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Sigma, PCP, and NMDA Receptors

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Sigma, PCP, and NMDA Receptors

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Contents

Phencyclidine Receptor Binding as a Probe of NMDA Receptor Functioning: Implications for Drug Abuse Research

Stephen R. Zukin and Daniel C. Javitt

INTRODUCTION

Phencyclidine (1 ,1-phenylcyclohexylpiperidine; PCP; “angel dust”), originally developed as a general anesthetic in the late 1950s was found to produce loss of consciousness and analgesia sufficient for the performance of surgical procedures while not causing significant respiratory or cardiovascular depression (Greifenstein et al. 1958; Meyer et al. 1959; Johnstone et al. 1958). However, a significant proportion of patients subjected to PCP anesthesia developed psychotic episodes typically lasting 12 to 72 hours but occasionally as long as 7 to 10 days. These episodes were characterized by excitation, unmanageability, paranoia, concreteness of thought (Greifenstein et al. 1958; Meyer et al. 1959), and “maniacal episodes” (Johnstone et al. 1958). Subanesthetic doses (0.1 mg/kg iv) were subsequently found to induce psychotic episodes in normal volunteers and to rekindle presenting symptomatology in recompensated schizophrenic subjects (Davies and Beech 1960; Luby et al. 1959, 1962). In addition, subanesthetic doses of PCP, but not lisergic acid diethylamide (LSD), amobarbital, or amphetamine, could induce abnormalities in tests of abstract reasoning, cognitive processing, attention, motor function, and proprioception in normal volunteers that closely resembled those seen in patients with chronic schizophrenia (Rosenbaum et al. 1959; Cohen et al. 1962; Domino and Luby 1981).

Despite evoking behavioral effects apparently so dysphoric, PCP emerged as a major drug of abuse during the 1970s (Petersen and Stillman 1978), and the rate of PCP abuse increased again during the 1980s (Crider 1986). PCP possesses powerful reinforcing and abuse-promoting effects (Balster 1986). The key to developing interventions targeting the clinical and public-health

problems posed by PCP-like drugs will be found in elucidating the mechanisms underlying their psychotomimetic and abuse-promoting properties.

PCP binds with high affinity to a specific brain PCP receptor (Zukin and Zukin 1979; Vincent et al. 1979; Reynolds et al. 1987). Several lines of evidence indicate that the PCP receptor is a site within the ion channel gated by the *N*-methyl-D-aspartate (NMDA)-type excitatory amino acid receptor. First, PCP and NMDA receptors are co-localized in the central nervous system (Maragos et al. 1988). Second, PCP receptor ligands have been shown to inhibit NMDA-receptor-mediated conductances noncompetitively (Anis et al. 1983) in a voltage- and use-dependent fashion (Honey et al. 1985; Huettner and Bean 1988). Finally, binding of PCP receptor ligands is enhanced by NMDA receptor agonists, such as L-glutamate or NMDA, and is diminished by competitive NMDA receptor antagonists, such as D(-)-2-amino-5-phosphonovaleric acid (D(-)AP5) (Javitt et al. 1987; Fagg 1987; Loo et al. 1987). Such data suggest a model in which NMDA and PCP receptors represent distinct sites associated with a supramolecular NMDA receptor complex. The identification of noncompetitive inhibition of NMDA receptor function as a possible mechanism underlying the psychotomimetic effects of PCP suggests that elucidation of the functioning of the NMDA receptor complex may reveal mechanisms relevant to the pathogenesis and treatment of schizophrenia.

MECHANISMS OF PCP-NMDA RECEPTOR INTERACTION

To shed light on the mechanisms underlying NMDA receptor activation, binding of the selective PCP receptor ligand [^{3}H]MK-801 was determined in the presence and absence of L-glutamate and either glycine or D-serine, agents that had previously been shown to stimulate binding to PCP receptors (Reynolds et al. 1987; Javitt and Zukin 1989*a).* L-glutamate mediates its actions as an agonist at the NMDA recognition site, whereas both glycine and D-serine mediate their actions at a non-strychnine-sensitive glycine recognition site associated with the NMDA receptor complex. For these studies, rat forebrain homogenates were subjected to extensive washing, freezing, and thawing to reduce the high endogenous concentrations of L-glutamate and glycine present in crude brain homogenate. Specific binding of [^{3}H]MK-801 was determined at 12 to 16 time points between 5 minutes and 24 hours using a filtration radioreceptor assay in the presence of a 5 mM Tris buffer system adjusted to pH 7.4 and a low (30 µM) concentration of Mg^{2+} (Javitt and Zukin 1989*b).*

These studies produced three novel findings. First, analysis of association curves using a computer-assisted, nonlinear-curve-fitting technique revealed

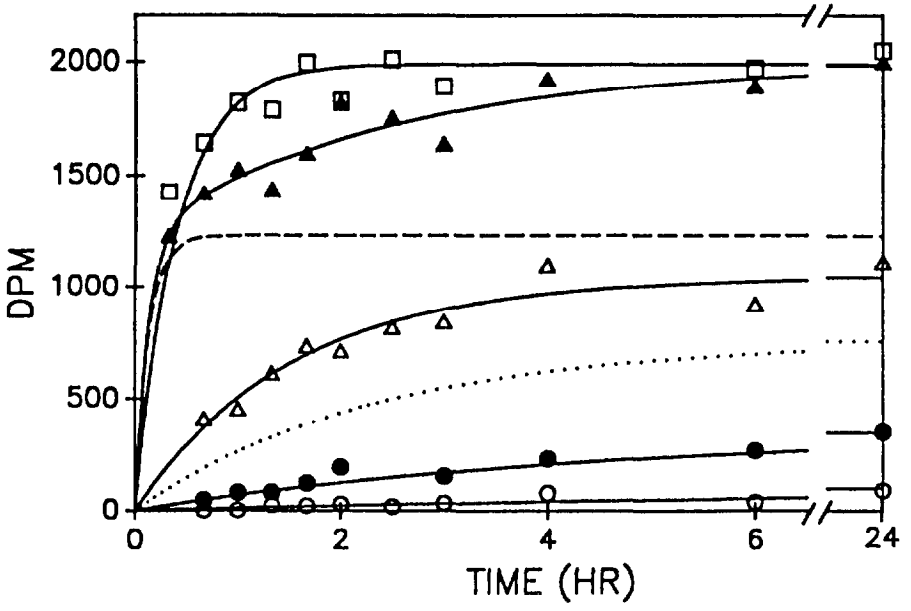

FIGURE 1. *Association curves of 1 nM [^{3}H]MK-801 under control conditions (filled circles) or in the presence of 10 µM concentrations of D(-)AP5 (open circles), glycine (open triangles), L-glutamate (filled triangles), or L-glutamate plus glycine (open squares). Under control conditions or in the presence of D(-)APS, binding was fit best by single exponentials with apparent $t_{1/2}$ values of 3.2 and 5.8 hours, respectively. In the presence of combined L-glutamate and glycine, binding was best fit by a single exponential with an apparent $t_{1/2}$ of 12 minutes. In the presence of L-glutamate alone, binding was best fit by dual exponentials with apparent $t_{1/2}$ values of 5.5 minutes and 1.6 hours for the fast (dashed line) and slow (dotted line) components, respectively. (Reprinted from Javitt and Zukin 1989b. Copyright 1989, American Society for Pharmacology and Experimental Therapeutics.)*

the presence of two distinct components of [^{3}H]MK-801 binding: a fast component with a $t_{1/2}$ of approximately 5 minutes and a slow component with a $t_{1/2}$ of approximately 3 hours (figure 1).

This finding suggests that PCP-like agents do not gain access to their receptor only via open channels, because channel-blocking drugs that interact exclusively with open channels should manifest single exponential association and dissociation (Starmer and Grant 1985). A model of PCP-NMDA receptor interaction consistent with biexponential association of [^{3}H]MK-801 would be one in which PCP-like agents can gain access to their recognition site via two distinct paths, each corresponding to one of the observed kinetic components of binding. It has been suggested that channel-blocking drugs having pK_a values near physiological pH may associate with their binding sites via both fast, hydrophilic and slow, hydrophobic paths (Starmer and Grant 1985). At pH 7.4, MK-801 (pK_a = 8.2 (Huettner and Bean 1988)) would exist in both deprotonated and protonated forms. The former would be capable of association via both hydrophilic and hydrophobic paths, while the latter would be capable of association only via a hydrophilic path.

When channels were maximally activated in the presence of combined L-glutamate and glycine, we found that more than 90 percent of [^{3}H]MK-801 binding displayed fast kinetics of association, suggesting that the fast path represents binding of [^{3}H]MK-801 to its receptor following diffusion to the binding site via a path corresponding to the open NMDA receptor channel. In the absence of added L-glutamate or in the presence of D(-)AP5, more than 99 percent of [^{3}H]MK-801 binding displayed slow kinetics of association, suggesting that the slow path represents binding of [^{3}H]MK-801 following diffusion to the binding site via a path associated with closed NMDA receptor channels. The latter path could involve diffusion of deprotonated [^{3}H]MK-801 through the lipid bilayer, through hydrophobic domains of the receptor complex, or through the (closed) NMDA-receptor gating mechanism. Biexponential kinetics of association of [^{3}H]MK-801 in the presence of L-glutamate alone indicate that association via fast and slow paths can occur simultaneously, thus supporting the concept that different underlying processes must be involved. The ability of PCP-like agents to reach their binding site within closed NMDA channels over the course of hours may be relevant to the course of PCP intoxication because it suggests that PCP can reach its site of action even in the absence of NMDA receptor activation.

