

## AN ABSTRACT OF THE THESIS OF

Jane E. Roth for the degree of Doctor of Philosophy in

Toxicology presented on 1st May, 1995.

Title: Interaction of Dextrorotatory Opioids with the N-Methyl-D-Aspartate  
Receptor

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Thomas F. Murray

The objective of these studies was to increase our understanding of the noncompetitive antagonist interactions of the dextrorotatory opioids 3-hydroxy-N-methylmorphinan (dextrorphan) and 3-methoxy-N-methylmorphinan (dextromethorphan) with the N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor. Dextrorphan and dextromethorphan potently suppressed convulsions induced by focal injection of (-)-bicuculline methiodide into the rat prepiriform cortex with complete efficacy. The anticonvulsant potency of an array of noncompetitive NMDA receptor antagonists was highly correlated with their respective affinities for [<sup>3</sup>H]dextrorphan-labelled NMDA receptors in rat forebrain membranes. These results suggest that noncompetitive antagonism of NMDA receptors underlies the anticonvulsant action of these compounds. In addition, 1,3-di(2-tolyl)guanidine (DTG) was found to be a potent anticonvulsant *in vivo*. This novel anticonvulsant action of DTG appears not to involve either sigma binding sites or the ion channel binding domain of the NMDA receptor.

Dextrorphan and dextromethorphan elicit stereotyped behaviour in rats that is indistinguishable from that produced by phencyclidine. Characterization of the behavioural potencies of several noncompetitive NMDA antagonists revealed a significant correlation with their respective affinities for the high affinity [<sup>3</sup>H]dextrorphan binding site in rat forebrain membranes. Furthermore, the affinity of dextromethorphan for NMDA receptors

adequately accounts for its ability to induce stereotyped behaviour. The common pharmacologic profiles of these compounds suggests that the abuse potential of dextromethorphan containing antitussive preparations is related to the noncompetitive NMDA antagonist activity of dextromethorphan and its primary metabolite dextrorphan.

The pharmacologic specificity and anatomical distribution of [ $^3\text{H}$ ]dextrorphan recognition sites in the rat brain was characterized by quantitative autoradiography. Equilibrium saturation analysis indicated that [ $^3\text{H}$ ]dextrorphan labelled a single population of high affinity binding sites. These sites are heterogeneously distributed throughout rat forebrain with the following order of binding densities: hippocampal formation > cerebral cortex > thalamic nuclei > striatum. The pharmacologic profile of [ $^3\text{H}$ ]dextrorphan binding sites in slide-mounted sections is consistent with the labelling of a noncompetitive antagonist domain of the NMDA receptor-channel complex. Furthermore, the distribution of [ $^3\text{H}$ ]dextrorphan binding sites in slide-mounted tissue is in excellent agreement with the distribution of NMDA receptors previously reported by autoradiographic visualization of NMDA receptor distribution using other radioligands. An exception was the cerebellar molecular layer, where the density of [ $^3\text{H}$ ]dextrorphan binding sites was particularly high. The anatomical distribution of [ $^3\text{H}$ ]dextrorphan binding sites does not correspond to the reported distributions of sigma binding sites.

**Interaction of Dextrorotatory Opioids with the  
N-Methyl-D-Aspartate Receptor**

**by  
Jane Roth**

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## CONTRIBUTION OF AUTHORS

Chapter 2 is reproduced with the written permission of Elsevier Science Publishers and *The European Journal of Pharmacology*. All of the experiments discussed in this chapter were performed and analysed by myself in the laboratory of Professor Thomas Murray. Dr. Paul Franklin and Professor Tom Murray provided guidance and assisted with interpretation of the data. These authors also provided editorial comments which resulted in useful revisions to the manuscript. Dr. Ge Zhang provided assistance and training in the technical aspects of this project.

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# **Interaction of Dextrorotatory Opioids with the N-Methyl-D-Aspartate Receptor**

## **Chapter 1**

### **Introduction**

Dextrorotatory enantiomers of the morphinan series include 3-hydroxy-N-methylmorphinan (dextrorphan) and 3-methoxy-N-methylmorphinan (dextromethorphan). Opioid receptors are highly stereoselective and unlike their levorotatory optical isomers, levorphanol and levomethorphan, the dextrorotatory opioids have virtually no affinity for opioid receptors. Despite their low affinity the dextrorotatory morphinans are considered opioid ligands; dextrorphan binds to opioid receptors with affinities approximately three orders of magnitude lower than levorphanol (Goldstein and Naidu, 1990).

A number of naturally occurring and synthetic opioids possess antitussive efficacy, and it is this property that is responsible for the widespread use of dextromethorphan as a non-prescription cough suppressant since the 1950s (Bem and Peck, 1992). Dextrorphan also possesses considerable antitussive efficacy and is thought to contribute significantly to the cough suppressant effects of dextromethorphan which is rapidly and extensively O-demethylated to dextrorphan in vivo (Kamm et al., 1967; Ramachander et al., 1977; Barnhart, 1980). More recently, however, the dextrorotatory opioids have been shown to have anticonvulsant and neuroprotective properties by virtue of their activity as noncompetitive antagonists of the N-methyl-D-aspartate (NMDA) subfamily of ionotropic glutamate receptors (Goldberg et al., 1987; Steinberg et al., 1988; Tortella et al., 1988; Wong et al., 1988).

## Glutamate Receptors

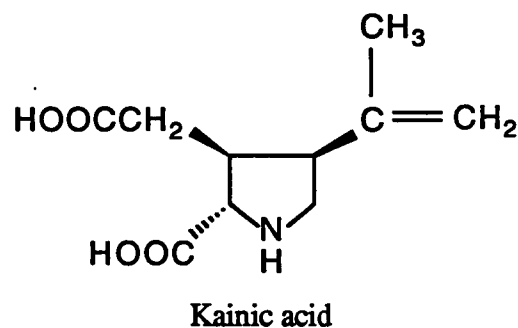
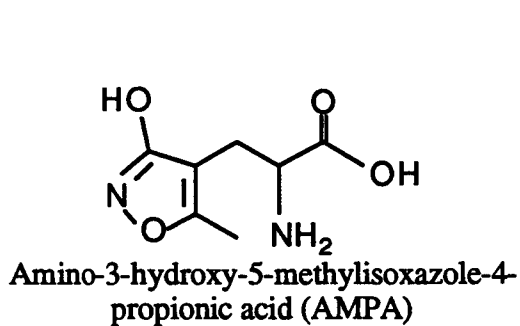
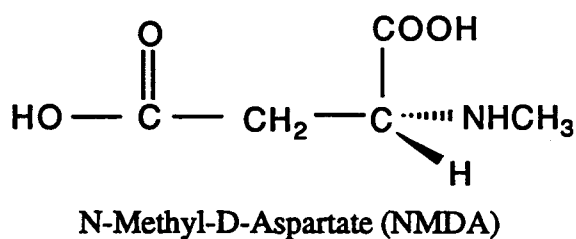
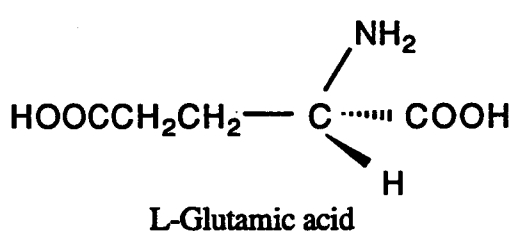
Most synapses in the central nervous system are excitatory and are believed to use L-glutamate as their neurotransmitter. Glutamate neurotransmission at these excitatory synapses is mediated via distinct receptors. Glutamate receptors not only mediate normal synaptic transmission via excitatory pathways, but also appear to play a key role in various forms of neuronal plasticity that are thought to underlie development, learning and memory. However, excessive glutamate release and subsequent activation of glutamate receptors contributes to pathologic processes such as epilepsy and neuronal degeneration associated with stroke, head trauma, sustained convulsions and hypoglycaemia.

The glutamate receptors can be divided into two major groups on the basis of their signal transduction mechanisms; the ionotropic receptors possess integral cation-specific ion channels, whereas the metabotropic receptors are coupled to G-protein transduction elements. Ionotropic glutamate receptors have traditionally been subclassified according to pharmacologic criteria: the N-methyl-D-aspartate (NMDA) receptors, which are activated by the agonist NMDA, and the non-NMDA receptors, which include those receptors sensitive to the agonists  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (Monaghan et al., 1989; Watkins et al., 1990) (Figure 1.1). The recent molecular cloning of glutamate receptor subtypes has greatly extended our understanding of the expression, structure and properties of glutamate receptors. To date, sixteen different subunits have been identified for the three glutamate-gated ion channels in rodent brain (NMDA, AMPA and high-affinity kainate receptors); Figure 1.1 depicts a classification scheme based on amino acid sequence comparison and functional properties of the ionotropic glutamate receptor subtypes (Sprengel and Seeburg, 1995). With respect to its pharmacology and physiology, the NMDA receptor-channel complex is the most thoroughly characterized of the glutamate receptors.

**FIGURE 1.1.**

**Ionotropic glutamate receptor subtypes and chemical structures of endogenous and exogenous agonists**

| <i>Receptor type</i> | <i>Exogenous agonist</i> | <i>Receptor subunit</i>               |
|----------------------|--------------------------|---------------------------------------|
| NMDA                 | NMDA                     | NR1<br>NR2A<br>NR2B<br>NR2C<br>NR2D   |
| Non-NMDA             | AMPA                     | GluRA<br>GluRB<br>GluRC<br>GluRD      |
|                      | Kainate                  | GluR5<br>GluR6<br>GluR7<br>KA1<br>KA2 |
|                      | Orphan                   | delta-1<br>delta-2                    |



## The N-Methyl-D-Aspartate Receptor

### General Pharmacology

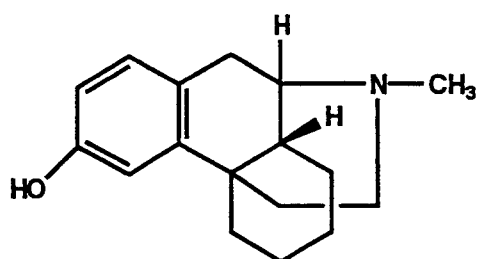
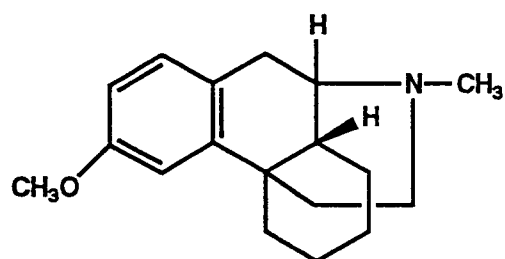
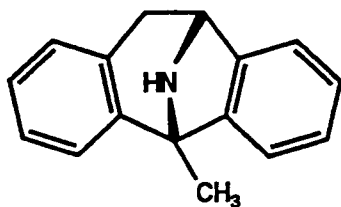
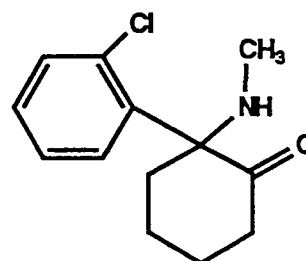
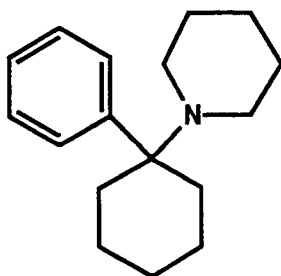
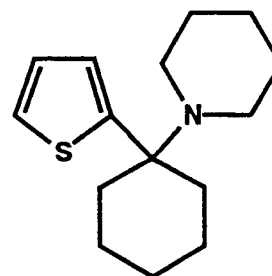
The NMDA receptor contains an integral channel that is highly permeable to  $\text{Ca}^{2+}$  and also permeable to  $\text{Na}^{+}$  and  $\text{K}^{+}$ . Increases in intracellular  $\text{Ca}^{2+}$  are thought to be responsible for both NMDA-receptor induced synaptic plasticity as well as NMDA-receptor mediated neuronal cell death (Monaghan et al., 1989). The NMDA receptor-channel complex appears to have several pharmacologically distinct modulatory domains:

- (1) a glutamate recognition site, at which NMDA is an agonist
- (2) a strychnine-insensitive glycine co-agonist site
- (3) a voltage-dependent  $\text{Mg}^{2+}$  site
- (4) a second divalent cation binding site defined by  $\text{Zn}^{2+}$
- (5) a polyamine site and
- (6) a recognition site within the ion channel that has historically been referred to as the phencyclidine (PCP) receptor.

The recognition site within the ion channel of the NMDA-receptor complex is the modulatory domain most relevant to the pharmacology of dextrorphan.

A structurally diverse group of compounds appear to bind within the integral cation-specific ion channel and exert a noncompetitive antagonism of the NMDA receptor. These compounds include (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), dissociative anaesthetics (PCP and ketamine) and dextrorotatory opioids from the morphinan (dextrorphan and dextromethorphan) and benzomorphan (cyclazocine and N-allylnormetazocine (SKF 10,047)) series. The structures of noncompetitive NMDA antagonists most germane to this research project are given in figure 1.2. The discovery by Lodge and coworkers in 1982 that PCP and ketamine acted as selective noncompetitive antagonists of the NMDA receptor led to a series of studies which established that the PCP receptor and the NMDA receptor coexist as a receptor/ionophore complex (Lodge and Anis, 1982).



**FIGURE 1.2.****Chemical structures of noncompetitive NMDA antagonists****dextrorphan****dextromethorphan****(+)MK-801****ketamine****phencyclidine****1-[1(2-Thienyl)cyclohexyl]piperidine**

The numerous reports of recreational dextromethorphan abuse (Degkwitz, 1964; McCarthy, 1971) also provide an early indication of a common pharmacologic basis for the effects of the dextrorotatory morphinans and PCP-like drugs. Furthermore, drug discrimination studies in a variety of species have suggested a common basis for the effects of dextrorotatory opioids and PCP-like compounds (Holtzman, 1980, 1994; Herling et al., 1981, 1983). This relationship was established electrophysiologically when dextrorphan, like PCP and ketamine (Anis et al., 1983), was reported to be a selective and potent antagonist of N-methylaspartate-induced excitation of spinal neurones (Church et al., 1985). Dextromethorphan also selectively antagonized N-methylaspartate-induced excitation but was 5-fold less potent than dextrorphan (Church et al., 1985). Many subsequent studies have indicated that although dextromethorphan is undoubtedly an effective noncompetitive antagonist of the NMDA receptor, it is consistently less potent than dextrorphan in many experimental models of NMDA receptor antagonism. Such reports include the ability of the dextrorotatory morphinans to attenuate hypoxic injury in neuronal cultures (Goldberg et al., 1987), antagonize epileptiform activity (Wong et al., 1988; Aram et al., 1989) and protect against seizures in vivo (Leander et al., 1988; Chapman and Meldrum, 1989). These findings are supported by radioligand binding data in that dextrorphan is 4-fold more potent than dextromethorphan as an inhibitor of [<sup>3</sup>H]PCP binding (Murray and Leid, 1984) and 8-fold more potent than dextromethorphan as an inhibitor of [<sup>3</sup>H]dextrorphan binding (Franklin and Murray, 1992) to rat brain membranes. Dextromethorphan, in addition, is thought to be prototypical of a unique class of anticonvulsants acting through a site labelled by [<sup>3</sup>H]dextromethorphan (Tortella et al., 1988; 1994). The high affinity of dextromethorphan for both [<sup>3</sup>H]dextromethorphan binding sites (Craviso and Musacchio, 1983b) and at sigma recognition sites (Walker et al., 1990) represent features that are unique to dextromethorphan, these pharmacologic characteristics are not shared by dextrorphan and are thought to be unrelated to the activity of dextromethorphan as an NMDA receptor antagonist.

MK-801 has been used as a prototypical ligand to study noncompetitive antagonist interactions with the channel binding domain of NMDA receptors (Wong et al., 1986). However, the results of both radioligand binding studies (Franklin and Murray, 1990; 1992) and electrophysiologic experiments (Cole et al., 1989) suggest that the mechanism of dextrorphan interaction with the NMDA receptor is distinctive and unlike that of MK-801. It is conceivable, therefore, that differences in noncompetitive NMDA antagonism may depend on both the mechanism of the interaction, and the specific domain of the NMDA receptor-channel complex with which a channel blocker interacts. The concept of multiple noncompetitive NMDA antagonist sites with affinity for this general class of drug, rather than the existence of a single binding site for all members of this drug class, has been suggested previously to explain the interaction of MK-801 and the noncompetitive NMDA antagonist desipramine on NMDA receptors (Serganor et al., 1989).

### Molecular Biology

The NMDA receptor-channel complex appears to exist as a hetero-oligomeric complex of subunits which co-assemble to form a functional channel. Two families of NMDA receptor subunits have recently been cloned and termed NMDAR1 (NR1) and NMDAR2 (NR2) in rat brain (Moriyoshi et al., 1991; Monyer et al., 1992; Ishii et al., 1993) (Figure 1.2), and designated  $\zeta$  and  $\epsilon$  in mouse brain (Yamazaki et al., 1992; Ikeda et al., 1992; Meguro et al., 1992; Kutsuwada et al., 1992). To date, eight different isoforms generated by alternative splicing of mRNA have been identified for the NR1 subunit (Sugihara et al., 1992; Nakanishi. et al., 1992; Hollman et al., 1993), whereas the NR2 family currently consists of four closely related subunits termed NR2A-NR2D (Monyer et al., 1992; Ishii et al., 1993). Homomeric expression of the NR1 subunit results in a functional receptor that exhibits the known characteristics of the native NMDA receptor-channel complex (Moriyoshi et al., 1991). This is in contrast to the NR2 subunits which show no conductance properties in response to glutamate and are thought not to form

functional channels in the homomeric state. Co-expression of NR1 with an NR2 subunit, however, greatly potentiates responses to glutamate or NMDA and produces functional variability depending on the subunit combination (Meguro et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993).

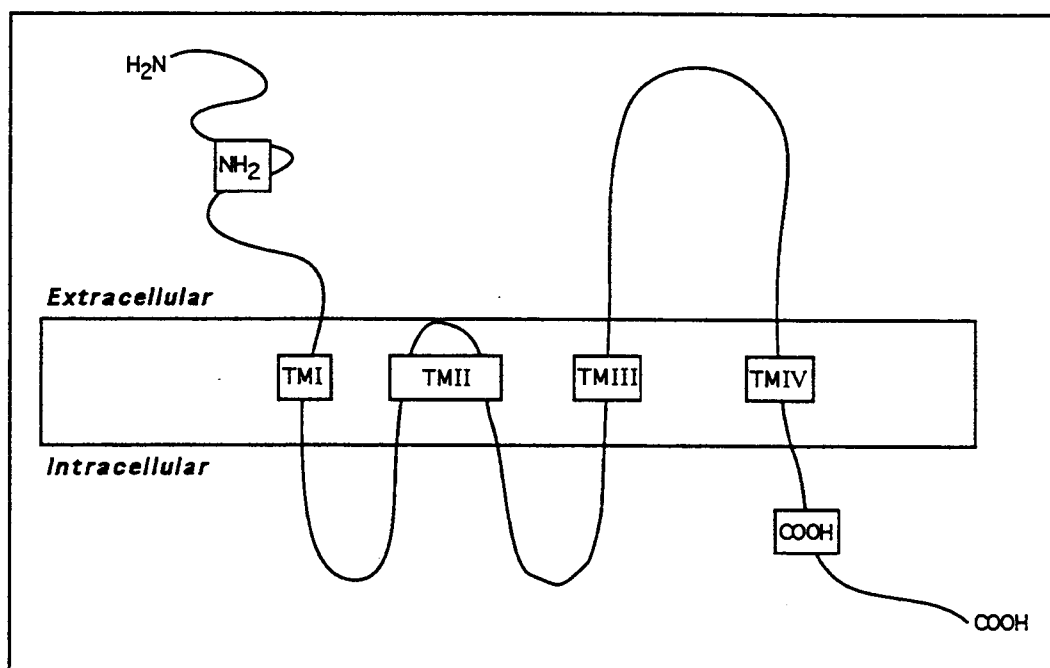
Localization of mRNA for NR1 by in situ hybridization techniques reveals an abundant and ubiquitous expression throughout the rat brain (Moriyoshi et al., 1991.), that is in marked contrast to the distinctive and restricted distribution of mRNA for NR2 subunits (reviewed by Nakanishi 1992). The potential for regional and functional NMDA receptor heterogeneity has now been further extended by the recent findings of Laurie and Seeburg (1994a). Using in situ hybridization techniques to detect the location of mRNA for the eight possible forms of the NR1 subunit these authors reported that although some splice forms occur extensively and homogeneously in the adult rat brain, the occurrence of other splice forms is restricted to specific brain regions. Furthermore, splice variants of the NR1 subunit have also been shown to generate functionally distinct NMDA receptors. NR1 homomeric NMDA receptor channels expressed in *Xenopus* oocytes display differential sensitivity to glutamate. This differential sensitivity is dependent on the presence or absence of a 63 nucleotide insertion in the DNA sequence that encodes an additional 21 amino acid residue in the proposed extracellular amino terminal region of the NR1 subunit (Nakanishi et al., 1992; Sugihara et al., 1992; Hollmann et al., 1993). Although the subunit composition of native NMDA receptors is currently unknown, different NR1 splice variant and NR2 subunit combinations could theoretically produce a large array of pharmacologically distinct receptor subtypes.

Although there is uncertainty with regard to the exact topology of NMDA receptor subunits, the following model (Figure 1.3) has been proposed based on the study of non-NMDA receptor subunits (Hollman et al., 1994; Bennet and Dingledine, 1995). All known glutamate receptor subunits are thought to contain four hydrophobic domains (TMI to TMIV) in their structure. Three of these domains (I, II and IV) appear to be true

transmembrane spanning domains, whilst the second (II) forms a reentrant loop with both ends facing the intracellular side of the membrane (Bennet and Dingledine, 1995). Based on models for other ligand-gated ion channels, several NMDA receptor subunits are thought to co-assemble to form a functional cation channel. The second membrane-dwelling domain (TMII) of each subunit is thought to be oriented in such a way that collectively the TMII regions of the subunits form the inside of the channel pore. Consequently, the second membrane-dwelling domain is thought to be important for the binding of noncompetitive antagonists to sites within the NMDA receptor channel.

**FIGURE 1.3.**

**Predicted structure of an NMDA receptor subunit**



Recent attempts to identify the location of a noncompetitive antagonist binding domain within the NMDA receptor channel have utilised recombinant DNA techniques with a particular emphasis on the second hydrophobic domain. All known NMDA receptor subunits have an asparagine residue in their putative channel-forming second membrane-dwelling segment, at a site that is homologous to the glutamine-arginine editing position (Q/R site) of the AMPA-kainate receptors (Nakanishi, 1992). Certain non-NMDA subunits possess a glutamine at the Q/R site, whilst others carry an arginine residue that appears to be a critical determinant of  $\text{Ca}^{2+}$  permeability. This arginine residue is not encoded in the subunit gene but is generated by an RNA editing process. Site-directed mutagenesis has revealed that substitution of this conserved asparagine residue in an NMDA receptor subunit, to glutamine or arginine, reduces the sensitivity of NMDA receptor channels to blockade by MK-801 (Mori et al., 1992; Sakurada et al., 1993). In a study by Kawajiri and Dingledine (1993), mutation of the same asparagine of NMDAR1 to an arginine residue resulted in a 200-fold reduction in the potency of TCP to block heteromeric NMDA receptors and abolished the use-dependent nature of the blockade. Furthermore, in support of the idea that noncompetitive antagonists may have more than one site of action on the NMDA receptor channel, these authors also reported a lower affinity, non use-dependent component of the blockade by TCP that was retained in the mutant receptor.

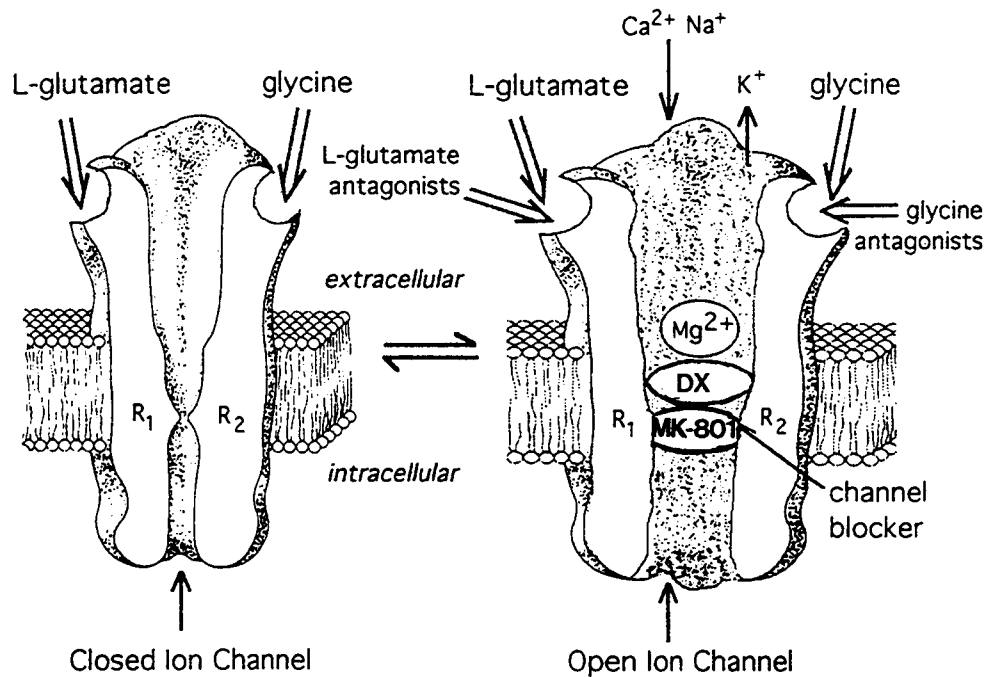
Although the conserved asparagine residue in the second membrane-dwelling region of NMDA receptor subunits appears to influence calcium permeability and  $\text{Mg}^{2+}$  blockade of the channel (Mori et al., 1992; Burashev et al., 1992) as well as noncompetitive antagonist binding, it has recently been proposed that the contribution of this residue may differ for various noncompetitive antagonists (Yamakura et al., 1993). In a comparison of four heteromeric murine NMDA receptors, wild-type channels differed in their sensitivity to blockade by MK-801. In the same study, mutation of the asparagine residue reduced the sensitivity of NMDA receptor channels to blockade by PCP, ketamine and SKF-10,047 to different extents. These findings are not incongruous with the

possibility of nonidentical binding sites with respect to the interaction of noncompetitive NMDA antagonists with the ion channel domain of the NMDA receptor .

### **Noncompetitive NMDA Receptor Antagonism**

Consonant with an interaction with a site within the ion channel domain of the NMDA receptor, the antagonism of NMDA-evoked currents by dissociative anaesthetics (PCP and ketamine) has been found to be highly voltage dependent (Honey et al., 1985). One explanation for the voltage-dependence of NMDA receptor blockade is that the binding site is within the ion channel; the channel blocker must therefore move through the membrane electric field to reach its site. Consequently, the association rate for binding of a blocker to a site within the channel would be voltage dependent. This mechanism was originally described by Woodhull et al. (1977) to explain the voltage dependence of the blockade of sodium channels by protons. More recently, however, the validity of this model to explain dissociative anaesthetic blockade of the NMDA receptor has been questioned, and a guarded receptor model has been proposed that takes into account the use dependent nature of dissociative anaesthetic blockade (MacDonald et al., 1987; 1991). Use dependence of channel blockade results in a requirement for agonist activation of the NMDA receptor-channel complex for antagonism to occur.

It is now generally accepted that noncompetitive antagonists attenuate NMDA receptor function by binding to a domain within the ion channel and that blockade can only occur, therefore, if the receptor is in the activated or open channel state as depicted in figure 1.4. The primary mediators of this activation are glutamate and glycine, endogenous agonists at the NMDA and strychnine-insensitive glycine co-agonist sites respectively. The mechanisms underlying regulation of noncompetitive antagonist binding by these agonists have not, however, been completely elucidated. It is possible that glycine and glutamate increase the rate of association and dissociation of noncompetitive antagonist

**FIGURE 1.4.****Model of the NMDA receptor depicting closed and open ion channel states**

Adapted from Neurotransmissions (Volume X, Number 1, 1994) and reproduced with the written permission of Research Biochemicals International, Natick, MA.

binding to an equal extent and thus have no net effect on equilibrium binding (Kloog et al., 1988a; 1988b; Bonhaus and McNamara, 1988). This model implies that access to a channel binding site, which may be unavailable under closed channel conditions, is the primary determinant of noncompetitive antagonist binding. A second possibility is that channel opening may be coupled to an increase in the affinity of a noncompetitive antagonist for its binding site. A combination of the above is also conceivable whereby a



noncompetitive antagonist has higher affinity for the open channel state of the NMDA receptor-channel complex at a site that is more accessible in the presence of glycine and glutamate (Foster and Wong, 1987; Reynolds et al., 1987; Johnson et al., 1988).

Previous studies indicate that [ $^3\text{H}$ ]dextrorphan labels a high affinity site in rat brain membranes corresponding to the noncompetitive antagonist binding domain of the activated state of the NMDA receptor-channel complex (Franklin and Murray, 1990; 1992). Although [ $^3\text{H}$ ]dextrorphan appears to recognize an "open channel" binding domain approximating the site or sites of other specific noncompetitive NMDA antagonists, the binding domain of [ $^3\text{H}$ ]dextrorphan may not be identical to the channel binding sites of either [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]1-[1-(2-thienyl)cyclohexyl]piperidine ([ $^3\text{H}$ ]TCP). Aspects of the regulation of [ $^3\text{H}$ ]dextrorphan binding by modulators of the NMDA receptor-channel complex distinguish it from [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]TCP and may be indicative of underlying differences in the mechanisms of noncompetitive antagonism among these compounds. Specifically, the regulation of [ $^3\text{H}$ ]dextrorphan binding by  $\text{Mg}^{2+}$  and the polyamine agonist spermidine is insensitive to the presence of glycine and glutamate. Whereas the potencies of  $\text{Mg}^{2+}$  and spermidine as modulators of [ $^3\text{H}$ ]TCP and [ $^3\text{H}$ ]MK-801 binding are significantly increased by the presence of exogenous glycine and glutamate in well washed membranes, their marked potencies as regulators of [ $^3\text{H}$ ]dextrorphan binding are unaffected by the presence or absence of these substances (Franklin and Murray, 1992).

It has recently been proposed that the dissociative anaesthetic binding site may be located in the vestibule of the NMDA channel pore and that the NMDA channel must be gated open before this binding site is exposed (MacDonald et al., 1991). Channel closure subsequent to the binding of a dissociative anaesthetic molecule, such as ketamine or PCP, results in the trapping of the blocker within the closed channel (MacDonald et al., 1991). It is therefore possible that the apparent indifference of [ $^3\text{H}$ ]dextrorphan binding, to the presence of glycine and glutamate, is a consequence of its occupation of a more

superficially located binding site, perhaps in the vestibule of the NMDA receptor ion channel, that is less sensitive to channel opening and closing. As a consequence [ $^3\text{H}$ ]dextrorphan binding may be less dependent on agonist activation of the receptor.

Further evidence for a difference in the nature of noncompetitive antagonism of NMDA-mediated responses by dextrorphan and MK-801 has been provided by electrophysiologic data (Cole et al., 1989). Unlike MK-801, which was found to act in clearly use-dependent manner, dextrorphan showed no evidence of use-dependency in producing a potent, dose-dependent and selective noncompetitive inhibition of NMDA-mediated depolarizations in the rat hippocampal slice (Cole et al., 1989). These findings are not inconsistent with the possibility of dextrorphan gaining access to a site within the ion channel domain that is relatively exterior to that of MK-801 and which may therefore be less influenced by the state of the channel. Consistent with this possibility, the onset of dextrorphan blockade 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 indicating a high degree of use-dependence (Cole et al., 1989). In addition to the differential sensitivity of dextrorphan and MK-801 to glycine and glutamate, the more rapid kinetics of channel blockade by dextrorphan may also contribute to its lack of use-dependency. The particularly slow time course and long duration of blockade of the NMDA receptor channel by MK-801 is thought to be related to its high degree of use-dependency (Wong et al., 1986).

## **Pharmacologic Consequences of NMDA Receptor Antagonism**

### Anticonvulsant Effects

Noncompetitive antagonists of the NMDA receptor possess anticonvulsant properties in a wide range of experimental seizure models (Aram et al., 1989; Chapman and Meldrum, 1989). Dextrorphan and dextromethorphan also possess significant antiepileptic

activity in vitro (Wong et al., 1988; Cole et al., 1989; Aram et al., 1989) and anticonvulsant efficacy in vivo (Ferkany et al., 1988; Leander et al., 1988; Tortella et al., 1988; Chapman and Meldrum, 1989). The observation by Tortella et al. (1988) is of particular interest, as dextrorphan was 2.5 times more potent than the prototypical anticonvulsant compound phenytoin, in suppressing maximal electroshock-induced convulsions in rats. Similarly, dextrorphan and other noncompetitive antagonists protect against sound-induced seizures in DBA/2 mice (Chapman and Meldrum, 1989). Moreover, for MK-801, PCP, dextrorphan, SKF 10,047 and ketamine an excellent correlation exists between their relative anticonvulsant potencies and their respective affinities as inhibitors of [ $^3\text{H}$ ]MK-801 binding to brain membranes (Chapman and Meldrum, 1989).

#### Neuroprotective Effects

The neurotoxic properties of glutamate have been proposed to contribute to the pathogenesis of human neuronal cell loss associated with several neurologic disease states (Rothman and Olney, 1987; Choi et al., 1988). The high calcium permeability of the NMDA subtype of glutamate receptors provides support for the hypothesis that glutamate neurotoxicity is largely mediated by an influx of calcium into the cell (Choi et al., 1988). It is not surprising, therefore, that selective NMDA receptor antagonists have been shown to have neuroprotective effects in both neuronal culture and animal models (Choi et al., 1988; Goldberg et al., 1988). Presumably by virtue of their NMDA antagonist activity, dextrorphan and dextromethorphan are also effective neuroprotectant agents and reduce the magnitude and extent of neuronal damage following ischaemic or traumatic brain insult in vivo (Steinberg et al., 1988, 1989; Faden et al., 1989), or that resulting from hypoxic, glutamate or hypoglycaemic challenge in vitro (Goldberg et al., 1987; Choi et al., 1987; Monyer and Choi, 1988).

Given the established safety record in humans of dextromethorphan at antitussive doses, the dextrorotatory opioids are considered attractive candidates for clinical investigation. It has been proposed that dextrorphan may of particular benefit for the treatment of patients with an acute stroke, a major neurological deficit, or those undergoing surgery carrying high risks of cerebral ischaemia (Steinberg et al., 1991).

### Psychoactive Effects

The dextrorotatory morphinans have virtually no affinity for opioid receptors and thus lack the potent analgesic properties, respiratory depressant effects and morphine-like addictive potential associated with related levorotatory opioid isomers (Isbell and Fraser, 1953). Although devoid of morphine-like effects and consequently considered non-addictive (Isbell and Fraser, 1953) the dextrorotatory morphinans, at levels exceeding the recommended antitussive dose, can induce a PCP-like syndrome presumably by virtue of their activity as noncompetitive antagonists of the NMDA (N-methyl-D-aspartate) subfamily of ionotropic glutamate receptors.

PCP is an intravenous anaesthetic agent that exhibits marked psychotomimetic activity in humans when administered at subanaesthetic doses (Luby et al., 1959). In 1957, PCP was introduced for clinical trials as a general intravenous anaesthetic (Greifenstein et al., 1958). Although PCP was found to be generally effective as an anaesthetic, its use was associated with a high incidence of postoperative emergence reactions. These emergence reactions included marked agitation, excitement, disorientation and hallucinations (Lear et al., 1959). The incidence of these reactions was considered clinically unacceptable and the subsequent search for a PCP analogue with a reduced propensity to produce emergence phenomena led to the development of ketamine.

Numerous reports of recreational dextromethorphan abuse (Degkwitz, 1964; McCarthy, 1971) provide an early indication of a common pharmacologic basis for the effects of the dextrorotatory morphinans and PCP-like drugs. In 1964 Degkwitz reported

sedative-hypnotic effects, slurred speech, pupillary dilation, sensory distortion, drunkenness and euphoria in patients that had ingested 300 mg (approximately 10 times the antitussive dose) of dextromethorphan. In a later study of the effects of dextromethorphan in humans, Jasinski et al. (1971) confirmed the presence of sedative-hypnotic and psychotomimetic effects following consumption of doses approximately 8 times higher than the recommended antitussive dose, and also noted that the subjective response to dextromethorphan was clearly different to that of morphine. Furthermore, the results of many drug discrimination studies have suggested a common mode of action for the effects of dextrorotatory opioids and PCP-like compounds Holtzman, 1980, 1994; Herling et al., 1981, 1983).

## Objectives

The intent of these studies was to increase our understanding of the interaction of dextrorotatory opioids with the NMDA receptor. Whole animal studies and quantitative receptor autoradiography were utilised in this research project, the specific aims of which are detailed below.

Chapter 2 describes studies to investigate the ability of dextrophan and dextromethorphan to suppress convulsions induced by focal injection of a chemoconvulsant into the rat prepiriform cortex. Intracerebral injection of chemoconvulsants into the prepiriform area initiates generalized motor seizures in the rat, thus providing a useful animal model with which to evaluate the protective effects of noncompetitive NMDA antagonists. In addition, the anticonvulsant effects of PCP, MK-801 and the sigma ( $\sigma$ ) ligand (+)-pentazocine were also studied as reference compounds in this model to allow comparison of the rank order of potency of these compounds with their respective affinities for the [ $^3\text{H}$ ]dextrophan binding site in vitro.

Chapter 3 documents a novel anticonvulsant action of the  $\sigma$  ligand 1,3-di(2-toly)guanidine (DTG). Sigma receptors were originally thought to be a type of opiate

receptor, however, two subsequent findings revealed that they are not: (1) naloxone is an ineffective antagonist of the effects of sigma ligands in vivo and in vitro and (2) whereas opiate receptors are selective for the levorotatory enantiomers of opium-derived compounds, sigma receptors are selective for dextrorotatory enantiomers. Initial studies suggested that sigma receptors were PCP receptors, however, the pharmacologic profile and anatomical distribution of sigma and PCP binding sites clearly demonstrates that they are not the same. Pharmacologic characterization of sigma binding sites remains ambiguous and their physiological relevance has yet to be established. DTG, however, displays a potent and efficacious anticonvulsant effect in the rat prepiriform cortex by a mechanism that does not involve  $\sigma$  receptors. Noncompetitive antagonism of NMDA receptor-mediated excitation at the MK-801 / PCP binding site is also unlikely to account for the protective effects of DTG reported in this chapter. (The site of injection in the prepiriform cortex for the compounds described in chapters 2 and 3 was sampled and examined histologically (Figure 1.5.)).

Chapter 4 describes studies in which the ability of dextrorphan and dextromethorphan to induce stereotyped behaviour in the rat were investigated. A possible correlation of the rank order of potencies of an array of noncompetitive NMDA antagonists to induce stereotyped behaviour in male Sprague-Dawley rats and their respective affinities for the high-affinity [ $^3\text{H}$ ]dextrorphan binding site in rat forebrain membranes was also assessed. To further investigate dextrorotatory opioid-induced stereotypies and to specifically address the propensity of dextromethorphan to induce this behaviour, studies were done using female Sprague-Dawley and female Dark Agouti rats. The female Dark Agouti rat is deficient in the cytochrome P450 enzyme 2D1 which catalyses the O-demethylation of dextromethorphan to dextrorphan in the Sprague-Dawley rat strain.

