* **85**:1307–1311 (1988).
52. MacDonald, J. F., Z. Miljkovic, and P. Pennefather. Use-dependent block of excitatory amino acid currents in cultured neurons by ketamine. *J. Neurophysiol.* **58**:251–266 (1987).
53. Javitt, D. C., and S. R. Zukin. Biexponential kinetics of [^{3}H]MK-801 binding: evidence for access to closed and open *N*-methyl-D-aspartate receptor channels. *Mol. Pharmacol.* **35**:387–393 (1989).
54. Kloog, Y., V. Nadler, and M. Sokolovsky. Mode of binding of [^{3}H]dibenzocycloalkenimine (MK-801) to the *N*-methyl-D-aspartate (NMDA) receptor and its therapeutic implication. *FEBS Lett.* **230**:167–170 (1988).
55. Kloog, Y., R. Haring, and M. Sokolovsky. Kinetic characterization of the phencyclidine-*N*-methyl-D-aspartate receptor interaction: evidence for a steric blockade of the channel. *Biochemistry* **27**:843–848 (1988).
56. Johnson, K. M., A. I. Sacaan, and L. D. Snell. Equilibrium analysis of [^{3}H] TCP binding: effects of glycine, magnesium and *N*-methyl-D-aspartate agonists. *Eur. J. Pharmacol.* **152**:141–146 (1988).
57. Bonhaus, D. W., and J. O. McNamara. *N*-Methyl-D-aspartate receptor regulation of uncompetitive antagonist binding in rat brain membranes: kinetic analysis. *Mol. Pharmacol.* **34**:250–255 (1988).
58. Lamdani-Itkin, H., Y. Kloog, and M. Sokolovsky. Modulation of glutamate-induced uncompetitive blocker binding to the NMDA receptor by temperature and by glycine. *Biochemistry* **29**:3987–3993 (1990).
59. Javitt, D. C., A. Jotkowitz, R. Sircar, and R. S. Zukin. Non-competitive regulation of phencyclidine/σ receptors by the *N*-methyl-D-aspartate receptor antagonist D-(−)2-amino-5-phosphonovaleric acid. *Neurosci. Lett.* **78**:193–198 (1987).
60. Kornhuber, J., F. Mack-Burkhardt, M. E. Kornhuber, and P. Riederer. [^{3}H] MK-801 binding sites in post-mortem human frontal cortex. *Eur. J. Pharmacol.* **162**:483–490 (1989).
61. Reynolds, I. J. Arcaine uncovers dual interactions of polyamines with the *N*-methyl-D-aspartate receptor. *J. Pharmacol. Exp. Ther.* **255**:1001–1007 (1990).
62. Church, J., and D. Lodge. Cyclazocine and pentazocine as *N*-methylaspartate antagonists on cat and rat spinal neurons *in vivo*. *J. Pharmacol. Exp. Ther.* **253**:636–645 (1990).
63. Craviso, G. L., and J. M. Musacchio. High-affinity dextromethorphan binding sites in guinea pig brain. I. Initial characterization. *Mol. Pharmacol.* **23**:619–628 (1983).
64. Leander, J. D., R. R. Lawson, P. L. Ornstein, and D. M. Zimmerman. *N*-Methyl-D-aspartic acid-induced lethality in mice: selective antagonism by phencyclidine-like drugs. *Brain Res.* **448**:115–120 (1988).
65. Klein, M., and J. M. Musacchio. High affinity dextromethorphan binding sites in guinea pig brain: effect of *sigma* ligands and other agents. *J. Pharmacol. Exp. Ther.* **251**:207–215 (1989).
66. Monaghan, D. T., H. J. Olverman, L. Nguyen, J. C. Watkins, and C. W. Cotman. Two classes of *N*-methyl-D-aspartate recognition sites: differential distribution and differential regulation by magnesium. *Proc. Natl. Acad. Sci. USA* **85**:9836–9840 (1988).
67. Yi, S. J., L. D. Snell, and K. M. Johnson. Linkage between phencyclidine (PCP) and *N*-methyl-D-aspartate (NMDA) receptors in the cerebellum. *Brain Res.* **445**:147–151 (1988).
68. Salem, H., and D. M. Aviado. *Antitussive Agents. International Encyclopedia of Pharmacology and Therapeutics*. Pergamon Press, Oxford, UK (1970).

Send reprint requests to: Paul H. Franklin, Ph.D., College of Pharmacy, Oregon State University, Corvallis, OR 97331.