A second finding of these studies (Javitt and Zukin 1989*b)* was that L-glutamate significantly increased total steady-state [^{3}H]MK-801 binding while D(-)AP5 significantly decreased total steady-state [^{3}H]MK-801 binding (figure 2) presumably by displacing endogenous agonists from the NMDA receptor. This finding differs from those of previous studies, which had reported no change in

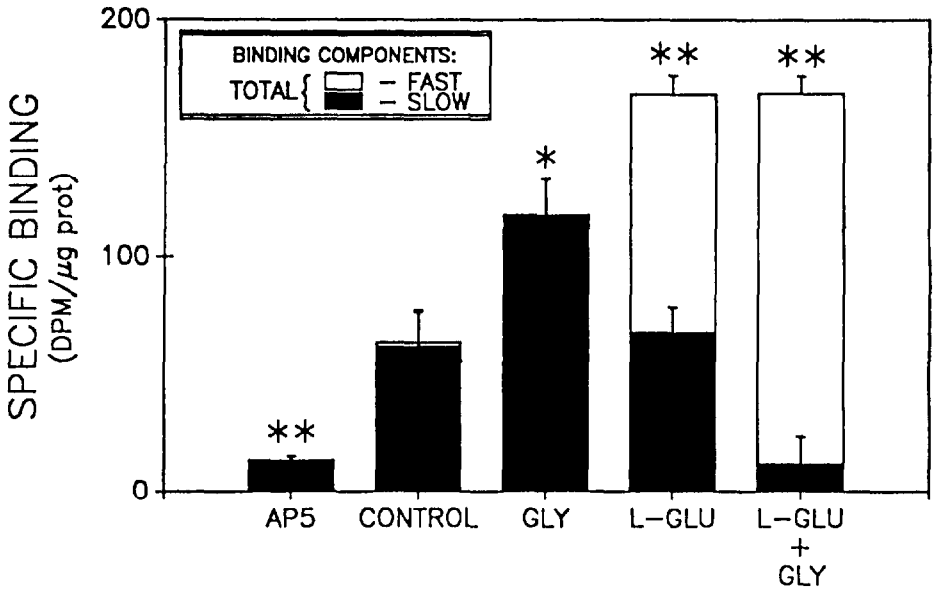

FIGURE 2. *Specific binding (mean ± SEM) of 1 nM [^{3}H]MK-801 to fast (open bar) and slow (filled bar) components under control conditions or in the presence of 10 μM concentrations of D(-)AP5 (AP5), glycine (GLY), L-glutamate (L-GLU), or L-glutamate plus glycine. Total bar height represents total steady-state binding of [^{3}H]MK-807 under conditions specified. Significant between-groups variation was found for fast ($p < .001$), slow ($p < .01$), and total ($p < .001$) steady-state binding. Values represent mean ± s.e.m. of 4 to 6 experiments. *$p < .05$ vs. control. ** $p < .01$ vs. control. (Reprinted from Javitt and Zukin 1989b. Copyright 1989, American Society for Pharmacology and Experimental Therapeutics.)*

equilibrium binding after the glutamate and glycine were added (Kloog et al. 1988; Bonhaus and McNamara 1988).

A third finding of our studies (Javitt and Zukin 1989*b)* was that the Hill coefficient for stimulation of [^{3}H]MK-801 binding by L-glutamate was significantly greater than unity (figure 3). Although glycine shifted the dose-response curve to the left, it did not alter the Hill coefficient significantly.

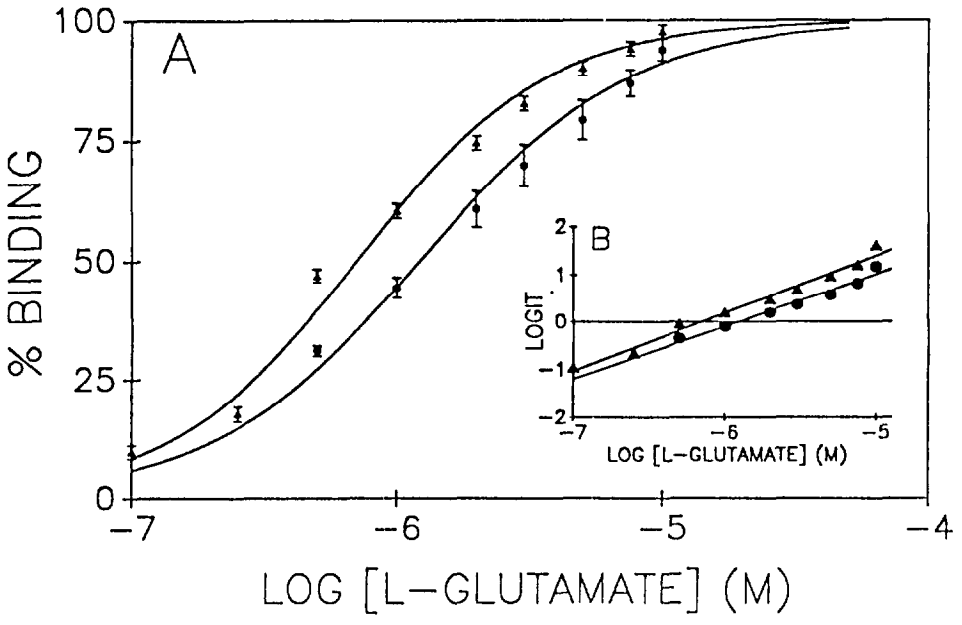

FIGURE 3. *Stimulation of specific 1 nM [^{3}H]MK-807 binding by L-glutamate in the absence (filled circles) or presence (filled triangles) of 10 µM glycine following 24-hour incubations. D(-)AP5 (50 µM) was added under all conditions to decrease basal binding. Inset, Hill plots of specific 1 nM [^{3}H]MK-801 binding. Correlation coefficients (r) were > .95 for both plots. In both the absence (filled circles) and presence (filled triangles) of 10 µM glycine, Hill coefficients were significant/y greater than unity (Reprinted from Javitt and Zukin 1989b. Copyright 1989, American Society for Pharmacology and Experimental Therapeutics.)*

This finding suggests that more than one molecule of agonist is required to induce NMDA channel activation.

A model of NMDA receptor functioning that could account for these findings is one involving the existence of two independent sets of agonist recognition sites within each functional NMDA receptor complex. Occupation of both sets by agonist would be required for channel activation and fast [^{3}H]MK-801 binding. Partial activation would not permit channel opening but would permit slow diffusion of [^{3}H]MK-801 to its binding site via a hydrophobic path. In the total

absence of agonist, however, the channel would remain closed and in a conformation to which PCP receptor ligands could not bind. This model is similar to those proposed to account for the functioning of nicotinic acetylcholine (Hess et al. 1983) and $GABA_A$ (Aoshima et al. 1987) receptors.

If future studies confirm a functional similarity between NMDA and nicotinic receptors, it may indicate that NMDA receptors are structurally homologous to receptors of the Class I superfamily of ligand-gated channels, which includes nicotinic, $GABA_A$, and strychnine-sensitive glycine receptors as well (Barnard et al. 1987).

ROLE OF NMDA RECEPTORS IN PSYCHOTOMIMETIC EFFECTS OF PCP

The ability of PCP-like agents to bind with high potency to a site within the NMDA channel suggests that PCP-induced NMDA channel blockade may be relevant to the clinical effects of PCP. The degree to which PCP receptors mediate the psychotomimetic effects of PCP has been a subject of controversy in the literature. Both haloperidol-sensitive σ sites (Su et al. 1988) and monoamine reuptake sites (Smith et al. 1977; Garey and Heath 1976) sites have been proposed as potential alternative receptors for mediation of the psychotomimetic effects of PCP.

Several lines of evidence, however, support a unique role of the PCP receptor in this regard. First, PCP receptors have been shown to mediate the discriminative stimulus effects of PCP in rodents (Browne 1986). The rank order of potency with which a large number of drugs from distinct chemical classes can induce the PCP response in animals trained to discriminate PCP from saline corresponds to their rank order of binding to PCP receptors and inducing NMDA receptor blockade (Browne 1986). By contrast, agents that selectively bind to the σ and/or dopamine reuptake sites neither induce PCP-like discriminative stimulus effects nor antagonize the discriminative stimulus effects induced by PCP receptor ligands (Browne 1986). These findings suggest that PCP receptors mediate the interoceptive cues induced by PCP and PCP-like agents.

Second, psychotomimetic effects similar to those induced by PCP can be induced by ketamine, a related arylcyclohexylamine derivative (Siegel 1978). The psychotomimetic effects of ketamine are induced by doses approximately tenfold greater than PCP (Siegel 1978), which is consistent with ketamine's tenfold lower potency of binding to PCP receptors (Zukin and Zukin 1979;

Vincent et al. 1979). By contrast, ketamine is essentially inactive at both dopamine reuptake (Vignon et al. 1988) and σ sites.

Finally, PCP-induced psychosis has been found to be associated with serum concentrations of PCP as low as 20 nM, while serum concentrations greater than 400 nM are associated with gross impairments in consciousness (Walberg et al. 1986). PCP receptors have been shown to bind PCP with an affinity of approximately 30 to 50 nM (Reynolds et al. 1987; Sircar et al. 1987; Wong et al. 1988) suggesting a highly significant degree of receptor occupancy by levels of PCP present during low-dose PCP psychosis. By contrast, σ binding sites and DA reuptake sites have been shown to bind PCP with affinities of approximately 600 nM (Wong et al. 1988) and 700 nM (Vignon et al. 1988) respectively. The affinity of PCP for these sites is thus significantly lower than its affinity for PCP receptors. Furthermore, the affinity of PCP for these sites suggests that they would be affected only to a limited extent by concentrations of PCP that have been demonstrated to cause robust psychotomimetic effects.

In summary, the unique behavioral effects of PCP and related drugs, which are accompanied by a high degree of abuse potential, appear to be mediated at the PCP receptor that is located within the NMDA-receptor-gated ion channel. Our data from experiments measuring the effects of NMDA receptor activation on binding of [^{3}H]MK-801 to PCP receptors support a model of NMDA receptor functioning in which two molecules of agonist are required for NMDA receptor activation. This model is similar to models that have been proposed for the nicotinic acetylcholine receptor, suggesting the possibility of functional and structural homology between NMDA receptors and members of the Class I superfamily of ligand-gated channels. The psychotomimetic effects of PCP are observed at serum concentrations similar to the concentration at which PCP binds to the NMDA-associated PCP receptor. It is hoped that future studies may clarify mechanisms whereby agents influencing NMDA receptor functioning may influence behaviors or lead to therapeutic approaches related to drug abuse.

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Pharmacologic Regulation of the NMDA Receptor-Ionophore Complex

Kenneth M. Johnson, Lawrence D. Snell, Aida I. Sacaan, and Susan M. Jones

INTRODUCTION

As Monaghan and colleagues (1989) pointed out in their recent review, the effects of excitatory amino acids such as glutamate and aspartate are mediated through at least five different receptor subtypes. Three are well known and have been defined by antagonist pharmacology and the distinct depolarizing actions of selective agonists (kainate, quisqualate, and *N*-methyl-D-aspartate [NMDA]). The fourth appears to be an inhibitory autoreceptor, selectively activated by AP4 (L-2-amino-4-phosphonobutyrate). AP4 has been demonstrated to inhibit synaptic activation of dentate granule cells (Koerner and Cotman 1981) olfactory cortex (Hearns et al. 1986), and hippocampal CA3 cells (Lanthorn et al. 1984). The fifth receptor subtype appears to activate an oscillating C1- current (Gundersen et al. 1984) that may be mediated by IP_3 (Parker and Miledi 1987). This response is blocked by pertussis toxin (Sugiyama et al. 1987). Glutamate and quisqualate-but not kainate, NMDA, or AP4—increase inositol phosphate formation (Nicoletti et al. 1986; Schoepp and Johnson 1988; Sladeczek et al. 1985) via a mechanism that is insensitive to the quisqualate antagonists, joro spider toxin (Jackson and Usherwood 1988), or 6-cyano-2, 3-dihydroxy- 7-nitro-quinoxaline (CNQX) (Palmer et al 1988). This unusual profile is amplified by the finding that the traditional quisqualate receptor agonist, DL-α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA), does not increase phosphatidyl inositol turnover: it is further enhanced by the observation that quisqualate-induced inositol phosphate formation is blocked by AP4 (Schoepp and Johnson 1988).