Chapter 5 describes studies that were undertaken to investigate the anatomical distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat brain using quantitative receptor autoradiography. A procedure for quantitative autoradiographic visualization of

[<sup>3</sup>H]dextrorphan binding sites in slide-mounted sections was defined and the specificity of the autoradiographic assay for the labelling of a site within the ion channel domain of the NMDA receptor confirmed. To gain further insight into the mechanism by which [<sup>3</sup>H]dextrorphan interacts with the NMDA receptor-operated cation channel, the regulation of [<sup>3</sup>H]dextrorphan binding by glycine and glutamate was assessed in several brain areas. In addition, the regional distribution of [<sup>3</sup>H]dextrorphan binding sites in the rat brain was compared to previously reported distributions of NMDA receptors determined by receptor autoradiography

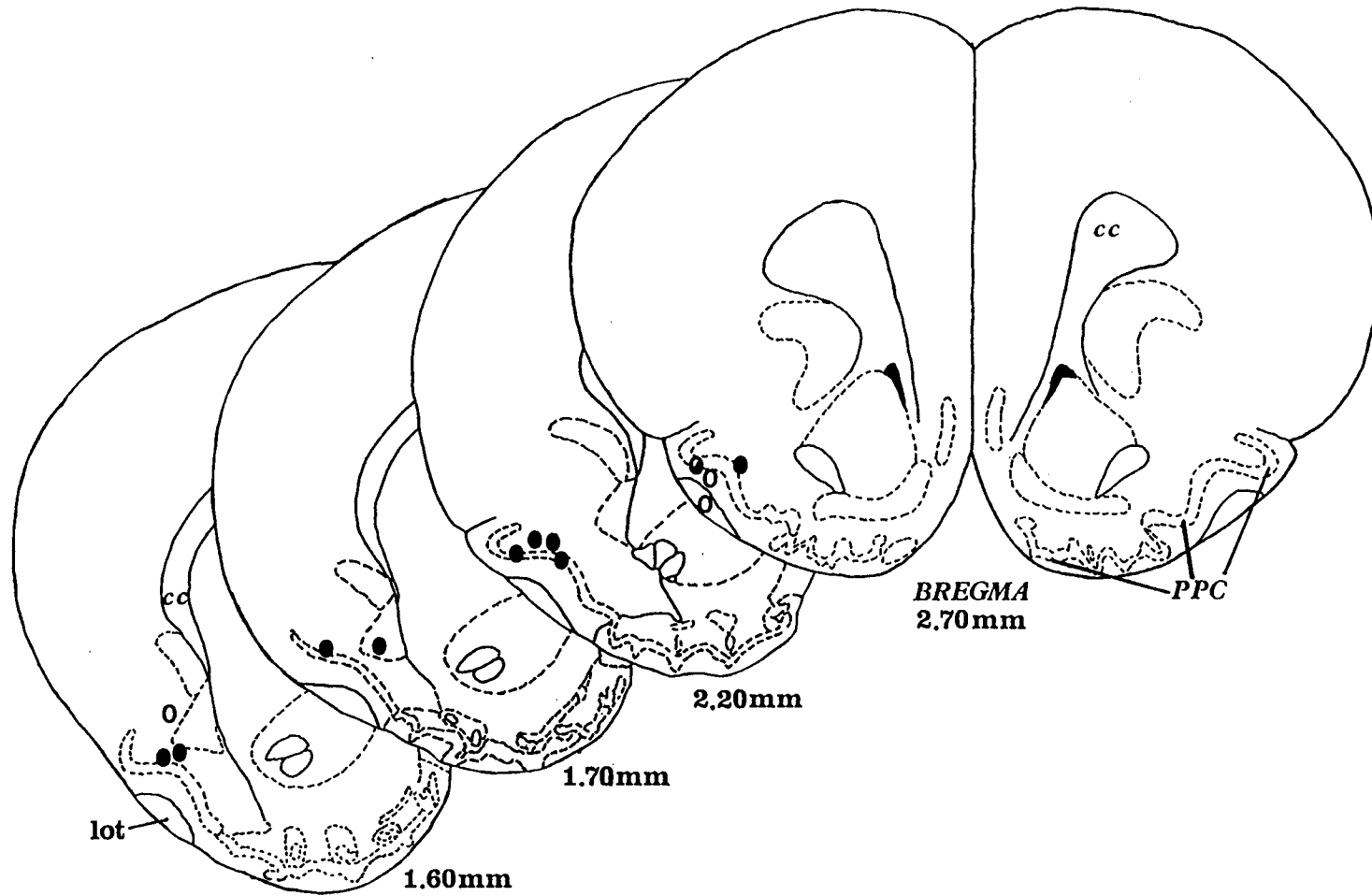
**FIGURE 1.5.**

**Schematic drawings of coronal rat brain sections showing the location of microinjection sites in the prepiriform cortex.**

The location of the cannula tip was determined histologically in a series of 16- $\mu$ m coronal sections stained with cresyl violet. Drawings are in accordance with the stereotaxic rat brain atlas of Paxinos and Watson (1986). Filled circles indicate sites most sensitive for evoking seizures (score  $\geq 4$ ) in response to unilateral focal injection of 118 pmol (-)-bicuculline methiodide (BMI). Half filled circles indicate partial response to BMI (score 1-3). Open circles indicate no response to BMI. The plane of the sections shown for histologic reference is the same as the plane of cannula penetration. Abbreviations: cc, corpus callosum; lot, lateral olfactory tract; PPC, prepiriform cortex.



**FIGURE 1.5.**



## **Chapter 2**

### **Dextrorotatory opioids and phencyclidine exert anticonvulsant action in prepiriform cortex**

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**215:293-296**

### Abstract

We have investigated the ability of an array of putative noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists to suppress convulsions induced by a unilateral, focal injection of (-)-bicuculline methiodide (118 pmol) into the rat prepiriform cortex. The anticonvulsant potency of these compounds, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) > dextrorphan  $\geq$  1-(1-phenylcyclohexyl)piperidine hydrochloride (PCP) > dextromethorphan > (+)-pentazocine, upon microinjection into the prepiriform cortex, was highly correlated ( $r=0.971$ ;  $P < 0.01$ ) with their respective affinities for the [ $^3\text{H}$ ]dextrorphan-labelled NMDA receptors in rat forebrain membranes. These results suggest that noncompetitive antagonism of NMDA receptors underlies the anticonvulsant action of these compounds.

## Introduction

Noncompetitive antagonists of the N-methyl-D-aspartate (NMDA) class of excitatory amino acid receptors, such as 1-(1-phenylcyclohexyl)piperidine hydrochloride (PCP) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801), are known to possess anticonvulsant properties. The dextrorotatory opioids, dextrorphan and its 3-methyl ether derivative dextromethorphan, also exhibit anticonvulsant activity in vivo in several seizure models (Ferkany et al., 1988; Leander et al., 1988; Tortella et al., 1988; Chapman and Meldrum, 1989). The observation by Tortella et al. (1988) is of particular interest, as dextrorphan was 2.5 times more potent than the prototypical anticonvulsant compound phenytoin, in suppressing maximal electroshock-induced convulsions in rats. In spite of the apparent similarity of dextrorphan and dextromethorphan to other noncompetitive NMDA antagonists, the basis for their anticonvulsant effects in vivo has not been clearly established.

We have recently described a specific binding site for [ $^3\text{H}$ ]dextrorphan in rat forebrain membrane preparations (Franklin and Murray, 1990). The pharmacological profile of [ $^3\text{H}$ ]dextrorphan binding and its regulation by modulators of NMDA receptor activity is consistent with the labelling of a site associated with the NMDA receptor cation channel complex.

The aim of this study was to investigate the ability of dextrorphan and dextromethorphan to suppress convulsions induced by a focal injection of (-)-bicuculline methiodide (BMI) into the rat prepiriform cortex. Initially reported by Pirreda and Gale (1985), the prepiriform area appears to play an important role in initiation of generalized motor seizures in the rat. In addition, the anticonvulsant effects of PCP, MK-801 and the  $\sigma$  ligand (+)-pentazocine were also studied as reference compounds in this model to allow comparison of the rank order of potency of these compounds with their respective affinities for the [ $^3\text{H}$ ]dextrorphan binding site in vitro.

## **Materials and Methods**

### **Stereotaxic surgery**

Male Sprague-Dawley rats (330–400 g; Simonsen Laboratories, Gilroy, CA) were maintained at 22°C on a standard 12 h light-dark schedule, with food and water available *ad libitum*. Animals, under Equithesin anesthesia (2.7 ml/kg *i.p.*), were placed in a Kopf small animal stereotaxic instrument and implanted with paired, 22 and 28 gauge guide and injection cannulas, respectively. With the incisor bar at -3.2 to -3.5 mm to achieve a 'flat brain' orientation in accordance with the stereotaxic atlas of Paxinos and Watson, the cannulas were aimed at a site in the anterior prepiriform cortex 2.0 mm anterior and 3.3 mm lateral to bregma, at a depth of 6.5 mm below dura as described in our previous studies (Franklin et al., 1989). Animals were housed individually and allowed at least 24 h recovery before experimentation.

### **Microinjection protocol**

Intracerebral microinjection procedure has been described previously (Franklin et al., 1989). All test compounds were dissolved in deionized water with the exception of (+)-pentazocine (100 mM phosphoric acid, final pH 5.0) and injected into the prepiriform cortex in a volume of 1  $\mu$ l at a rate of 4 nl/s. Limited aqueous solubility of dextromethorphan and (+)-pentazocine in their injection vehicles prevented microinjection of doses higher than 85 and 105 nmol, respectively. All experiments were done during the 12 h light cycle.

### **Evaluation of anticonvulsant efficacy**

Cannula placement in the prepiriform cortex was verified by the behavioural response of each animal to a 118 pmol dose of BMI on day 1. Animals were observed 30 min and stereotypical seizure behaviour quantified by assignment of a maximum seizure score ranging from 0 to 5 on a scale of increasing seizure severity. This scale is derived

from that of Racine and described in detail elsewhere (Franklin et al., 1989). Only animals showing seizure scores of 4 or 5 on the first day, were included in the study.

Anticonvulsant activity of test compounds was evaluated on the following day by microinjection 15 min prior to the chemoconvulsant. Absence or reduction of BMI-induced seizure behaviour after drug treatment was only confirmed as an anticonvulsant response, if the same dose of BMI elicited a positive response in post-test on the third day. Cannula placement was routinely confirmed by histological examination in this population of animals at the end of their participation in experiments and also in animals failing to show an initial sensitivity to BMI on day 1.

Protective effects were determined as the percentage reduction in mean seizure score from control for groups of animals at each dose level. Dose-response curves were fit to data sets by means of an iterative nonlinear least-squares regression analysis to a rectangular hyperbola defined by the following equation:

$$E = \frac{E_{\max} * D}{ED_{50} + D}$$

where E is the % reduction in seizure severity and D represents the dose ( $\mu$ g).

## Results

Unilateral focal injection of each test compound produced no change in behaviour during the 15 min pretreatment epoch. As shown in figure 2.1, all compounds protected against BMI-induced convulsions in the prepiriform cortex in a dose dependent manner. MK-801 was the most potent anticonvulsant tested, with an ED<sub>50</sub> value ( $\pm$  S.E.) of  $4.07 \pm 0.8$  nmol. Dextrorphan and PCP also potently suppressed convulsions with complete efficacy; ED<sub>50</sub> values were  $17.08 \pm 7.9$  and  $20.30 \pm 6.9$  nmol respectively. Dextromethorphan was approximately 4-fold less potent than dextrorphan, with an ED<sub>50</sub>

**FIGURE 2.1.**

**Protective effects of MK-801, dextrorphan (DX), phencyclidine (PCP), dextromethorphan (DXM) and (+)-pentazocine ((+)PEN) against seizures induced by (-)-bicuculline methiodide (BMI) in the rat prepiriform cortex.**

All compounds were injected unilaterally, at the doses indicated, 15 min prior to a challenging dose of BMI (118 pmol). Animals were observed for 30 min and the highest seizure score determined for each animal. For a representative compound such as dextrorphan the coefficient of variation for mean seizure scores ranged from 11.8 % at a dose of 0.3 nmol to 0 % at the 25 nmol dose. Values represent the reduction in seizure score from the mean control response to BMI at each dose group (n = 3-10).

**FIGURE 2.2.**

**Correlation between the potencies of noncompetitive NMDA antagonists as anticonvulsants and their respective affinities for the [<sup>3</sup>H]dextrorphan binding site in rat forebrain membranes.**

The potencies (nmol) of MK-801, dextrorphan (DX), phencyclidine (PCP), dextromethorphan (DXM) and (+)-pentazocine ((+)PEN) to protect against BMI-induced seizures were highly correlated ( $r = 0.971$ ;  $P < 0.01$ ) with their respective affinities for the [<sup>3</sup>H]dextrorphan binding site in rat forebrain membranes. IC<sub>50</sub> values (nM) are taken from Franklin and Murray (1992), except (+)PEN (unpublished observation).

FIGURE 2.1.

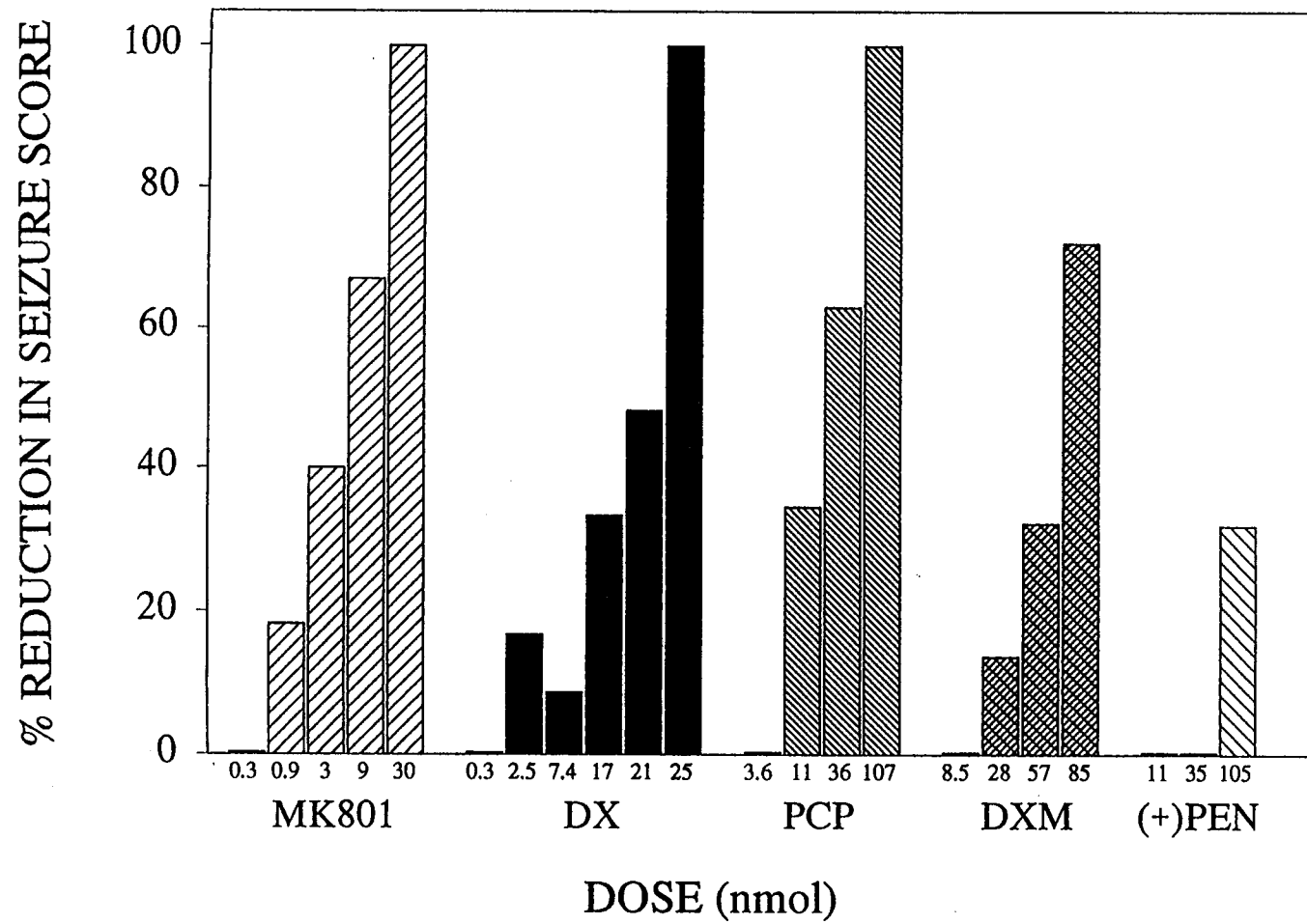
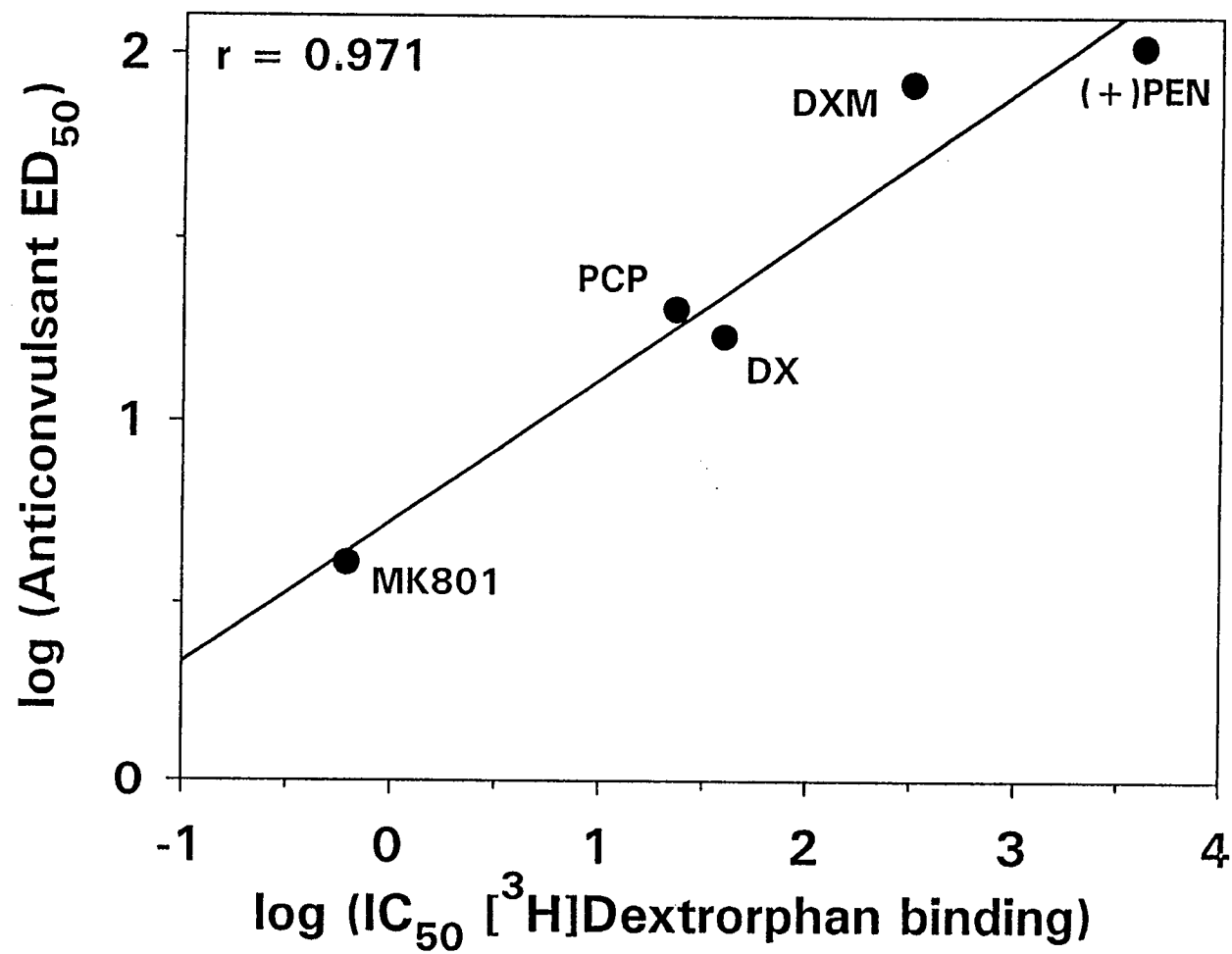




FIGURE 2.2.



value of  $81.57 \pm 32.0$  nmol. The ED<sub>50</sub> value of (+)-pentazocine could not be determined due to the marginal efficacy of this compound; a dose of 105 nmol afforded only 31% of maximum protection.

The correlation between the potency of compounds to protect against convulsions induced by BMI in the prepiriform cortex and their ability to inhibit [<sup>3</sup>H]dextrorphan binding in rat forebrain membranes is shown in figure 2.2. Linear regression of the log transformed data gave a correlation coefficient of 0.971 ( $P < 0.01$ ). These data suggest that cortical NMDA receptors mediate the observed anticonvulsant actions of the compounds tested.

## Discussion

Although the anticonvulsant activity of dextrorphan and dextromethorphan is comparable to that of other noncompetitive NMDA antagonists, the underlying mechanism of action has remained less certain. The rapid metabolism of dextromethorphan to dextrorphan following peripheral administration, has been a complicating factor in the interpretation of some studies in vivo. In addition, binding sites for [<sup>3</sup>H]dextromethorphan have been identified and characterized in guinea pig brain, but appear to be pharmacologically unrelated to the NMDA antagonist activity of dextromethorphan (Craviso and Musacchio, 1983b).  $\sigma$  Ligands such as (+)-3-PPP ((+)-3-(hydroxyphenyl)-N-propylpiperidine hydrochloride), (+)-SKF-10047 ((+)-N-allyl-N-normetazocine hydrochloride) and haloperidol have been shown to inhibit [<sup>3</sup>H]dextromethorphan binding; however, dextrorphan interacts at this site with a very modest affinity. Although [<sup>3</sup>H]dextromethorphan binding is positively modulated by phenytoin, and phenytoin appears to increase the anticonvulsant potency of dextrorphan against maximal

electroshock-induced seizures, the role of this site in the anticonvulsant actions of dextrorphan and dextromethorphan remains to be established.

From our findings it is apparent that the anticonvulsant efficacy of dextrorphan and dextromethorphan, in the prepiriform cortex, is comparable to that of PCP and MK-801. Furthermore, the anticonvulsant potencies of the compounds tested were highly correlated with their respective affinities for the [ $^3\text{H}$ ]dextrorphan-labelled NMDA receptors in rat forebrain membrane preparations (Franklin and Murray, 1992). A 4-fold difference in the anticonvulsant potency of dextrorphan and dextromethorphan, reported herein, compares favourably to an 8-fold greater potency of dextrorphan over dextromethorphan to inhibit [ $^3\text{H}$ ]dextrorphan binding. This correlation is consonant with an NMDA receptor-mediated anticonvulsant action of these compounds. Moreover, this observation is consistent with a proposed NMDA receptor-modulated output pathway from the prepiriform cortex, thought to be essential for the generation of seizures from this brain area.

These data are in agreement with other studies that have shown dextromethorphan to be less potent than dextrorphan in experimental models of NMDA antagonism *in vivo*. The anticonvulsant doses in the prepiriform cortex, reported herein, were comparable to *i.c.v.* doses required to protect against sound-induced seizures in DBA/2 mice (Chapman and Meldrum, 1989). Moreover, dextromethorphan was also less potent than dextrorphan in protecting against maximal electroshock-induced seizures in mice (Leander et al., 1988). The relatively weak action of (+)-pentazocine lends further support for an association between anticonvulsant activity and noncompetitive antagonism of NMDA receptors. Although reported to have weak NMDA antagonist activity, the (+) isomer of pentazocine has high affinity for the  $\sigma$  receptor (Church and Lodge, 1990).

In summary, by direct intracranial administration we have studied the ability of a series of putative NMDA antagonists, in the absence of dispositional influences, to suppress BMI-induced convulsions following focal injection into the prepiriform cortex. The correlation of this pharmacological profile with that defined by [ $^3\text{H}$ ]dextrorphan

equilibrium competition binding studies suggests that interaction with the NMDA receptor cation channel complex mediates the anticonvulsant actions of the dextrorotatory opioids, PCP and MK-801 in the prepiriform cortex. Further characterization of the anticonvulsant effects of noncompetitive NMDA antagonists in this brain region will provide a better understanding of both their underlying mechanisms of action and promise as therapeutic agents in the treatment of epilepsy.

### **Acknowledgement**

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## **Chapter 3**

**The  $\sigma$  receptor ligand 1,3-di(2-tolyl)guanidine is anticonvulsant  
in the rat prepiriform cortex**

**Jane E. Roth, Paul H. Franklin and Thomas F. Murray**

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### **Abstract**

Unilateral focal injection of 1,3-di(2-tolyl)guanidine (DTG) caused a dose-dependent and potent ( $ED_{50} = 5.25$  nmol, 95 % confidence limits 1.1 to 25.0 nmol) suppression of generalized motor seizures induced by (-)-bicuculline methiodide in the rat prepiriform cortex. These findings indicate that DTG is equipotent to the noncompetitive NMDA receptor antagonist MK-801 ((+)-5-methyl-10, 11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate) as an anticonvulsant. This potent pharmacological effect DTG distinguishes it from two other prototypic  $\sigma$  ligands, haloperidol and (+)-pentazocine, which are ineffective as anticonvulsants. Pretreatment of animal with haloperidol failed to block the anticonvulsant effects of DTG. These data therefore document a novel anticonvulsant action of DTG in vivo that does not involve  $\sigma$  receptors.

## Introduction

1,3-Di(2-toly)guanidine (DTG) was synthesized by Weber and coworkers (1986) and has been considered a prototypic ligand for  $\sigma$  binding sites. Although regarded as distinct from the phencyclidine (PCP) binding site of the N-methyl-D-aspartate (NMDA) receptor cation channel complex, pharmacological characterization of  $\sigma$  binding sites remains ambiguous and their physiological relevance has yet to be established. In particular, progress has been hindered both by the absence of a consistent agonist / antagonist profile and the heterogeneity of binding characteristics amongst currently available  $\sigma$  ligands.

Sigma ( $\sigma$ ) sites have recently been subclassified, largely on the basis of biochemical and radioligand binding data, further underscoring the importance of DTG as a pharmacological tool. Under the proposed classification scheme DTG and haloperidol do not discriminate between  $\sigma_1$  and  $\sigma_2$  subtypes and therefore, in the absence of selective  $\sigma_2$  ligands, DTG has become especially valuable in the study of the putative  $\sigma_2$  site.

Additionally, there have been several reports documenting effects of DTG that may not be attributed to its  $\sigma$  binding properties, and have thus raised the possibility that DTG may have pharmacological activity independent of its action at  $\sigma$  sites (Holtzman, 1989; Walker et al., 1990; Connick et al., 1992). We now report that DTG potently protects against convulsions induced by (-)-bicuculline methiodide in the rat prepiriform cortex in a manner that its neither mimicked nor blocked by haloperidol.

## Materials and Methods

Male Sprague-Dawley rats (340-390 g; Simonsen Laboratories, Gilroy, CA) were anaesthetised and stereotaxically implanted with paired guide and injection cannulas, aimed at a site in the anterior prepiriform cortex as described previously (Roth et al., 1992). Cannula placement was verified 24-48 h later by the behavioural response of each animal to

a single microinjection of 118 pmol of (-)-bicuculline methiodide, dissolved in normal saline and administered at a rate of 0.9 nl/s in a volume of 120 nl. Following injection rats were observed for 30 min and stereotypical seizure behaviour quantified by a seizure scoring system derived from that of Racine (1972). A maximum seizure score ranging from 0 to 5 was assigned to each animal as follows: (0) no seizure; (1) myoclonic jerks of the forelimb contralateral to the injection; (2) mild forelimb clonus ( $\pm$  mouth and facial movements-clonus of jaw and head nodding) lasting at least 5 s; (3) severe forelimb clonus lasting more than 15 s; (4) rearing concomitant to or succeeding an instance of forelimb clonus; (5) loss of balance or falling concomitant to or succeeding an incident of rearing. Only rats showing seizure scores 4 or 5 on the first (control) day were used for subsequent studies.

The following day DTG was focally injected 15 min before (-)-bicuculline methiodide. If animals showed a reduced seizure score, an anticonvulsant response of DTG pretreatment was confirmed by post-test with (-)-bicuculline methiodide on the third day. Any animal showing a loss of sensitivity to (-)-bicuculline methiodide (seizure score less than 4 or 5) was excluded from the study. Protective effects were calculated as the percentage reduction in seizure score from the mean control response to (-)-bicuculline methiodide at each dose group. The ED<sub>50</sub> value was calculated using the method of Litchfield and Wilcoxon (1949), a minimum of four animals was used for each dose group. Dose-response data were further analysed by iterative nonlinear least-squares regression analysis to generate a sigmoid curve defined by the logistic equation:

$$E = \frac{E_{\max}}{1 + (ED_{50} / [D])}$$

where E represents the % reduction in seizure severity and D is the dose (nmol).

To investigate the effects of haloperidol on the protective action of DTG against (-)-bicuculline methiodide-induced seizures, animals from two separate groups received a focal injection of haloperidol (10.6 nmol) either alone or followed 15 min later by a single dose



of DTG (12.5 nmol), 30 min prior to (-)-bicuculline methiodide. In all studies DTG and haloperidol were dissolved in 60 mM phosphoric acid and the pH adjusted to 5.0 with sodium hydroxide. Both compounds were injected at a rate of 4 nl/s in a total volume of 1  $\mu$ l.

## Results

DTG protected against convulsions induced by (-)-bicuculline methiodide in the rat prepiriform cortex in a highly potent manner; the ED<sub>50</sub> value, with 95% confidence limits, was 5.25 (1.1 to 25.0) nmol. As shown in figure 3.1, DTG afforded a dose-dependent reduction in seizure behaviour that reached 81% efficacy at the highest dose tested. The limited aqueous solubility precluded testing of higher doses of DTG. Unilateral focal injection of DTG into the prepiriform cortex produced no change in behaviour during the 15 min pretreatment epoch, nor did it induce any signs of motor dysfunction during the course of the study. A separate group of animals pretreated with vehicle showed no reduction in seizure score (Table 3.1).

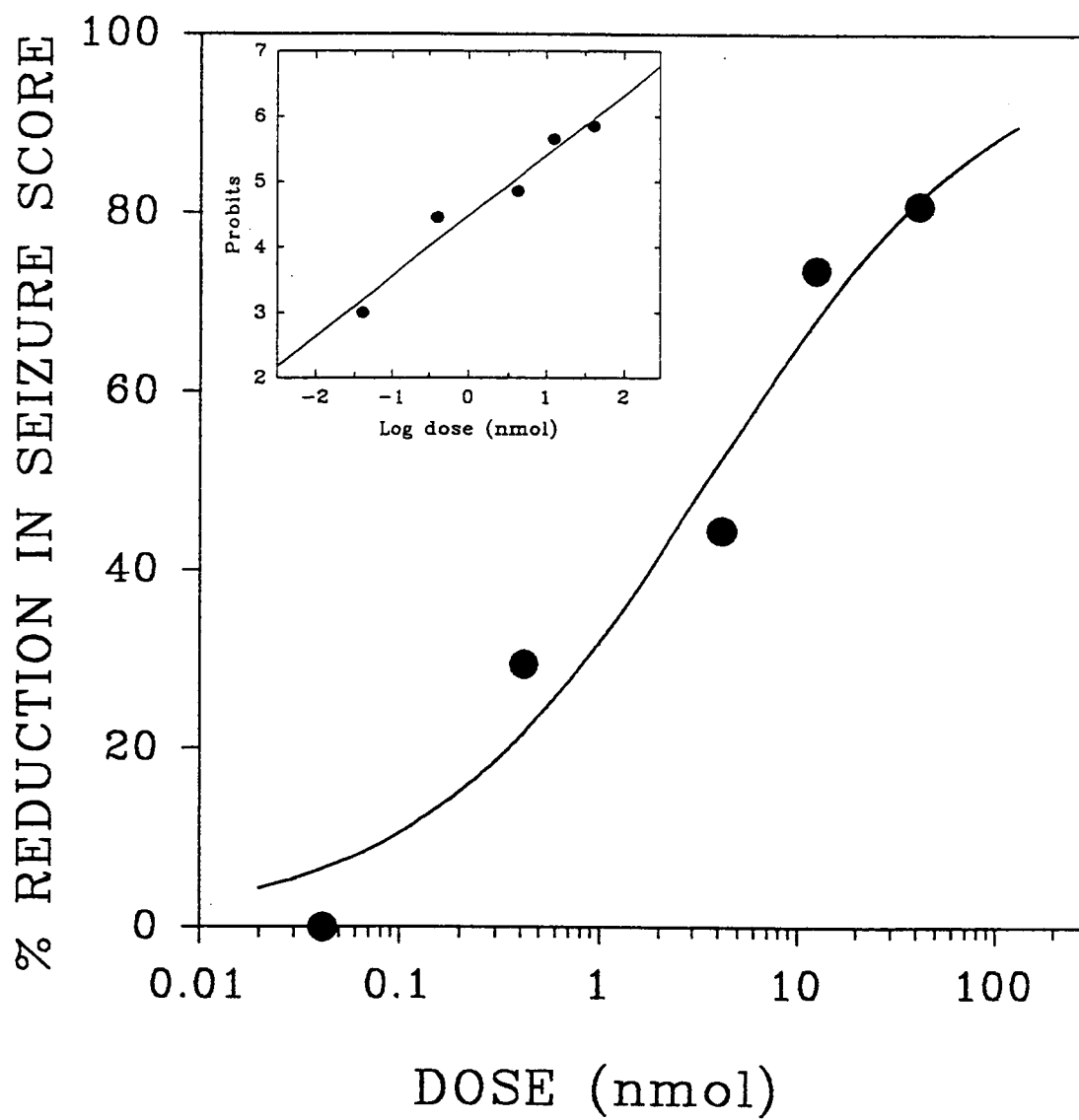
In contrast to the effects of DTG, unilateral focal injection haloperidol (10.6 nmol) into the prepiriform cortex failed to protect against (-)-bicuculline methiodide-induced seizures (Table 3.1). When haloperidol was administered 15 min prior to a known anticonvulsant dose of DTG, the effects of DTG were not changed. As shown in table 3.1, 12.5 nmol of DTG still afforded 79% protection against (-)-bicuculline methiodide-induced seizures in animals pretreated with haloperidol. Hence haloperidol neither mimics nor antagonizes the anticonvulsant action of DTG.

**FIGURE 3.1.**

**Anticonvulsant effect of DTG against seizures induced by (-)-bicuculline methiodide in the rat prepiriform cortex.**

On day 1 animals received a single microinjection of (-)-bicuculline methiodide (118 pmol) and were observed for 30 min. Stereotypical seizure behaviour was quantified by assignment of a score ranging from 0 to 5 on a scale of increasing seizure severity; only rats showing scores of 4 or 5 were used for subsequent studies. The following day DTG was focally injected 15 min prior to (-)-bicuculline methiodide (118 pmol) and the highest seizure score determined for each animal during a 30 min epoch. Ordinate values represent the reduction in seizure score from the mean control response to (-)-bicuculline methiodide at each dose group (n = 4-6). The symbols describe the actual data points and the curve represents the dose-response relationship described by the parameter estimates obtained by fitting a logistic equation to the data by iterative nonlinear least-squares regression analysis. Inset shows the regression line of probit values versus log dose (nmol).

FIGURE 3.1.



**TABLE 3.1.**

**Failure of haloperidol to protect against (-)-bicuculline methiodide-induced seizures or antagonize the anticonvulsant action of DTG in the rat prepiriform cortex**

| Treatment                      | <u>Distribution of Seizure Scores</u> |   |   |   |   |   | Mean Seizure Score (n) | % Protection |
|--------------------------------|---------------------------------------|---|---|---|---|---|------------------------|--------------|
|                                | 0                                     | 1 | 2 | 3 | 4 | 5 |                        |              |
| Control                        |                                       |   |   |   | 4 | 2 | 4.33                   |              |
| Vehicle                        |                                       |   |   |   |   | 6 | 5.00 (6)               | 0            |
| Control                        |                                       |   |   |   | 1 | 3 | 4.75                   |              |
| DTG                            | 3                                     |   |   |   |   | 1 | 1.25 (4)               | 74           |
| Post-test                      |                                       |   |   |   | 2 | 1 | 4.33                   |              |
| Control                        |                                       |   |   |   | 1 | 4 | 4.80                   |              |
| Haloperidol                    |                                       |   |   |   | 1 | 4 | 4.80 (5)               | 0            |
| Control                        |                                       |   |   |   | 1 | 4 | 4.80                   |              |
| Haloperidol + DTG <sup>a</sup> | 4                                     |   |   |   |   | 1 | 1.00 (5)               | 79           |
| Post-test                      |                                       |   |   |   | 2 | 2 | 4.50                   |              |

All compounds were microinjected unilaterally into the prepiriform cortex; <sup>a</sup> haloperidol (10.6 nmol) was administered 15 min prior to DTG (12.5 nmol), which was given 15 min before (-)-bicuculline methiodide. Animals were observed for a 30 min epoch following (-)-bicuculline methiodide and the highest seizure score attained for each animal. The distribution of seizure scores, for animals within each treatment group of size (n), are tallied under numerical columns corresponding to each level of seizure severity as defined in the text. Responsiveness to (-)-bicuculline methiodide (118 pmol) was confirmed in all animals showing a reduced seizure score by post-test the following day. Percent protection reflects the reduction in mean seizure score from the control response to 118 pmol (-)-bicuculline methiodide.

## Discussion

We have recently shown that antagonism of NMDA receptors underlies the ability of an array of dextrorotatory opioids and PCP to protect against (-)-bicuculline methiodide-induced convulsions in the rat prepiriform cortex (Roth et al., 1992). In that study the selective  $\sigma_1$  ligand (+)-pentazocine had limited anticonvulsant efficacy, thereby arguing against a role for  $\sigma_1$  sites in the mediation of this response; a dose of 105 nmol (+)-pentazocine afforded only 31% of maximal protection. In contrast, the results of the present study indicate that DTG is both an efficacious and potent anticonvulsant against (-)-bicuculline methiodide-induced seizures. By comparison, DTG ( $ED_{50}$  = 5.25 nmol) was equipotent to MK-801, the prototypical noncompetitive NMDA antagonist, and greater than 20-fold more potent than (+)-pentazocine; whereas DTG and (+)-pentazocine are essentially equipotent at the [ $^3H$ ]DTG-labelled  $\sigma$  binding site (Weber et al., 1986).

The clear disparity between the respective affinities of DTG and (+)-pentazocine for  $\sigma$  sites *in vitro* and their anticonvulsant activities *in vivo* led us to investigate the ability of haloperidol to modulate (-)-bicuculline methiodide-induced seizures in the prepiriform cortex. Haloperidol is a reference compound with a high affinity for  $\sigma$  sites labelled by a variety of ligands such as [ $^3H$ ](+)-SKF 10,047 ([ $^3H$ ](+)-N-allylnormetazocine), [ $^3H$ ](+)-pentazocine, [ $^3H$ ]3-PPP ([ $^3H$ ]3-(3-hydroxyphenyl)-N-(1-propyl)piperidine) and [ $^3H$ ] DTG (Walker et al., 1990). Consequently, a high affinity for haloperidol has become one of the hallmarks of  $\sigma$  site characterization. In addition, haloperidol, like DTG, is thought to act with high affinity at the  $\sigma_1$  and  $\sigma_2$  binding sites. The results of our present study indicate, however, that haloperidol does not exhibit the marked seizure suppressant effects shown by DTG. Focal administration of haloperidol in a dose (10.6 nmol) 2-fold greater than the  $ED_{50}$  dose of DTG, failed to produce any anticonvulsant response against (-)-bicuculline methiodide-induced seizures. It therefore seems unlikely that the anticonvulsant action of DTG is mediated by either  $\sigma_1$  or  $\sigma_2$  sites inasmuch as haloperidol has 40-fold greater

affinity than DTG for the [ $^3\text{H}$ ]DTG labelled  $\sigma_1$  binding site and is equipotent to DTG at the [ $^3\text{H}$ ]DTG labelled  $\sigma_2$  binding site (Rothman et al., 1991).

We also pretreated animals with haloperidol in an attempt to block the anticonvulsant effects of DTG, but were unable to demonstrate  $\sigma$  antagonist properties of haloperidol similar to those recently described by Monnet et al. (1992). These authors distinguished two classes of  $\sigma$  ligands based on their ability to modulate NMDA-evoked excitation of rat CA<sub>3</sub> hippocampal neurones:  $\sigma$  agonists produced a selective potentiation of the NMDA response;  $\sigma$  antagonists, which included haloperidol, had no effect alone but suppressed the  $\sigma$  agonist-induced potentiation of the NMDA response. Although identification of selective agonists and antagonists will eventually be an integral part of determining the functional relevance of  $\sigma$  sites, the proposed  $\sigma$  antagonist status of haloperidol should be viewed with caution as the majority of reports in the  $\sigma$  literature have shown haloperidol to be a  $\sigma$  agonist. In particular, previous attempts to correlate the binding affinity of  $\sigma$  ligands with activity in a functional assay, such as the ability to cause dystonia in the rat following injection into the red nucleus, inhibition of agonist-stimulated phosphoinositide turnover and inhibition of electrically or serotonin-evoked contractions of the guinea-pig ileum, have all shown haloperidol to be a more potent  $\sigma$  agonist ligand than DTG (Walker et al., 1990). The general inability of haloperidol to modulate (-)-bicuculline methiodide-induced seizures further argues against an association between  $\sigma$  activity and the anticonvulsant effect of DTG in the prepiriform cortex.

Noncompetitive antagonism of NMDA receptor-mediated excitation at the MK-801 / PCP binding site is also unlikely to account for the protective effects of DTG reported herein. Radioligand binding experiments have shown DTG to have low affinity for [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]TCP 1-[1-(2-thienyl)cyclohexyl]piperidine-labelled NMDA receptors in rat and guinea-pig brain membranes (Keana et al., 1989). Furthermore, DTG was 500 times less potent than MK-801 as an antagonist of NMDA-evoked depolarizations in rat neocortical slices (Aram et al., 1989). It is interesting, however, to note that DTG

was equipotent to MK-801 in its ability to induce PCP-like stereotyped behaviour and ataxia in rats following i.c.v. administration (Contreras et al., 1988). This rather surprising observation could not be explained by the relative order of potency for DTG and MK-801 to displace [ $^3\text{H}$ ]TCP, [ $^3\text{H}$ ](+)-SKF 10,047 or [ $^3\text{H}$ ](+)-3-PPP from rat whole brain membrane preparations. Thus the mechanism of DTG-induced stereotyped behaviour remains to be delineated. Consistent with our own finding in vivo, the pharmacological profile for DTG-like discriminative effects was also not predicted by affinity for either PCP or  $\sigma$  binding sites (Holtzman, 1989).

Of potential relevance to the anticonvulsant response observed in our investigations, there are now a number of reports showing that DTG has a potent inhibitory effect on both spontaneous and induced neuronal firing; none of which can be definitively related to a direct action at the putative  $\sigma$  receptor (Walker et al., 1990). In a recent study by Connick et al. (1992), DTG reversibly inhibited NMDA and quisqualate induced firing in cortical and hippocampal neurones in a haloperidol insensitive manner. Similarly, Fletcher et al. (1992) found DTG to be a potent and voltage-dependent antagonist of NMDA-evoked depolarizations in rat hippocampal neurones. Other investigators have found that DTG exhibits a distinct pharmacological profile when compared to other  $\sigma$  ligands in a variety of experimental paradigms. French and Ceci (1990) reported that DTG produced a 10% depression in the firing rate of A<sub>10</sub> dopamine neurones which was in marked contrast to the stimulatory action of (+)-pentazocine. In addition, DTG has been found to have anomalous excitatory properties in that it was 3-fold more efficacious than other  $\sigma$  ligands, including (+)-pentazocine, in potentiating NMDA-induced excitation of rat CA<sub>3</sub> hippocampal neurones (Monnet et al., 1992). These latter authors also reported an inhibitory effect of DTG at high doses but not for other  $\sigma$  ligands. Atypical responses to DTG have also occurred in peripheral tissues. Kennedy and Henderson (1989) noted that, unlike other  $\sigma$  ligands, DTG failed to potentiate electrically evoked neurogenic twitch contractions of the mouse isolated vas deferens and in fact produced inhibition of the response at high doses.

Excitatory mechanisms are thought to play an integral role in the generation of seizures from the prepiriform cortex. The suppression of such excitatory amino acid transmission by DTG, perhaps by a direct receptor level antagonism of depolarization mediated by activation of excitatory amino acid receptors, or indirectly through inhibition of excitatory neurotransmitter release, offers a possible explanation for the anticonvulsant response observed in vivo and is consistent with the inhibitory properties frequently observed with DTG. Whilst it is also interesting to note that autoradiographic studies of [<sup>3</sup>H]DTG binding to rat and guinea-pig brain sections revealed dense binding in the piriform cortex (Weber et al., 1986), it would be unwarranted, given the distinct pharmacological profile of DTG in this model, to attribute its anticonvulsant effect to a specific action at  $\sigma$  sites.

Given the unique profile of DTG in a variety of experimental systems, pharmacologic effects of DTG independent of its activity at  $\sigma$  sites are not uncommon. In this context, the high affinity of DTG for  $\sigma$  binding sites can only be considered a small part of the diverse and largely unexplored pharmacology of this interesting compound. A better understanding of the mechanism by which DTG exerts its potent and efficacious anticonvulsant effect in the prepiriform cortex will be of future importance.

### **Acknowledgement**

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## **Chapter 4**

### **Dextrorotatory opioids induce stereotyped behaviour in Sprague-Dawley and Dark Agouti rats**

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### **Abstract**

Dextrorphan and dextromethorphan elicit a stereotyped behavioural syndrome in rats that is indistinguishable from that produced by PCP and other noncompetitive NMDA antagonists. The rank order of potency for the induction of stereotyped behaviour was MK-801 > PCP > (±)cyclazocine > dextrorphan > ketamine > dextromethorphan. These behavioural potencies were significantly correlated (0.94;  $P > 0.01$ ) with their respective affinities for the high affinity [ $^3\text{H}$ ]dextrorphan binding site in rat forebrain membranes. To specifically address the propensity of dextromethorphan to induce adverse behavioural effects, dextrorotatory-opioid induced stereotypies were investigated in the female Dark Agouti and female Sprague-Dawley rats. The female Dark Agouti rat lacks cytochrome P450 2D1 which catalyses the O-demethylation of dextromethorphan to dextrorphan. There were no differences in either the potencies or time to peak effect of dextrorphan and dextromethorphan to induce stereotyped behaviour in either of the two rat strains. These data suggest that the affinity of dextromethorphan for NMDA receptors adequately accounts for its ability to induce stereotyped behaviour. Following dextrorphan administration female Dark Agouti rats showed a greater incidence of stereotyped behaviour than Sprague-Dawley rats, which may be indicative of strain differences in dextrorphan metabolism. In addition, dextrorotatory opioids were more potent inducers of stereotyped behaviour in female than male Sprague-Dawley rats. The commonality between the pharmacologic profiles of these compounds suggests that the abuse potential of dextromethorphan containing antitussive preparations is related to the noncompetitive NMDA antagonist activity of dextromethorphan and its primary metabolite dextrorphan.

## Introduction

The dextrorotatory opioids, dextrorphan and its 3-methyl ether derivative dextromethorphan, have virtually no affinity for opioid receptors and thus lack the potent analgesic properties, respiratory depressant effects and morphine-like addictive potential associated with related levorotatory opioid isomers (Isbell and Fraser, 1953). A number of opioids possess antitussive efficacy and it is this property that is responsible for the long and widespread use of dextromethorphan as a non-prescription cough suppressant in over sixty countries (Bem and Peck, 1992). Although devoid of morphine-like effects and consequently considered non-addictive (Isbell and Fraser, 1953), the dextrorotatory morphinans, at levels exceeding the recommended antitussive dose, can induce a phencyclidine (PCP)-like syndrome presumably by virtue of their activity as noncompetitive antagonists of the NMDA (N-methyl-D-aspartate) subfamily of ionotropic glutamate receptors.

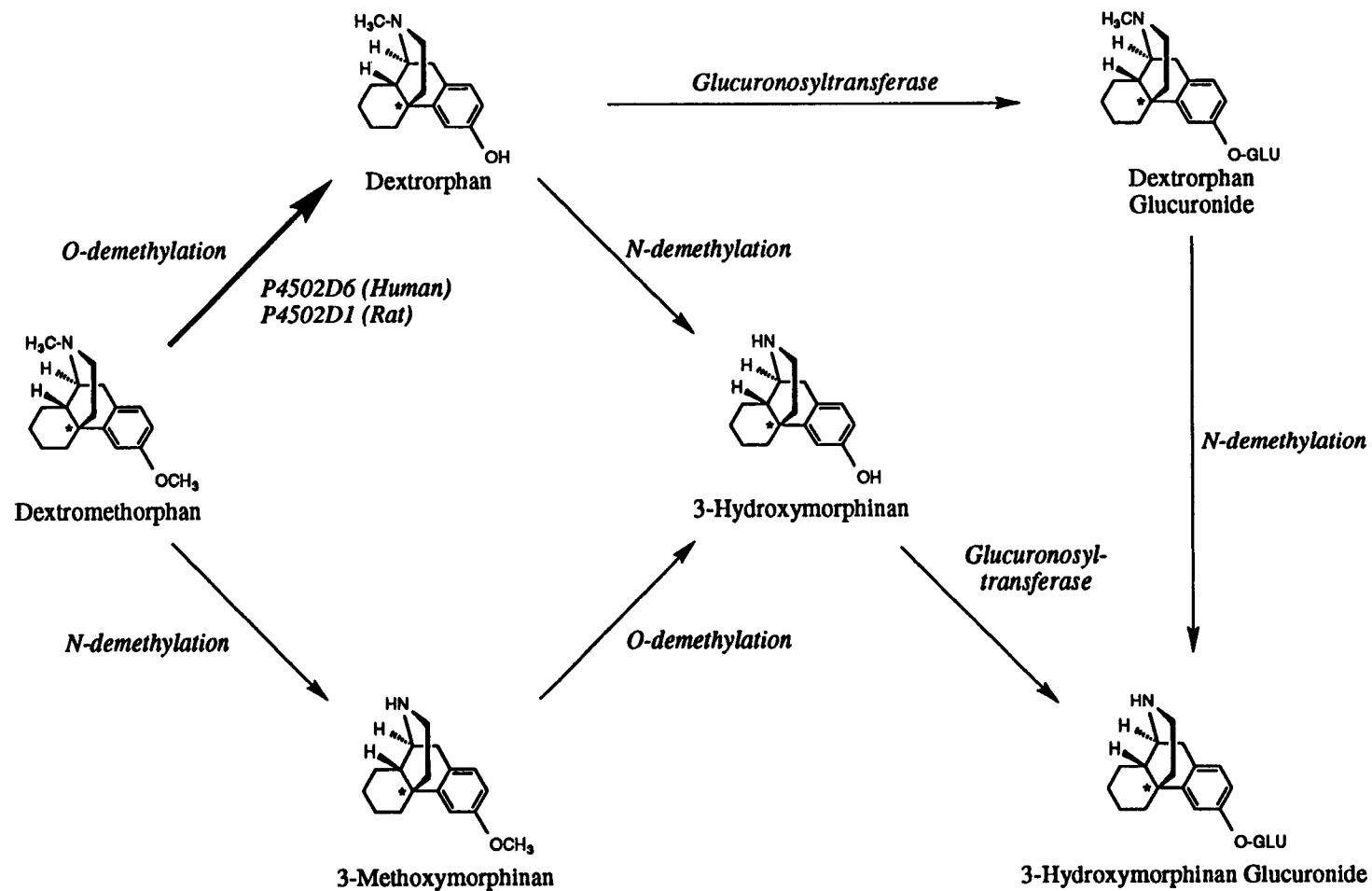
The numerous reports of recreational dextromethorphan abuse provide an early indication of a common pharmacologic basis of action for dextrorotatory opioids and PCP-like drugs (Degkwitz, 1964; McCarthy, 1971). In 1964 Degkwitz reported sedative-hypnotic effects, slurred speech, pupillary dilation, sensory distortion, drunkenness and euphoria in patients following ingestion of 300 mg (approximately ten times the antitussive dose) of dextromethorphan. In a later study in humans, Jasinski et al. (1971) reported sedative-hypnotic and psychotomimetic effects in subjects following consumption of doses approximately eight times higher than the recommended antitussive dose, and also noted that the subjective response to dextromethorphan was clearly different to that of morphine. Furthermore, drug discrimination studies in a variety of species have suggested a common basis for the effects of dextrorotatory opioids and PCP-like compounds (Holtzman, 1980, 1994; Herling et al., 1981, 1983). This relationship was established electrophysiologically when dextrorphan and dextromethorphan were reported to be selective antagonists of N-methylaspartate-induced excitation of spinal neurones (Church et al., 1985).

We have recently identified high affinity [ $^3\text{H}$ ]dextrorphan binding sites in rat forebrain that appear to be localized to a site within the ion channel domain of the activated NMDA receptor (Franklin and Murray, 1992). Although dextrorphan is undoubtedly a potent and selective noncompetitive NMDA receptor antagonist, the pharmacologic profile of dextromethorphan has remained less clear. Dextromethorphan has been suggested to be prototypical of a unique class of anticonvulsants that interact with high affinity at a site labelled by [ $^3\text{H}$ ]dextromethorphan (Tortella et al., 1988; 1994). The high affinity of dextromethorphan for both [ $^3\text{H}$ ]dextromethorphan binding sites (Craviso and Musacchio, 1982b) and at sigma recognition sites (Walker et al., 1990) are pharmacologic characteristics that are thought to be unrelated to the activity of dextromethorphan at NMDA receptors. This distinctive dextromethorphan pharmacology is not shared by dextrorphan, which is consistently the more potent of the two compounds in many experimental models of NMDA receptor antagonism. Such studies include the ability of dextrorotatory morphinans to attenuate hypoxic injury in neuronal cultures (Goldberg et al., 1987) antagonize epileptiform activity (Wong et al., 1988; Aram et al., 1989) and protect against seizures in vivo (Leander et al., 1988; Tortella et al., 1988; Chapman and Meldrum, 1989; Roth et al., 1992). However, the rapid metabolism of dextromethorphan following systemic administration confounds the interpretation of many studies of the effects of dextromethorphan in vivo. Of particular relevance to the present study, adverse behavioural effects of dextromethorphan have been attributed solely to the presence of its main metabolite dextrorphan, with the parent compound considered to be devoid of PCP-like psychotomimetic effects (Szekely, 1991; Loscher et al., 1994).

Dextromethorphan is metabolized via two different pathways: O-demethylation to dextrorphan and N-demethylation to methoxymorphinan as shown in figure 4.1. Plasma and urine analysis following dextromethorphan administration in a variety of species indicates that of these two metabolic pathways, conversion to dextrorphan represents the

**FIGURE 4.1.**

**Metabolism of dextromethorphan in humans and rats**



predominant route (Kamm et al., 1967; Ramachander et al., 1977; Barnhart, 1980; Fossati et al., 1993). Dextrorphan may be excreted in its conjugated form or N-demethylated to 3-hydroxymorphinan. In addition, 3-hydroxymorphinan can be formed by O-demethylation of methoxymorphinan (Figure 4.1). The O-demethylation of dextromethorphan is well characterized in humans and, like the antihypertensive debrisoquine, is catalysed by the polymorphic cytochrome P450 enzyme 2D6 (Cholerton et al., 1992). Cytochrome P4502D6 polymorphism in humans is genetically determined; in fact, dextromethorphan is considered a safe probe for identifying poor metabolizers (PM) and extensive metabolizers (EM) of P4502D6 substrates in human populations (Kupfer et al., 1986). In rodents, female Dark Agouti and female Sprague-Dawley rats have been shown to be suitable animal models for the study of human PM and EM phenotypes respectively (Al-Dabbagh et al., 1980; Zysset et al., 1988). The female Dark Agouti rat does not express CYP2D1 (Matsunaga et al., 1989) and is consequently deficient in P4502D1 which catalyses the O-demethylation of dextromethorphan to dextrorphan in Sprague-Dawley rats (Figure 4.1).

The aim of the present study was to investigate the ability of dextrorphan and dextromethorphan to induce stereotyped behaviour in the rat. We have assessed the propensity of the dextrorotatory morphinans and other noncompetitive NMDA antagonists, including MK-801 and PCP, to induce stereotypies in male Sprague-Dawley rats and compared the rank order of potency of these compounds with their respective affinities for the high-affinity [<sup>3</sup>H]dextrorphan binding site in rat forebrain membranes (Franklin and Murray, 1992). To further investigate dextrorotatory-induced stereotypies and to specifically address the propensity of dextromethorphan to induce these behaviours, we have compared the effects of these compounds in female Sprague-Dawley and female P4502D1 deficient Dark Agouti rats. In addition, the ability of dextrorphan and dextromethorphan to impair motor coordination was also assessed in both rat strains.

## **Materials and Methods**

### **Animals**

Male Sprague-Dawley rats (180-230 g) were purchased from Simonsen Laboratories, Gilroy, CA. Female Dark Agouti rats (150-170 g) and female Sprague-Dawley rats (160-200 g) were purchased from Bantin and Kingman, Fremont, CA. Animals were housed in groups of four to six at 22°C on a standard 12 hour light-dark schedule. Food and water were available ad libitum. Animals were allowed to acclimatize to the test room for at least one hour prior to an experimental session and were only used once. All testing was took place between 1 p.m. and 7 p.m. each day. Effects of dextrorotatory opioids in female Dark Agouti and female Sprague-Dawley rats were always studied in pairs of animals (one of each strain) that had been weight-matched as closely as possible.

### **Drugs.**

Dextrorphan, MK-801, ketamine and dextromethorphan were purchased from Research Biochemicals International (Antic, MA). PCP and (±)cyclazocine were provided by the National Institute on Drug Abuse. All compounds were dissolved in deionized water with the exception of (±)cyclazocine which was dissolved in a small volume of 0.1N HCl, diluted with deionized water and the pH adjusted to 6.0 with 0.5N NaOH. All drug solutions were administered i.p. in a constant injection volume of 5ml/kg.

### **Evaluation of Stereotyped Behaviour.**

This procedure has been described in detail elsewhere (Murray and Horita, 1979). Briefly, following i.p. injection of vehicle (control) or drug solution (test) animals were immediately transferred into large, individual Plexiglass testing chambers and observed for 2 hours. The presence or absence of any of the following three stereotyped behaviours was recorded during a one minute epoch every five minutes: (1) Repetitive lateral head

swaying, (2) Circling (pivoting around the hind limbs in either direction) and (3) Backward walking (involving all four limbs). The total incidence of each behaviour was determined for each animal over the two hour observation period. Mean total stereotypy scores were calculated for each dose group by summing the scores for the three individual behaviours.

### **Evaluation of Rotorod Performance.**

Animals were trained one to two hours before the experimental session to remain on a rotating rod of 7 cm diameter, rotating at 6 revolutions per minute. Rats were allowed three practice trials, after which all animals were able to remain on the rotorod for at least 60 sec. Animals were injected with deionized water, dextrorphan or dextromethorphan and returned to their individual cages. Thirty minutes post-injection (dextrorphan or control) or forty-five minutes post-injection (dextromethorphan or control), animals were placed on the rotating rod. Animals that were unable to remain on the rotating rod for a 60 sec testing period were considered to have failed the rotorod test.

### **Data Analysis.**

Dose-response data were analyzed by iterative non-linear least squares regression analysis to generate a sigmoid curve defined by the following four parameter logistic equation:

$$E = \frac{E_{\max}}{1 + (ED_{50} / [D])^n}$$

where E = response,  $E_{\max}$  = maximal response, D = dose and  $n$  = a slope factor.



## Results

The stereotyped behavioural syndrome elicited by dextrorphan and dextromethorphan in male Sprague-Dawley rats was indistinguishable from that produced by MK-801, PCP, ( $\pm$ )cyclazocine and ketamine. Control animals treated with deionized water or vehicle failed to show stereotyped behaviour. As shown in figure 4.2, all compounds induced stereotypies in a dose-dependent manner. Although there was a dose-dependent increase in all three behaviours, repetitive lateral head swaying was the predominant stereotypy, followed by circling which was more common than backward walking at all dose levels. Ketamine-treated rats showed varying degrees of ataxia at doses of 60 mg/kg and above, whilst dextromethorphan induced convulsions in 1 rat at 60 mg/kg and 2 rats at 100 mg/kg. Both these conditions impaired the ability of animals in these dose groups to perform stereotyped or normal behaviours, thereby influencing the total stereotypy score. Differences in drug disposition over the two hour observation period may also contribute to the seemingly limited efficacy of some of these compounds.

The rank order of potency for induction of stereotyped behaviour was: MK-801 > PCP > ( $\pm$ )cyclazocine > dextrorphan > ketamine > dextromethorphan (Table 4.1). The correlation between the potencies of these compounds to induce stereotyped behaviour and their respective affinities as inhibitors of high-affinity [ $^3$ H]dextrorphan binding in rat forebrain membranes is shown in figure 4.3. Linear regression of the log transformed data gave a correlation coefficient of 0.937 ( $P > 0.01$ ). These data suggest that the ability of these compounds to act as noncompetitive NMDA antagonists appears to underlie their ability to elicit stereotyped behaviour in these animals.

The rapid metabolism of dextromethorphan to dextrorphan is a complicating factor in the interpretation of many in vivo studies investigating the noncompetitive NMDA antagonist activity of dextromethorphan. In order to study dextrorotatory opioid-induced stereotypies under conditions of enhanced dextromethorphan bioavailability, the behavioural effects of these compounds in female Sprague-Dawley rats and cytochrome

**FIGURE 4.2.**

**Dose response relationships for MK-801, PCP, ( $\pm$ )cyclazocine, ketamine, dextrorphan (DX) and dextromethorphan (DXM)-induced stereotyped behaviour in male Sprague-Dawley rats.**

Following i.p. administration of a test compound rats were placed in an open field arena and observed for 2 hours. The presence or absence of repetitive lateral head swaying, circling and backward walking was recorded during a 1 min epoch every 5 min. Symbols represent mean total stereotypy scores at each dose group ( $n = 4$  animals) and the respective curves are expanded from the parameter estimates derived from fitting a logistic equation to the data by means of iterative nonlinear least squares regression analysis.

**FIGURE 4.3.**

**Correlation between the potencies of noncompetitive antagonists to induce stereotyped behaviour in male Sprague-Dawley rats and their respective affinities for the high-affinity [ $^3$ H]dextrorphan binding site in rat forebrain membranes.**

The potencies ( $\mu$ mol) of MK-801, PCP, ( $\pm$ )cyclazocine, ketamine, dextrorphan (DX) and dextromethorphan (DXM) to induce stereotyped behaviour in male Sprague-Dawley rats were highly correlated ( $r = 0.937$ ;  $p < 0.01$ ) with their respective affinities as inhibitors of high-affinity [ $^3$ H]dextrorphan binding in rat forebrain membrane preparations.  $IC_{50}$  values (nM) taken from Franklin and Murray (1992), except ( $\pm$ )cyclazocine (unpublished data). The significance of the correlation was evaluated by  $t$  test of the probability of  $r=0$ .

FIGURE 4.2.

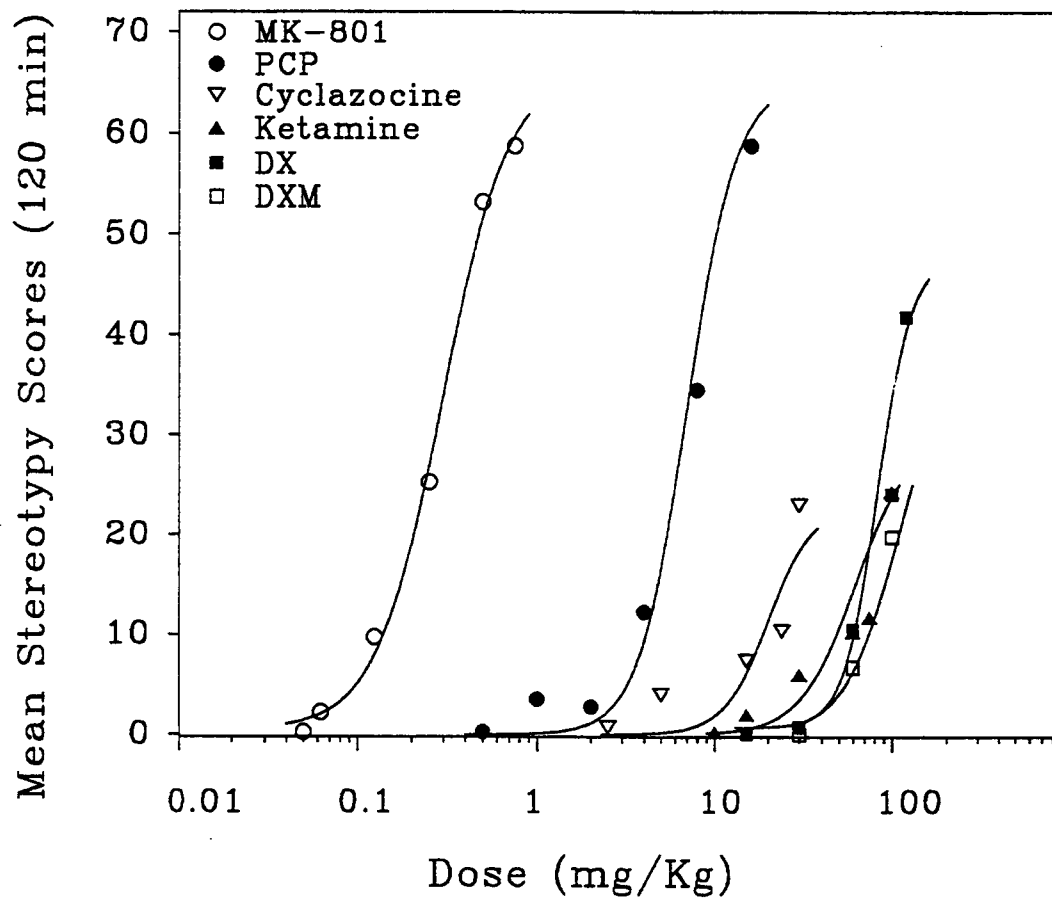
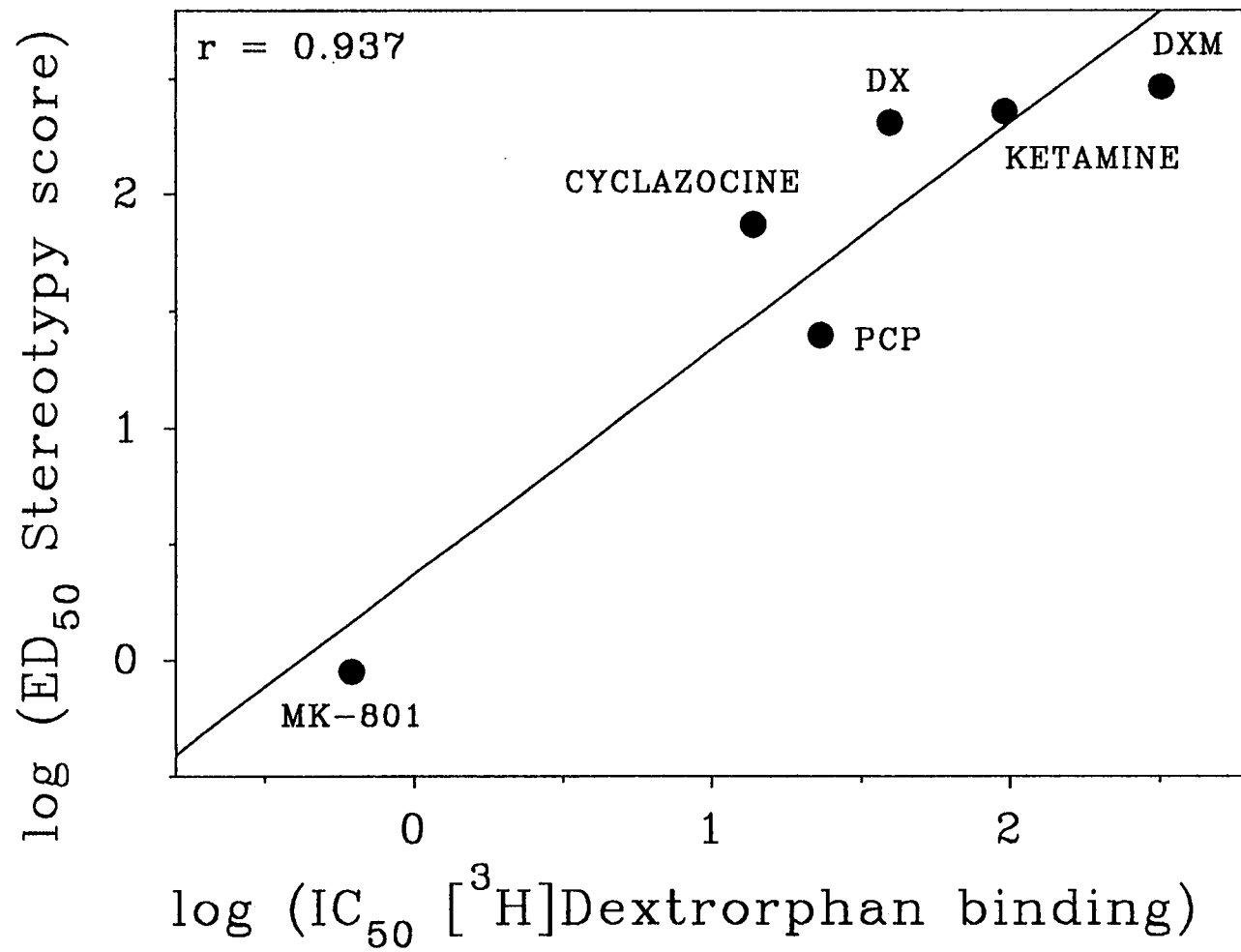


FIGURE 4.3.



**TABLE 4.1.**

**Potencies of noncompetitive NMDA antagonists to induce stereotyped behaviour in male Sprague-Dawley rats**

| Compound         | ED <sub>50</sub> ± S.E. |         |
|------------------|-------------------------|---------|
|                  | mg/kg                   | μmol/kg |
| MK-801           | 0.3 ± 1.1               | 0.89    |
| PCP              | 7.0 ± 1.1               | 25      |
| (±)Cyclazocine   | 20.1 ± 16.1             | 74      |
| Dextrorphan      | 82.7 ± 1.2              | 203     |
| Ketamine         | 62.2 ± 1.2              | 227     |
| Dextromethorphan | 103 ± 1.0               | 291     |

Rats were injected i.p. with a single dose of the appropriate test compound immediately before being placed in an open field arena. The presence or absence of repetitive lateral head swaying, circling and backward walking was recorded during a 1 min epoch every 5 min over a 2 hour observation period. Group mean stereotypy scores were calculated for each dose by summing the scores of the 3 individual behaviours (n= 4 animals).

P450 2D1 deficient female Dark Agouti rats were compared. As shown in figure 4.4, dextrorphan and dextromethorphan induced stereotyped behaviour in female Dark Agouti rats and female Sprague-Dawley rats in a dose-dependent manner. As noted for male Sprague-Dawley rats, repetitive lateral head swaying was the predominant stereotypy in both rat strains. Control animals treated with deionized water failed to show stereotyped behaviour. Both compounds were more potent inducers of stereotypies in female animals than in male Sprague-Dawley rats, however, there were no differences in the potencies of dextrorphan or dextromethorphan to elicit stereotyped behaviour in either of the female rat strains (Table 4.2 ). Although dextrorphan was equipotent in its ability to induce stereotypies in both rat strains, Dark Agouti rats showed a significantly greater incidence of stereotyped behaviour at higher doses (45 and 60 mg/kg) (Figure 4.4).

**TABLE 4.2.**

**Potencies of dextrorphan and dextromethorphan to induce stereotyped behaviour in female Sprague-Dawley and female Dark Agouti rats**

| Compound         | <u>Sprague Dawley</u>   |         | <u>Dark Agouti</u>      |         |
|------------------|-------------------------|---------|-------------------------|---------|
|                  | ED <sub>50</sub> ± S.E. |         | ED <sub>50</sub> ± S.E. |         |
|                  | mg/kg                   | μmol/kg | mg/kg                   | μmol/kg |
| Dextrorphan      | 44.0 ± 1.1              | 108     | 40.0 ± 1.0              | 98      |
| Dextromethorphan | 55.0 ± 1.1              | 157     | 52.0 ± 1.0              | 148     |

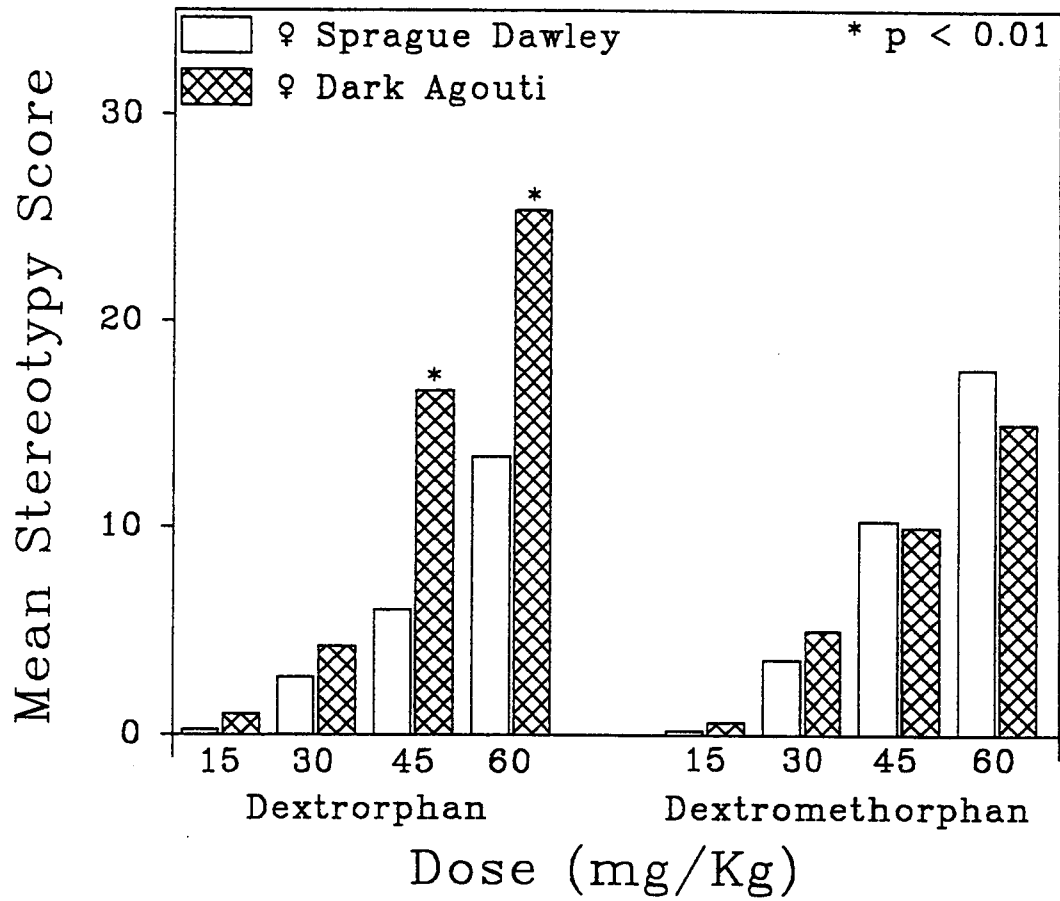
Rats were injected i.p. with a single dose of dextrorphan or dextromethorphan immediately before being placed in an open field arena. The presence or absence of repetitive lateral head swaying, circling and backward walking was recorded during a 1 min epoch every 5 min over a 2 hour observation period. Group mean stereotypy scores were calculated for each dose by summing the scores of the 3 individual behaviours (n = 4-6 animals).

**FIGURE 4.4.**

**Dose response relationships for dextrorphan and dextromethorphan-induced stereotyped behaviour in female Dark Agouti and female Sprague-Dawley rats.**

Following i.p. administration of a test compound, rats were placed in an open field arena and the presence or absence of repetitive lateral head swaying, circling and backward walking was recorded during a 1 min epoch every 5 min. Values represent mean total stereotypy scores at each dose group (n = 4-6 animals) over a 2 hour observation period. \* Mean total stereotypy score was significantly different from Sprague-Dawley dose group (Student's t test,  $P < 0.01$ ).

FIGURE 4.4.





The duration of dextrorotatory opioid-induced stereotyped behaviour was a function of dose. As shown in figure 4.5, the peak effect of dextrorphan occurred during the 20 to 30 min epochs, with the intensity and duration of the animal's behaviour being more prolonged following a 60 mg/kg dose (lower panel). The peak effect of dextromethorphan-induced stereotypies was the same in female Dark Agouti and female Sprague-Dawley rats and occurred during the 35 to 45 min epochs (figure 4.6). After an initial rise, the transient decline in stereotyped behaviour in both rat strains 20 to 30 min following a 60 mg/kg dose of dextromethorphan (lower panel) was a direct result of the ability of dextromethorphan to induce convulsions in the majority of animals at this dose level, and thus impair their capacity to perform other behaviours. Total stereotypy scores increased at later time points following a short recovery period. Dark Agouti rats continued to display stereotyped behaviour 120 min post-injection (60 mg/kg).

To further assess the adverse behavioural effects of these compounds, the ability of dextrorphan and dextromethorphan to disrupt motor coordination was studied in female Dark Agouti and female Sprague-Dawley rats. Dextrorphan and dextromethorphan, measured 30 and 45 min post-injection respectively, impaired rotorod performance in a dose-dependent manner in both rat strains (Figure 4.7). Dextrorotatory opioids were more potent in their ability to disrupt motor coordination than induce stereotyped behaviour in all test groups with the exception of the effects of dextromethorphan in female Sprague-Dawley rats (Table 4.3). Dextrorphan was more potent than dextromethorphan in its ability to disrupt rotorod performance. In addition, both dextrorphan and dextromethorphan were more potent in their ability to impair rotorod performance in female Dark Agouti rats than in female Sprague-Dawley rats (Table 4.3 ).

**FIGURE 4.5.**

**Time course of stereotyped behaviour induced by 45 mg/kg and 60 mg/kg doses of dextrorphan in female Sprague-Dawley and female Dark Agouti rats.**

Following i.p. administration of dextrorphan, 45 mg/kg (upper panel) and 60 mg/kg (lower panel), rats were placed in an open field arena and the presence or absence of repetitive lateral head swaying, circling and backward walking recorded during a 1 min epoch every 5 min. Values represent total stereotypy scores (n = 5 animals) over a 2 hour observation period.

**FIGURE 4.6.**

**Time course of stereotyped behaviour induced by 45 mg/kg and 60 mg/kg doses of dextromethorphan in female Sprague-Dawley and female Dark Agouti rats.**

Following i.p. administration of dextromethorphan, 45 mg/kg (upper panel) and 60 mg/kg (lower panel), rats were placed in an open field arena and the presence or absence of repetitive lateral head swaying, circling and backward walking recorded during a 1 min epoch every 5 min. Values represent total stereotypy scores (n = 6 animals) over a 2 hour observation period.

FIGURE 4.5.

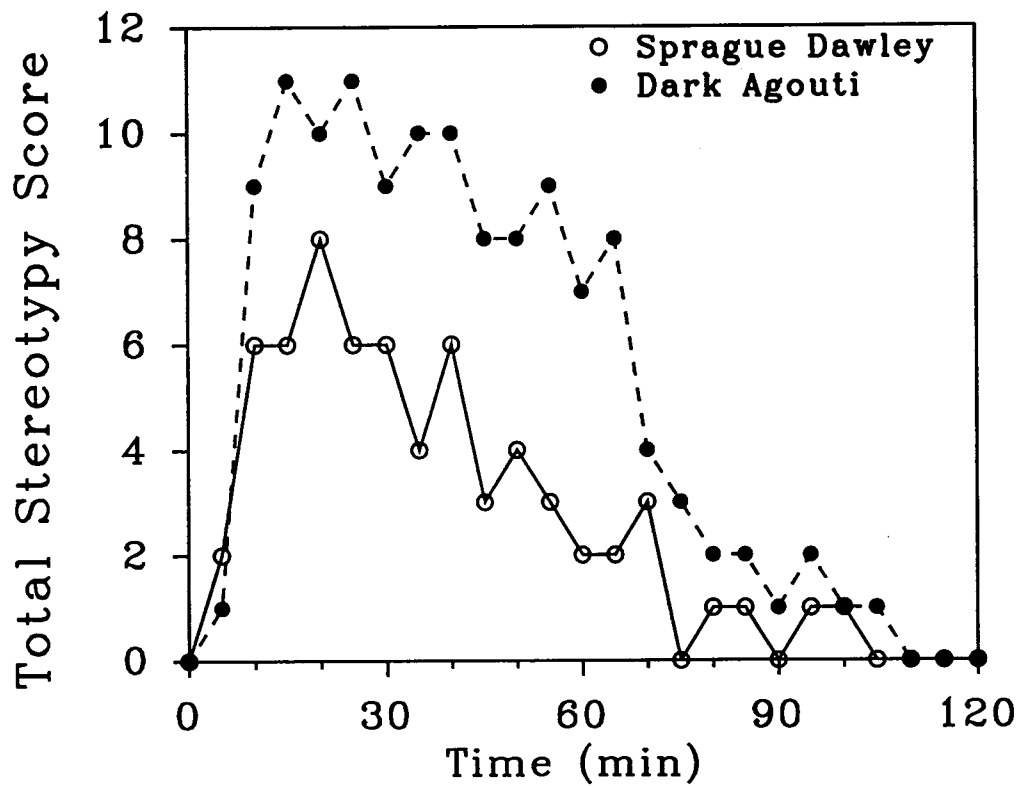
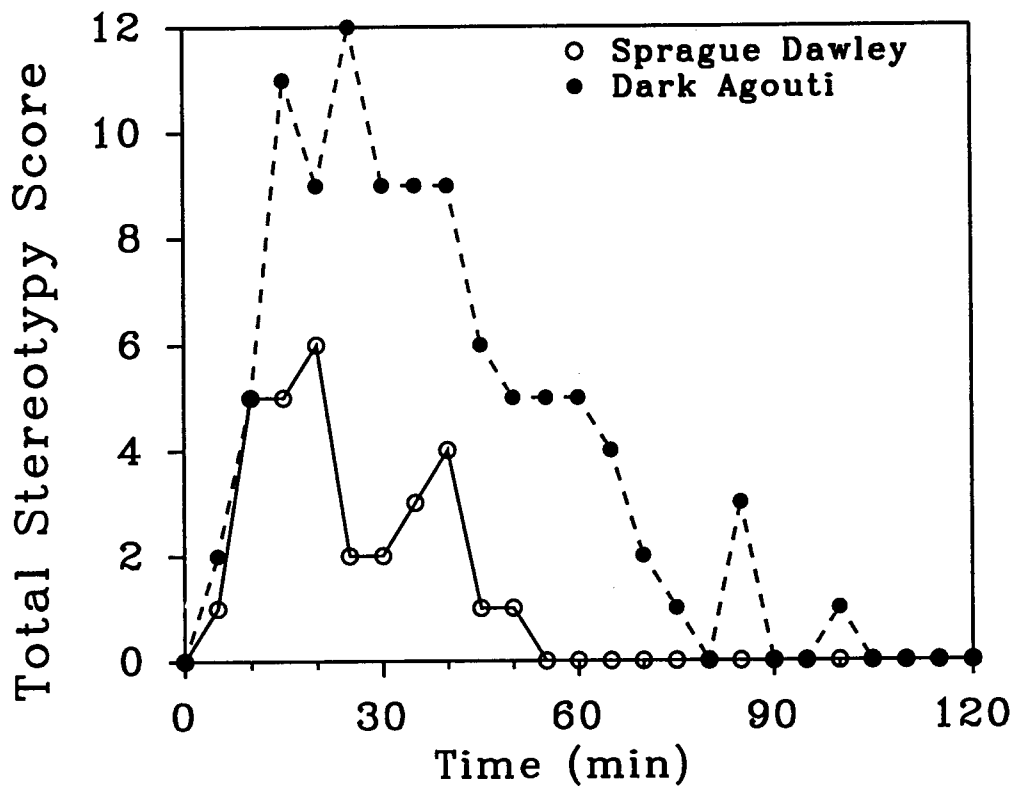
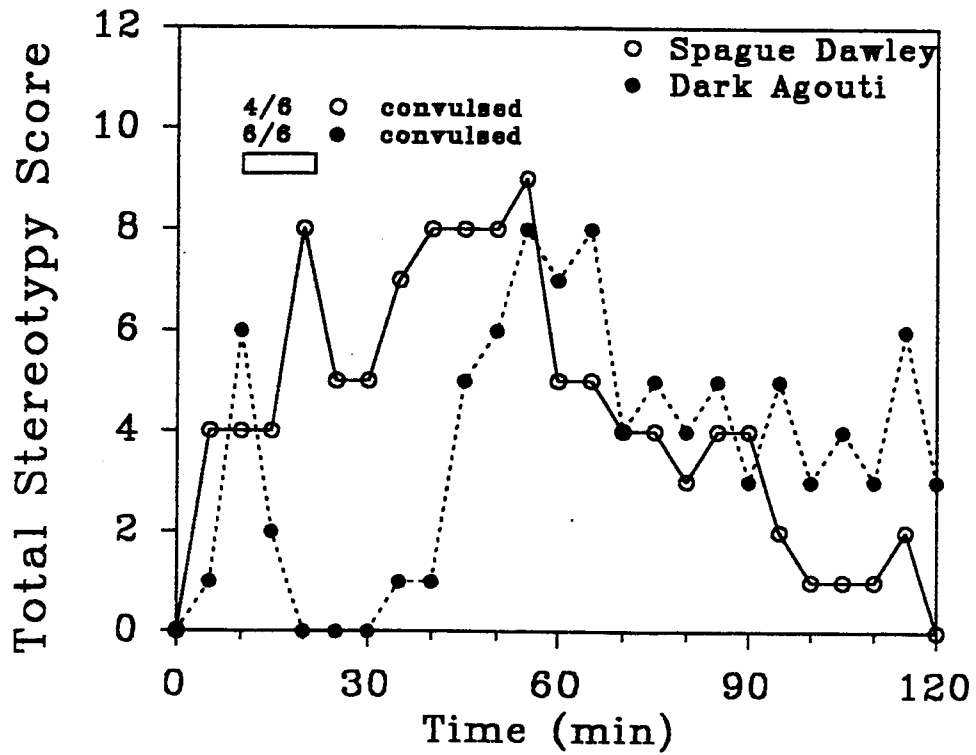
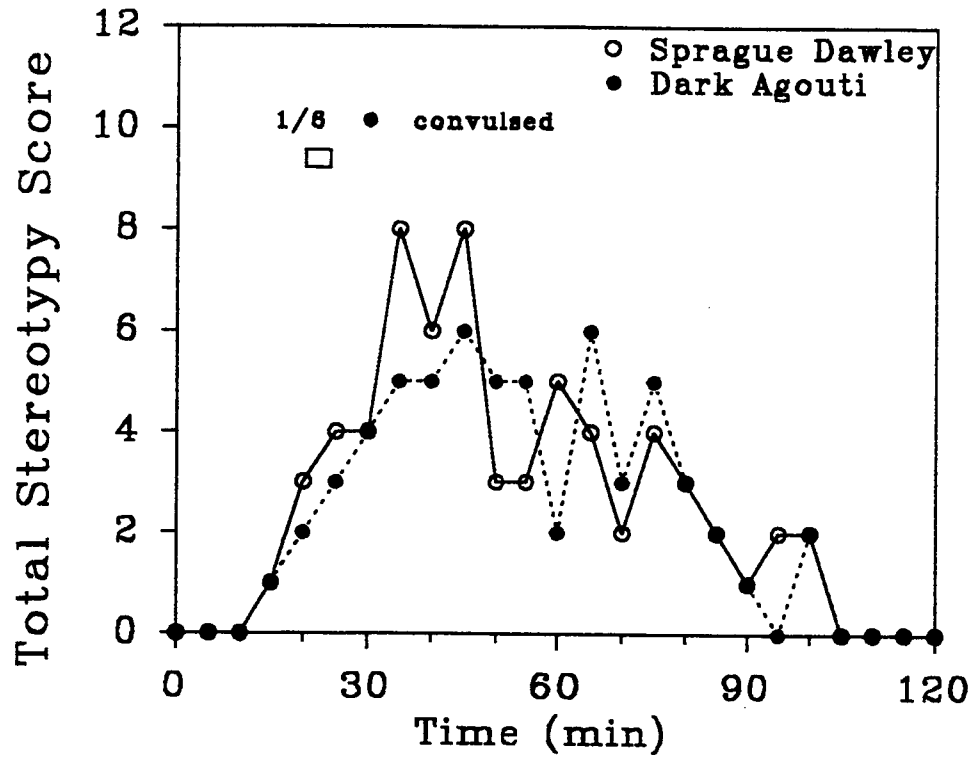


FIGURE 4.6.