The NMDA receptor mediates ion flux through a channel permeable to Na^+, K^+, and Ca^{++}. Ion flux is voltage dependent and gated by Mg^{++} and phencyclidine (PCP), which act at distinct sites within the channel itself. An additional role for

divalent cations has been illustrated by the voltage-independent inhibition of NMDA by Zn^{++}. Glycine also modulates channel activation by NMDA agonists and may be required for the expression of receptor function. Recent work has shown that polyamines act on two or more sites to regulate both glycine binding and channel activation. In addition, both the NMDA and glycine receptor recognition sites may exist in agonist- and antagonist-preferring conformations, and the receptor state may be regulated by the relative concentrations of glutamate, glycine, and polyamines. Thus, the actions of PCP in this complex depend on the degree of activation of the ion channel, which in turn depends on the relationship between at least five other receptors or binding sites. Therefore, understanding the mechanism of action of PCP requires understanding the various components of the complex and how they relate to one another. An overview of the state of knowledge of this rapidly changing field is given below. For information on other aspects of PCP pharmacology, see Johnson and Jones (1990).

THE NMDA RECOGNITION SITE

[^{3}H]Glutamate has been widely used to specifically label each of the glutamate receptors, including the NMDA subtype (Foster and Fagg 1984). More selective NMDA agonists and antagonists have also been utilized to label the NMDA receptor, but their use has been somewhat limited by their relatively poor affinity. That is, the agonist [^{3}H]NMDA (Foster and Fagg 1987) and the antagonists [^{3}H]D-AP5 (Olverman et al. 1988) [^{3}H]DL-AP7 (Manallack and Beart 1986; Ferkany and Coyle 1983) and [^{3}H]3-[(±)-2-carboxypiperazine-4-yl]propyl-1-phosphonate (CPP) (Harris et al. 1986; Murphy et al. 1987) have equilibrium dissociation constants in the 200 to 2,000 nM range. However, a recently synthesized piperidine analog of CPP, *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755), appears to be a selective and more potent NMDA antagonist (K_i of 50 nM against [^{3}H]CPP binding [Murphy et al. 1987]), and early indications are that [^{3}H]CGS 19755 is the best radioligand available at this time, at least for the antagonist-preferring state of the NMDA receptor (see below).

Despite its lack of selectivity, L-[^{3}H]glutamate is still the agonist of choice for studies of the NMDA recognition site. Depending on the membrane preparation (and perhaps the extent to which endogenous glutamate and aspartate are removed), NMDA-displaceable L-[^{3}H]glutamate binding varies between 25 and 90 percent of the total binding and has a Kc value between 50 and 200 nM (Fagg and Baud 1988; Fagg and Matus 1984; Monahan and Michel 1987;

Monahan et al. 1988). Recently, the authors' laboratory has described high-affinity (36 nM K_D), NMDA-displaceable L-[^{3}H]glutamate binding that constituted about 85 percent of the total binding in an easily prepared buffy coat membrane preparation that can also be utilized for the study of [^{3}H]1 -[1-(2-thienyl)cyclohexyl]piperidine (TCP) and [^{3}H]glycine binding (Jones et al. 1989).

Monaghan and colleagues (1988) have studied the autoradiographic distribution of [^{3}H]CPP and NMDA-sensitive [^{3}H]glutamate binding sites and have found their distribution to be heterogeneous. L[^{3}H]glutamate-specific binding was relatively higher than that of [^{3}H]CPP in the striatum and relatively lower in portions of the thalamus and cortex. Glycine increased [^{3}H]glutamate binding but decreased [^{3}H]CPP binding. These data led the authors to postulate that the NMDA receptor exists in both agonist- and antagonist-preferring states and that glycine may convert the antagonist-preferring state to the agonist-preferring state.

In an attempt to verify this hypothesis, the effect of glycine and D-serine (0.3 to 100 μM) on [^{3}H]CPP binding in cortical buffy coat membranes was determined. No effect was observed (data not shown). Furthermore, the IC_{50} values for inhibition of NMDA-sensitive L-[^{3}H]glutamate binding to thalamic and striatal membranes was determined for both NMDA and CPP. As can be seen in table 1, 10 μM glycine had no effect on the apparent affinity of either agonist or antagonist binding to membranes from the striatum (an area rich in agonist-preferring sites) or the thalamus (an antagonist-preferring area). Thus, it

TABLE 1. *Effect of 10 μM glycine on the inhibition of NMDA-specific [^{3}H]glutamate binding by an agonist and antagonist in brain areas relatively rich in agonist sites (striatum) and antagonist sites (thalamus)*

	IC_{50} (μM)			
	Striatum		Thalamus	
Addition	NMDA	CPP	NMDA	CPP
Control	1.45±0.17	1.47±0.20	1.85±1.20	1.90±0.26
10 μM glycine	1.80±0.46	1.20±0.49	2.10±0.45	1.39±0.61

NOTE: These data represent the mean±SE of two to four independent experiments carried out in triplicate with six concentrations of displacing drug.

TABLE 2. *Comparison of the affinity constants of NMDA agonists and antagonists for [^{3}H]glutamate and [^{3}H]CPP binding sites in cortical membranes*

	K_i (μM)		
Agonist/Antagonist	[^{3}H]glutamate	[^{3}H]CPP	Agonist ratio*
L-glutamate	0.025	0.081	3.24
L-homocysteate	0.214	0.582	2.72
NMDA	1.80	2.90	1.61
CGS 19755	0.195	0.048	0.246
CPP	1.50	0.117	0.078

* The higher number Indicates a greater relative affinity for the agonist-preferring NMDA receptor.

is unlikely that the data of Monaghan and coworkers (1988) can be explained by an affinity shift in the NMDA recognition site.

That agonist- and antagonist-preferring states of the NMDA receptor exist was first postulated by Fagg and Baud (1988). As shown in table 2, this postulate has been verified by showing that agonists have a greater relative affinity for sites labeled by [^{3}H]glutamate, whereas antagonists have a higher relative affinity for [^{3}H]CPP-labeled sites.

THE PHENCYCLIDINE BINDING SITE

Lodge and colleagues first demonstrated that PCP and the dissociative anesthetic ketamine blocked NMDA-induced depolarization of spinal neurons while having no effect on either kainate- or quisqualate-induced depolarization (Lodge et al. 1982; Anis et al. 1983). This finding was critical to understanding the mechanism of action of PCP.

The mechanism by which PCP inhibits NMDA-mediated responses is noncompetitive with respect to NMDA (Harrison and Simmonds 1985; Snell and Johnson 1985; Jones et al. 1989). The block also exhibits voltage and use dependence (Kemp et al. 1988; Honey et al. 1985; Snell et al. 1987; Mayer et al. 1988; MacDonald et al. 1987; Woodruff et al. 1987). For example, using whole-cell patch-clamp techniques in dissociated cultures of mouse hippocampal neurons, Mayer and colleagues (1988) reported that NMDA-activated channels have a mean open time of 5 to 6 ms. In contrast to

the rapid-onset blockade caused by the competitive antagonist AP5 (which had no effect on mean open time), ketamine produced a slowly developing reduction in mean channel open time (from about 6 ms to 3 ms). This effect presumably reflects the high probability that open channels will be blocked by ketamine before they would normally close. The recovery from ketamine antagonism was also very slow and dependent on the presence of NMDA, suggesting that ketamine can enter the open channel and be trapped unless NMDA agonists are available to reopen the channel (Mayer et al. 1988; MacDonald et al. 1987). This use dependence has also been noted for other PCP-like ligands, including cyclazocine and MK-801 (Davies et al. 1988). Use dependence was greatest for MK-801, followed by PCP and cyclazocine, and then ketamine (which showed very little use dependence in a cortical wedge preparation). The reason for these differences is unclear, but it may have to do with the size and relative flexibility of the molecules; for example, MK-801 is a rigid, planar, three-ring structure with a nitrogen bridge oriented perpendicular to the central ring, whereas ketamine is a flexible, bicyclic structure.

A drug that exhibits substantial use dependence relative to its onset of action could conceivably be utilized clinically as a pro-drug in that its antagonistic effects would not be realized until endogenous NMDA agonists opened the channel binding site. This possibility has been examined in vivo in rat spinal cord neurons (Davies et al. 1988). However, in this paradigm the onset of block by intravenous MK-801 was not altered by iontophoretic application of NMDA, although the rate of recovery from MK-801 block was enhanced by NMDA application. (The voltage dependence of the binding also partially accounts for the accelerated rate of recovery from MK-801 block observed in cells held at +30 mV [Huettner and Bean 1988].) This suggests that there may be enough endogenous NMDA tone in vivo to allow MK-801 to act immediately as an open-channel blocker or that MK-801 may gain access to its channel binding site by rapidly diffusing through the membrane lipid bilayer.

Studies of the voltage dependence of the NMDA block by PCP and ketamine have provided additional evidence for the channel localization of the PCP binding site (Honey et al. 1985; Mayer et al. 1988; MacDonald et al. 1987; Huettner and Bean 1988). In cultured hippocampal cells, blockade of NMDA responses is much greater at hyperpolarized membrane potentials (-60 mV to -70 mV) than at more depolarized potentials, and virtually no block is seen at holding potentials greater than +30 mV. Equilibrium analysis of the ketamine block at -60 mV revealed a K_i of 1.5 μM, whereas at +40 mV the K_i was calculated at 49 μM (Mayer et al. 1988). Using these data, Mayer and

colleagues (1988) calculated that the fraction of the membrane electric field that influences ketamine binding is close to 1.0. Although recently it has been argued that the models used for this calculation may be inappropriate (MacDonald and Nowak 1990), this suggests that the ketamine binding site is deep within the channel, close to the cytoplasmic surface of the membrane.