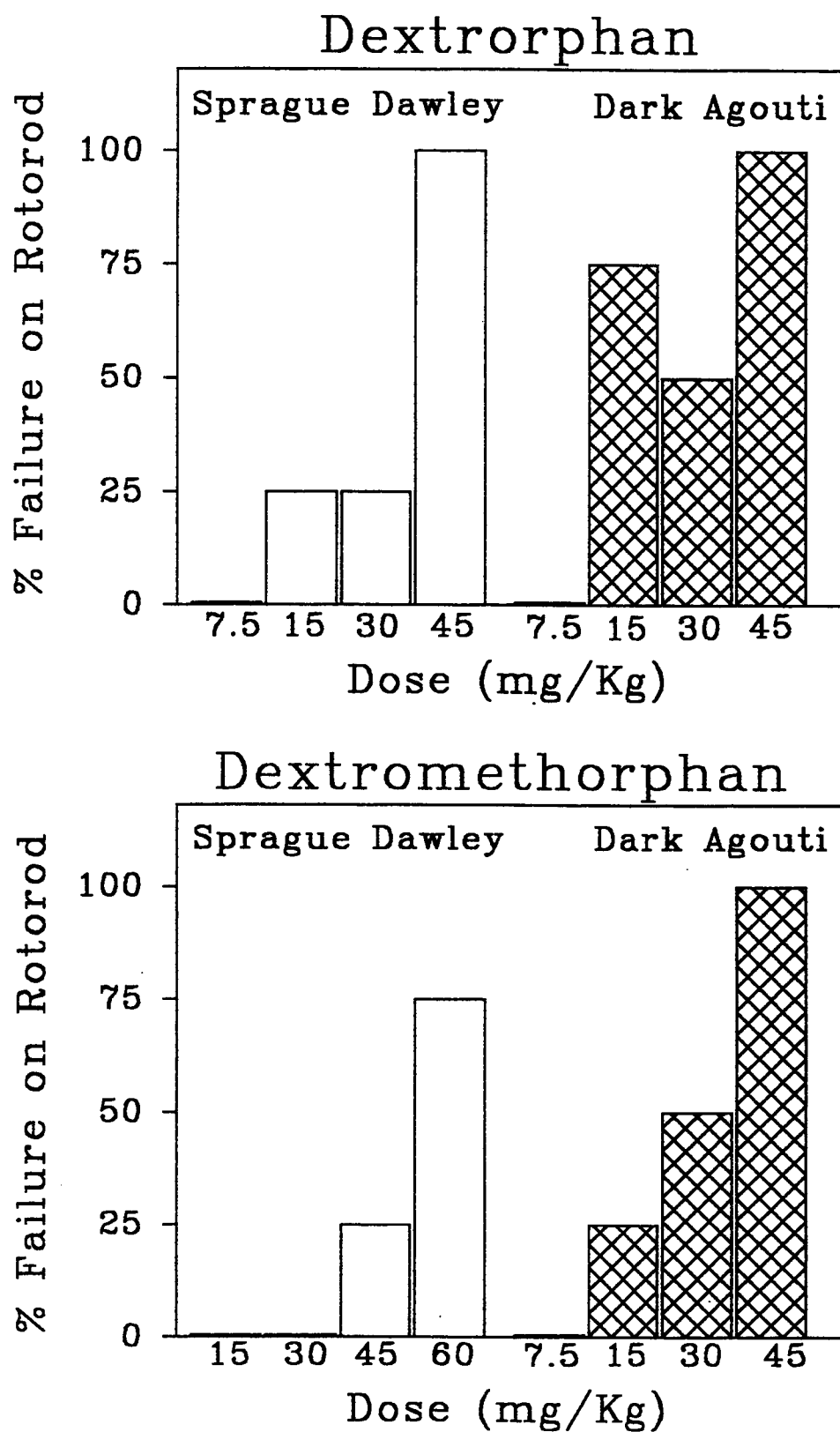


**FIGURE 4.7.**

**Ability of dextrorphan and dextromethorphan to impair rotorod performance in female Sprague-Dawley and female Dark Agouti rats.**

Dose response relationships showing the ability of dextrorphan (upper panel) and dextromethorphan (lower panel) to impair rotorod performance in female Sprague-Dawley and female Dark Agouti rats. Performance was evaluated 30 min (dextrorphan) or 45 min (dextromethorphan) following i.p. administration of the test compound. Percent failure indicates % of animals in each dose group (n = 3-4) failing to remain on the rotorod for 60 sec.

FIGURE 4.7.



**TABLE 4.3.**

**Potencies of dextrorphan and dextromethorphan to impair rotorod performance in female Sprague-Dawley and female Dark Agouti rats**

| Compound         | <u>Sprague Dawley</u><br>ED <sub>50</sub> ± S.E. |         | <u>Dark Agouti</u><br>ED <sub>50</sub> ± S.E. |         |
|------------------|--|---------|---|---------|
|                  | mg/kg  | μmol/kg | mg/kg   | μmol/kg |
| Dextrorphan      | 32.0 ± 3.0                                       | 79      | 16.0 ± 1.6                                    | 39      |
| Dextromethorphan | 52.0 ± 1.0                                       | 148     | 25.0 ± 1.2                                    | 71      |

Rotorod performance was evaluated 30 min (dextrorphan) or 45 min (dextromethorphan) following i.p. administration of the test compound. Percent failure was calculated for each dose group (n = 3-4 animals) as the % of animals failing to remain on the rotorod for 60 sec.

### Discussion

The ability of PCP to produce a characteristic and reproducible stereotyped behavioural syndrome in rats has been known for some time (Murray and Horita, 1979). Other noncompetitive NMDA antagonists, including dextrorphan and dextromethorphan, have also been shown to produce PCP-like effects in laboratory rodents and other species (Murray et al., 1982; Koek et al., 1988; Skekely et al., 1991). In the present study dextrorphan and dextromethorphan induced a stereotyped behavioural syndrome in rats that was indistinguishable from that produced by MK-801 and PCP. This dextrorotatory opioid-induced hyperactivity was characterized by repetitive head swaying, with circling and backward walking occurring at higher doses. Furthermore, the potencies of an array of noncompetitive NMDA antagonists to produce these behaviours were significantly correlated with their respective affinities as inhibitors of high affinity [<sup>3</sup>H]dextrorphan

binding to rat forebrain membrane preparations (Franklin and Murray, 1992). The ability of these compounds to act as noncompetitive NMDA antagonists therefore appears to underlie their propensity to produce stereotyped behaviour in rats.

The finding that dextrorphan was more potent than dextromethorphan in these studies is consistent with radioligand binding data. Dextrorphan is more potent than dextromethorphan as an inhibitor of [ $^3\text{H}$ ]PCP binding (Murray and Leid, 1984) and [ $^3\text{H}$ ]dextrorphan binding (Franklin and Murray, 1992) to rat brain membranes. Although these data strongly suggest that interaction with NMDA receptors mediates the observed psychoactive properties of the dextrorotatory morphinans, the well documented O-demethylation of dextromethorphan to dextrorphan (Figure 1) is a confounding factor in the interpretation of the effects of dextromethorphan *in vivo*. The results of our studies with female Dark Agouti rats, however, indicate that dextromethorphan also has psychoactive properties.

There was no difference in the potency of dextromethorphan to induce stereotyped behaviour in female Dark Agouti and female Sprague-Dawley rats. Furthermore, over a two hour observation period dextromethorphan showed the same time to peak effect in both rat strains. Presumably if dextromethorphan had to first be metabolized to dextrorphan in order to induce its effect, the onset of stereotyped behaviour would be different for the Dark Agouti group. It therefore appears that the stereotyped behavioural response observed in female Dark Agouti rats is induced by dextromethorphan. Metabolic fate studies conducted both *in vitro* in liver microsomes (Zysset et al., 1988; Kerry et al., 1993) and *in vivo* (Zysset et al., 1988; Bochner et al., 1994) indicate that the clearance rate of dextromethorphan is greatly reduced in the female Dark Agouti rat compared to the female Sprague-Dawley. Consistent with these observations, Dark Agouti rats excrete a greater percentage of unchanged dextromethorphan 48 hours (Bochner et al., 1994) or 72 hours (Zysset et al., 1988) after administration of the parent compound than Sprague-Dawley rats. As a compensatory mechanism the N-demethylation of dextromethorphan, which is a



mediated by different cytochrome P450 enzymes and thought to be a minor pathway in the Sprague-Dawley rat, appears to be increased in the Dark Agouti strain (Kerry et al., 1993). Consequently, a larger percentage of the total recovered dose is excreted as 3-methoxymorphinan in the Dark Agouti rat (Zysset et al., 1988; Bochner et al., 1994). Alternative O-demethylation pathways are also likely to compensate for the absence of P4502D1 in the female Dark Agouti rat. However, based on the study of debrisoquine metabolizing P450s, it has been suggested that other enzymes probably have reduced affinity for P4502D1 substrates in vivo (Matsunaga et al., 1989). It is therefore assumed that alternative pathways do not make a significant contribution to the formation of dextrorphan in female Dark Agouti rats over the time course of our studies.

Dextrorphan was also equipotent in its ability to induce stereotypies in female Dark Agouti and Sprague-Dawley rats, yet the Dark Agouti rats showed a significantly greater incidence of stereotyped behaviour at the higher doses tested. In vitro studies have indicated that there are no differences between the two rat strains with respect to the N-demethylation of dextrorphan (Kerry et al., 1993). It is possible therefore that the two strains differ in their ability to perform glucuronide conjugation reactions even though this appears not to be the case in humans. Duché et al. (1993) recently reported that dextrorphan was normally glucuronidated in a population of poor metabolizers. However, given the wide species variation in drug metabolizing enzymes, this finding does not necessarily apply to the female Dark Agouti rat. It has been proposed that sex-dependent differences in glucuronidation may account for the striking differences observed in the pharmacokinetics of dextrorphan in male and female Sprague-Dawley rats. Ramachander et al. (1978) noted that following oral administration of dextromethorphan, the plasma half-life of dextrorphan in male rats was approximately half that observed in females. This appears not to be the case in human populations; no sex-dependent differences were observed in the pharmacokinetics of dextromethorphan and dextrorphan in poor and extensive metabolizers (Kupffer et al., 1986). Given the observations by Ramachander et

al. (1978), enhanced bioavailability may explain the increased potency of the dextrorotatory opioids to induce stereotyped behaviour in female rats compared to male rats reported herein.

The spectrum of behavioural effects observed following administration of a noncompetitive NMDA antagonist is clearly dose-dependent (Murray, 1994). It is not surprising, therefore, that motor coordination was impaired by dextrorphan and dextromethorphan in both rat strains. In particular, the ability of dextromethorphan to potently diminish rotorod performance in female Dark Agouti rats lends further support for NMDA receptor-mediated adverse behavioural effects of dextromethorphan. Moreover, the propensity of dextrorphan to markedly impair motor coordination in female Dark Agouti rats is consonant with a possible difference in the ability of this strain to metabolize dextrorphan. In the Sprague-Dawley rat, dextrorphan is largely excreted in its conjugated form following i.p. administration of dextrorphan (Bochner et al., 1994). Unfortunately, these authors did not compare the metabolic fate of dextrorphan in Dark Agouti and Sprague-Dawley rat strains following dextrorphan administration .

The results of the present study suggest that the affinity of dextromethorphan for NMDA receptors can adequately account for its propensity to induce stereotyped behaviour in the rat. These data are in agreement with numerous studies of the effects of dextromethorphan in vitro where metabolism to dextrorphan is not a confounding factor in the interpretation of the noncompetitive NMDA antagonist activity of dextromethorphan (Goldberg et al., 1987; Wong et al., 1988; Aram et al., 1989). Studies of the effects of dextromethorphan in vivo, that have been designed to limit metabolite formation, also demonstrate that noncompetitive antagonism of NMDA receptors mediates the behavioural effects of dextromethorphan. Holtzman (1994) recently reported that the discriminative stimulus effects of s.c. dextromethorphan are clearly PCP-like and not mediated by the high affinity [ $^3\text{H}$ ]dextromethorphan binding site. Furthermore, in a study of the therapeutic efficacy of dextromethorphan in humans, patients were given the selective

cytochrome P4502D6 inhibitor quinidine to inhibit dextromethorphan metabolism and thus enhance its bioavailability (Zhang et al., 1992). High plasma concentrations of dextromethorphan were not well tolerated by all patients; one patient experienced a severe PCP-like reaction following a 60 mg oral dose in the presence of 150 mg/day quinidine. Other patients who failed to complete the drug trial complained of adverse side effects such as nervousness, tremors, fatigue, unsteady gait, dizziness and confusion (Zhang et al., 1992).

In summary, dextrorphan and dextromethorphan produced a stereotyped behavioural response in rats that was indistinguishable from that produced by PCP and other noncompetitive NMDA antagonists. The correlation of this pharmacologic profile with that defined by [ $^3\text{H}$ ]dextrorphan equilibrium competition binding studies suggests that interaction with the NMDA receptor-channel complex mediates the stereotyped behavioural effects of these compounds. Dextromethorphan was equipotent in its ability to induce stereotypies in female Dark Agouti and female Sprague-Dawley rats. Moreover, following dextromethorphan administration there was no difference in the time to peak effect between the two rat strains. Together, these results suggest that the affinity of dextromethorphan for NMDA receptors adequately accounts for its propensity to induce stereotyped behaviour. Although dextromethorphan has a distinct pharmacology that is thought to be unrelated to its activity as an NMDA receptor antagonist and may explain some of its effects in vivo (Tortella et al., 1988, 1994; Szekely, 1991; Loscher and Honack, 1993), it would be premature to consider it completely devoid of the adverse behavioural effects associated with noncompetitive NMDA antagonists.

### **Acknowledgement**

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## **Chapter 5**

### **Regional distribution and characterization of [ $^3\text{H}$ ]dextrorphan binding sites in rat brain determined by quantitative autoradiography**

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### Abstract

The pharmacologic specificity and anatomical distribution of [ $^3\text{H}$ ]dextrorphan recognition sites in the rat brain was characterized by quantitative autoradiography. Equilibrium saturation analysis indicated that [ $^3\text{H}$ ]dextrorphan labelled a single population of high affinity binding sites. These sites are heterogeneously distributed throughout rat forebrain with the following order of binding densities: hippocampal formation > cerebral cortex > thalamic nuclei > striatum. The association rate of [ $^3\text{H}$ ]dextrorphan with its binding site in area stratum radiatum of CA1 is accelerated by the addition of glycine and glutamate. [ $^3\text{H}$ ]Dextrorphan binding is, however, relatively insensitive to glycine and glutamate under equilibrium conditions, despite extensive pre-washing procedures to deplete endogenous levels of these substances. The competitive N-methyl-D-aspartate (NMDA) antagonist D(-)-2-amino-5-phosphonopentanoic acid (D-AP5) and the glycine site antagonist 7-chlorokynurenic acid completely inhibit specific [ $^3\text{H}$ ]dextrorphan binding. D-AP5 suppresses [ $^3\text{H}$ ]dextrorphan binding in a regionally distinctive manner; a population of binding sites is weakly inhibited by D-AP5 in the lateral thalamic nuclei, whereas D-AP5 potently inhibits [ $^3\text{H}$ ]dextrorphan binding in the cerebral cortex. The rank order of potencies of an array of noncompetitive antagonists to inhibit [ $^3\text{H}$ ]dextrorphan binding unambiguously displays the pharmacologic profile of the noncompetitive antagonist domain of the NMDA receptor-channel complex. Furthermore, the distribution of [ $^3\text{H}$ ]dextrorphan binding sites in slide-mounted tissue is in excellent agreement with the distribution of NMDA receptors previously reported using NMDA-displacement of [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ](+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) and [ $^3\text{H}$ ]1-[1-(2-thienyl)cyclohexyl]piperadine (TCP). An exception was the molecular layer of the cerebellum, where the density of [ $^3\text{H}$ ]dextrorphan binding sites was particularly high. The regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat brain does not correspond to the reported distributions of [ $^3\text{H}$ ]dextromethorphan or sigma binding sites.

## Introduction

Dextrorphan, (+)-3-hydroxy-N-methyl-morphinan, is a selective noncompetitive antagonist of the NMDA (N-methyl-D-aspartate) subfamily of ionotropic glutamate receptors. Dextromethorphan, the 3-methyl derivative of dextrorphan, which has found widespread use as a non-prescription antitussive is also an effective noncompetitive NMDA antagonist but is less potent than dextrorphan in a variety of experimental models of NMDA receptor antagonism. These studies document the activity of dextrorotatory morphinans to attenuate hypoxic injury in neuronal cultures (Goldberg et al., 1987), antagonize epileptiform activity (Wong et al., 1988; Aram et al., 1989) and protect against seizures in vivo (Leander et al., 1988; Chapman and Meldrum, 1989; Roth et al., 1992).

Ionotropic glutamate receptors have traditionally been subclassified according to pharmacologic criteria: the NMDA receptors, which are activated by the agonist NMDA, and the non-NMDA receptors, which include those receptors sensitive to the agonists  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (Monaghan et al., 1989; Watkins et al., 1990). With respect to its pharmacology and physiology, the NMDA receptor-channel complex is the most thoroughly characterized of the glutamate receptors and appears to have several distinguishing features. The NMDA receptor channel is highly permeable to  $\text{Ca}^{2+}$ , has an NMDA agonist recognition site, a strychnine insensitive glycine co-agonist site, is blocked by  $\text{Mg}^{2+}$  in a voltage-dependent manner and also has binding sites for  $\text{Zn}^{2+}$  and polyamines. Finally, and most germane to the pharmacology of dextrorphan, the activated NMDA receptor is noncompetitively blocked by a structurally diverse group of compounds that are thought to bind within the ion channel. These compounds include (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), dissociative anaesthetics, phencyclidine (PCP) and ketamine, dextrorotatory morphinans, dextrorphan and dextromethorphan, and benzomorphans such as cyclazocine and N-allylnormetazocine (SKF 10,047).

Early reports of recreational dextromethorphan abuse (Degkwitz, 1964; McCarthy, 1971) provided an indication of a common pharmacologic basis for the effects of the dextrorotatory morphinans and PCP-like drugs. Furthermore, the results of many drug discrimination studies have suggested a common mode of action for the effects of dextrophan and PCP-like compounds. Dextrophan will substitute for PCP (Holtzman, 1980), ketamine (Herling et al., 1981), dextromethorphan (Holtzman, 1994), cyclazocine (Teal and Holtzman, 1980; Herling et al., 1981) and SKF 10,047 (Shannon, 1983) in a variety of species. Similarly, dextromethorphan, cyclazocine and SKF 10,047 produce dose-dependent dextrophan-appropriate responses in animals trained to discriminate dextrophan from saline (Herling et al., 1983). The relationship between these compounds was established electrophysiologically when dextrophan, like PCP and ketamine (Anis et al., 1983), was reported to be a selective antagonist of N-methylaspartate-induced excitation of spinal neurones (Church et al., 1985). Dextromethorphan also selectively antagonized N-methylaspartate-induced excitation but was 5-fold less potent than dextrophan (Church et al., 1985). Dextromethorphan, in addition, is thought to be prototypical of a unique class of anticonvulsants acting through a site labelled by [ $^3\text{H}$ ]dextromethorphan (Tortella et al., 1988; 1994). The high affinity of dextromethorphan for both [ $^3\text{H}$ ]dextromethorphan binding sites (Craviso and Musacchio, 1983b) and the sigma-1 recognition site (Walker et al., 1990) represent features that are unique to dextromethorphan, these pharmacologic characteristics are not shared by dextrophan and are thought to be unrelated to the activity of dextromethorphan as an NMDA receptor antagonist.

The NMDA receptor appears to be composed of several subunits which co-assemble to form a functional channel. Two families of NMDA receptor subunits have recently been cloned and termed NMDAR1 (NR1) and NMDAR2 (NR2) in rat brain (Moriyoshi et al., 1991; Monyer et al., 1992; Ishii et al., 1993), and designated  $\zeta$  and  $\epsilon$  in mouse brain (Yamazaki et al., 1992; Ikeda et al., 1992; Meguro et al., 1992; Kutsuwada et

al., 1992). Eight different isoforms generated by alternative splicing of mRNA have been identified for the NR1 subunit (Sugihara et al., 1992; Nakanishi. et al., 1992; Hollman et al., 1993), whereas the NR2 family currently consists of four closely related subunits termed NR2A-NR2D (Monyer et al., 1992; Ishii et al., 1993). Although the NR1 subunit is able to form functional homo-oligomeric receptors (Moriyoshi et al., 1991), co-expression of NR1 with an NR2 subunit greatly potentiates responses to glutamate or NMDA and produces functional variability depending on the subunit combination (Meguro et al., 1992; Kutsuwada et al., 1992; Monyer et al., 1992; Ishii et al., 1993). Localization of mRNA for NR1 by in situ hybridization techniques reveals an abundant and ubiquitous expression throughout the rat brain (Moriyoshi et al., 1991.), that is in marked contrast to the distinctive and restricted distribution of mRNA for NR2 subunits (reviewed in Nakanishi, 1992). The potential for regional and functional NMDA receptor heterogeneity has now been further extended by the recent findings of Laurie and Seeburg (1994a). Using in situ hybridization techniques to detect the location of mRNA for the eight possible forms of the NR1 subunit these authors reported that although some splice forms occur extensively and homogeneously in the adult rat brain, the occurrence of other splice forms is restricted to specific brain regions.

In order to determine the functional significance of various combinations of recombinant NMDA receptor subunits and splice variants, there is an increasing need for a better understanding of the regional distribution and properties of native NMDA receptors. Receptor autoradiography has already provided some evidence for regionally heterogeneous populations of native NMDA receptors in the rat forebrain based on differential activation of the NMDA receptor-channel complex by [ $^3\text{H}$ ]glutamate, binding of the competitive glutamate antagonist [ $^3\text{H}$ ]3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid ([ $^3\text{H}$ ]CPP) or regulation of [ $^3\text{H}$ ]MK-801 binding by NMDA receptor agonists and antagonists (Monaghan et al., 1988; Monaghan 1991; Beaton et al., 1992; Sakurai et al., 1993). In addition, a pharmacologically distinct NMDA receptor has been identified in the



cerebellum that appears to have, as one of its characteristics, a particularly low affinity for MK-801 (Maurice and Vignon 1990; Quarum et al., 1990; Ebert et al., 1991; Beaton et al., 1992). Further support for NMDA receptor heterogeneity amongst native NMDA receptors of unknown subunit composition is provided by data derived from the study of recombinantly expressed heteromeric NMDA receptors. With respect to the pharmacology of noncompetitive antagonists of the NMDA receptor, differences in the characteristics of channel blockade (Kutsuwada et al., 1992; Lynch et al., 1993; Yamakura et al., 1993) and the binding affinity of [ $^3\text{H}$ ]MK-801 (Cik et al., 1993; Chazot et al., 1994; Laurie and Seeburg, 1994b) have been shown to vary as a function of subunit composition.

Our previous studies indicate that [ $^3\text{H}$ ]dextrorphan labels a high affinity site in rat brain membranes corresponding to the noncompetitive antagonist binding domain of the activated state of the NMDA receptor-channel complex (Franklin and Murray, 1990; Franklin and Murray, 1992). Although [ $^3\text{H}$ ]dextrorphan appears to recognize an "open channel" binding domain approximating the site or sites of other specific noncompetitive NMDA antagonists, the binding domain of [ $^3\text{H}$ ]dextrorphan may not be identical to the channel binding sites of either [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]1-[1-(2-thienyl)cyclohexyl]piperidine ([ $^3\text{H}$ ]TCP). Specific aspects of the regulation of [ $^3\text{H}$ ]dextrorphan binding by modulators of the NMDA receptor-channel complex distinguish it from [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]TCP and may be indicative of underlying differences in the mechanisms of noncompetitive antagonism among these compounds. [ $^3\text{H}$ ]Dextrorphan may therefore represent a unique ligand for the NMDA receptor-channel complex that could facilitate the identification of functionally and regionally distinct populations of native NMDA receptors.

In the present study we have defined a procedure for quantitative autoradiographic visualization of [ $^3\text{H}$ ]dextrorphan binding sites in slide-mounted sections and confirmed the specificity of this autoradiographic assay for the labelling of a site within the ion channel domain of the NMDA receptor. To gain further insight into the mechanism by which [ $^3\text{H}$ ]dextrorphan interacts with the NMDA receptor-operated cation channel, we have

specifically assessed the regulation of [ $^3\text{H}$ ]dextrorphan binding by glycine and glutamate in several brain areas. In addition, we have studied the regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites in the rat brain and compared the relative distribution with previously reported distributions determined by autoradiographic visualization of NMDA receptors.

## **Materials and Methods**

### **Materials**

[ $^3\text{H}$ ]Dextrorphan (47.5 Ci/mmol) was provided by Dupont-New England Nuclear (Boston, MA). Dextrorphan, MK-801, TCP, ketamine, dextromethorphan and 7-chlorokynurenic acid were purchased from Research Biochemicals International (Natick, MA). D(-)-AP5 was purchased from Tocris Neuramin (Essex, U.K.). PCP was provided by the National Institute on Drug Abuse. 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) hemisodium salt was purchased from Research Organics Inc. (Cleveland, OH). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

### **Tissue preparation**

Male Sprague-Dawley rats (Simonsen Labs., Gilroy, CA; 250-350 g) were decapitated under  $\text{CO}_2$ -induced narcosis. Brains were rapidly removed, frozen by immersion in isopentane at  $-20^\circ\text{C}$  and stored at  $-70^\circ\text{C}$  until use. Frozen brains were warmed sufficiently in a cryostat (Minotome, IEC) to allow them to be blocked and freeze-mounted to brass microtome chucks with Tissue Tek (Miles, Elkhart, IN) embedding medium. Serial  $16\text{-}\mu\text{m}$  sections were cut on the cryostat at  $-17^\circ\text{C}$  and thaw-mounted onto gelatin-coated (0.5%; porcine 300 bloom) microscope slides. Slide-mounted brain sections, two per slide, were desiccated at  $4^\circ\text{C}$  under vacuum for 24 h and then stored at  $-20^\circ\text{C}$  for up to three weeks.

## **Radioligand binding**

Slide-mounted sections were allowed to thaw at room temperature for 30-45 min. Sections were then pre-washed (3 x 20 min) in a large volume of 5 mM HEPES buffered to pH 7.4 at 20°C. In studies to investigate the effect of prolonged washing, sections were immersed in 5 mM HEPES at 4°C (pH 7.4) for 16 h prior to this procedure. Excess buffer was aspirated from each slide before allowing the sections to air dry; at this time each section was circumscribed by a barrier drawn with a wax pencil. [<sup>3</sup>H]Dextrorphan binding was routinely determined by covering each section with a droplet (430 µl for coronal and sagittal sections or 480 µl for horizontal sections) of 5 mM HEPES pH 7.4 containing 40 nM [<sup>3</sup>H]dextrorphan, 100 µM glycine, 1 µM glutamate and 1 mM EDTA. Nonspecific binding was defined by the presence of 100 µM MK-801 in adjacent brain sections. Under these conditions at 20°C the [<sup>3</sup>H]dextrorphan association reaction is >95% complete by 4 h. An H<sub>2</sub>O vapour-saturated environment was employed during the long incubation period in order to minimize droplet evaporation (approximately 1% after 8 h). After routine incubation for 4 h, the reaction was terminated by draining the incubation droplets and rapidly dipping each slide in a large (400 ml) volume of 5 mM HEPES buffer containing 1 mM EDTA (pH 7.4 at 20°C) for 10 s. Each slide was then placed in 100 ml of the same HEPES/EDTA buffer for a further 90 min at 20°C. At the end of this postincubation wash period slides were rapidly dipped in deionized water to remove buffer salts and the excess liquid aspirated from around each section. Slides were finally dried on a slide warmer (37°C) under a flow of room temperature air.

The pharmacologic profile of [<sup>3</sup>H]dextrorphan binding sites was characterized in coronal sections taken from bregma -2.8 mm to -4.8 mm (Paxinos and Watson, 1986). For assessing the regional distribution of [<sup>3</sup>H]dextrorphan binding whole brains sectioned in horizontal, sagittal and coronal planes were studied. Representative sections were later stained with cresyl violet acetate to facilitate the identification of various brain regions in accordance with the atlas of Paxinos and Watson, 1986). With the exception of studies to

determine the effects of 7-chlorokynurenic acid and D-AP5 on [ $^3\text{H}$ ]dextrorphan binding, which were done in the absence of added glutamate and glycine, standard conditions, as described above, were employed in all experiments. For equilibrium saturation studies, samples were taken from the incubation droplet prior to termination of the 4 h incubation period in order to accurately assess the final concentration of unbound radioligand.

### **Autoradiography and data analysis**

Dried sections were placed in x-ray cassettes and apposed to Hyperfilm- $^3\text{H}$  (Amersham; Arlington Heights, IL); plastic tritium standards ([ $^3\text{H}$ ]Micro-scales; Amersham) were included with each film. After a 13 to 15 day exposure period at 20°C, films were developed in D-19 (Kodak; Rochester, New York) for 4 min, fixed in Kodak Rapid Fixer, rinsed and allowed to air dry. Autoradiograms were analyzed by computer-assisted densitometry with a MCID image analysis system (Imaging Research Inc., St. Catharines, Ontario, Canada). [ $^3\text{H}$ ]Dextrorphan concentrations were determined from a standard curve constructed from the tissue equivalent concentrations of the images of the [ $^3\text{H}$ ]plastic standards (Geary and Wooten, 1983). Multiple sites (10-20 readings) were sampled in each brain area from quadruplicate sections (or single sections for time course studies). Specific [ $^3\text{H}$ ]dextrorphan binding was determined by subtracting mean binding in the presence of 100  $\mu\text{M}$  MK-801 from mean total binding values for each particular brain area.

For saturation analysis of [ $^3\text{H}$ ]dextrorphan binding, free radioligand concentrations (1 to 60 nM) were fit to nonspecific binding values for each brain area by linear least squares regression analysis. Corresponding specific binding isotherms were fit to a hyperbolic equation using iterative nonlinear least squares regression analysis. The

parameters of [<sup>3</sup>H]dextrorphan association and dissociation kinetics were determined by fitting the following exponential equations:

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

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

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 constant. Competition data were also analysed by computer-assisted nonlinear least squares regression analysis. Binding potencies ( $IC_{50}$ ) were determined by fitting the following logistic equation to the data:

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

where *E* and  $E_{max}$  are the response and maximal response, respectively, *K* is the  $IC_{50}$ , *X* is the dose and *n* is a slope factor.  $K_i$  values were calculated from  $IC_{50}$  values based on the Cheng-Prusoff equation :  $K_i = IC_{50} / (1 + [L]/K_D)$  where [*L*] is the concentration of [<sup>3</sup>H]dextrorphan and  $K_D$  is the dissociation constant for the particular brain region of interest. Regional differences in [<sup>3</sup>H]dextrorphan binding affinity and the effects of D-AP5 and 7-chlorokynurenic acid on [<sup>3</sup>H]dextrorphan binding were statistically analysed by one-way analysis of variance (ANOVA) followed by post hoc comparisons using the method of Bonferroni.

## Results

### Characterization of [ $^3\text{H}$ ]dextrorphan binding to slide-mounted rat brain sections

A study of preliminary autoradiographic images revealed a particularly high density of specific [ $^3\text{H}$ ]dextrorphan binding sites in the stratum radiatum of the CA1 subfield of the hippocampal formation. As the stratum radiatum of CA1 is not only a discrete and easily quantifiable brain area, but also appears to represent a region of high specific [ $^3\text{H}$ ]dextrorphan binding, this region of the hippocampus was specifically chosen for the initial characterization of [ $^3\text{H}$ ]dextrorphan binding parameters in slide-mounted rat brain sections. Preliminary images also showed that specific [ $^3\text{H}$ ]dextrorphan binding was greatly enhanced in all brain regions when assayed in low ionic strength buffer in tissue depleted of divalent cations. These findings with regard to both the choice of incubation buffer (5 mM HEPES, pH 7.4) and the high binding signal in the hippocampus are consistent with our earlier reports demonstrating [ $^3\text{H}$ ]dextrorphan binding in membranes prepared from rat brain homogenates (Franklin and Murray, 1990; Franklin and Murray, 1992).

In defining a procedure for autoradiographic visualization of [ $^3\text{H}$ ]dextrorphan binding sites initial kinetic studies were undertaken to determine the time course of [ $^3\text{H}$ ]dextrorphan association with its recognition sites in the stratum radiatum of CA1. In the absence of exogenously added glycine and glutamate specific [ $^3\text{H}$ ]dextrorphan binding required approximately 4 h to reach equilibrium at 20°C. Based on this information, sections were allowed to incubate with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h prior to immersion in a large volume (100 ml) of buffer in order to determine the optimum washout time to maximize the ratio of specific to nonspecific binding. The use of an excess volume to initiate dissociation of nonspecifically bound [ $^3\text{H}$ ]dextrorphan from sections (2000-fold dilution assuming 50  $\mu\text{l}$  remains after the incubation droplet has been drained from the section) effectively eliminated rebinding of the radioligand. As shown in figure 5.1 (panel

A), the fraction of nonspecific binding in the stratum radiatum of CA1 was reduced in a time-dependent manner by rinsing sections at 20°C. This kind of profile has been described previously for [<sup>3</sup>H]MK-801 binding in sections of brain mash (Subramaniam and McGonigle, 1991). As highlighted in figure 5.1 (panel B), specific [<sup>3</sup>H]dextrorphan binding could not be detected following postincubation wash times of less than 10 min at 20°C, but did manifest at wash times greater than 10 min. Maximum signal to noise ratios were obtained in the stratum radiatum of the CA1 subfield of the hippocampus by approximately 75 min and remained stable for up to 260 min. Reducing the temperature of the postincubation wash procedure to 4°C slowed the dissociation rate of nonspecifically bound [<sup>3</sup>H]dextrorphan considerably; maximum specific to nonspecific binding ratios were achieved at 180 min (data not shown). Attempts to expose slide-mounted sections, under our standard binding conditions, to wash temperatures of 37°C resulted in damage to the sections. Based on these data, a postincubation wash time of 90 min at 20°C was used for all subsequent studies. Independent determination of the dissociation rate of specific [<sup>3</sup>H]dextrorphan binding from the stratum radiatum of the CA1 subfield of the hippocampus by nonlinear regression analysis revealed a monophasic process with an estimated dissociation rate constant of  $0.005 \pm 0.001 \text{ min}^{-1}$  at 20°C (data not shown).

Using the optimized assay conditions the association kinetics of specific [<sup>3</sup>H]dextrorphan binding were studied in the presence and absence of exogenous glycine and glutamate (Figure 5.2). In the absence of exogenous glycine and glutamate at 20°C the association reaction of [<sup>3</sup>H]dextrorphan in the stratum radiatum of the CA1 subfield of the hippocampus was found to be monophasic by nonlinear regression analysis with an apparent  $t_{0.5}$  of 46 min. Equilibrium, was obtained under these conditions at approximately 4 h and then remained stable until at least 8 h. The inclusion of 100  $\mu\text{M}$  glycine and 1  $\mu\text{M}$  glutamate in the incubation droplet produced a marked increase in the apparent association rate of [<sup>3</sup>H]dextrorphan with its binding sites, decreasing the  $t_{0.5}$  to 21 min. Maximal specific [<sup>3</sup>H]dextrorphan binding levels in the stratum radiatum of CA1

were unchanged in the presence of exogenous glycine and glutamate; however they were routinely included in the binding assay in order to ensure that equilibrium was reached by 4 h.

To address the regional distributions of [ $^3\text{H}$ ]dextrorphan binding sites in brain, saturation analysis of [ $^3\text{H}$ ]dextrorphan binding was performed. Specific binding in the stratum radiatum of the CA1 region of the hippocampus accounted for approximately 64-45 % of total binding at [ $^3\text{H}$ ]dextrorphan concentrations ranging 5-60 nM. Nonlinear regression analysis of equilibrium saturation binding isotherms from a number of different brain regions indicated that in each region [ $^3\text{H}$ ]dextrorphan appears to label a single population of high affinity binding sites (Figure 5.3). Binding affinities ranged from  $25.3 \pm 8.8$  nM in the inner frontoparietal cortex (layers IV-VI) to  $48.7 \pm 12.2$  nM in the stratum radiatum of CA1 (Table 5.1). Significant variation among brain regions was found in the densities of [ $^3\text{H}$ ]dextrorphan binding sites, which ranged from  $490 \pm 68$  fmol/mg tissue in the stratum radiatum of CA1 to  $113 \pm 16$  fmol/mg tissue in the lateral thalamic nuclei (Table 5.1 and Figure 5.3).

### **Regulation of [ $^3\text{H}$ ]dextrorphan binding by glycine and glutamate**

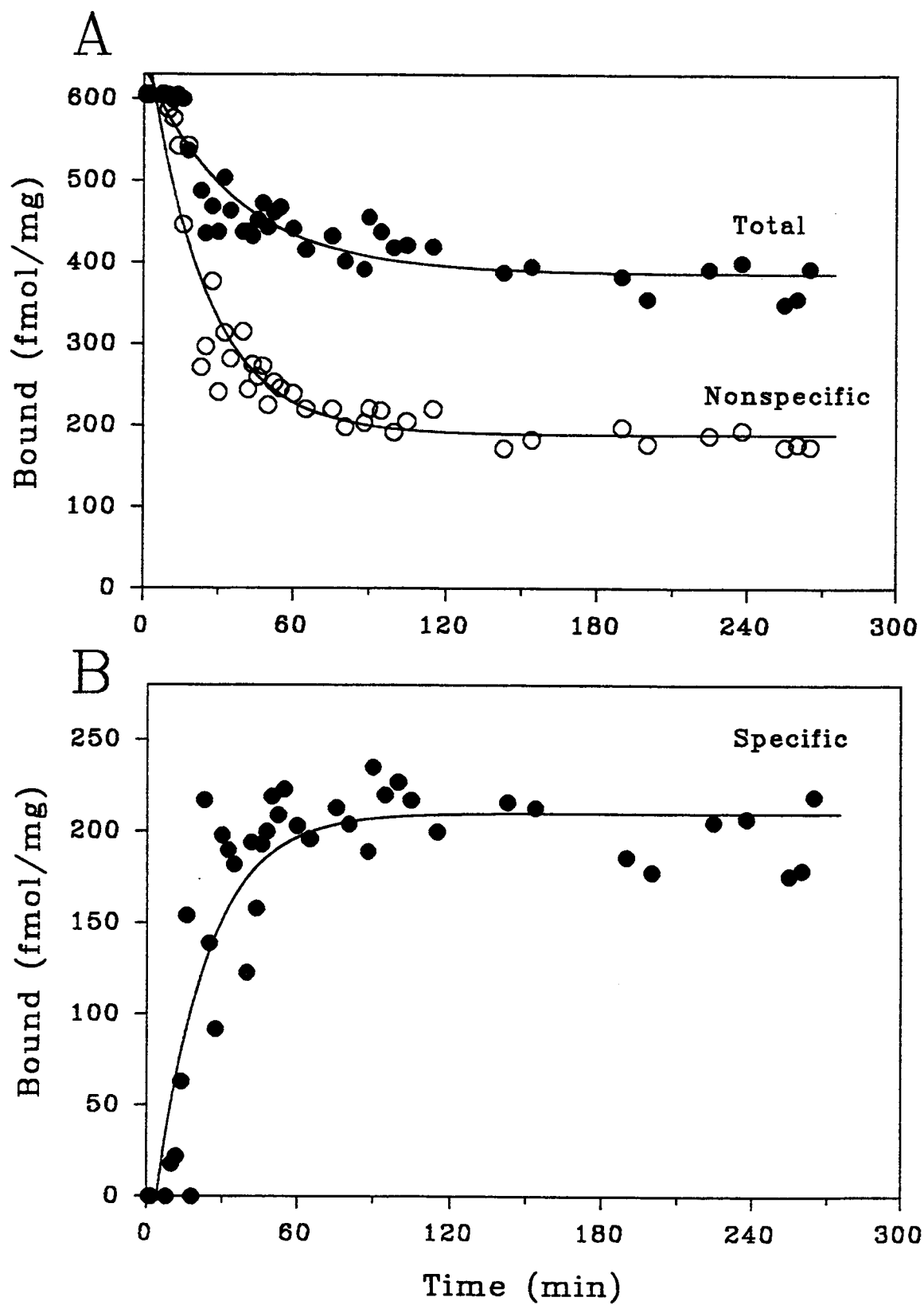
Our previous studies in membrane homogenates have shown that the regulation of [ $^3\text{H}$ ]dextrorphan binding by  $\text{Mg}^{2+}$  and the polyamine agonist spermidine is insensitive to the presence of glycine and glutamate (Franklin and Murray, 1992). Whereas the potencies of  $\text{Mg}^{2+}$  and spermidine as modulators of [ $^3\text{H}$ ]TCP and [ $^3\text{H}$ ]MK-801 binding are significantly increased by the presence of exogenous glycine and glutamate in well washed membranes, their marked potencies as regulators of [ $^3\text{H}$ ]dextrorphan binding are unaffected by the presence or absence of these substances.



**FIGURE 5.1.****Determination of optimal postincubation wash time.**

The effect of rinse time on [ $^3\text{H}$ ]dextrorphan binding was measured in the stratum radiatum of the CA1 region of the rat hippocampus. Coronal sections (16- $\mu\text{m}$ ) were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h at 20°C in the presence of glycine (100  $\mu\text{M}$ ) and glutamate (1  $\mu\text{M}$ ). Following incubation sections were washed at 20°C in 5 mM HEPES buffer containing 1 mM EDTA for various time periods as indicated on the abscissa. (A) Time course of total [ $^3\text{H}$ ]dextrorphan binding (closed circles) and time course of nonspecific binding (open circles) defined in the presence of 100  $\mu\text{M}$  MK-801. Each point represents mean [ $^3\text{H}$ ]dextrorphan binding determined in single sections from a representative experiment. (B) Specific [ $^3\text{H}$ ]dextrorphan binding profile (total minus nonspecific binding).

FIGURE 5.1.

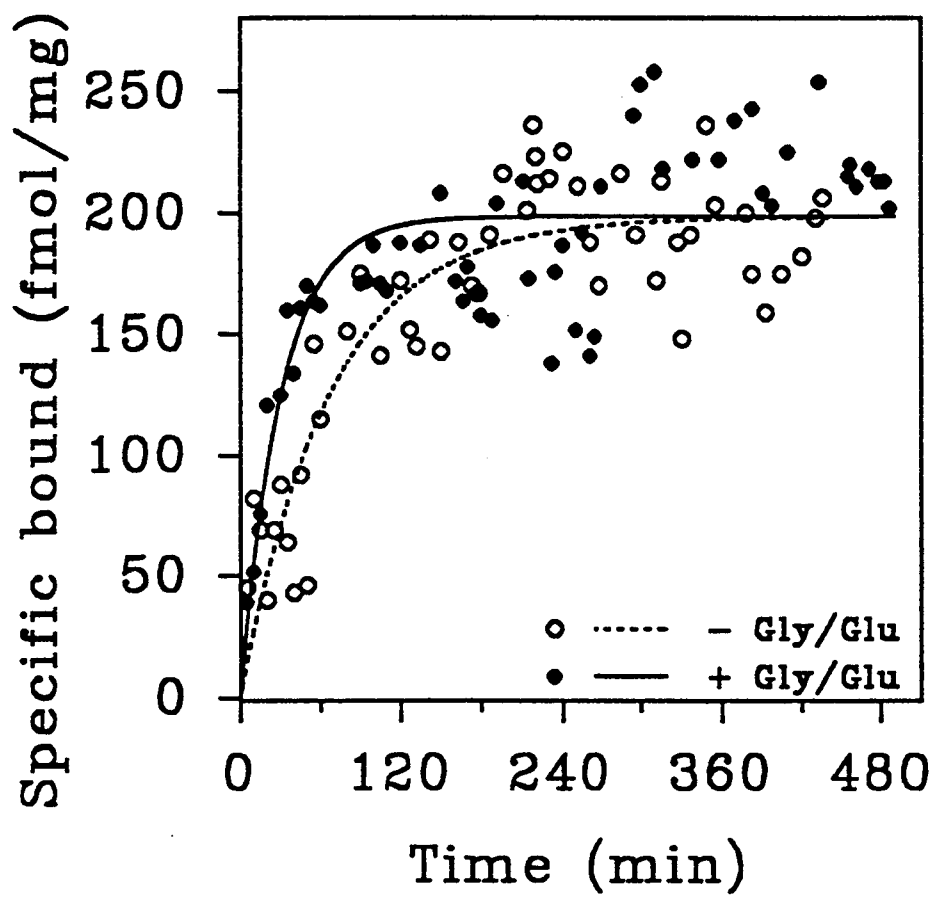


**FIGURE 5.2.**

**Time course of association of specific [ $^3\text{H}$ ]dextrorphan binding to stratum radiatum of the CA1 region of the rat hippocampus.**

Coronal sections (two animals) were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan either in the absence (open circle/dotted line) or presence (closed circles/solid line) of glycine (100  $\mu\text{M}$ ) and glutamate (1  $\mu\text{M}$ ). Incubation was allowed to proceed under humid conditions at 20°C for various time points as indicated on the abscissa. Sections were then washed for 90 min at 20°C in 5mM HEPES buffer containing 1 mM EDTA. Each point represents specific [ $^3\text{H}$ ]dextrorphan binding in single brain sections from a representative experiment. Nonspecific binding was defined by 100  $\mu\text{M}$  MK-801.

FIGURE 5.2.

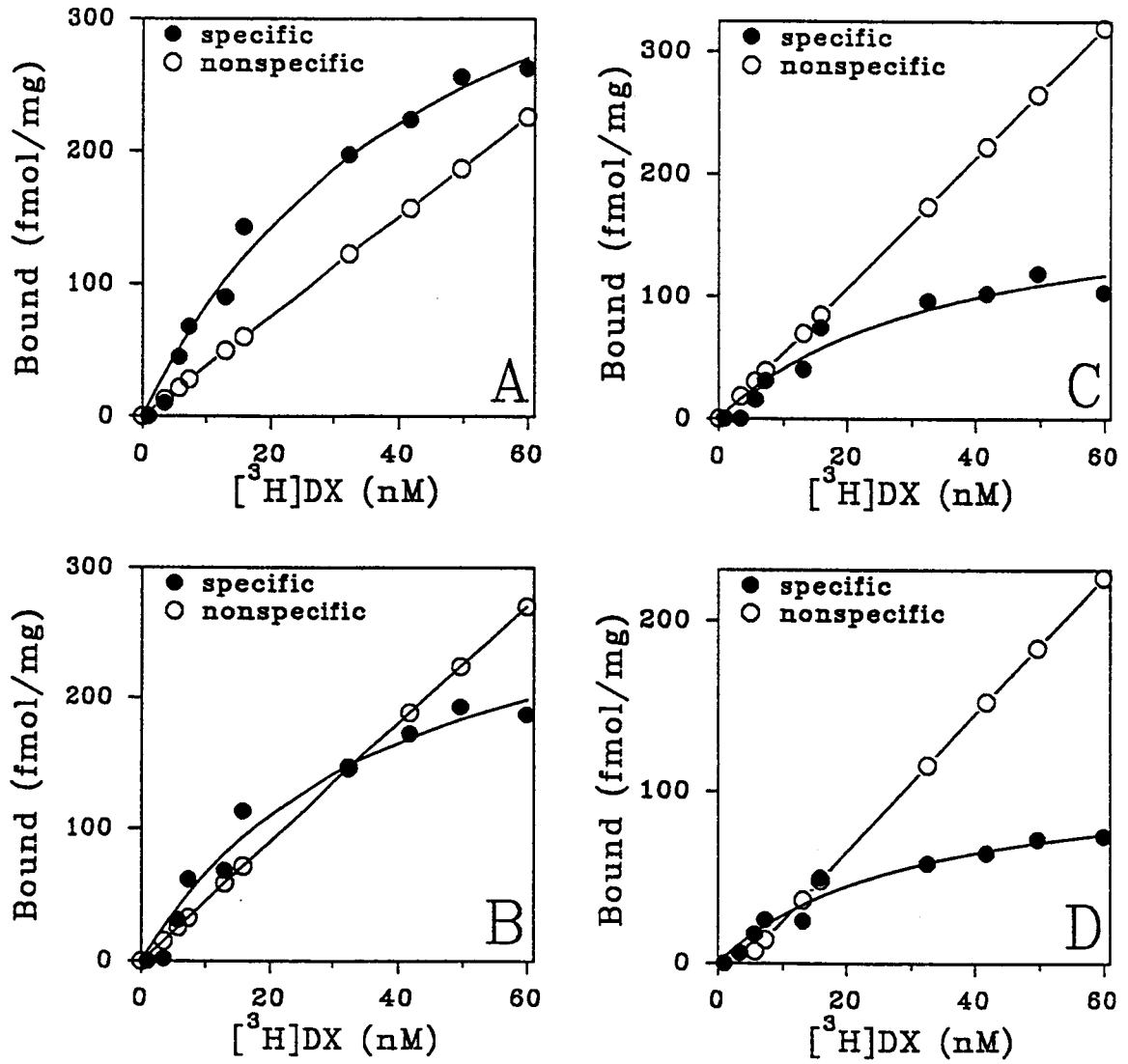


**FIGURE 5.3.**

**[<sup>3</sup>H]Dextrorphan equilibrium saturation binding isotherms from rat brain at the level of: (A) stratum radiatum of the CA1 region of the hippocampus, (B) dentate gyrus (molecular layer), (C) outer frontoparietal cortex (layers II-III) and (D) lateral thalamus.**

Coronal sections were incubated with increasing concentrations of [<sup>3</sup>H]dextrorphan for 4 h at 20°C in the presence of added glycine (100 µM) and glutamate (1µM). Incubation droplets were sampled at 4 h to determine the free radioligand concentration as indicated on the abscissa. Sections were washed for 90 min at 20°C in 5 mM HEPES buffer containing 1 mM EDTA. Each point represents the mean [<sup>3</sup>H]dextrorphan binding determined in quadruplicate brain sections from a representative experiment repeated twice with similar results. Nonspecific binding was defined by 100 µM MK-801.

FIGURE 5.3.



**TABLE 5.1.**

**Equilibrium saturation binding of [<sup>3</sup>H]dextrorphan in various brain regions**

| Brain region                    | $K_D \pm \text{S.E.}$<br>nM | $B_{\max} \pm \text{S.E.}^a$<br>fmol/mg |
|---------------------------------|-----------------------------|---|
| Hippocampus (CA1 radiatum)      | $48.7 \pm 12.2$             | $490 \pm 68$                            |
| Dentate gyrus (molecular layer) | $40.5 \pm 13.5$             | $332 \pm 58$                            |
| Outer cortex (layers II-III)    | $36.2 \pm 16.1$             | $189 \pm 42$                            |
| Inner cortex (layers IV-VI)     | $25.3 \pm 8.8$              | $134 \pm 20$                            |
| Lateral thalamus <sup>b</sup>   | $30.3 \pm 9.4$              | $113 \pm 16$                            |

[<sup>3</sup>H]Dextrorphan binding and autoradiographic procedures were followed as described in Materials and Methods.  $K_D$  and  $B_{\max}$  values are derived from nonlinear regression analysis-based fitting of a hyperbolic equation to the data.

<sup>a</sup> Regional density values are expressed as [<sup>3</sup>H]dextrorphan bound in fmol/mg tissue (wet weight) by calibrating the images produced by the tritium-embedded plastic standards for each film to their known tissue equivalent concentrations.

<sup>b</sup> Includes ventroposteromedial, ventral posterolateral and ventrolateral thalamic nuclei.

The inability of exogenously added glycine and glutamate, at equilibrium, to significantly enhance maximal specific binding of noncompetitive NMDA antagonists in slide-mounted brain sections has been attributed to the presence of high levels of endogenous glycine and glutamate remaining in the sections. It is possible, therefore, that the effects of exogenous glycine and glutamate on [<sup>3</sup>H]dextrorphan binding are blunted under our standard assay conditions. In practice, it is not feasible to deplete slide-mounted of endogenous glycine and glutamate as washing procedures have to be curbed in the interests of preserving the anatomical integrity of the tissue.

To address this issue we compared the effects of glycine and glutamate in sections that, prior to the radioligand incubation, had been pre-washed for 1 h at 20°C or extensively washed for an additional 16 h at 4°C. The magnitude of specific [ $^3\text{H}$ ]dextrorphan binding at equilibrium appears, however, to be relatively insensitive to added glycine and glutamate even after prolonged pre-washing of the sections (Figure 5.4). Under standard pre-wash conditions some concentrations of glutamate modestly enhanced specific [ $^3\text{H}$ ]dextrorphan binding in the stratum radiatum of CA1 by 20 to 35 %, but these increases were not significant. Following extensive pre-washing of sections, the addition of glutamate consistently produced very modest increments in [ $^3\text{H}$ ]dextrorphan binding which were not statistically significant (Figure 5.4A). Exogenous glycine produced no significant change in specific [ $^3\text{H}$ ]dextrorphan binding levels in the stratum radiatum of CA1 following standard or prolonged pre-washing of brain sections (Figure 5.4B). Other brain regions were equally insensitive to the addition of glycine and glutamate despite attempts to remove endogenous factors by extensive pre-washing of the sections. Specific [ $^3\text{H}$ ]dextrorphan binding, measured under equilibrium conditions, in the molecular layer of the dentate gyrus, outer (II-III) and inner (IV-VI) layers of the frontoparietal cortex and the lateral thalamic nuclei was not significantly changed by the addition of glycine or glutamate (data not shown).

The modest stimulation of [ $^3\text{H}$ ]dextrorphan binding by glycine and glutamate following extensive washing protocols suggests that specific [ $^3\text{H}$ ]dextrorphan binding is unusually insensitive to glycine and glutamate. This is in marked contrast to the binding properties of [ $^3\text{H}$ ]MK-801 characterized under equilibrium conditions by Sakurai et al. (1991) in extensively washed sections. In that study, 100  $\mu\text{M}$  NMDA was reported to enhance [ $^3\text{H}$ ]MK-801 binding in the stratum radiatum of the CA1 subfield of the hippocampus and molecular layer of the dentate gyrus by 181 % and 214 % respectively, whilst 100  $\mu\text{M}$  glycine alone was reported to increase [ $^3\text{H}$ ]MK-801 binding 255 % in the molecular layer of the dentate gyrus.

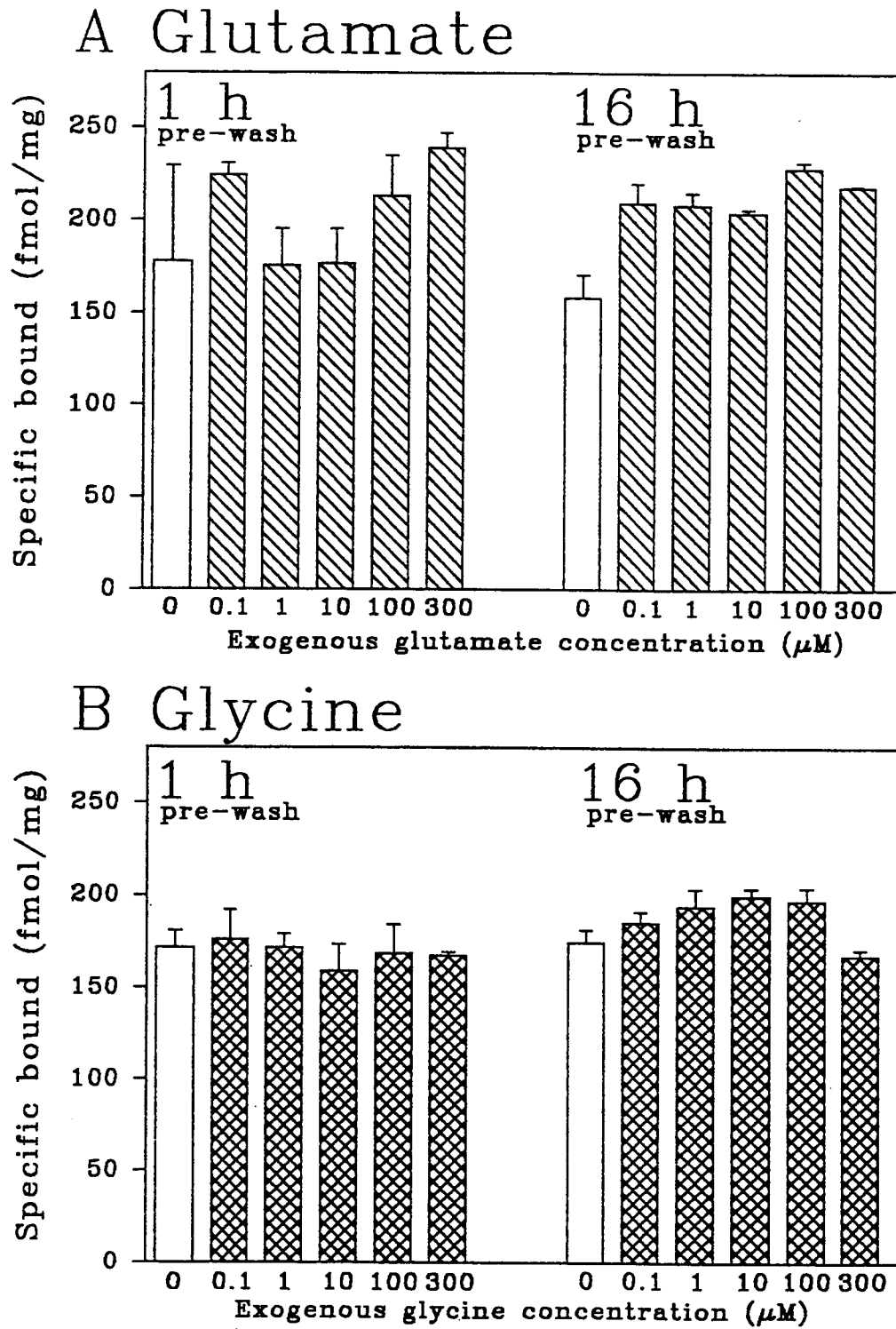


**FIGURE 5.4.**

**Effect of (A) glutamate and (B) glycine on specific [ $^3\text{H}$ ]dextrorphan binding to stratum radiatum of the CA1 region of the hippocampus following standard (1 h) or prolonged (16 h) pre-washing of slide-mounted sections.**

Coronal rat brain sections were washed in 5 mM HEPES (pH 7.4) either for 3 x 20 min at 20°C or for an extra 16 h at 4°C prior to the standard pre-washing procedure before being allowed to air dry. Pre-washed sections were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h either in the absence (white bars) or in the presence (hatched bars) of increasing concentrations (0.1-300  $\mu\text{M}$ ) of glutamate or glycine. Bars represent mean specific [ $^3\text{H}$ ]dextrorphan binding  $\pm$  S.E. determined in quadruplicate sections from a representative experiment. Nonspecific binding was defined by 100  $\mu\text{M}$  MK-801.

FIGURE 5.4.



In contrast to the lack of effect of exogenous glycine and glutamate, [ $^3\text{H}$ ]dextrorphan binding was potently inhibited in a concentration-dependent manner both by the strychnine-insensitive glycine site antagonist 7-chlorokynurenic acid (Figure 5.5) and the competitive NMDA antagonist D-AP5 (Figure 5.6) when measured in the absence of added glycine and glutamate. These data suggest that the NMDA receptor agonist and co-agonist sites are saturated by endogenous glutamate and glycine respectively and that activation of these sites is an absolute requirement for specific [ $^3\text{H}$ ]dextrorphan binding to occur. This occupancy of the agonist and co-agonist sites by endogenous glutamate and glycine is surmountable in the presence of increasing concentrations of 7-chlorokynurenic acid and D-AP5

The inhibition of [ $^3\text{H}$ ]dextrorphan binding by 7-chlorokynurenic acid was studied in detail in four different brain areas (Figure 5.5). The inhibitory potencies of 7-chlorokynurenic acid to suppress [ $^3\text{H}$ ]dextrorphan binding in the stratum radiatum of the CA1 region of the hippocampus, lateral thalamus or outer (II-III) and inner (IV-VI) layers of the frontoparietal cortex were similar (Table 5.2). The potency of 7-chlorokynurenic acid to inhibit [ $^3\text{H}$ ]dextrorphan binding in the inner cortex ( $K_i = 2.8 \pm 0.7 \mu\text{M}$ ) was, however, significantly different from that of the stratum radiatum of hippocampal area CA1 ( $6.8 \pm 0.6 \mu\text{M}$ ) ( $P < 0.05$ , Bonferroni procedure). D-AP5, moreover, exhibited a moremarked regional variation ( $P < 0.0001$ , ANOVA) in its ability to inhibit [ $^3\text{H}$ ]dextrorphan binding when studied in the same four brain areas (Figure 5.6 and Table 5.2). Specific [ $^3\text{H}$ ]dextrorphan binding was most potently inhibited by D-AP5 in the cortical layers, with  $K_i$  values of  $0.89 \pm 0.8 \mu\text{M}$  (layers II-III) and  $0.85 \pm 0.6 \mu\text{M}$  (layers IV-VI). D-AP5 inhibition of [ $^3\text{H}$ ]dextrorphan binding was significantly less potent than cortex ( $P < 0.001$ , Bonferroni procedure) in the stratum radiatum of CA1 ( $11.2 \pm 0.8 \mu\text{M}$ ) and approximately 20-fold less potent ( $P < 0.001$ , Bonferroni procedure) than the cortex in the lateral thalamic nuclei ( $20.0 \pm 0.8 \mu\text{M}$ ).

**FIGURE 5.5.****Regional inhibition of specific [ $^3\text{H}$ ]dextrorphan binding by 7-chlorokynurenic acid in rat brain.**

Coronal sections were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h at 20°C in the presence of increasing concentrations of 7-chlorokynurenic acid (7-Cl-KYNA) as indicated on the abscissa. Sections were then washed for 90 min at 20°C in 5 mM HEPES buffer containing 1 mM EDTA. The symbols describe the actual data points for each brain region and the curves are expanded from the parameter estimates derived from fitting a logistic equation to the data by means of iterative nonlinear least squares regression analysis. Each point represents mean specific [ $^3\text{H}$ ]dextrorphan binding  $\pm$  S.E. determined in quadruplicate sections from a representative experiment. Nonspecific binding was defined by 100  $\mu\text{M}$  MK-801.

**FIGURE 5.6.****Regional inhibition of specific [ $^3\text{H}$ ]dextrorphan binding by D-AP5 in rat brain.**

Coronal sections were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h at 20°C in the presence of increasing concentrations of D-AP5 as indicated on the abscissa. Sections were then washed for 90 min at 20°C in 5 mM HEPES buffer containing 1 mM EDTA. The symbols describe the actual data points for each brain region and the curves are expanded from the parameter estimates derived from fitting a logistic equation to the data by means of iterative nonlinear least squares regression analysis. Each point represents mean specific [ $^3\text{H}$ ]dextrorphan binding  $\pm$  S.E. determined in quadruplicate sections from a representative experiment. Nonspecific binding was defined by 100  $\mu\text{M}$  MK-801.

FIGURE 5.5.

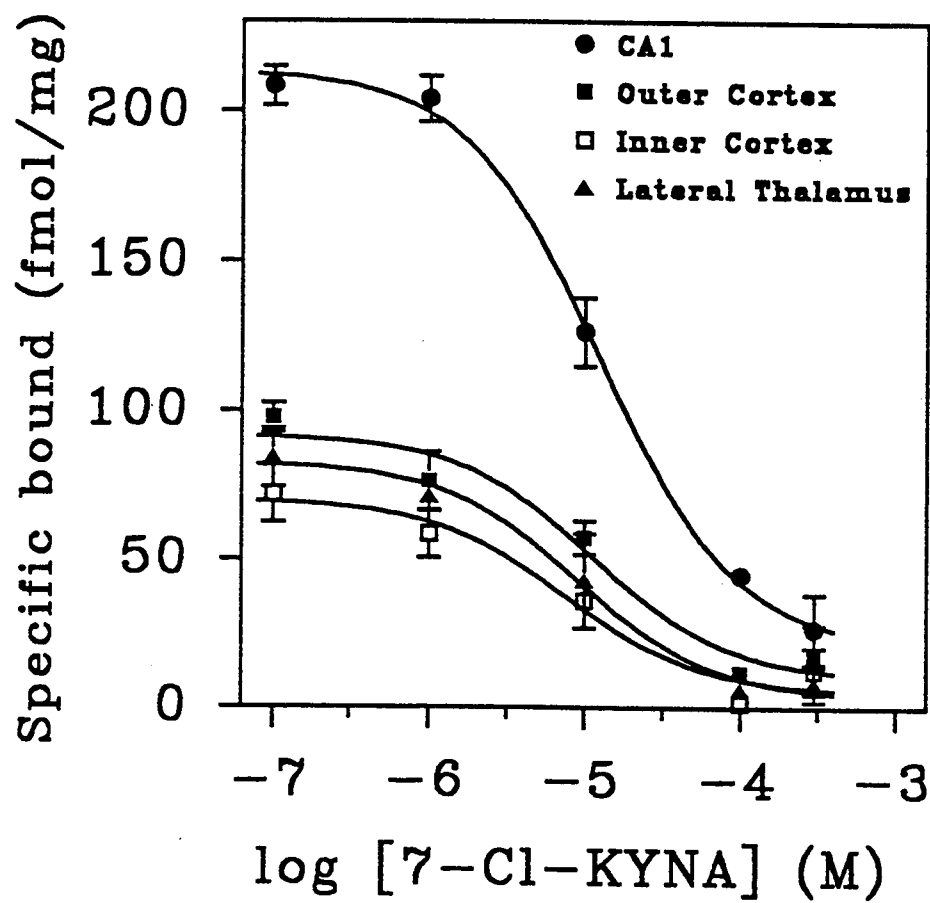
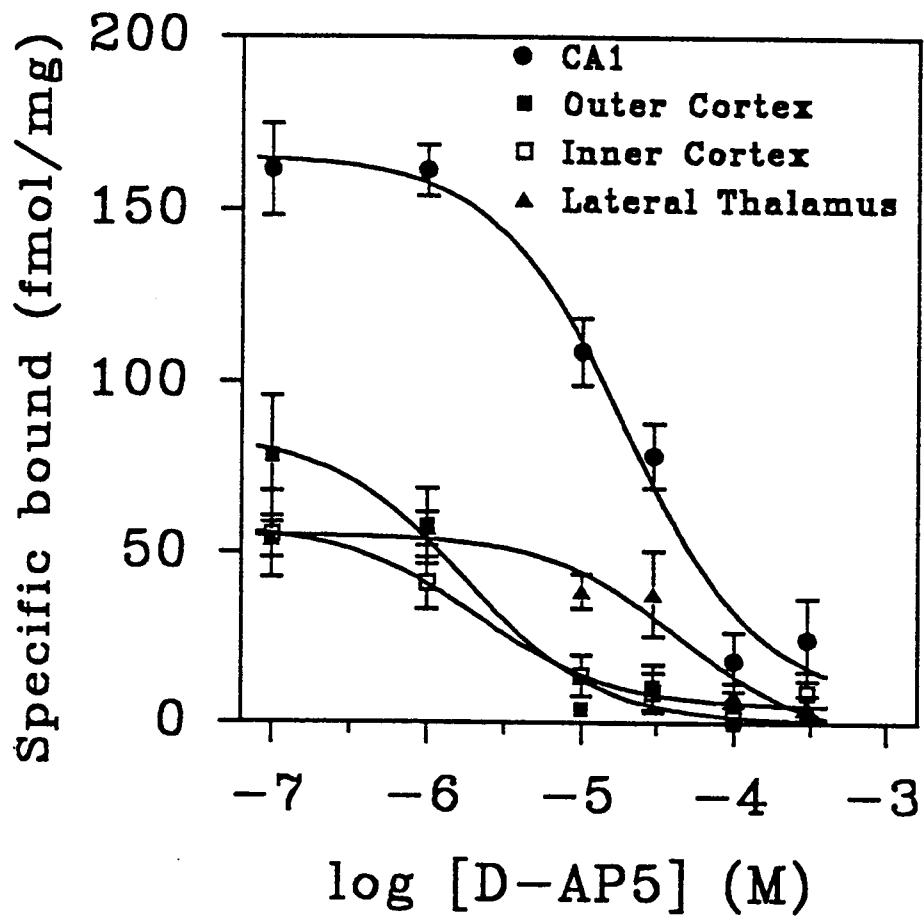


FIGURE 5.6.



**TABLE 5.2.**

**Potencies of 7-chlorokynurenic acid and D-AP5 to inhibit  
[<sup>3</sup>H]dextrorphan binding in various brain regions**

| Brain region                  | 7-Cl-KA                                | D-AP5                                  |
|-------------------------------|--|--|
|                               | $K_i \pm \text{S.E.}$<br>$\mu\text{M}$ | $K_i \pm \text{S.E.}$<br>$\mu\text{M}$ |
| Hippocampus (CA1 radiatum)    | $6.8 \pm 0.6^a$                        | $11.2 \pm 0.8^b$                       |
| Outer cortex (layers II-III)  | $5.2 \pm 0.8$                          | $0.89 \pm 0.8$                         |
| Inner cortex (layers IV-VI)   | $2.8 \pm 0.7$                          | $0.85 \pm 0.6$                         |
| Lateral thalamus <sup>d</sup> | $3.8 \pm 0.5$                          | $20.0 \pm 0.8^c$                       |

[<sup>3</sup>H]Dextrorphan binding and autoradiographic procedures were followed as described in Materials and Methods. Data were fit to a one-site logistic equation by iterative nonlinear least squares regression analysis. Differences between  $K_i$  values were significantly different for 7-chlorokynurenic acid at  $P < 0.001$  and D-AP5 at  $P < 0.0001$  by ANOVA and post tests using the method of Bonferonni as indicated.

<sup>a</sup> Significantly different than inner cortex ( $P < 0.05$ ).

<sup>b</sup> Significantly different than cortical areas ( $P < 0.001$ ).

<sup>c</sup> Significantly different than stratum radiatum of CA1 ( $P < 0.01$ ) and cortical areas ( $P < 0.001$ ).

<sup>d</sup> Includes ventroposteromedial, ventral posterolateral and ventrolateral thalamic nuclei.

### **Pharmacologic profile of [<sup>3</sup>H]dextrorphan binding**

In order to establish the pharmacologic specificity of [<sup>3</sup>H]dextrorphan binding, we evaluated concentration-response relationships of a number of noncompetitive NMDA antagonists as competitors for specific [<sup>3</sup>H]dextrorphan equilibrium binding. Competition curves are shown for two brain areas that exhibit high densities of [<sup>3</sup>H]dextrorphan binding sites, the stratum radiatum of hippocampal area CA1 (Figure 5.7) and the outer layers of the frontoparietal cortex (Figure 5.8). In contrast to our findings in membrane

homogenates (Franklin and Murray, 1992), all compounds displaced [ $^3\text{H}$ ]dextrorphan in a monophasic manner suggesting interaction with a single high-affinity binding site in both brain areas. Although quantitative receptor autoradiography permits the pharmacologic characterization of discrete brain regions, long postincubation wash periods such as we employed here may not permit detection of a low affinity [ $^3\text{H}$ ]dextrorphan binding sites in slide-mounted tissue.

With the exception of unlabelled dextrorphan, all compounds were either similar (MK-801, TCP) or less potent (PCP, dextromethorphan) inhibitors of [ $^3\text{H}$ ]dextrorphan binding in the outer cortex than in the stratum radiatum of CA1 (Table 5.3). MK-801 was the most potent inhibitor of [ $^3\text{H}$ ]dextrorphan binding both in the stratum radiatum of hippocampal area CA1 and the outer cortex, with  $K_i$  values of  $2.5 \pm 0.6$  nM and  $2.7 \pm 0.6$  nM respectively. The rank order of potency for displacement of [ $^3\text{H}$ ]dextrorphan binding in the stratum radiatum of CA1 was as follows: MK-801 > TCP > PCP > dextrorphan > ketamine > dextromethorphan (Table 5.3). A similar pattern was observed in the outer cortex: MK-801 > TCP > dextrorphan > PCP > ketamine > dextromethorphan, but with dextrorphan being approximately 2-fold more potent than PCP,  $K_i$  values were  $33.5 \pm 0.7$  nM and  $60.1 \pm 0.6$  nM respectively (Table 5.3). Of the compounds tested dextromethorphan was the least potent inhibitor of [ $^3\text{H}$ ]dextrorphan binding; dextromethorphan was over 6-fold less potent than dextrorphan in the stratum radiatum of CA1. The potent inhibitory effects of dextrorphan in the outer cortex, however, render dextromethorphan approximately 30-fold less potent than dextrorphan in this brain area.



**FIGURE 5.7.**

**[<sup>3</sup>H]Dextrorphan competition binding in stratum radiatum of the CA1 region of rat hippocampus.**

Sections were incubated with 40 nM [<sup>3</sup>H]dextrorphan for 4 h at 20°C in the presence of added glycine (100 µM) and glutamate (1 µM). Sections were then washed for 90 min in 5 mM HEPES buffer containing 1 mM EDTA. Binding of MK-801, TCP, PCP, dextrorphan (DX), ketamine and dextromethorphan (DXM) is depicted by plots of the actual data points. The respective curves are expanded from the parameter estimates derived from fitting a logistic equation to the data by means of iterative nonlinear least squares regression analysis. Each point represents mean specific [<sup>3</sup>H]dextrorphan binding determined in quadruplicate sections from a representative experiment. The S.E. for each point was less than 10 % of the mean. Nonspecific binding was defined by 100 µM MK-801.

**FIGURE 5.8.**

**[<sup>3</sup>H]Dextrorphan competition binding in outer frontoparietal cortical layers (II-III) of rat brain.**

Sections were incubated with 40 nM [<sup>3</sup>H]dextrorphan for 4 h at 20°C in the presence of added glycine (100 µM) and glutamate (1 µM). Sections were then washed for 90 min in 5 mM HEPES buffer containing 1 mM EDTA. Binding of MK-801, TCP, dextrorphan (DX), PCP, ketamine and dextromethorphan (DXM) is depicted by plots of the actual data points. The respective curves are expanded from the parameter estimates derived from fitting a logistic equation to the data by means of iterative nonlinear least squares regression analysis. Each point represents mean specific [<sup>3</sup>H]dextrorphan binding determined in quadruplicate sections from a representative experiment. The S.E. for each point was less than 10 % of the mean. Nonspecific binding was defined by 100 µM MK-801.

FIGURE 5.7.

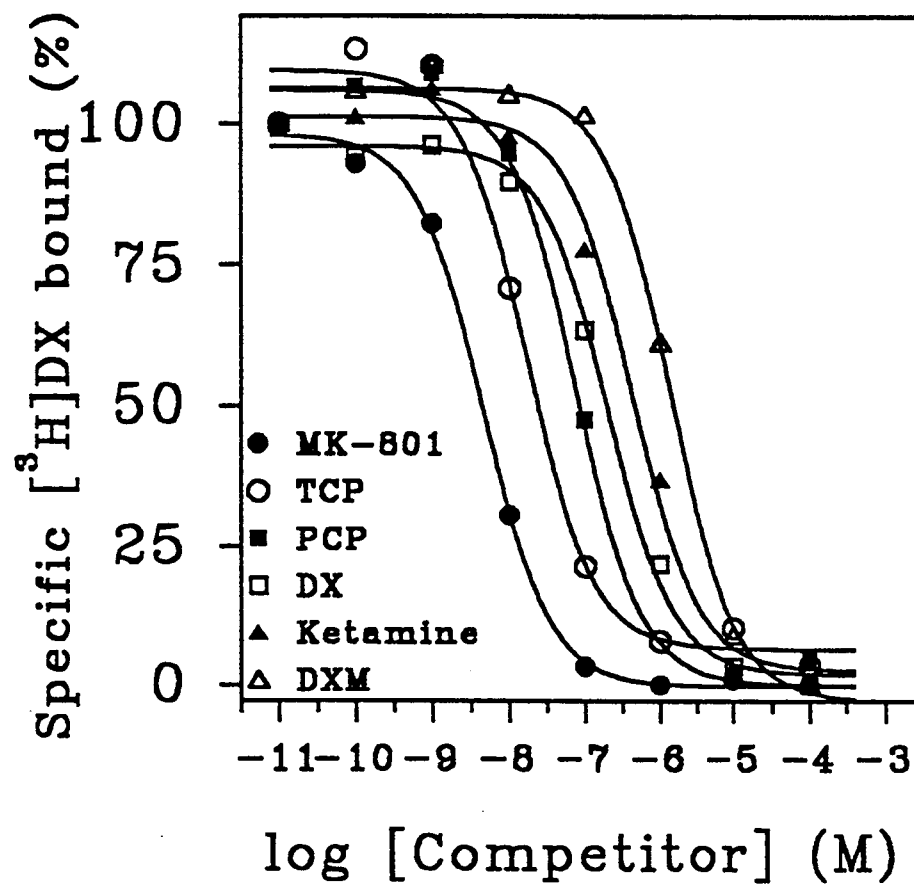
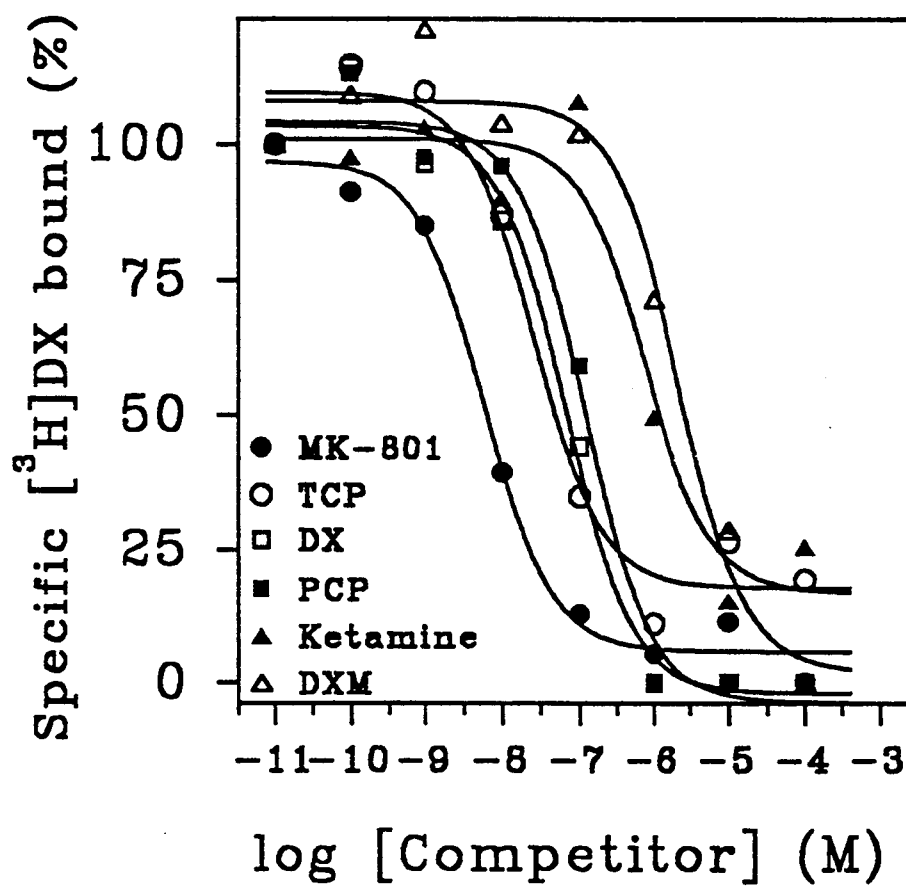


FIGURE 5.8.



**TABLE 5.3.**

**Potencies of competitors for [<sup>3</sup>H]dextrorphan binding to stratum radiatum of the CA1 region of the hippocampus and outer cortical layers (II-III)**

| Compound         | CA1<br>$K_i \pm \text{S.E}$<br>nM | Outer cortex<br>$K_i \pm \text{S.E.}$<br>nM |
|------------------|-----------------------------------|---|
| MK-801           | $2.5 \pm 0.6$                     | $2.7 \pm 0.6$                               |
| TCP              | $9.4 \pm 0.6$                     | $12.2 \pm 0.7$                              |
| PCP              | $43.3 \pm 0.7$                    | $60.1 \pm 0.6$                              |
| Dextrorphan      | $116.6 \pm 0.6$                   | $33.5 \pm 0.7$                              |
| Ketamine         | $231.7 \pm 0.7$                   | $360.0 \pm 0.9$                             |
| Dextromethorphan | $760 \pm 0.6$                     | $1037 \pm 0.3$                              |

[<sup>3</sup>H]Dextrorphan binding and autoradiographic procedures were followed as described in Materials and Methods. Data were fit to a one-site logistic equation by iterative nonlinear least squares regression analysis.