Together, the voltage- and use-dependent open-channel block (and unblock) may contribute to the pharmacologic uniqueness of the various drugs that act at the PCP site. Although not well studied, it appears that voltage and use dependence may be independent properties. For example, the ketamine block is clearly voltage dependent and is similar to PCP in this regard (Honey et al. 1985) but shows relatively little use dependence (Davies et al. 1988). MK-801 blockade shows little voltage dependence during its onset, but the recovery of blockade in the presence of NMDA does show marked voltage dependence (Huettner and Bean 1988). MK-801 shows greater use dependence than PCP and considerably more than ketamine (Davies et al. 1988). The relative importance of these two properties is difficult to assess, but their significance can be understood by realizing that these two properties tend to offset each other in a functional sense. That is, inhibition of NMDA responses by PCP depends on the presence of an NMDA agonist, but NMDA also depolarizes the membrane, effectively decreasing the action of a given concentration of PCP. Therefore, a compound that exhibits high use dependence relative to voltage dependence might be a more selective and potent NMDA antagonist than one that shows low use dependence but high voltage dependence.

The voltage-dependent properties of PCP blockade of the NMDA receptor-ionophore complex could also contribute to the uniqueness of PCP pharmacology in a way that is interdependent with the selectivity of endogenous excitatory amino acids for the several receptor subtypes. That is, because glutamate can act at kainate, quisqualate, and NMDA receptors, its ability to depolarize the cell membrane through quisqualate or kainate receptors would theoretically partially negate the effects of PCP-like compounds acting at NMDA receptors. However, aspartate, and particularly homocysteic acid, may have more selectivity for NMDA receptors. Thus, the authors predict that PCP would be a more potent antagonist of aspartate than of glutamate responses. Such a finding would be important because these amino acids probably mediate synaptic transmission in independent neuronal pathways, thus affording an additional mechanism for PCP selectivity.

The localization of the PCP binding site within the NMDA receptor-ionophore complex has received considerable support from binding studies. Initially, it was reported that the specific binding of [^{3}H]TCP was reduced by progressively washing brain membranes and that this reduction could be reversed by adding glutamate back to the medium (Loo et al. 1986). Subsequent studies demonstrated an excellent correlation between the potency of ligands in activating NMDA receptors and in increasing [^{3}H]TCP or [^{3}H]MK-801 binding in well-washed membranes (Foster and Wong 1987; Fagg 1987). This relationship was further strengthened by reports that NMDA receptor antagonists inhibited [^{3}H]TCP or [^{3}H]MK-801 binding (Johnson et al. 1988a; Loo et al. 1987; Javitt et al. 1987; Reynolds et al. 1987).

In addition, autoradiographic studies of [^{3}H]TCP together with NMDA-displaceable [^{3}H]glutamate, [^{3}H]CPP, or [^{3}H]AP5 binding in brain slices have shown a very high degree of co-localization of NMDA receptor sites and PCP binding sites (Monaghan et al. 1988; Maragos et al. 1986, 1988; Jarvis et al. 1987). Binding of ligands to both sites was the highest in the stratum radiatum and stratum oriens of hippocampal area CA1 , followed by the dentate gyrus, entorhinal cortex, and layers I and II of the somatosensory and motor cortex (Monaghan et al. 1988; Maragos et al. 1988). Intermediate levels were found in the olfactory region, amygdala, thalamus, lateral septum, hippocampal area CA3, and the caudate nucleus. Approximately 10 percent or less of the binding found in area CA1 was found in the globus pallidus, medial septum, habenula, hypothalamus, diagonal band, and cerebellum. The granule cell layer of the cerebellum was the only area reported to be discordant in binding of the two ligands (Maragos et al. 1988).

The mechanism by which NMDA agonists selectively stimulate ligand binding to the receptor-ionophore complex has not been completely resolved. Initial studies suggested that glutamate and NMDA either increased the affinity (Foster and Fagg 1987; Loo et al. 1987; Javitt et al. 1987; Johnson et al. 1988 *b)* or the apparent site density (Javitt et al. 1987) for [^{3}H]TCP or [^{3}H]MK-801 binding. Subsequently, it was determined that the time required for either ligand to reach true equilibrium in highly washed membranes (nominally free of glutamate) was 5 to 24 hours, depending on the ligand used and the thoroughness with which the membranes were washed (Bonhaus and McNamara 1988; Kloog et al. 1988*a,* 1988*b;* Johnson et al. 1989). Thus, earlier studies using shorter incubation times were actually conducted at 50 to 70 percent of true equilibrium, making inferences from these experiments difficult.

More recent studies have suggested that glutamate and other NMDA agonists increase both the rate of association and dissociation of [^{3}H]TCP or [^{3}H]MK-801 (Kloog et al. 1988*a;* Reynolds and Miller 1988) without altering the equilibrium dissociation constant (Kloog et al. 1988*a,* 1988*b).* Although it has been stated that the equilibrium Ko is similar to the kinetically determined Kc (Kloog et al. 1988*a),* closer examination of the kinetic data (Bonhaus and McNamara 1988; Sacaan and Johnson 1990*a)* suggests that both association and dissociation are multiphasic, making the K_D (assuming classical bimolecular kinetics) somewhat difficult to interpret. This is further complicated when considered in the light of the effects that glycine has on channel kinetics (see below).

Nevertheless, the data thus far have been widely interpreted in a manner consistent with open channel block by PCP-like ligands. That is, the increase in the rates of binding and unbinding induced by NMDA agonists is due to an increase in the fraction of time the channel binding site is accessible to [^{3}H]TCP or [^{3}H]MK-801. This finding is supported by the observation that NMDA antagonists such as AP5 decrease the rates of association and dissociation (Bonhaus and McNamara 1988; Kloog et al. 1988*a;* Reynolds and Miller 1988). Thus, there appears to be overwhelming evidence that NMDA agonists regulate the binding of PCP-like ligands to the channel binding site by increasing channel opening. However, whether NMDA agonists alone can induce a conformational change in the channel that is associated with a conductance increase or an affinity change in the PCP binding site or both is still a matter of debate that may be settled only with the precise understanding of the role of glycine.

The authors' laboratory has consistently observed that glutamate and glycine addition results in apparent increases in affinity of [^{3}H]TCP binding (Johnson et al. 1988*b* Jones et al. 1989). Although these data (under control conditions) were collected before true equilibrium was attained, arguments have been presented that suggest that this does not undermine the interpretation that glutamate (in the presence of glycine) opens the NMDA-operated ion channel, increases the accessibility of [^{3}H]TCP to its binding sites, *and* alters the conformation of the channel-associated PCP binding site (Johnson and Jones 1990).

One of the difficulties inherent in experiments trying to delineate the individual actions of glutamate and glycine is their presence even in well-washed membrane preparations. To overcome this problem the authors have conducted equilibrium, saturation isotherms in the presence of a glycine antagonist

(7-chlorokynurenate) and an NMDA antagonist (CPP). These data are summarized in table 3. In the presence of saturating concentrations of 7-chlorokynurenate (7CIKYN) and CPP, [^{3}H]TCP binds with low affinity (Kc of 95 nM) to cortical membrane sites that have an apparent density of 1.2 pmol/mg protein (table 3). Addition of L-glutamate produces a concentration-dependent increase (about fourfold) in apparent site density but does not alter the K_D. The addition of 1 μM glycine produces no further increase in site density but increases the binding affinity about fivefold. These data are very similar to those recorded in the absence of antagonists and in the presence of maximally effective concentrations of glutamate and glycine.

TABLE 3. *Effect of glutamate and glycine on [^{3}H]TCP binding at equilibrium in the presence and absence of NMDA and glycine antagonists*

Condition	K_D (nM)	B_{max} (pmol/mg)
100 μM CPP+3 μM 7CIKYN	95±56	1.2±0.3
CPP+7CIKYN+1 μM glutamate	110±3	2.0±0.3
CPP+7CIKYN+10 μM glutamate	124±19	4.3±0.4
CPP+7CIKYN+1 μM glutamate +10 μM glycine	21±2	4.2±0.2
10 μM glutamate+10 μM glycine	11±0.5	4.2±0.1

NOTE: Experiments were conducted at 4°C over a 48-hour incubation period. Approximate K_D, values for glutamate and glycine in this preparation are 36 and 133 nM, respectively. K_i values for CPP and 7-chlorokynurenate are 1.5 μM and 0.27 μM, respectively.

The authors interpret these data in the following manner. In the absence of glutamate or glycine, [^{3}H]TCP binds to approximately one-fourth of the available channel sites. These channels are in a nonconducting, low-affinity state. If these channels are continuously closed, then [^{3}H]TCP must gain access through the lipid bilayer (hydrophobic path). Alternatively, binding under these conditions could represent binding to spontaneously active channels. In this case, the hydrophobic path need not be postulated, as access would be afforded through the open channel (hydrophilic path).

The presence of glutamate increases the apparent site density by increasing the number of open channels and, hence, the overall accessibility to the channel population. However, binding under these conditions is still to a low-affinity site. The addition of glycine converts this population of sites from low to high affinity. We postulate that this conversion results from a

glycine-induced conformational change in the channel as it goes from a nonconducting to a conducting state. This change requires glutamate, as 10 μM glycine has no significant effect on the K_D in the presence of CPP alone (data not shown). This concept is modeled in figure 1 and should be compared to that derived by Zukin and Javitt elsewhere in this volume.

At this time, very little is known about the structure of the NMDA receptor-ionophore complex. Radiation inactivation analysis has indicated that the sites labeled by [^{3}H]TCP, [^{3}H]glutamate (NMDA specific), and [^{3}H]glycine have