### **Regional distribution of [<sup>3</sup>H]dextrorphan binding sites**

To further assess the regional distribution of [<sup>3</sup>H]dextrorphan binding sites the specific binding of a fixed concentration of the radioligand was determined in 23 brain structures. The representative autoradiograms shown in figure 5.9 were generated from adjacent coronal brain sections; total [<sup>3</sup>H]dextrorphan binding is shown in panel A and nonspecific binding measured in the presence of 100  $\mu\text{M}$  MK-801 in panel B. These colour-coded images clearly show high densities of [<sup>3</sup>H]dextrorphan binding sites localized in distinct regions of the hippocampal formation and the superficial layers of the cortex. In

addition, moderate levels of [ $^3\text{H}$ ]dextrorphan binding are found in the deeper layers of the cerebral cortex and the thalamic nuclei.

A summary of the neuroanatomical distribution of [ $^3\text{H}$ ]dextrorphan binding sites is presented in Table 5.4. These data are in agreement with the differences observed in relative densities of [ $^3\text{H}$ ]dextrorphan binding sites determined by equilibrium saturation analysis: stratum radiatum (CA1) > dentate gyrus > outer cortex > inner cortex > thalamus (Table 5.1). The highest density of specific [ $^3\text{H}$ ]dextrorphan binding was seen in the stratum radiatum of the CA1 region of the hippocampus ( $181.5 \pm 14.6$  fmol/mg). Binding densities were also high in the stratum oriens of CA1 and the molecular layer of the dentate gyrus, and somewhat lower in the stratum lacunosum moleculare of CA1, stratum radiatum of CA3 and stratum oriens of CA3 as shown in detail in figure 5.10 (A-C). In contrast, the stratum pyramidale and the granule cell layer of the dentate gyrus, which correspond to the dense cell body layers of these areas, showed lower levels of [ $^3\text{H}$ ]dextrorphan binding (Figure 5.10 A-C).

Two other areas of relatively high specific [ $^3\text{H}$ ]dextrorphan binding were the outer layers of the cortex (II-III) and the molecular layer of the cerebellum (Table 5.4). In both these brain regions the density of binding sites was approximately 50 % of that measured in the stratum radiatum of the CA1 region of the hippocampus. Deeper layers (IV-VI) of the cortex showed less [ $^3\text{H}$ ]dextrorphan binding than the outer layers (II-III). Whilst the high levels of [ $^3\text{H}$ ]dextrorphan binding observed in the superficial layers of the cortex, together with the pronounced distribution of sites in the hippocampal formation, are largely consistent with other autoradiographic studies that have employed a variety of radioligands to label the NMDA receptor, our findings with regard to the cerebellum represent a pattern unique to [ $^3\text{H}$ ]dextrorphan binding.

**FIGURE 5.9.**

**Representative autoradiograms depicting (A) total [ $^3\text{H}$ ]dextrorphan binding and (B) nonspecific binding measured in the presence of 100  $\mu\text{M}$  MK-801.**

Adjacent coronal rat brain sections (16- $\mu\text{m}$ ) were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h at 20°C in the presence of glycine (100  $\mu\text{M}$ ) and glutamate (1  $\mu\text{M}$ ). Sections were then washed for 90 min at 20°C in 5 mM HEPES buffer containing 1 mM EDTA, dried and apposed to tritium-sensitive film for 13 days with a complete set of tritium-standards of known radioactivity. Colour has been assigned to the scale representing binding site density so that the highest levels of [ $^3\text{H}$ ]dextrorphan binding appear red to orange and the lowest levels appear blue to pink. The dense sporadic labelling around the outer edges of the sections is also seen in areas where the tissue section has been damaged or in and around the ventricles and is an artifact of interaction of [ $^3\text{H}$ ]dextrorphan with the embedding medium. This phenomenon is not unique to [ $^3\text{H}$ ]dextrorphan and has been described previously for other ligands such as [ $^3\text{H}$ ]TCP (McDonald et al., 1989).

FIGURE 5.9.

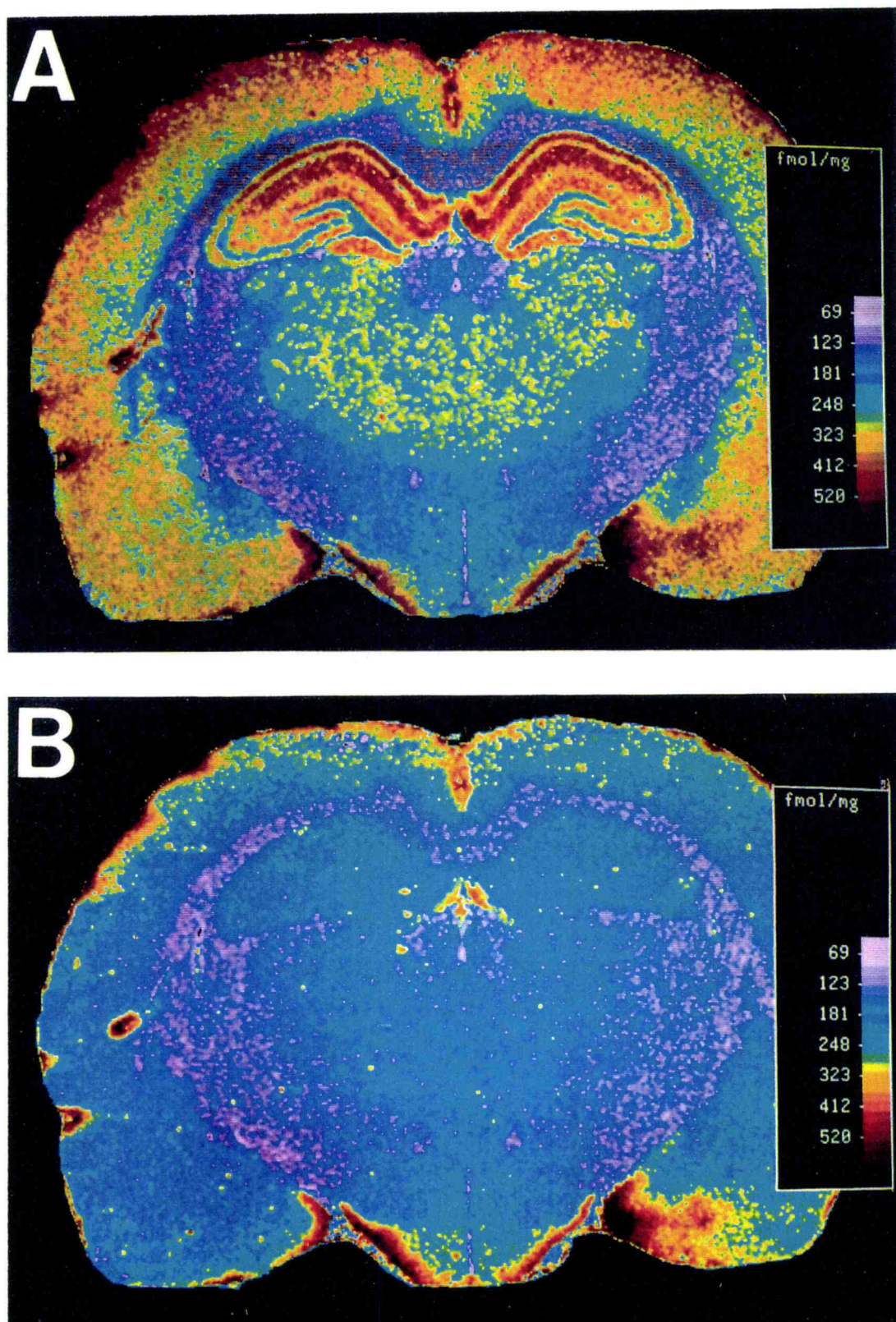


TABLE 5.4.

Regional distribution of [ $^3\text{H}$ ]dextrorphan ([ $^3\text{H}$ ]DX) binding sites in the rat brain

| Brain region (abbreviation)                | [ $^3\text{H}$ ]Dextrorphan bound (fmol/mg) <sup>a</sup> |                                      |
|--|--|--------------------------------------|
|  | Mean $\pm$ S.E.  | Relative to CA1 stratum radiatum (%) |
| <b>Cortex</b>                              |  |                                      |
| Frontal layers II-III (Fr II-III)          | 89.8 $\pm$ 7.5   | 49.5                                 |
| Frontal layers IV-VI (Fr IV-VI)            | 62.5 $\pm$ 7.8   | 34.4                                 |
| Frontoparietal layers II-III (FrPa II-III) | 89.5 $\pm$ 4.2   | 49.3                                 |
| Frontoparietal layers IV-VI (FrPa IV-VI)   | 61.0 $\pm$ 3.8   | 33.6                                 |
| Entorhinal layers II-III (Ent II-III)      | 82.0 $\pm$ 4.9   | 45.2                                 |
| Entorhinal layers IV-VI (Ent IV-VI)        | 49.2 $\pm$ 5.0   | 27.1                                 |
| <b>Hippocampal formation</b>               |  |                                      |
| CA1-stratum lacunosum moleculare (LMol)    | 119.3 $\pm$ 7.4  | 65.7                                 |
| CA1-stratum radiatum (Rad)                 | 181.5 $\pm$ 14.6   | 100.0                                |
| CA1-stratum pyramidale (Py)                | 79.8 $\pm$ 6.2   | 44.0                                 |
| CA1-stratum oriens (Or)                    | 167.6 $\pm$ 17.0   | 92.3                                 |
| CA3-stratum radiatum                       | 119.3 $\pm$ 10.6   | 65.7                                 |
| CA3-stratum pyramidale                     | 46.2 $\pm$ 4.9   | 25.5                                 |
| CA3-stratum oriens                         | 121.8 $\pm$ 11.1   | 67.1                                 |
| Dentate gyrus-stratum moleculare (DG-Mol)  | 159.1 $\pm$ 10.7   | 87.7                                 |
| <b>Thalamic nuclei</b>                     |  |                                      |
| laterodorsal (LD)                          | 65.5 $\pm$ 4.8   | 36.1                                 |
| mediodorsal (MD)                           | 50.0 $\pm$ 7.6   | 27.6                                 |
| medial geniculate (MG)                     | 39.6 $\pm$ 4.2   | 21.8                                 |
| ventroposterior (VP)                       | 63.2 $\pm$ 4.3   | 34.8                                 |
| <b>Cerebellum <sup>b</sup></b>             |  |                                      |
| Molecular layer (Cb-Mol)                   | 87.0 $\pm$ 11.4  | 47.9                                 |
| Granule layer (Cb-Gr)                      | 27.3 $\pm$ 8.3   | 15.0                                 |
| Caudate putamen (CPu)                      | 52.8 $\pm$ 2.9   | 29.1                                 |
| Central gray (CG)                          | 10.8 $\pm$ 3.0   | 6.0                                  |
| Corpus callosum (cc)                       | 0.3 $\pm$ 0.2  | 0.2                                  |

<sup>a</sup> Values represent mean specific binding  $\pm$  S.E. of measurements made from autoradiograms generated from quadruplicate sections at each brain level from three animals.

<sup>b</sup> Specific [ $^3\text{H}$ ]DX binding was calculated using representative values from hippocampus (CA1), as nonspecific [ $^3\text{H}$ ]DX binding in the cerebellum is inadequately defined by 100  $\mu\text{M}$  MK-801.



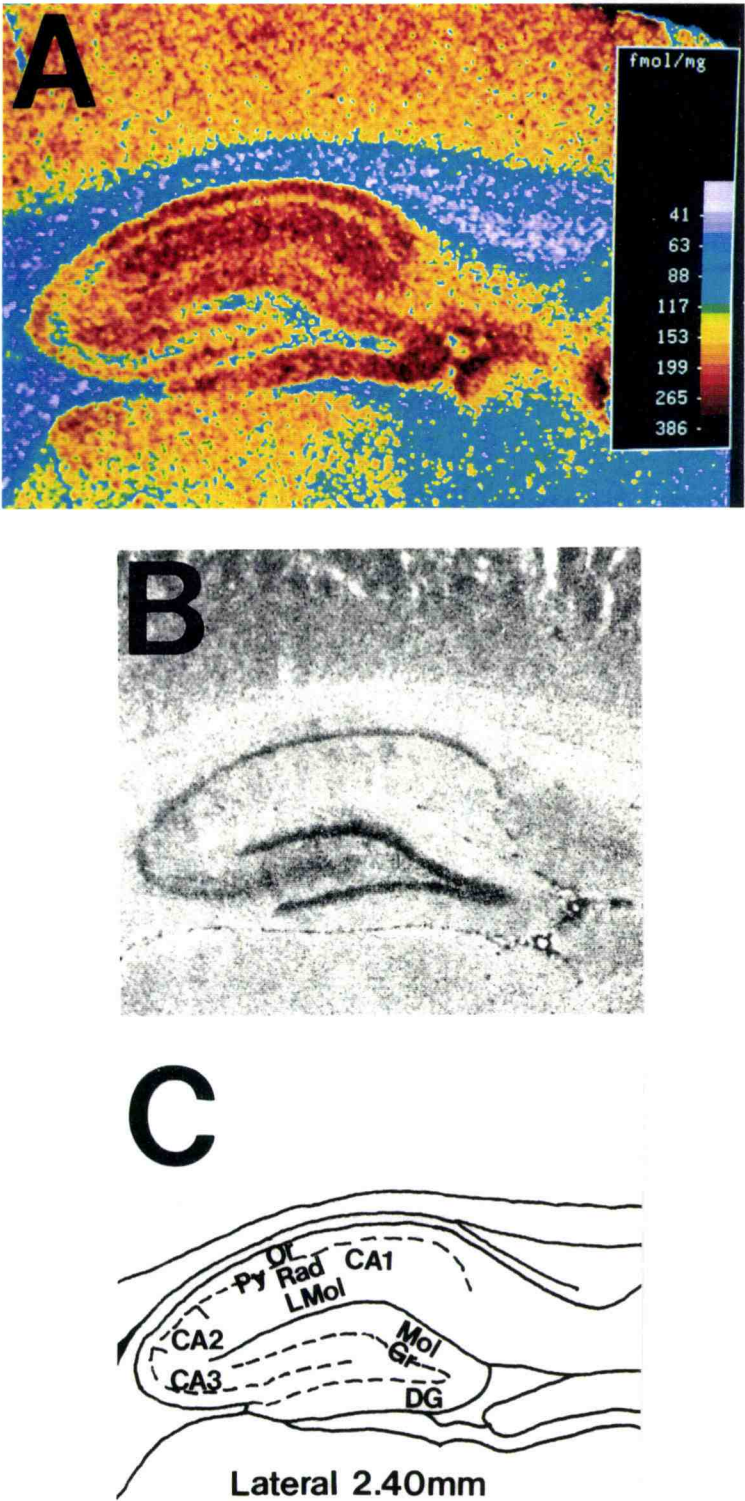
**FIGURE 5.10.****Detail of the rat hippocampal formation in the sagittal plane.**

(A) Autoradiogram showing the distinct laminar distribution of [<sup>3</sup>H]dextrorphan binding sites under the same conditions as Fig. 9. [<sup>3</sup>H]Dextrorphan binding is particularly dense in the stratum oriens and stratum radiatum of CA1 and the molecular layer of the dentate gyrus.

(B) Section stained with cresyl violet acetate for comparison with the autoradiogram. Dark bands correspond to the pyramidal cell layer of the hippocampus and granule cell layer of the dentate gyrus.

(C) Line drawing corresponds to Lateral 2.40 mm in accordance with the atlas of Paxinos and Watson (1986). Abbreviations are as follows: CA1-3, fields CA1-3 of Ammon's horn; Or, oriens layer; Py, pyramidal cell layer; Rad, stratum radiatum; LMol, lacunosum moleculare of hippocampus; DG, dentate gyrus; Gr, granule layer of dentate gyrus; Mol, molecular layer of dentate gyrus.

FIGURE 5.10.



Autoradiographic visualization of specific [ $^3\text{H}$ ]dextrorphan binding sites in the cerebellum revealed a distinct pattern of distribution. [ $^3\text{H}$ ]Dextrorphan binding was particularly concentrated in the cerebellar molecular layer where there was over a three-fold greater density of labelled sites than in the granule cell layer (Table 5.4). The location of the outer molecular layer and the innermost granule cell layer of the cerebellar cortex is shown in figure 5.11. The representative autoradiograms shown in figures 5.12 and 5.13 were generated from two sets of adjacent sagittal sections of cerebellum. Total [ $^3\text{H}$ ]dextrorphan binding is shown in figure 5.12 panel A and nonspecific binding measured in the presence of 100  $\mu\text{M}$  unlabelled dextrorphan in figure 5.12 panel B. [ $^3\text{H}$ ]Dextrorphan binding is concentrated in the molecular cell layer and becomes more diffuse in the granule cell layer of all folia. Total [ $^3\text{H}$ ]dextrorphan binding is also shown in figure 5.13 panel A and, from the corresponding adjacent section, figure 5.13 panel B shows [ $^3\text{H}$ ]dextrorphan binding in the presence of 100  $\mu\text{M}$  MK-801. As highlighted in figure 5.13, images produced in the presence of unlabelled MK-801 (panel B) are largely indistinguishable from total [ $^3\text{H}$ ]dextrorphan binding (panel A of figures 5.12 and 5.13 ).

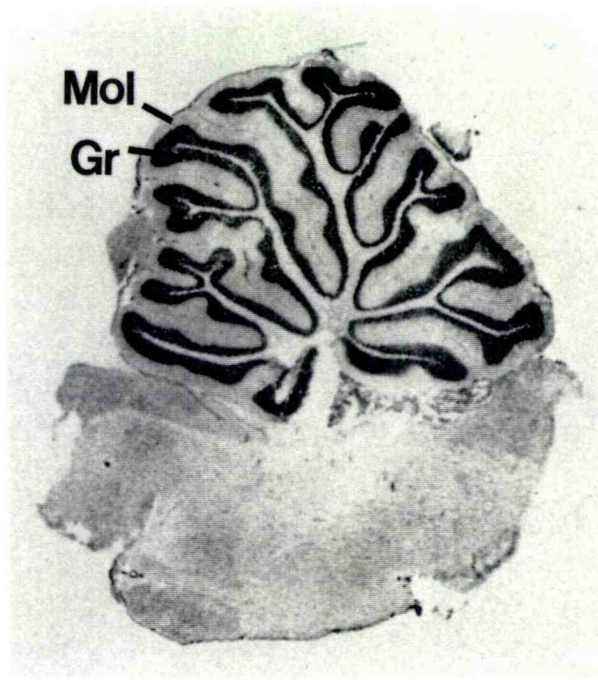
Competition analysis revealed that unlabelled dextrorphan displaces [ $^3\text{H}$ ]dextrorphan from the molecular cell layer and granule cell layer in a monophasic manner suggesting interaction with a single binding site (Figure 5.14);  $\text{IC}_{50}$  values =  $2.7 \pm 0.7 \mu\text{M}$  and  $5.9 \pm 0.9 \mu\text{M}$  for the molecular and granule cell layers respectively. In self-competition studies, dextrorphan was approximately 20-fold less potent in the molecular layer and 50-fold less potent in the granule cell layer of the cerebellum than in the stratum radiatum of the CA1 region of the hippocampus. These differences are even more pronounced when comparing the potency of unlabelled dextrorphan to displace [ $^3\text{H}$ ]dextrorphan from the cerebellum and the outer cortical layers (Table 5.3). MK-801, however, does not compete for sites labelled by [ $^3\text{H}$ ]dextrorphan in either the molecular cell layer or the granule cell layer of the cerebellum (Figure 5.14) .

Our previous studies in membrane homogenates (Franklin and Murray, 1992) indicated that [ $^3\text{H}$ ]dextrorphan labels a population of binding sites in the cerebellum that display lower affinity for MK-801 and TCP than do forebrain [ $^3\text{H}$ ]dextrorphan sites. Based on our earlier characterization of [ $^3\text{H}$ ]dextrorphan binding in cerebellar membranes, together with the fact that [ $^3\text{H}$ ]MK-801 has such low affinity for the NMDA receptors in the cerebellum that specific binding is often undetectable by receptor autoradiography in this brain region (Bowery et al. 1988; Monaghan, 1991; Sakurai et al., 1991), it would appear that the [ $^3\text{H}$ ]dextrorphan binding site in cerebellum is distinct from forebrain NMDA receptors which recognize [ $^3\text{H}$ ]MK-801 with high affinity in this brain area.

Moderate levels of [ $^3\text{H}$ ]dextrorphan binding were seen in the thalamus (Table 5.4), with highest densities in the dorsolateral and ventral posterior thalamic nuclei. The caudate-putamen also showed moderate levels of specific [ $^3\text{H}$ ]dextrorphan binding, approximately 30% of that measured in the stratum radiatum of the CA1 region of the hippocampus. Of the areas studied, the lowest levels of [ $^3\text{H}$ ]dextrorphan binding were seen in the granule cell layer of the cerebellum and the central grey. White matter tracts such as the corpus callosum and the white matter of the cerebellum showed practically no specific [ $^3\text{H}$ ]dextrorphan binding. White matter does demonstrate greater self-absorption of beta-particles emitted by tritium than gray matter (Alexander et al., 1981). Consequently, quenching of tritium-labelled ligands has been shown to change in relation to the white matter content of a particular brain region (Rainbow et al., 1984). The low level of [ $^3\text{H}$ ]dextrorphan binding observed in white matter tracts is, however, consistent with other descriptions of NMDA receptor distribution.

We have compared the regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites obtained in the present study with previously reported distributions of NMDA receptors determined by ligand autoradiography. To facilitate these comparisons, density values were expressed as the percentage of binding relative to the stratum radiatum of the CA1 region of the hippocampus reported for each radioligand. Support for a relationship

**FIGURE 5.11.**  
**Stained sagittal section of rat cerebellum.**



Sagittal section of rat cerebellum stained with cresyl violet acetate for comparison with autoradiograms depicted in figures 5.12. and 5.13. Dark bands correspond to the granule (Gr) cell layer characterized by densely packed cell bodies. The outer molecular (Mol) cell layer consists largely of Purkinje cell dendrites.

**FIGURE 5.12.**

**Representative autoradiograms derived from sagittal sections of rat cerebellum depicting (A) total [ $^3\text{H}$ ]dextrorphan binding and (B) nonspecific binding measured in the presence of 100  $\mu\text{M}$  unlabelled dextrorphan.**

Autoradiograms derived from rat cerebellum depicting: (A) total [ $^3\text{H}$ ]dextrorphan binding and (B) nonspecific binding measured in the presence of 100  $\mu\text{M}$  dextrorphan. Sagittal sections (16- $\mu\text{m}$ ) were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h at 20°C in the presence of glycine (100  $\mu\text{M}$ ) and glutamate (1  $\mu\text{M}$ ). Sections were then washed for 90 min at 20°C in 5 mM HEPES buffer containing 1 mM EDTA, dried and apposed to tritium-sensitive film for 13 days with a complete set of tritium-standards of known radioactivity. Colour has been assigned to the scale representing binding site density so that the highest levels of [ $^3\text{H}$ ]dextrorphan binding appear red to orange and the lowest levels appear blue to pink.

**FIGURE 5.13.**

**Representative autoradiograms derived from sagittal sections of rat cerebellum depicting (A) total [ $^3\text{H}$ ]dextrorphan binding and (B) [ $^3\text{H}$ ]dextrorphan binding measured in the presence of 100  $\mu\text{M}$  MK-801.**

Autoradiograms derived from rat cerebellum depicting: (A) total [ $^3\text{H}$ ]dextrorphan binding and (B) [ $^3\text{H}$ ]dextrorphan binding measured in the presence of 100  $\mu\text{M}$  MK-801. Sagittal sections (16- $\mu\text{m}$ ) were incubated with 40 nM [ $^3\text{H}$ ]dextrorphan for 4 h at 20°C in the presence of glycine (100  $\mu\text{M}$ ) and glutamate (1  $\mu\text{M}$ ). Sections were then washed for 90 min at 20°C in 5 mM HEPES buffer containing 1 mM EDTA, dried and apposed to tritium-sensitive film for 13 days with a complete set of tritium-standards of known radioactivity. Colour has been assigned to the scale representing binding site density so that the highest levels of [ $^3\text{H}$ ]dextrorphan binding appear red to orange and the lowest levels appear blue to pink.

FIGURE 5.12.

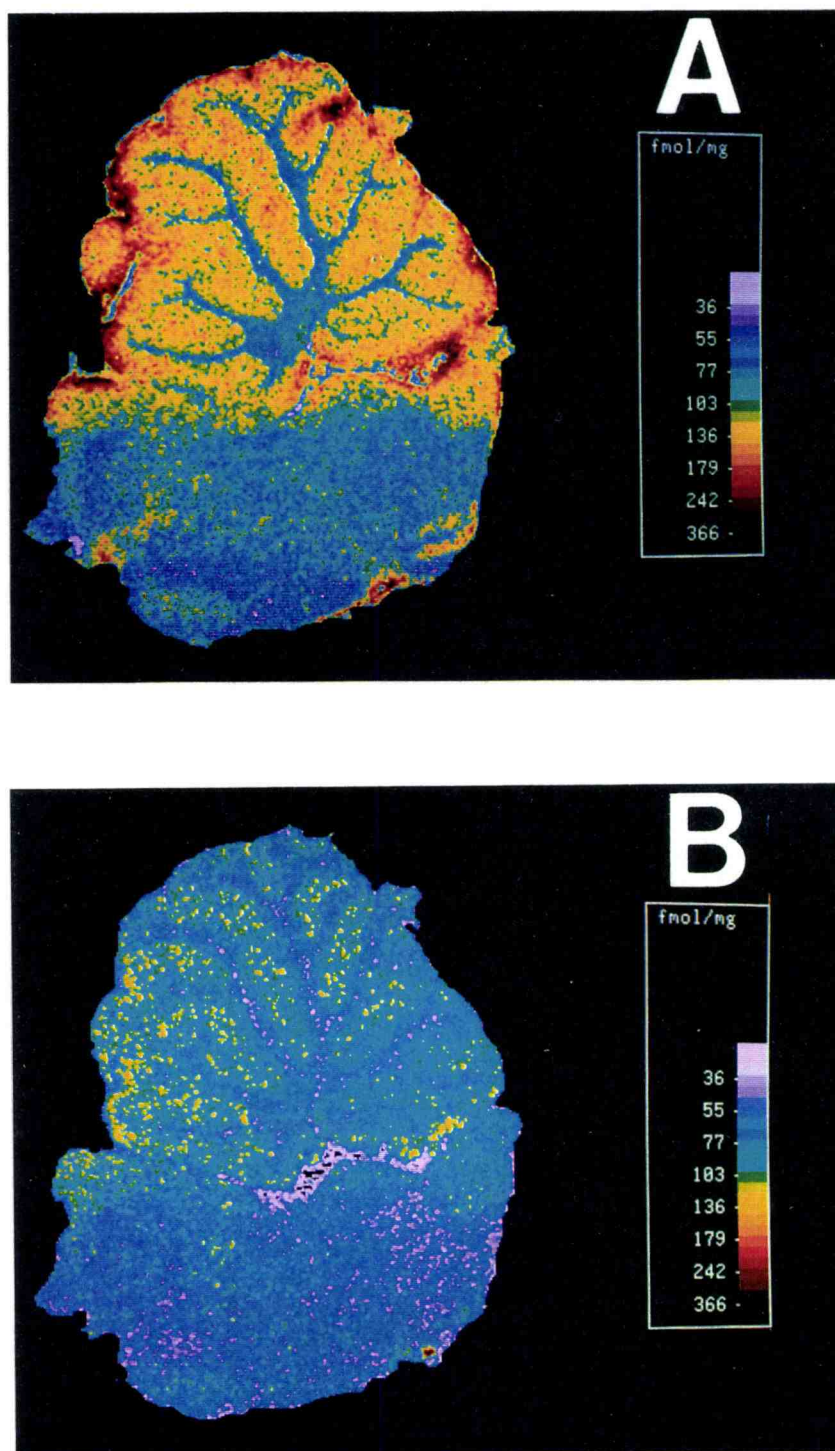
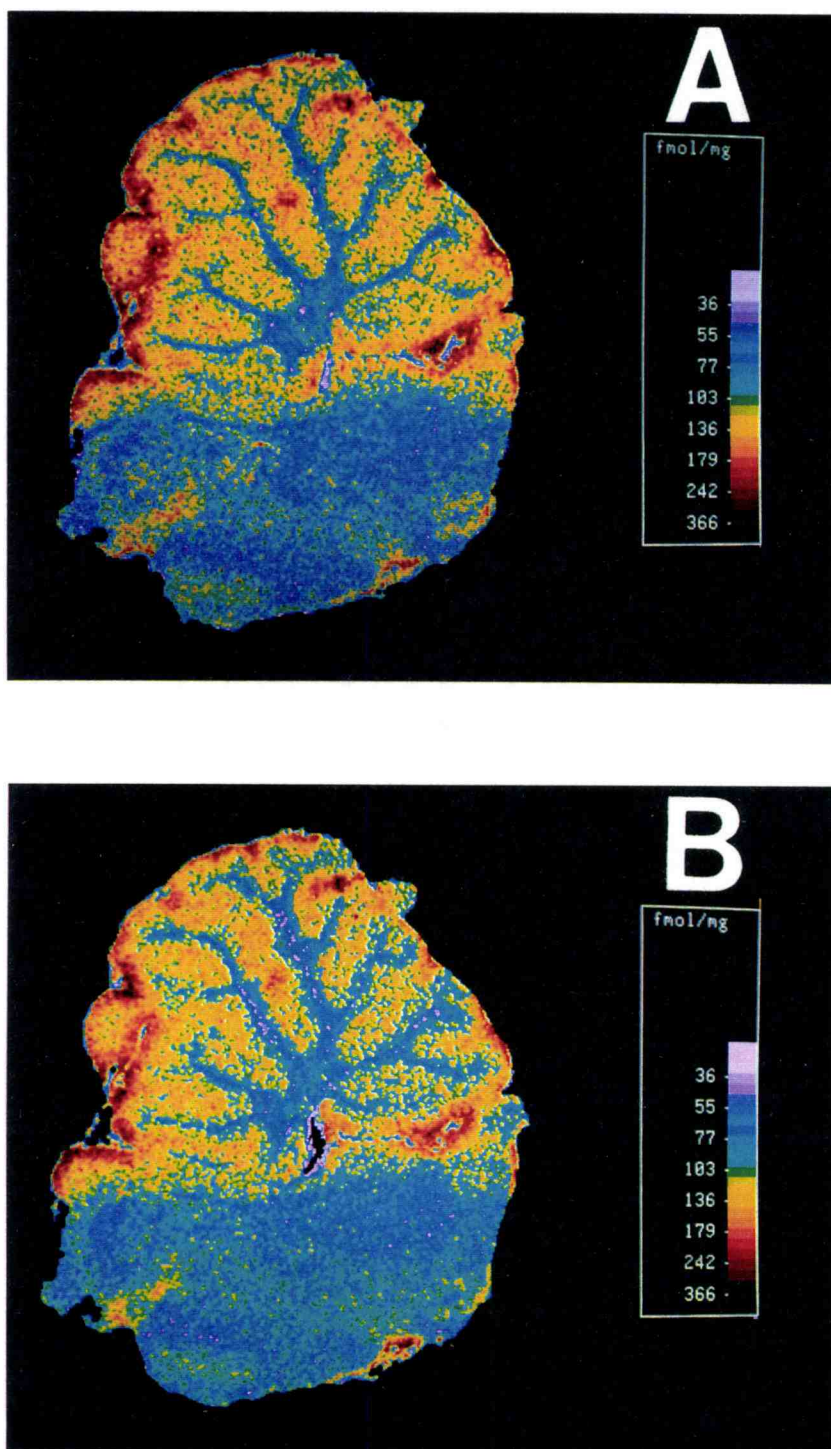




FIGURE 5.13.



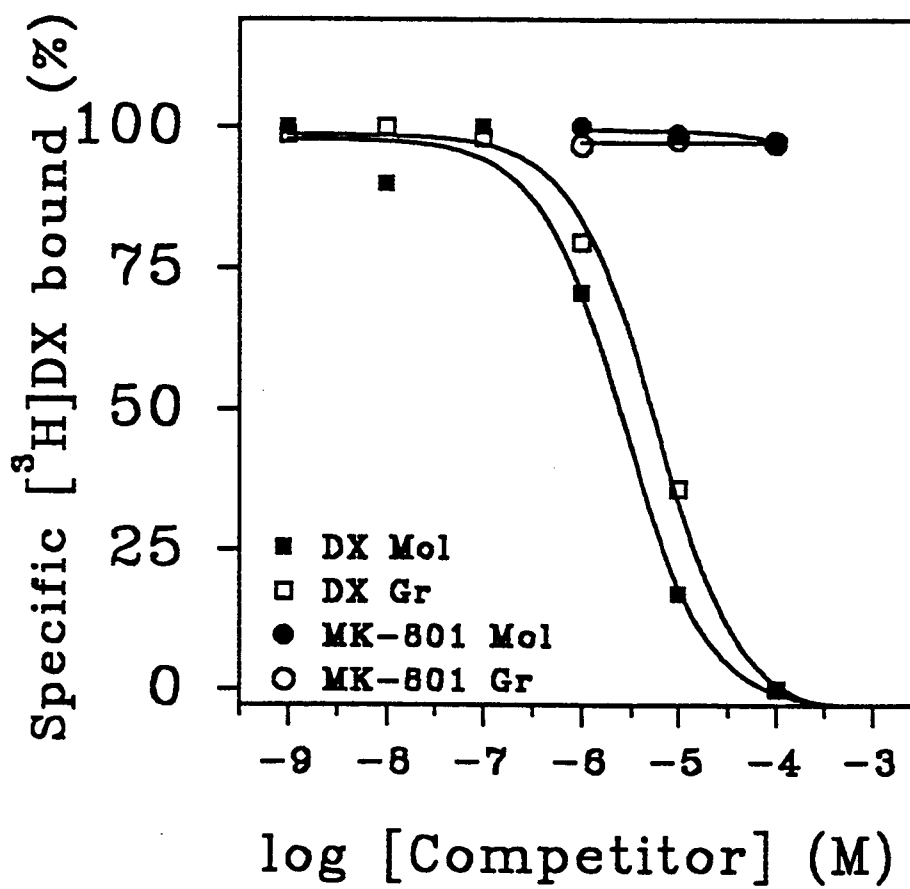


**FIGURE 5.14.**

**[<sup>3</sup>H]Dextrorphan competition binding in the molecular and granule cell layers of the rat cerebellum.**

Competition binding in the molecular (Mol) and granule (Gr) cell layers of the rat cerebellum. Sections were incubated with 40 nM [<sup>3</sup>H]dextrorphan for 4 h at 20°C in the presence of added glycine (100 μM) and glutamate (1 μM). Sections were then washed for 90 min in 5 mM HEPES buffer containing 1 mM EDTA. Binding of dextrorphan (DX) and MK-801 is depicted by plots of the actual data points. The respective curves are expanded from the parameter estimates derived from fitting a logistic equation to the data by means of iterative nonlinear least squares regression analysis. Each point represents mean specific [<sup>3</sup>H]dextrorphan binding determined in quadruplicate sections from a representative experiment. The S.E. for each point was less than 10 % of the mean. Nonspecific binding was defined by 100 μM dextrorphan.

FIGURE 5.14.



between the regional distribution of NMDA receptors and [ $^3\text{H}$ ]dextrorphan binding sites is provided by the strong correlation ( $r=0.78$ ,  $P < 0.002$ ) between the occurrence of [ $^3\text{H}$ ]dextrorphan binding sites and the regional distribution of NMDA-displaceable [ $^3\text{H}$ ]glutamate binding sites described by Monaghan and Cotman (1985) (Figure 5.15A). Furthermore, there is an excellent correlation between the distribution of [ $^3\text{H}$ ]dextrorphan binding sites and those sites labelled by other noncompetitive NMDA antagonists. The regional distribution of [ $^3\text{H}$ ]dextrorphan labelled NMDA receptors was significantly correlated ( $r=0.90$ ,  $P < 0.0001$ ) with [ $^3\text{H}$ ]TCP binding sites described by Gundlach et al. (1986) (Figure 5.15B) and also with [ $^3\text{H}$ ]MK-801 binding sites ( $r=0.90$ ,  $P < 0.0001$ ) described by Sakurai et al. (1991) (Figure 5.15C).

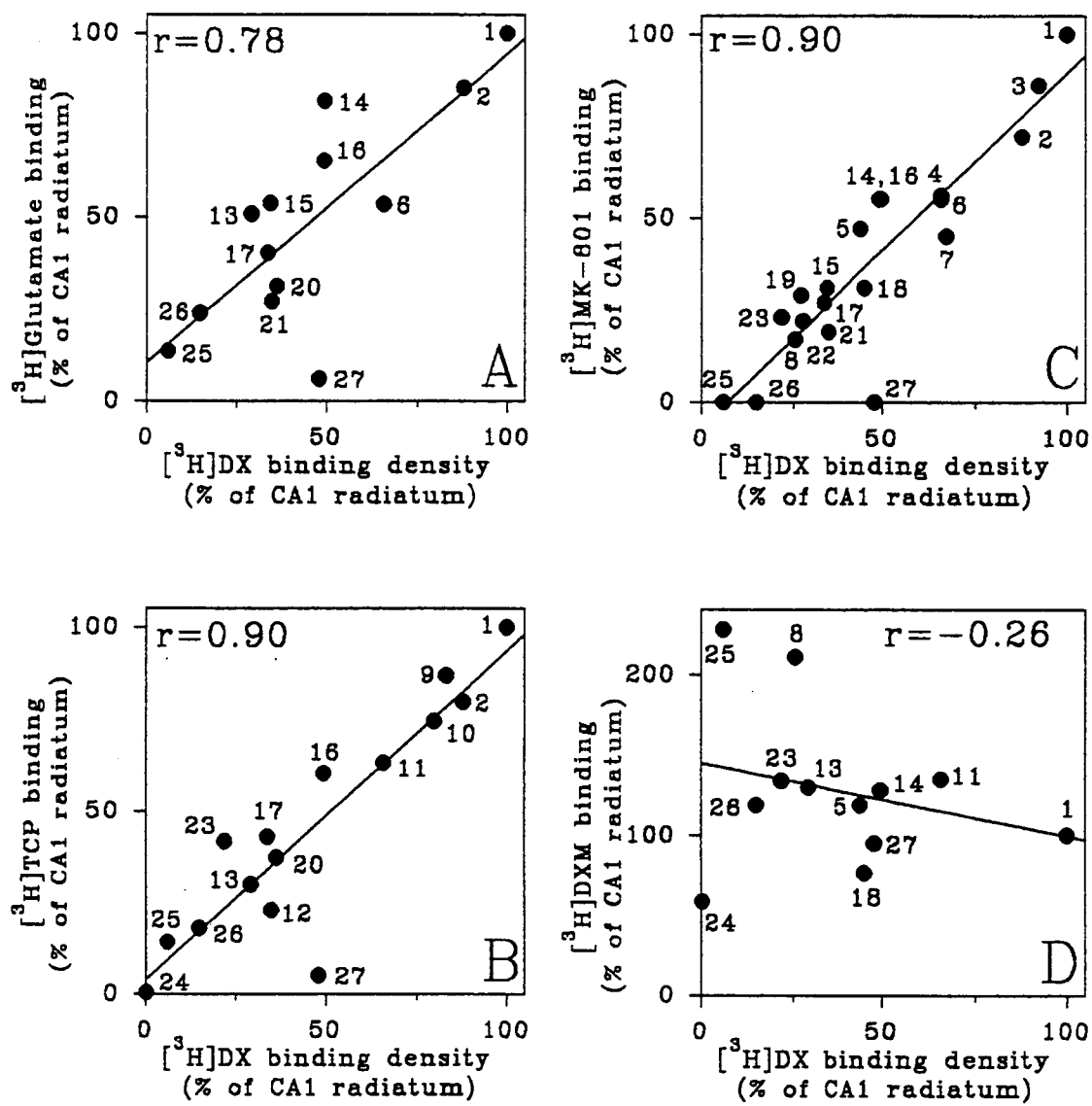
The distinct laminar pattern of [ $^3\text{H}$ ]dextrorphan binding within the hippocampal formation was in marked concordance with the distribution of [ $^3\text{H}$ ]MK-801, [ $^3\text{H}$ ]TCP and NMDA-displaceable [ $^3\text{H}$ ]glutamate binding sites in the hippocampus. Despite the apparent similarities in the regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites and those sites labelled by [ $^3\text{H}$ ]MK-801, [ $^3\text{H}$ ]TCP and NMDA-sensitive [ $^3\text{H}$ ]glutamate, some differences were observed particularly in the relative percentages of binding in various brain regions. NMDA-displaceable [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ]TCP and to a lesser extent [ $^3\text{H}$ ]MK-801, label a comparatively greater fraction of binding sites in the superficial layers of the cerebral cortex than [ $^3\text{H}$ ]dextrorphan which shows approximately 50 % of the binding levels seen in the stratum radiatum of CA1. The most striking disparity, however, between [ $^3\text{H}$ ]dextrorphan binding sites and those sites labelled by NMDA-displaceable [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ]TCP or [ $^3\text{H}$ ]MK-801, is in the molecular layer of the cerebellum (Figure 5.15, point 27). In fact, if data from the cerebellum are omitted, linear-regression analysis reveals an improvement in the correlation between [ $^3\text{H}$ ]dextrorphan binding site distribution and those sites labelled by NMDA-displaceable [ $^3\text{H}$ ]glutamate ( $r=0.87$ ,  $P < 0.001$ ), [ $^3\text{H}$ ]MK-801 ( $r=0.95$ ,  $P < 0.0001$ ) or [ $^3\text{H}$ ]TCP ( $r=0.97$ ,  $P < 0.0001$ ) (data not shown).

**FIGURE 5.15.**

**Correlation between the regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat brain and those sites labelled by NMDA-sensitive [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ]TCP, [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]dextromethorphan.**

Correlation between the regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat brain and (A) NMDA-sensitive [ $^3\text{H}$ ]glutamate sites in rat brain ( $r=0.78$ ,  $P < 0.002$ ) from the data of Monaghan and Cotman (1985); (B) sites labelled by [ $^3\text{H}$ ]TCP in rat brain ( $r=0.90$ ,  $P < 0.0001$ ) from the data of Gundlach et al. (1986); (C) sites labelled by [ $^3\text{H}$ ]MK-801 in rat brain ( $r=0.90$ ,  $P < 0.0001$ ) from the data of Sakurai et al. (1991) and (D) sites labelled by [ $^3\text{H}$ ]dextromethorphan in guinea pig brain ( $r=-0.26$ ,  $P < 0.5$ ) from the data of Canoll et al. (1989). The significance of the correlations was evaluated by  $t$  test of the probability of  $r=0$  for each of the comparisons. All radioligand binding values are expressed as the percentage of binding relative to the stratum radiatum of the CA1 region of the hippocampus. Abbreviations are taken from Table 4: (1) CA1-Rad, (2) DG-Mol, (3) CA1-Or, (4) CA1-LMol, (5) CA1-Py, (6) CA3-Rad, (7) CA3-Or, (8) CA3-Py, (9) Rad, (10) Or, (11) LMol, (12) Py, (13) CPu, (14) Fr II-III, (15) Fr IV-VI, (16) FrPa II-III, (17) FrPa IV-VI, (18) Ent II-III, (19) Ent IV-VI, (20) LD, (21) MD, (22) MG, (23) VP, (24) cc, (25) CG, (26) Cb-Gr, (27) Cb-Mol.

FIGURE 5.15.



In contrast, there was no significant correlation ( $r=-0.26$ ,  $P < 0.5$ ) with the regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites described herein and those labelled with [ $^3\text{H}$ ]dextromethorphan in guinea pig brain reported by Canoll et al. (1989) (Figure 5.15D). [ $^3\text{H}$ ]Dextromethorphan recognition sites in rat brain (Tortella et al., 1989) follow a similar pattern of distribution to binding sites labelled in guinea pig brain and also appear to be unrelated to [ $^3\text{H}$ ]dextrorphan-labelled NMDA receptors (data not shown).

## Discussion

We have characterized [ $^3\text{H}$ ]dextrorphan binding sites in slide-mounted rat brain sections and ascertained their regional distribution using quantitative receptor autoradiography. The present studies extend our previous findings in membrane homogenates (Franklin and Murray, 1990; Franklin and Murray, 1992) in that [ $^3\text{H}$ ]dextrorphan appears to selectively label a high affinity site localized within an open channel domain of the NMDA receptor. The [ $^3\text{H}$ ]dextrorphan binding site may not, however, be identical to ion channel binding sites labelled by other noncompetitive antagonists. Specifically, differences in the regulation of [ $^3\text{H}$ ]dextrorphan binding by glycine and glutamate and the labelling of high affinity sites in the cerebellum distinguish [ $^3\text{H}$ ]dextrorphan binding from that of either [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]TCP.

In agreement with our observations in membrane homogenates (Franklin and Murray, 1992), the association kinetics of [ $^3\text{H}$ ]dextrorphan with its binding site are slow in slide-mounted brain sections following standard washing conditions designed to deplete endogenous glycine and glutamate from the sections. In the absence of supplementary glycine and glutamate, an incubation period of at least 4 hours at room temperature is required to reach. The addition of exogenous glycine and glutamate to the incubation droplet, however, increases the apparent association rate of [ $^3\text{H}$ ]dextrorphan without any demonstrable change in the number of binding sites in CA1; equilibrium is reached in

150 min. An acceleration in the association rate of [ $^3\text{H}$ ]dextrorphan is presumably a result of the ability of glycine and glutamate to facilitate access of [ $^3\text{H}$ ]dextrorphan to a binding domain within the activated NMDA receptor ion channel. Access to such a site would be determined by the fractional open time of the channel and is thus consistent with the possibility of a guarded or transiently available [ $^3\text{H}$ ]dextrorphan binding site. In addition, the results of the present study indicate that specific [ $^3\text{H}$ ]dextrorphan also has a slow rate of dissociation in the absence of exogenous glycine and glutamate in slide-mounted brain sections. These data are in agreement with previous reports demonstrating NMDA receptor agonist induced increases in both the association and dissociation rates of [ $^3\text{H}$ ]TCP (Kloog et al., 1988a; Bonhaus and McNamara, 1988; Kessler et al., 1989) and [ $^3\text{H}$ ]MK-801 (Kloog et al., 1988b; Kornhuber et al., 1989) in well washed membrane preparations.

Although binding kinetics are likely to be greatly influenced by specific assay conditions, if access to the NMDA receptor channel binding domain is a major hindrance noncompetitive antagonist binding, the time required for ligand association and dissociation with its binding site is likely to be longer in tissue that has been partly depleted of endogenous glycine and glutamate. Consistent with this possibility, kinetic studies have indicated that in the absence of exogenous NMDA agonists, [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]TCP also require a long time to reach equilibrium in extensively washed membrane homogenates (Kloog et al., 1988a,b; Kornhuber et al., 1989; Johnson et al., 1989). Furthermore, slow association and dissociation rates have also been reported in quantitative autoradiographic studies of [ $^3\text{H}$ ]MK-801 binding to rat brain sections (Subramanian and McGonigle, 1991; Sakurai et al. 1991).

We have previously shown that the magnitude of the increase in [ $^3\text{H}$ ]dextrorphan binding produced by exogenous glycine and glutamate in membrane homogenates dependent on the degree to which endogenous glycine and glutamate have been depleted from repetitively washed tissue (Franklin and Murray, 1992). In a similar fashion, exogenously added NMDA receptor agonists have been reported to enhance both [ $^3\text{H}$ ]TCP

(Loo et al., 1986; Snell et al., 1987) and [ $^3\text{H}$ ]MK-801 (Foster and Wong, 1987; Reynolds et al., 1987) binding in extensively washed membrane homogenates. NMDA receptor agonist-induced increases in noncompetitive antagonist binding has proven to be more difficult to demonstrate directly by receptor autoradiography. Glutamate is the major neurotransmitter at excitatory synapses in the vertebrate central nervous system and is also a key amino acid in intermediary metabolism. It is not surprising, therefore, that rigorous washing procedures are required to lower concentrations of glutamate and other endogenous amino acids from brain tissue. The removal of endogenous glycine and glutamate from slide-mounted brain sections is especially difficult given the existence of diffusion barriers and because washing procedures have to be limited in the interests of preserving the anatomical integrity of the tissue section. Some investigators have instead employed indirect methods to observe NMDA receptor agonist-induced increases in noncompetitive antagonist binding by measuring binding in the presence of a competitive NMDA or glycine site antagonist (Bowery et al., 1988; Monaghan, 1991). Others have used non-equilibrium conditions to show large stimulations of binding by exogenously added agonists. In a report by Hosford et al. (1990), a 5 min incubation period was specifically chosen to demonstrate enhanced [ $^3\text{H}$ ]TCP binding by glycine and NMDA. Non-equilibrium conditions may also have contributed to the large stimulations in [ $^3\text{H}$ ]MK-801 binding observed by Fletcher and Bowery (1991), however, these investigators clearly demonstrated that the ability of glycine and glutamate to increase [ $^3\text{H}$ ]MK-801 binding could be altered by pre-washing the brain sections. In that study [ $^3\text{H}$ ]MK-801 binding in the stratum radiatum of the CA1 region of the hippocampus was enhanced by glycine (10  $\mu\text{M}$ ) and glutamate (1  $\mu\text{M}$ ) to 187 % of control levels following a 2 h pre-wash at room temperature. In the same study, the addition of glycine and glutamate following a 12 h pre-wash enhanced [ $^3\text{H}$ ]MK-801 binding further to 285 % of control levels in the CA1.

Although some pre-washing procedure is usually employed prior to the autoradiographic study of NMDA receptor modulation, subtle variations in methodology



are likely to produce variations in baseline levels of endogenous glycine and glutamate in the tissue sections. In particular, differences in section thickness, temperature of the buffer and the wash time may all contribute to the efficiency of the wash procedure and the final condition of the tissue sections. Nevertheless, in an autoradiographic study of [ $^3\text{H}$ ]MK-801 binding by Sakurai et al. (1991) the apparent lack of stimulation of [ $^3\text{H}$ ]MK-801 binding by added NMDA and glycine under equilibrium conditions was attributed to high residual levels of glycine and glutamate in the brain sections. Following a more rigorous pre-washing procedure, 100  $\mu\text{M}$  NMDA was reported to enhance [ $^3\text{H}$ ]MK-801 binding in the stratum radiatum of the CA1 subfield of the hippocampus and molecular layer of the dentate gyrus by 181 % and 214 % respectively, whilst 100  $\mu\text{M}$  glycine alone was reported to increase [ $^3\text{H}$ ]MK-801 binding 255 % in the molecular layer of the dentate gyrus (Sakurai et al., 1991). In another recent report by Subramanian and McGonigle (1993), washing was reported to have regionally specific stimulatory effects on [ $^3\text{H}$ ]MK-801 binding in the inner (III-V) layers of the frontoparietal cortex and the dorsolateral caudate putamen.

Following our standard pre-wash procedure, specific [ $^3\text{H}$ ]dextrorphan binding in the stratum radiatum of CA1 was modestly enhanced (20 - 35 %) by glutamate and unstimulated by glycine under equilibrium conditions. In contrast to the reports of [ $^3\text{H}$ ]MK-801 binding mentioned above, this profile was basically unchanged by a prolonged washing procedure designed to lower endogenous levels of glycine and glutamate in the tissue sections. The study of other brain areas revealed the same pattern; [ $^3\text{H}$ ]dextrorphan binding was not significantly enhanced by exogenously added glycine or glutamate in the molecular layer of the dentate gyrus, outer (II-III) and inner (IV-VI) layers of the frontoparietal cortex or the lateral thalamic nuclei following standard or prolonged pre-washing of the tissue sections. In light of the fact that we were able to detect a substantial increase in the association rate of [ $^3\text{H}$ ]dextrorphan by the addition of glycine (100  $\mu\text{M}$ ) and glutamate (1  $\mu\text{M}$ ) to the incubation droplet, it seems unlikely that saturating concentrations of the endogenous agonists are still present following our prolonged pre-

wash procedure. It therefore appears that [ $^3\text{H}$ ]dextrorphan is far less sensitive to the modulatory effects of glycine and glutamate than [ $^3\text{H}$ ]MK-801.

The relative insensitivity of [ $^3\text{H}$ ]dextrorphan binding to glycine and glutamate may be provisionally explained by the possible existence of a [ $^3\text{H}$ ]dextrorphan binding site that to some degree is nonidentical with the binding sites of [ $^3\text{H}$ ]TCP and [ $^3\text{H}$ ]MK-801, rather than the unique regulation of [ $^3\text{H}$ ]dextrorphan at a common noncompetitive antagonist binding site. The concept of multiple noncompetitive NMDA antagonist sites with affinity for this general class of drug, rather than the existence of a single binding site for all members of this drug class, has been suggested previously to explain the interaction of desipramine and MK-801 with NMDA receptors (Serganor et al., 1989). It has recently been proposed that the dissociative anaesthetic binding site may be located in the vestibule of the NMDA channel pore and that the NMDA channel must be gated open before this binding site is exposed (MacDonald et al., 1991). Channel closure subsequent to the binding of a dissociative anaesthetic molecule, such as MK-801, ketamine or PCP, results in the trapping of the blocker within the closed channel (MacDonald et al., 1991). It is therefore possible that the apparent indifference of [ $^3\text{H}$ ]dextrorphan binding, to the presence of glycine and glutamate, is a consequence of its occupation of a more superficially located binding site, perhaps in the vestibule of the NMDA receptor ion channel, that is less sensitive to channel opening and closing. As a consequence [ $^3\text{H}$ ]dextrorphan binding may be less dependent on agonist activation of the receptor.

Recent attempts to identify the location of a noncompetitive antagonist binding domain within the NMDA receptor channel have utilised recombinant DNA techniques. All known NMDA receptor subunits have an asparagine residue in their putative channel-forming second membrane-dwelling segment at a site that is homologous to the glutamine-arginine editing position (Q/R site) of the AMPA-kainate receptors (Nakanishi, 1992). Certain non-NMDA subunits possess a glutamine at the Q/R site, whilst others carry an arginine residue that appears to be a critical determinant of  $\text{Ca}^{2+}$  permeability. This

arginine residue is not encoded in the subunit gene but is generated by an RNA editing process. Site-directed mutagenesis has revealed that substitution of this conserved asparagine residue in an NMDA receptor subunit, to glutamine or arginine reduces the sensitivity of NMDA receptor channels to blockade by MK-801 (Mori et al., 1992; Sakurada et al., 1993). In a study by Kawajiri and Dingledine (1993), mutation of the same asparagine of NMDAR1 to an arginine residue resulted in a 200-fold reduction in the potency of TCP to block heteromeric NMDA receptors and abolished the use-dependent nature of the blockade. Furthermore, in support of the idea that noncompetitive antagonists may have more than one site of action on the NMDA receptor channel, these authors also reported a lower affinity, non use-dependent component of the blockade by TCP that was retained in the mutant receptor. Although the conserved asparagine residue in the second membrane-dwelling region of NMDA receptor subunits appears to influence calcium permeability and  $Mg^{2+}$  blockade of the channel (Mori et al., 1992; Burashev et al., 1992) as well as noncompetitive antagonist binding, it has recently been proposed that the contribution of this residue may differ for various noncompetitive antagonists (Yamakura et al., 1993). In a comparison of four heteromeric murine NMDA receptors, wild-type NMDA receptor channels differed in their sensitivity to blockade by MK-801. In the same study, mutation of the asparagine residue reduced the sensitivity of NMDA receptor channels to blockade by PCP, ketamine and SKF-10,047 to different extents. These findings are not incongruous with our positing of nonidentical sites with respect to noncompetitive antagonist binding.

Further evidence for a difference in the nature of noncompetitive antagonism of NMDA-mediated responses by dextrorphan and MK-801 has been provided by electrophysiologic data (Cole et al., 1989). Unlike MK-801, which was found to act in clearly use-dependent manner, dextrorphan showed no evidence of use-dependency in producing a potent, dose-dependent and selective noncompetitive inhibition of NMDA-mediated depolarizations in the rat hippocampal slice (Cole et al., 1989). These findings

are not inconsistent with the possibility of dextrorphan gaining access to a site within the ion channel domain that is relatively exterior to that of MK-801 and which may therefore be less influenced by the state of the channel. Consistent with this possibility, the onset of dextrorphan blockade 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 indicating a high degree of use-dependence (Cole et al., 1989). In addition to the differential sensitivity of dextrorphan and MK-801 to glycine and glutamate, the more rapid kinetics of channel blockade by dextrorphan may also contribute to its lack of use-dependency. The particularly slow time course and long duration of blockade of the NMDA receptor channel by MK-801 is thought to be related to its high degree of use-dependency (Wong et al., 1986).

Although [ $^3\text{H}$ ]dextrorphan binding appears to be regulated in a distinctive manner by glycine and glutamate, the presence of both agonists is still required for binding to occur. Consistent with a dependence of [ $^3\text{H}$ ]dextrorphan binding upon activation of the NMDA receptor-complex, basal specific binding was sensitive to D-AP5, a competitive NMDA antagonist, and 7-chlorokynurenic acid, a strychnine-insensitive glycine antagonist. Following standard pre-wash conditions in the absence of exogenous glycine and glutamate, specific [ $^3\text{H}$ ]dextrorphan binding was completely inhibited by the presence of the either antagonist in a concentration dependent manner. These data confirm our previous findings in membrane homogenates (Franklin and Murray, 1992) and thus lend further support for a relationship between [ $^3\text{H}$ ]dextrorphan binding and activation of the NMDA receptor complex. These data are also consistent with our demonstration of glutamate and glycine augmentation of the [ $^3\text{H}$ ]dextrorphan association rate in brain sections. The ability of 7-chlorokynurenic acid and D-AP5 to inhibit [ $^3\text{H}$ ]dextrorphan binding is therefore a result of their competitive surmounting of the activation of NMDA receptors by endogenous glutamate and glycine.

The present findings are also in agreement with the results of other autoradiographic studies which have used different radioligands to visualize the NMDA receptor. Complete inhibition of specific [ $^3\text{H}$ ]MK-801 binding has been reported in the presence of D-AP5 (Bowery et al., 1988; Sakurai et al., 1991; Beaton et al., 1992) and in the presence of 7-chlorokynurenic acid (Sakurai et al., 1991; Sakurai et al., 1993; Taconi et al., 1993). In addition, D-AP5 will afford complete inhibition of both specific [ $^3\text{H}$ ]TCP (Hosford et al., 1990) and NMDA-sensitive [ $^3\text{H}$ ]glutamate (Greenamyre et al., 1985; Beaton et al., 1992) binding.

Recent evidence has suggested that regionally distinct populations of NMDA receptors can be distinguished autoradiographically on the basis of inhibition of binding by competitive and noncompetitive NMDA receptor antagonists (Beaton et al., 1992; Sakurai et al., 1993). It is interesting in this context that [ $^3\text{H}$ ]dextrorphan binding was inhibited by 7-chlorokynurenic acid with similar potency in the brain areas that we studied, yet the competitive antagonist D-AP5 showed a more marked variation in its potency to inhibit [ $^3\text{H}$ ]dextrorphan binding over the same areas. Furthermore, regional distinctions in the inhibition of [ $^3\text{H}$ ]dextrorphan binding by D-AP5 did not follow the same patterns reported for inhibition of [ $^3\text{H}$ ]MK-801 binding by the competitive NMDA antagonist 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) (Beaton et al., 1992; Sakurai et al., 1993). Inhibition of [ $^3\text{H}$ ]dextrorphan binding by D-AP5 seems to distinguish a distinct population of sites that are weakly inhibited by D-AP5 in the lateral thalamic nuclei. Buller et al. (1994) have recently shown that populations of receptors displaying high affinity for [ $^3\text{H}$ ]CPP, typified by the lateral thalamus, are localized to brain regions expressing high densities of NMDAR2A (NR2A) mRNA but not NR2B mRNA. The ratio of NR2A to NR2B mRNA is greater in cortex than lateral thalamus (Buller et al., 1994); thus, a preponderance of the NR2A subunit may confer the much higher affinity of D-AP5 for [ $^3\text{H}$ ]dextrorphan binding sites in this area relative to lateral thalamus. It is also interesting to note that the lateral thalamus and cortex have previously been distinguished from other

brain areas in that [ $^3\text{H}$ ]MK-801 binding appears to be less sensitive to stimulation by L-glutamate in these regions (Monaghan, 1991).

At this point the possibility of regional differences in the concentration of endogenous glutamate should not be ruled out as a potential source of variation in the relative potencies of [ $^3\text{H}$ ]dextrorphan inhibition by D-AP5. It seems unusual, however, that endogenous factors would not also differentially influence the effects of 7-chlorokynurenic acid in the same experimental paradigm. At present the wide variations in methodology permit only general comparison of our findings with other reported NMDA receptor populations distinguished by this kind of autoradiographic study. The binding profile of [ $^3\text{H}$ ]dextrorphan, however, is clearly different from that reported for [ $^3\text{H}$ ]MK-801 (Sakurai et al., 1993). Given the increasing evidence for NMDA receptor heterogeneity, it would not be unreasonable to expect different combinations of subtypes to also differ with respect to both their competitive and noncompetitive antagonist pharmacology.

Of relevance to the problem of residual glycine and glutamate in slide-mounted sections, our study of [ $^3\text{H}$ ]dextrorphan binding in the presence of D-AP5 and 7-chlorokynurenic acid also confirms that after our standard pre-wash conditions, in a large volume of 5 mM HEPES (pH 7.4) at 20°C for 3 x 20 min, endogenous glutamate is certainly not removed from the 16  $\mu\text{m}$  sections entirely. In a recent study by Jaarsma et al. (1993), the concentration of residual glutamate was measured in 15  $\mu\text{m}$  sections that had been pre-washed in 50 mM Tris-HCL buffer (pH 8.0) for 3 x 20 min at room temperature. Following a 1 h incubation at 2°C with incubation buffer alone, a free L-glutamate concentration of  $42 \pm 4$  nM was detected in the 300  $\mu\text{l}$  incubation droplet. Although in our own studies we routinely use a larger incubation droplet (430  $\mu\text{l}$ ) to help lower the free glutamate concentration and guard against ligand depletion, it is likely that at least this much glutamate will leak from the section following a 4 h incubation period at room temperature. Since D-AP5 and 7-chlorokynurenic acid were able to completely inhibit basal

[<sup>3</sup>H]dextrorphan binding, the endogenous agonists must certainly be present at a concentration sufficient for NMDA receptor activation to occur.

The rank order of potencies of an array of noncompetitive NMDA receptor antagonists tested in the present study as inhibitors of [<sup>3</sup>H]dextrorphan binding in the stratum radiatum of the CA1 subfield of the hippocampus and the outer layers of the frontoparietal cortex, is in accordance with the pharmacologic profile for the NMDA receptor site labelled by [<sup>3</sup>H]dextrorphan in membrane homogenates prepared from rat forebrain (Franklin and Murray, 1992). These competition data provide additional evidence that the site labelled by [<sup>3</sup>H]dextrorphan in slide-mounted sections is localized to a noncompetitive antagonist domain of the NMDA receptor-channel complex. In contrast to our findings in membrane homogenates, all compounds recognized a single high-affinity [<sup>3</sup>H]dextrorphan binding site in both the stratum radiatum of CA1 and the outer cortical layers. Although quantitative receptor autoradiography permits the pharmacologic characterization of discrete brain regions, long postincubation wash periods such as the one employed in the present study may not permit detection of lower affinity [<sup>3</sup>H]dextrorphan binding sites in slide-mounted tissue.

In accordance with pharmacologic signature of sites labelled by [<sup>3</sup>H]PCP (Murray and Leid, 1984) and [<sup>3</sup>H]dextrorphan (Franklin and Murray, 1992) in rat brain membranes, dextromethorphan was less potent than dextrorphan as a competitor for [<sup>3</sup>H]dextrorphan binding sites in slide-mounted sections. The rapid metabolism of dextromethorphan to dextrorphan following its peripheral administration (Barnhart, 1980) is often a complicating factor in the interpretation of many experimental models of NMDA antagonism by dextromethorphan in vivo. Craviso and Musacchio (1983a), however, found no evidence for dextromethorphan metabolism in brain microsomes in vitro and it is therefore unlikely that the binding properties of dextromethorphan in vitro are confounded by the presence of dextrorphan. Whilst showing complete efficacy for [<sup>3</sup>H]dextrorphan binding sites in the stratum radiatum of the CA1 region of the hippocampus and the outer

layers of the frontoparietal cortex, dextromethorphan was over 6-fold less potent than dextrorphan in the CA1 and approximately 30-fold less potent than in the outer cortical layers. Given the recent evidence for differential sensitivity of recombinant heteromeric receptors to block by MK-801 (Yamakura et al., 1993), it is intriguing to speculate that the wide variation in relative potencies of dextrorphan and dextromethorphan for sites labelled by [ $^3\text{H}$ ]dextrorphan may reflect differences in the properties of native NMDA receptors located in the stratum radiatum of CA1 and the outer frontoparietal cortex.

[ $^3\text{H}$ ]Dextrorphan binding sites are heterogeneously distributed throughout the rat brain with a marked anatomical specificity that unambiguously corresponds to the known distribution of NMDA receptors. Equilibrium saturation binding data from several brain areas confirms the presence of a differential distribution of recognition sites and suggests that [ $^3\text{H}$ ]dextrorphan binds to a single population of high affinity sites under the assay conditions employed. Since the  $K_D$  values of these binding sites do not vary significantly, the regional heterogeneity of [ $^3\text{H}$ ]dextrorphan binding sites, as determined by single [ $^3\text{H}$ ]dextrorphan concentration equilibrium binding values, can be regressed to differences in the absolute receptor densities in the regions evaluated.

The highest levels of [ $^3\text{H}$ ]dextrorphan binding were found in the stratum radiatum and stratum oriens of the CA1 subfield of the hippocampus. These areas correspond to the target zones of ipsilateral and commissural axons of glutamatergic pyramidal neurones originating in the CA3 region of the hippocampus. In contrast, low levels of [ $^3\text{H}$ ]dextrorphan binding were found in the stratum pyramidale of hippocampal region CA1 corresponding to the pyramidal cell body layer of this area. Similarly, low levels of binding were detected in the stratum pyramidale and stratum lucidum of area CA3, representing the target zones of mossy fibres which are thought to have low densities of NMDA receptors (Cotman et al., 1987). The granule cell layer of the dentate gyrus, which contains the densely packed cell bodies of granule cell neurones, was virtually devoid of specific [ $^3\text{H}$ ]dextrorphan binding. The molecular layer of the dentate gyrus, however,



displayed high levels of binding presumably corresponding to NMDA receptors located on the apical dendrites of pyramidal cells which form synaptic connections with input fibres from the perforant pathway. The outer layers of the cerebral cortical regions also displayed moderate to high levels of [ $^3\text{H}$ ]dextrorphan binding consistent with the dense populations of NMDA receptors located in cortical layers I to III (Cotman et al., 1987). Somewhat lower densities of [ $^3\text{H}$ ]dextrorphan binding were detected in the striatum and lateral thalamic nuclei.

The relative distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat forebrain is consistent with the reported distribution of NMDA-sensitive [ $^3\text{H}$ ]glutamate binding sites (Greenamyre et al., 1985; Monaghan and Cotman, 1985). Furthermore, the autoradiographic pattern of [ $^3\text{H}$ ]dextrorphan binding sites in the forebrain is in excellent agreement with previously reported distributions of [ $^3\text{H}$ ]MK-801 (Bowery et al., 1988; Subramaniam and McGonigle, 1991; Sakurai et al., 1991) and [ $^3\text{H}$ ]TCP (Gundlach et al., 1986; Maragos et al., 1988) binding sites. As the relative distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat brain bears no resemblance to the previously reported distributions of [ $^3\text{H}$ ]dextromethorphan binding sites in rat or guinea pig brain (Canoll et al., 1989; Tortella et al., 1989), it appears that [ $^3\text{H}$ ]dextrorphan binding sites are unrelated to [ $^3\text{H}$ ]dextromethorphan or sigma binding sites. These data lend further support for a distinct pharmacology of dextromethorphan that is unrelated to its affinity for a noncompetitive antagonist domain of the NMDA receptor.

One brain area where the relative distribution of [ $^3\text{H}$ ]dextrorphan binding sites does not match the reported pattern of sites labelled by either NMDA-sensitive [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]TCP is in the cerebellum. [ $^3\text{H}$ ]Dextrorphan binds with a 3-fold greater density to the molecular layer of the cerebellum than the granule cell layer. Although a large percentage of glutamate receptors in the molecular layer of the cerebellum are thought to be of the non-NMDA receptor subtype, a population of NMDA-sensitive [ $^3\text{H}$ ]glutamate binding sites is certainly present in this brain area. Greenamyre et al. (1985)

reported that 25-30 % of [ $^3\text{H}$ ]glutamate binding in the molecular layer of the cerebellum could be inhibited by NMDA receptor-specific agents. Monaghan and Cotman (1985) also detected NMDA-sensitive binding in the molecular layer of the cerebellum that was approximately 25 % of that seen in the granule cell layer. Furthermore, autoradiographic visualization of [ $^3\text{H}$ ]PCP binding sites by Quirion et al. (1981) in the rat brain revealed preferential labelling of the molecular layer of the cerebellum. The discovery that [ $^3\text{H}$ ]TCP preferentially binds to the granule cell layer of the cerebellum gave an early indication of potential differences in the binding properties of [ $^3\text{H}$ ]PCP and [ $^3\text{H}$ ]TCP in the cerebellum and led to the proposal that the two ligands had differing anatomical specificities (Vignon et al., 1986). It is possible, therefore, that the high density of [ $^3\text{H}$ ]dextrorphan binding sites in the molecular layer of the cerebellum, and possibly those sites labelled by [ $^3\text{H}$ ]PCP (Quirion et al., 1981), correspond to autoradiographic localization of NMDA receptors at excitatory synapses on the dendrites of Purkinje cells in this brain region. Immunocytochemistry has recently revealed NMDAR1 specific labelling of Purkinje cell dendrites in the molecular layer of the cerebellar cortex (Brose et al., 1993). Furthermore, in situ hybridization studies have localized NMDAR1 mRNA in Purkinje cell bodies of adult rat cerebellum (Moriyoshi et al., 1991; Laurie and Seeburg, 1994a).

A consensus of electrophysiological and binding data provide evidence for NMDA receptors in the cerebellum that are pharmacologically distinct from those in forebrain. Cerebellar NMDA receptors have been shown to differ with respect to their responses to regulation via the glycine and glutamate modulatory domains of the NMDA receptor (Monaghan, 1988; Monaghan and Beaton, 1991; Priestly and Kemp, 1993). In addition, these receptors appear to have a relatively low affinity for MK-801 (Quarum et al., 1990; Ebert et al., 1991; Beaton et al., 1992) and TCP (Vignon et al., 1986; Maurice and Vignon, 1990). Receptor autoradiography of the cerebellum has revealed that NMDA-sensitive [ $^3\text{H}$ ]glutamate binding is predominantly found in the granule cell layer (Greenamyre et al., 1985; Monaghan and Cotman, 1985). In accordance with this distribution, [ $^3\text{H}$ ]TCP

binding sites predominate in the granule cell layer of the cerebellum (Gundlach et al., 1986; Vignon et al., 1986) but have been reported to be much fewer than those sites labelled by NMDA-displaceable [ $^3\text{H}$ ]glutamate (Maragos et al., 1988) or the competitive NMDA antagonist [ $^3\text{H}$ ]CPP (Jarvis et al., 1987). Similarly, low levels of [ $^3\text{H}$ ]MK-801 binding have been reported in the granule cell layer of the cerebellum (Subramaniam and McGonigle, 1991; Beaton et al., 1992); in some studies, however, the low affinity of MK-801 for cerebellar NMDA receptors has been offered as an explanation for the fact that [ $^3\text{H}$ ]MK-801 binding can not be detected in the cerebellum by receptor autoradiography (Bowery et al., 1988; Monaghan, 1991; Sakurai et al., 1991). Support for the idea of regional heterogeneity amongst native NMDA receptors is also provided by data derived from the study of recombinantly expressed heteromeric NMDA receptors. Given that mRNAs encoding specific splice variants of NR1 (Laurie and Seeburg, 1994a) and particular subunits of NR2, ( $\epsilon 3$  / NR2C) (Kutsuwada et al., 1992; Monyer et al., 1992) are predominantly found in the cerebellum, it would not be unreasonable to expect variance in subunit composition to underlie pharmacologic differences in native cerebellar NMDA receptors.

The regional distribution of [ $^3\text{H}$ ]dextrorphan binding sites clearly distinguishes [ $^3\text{H}$ ]dextrorphan sites from those sites labelled by [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]TCP in the cerebellum lending further support for regional heterogeneity amongst native NMDA receptors. We have previously identified differences in populations of NMDA receptors labelled by [ $^3\text{H}$ ]dextrorphan in rat forebrain and cerebellum (Franklin and Murray, 1992). In slide-mounted sections dextrorphan was a less potent inhibitor of [ $^3\text{H}$ ]dextrorphan binding in the molecular and granule cell layers of the cerebellum than in forebrain regions. [ $^3\text{H}$ ]Dextrorphan labels high and low affinity sites in forebrain and cerebellar membranes, however, a larger fraction of sites in the cerebellum are of low affinity. Although we are unable to resolve two [ $^3\text{H}$ ]dextrorphan affinity states in slide-mounted tissue this low affinity population of sites may contribute to the reduced potency reported herein. Further

support for a distinction between forebrain and cerebellar [ $^3\text{H}$ ]dextrorphan binding sites is provided by the fact that MK-801 was an ineffective competitor for [ $^3\text{H}$ ]dextrorphan sites in the cerebellar molecular and granule cell layers. It is interesting to note that in an in vivo study in the mouse, Maurice and Vignon (1990) found MK-801 to be the most potent inhibitor of [ $^3\text{H}$ ]TCP binding in the cerebrum and hippocampus but an ineffective competitor for [ $^3\text{H}$ ]TCP binding sites in cerebellum. These observations may be indicative of some degree of non-identity between the low affinity MK-801 and TCP recognition sites in the cerebellum and provide additional confirmation of the unsuitability of MK-801 to define nonspecific [ $^3\text{H}$ ]dextrorphan binding in this brain region.

In summary, the anatomical distribution of [ $^3\text{H}$ ]dextrorphan recognition sites in rat forebrain corresponds to the known distribution of NMDA receptors labelled by NMDA-sensitive [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]TCP. Furthermore, the pharmacologic profile of these binding sites is consistent with an association of [ $^3\text{H}$ ]dextrorphan with a noncompetitive antagonist domain of the NMDA receptor-channel complex. We have, however, identified both regional and pharmacologic heterogeneity in the binding characteristics of [ $^3\text{H}$ ]dextrorphan which appears to reflect some degree of nonidentity between [ $^3\text{H}$ ]dextrorphan binding sites and those sites labelled by other noncompetitive antagonists. Given the increasing evidence for NMDA receptor heterogeneity, ligand autoradiography represents a powerful technique with the potential to selectively identify distinct populations of native NMDA receptors.

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## Chapter 6

### Discussion

The pharmacologic properties of the dextrorotatory morphinans, dextrorphan and dextromethorphan, have been studied since the early 1950s when they became widely used as antitussive agents. Dextromethorphan is now marketed as a safe and effective non-prescription cough suppressant in more than sixty countries under many different proprietary names. More recently, however, dextrorphan and dextromethorphan have been shown to have anticonvulsant and neuroprotective properties by virtue of their activity as noncompetitive antagonists of the N-methyl-D-aspartate (NMDA) subfamily of ionotropic glutamate receptors. The aim of this research project was to gain a better understanding of the interaction of dextrorotatory opioids with the NMDA receptor-operated ion channel. Initial studies were undertaken in the whole animal to specifically investigate the anticonvulsant and psychoactive effects of dextrorphan and dextromethorphan. Later studies were done in vitro, using radiolabelled dextrorphan to determine the anatomical distribution of dextrorphan binding sites in the rat brain by quantitative receptor autoradiography. Although the NMDA receptor is the most thoroughly characterized of the glutamate receptors with respect to its pharmacology and physiology, the cloning of the first NMDA-gated ion channel was not accomplished until the end of 1991 (Moriyoshi et al., 1991). It is hoped that a more complete mechanistic understanding of the function of native NMDA receptors will eventually allow this receptor system to be better manipulated for therapeutic gain in the treatment of epilepsy and to reduce neuronal injury associated with stroke, head trauma, sustained convulsions and hypoglycaemia.

Although the anticonvulsant activity of dextrorphan and dextromethorphan has been documented and is comparable to that of other noncompetitive NMDA antagonists, the

underlying mechanism of action has remained less certain. The rapid O-demethylation of dextromethorphan to dextrorphan following peripheral administration, has been a complicating factor in the interpretation of some studies *in vivo*. The ability of dextrorphan and dextromethorphan to suppress convulsions was therefore assessed using an animal model in which generalized motor seizures can be generated by unilateral focal injection of a chemoconvulsant in the rat prepiriform. In this model, test compounds are also delivered to the same locus of the prepiriform cortex by direct intracerebral administration thereby limiting dispositional influences. The anticonvulsant efficacy of dextrorphan and dextromethorphan in the prepiriform cortex was comparable to that of the noncompetitive NMDA antagonists phencyclidine (PCP) and MK-801. Furthermore, the anticonvulsant potencies of the compounds tested were highly correlated with their respective affinities for the [ $^3\text{H}$ ]dextrorphan-labelled NMDA receptors in rat forebrain membrane preparations (Franklin and Murray, 1992). The correlation of this pharmacological profile with that defined by [ $^3\text{H}$ ]dextrorphan equilibrium competition binding studies suggests that interaction with the NMDA receptor cation channel complex mediates the anticonvulsant actions of the dextrorotatory opioids.

Whilst investigating the anticonvulsant effects of the dextrorotatory opioids in the prepiriform cortex, a novel anticonvulsant action of 1,3-di(2-tolyl)guanidine (DTG) was discovered. This compound was initially included as a reference sigma ( $\sigma$ ) compound in these studies and was therefore predicted to have little effect. DTG, however, was a potent and efficacious anticonvulsant in the rat prepiriform cortex by a mechanism that does not involve  $\sigma$  receptors. Noncompetitive antagonism of NMDA receptor-mediated excitation at the MK-801 / PCP binding site is also unlikely to account for the protective effects of DTG.

Although devoid of morphine-like effects and consequently considered non-addictive (Isbell and Fraser, 1953), the dextrorotatory morphinans, at levels exceeding the recommended antitussive dose, can induce a PCP-like syndrome. Dextrorphan and

dextromethorphan were found to induce a stereotyped behavioural response in rats that was indistinguishable from that produced by PCP and other noncompetitive NMDA antagonists. The correlation of this pharmacologic profile with that defined by [ $^3\text{H}$ ]dextrorphan equilibrium competition binding studies suggests that interaction with the NMDA receptor-channel complex mediates the stereotyped behavioural effects of these compounds.

To specifically address the propensity of dextromethorphan to induce these behaviours, dextrorotatory opioid-induced stereotypies were compared in female Sprague-Dawley and female Dark Agouti rats. The female Dark Agouti rat lacks cytochrome P450 2D1 which catalyses the O-demethylation of dextromethorphan to dextrorphan. There were no differences in either the potencies or time to peak effect of dextrorphan and dextromethorphan to induce stereotyped behaviour in either of the two rat strains. These data suggest that the affinity of dextromethorphan for NMDA receptors adequately accounts for its ability to induce stereotyped behaviour.

In order to determine the functional significance of various combinations of recombinant NMDA receptor subunits and splice variants, there is an increasing need for a better understanding of the regional distribution and properties of native NMDA receptors. Receptor autoradiography has provided evidence for regionally heterogeneous populations of native NMDA receptors in the rat forebrain based on differential activation of the NMDA receptor-channel complex by [ $^3\text{H}$ ]glutamate, binding of the competitive glutamate antagonist [ $^3\text{H}$ ]3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid ([ $^3\text{H}$ ]CPP) or regulation of [ $^3\text{H}$ ]MK-801 binding by NMDA receptor agonists and antagonists (Monaghan et al., 1988; Monaghan 1991; Beaton et al., 1992; Sakurai et al., 1993). In addition, a pharmacologically distinct NMDA receptor has been identified in the cerebellum that appears to have, as one of its characteristics, a particularly low affinity for MK-801 (Maurice and Vignon 1990; Quarum et al., 1990; Ebert et al., 1991; Beaton et al., 1992).

Previous studies indicate that [ $^3\text{H}$ ]dextrorphan labels a high affinity site in rat brain membranes corresponding to the noncompetitive antagonist binding domain of the NMDA receptor-channel complex (Franklin and Murray, 1990; 1992). However, the binding domain of [ $^3\text{H}$ ]dextrorphan may not be identical to the channel binding sites of either [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]1-[1-(2-thienyl)cyclohexyl]piperidine ([ $^3\text{H}$ ]TCP). Specific aspects of the regulation of [ $^3\text{H}$ ]dextrorphan binding by modulators of the NMDA receptor-channel complex distinguish it from [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]TCP and may be indicative of underlying differences in the mechanisms of their noncompetitive antagonism. [ $^3\text{H}$ ]Dextrorphan may therefore represent a unique ligand for the NMDA receptor that could facilitate the identification of functionally and regionally distinct populations of native NMDA receptors.

[ $^3\text{H}$ ]Dextrorphan binding sites were characterized in slide-mounted rat brain sections and their regional distribution ascertained using quantitative receptor autoradiography. These studies extend our previous findings in membrane homogenates (Franklin and Murray, 1990; 1992) in that [ $^3\text{H}$ ]dextrorphan appears to selectively label a high affinity site localized within an open channel domain of the NMDA receptor. The [ $^3\text{H}$ ]dextrorphan binding site may not, however, be identical to ion channel binding sites labelled by other noncompetitive antagonists. Specifically, differences in the regulation of [ $^3\text{H}$ ]dextrorphan binding by glycine and glutamate and the labelling of high affinity sites in the cerebellum distinguish [ $^3\text{H}$ ]dextrorphan binding from that of either [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]TCP.

The kinetics of association and dissociation of [ $^3\text{H}$ ]dextrorphan with its binding site are slow in slide-mounted brain sections. The addition of glycine and glutamate, however, increases the apparent association rate of [ $^3\text{H}$ ]dextrorphan, presumably a result of their ability to facilitate access of [ $^3\text{H}$ ]dextrorphan to a guarded or transiently available binding domain within the activated NMDA receptor ion channel. In contrast to reports of [ $^3\text{H}$ ]MK-801 binding in slide-mounted sections (Fletcher and Bowery, 1991; Sakurai et



al., 1991), we were unable to demonstrate agonist-induced increases in [ $^3$ H]dextrorphan binding following a prolonged washing procedure designed to lower endogenous levels of glycine and glutamate in the sections. As the addition of glycine and glutamate produced a substantial increase in the association rate of [ $^3$ H]dextrorphan following standard pre-washing conditions, it seems unlikely that saturating concentrations of the endogenous agonists are still present following prolonged pre-washing. Together with our previous findings in membrane homogenates (Franklin and Murray, 1992), these data suggest that [ $^3$ H]dextrorphan is less sensitive to the modulatory effects of glycine and glutamate than [ $^3$ H]MK-801. Although it would be premature at this point to attempt to assign a functional significance to our observations, the relative insensitivity of [ $^3$ H]dextrorphan binding to glycine and glutamate may provisionally be explained by the possible existence of a [ $^3$ H]dextrorphan binding site that to some degree is nonidentical to the binding site of [ $^3$ H]MK-801, rather than the unique regulation of [ $^3$ H]dextrorphan at a common noncompetitive antagonist binding site. The apparent indifference of dextrorphan to the presence of glycine and glutamate may be a consequence of its occupation of a more superficially located binding site than that of MK-801 that is less dependent on agonist activation of the receptor.