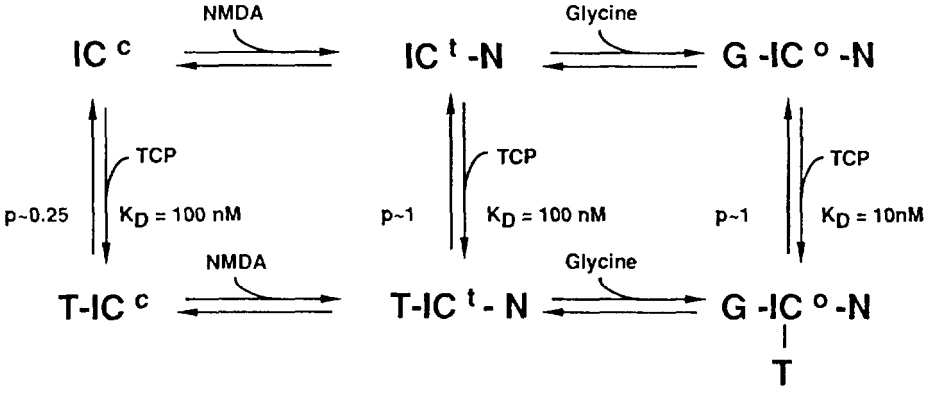

FIGURE 1. *Proposed model of how NMDA (N) and glycine (G) affect the binding of TCP (T) to the NMDA-operated ion channel (IC). IC can exist in a closed, nonconducting state (IC^C). Binding of TCP to this state is low because of the low probability (p=0.25) that the channel will open in the absence of NMDA agonists. This probability factor also includes the possibility that [^{3}H]TCP may gain access to its binding site by diffusion through a hydrophobic path. NMDA shifts the equilibrium to a transition state, open with respect to TCP (IC^t-N), but still nonconducting. TCP binds to this state with a relatively low affinity (K_D=100 nM), but with a high probability (p=1). Glycine does not produce a further increase in accessibility (p), but converts the channel to an active, open, conductive state (G-IC^o-N). This opening is associated with a conformational change in the channel (and in the PCP binding site) that is reflected by an increased binding affinity (K_D=10 nM). The postulated two binding sites each for glutamate and glycine were deleted for simplicity.*

molecular masses in the range of 115 kDa to 125 kDa (Honore et al. 1989). Curiously, the site labeled by [^{3}H]CPP appears to have a molecular mass of 209 kDa. This suggests that the recognition sites for NMDA and glycine are part of the same protein that constitutes the channel binding site for PCP, but that CPP may bind to a distinct but tightly coupled protein (Honore et al. 1989). Receptor solubilization studies also suggest that the NMDA, glycine, and PCP binding sites are tightly coupled, as the binding of [^{3}H]TCP and[^{3}H]MK-801 to a solubilized protein is enhanced by NMDA agonists, glycine, and divalent cations (Ambar et al. 1988; McKernan et al. 1989). Furthermore, injection of crude poly (A) mRNA from rat brain into *Xenopus* oocytes results in the expression of a cation channel opened by NMDA agonists (in the presence of glycine) and blocked by PCP, MK-801, and Mg^{++} (Kleckner and Dingledine 1988; Kushner et al. 1988; Fong et al. 1988). The NMDA complex is expressed by mRNA in the range of 4.2 to 7.7 kb, suggesting that the complex consists of several homologous subunits that might be encoded by mRNAs of different length, presumably because of differences in the length of untranslated regions (Fong et al. 1988). It seems likely that advances in knowledge of the molecular biology of this complex will occur soon. It will be most interesting to see whether the widely anticipated similarities with other receptor-operated ion channels become a reality.

THE GLYCINE RECEPTOR

In studies of the effects of excitatory amino acids on cultured neurons, Johnson and Ascher (1987) observed that glycine potentiated the effects of NMDA in a strychnine-insensitive manner. The effects of glycine were mimicked by D-serine and D-alanine and were completely blocked by NMDA antagonists, suggesting that glycine was acting through a unique receptor to facilitate, or perhaps to permit, the actions of NMDA. Patch-clamp analysis revealed that the primary effect of glycine was to increase the frequency of channel opening without changing the channel-open time or channel conductance (Johnson and Ascher 1987) suggesting that glycine could regulate transitions to intermediate states between binding NMDA agonists and ion channel gating (Mayer et al. 1989). Consistent with this suggestion, Javitt and Zukin (1989) have presented a model based on near equilibrium binding of [^{3}H]MK-801, which postulates that glycine converts a glutamate-associated, closed conformation of the channel (with a low affinity for [^{3}H]MK-801) to a high-affinity, open conformation. This is similar to the model derived above based on equilibrium analysis in the presence of NMDA and glycine antagonists.

Another prominent effect of glycine appears to be a reduction in the rapid desensitization of NMDA receptors (Mayer et al. 1989). In other experiments in which ambient glycine concentration was minimized, no NMDA response could be obtained, suggesting an obligatory role for glycine (Kleckner and Dingledine 1988). However, Mayer and colleagues (1989) have pointed out that these measurements were made under conditions insufficient to detect rapid changes in receptor gating, and thus the apparent requirement for glycine may be due in part to the ability of glycine to retard the rate of receptor desensitization.

The strychnine-insensitive glycine receptor has been studied using [^{3}H]glycine binding (Kishimoto et al. 1981) and autoradiography (Bristow et al. 1986). The glycine receptor has a K_D of about 100 to 250 nM for [^{3}H]glycine. The relatively high concentrations reported for cerebrospinal fluid (Connick and Stone 1988) suggest that this receptor may be normally saturated. This hypothesis is supported by the relatively low-affinity transport systems for glycine in forebrain (Debler and Lajtha 1987) and by the lack of effect of exogenously applied glycine on NMDA responses in cortical wedges (Fletcher and Lodge 1988; Kemp et al. 1988). However, a recent study has found that iontophoretic application of glycine selectively facilitated NMDA responses in neocortical neurons (Thomson et al. 1989). Thus, the saturation of glycine receptors may vary widely and may depend on the particular synapse. There may also be pathological conditions in which the local glycine concentration is abnormal, thereby adversely affecting normal synaptic transmission mediated by NMDA receptors.

The structural requirements for activation of the glycine receptor have been determined by measuring the effects of glycine and various analogs on [^{3}H]TCP binding under nonequilibrium conditions (Snell et al. 1988). The rank order potency for simple amino acids is as follows:

glycine = D-serine > D-alanine > L-serine > L-alanine = L-valine >> D-valine

This rank order was found to correlate precisely with affinity for the site labeled by [^{3}H]glycine (Snell et al. 1988). Comparison with other analogs including cysteine (Wong et al. 1987) phenylalanine, GABA, ß-alanine, glycine methyl ester, glycinamide, and *N*-methylglycine revealed a strict structural specificity, suggesting that the glycine receptor contains three points of attachment for agonists: negative and positive ionic sites (corresponding to the α-amino and α-carboxyl groups of glycine) and a hydrogen bond-donating site (corresponding to the hydroxyl group of D-setine) (McBain et al. 1989; Snell et al. 1988).

Recently, another cyclic glycine analog, 1-hydroxy-3-aminopyrolid-2-one (HA-966) was reported to antagonize NMDA-induced depolarization in a manner that was reversible by glycine (Fletcher and Lodge 1988). This compound completely inhibits [^{3}H]glycine binding with a K_i of about 5 μM and has no effect on the NMDA recognition site at concentrations up to 100 μM (L.D. Snell and K.M. Johnson, unpublished observations). HA-966 is remarkably similar in structure to D-cycloserine, a moderately potent glycine agonist (Johnson et al. 1988a), perhaps accounting for its inability to completely inhibit NMDA responses. Alternatively, this finding may suggest that glycine is not obligatory for NMDA function.

Kynurenic acid is a well-known, rather nonselective antagonist of NMDA, kainate, and quisqualate receptors. The 7-chloro derivative of kynurenic acid is a potent (K_i = 0.5 μM) and selective glycine antagonist (Kemp et al. 1988). Kynurenic acid also inhibits [^{3}H]glycine binding but is about 75 times less potent and 70 times less selective (relative to the NMDA receptor) than 7-chlorokynurenic acid (Kemp et al. 1988). Comparisons with HA-966 suggest that 7-chlorokynurenic acid may be an inverse agonist rather than a simple antagonist (Foster and Kemp 1989). Recently, indole-2-carboxylic acid and its 5-chloro derivative have been reported to be selective but somewhat less potent glycine antagonists (Huettner 1989).

INTERACTIONS BETWEEN NMDA AND GLYCINE RECEPTORS

NMDA agonists and antagonists have been shown to increase and decrease, respectively, [^{3}H]glycine binding to a strychnine-insensitive site. Conversely, glycine agonists tend to increase NMDA-sensitive [^{3}H]glutamate binding, and glycine antagonists decrease it. These effects are not due to pharmacologic nonspecificity, as NMDA and glycine agonists reverse the effects of NMDA and glycine antagonists on [^{3}H]glycine and NMDA-sensitive [^{3}H]glutamate binding, respectively. The mechanisms responsible for this reciprocal interaction are unclear, but some investigators found that changes in affinity accounted for the effects of glycine (Fadda et al. 1988) and NMDA antagonists (Hood et al. 1990). This finding suggests that the effects of NMDA and glycine agonists, as well as antagonists, are potentiated via allosteric increases or decreases in the binding affinity of the other receptor. This point has been shown in one study to have a functional consequence in that the potency of glycine agonists for stimulation of [^{3}H]TCP binding increases in the presence of glutamate (Hood et al. 1990).

As mentioned previously, there is evidence that the site labeled by [^{3}H]CPP may not be the same as that labeled by [^{3}H]glutamate. Unpublished data from this laboratory confirm the uniqueness of this site. Both glycine and D-serine have no effect on [^{3}H]CPP binding (L.D. Snell and K.M. Johnson, unpublished observations) but decrease [^{3}H]CGS 19755 binding (Kaplita and Ferkany 1990). This decrease is in direct contrast to their stimulatory effect on NMDA-sensitive [^{3}H]glutamate binding (Fadda et al. 1988; L.D. Snell and K.M. Johnson, unpublished observations). Furthermore, the glycine antagonists 7-chlorokynurenate and HA-966 have differential effects on agonist and antagonist binding. 7-Chlorokynurenate inhibits both NMDA-sensitive [^{3}H]glutamate and [^{3}H]CPP binding (L.D. Snell and K.M. Johnson, unpublished observations), but HA-966 stimulates [^{3}H]CPP binding (Compton et al. 1990; L.D. Snell and K.M. Johnson, unpublished observations) and [^{3}H]CGS 19755 binding (Kaplita and Ferkany 1990).

Further complexity is revealed by the observation that glycine inhibits [^{3}H]CGS 19755 binding but not [^{3}H]CPP binding (Kaplita and Ferkany 1990). Thus, at this time it is exceedingly difficult to model the interactions between NMDA and glycine receptors, particularly when the differential effects of glycine, HA-966, and 7-chlorokynurenate on NMDA agonist and antagonist binding sites are considered. It is also difficult to imagine the physiological significance of distinct antagonist binding sites. Perhaps they are involved in mediating the effects of endogenous antagonist molecules. The various interactions between glycine and NMDA ligands are summarized in table 4.

ROLE OF POLYAMINES

Putrescine is derived from the decarboxylation of ornithine. This divalent diamine is metabolized to the triamine spermidine, which in turn is converted to spermine, a primary amine with the following structure: NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3NH_2$. Ransom and Stec (1988) reported that spermidine and spermine, but not putrescine, increased [^{3}H]MK-801 binding by a mechanism that was enhanced by both glutamate and glycine and blocked by NMDA antagonists. This report suggested that certain polyamines may modulate NMDA receptor function through an intracellular mechanism. This finding became more intriguing with the preliminary report that the effects of NMDA on Ca^{2+} flux and neurotransmitter release were blocked by an irreversible inhibitor of omithine decarboxylase (ODC) in a manner reversible by addition of putrescine (Siddiqui et al. 1988). Furthermore, these investigators also reported that NMDA produced a rapid, transient increase in ODC. Together, these data

TABLE 4. *Summary of actions of NMDA and glycine ligands on binding at several recognition sites*

[^{3}H]-Ligand	NMDA/Glutamate	CPP	CGS 19755, AP5
[^{3}H]glycine	O[a, m] or ↑[j]	O[c, h] or ↓[a, f]	Å[a, l, f, l]
	Glycine	HA-966	7Cl-KYN
[^{3}H]glutamate	↑[a, e, k]	O[a, g, h]	↓[a, d]
[^{3}H]CGS	↓[b, i]	O[b, f]	↑[f]
[^{3}H]CPP	O[a, b, f]	↑[a, b, f]	↓[a, d]

KEY: ↑=compound increases binding; ↓=compound decreases binding; O=no effect

[a]L.D. Snell and K.N. Johnson, unpublished observations
[b]Compton et al. 1989
[c]Danysz et al. 1989a
[d]Danysz et al. 1989b
[e]Fadda et al. 1988
[f]Kaplita and Ferkany 1990
[g]Foster and Kemp 1989
[h]Hood et al. 1989a
[i]Hood et al. 1989b
[j]Kessler et al. 1989
[k]Monaghan et al. 1988
[l]Monahan et al. 1988
[m]White et al. 1989

suggest that the actions of NMDA may be partially mediated by spermidine or spermine or both.

As of this writing, the effect of NMDA on ODC activity and the effect of ODC inhibition on NMDA responses have not yet been confirmed, but the effects of spermine and spermidine on [^{3}H]MK-801 binding have been confirmed (Reynolds 1990). The authors' laboratory has also confirmed this finding using [^{3}H]TCP and has extended it to several synthetic polyamine analogs in an effort to determine the structural requirements for this unique modulatory site (Sacaan and Johnson 1990a). The number of nitrogen atoms in the polyamine backbone have been found to play a major role in determining the efficacy of receptor activation, and the number of methylene groups separating the nitrogens were found to be a major determinant of affinity. Furthermore, it has been determined that several diamine analogs, including putrescine, antagonize spermidine-induced [^{3}H]TCP binding in a manner consistent with a noncompetitive mechanism (Sacaan and Johnson 1990*a).* Because the metabolism of

putrescine to spermidine and spermine is reversible, these data imply that the regulation of polyamine metabolism could be intimately involved in a complex regulation of NMDA receptor function. It has been reported that SL 82,0715 and ifenprodil may be antagonists at this polyamine site (Carter et al. 1988), but Reynolds and Miller (1989) concluded that ifenprodil is a noncompetitive antagonist at this site. Comparisons with the authors' putrescine data suggest that ifenprodil is about 100 times more potent and possibly more so, depending on the concentration of glutamate and glycine (Reynolds and Miller 1989). Ifenprodil is of additional interest in this context because of its neuroprotective effects in focal cerebral ischemia (Gotti et al. 1988).

Even more recently a spermidine analog, diethylenetriamine (Williams et al. 1990), and a putrescine analog, diguanidinobutane (Reynolds 1990) were identified as putative competitive spermidine antagonists. This laboratory has confirmed that arcaine (diguanidinobutane) competitively inhibits spermidine-induced [^{3}H]TCP binding (Sacaan and Johnson 1990*b).* It was also observed that arcaine competitively antagonizes Mg^{2+}-induced [^{3}H]TCP binding, thereby suggesting that this previously poorly understood effect of Mg^{2+} was mediated through the spermidine site, where it acts as a partial agonist (Sacaan and Johnson 1990*b).* An obligatory role for polyamines was suggested by the observation that arcaine completely inhibited both [^{3}H]TCP binding and NMDA-induced [^{3}H]NE release from hippocampal slices (Sacaan and Johnson 1990*c).* The specificity of arcaine was demonstrated by the observations that (1) it had no effect on either K^+- or kainate-stimulated release, (2) it inhibited NMDA-induced release noncompetitively, and (3) its inhibition of NMDA-induced release was reversed by spermidine (Sacaan and Johnson 1990*c).* The reversal by spermidine was also interesting because it occurred only in buffer containing 5 percent dimethyl sulfoxide (DMSO). It is postulated that the spermidine site is intracellular and that DMSO increases the transport or diffusion of spermidine across the neuronal membrane. Alternatively, the site could be extracellular and the effect of DMSO could be due to its ability to increase spermidine diffusion through the slice.

Further complexity is suggested by preliminary studies of polyamines on [^{3}H]glycine binding (Johnson and Sacaan 1989). These experiments revealed that spermine enhanced [^{3}H]glycine binding by increasing the binding affinity threefold. The structural requirements for activating the glycine receptor were completely different from those for activating the NMDA ionophore, suggesting the existence of at least two independent sites for polyamine modulation of the NMDA receptor ionophore complex. It also was found that the spermine-

induced change in glycine receptor affinity was selective for agonists (i.e., spermine shifted the [^{3}H]glycine displacement curve threefold to the left for glycine and D-serine but had no effect on the displacement by the antagonists 7-chlorokynurenic acid and cycloleucine) (Sacaan and Johnson 1989). This finding suggests that the glycine receptor may exist in agonist- and antagonist-preferring conformations. We propose that this effect of spermine may be important physiologically in regulating the effects of an endogenous antagonist, such as kynurenic acid.

CONCLUSIONS

As covered extensively in a recent review (Johnson and Jones 1990), the mechanisms of action of PCP are extremely complex. Although it is not certain exactly what role blockade of the NMDA receptor-ionophore plays in the psychotomimetic and reinforcing effects of PCP, this mechanism clearly is central to its anticonvulsant and neuroprotective effects in acute trauma, anoxia, and hypoglycemia. Thus, if PCP (or related compounds) ultimately has any impact in the therapy of chronic illnesses such as epilepsy, it will be necessary to isolate pharmacologically the therapeutic effects from the psychoactive “side effects.” It is unknown at this time if this is possible, but it is conceivable that, in situations of acute trauma or stroke, the potential therapeutic benefit of PCP-like drugs may outweigh these concerns.

Further research on PCP and similar drugs will be required to elucidate the actions of these drugs and to understand the regulation of the NMDA ionophore. To date, this complex has been shown to be regulated by several mechanisms and by a diverse spectrum of effector molecules (figure 2). This complexity affords an opportunity to subtly control receptor function, possibly according to therapeutic needs. In addition to the voltage- and use-dependent nature of the blockade by PCP-like drugs, the possibility that the makeup and organization of NMDA receptor complexes vary regionally also provides an opportunity for more selective modulation of receptor function. Thus, the possibility of selectively targeting specific pathways for therapeutic intervention is exciting and should be a fruitful area for future research.

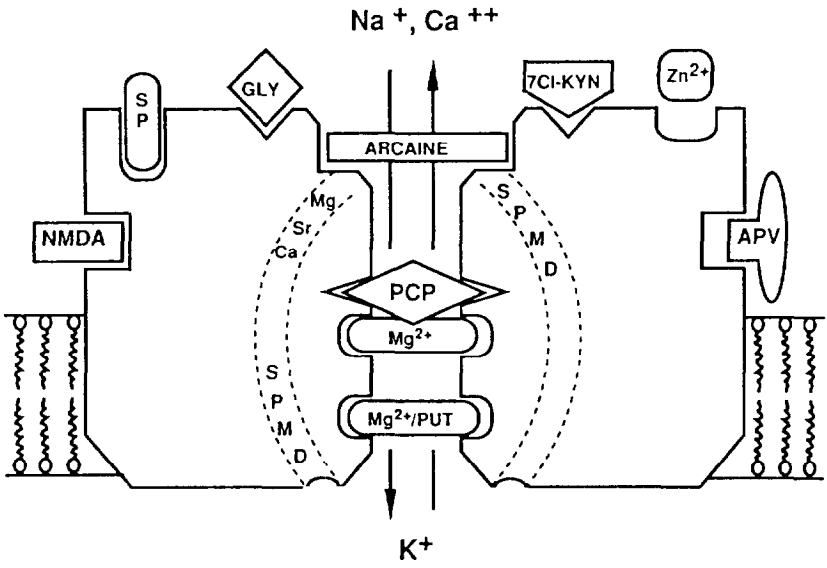

FIGURE 2. *Proposed model of the NMDA receptor ionophore complex. Antagonists are shown on the right half of the complex or in the channel itself; agonists are shown on the left half. There is no known Zn^{2+} site or antagonist for the putative extracellular spermine (SP) site. Occupation of the latter site by SP induces a conformational change in the glycine (GLY) site that is seen as an increased affinity for agonists, but not antagonists (Sacaan and Johnson 7989). This site is not obligatory and has a distinct pharmacology from the obligatory intracellular site activated by Mg^{2+}, Ca^{2+}, Sr^{2+}, and spermidine (SPMD). Activation of this site results in an increased affinity for [^{3}H]TCP or [^{3}H]MK-801 binding to the PCP site, as well as for Mg^{2+} and putrescine (PUT) binding to their inhibitoty site that is thought to be in the channel. Magnesium can also act as a partial agonist at the intracellular SPD site, but at high receptor occupancy levels (by SPD) magnesium acts as an antagonist at this site as well. At concentrations above 1 mM, SPD acts at a distinct site as an antagonist. The binding domains for SPD are depicted between dashed lines to indicate the uncertainty of their locations. The proposed transitional state induced by NMDA agonists in the absence of glycine, as well as the allosteric interactions between the NMDA and glycine recognition sites, are not included for ease of presentation. APV= 2-amino-5-phosphonovaleric acid.*

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Pharmacologic Characterizations of σ Receptors

Tsung-Ping Su

Two binding sites, σ receptor and PCP receptor, have been proposed as mediators of psychotomimetic effects exerted by certain benzomorphans and phencyclidine (PCP) (Vincent et al. 1979; Zukin and Zukin 1979; Su 1981; Su 1982; Quirion et al. 1987). Exactly which receptor is responsible for the effects is still unknown, however. Although a large number of reports have presented pharmacological and behavioral results that indicated the activation of PCP receptors (e.g., Shannon 1982; Brady et al. 1982; Johnson 1987), many studies have also shown that σ receptors may mediate certain behavioral and biochemical responses (Ceci et al. 1988; Steinfels et al. 1988; Bowen et al. 1988; Walker et al. 1988; Iwamoto 1989*a,* 1989*b;* Woods, this volume). Because no animal model can yet be said to definitively represent psychotomimetic disturbances in humans, the results of those studies may be interpreted as suggesting that both receptors may be related to psychotomimesis or other psychological disturbances. It is noteworthy, however, that at least seven potential antipsychotic drugs, which are efficacious in preclinical tests, all possess high affinity for the σ receptor while exhibiting minimal affinity for the PCP receptors (Largent et al. 1988). Our understanding of the exact relationship between the two receptors and psychological disturbances in humans can be clarified only when we obtain results from clinical trials using drugs specific for each type of receptor.