Although [ $^3$ H]dextrorphan binding appears to be regulated in a distinctive manner by glycine and glutamate, the presence of both agonists is still required for binding to occur. Basal specific [ $^3$ H]dextrorphan binding was sensitive to D-AP5, a competitive NMDA antagonist, and 7-chlorokynurenic acid, a strychnine-insensitive glycine antagonist, presumably a result of their competitive surmounting of the activation of NMDA receptors by endogenous glutamate and glycine. Interestingly, D-AP5 showed a marked regional variation in its potency to inhibit [ $^3$ H]dextrorphan binding which did not follow patterns previously reported for inhibition of [ $^3$ H]MK-801 binding by CPP (Beaton et al., 1992; Sakurai et al., 1993). In particular, a distinct population of [ $^3$ H]dextrorphan binding sites was weakly inhibited by D-AP5 in the lateral thalamus. Buller et al. (1994) have recently

shown that populations of receptors displaying high affinity for [ $^3$ H]CPP, typified by the lateral thalamus, are localized to brain regions expressing high densities of NMDAR2A (NR2A) mRNA but not NR2B mRNA. The ratio of NR2A to NR2B mRNA is greater in cortex than lateral thalamus (Buller et al., 1994); thus, a preponderance of the NR2A subunit may confer the much higher affinity of D-AP5 for [ $^3$ H]dextrorphan binding sites in this area relative to lateral thalamus.

The rank order of potencies of an array of noncompetitive NMDA receptor antagonists as inhibitors of [ $^3$ H]dextrorphan binding in CA1 and outer cortex, provide additional evidence that the site labelled by [ $^3$ H]dextrorphan in slide-mounted sections is localized to a noncompetitive antagonist domain of the NMDA receptor. In accordance with the pharmacologic signature of sites labelled by [ $^3$ H]PCP (Murray and Leid, 1984) and [ $^3$ H]dextrorphan (Franklin and Murray, 1992) in brain membranes, dextromethorphan was less potent than dextrorphan as a competitor for [ $^3$ H]dextrorphan binding sites in slide-mounted sections. Given recent evidence for differential sensitivity of recombinant heteromeric receptors to block by MK-801 (Yamakura et al., 1993), variation in the relative potencies of dextrorphan and dextromethorphan for sites labelled by [ $^3$ H]dextrorphan may reflect differences in the properties of native NMDA receptors located in CA1 and outer cortex.

[ $^3$ H]Dextrorphan binding sites are heterogeneously distributed throughout the rat brain with a marked anatomical specificity that unambiguously corresponds to the known distribution of NMDA receptors (Cotman et al., 1987). Equilibrium saturation binding data from several brain areas confirms the presence of a differential distribution of recognition sites and suggests that [ $^3$ H]dextrorphan binds to a single population of high affinity sites under the assay conditions employed. Since the  $K_D$  values of these binding sites do not vary significantly, the regional heterogeneity of [ $^3$ H]dextrorphan binding sites, as determined by single [ $^3$ H]dextrorphan concentration equilibrium binding values, can be regressed to differences in the absolute receptor densities in the regions evaluated.

The relative distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat forebrain is consistent with the reported distribution of NMDA-sensitive [ $^3\text{H}$ ]glutamate binding sites (Greenamyre et al., 1985; Monaghan and Cotman, 1985). Furthermore, the autoradiographic pattern of [ $^3\text{H}$ ]dextrorphan binding sites in the forebrain is in excellent agreement with previously reported distributions of [ $^3\text{H}$ ]MK-801 (Bowery et al., 1988; Subramaniam and McGonigle, 1991; Sakurai et al., 1991) and [ $^3\text{H}$ ]TCP (Gundlach et al., 1986; Maragos et al., 1988) binding sites. As the relative distribution of [ $^3\text{H}$ ]dextrorphan binding sites in rat brain bears no resemblance to the previously reported distributions of [ $^3\text{H}$ ]dextromethorphan binding sites in rat or guinea pig brain (Canoll et al., 1989; Tortella et al., 1989), it appears that [ $^3\text{H}$ ]dextrorphan binding sites are unrelated to either [ $^3\text{H}$ ]dextromethorphan binding sites or sigma binding sites (Walker et al., 1990). These data lend further support for a distinct pharmacology of dextromethorphan that is unrelated to its affinity for the noncompetitive antagonist domain of the NMDA receptor.

One brain area where the relative distribution of [ $^3\text{H}$ ]dextrorphan binding sites does not match the reported pattern of sites labelled by either NMDA-sensitive [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ]MK-801 or [ $^3\text{H}$ ]TCP is the cerebellum. The density of [ $^3\text{H}$ ]dextrorphan sites in the cerebellar molecular layer exceeds that of the granule cell layer by a factor of three. Receptor autoradiography of the cerebellum has revealed NMDA-sensitive [ $^3\text{H}$ ]glutamate binding predominantly in the granule cell layer (Greenamyre et al., 1985; Monaghan and Cotman, 1985). In accordance with this distribution, [ $^3\text{H}$ ]TCP binding sites predominate in the granule cell layer (Gundlach et al., 1986; Vignon et al., 1986) but have been reported to represent a much lower density of sites than those labelled by NMDA-displaceable [ $^3\text{H}$ ]glutamate (Maragos et al., 1988) or [ $^3\text{H}$ ]CPP (Jarvis et al., 1987). Similarly, low levels of [ $^3\text{H}$ ]MK-801 binding have been reported in the granule cell layer (Subramaniam and McGonigle, 1991; Beaton et al., 1992); in some studies, however, the low affinity of MK-801 for cerebellar NMDA receptors has been offered as an explanation for the fact that [ $^3\text{H}$ ]MK-801 binding is frequently undetected in the cerebellum by receptor

autoradiography (Bowery et al., 1988; Monaghan, 1991; Sakurai et al., 1991).

Although non-NMDA receptors are thought to predominate in the cerebellar molecular layer, a population of NMDA-sensitive [ $^3\text{H}$ ]glutamate binding sites is certainly present in this brain area (Monaghan and Cotman, 1985). Greenamyre et al. (1985) also reported 25-30 % of [ $^3\text{H}$ ]glutamate binding in the molecular layer of the cerebellum could be inhibited by NMDA receptor-specific agents. Furthermore, autoradiography of [ $^3\text{H}$ ]PCP binding sites by Quirion et al. (1981) revealed preferential labelling of the cerebellar molecular layer. The discovery that [ $^3\text{H}$ ]TCP preferentially bound to the cerebellar granule cell layer gave an early indication of potential differences in the binding properties of [ $^3\text{H}$ ]PCP and [ $^3\text{H}$ ]TCP in the cerebellum and led to the proposal that the two ligands had differing anatomical specificities (Vignon et al., 1986). It is possible, therefore, that the high density of [ $^3\text{H}$ ]dextrorphan binding sites in the molecular layer correspond to autoradiographic localization of NMDA receptors at excitatory synapses on the dendrites of Purkinje cells in this brain region. Immunocytochemistry has recently revealed NMDAR1 specific labelling of Purkinje cell dendrites in the molecular layer of the cerebellar cortex (Brose et al., 1993). Furthermore, in situ hybridization studies have localized NMDAR1 mRNA in Purkinje cell bodies of adult rat cerebellum (Moriyoshi et al., 1991; Laurie and Seeburg, 1994a).

In summary, the anatomical distribution of [ $^3\text{H}$ ]dextrorphan recognition sites in rat forebrain corresponds to the known distribution of NMDA receptors labelled by NMDA-sensitive [ $^3\text{H}$ ]glutamate, [ $^3\text{H}$ ]MK-801 and [ $^3\text{H}$ ]TCP. We have, however, identified both regional and pharmacologic heterogeneity in the binding characteristics of [ $^3\text{H}$ ]dextrorphan which appears to reflect some degree of nonidentity between [ $^3\text{H}$ ]dextrorphan binding sites and those sites labelled by other noncompetitive antagonists. Given the increasing evidence for NMDA receptor heterogeneity, ligand autoradiography represents a powerful technique with the potential to resolve distinct populations of native NMDA receptors.

## Bibliography

- Al-Dabbagh, S.G., Idle, J.R. and Smith, R.L.: Animal modelling of human polymorphic drug oxidation-the metabolism of debrisoquine and phenacetin in rat inbred strains. *J. Pharm. Pharmacol.* 33: 161-164, 1981.
- Alexander, G.M., Schwartzman, R.J., Bell, R.D., Yu, J. and Renthal, A.: Quantitative measurement of local cerebral metabolic rate for glucose utilizing tritiated 2-deoxyglucose. *Brain Res.* 223: 59-67, 1981.
- Anis, N.A., Berry, S.C., Burton, N.R. and Lodge, D.: The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurones by N-methyl-aspartate. *Br. J. Pharmacol.* 79: 565-575, 1983.
- Aram, J.A., Martin, D., Tomczyk, M., Zeman, S., Millar, J., Pohler, G. and Lodge, D.: Neocortical epileptogenesis in vitro: studies with N-methyl-D-aspartate, phencyclidine, sigma and dextromethorphan receptor ligands. *J. Pharmacol. Exp. Ther.* 248: 320-328, 1989.
- Barnhart, J.W.: The urinary excretion of dextromethorphan and three metabolites in dogs and humans. *Toxicol. Appl. Pharmacol.* 55: 43-48, 1980.
- Beaton, J.A., Stemsrud, K. and Monaghan, D.T.: Identification of a novel N-methyl-D-aspartate receptor population in the rat medial thalamus. *J. Neurochem.* 59: 754-757, 1992.
- Bem, J.L. and Peck, R.: Dextromethorphan: An Overview of Safety Issues. *Drug Safety.* 7: 190-199, 1992.
- Bennett, J.A. and Dingledine R.: Topology profile for a glutamate receptor: three transmembrane domains and a channel-lining reentrant loop. *Neuron.* 14: 373-384, 1995.
- Bochner, F., Somogyi, A.A. and Chen, Z.R.: Dextromethorphan metabolism in rat: interstrain differences and the fate of individually administered oxidative metabolites. *Xenobiotica.* 24: 543-552, 1994.
- Bonhaus, D.W. and McNamara, J.O.: N-Methyl-D-aspartate receptor regulation of uncompetitive antagonist binding in rat brain membranes: kinetic analysis. *Mol. Pharmacol.* 34: 250-255, 1988.
- Bowery, N.G., Wong, E.H.F. and Hudson, A.L.: Quantitative autoradiography of [<sup>3</sup>H]MK-801 binding sites in mammalian brain. *Br. J. Pharmacol.* 93: 944-954, 1988.
- Brose, N., Gasic, G.P., Vetter, D.E., Sullivan, J.M. and Heinemann, S.F.: Protein chemical characterization and immunocytochemical localization of the NMDA receptor subunit NMDA R1. *J. Biol. Chem.* 268: 22663-22671, 1993.
- Buller, A.L., Larson, H.C., Schneider, B.E., Beaton, J.A., Morrisett, R.A. and Monaghan, D.T.: The molecular basis of NMDA receptor subtypes: Native receptor diversity is predicted by subunit composition. *J. Neurosci.* 14: 5471-5484, 1994.
- Burnashev, N., Schoepfer, R., Monyer, H., Ruppersberg, J.P., Günther, W., Seeburg, P.H. and Sakmann, B.: Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA Receptor. *Science.* 257: 1415-1419, 1992.

- Canoll, P.D., Smith, P.R., Gottesman, S. and Musacchio, J.M.: Autoradiographic localization of [ $^3$ H]dextromethorphan in guinea pig brain: allosteric enhancement by rozipine. *J. Neurosci. Res.* 24: 311-328, 1989.
- Chapman, A.G. and Meldrum, B.S.: Non-competitive N-methyl-D-aspartate antagonists protect against sound-induced seizures in DBA / 2 mice. *Eur. J. Pharmacol.* 166: 201-211, 1989.
- Chazot, P.L., Coleman, S.K., Cik, M. and Stephenson, F.A.: Molecular characterization of N-methyl-D-aspartate receptors expressed in mammalian cells yields evidence for the coexistence of three subunit types within a discrete receptor molecule. *J. Biol. Chem.* 269: 24403-24409, 1994.
- Choi, D.W., Koh, J. and Peters, S.: Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. *J. Neurosci.* 8: 185-196, 1988.
- Choi, D.W., Peters, S. and Visekul, V.: Dextrorphan and levorphanol selectively block N-methyl-D-aspartate receptor-mediated neurotoxicity on cortical neurons. *J. Pharmacol. Exp. Ther.* 242: 713-720, 1987.
- Cholerton, S., Daly, A.K. and Idle, J.R.: The role of individual human cytochrome P450 in drug metabolism and clinical response. *Trends Pharmacol Sci.* 13: ,1992.
- Church, J., Lodge, D. and Berry, S.C.: Differential effects of dextrorphan and levorphanol on the excitation of rat spinal neurons by amino acids. *Eur. J. Pharmacol.* 111: 185-190, 1985.
- Church, J. and Lodge, D.: Cyclazocine and pentazocine as N-methylaspartate antagonists on cat and rat spinal neurons in vivo. *J. Pharmacol. Exp. Ther.* 253: 636-645, 1990.
- Cik, M., Chazot, P.L. and Stephenson, F.A.: Optimal expression of cloned NMDAR1/NMDAR2 heteromeric glutamate receptors: a biochemical characterization. *Biochem. J.* 296: 877-883, 1993.
- Cole, A.E., Eccles, C.U., Aryanpur, J.J., and Fisher, R.S.: Selective Depression of N-Methyl-D-aspartate-mediated responses by dextrorphan in the hippocampal slice in rat. *Neuropharmacology.* 28: 249-254, 1989.
- Connick, J.H., Addae, J.I., Nicholson, C.D. and Stone T.W.: The  $\sigma$  ligand 1,3-di-o-tolylguanidine depresses amino acid induced excitation non-selectively in rat brain, *Eur J. Pharmacol.* 214 :169, 1992.
- Contreras, P.A., Contreras, M.L., O'Donohue, T.L. and Lair, C.C.: Biochemical and behavioral effects of sigma and PCP ligands. *Synapse.* 2: 240, 1988.
- Cotman, C.W., Monaghan, D.T., Otterson, O.P. and Storm-Mathisen, J.: Anatomical organization of excitatory amino acid receptors and their pathways. *Trends Neurosci.* 10: 273-280, 1987.
- Craviso, G.L. and Musacchio, J.M.: High-affinity dextromethorphan binding sites in guinea pig brain. I. Initial characterization. *Mol. Pharmacol.* 23: 619-628, 1983a.
- Craviso, G.L. and Musacchio, J.M.: High-affinity dextromethorphan binding sites in guinea pig brain. II. Competition experiments. *Mol. Pharmacol.* 23: 629-640, 1983b.

- Degkwitz, R.: Dextromethorphan (Romilar) as an intoxicant. *Nervenarzt*. 35: 412-414, 1964.
- Duché, J.C., Quérol-Ferrer, V., Barré, J., Mésangeau, M. and Tillement, J.P.: Dextromethorphan O-demethylation and dextrorphan glucuronidation in a French population. *Intl. J. Clin. Pharmacol., Therapy and Toxicol.* 31: 392-398, 1993.
- Ebert, B., Wong, E.H.F. and Krogsgaard-Larsen, P.: Identification of a novel NMDA receptor in rat cerebellum. *Eur. J. Pharmacol.-Mol. Pharmacol. Section*. 208: 49-52, 1991.
- Faden, A.I., Demediuk, S.S., Panter, S.S. and Vink, R.: The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science*. 244: 798-800, 1989.
- Ferkany, J.W., Borosky, S.A., Clissold, D.B. and Pontecorvo, M.J.: Dextromethorphan inhibits NMDA-induced convulsions. *Eur. J. Pharmacol.* 151: 151-154, 1988.
- Fletcher, A.E. and Bowery, N.G.: Autoradiographical determination of strychnine-insensitive glycine receptor modulation of [ $^3$ H]MK-801 binding in rat CNS. *Br J. Pharmacol.* 102: 70P.
- Fletcher, E.J., Church, J. and MacDonald, J.F.: Sigma receptor ligands reduce NMDA-evoked depolarizations and elevations of intracellular calcium in hippocampal neurons. *Soc. Neurosci. Abstr.* 18: 1153, 1992.
- Fossati, A., Vimercati, M.G., Caputo, R., Citerio, L., Ceriani, R. and Valenti, M.: Comparative pharmacokinetics of oral dextromethorphan and dextrorphan in the rabbit. *Drug Res.* 43: 1337-1340, 1993.
- Foster, A.C. and Wong, E.H.F.: 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.
- Franklin, P.H. and Murray, T.F.: Identification and initial characterization of high-affinity [ $^3$ H]dextrorphan binding sites in rat brain. *Eur. J. Pharmacol.-Mol. Pharmacol. Section*. 189: 89-93, 1990.
- Franklin, P.H. and Murray, T.F.: High affinity [ $^3$ H]dextrorphan binding in rat brain is localized to a noncompetitive antagonist site of the activated N-methyl-D-aspartate receptor-activation channel. *Mol. Pharmacol.* 41: 134-146, 1992.
- Franklin, P.H., Zhang, G., Tripp, E.H. and Murray, T.F.: Adenosine A<sub>1</sub> receptor activation mediates the suppression of (-)-bicuculline methiodide-induced seizures in the rat prepiriform cortex. *J. Pharmacol. Exp. Ther.* 251: 1229, 1989.
- French, E.D. and Ceci, A.: Non-competitive N-methyl-D-aspartate antagonists are potent activators of ventral tegmental A<sub>10</sub> dopamine neurons. *Neurosci. Letters*. 119: 159, 1990.
- Geary, W.A. and Wooten, G.F.: Quantitative film autoradiography of opiate agonist and antagonist binding in rat brain. *J. Pharmacol. Exp. Ther.* 225: 234-240, 1983.
- Goldberg, M.P., Pham, P.C. and Choi, D.W.: Dextrorphan and dextromethorphan attenuate hypoxic injury in neuronal culture. *Neurosci. Letters*. 80: 11-15, 1987.

Goldstein, A. and Naidu, A.: Dextrorphan binds to opioid receptors in guinea-pig brain membranes and is an antagonist at opioid receptors in myenteric plexus. *Proc. Natl. Acad. Sci. USA.* 87: 1629-1632, 1990.

Greenamyre, J.T., Olsen, J.M.M., Penny, J.B. and Young, A.B.: Autoradiographic characterization of N-methyl-D-aspartate-, quisqualate- and kainate-sensitive glutamate binding. *J. Pharmacol. Exp. Ther.* 233: 254-263, 1985.

Greifenstein, F.E., DeVault, M., Yoshitake, J. and Gajewski, J.R.: A study of 1-arylcylohexyl amine for anesthesia. *Anesth. Analg.* 37: 283, 1959.

Gundlach, A.L., Largent, B.L. and Snyder, S.H.: Phencyclidine (PCP) receptors: autoradiographic localization in brain with the selective ligand, [ $^3\text{H}$ ]TCP. *Brain Res.* 386: 266-279, 1986.

Herling, S., Coale, E.H., Hein, D.W., Winger G. and Woods, J.H.: Similarity of the discriminative stimulus effects of ketamine, cyclazocine and dextrorphan in the pigeon. *Psychopharmacology.* 73: 286-291, 1981.

Herling, S., Solomon, R.E. and Woods J.H.: Discriminative stimulus effect of dextrorphan in pigeons. *J. Pharmacol. Exp. Ther.* 227: 723-731, 1983.

Hollmann, M., Boulter, J., Maron, C., Beasley, L., Sullivan, J., Pecht, G. and Heinemann, S.: Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. *Neuron.* 10: 943-954, 1993.

Hollmann, M., Maron, C., and Heinemann, S.: N-Glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. *Neuron.* 13: 1331-1343, 1994.

Holtzman, S.G.: Phencyclidine-like discriminative effects of opioids in the rat. *J. Pharmacol. Exp. Ther.* 214: 614-619, 1980.

Holtzman, S.G.: Opioid and phencyclidine-like discriminative effects of ditolylguanidine, a selective sigma ligand. *J. Pharmacol. Exp. Ther.* 248: 1054, 1989.

Holtzman, S.G.: Discriminative stimulus effects of dextromethorphan in the rat. *Psychopharmacology.* 116: 249-254, 1994.

Honey, C.R., Miljovic, Z. and MacDonald, J.F.: Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid. *Neurosci. Letters.* 61: 135-139, 1985.

Hosford, D.A., Bonhaus, D.W. and McNamara, J.O.: A radiohistochemical measure of [ $^3\text{H}$ ]TCP binding to the activated NMDA-receptor-gated ion channel in rat brain. *Brain Res.* 516: 192-200, 1990.

Ikeda, K., Nagasawa, M., Mori, H., Araki, K., Watanabe, M., Inoue, Y. and Mishina, M.: Cloning and expression of the  $\epsilon 4$  subunit of the NMDA receptor channel. *FEBS Letters.* 313: 34-38, 1992.

Isbell, H. and Fraser, H.F.: Actions and addiction liabilities of dromoran derivatives in man. *J. Pharmacol. Exp. Ther.* 107: 524-530, 1953.



Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M. and Nakanishi, S.: Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *J.Biol.Chem.* 268: 2836-2843, 1993.

Jaarsma, D., Sebens, J.B. and Korf, J.: Glutamate dehydrogenase improves binding of [<sup>3</sup>H]CGP39653 to NMDA receptors in the autoradiographic assay. *J. Neurosci. Res.* 46: 133-138, 1993.

Jarvis, M.F., Murphy, D.E. and Williams, M.: Quantitative autoradiographic localization of NMDA receptors in rat brain using [<sup>3</sup>H]TCP binding sites. *Eur. J. Pharmacol.* 141: 149-152, 1987.

Jasinski, D.R., Martin, W.R. and Mansky, P.A.: Progress report on the assessment of the antagonists nalbuphine and GPA-2087 for abuse potential and studies of the effects of dextromethorphan in man. Report of the 33rd Meeting, Committee on Problems of Drug Dependence. 143-178, 1971.

Johnson, K.M., Snell, L.D., Sacca, A.I. and Jones, S.: 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.

Kamm, J.J., Taddeo, A.B. and Van Loon, E.J.: Metabolism and excretion of tritiated dextromethorphan by the rat. *J. Pharmacol. Exp. Ther.* 158: 437-444, 1967.

Kawajiri, S. and Dingledine, R.: Multiple structural determinants of voltage-dependent magnesium block in recombinant NMDA receptors. *Neuropharmacology.* 32: 1203-1211, 1993.

Keana, J.F.W., McBurney, R.N., Scherz, M.W., Fischer, J.B., Hamilton, P.N., Smith, S.M., Server, A.C., Finkbeiner, S., Stevens, C.F., Jahr, C. and Weber, E.: Synthesis and characterization of a series of diarylguanidines that are noncompetitive N-methyl-D-aspartate receptor antagonists with neuroprotective properties. *Proc. Natl. Acad. Sci. USA.* 86: 5631, 1989.

Kennedy, C. and Henderson, G.: An examination of the putative  $\sigma$ -receptor in the mouse isolated vas deferens. *Br. J. Pharmacol.* 98: 429, 1989.

Kerry, N.L., Somogyi, A.A., Mikus, G. and Bochner, F.: Primary and secondary oxidative metabolism of dextromethorphan. In vitro studies with female Sprague-Dawley and Dark Agouti rat liver microsomes. *Biochem. Pharmacol.* 45: 833-839, 1993.

Kessler, M., Terramani, T., Lynch, G. and Baudry, M.: A glycine site associated with N-methyl-D-aspartic acid receptors: characterization and identification of a new class of antagonists. *J. Neurochem.* 52: 1319-1328, 1989.

Kloog, Y., Haring, R. and Sokolovsky, M.: Kinetic characterization of the phencyclidine-N-methyl-D-aspartate receptor interaction: evidence for a steric blockade of the channel. *Biochemistry.* 27: 843-848, 1988a.

Kloog, Y., Nadler, V. and Sokolovsky, M.: Mode of binding of [<sup>3</sup>H]dibenzocycloalkenimine (MK-801) to the N-methyl-D-aspartate (NMDA) receptor and its therapeutic implication. *FEBS Letters.* 230: 167-170, 1988b.

Koek, W., Woods, J.H. and Winger, G.D.: MK-801, a proposed noncompetitive antagonist of excitatory amino acid neurotransmission, produces phencyclidine-like behavioral effects in pigeons, rats and rhesus monkeys. *J. Pharmacol. Exp. Ther.* 245: 969-974, 1988.

Kornhuber, J., Mack-Burkhardt, F., Kornhuber, M.E. and Riederer, P.: [ $^3\text{H}$ ]MK-801 binding sites in post-mortem human frontal cortex. *Eur. J. Pharmacol.* 162: 483-490, 1989.

Kupfer, A., Schmid, B. and Pfaff, G.: Pharmacogenetics of dextromethorphan O-demethylation in man. *Xenobiotica*. 16: 421-433, 1986.

Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiya, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M. and Mishina, M.: Molecular diversity of the NMDA receptor channel. *Nature*. 358: 36-41, 1992.

Laurie, D.J., and Seeburg, P.H.: Regional and developmental heterogeneity in splicing of the rat brain NMDAR1 mRNA. *J. Neurosci.* 14: 3180-3194, 1994a.

Laurie, D.J., and Seeburg, P.H.: Ligand affinities at recombinant N-methyl-D-aspartate receptors depend on subunit composition. *Eur. J. Pharmacol.* 268: 335-345, 1994b.

Leander, J.D., Rathbun, R.C. and Zimmerman, D.M.: Anticonvulsant effects of phencyclidine-like drugs: relation to N-methyl-D-aspartic acid antagonism. *Brain Res.* 454: 368-372, 1988.

Lear, E., Suntay, R., Pallin, I.M. and Chiron, A.E.: Cyclohexamine (CI-400) a new intravenous agent. *Anesthesiology*. 20: 330, 1953.

Litchfield, J.T. and Wilcoxon, F.: A simplified method of evaluating dose-effect experiments. *J. Pharmacol. Exp. Ther.* 96: 99, 1949.

Lodge, D. and Anis, N.A.: Effects of phencyclidine on excitatory amino acid activation of spinal interneurons in the cat. *Eur. J. Pharmacol.* 77: 203-204, 1992.

Loo, P., Braunwalder, A., Lehmann, J. and Williams, M.: Radioligand binding to central phencyclidine recognition sites is dependent on excitatory amino acid receptor agonists. *Eur. J. Pharmacol.* 123: 467-468, 1986.

Loscher, W. and Honack, D.: Differences in the anticonvulsant potency and adverse effects between dextromethorphan and dextrorphan in amygdala-kindled and non-kindled rats. *Eur. J. Pharmacol.* 238: 191-200, 1993.

Loscher, W., Netzer, R. and Schmitt, B.: Dextromethorphan. *Neurology*. 44: 582-583, 1994.

Luby, E.D., Cohen, B.D., Rosenbaum, J.S., Gottlieb, J.S. and Kelly, R.: Study of a new psychotomimetic drug-Sernyl. *Arch. Neurol. Psychiatry*. 81: 113-119, 1959.

Lynch, D.R., Anegawa, N.J., Verdoorn, T. and Pritchett, D.B.: N-methyl-D-aspartate receptors: different subunit requirements for binding of glutamate antagonists, glycine antagonists and channel-blocking agents. *Mol. Pharmacol.* 45: 540-545, 1994.

- MacDonald, J.F., Bartlett, M.C., Mody, I., Pahapill, P., Reynolds, J.N., Salter, M.W., Schneiderman, J.H. and Pennefather, P.S.: Actions of ketamine, phencyclidine and MK-801 on NMDA receptor currents in cultured mouse hippocampal neurones. *J. Physiol.* 432: 483-508, 1991.
- MacDonald, Miljkovic, Z. and Pennefather, P.S.: Use-dependent block of excitatory amino acid currents in cultured neurones by ketamine. *J. Neurophysiol.* 58: 251, 1987.
- Maragos, W.F., Penny, J.B. and Young, A.B.: Anatomic correlation of NMDA and  $^3\text{H}$ -TCP -labeled receptors in rat brain. *J. Neurosci.* 8: 493-501, 1988.
- Matsunaga, E., Zanger, U.M., Hardwick, J.P., Gelboin, H.V., Meyer, U.A. and Gonzalez, F.J.: The CYP2D gene subfamily: Analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry.* 28: 7349-7355, 1989.
- Maurice, T. and Vignon, J.: In vivo labeling of phencyclidine (PCP) receptors with  $^3\text{H}$ -TCP in the mouse brain. *J. Neurosci. Res.* 26: 377-385, 1990.
- McCarthy, J.P.: Some less familiar drugs of abuse. *Med. J. Austral.* 2: 1078-1081, 1971.
- McDonald, J.W., Cline, H.T., Constantine-Paton, M., Maragos, W.F., Johnston, M.V. and Young A.B.: Quantitative autoradiographic localization of NMDA, quisqualate and PCP receptors in the frog tectum. *Brain Research.* 482: 155-158, 1989.
- Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K. and Mishina, M.: Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature.* 357: 70-74, 1992.
- Monaghan, D.T.: Differential stimulation of [ $^3\text{H}$ ]MK-801 binding to subpopulations of NMDA receptors. *Neurosci. Lett.* 122: 21-24, 1991.
- Monaghan, D.T. and Beaton, J.A.: Quinolinate differentiates between forebrain and cerebellar NMDA receptors. *Eur. J. Pharmacol.* 194: 123-125, 1991.
- Mongahan, D.T., Bridges, R.J. and Cotman, C.W.: The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.* 29: 365-402, 1989.
- Monaghan, D.T. and Cotman, C.W.: Distribution of N-methyl-D-aspartate-sensitive L-[ $^3\text{H}$ ]glutamate-binding sites in rat brain. *J. Neurosci.* 5: 2909-2919, 1985.
- Monaghan, D.T., Olverman, H.J., Nguyen, L., Watkins, J.C. and Cotman, C.W.: Two classes of N-methyl-D-aspartate recognition sites: differential distribution and differential regulation by glycine. *Proc. Natl. Acad. Sci. USA.* 85: 9836-9840, 1988.
- Monnet, F.P., Debonnel, G. and DeMontigny, C.: In vivo and electrophysiological evidence for a selective modulation of N-methyl-D-aspartate-induced neuronal activation in rat CA<sub>3</sub> dorsal hippocampus by sigma ligands. *J. Pharmacol. Exp. Ther.* 261: 123, 1992.
- Monyer, H. and Choi, D.W.: Morphinans attenuate cortical neuronal injury induced by glucose deprivation in vitro. *Brain Res.* 446: 144-148, 1988.

Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B. and Seeburg, P.H.: Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science*. 256: 1217-1221, 1992.

Mori, H., Masaki, H., Yamakura, T. and Mishina, M.: Identification by mutagenesis of a  $Mg^{2+}$ -block site of the NMDA receptor channel. *Nature*. 358: 673-675, 1992.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N. and Nakanishi, S.: Molecular cloning and characterization of the rat NMDA receptor. *Nature*. 354: 31-37, 1991.

Murray, T.F.: Basic Pharmacology of Ketamine. In: *The Pharmacologic Basis of Anesthesiology*, eds. T.A. Bowdle, A. Horita and E.D. Kharasch. pp 337-355, Churchill Livingstone, 1994.

Murray, T.F. and Horita, A.: Phencyclidine-induced stereotyped behavior in rats: dose response effects and antagonism by neuroleptics. *Life Sci*. 24: 2217-2226, 1979.

Murray, T.F. and Leid, M.E.: Interaction of dextrorotatory opioids with phencyclidine recognition sites in rat brain membranes. *Life Sci*. 34: 1899-1911, 1984.

Murray, T.F., Leid, M.E., Zaro, B.J. and Klavano, P.A.: *Soc. Neurosci. Abstr.* 8: 388, 1982.

Nakanishi, N., Axel, R. and Shneider, N.A.: Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors. *Proc. Natl. Acad. Sci. USA*. 89: 8552-8556, 1992.

Nakanishi, S.: Molecular diversity of glutamate receptors and implications for brain function. *Science*. 258: 597-603, 1992.

Paxinos, G. and Watson, C.: *The rat brain in stereotaxic coordinates*. Academic Press, New York, 1986.

Pirreda, S. and Gale, K.: A crucial epileptogenic site in the deep prepiriform cortex. *Nature*. 317: 623-625, 1985.

Priestly, T and Kemp, J.: Agonist response kinetics of N-methyl-D-aspartate receptors in neurons cultured from rat cerebral cortex and cerebellum: evidence for receptor heterogeneity. *J. Pharmacol. Exp. Ther.* 44: 1252-1257, 1993.

Quarum, M.L., Parker, J.D., Keana, J.F.W. and Weber, E.: (+)-[ $^3H$ ]MK-801 binding sites in postmortem human brain. *J. Neurochem.* 54: 1163-1168, 1990.

Quirion, R., Hammer, P.P., Jr., Herkenham, M. and Pert, C.B.: Phencyclidine (angel dust) /  $\sigma$  "opiate" receptor: visualization by tritium-sensitive film. *Proc. Natl. Acad. Sci. USA*. 78: 5881-5885, 1981.

Racine, R.J.: Modification of seizure activity by electrical stimulation. II. Motor Seizures. *Electroencephalogr. Clin. Neurophysiol.* 32: 281, 1972.

Rainbow, T.C., Biegon, A. and Berck, D.J.: Quantitative receptor autoradiography with tritium-labeled ligands: comparison of biochemical and densitometric measurements. *J.*

Neurosci. Methods. 11: 231-241, 1984.

Ramachander, G., Bapatla, K.R. and Emele, J.F.: Sex differences in plasma half-life of dextrophan in rats administered dextromethorphan. *J. Pharm. Sci.* 67: 1326-1327, 1978.

Ramachander, G., Williams, F.D. and Emele, J.F.: Determination of dextrophan in plasma and evaluation of bioavailability of dextromethorphan hydrobromide in humans. *J. Pharm. Sci.* 66: 1047-1048, 1977.

Reynolds, I.J., Murphy, S.N. and Miller R.J.:  $^3\text{H}$ -labeled MK801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. *Proc. Natl. Acad. Sci. USA.* 84: 7744-7748, 1987.

Roth, J.E., Zhang, G., Murray, T.F. and Franklin, P.H.: Dextrorotatory opioids and phencyclidine exert anticonvulsant action in prepiriform cortex. *Eur. J. Pharmacol.* 215: 293-296, 1992.

Rothman, R.B., Reid, A., Mahboubi, A., Kim, C., DeCosta, B., Jacobson, A.E. and Rice, K.C.: Labeling by [ $^3\text{H}$ ]1,3-di(2-tolyl)guanidine of two high affinity sites in guinea pig brain: evidence for allosteric regulation by calcium channel antagonists and pseudoallosteric modulation of  $\sigma$  ligands. *Mol. Pharmacol.* 39: 222, 1991.

Rothman, S.M. and Olney, J.W.: Excitotoxicity and the NMDA receptor. *Trends Neurosci.* 10: 229-302, 1987.

Sakaruda, K., Masu, M. and Nakanishi, S.: Alteration of  $\text{Ca}^{2+}$  permeability and sensitivity to  $\text{Mg}^{2+}$  and channel blockers by a single amino acid substitution in the N-methyl-D-aspartate receptor. *J. Biol. Chem.* 268: 410-415, 1993.

Sakurai, S.Y., Cha, J.H.J., Penny, J.B. and Young, A.B.: Regional distribution and properties of [ $^3\text{H}$ ]MK-801 binding sites determined by autoradiography in rat brain. *Neuroscience*.40: 533-543, 1991.

Sakurai, S.Y., Penny, J.B. and Young, A.B.: Regionally distinct N-methyl-D-aspartate receptors distinguished by quantitative autoradiography of [ $^3\text{H}$ ]MK-801 binding in rat brain. *J. Neurochem.* 60: 1344-1353, 1993.

Sernagor, E., Kuhn, D., Vyklicky, L., Jr., and Mayer, M.L.: Open channel block of NMDA receptor responses evoked by tricyclic antidepressants. *Neuron.* 2: 1221-1227, 1989.

Shannon, H.E.: Pharmacological evaluation of N-allylnormetazocine (SKF 10,047) on the basis of its discriminative stimulus properties in the rat. 1983. *J. Pharmacol. Exp. Ther.* 255: 144-152, 1983.

Snell, L.D., Morter, R.S. and Johnson K.M.: Glycine potentiates N-methyl-D-aspartate-induced [ $^3\text{H}$ ]TCP binding to rat cortical membranes. *Neurosci. Letters.* 83: 313-317, 1987.

Sprengel, R. and Seeburg, P.H.: Ionotropic Glutamate Receptors. In *Handbook of receptors and channels: Ligand- and Voltage-gated ion channels*, ed. by R.A. North, pp 213-263, CRC Press, Inc. 1995.

Steinberg, G.K., Kunis, D., Saleh, J. and DeLaPaz, R.: Protection after transient focal cerebral ischemia by the N-methyl-D-aspartate antagonist dextrorphan is dependent upon plasma and brain levels.: *J. Cereb. Blood Flow Metab.* 11: 1015-1024, 1991.

Steinberg, G.K., Saleh, J. and Kunis, D.: Delayed treatment with dextromethorphan and dextrorphan reduces cerebral damage after transient focal ischemia. *Neurosci. Letters.* 89: 193-197, 1988.

Steinberg, G.K., Saleh, J. and Kunis, D., DeLaPaz, R. and Zarnegar, S.R.: Protective effect of N-methyl-D-aspartate antagonists after focal cerebral ischemia in rabbits. *Stroke.* 20: 1247-1252, 1989.

Subramaniam, S. and McGonigle, P.: Quantitative autoradiographic characterization of the binding of (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine ([<sup>3</sup>H]MK-801) in rat brain: regional effects of polyamines. *J. Pharmacol. Exp. Ther.* 256: 811-819, 1991.

Subramaniam, S. and McGonigle, P.: Regional heterogeneity of polyamine effects on the N-methyl-D-aspartate receptor in rat brain. *J. Neurochem.* 60: 2276-2284, 1993.

Sugihara, H., Moriyoshi, K., Ishii, T., Masu, M. and Nakanishi, S.: Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. *Biochem. Biophys. Res. Commun.* 185: 826-832, 1992.

Szekely, J.I., Sharpe, L.G. and Jaffe, J.H.: Induction of phencyclidine-like behaviour in rats by dextrorphan but not dextromethorphan. *Pharmac. Biochem. Behav.* 40: 381-386, 1991.

Tacconi, S., Ratti, E., Marien, M.R. Gaviraghi, G. and Bowery, N.G.: Inhibition of [<sup>3</sup>H]-(+)-MK-801 binding to rat brain sections by CPP and 7-chlorokynurenic acid. *Br. J. Pharmacol.* 108: 577-582, 1993.

Teal, J.J. and Holtzman, S.G.: Stereoselectivity of the stimulus effects of morphine and cyclazocine in the squirrel monkey. *J. Pharmacol. Exp. Ther.* 215: 369- 376, 1980.

Tortella, F.C., Ferkany, J.W. and Pontecorvo, M.J.: Anticonvulsant effects of dextrorphan in rats: possible involvement in dextromethorphan-induced seizure protection. *Life Sci.* 42: 2509-2514, 1988.

Tortella, F.C., Pellicano, M. and Bowery, N.G.: Dextromethorphan and neuromodulation: old drug coughs up new activities. *Trends Pharmacol.* 10: 501-507, 1989.

Tortella, F.C., Robles, L., Witkin, J.M. and Newman, A.H.: Novel anticonvulsant analogs of dextromethorphan: improved efficacy, potency, duration and side-effect profile. *J. Pharmacol. Exp. Ther.* 268: 727-733, 1994.

Vignon, J., Privat, A., Chaudieu, I., Thierry, A., Kamenka, J-M. and Chicheportiche, R.: [<sup>3</sup>H]Thienyl-phencyclidine ([<sup>3</sup>H]TCP) binds to two different sites in rat brain. Localization by autoradiographic and biochemical techniques. *Brain Res.* 378: 133-141, 1986.

Walker, J.M., Bowen, W.D., Walker, F.O., Matsumoto, R.R., De Costa, B. and Rice, K.C.: Sigma receptors: biology and function. *Pharmacol. Rev.* 42: 355-402, 1990.

Watkins, J.C., Krogsgaard-Larsen, P. and Honoré, T.: Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharmacol. Sci.* 11: 25-33, 1990.

Weber, E., Sonders, M., Quarum, M., McLean, S., Pou, S. and Keana, J.F.W.: 1,3-Di(2-[5-<sup>3</sup>H]tolyl)guanidine: a selective ligand that labels for  $\sigma$ -type receptors for psychotomimetic opiates and antipsychotic drugs. *Proc. Natl. Acad. Sci. USA* . 83: 8784, 1986.

Wong, B.W., Coulter, D.A., Choi, D.W. and Prince, D.A.: Dextrorphan and dextromethorphan, common antitussives, are antiepileptic and antagonize N-methyl-D-aspartate in brain slices. *Neurosci. Lett.* 85: 261-266, 1988.

Wong, E.H.F., Kemp, J.A., Priestley, T., Knight, A.R., Woodruff, G.N. and Iversen, L.L.: The anticonvulsant MK-801 is a potent N-methyl-D-aspartate antagonist. *Proc. Natl. Acad. Sci. USA* . 83: 7104-7108, 1986.

Woodhull, A.M.: Ionic blockade of sodium channels in nerve. *J. Gen. Physiol.* 61: 687, 1973.

Yamakura T., Mori, H., Masaki, H., Shimoji, K. and Mishina, M.: Different sensitivities of NMDA receptor channel subtypes to non-competitive antagonists. *NeuroReport*. 4: 687-690, 1993.

Yamazaki, M., Mori, H., Araki, K., Kazuhiro, K.J. and Mishina, M.: Cloning, expression and modulation of a mouse NMDA receptor subunit. *FEBS Letters*. 300: 39-45, 1992.

Zhang, Y., Britto, M.R., Valderhaug, K.L., Wedlund, P.J. and Smith, R.A.: Dextromethorphan: Enhancing its systemic availability by way of low-dose quinidine-mediated inhibition of cytochrome P4502D6. *Clin. Pharmacol. Ther.* 51: 647-655, 1992.

Zysset, T., Zeugin, T. and Kupfer, A.: In-vivo and in-vitro dextromethorphan metabolism in SD and DA rat. An animal model of the debrisoquine-type polymorphic oxidation in man. *Biochem. Pharmacol.* 37: 3155-3160, 1988.