In this chapter three areas concerning σ receptors will be addressed: (1) endogenous ligands for σ receptors; (2) the structure-activity relationship of a-selective compounds; and (3) the subcellular distribution of σ receptors.

ENDOGENOUS LIGANDS FOR σ RECEPTORS

The unique ligand specificity and distinctive central nervous system distribution of σ receptors (Gundlach et al. 1986; Largent et al. 1986) have suggested that

endogenous ligand(s) for this receptor might exist. An early attempt to identify endogenous ligands for the σ receptor employed a receptor binding assay and yielded two putative endogenous σ ligands of about 10,000 and 4,000 daltons (Su et al. 1986). Because many factors may interfere unpredictably with receptor binding, we developed a bioassay system that, when coupled with binding assays, would serve as a more selective tool for examining endogenous ligands. This bioassay was a guinea pig vas deferens preparation.

An endogenous a ligand should be active in both the receptor binding assay and the guinea pig vas deferens bioassay. The electrically induced twitches of the guinea pig vas deferens were potentiated by σ drugs such as *d*-SKF-10,047, *d*-pentazocine, and *d*-ketocyclazocine, whereas non-a drugs such as morphine, DADLE, and *l*-ketocyclazocine were inactive (Vaupel and Su 1987). Further, the potentiation induced by' σ drugs was blocked by putative σ antagonists haloperidol and BW234U (Vaupel and Su 1987). Although this bioassay system was not a very specific assay for σ drugs because the less o-selective drugs such as PCP and *l*-SKF-10,047 were also active, the assay did provide an adjunct tool for examining potential endogenous σ ligands.

Using the two σ assays, we identified from guinea pig brain extract an endogenous σ I ligand of about 485 daltons (Su and Vaupel 1988). The larger molecular-weight substances found in the earlier study (i.e., 10,000 and 4,000 daltons from the receptor binding assay (Su et al. 1986) were inactive in the guinea pig vas deferens assay (Su, unpublished observation). The 485-dalton material was obtained by fractionating a 700 to 200 molecular weight mixture from a Sephadex G-50 column, the technique used in the earlier study (Su et al. 1986). The mixture was then absorbed onto a Bio-Rex70 cation exchanging resin (equilibrated in 0.05 M ammonium acetate), washed with a linear gradient of 0.05 M to 1 M ammonium acetate, and then eluted with 1 N acetic acid. The 1 N acetic-aid-eluted fraction was found to be active not only in the receptor binding assay but also in the guinea pig vas deferens bioassay (Su and Vaupel 1988). The active fraction from the Bio-Rex70 column was further separated in a Sephadex G-15 column and the 485-dalton material obtained (figure 1). Characterization of this material indicated that it is nonpeptidic and may not contain nitrogen. Although this material may not be pure at present, preliminary NMR data indicated that it may contain repetitive methylene group. Final purification of this material, if successful, may provide an interesting clue to our understanding of σ receptors.

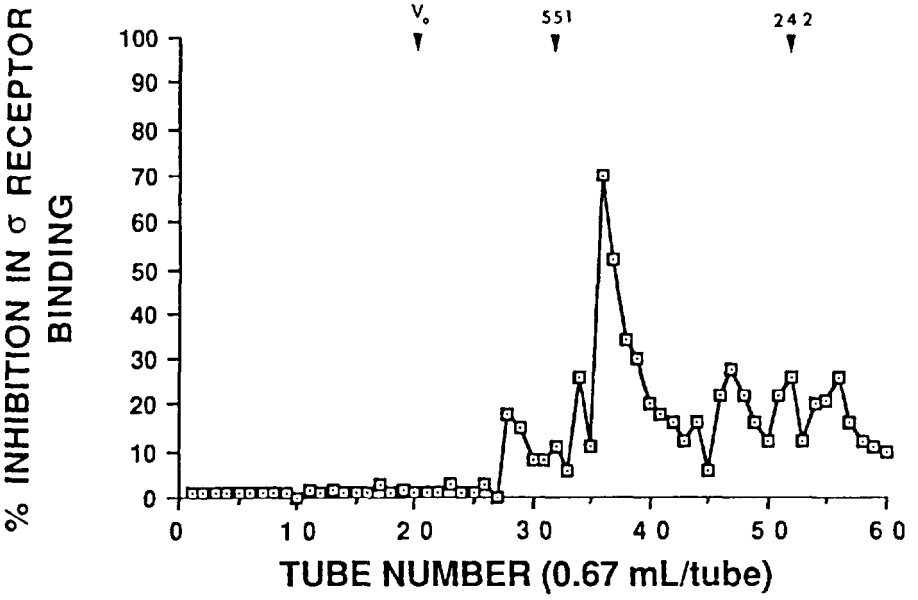

FIGURE 1. *Fractionation of an endogenous σ ligand in a Sephadex G-15 column. Samples applied to the column were obtained from the guinea pig brain extract after a partial purification using Sephadex G-50 and Bio-Rex70 columns. Twenty-five mg of the lyophilized powder of the sample was applied to the column that was preequilibrated in 0.1 M ammonium acetate buffer (pH 5.4). The column (7.6 cm x 90 cm) was eluted with the same buffer and fractions of 0.67 mL were collected. Each fraction was lyophilized and tested in σ receptor binding assay using 2 nM of [^{3}H]d-SKF-10,047. Percent inhibitions are shown on the y-axis. Positions of molecular weight standards are indicated. V_0 indicated void volume of the column.*

The information collected initially suggested that the 485-dalton endogenous σ ligand could bear some structural similarity to steroids. For example, the material was not peptidic and presumably contained no nitrogen while containing repetitive methylene groups. Further, the 485-dalton material was active in an endocrine tissue, the guinea pig vas deferens (Su and Vaupel 1988). These properties suggested that some steroids could act at σ receptors and be related to an endogenous σ ligand.

Our examination of a wide variety of steroids indicated that certain steroids were indeed potent σ ligands (Su et al. 1988), with the gonadal steroids progesterone and testosterone among the most potent (Su et al. 1988). The affinity of progesterone for the σ receptor was about half that of the prototypic σ drug *d*-SKF-10,047 (268 nM and 134 nM, respectively). Other steroids possessing appreciable affinity for the σ receptor include deoxycorticosterone, 11ß-hydroxyprogesterone, pregnenolone sulfate, and corticosterone.

Steroids have been well known to bind at "steroid" receptors in peripheral as well as neuronal tissues. The σ receptor, however, differs from traditionally recognized "steroid" receptors. The "steroid" receptor is cytosolic and not localized on cellular membranes, while σ receptors are membrane bound. There are also other differences between these two receptors. For example, potent, specific steroid ligands such as promegestone, RU 27987, estradiol, and estriol, which bind at steroid receptors, were all inactive in the σ receptor binding assay (Su et al. 1988). Further, a progesterone antagonist, RU 486, which interacts specifically with the cytosolic progesterone receptor (Baulieu 1989) was inactive in receptor assays (Su, unpublished observation). The K_D values of the steroids binding to cytosolic steroid receptors were typically in the range of about 0.1 to 10 nM (Gorski and Gannon 1976) while much higher K_D values were found for steroids binding to σ receptors (Su et al. 1988). Also, steroids that were active at σ receptors in the brain were also active at o receptors in the spleen cells (Su et al. 1988).

A question arises: What is the functional significance of the interaction of steroids with σ receptors in the brain? or in the cells from the immune system? All possible answers are purely speculative at this time. Because σ receptors may mediate certain aspects of psychiatric disturbances, it is not unreasonable to speculate that steroids may produce psychiatric disturbances by acting at σ receptors.

Steroid abuse has recently aroused public awareness, especially abuse among athletes. The adverse medical effects and major psychiatric disturbances caused by steroid abuse are well documented (Annitto and Layman 1980; Freinhar and Alvarez 1985; Wilson et al. 1974; Pope and Katz 1987, 1988). The effects are even more dramatic because typical steroid abusers may routinely use doses 10 to 100 times greater than those used clinically (Pope and Katz 1988). The possibility certainly exists that the σ receptor mediates steroid-induced psychiatric disturbances, and, conversely, that some of those behavioral effects may contribute to the abuse of steroids.

Steroids may also affect immune responses through interaction at σ receptors in immune cells; σ receptors have been found in spleen cells and lymphocytes (Su and Vaupel 1988; Su et al. 1988; Wolfe et al. 1988). The functional role of σ receptors in these cells is the subject of speculation. These tissues contain immune cells, so it is tempting to speculate that σ receptors in these cells may be involved in cellular immune responses. If this speculation is true, then steroids active at σ receptors should also be active in certain immunologic events. Indeed, an interesting correlation was observed when the efficacies of steroids in anti-inflammatory tests (Siiteri et al. 1977) were compared with their affinities at σ receptors (Su et al. 1988). (A direct comparison of the potencies could not be made because, in the anti-inflammatory tests, steroids were delivered through silastic tubing and, thus, no determination of the local concentration of the test steroids was made. However, the qualitative correlation is striking.) Steroids that were active in the anti-inflammatory tests were also active in σ receptor binding, including progesterone, deoxycorticosterone, and corticosterone. Many steroids that were inactive in the anti-inflammatory tests were also inactive in the σ receptor binding assay. Those included estriol, estrone 3-hemisuccinate, 17ß-estradiol-17-hemisuccinate, hydrocortisone, 17α-hydroxyprogesterone, and pregnenolone (Su et al. 1988). However, there were exceptions. For example, although testosterone was one of the most potent in the σ receptor binding assay, it was inactive in the anti-inflammatory test. These results suggest that σ receptors may be involved in immune responses. Because σ receptors have also been found in many endocrine tissues (Wolfe et al. 1989) it has been suggested that σ receptors may provide a link between immune, endocrine, and central nervous systems (Su et al. 1988). Therefore, σ ligands may have a wide spectrum of therapeutic uses (Su et al. 1988).

STRUCTURE-ACTIVITY RELATIONSHIP OF σ-SELECTIVE COMPOUNDS

Drugs that bind and are active at σ receptors encompass diverse classes of pharmacological agents. The σ-active compounds include neuroleptics such as haloperiodol, chlopromazine, and fluphenazine (Su 1982; Tam and Cook 1984; Largent et al. 1984; Martin et al. 1984; Weber et al. 1986; Contreras et al. 1987); benzomorphans such as *d*-pentazocine, *l*-pentazocine, *d*-cyclazocine, and *d*-SKF-10,047 (Su 1982; Tam 1983; Largent et al. 1984; Martin et al. 1984; Weber et al. 1986); antitussive agents such as dextromethorphan (Su 1982; Largent et al. 1984); dissociative anesthetics such as phencyclidine (Su 1982; Tam 1983; Largent et al. 1984; Martin et al. 1984); antihypertensives such as propranolol (Su 1982; Tam 1983; Martin et al. 1984); antidepressants such as

imipramine (Su 1982; Largent et al. 1987) and sertraline (Schmidt et al. 1989); a polymerization catalyzing agent, ditolyl-guanidine (Weber et al. 1986); a compound structurally related to dopamine, (+)-3-PPP((+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine) (Largent et al. 1984); and steroids such as progesterone (Su et al. 1988).

What do these compounds have in common, structurally, that leads to high affinities at σ receptors? By synthesizing a large number of compounds and comparing their activities in the σ receptor binding assay, Largent et al. (1987) proposed that the basic pharmacophore for drugs to possess affinity at a σ receptor is a phenylpiperidine with a lipophilic nitrogen substituent. Using calculations based on energy and geometric fit of a series of o-selective drugs, others reached the same conclusion, that the basic requirement for a o-selective ligand is a substituted nitrogen and a phenyl hydrophilic center separated by an intermediate chain (Manall`ack and Beart 1987; Manallack et al. 1988). It was also proposed in these reports that a σ receptor may interact with a σ ligand through three hypothetical points on the receptor (Manallack and Beart 1987; Manallack et al. 1988). An R1 and an R2 would each perpendicularly project to the opposite side of the flat phenyl ring. An R3 site that was relatively parallel to the R1-phenyl projection was proposed to receive the vector provided by the lone pair electron on the nitrogen atom.

Similar results were obtained when several a-selective ligands such as *d*-pentazocine, *d*-SKF-10,047, haloperidol, BW 234U, BMY 14,802, remoxipride, and progesterone were examined based on molecular modeling and electrostatic potential calculations (Gund et al. 1991). (Progesterone was included in the study-although it does not contain nitrogen, because it is a potent σ ligand.) The study suggested that the oxygen atom of the 20-carbonyl group on progesterone must be the vector to the R3 hypothetical receptor point. Further, the R1 and R2 receptor pocket can accommodate quite a variety of lipophilic rings. The lipophilic ring does not have to be a phenyl ring, because ring B of progesterone (a cyclohexyl ring) can substitute for a phenyl ring. Also, the R1-R2 receptor pocket may tolerate lipophilic groups of quite different sizes, because ring A of a steroid, immediately adjacent to ring B, does not destroy progesterone activity at a σ receptor.

An important aspect of the results of the study by Gund et al. (1989) was obtained from the calculation of the electrostatic potentials (ESP) on the van der Waals surfaces of these o-selective ligands. ESP on progesterone indicated a highly positively charged center around ring B of the molecule, i.e.,

the lipophilic area interacting with the R1-R2 hypothetical receptor points. However, the ESP of other σ-selective ligands, when examined in their protonated forms, had a positive center surrounding the nitrogen atoms. These results represent a potential contradiction, as one would expect that the highly positively charged centers of all these drugs would be located at the same end of the molecules, where they share a common chemical-physical property to interact with a common site on the receptor. The contradiction was resolved when ESPs were examined on the unprotonated forms of the σ ligands. A highly positively charged center on each drug was found to localize around the lipophilic pocket-similar to the ESP on progesterone. These results indicated that the unprotonated form of a σ drug is probably the molecular species that interacts with σ receptors.

Ever since the demonstration of separate PCP and σ receptors, it has been noted that phencyclidine, although more specific toward the PCP receptor, can still interact with the σ rreceptor. The IC_{50} ratio of phencyclidine for the two receptors is about 1/10 (PCP/σ). An interesting question can be asked: Is it possible to modify the phencyclidine molecule to obtain compounds that are more selective for the σ receptor? After an extra methylene group was inserted into phencyclidine between the cyclohexyl and piperidine rings, the modified compound became more selective than the parent compound for the σ receptor (figure 2; Su and Parish, unpublished observation). The IC_{50} ratio of this new compound for the two receptors is 17.8/1 (PCP/σ). The results suggest that by increasing the length between the cyclohexyl ring and the amine moeity, one may change PCP-selective compounds into σ-selective drugs. These results indicate that the intermediate chain between the lipophilic ring and amine group is probably essential for σ-selective compounds.

SUBCELLULAR DISTRIBUTION OF σ RECEPTORS

An examination of the subcellular distribution of σ receptor activity in rat brain revealed that σ receptors were concentrated in the microsomal fraction (i.e., P_3) when compared with the crude synaptosomal fraction (i.e., P_2) (McCann et al. 1989). Thus, σ receptors may not be localized in the synaptic area of plasma membranes. Because microsomes contain many drug-metabolizing enzymes, it was possible that the σ receptor may be an enzyme (McCann et al. 1989). However, that speculation was made from data obtained from a relatively "rough" fractionation of subcellular structures, and the authors also suggested other possibilities such as localization on the nonsynaptic regions of plasma membrane. The proof of cellular localization requires further fractionation and

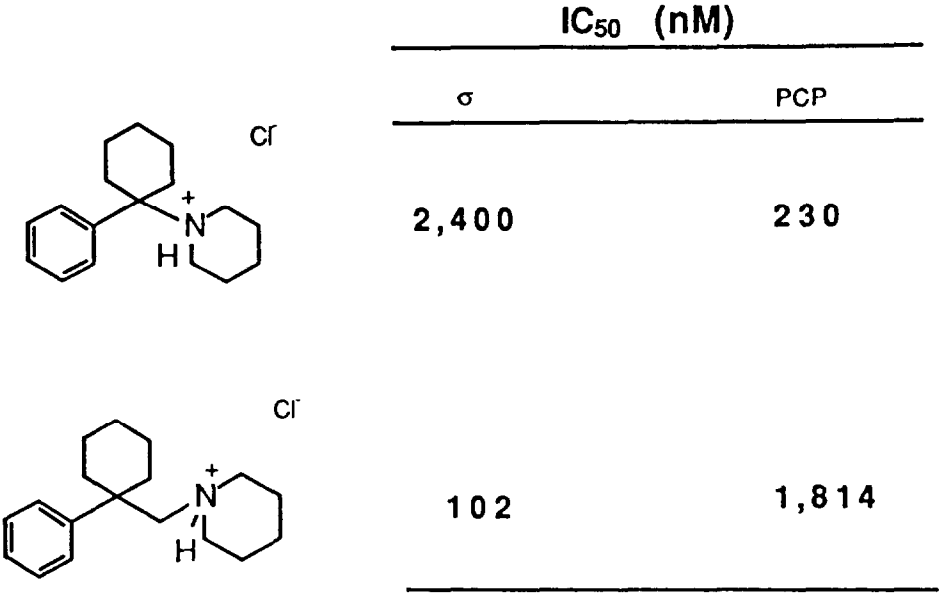

FIGURE 2. *Effect of inserting a methylene group between the arylcyclohexyl ring and amine group of phencyclidine on the relative affinities for σ and PCP receptors. The compound with a methylene insertion was synthesized from phenylacetonitrile and 1,5-dihaloalkane followed by an amine (Parish et al., in preparation). σ and PCP receptor binding assays were performed using guinea pig brain homogenates with 2 nM [^{3}H]d-SKF-10,047 and 1 nM [^{3}H]TCP as the radioligands, respectively. IC_{50}s represent the average from three experiments, each assayed in quadruplicate.*

comparison with known markers (McCann et al. 1989). In further studies, it was found that the distribution patterns of σ receptors paralleled the patterns of a general plasma membrane marker, 5' nucleotidase, indicating the possibility that σ receptors may reside in the nonsynaptic portion of the plasma membrane (McCann and Su 1990). The distribution of σ receptors also differed from the distribution of a marker for a major microsomal constituent-endoplasmic reticulum (McCann and Su 1990). A better understanding of the exact localization of the σ receptor may have to wait on examinations using electronmicroscopic techniques coupled with immunocytochemistry.

Whether the σ receptor is an enzyme or not is by itself an interesting question. It would be very revealing indeed (if the σ receptor turns out to be an enzyme) to discover how an enzyme can play such interesting pharmacologic roles. So far, however, no enzymatic activity has been demonstrated in σ receptor preparations. In fact, in a report using liver homogenates, where there is an abundance of drug-metabolizing enzymes, [^{3}H]*d*-SKF-1 0,047 was found unchanged after incubation (Samovilova et al. 1988). This suggests that the σ receptor may not be an enzyme. The biochemical nature of σ receptors certainly deserves further investigation,

In conclusion, much remains to be learned about σ receptors. Our understanding of this receptor is just beginning. Because of the potential relation of σ receptor activity to psychiatric disturbances in humans, or other as yet to be fully revealed actions, the studies reviewed in this chapter provide significant information.

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Studies of σ Receptors and Metabolic Responses to σ Ligands in the Brain

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σ Binding sites were first characterized in rodent brain by using racemic tritiated N-allylnormetazocine (NANM, SKF 10,047) (Su 1981, 1982), the prototypical σ ligand (Martin et al. 1976). More recent studies of σ receptors have employed tritiated d-NANM (Tam 1983; Martin et al. 1984; ltzhak et al. 1985; Mendelsohn et al. 1985; Mickelson and Lahti 1985; Largent et al. 1986; Sircar et al. 1986), (+)3-(3-hydroxyphenyl)-N-(1-propyl)-piperidine (PPP) (Largent et al. 1984), di-o-tolylguanidine (DGT) (Weber et al. 1986), and haloperidol (HAL) (Tam and Cook 1984; Downes et al. 1986; Contreras et al. 1987). In the studies discussed below, our laboratory used [^{3}H]HAL as a σ receptor ligand because of its greater affinity for σ receptors than d-NANM, its negligible affinity for phencyclidine (PCP) receptors, and because HAL has the potential for use in in vivo human studies when labelled with the positron-emitting radionuclide F-l 8.

Although HAL binds to dopaminergic and α_1-adrenergic receptors, we blocked radioligand binding to these sites by adding spiperone to incubations. Assays using various concentrations of spiperone to block the binding of 1 nM HAL showed that 75 nM spiperone blocked 95 percent of the binding to one population of sites without affecting binding to a lower affinity population in the guinea pig brain; in samples from other subhuman species, 100 nM spiperone was an optimal concentration for blocking binding to the higher affinity population (Vu et al. 1990). In the human cerebellum, 50 nM spiperone completely inhibited binding to one population of sites without appreciably affecting another population of sites (Weissman et al. 1988*b).* The K_i values of spiperone in competing for HAL binding to dopaminergic and α_1-adrenergic receptors are 0.25 nM (Bun et al. 1976) and about 4 nM (U'Prichard et al. 1977) respectively. In contrast, spiperone shows an IC_{50} value between 10^{-7} and 10^{-6} M in inhibiting HAL binding to σ receptor (Tam and Cook 1984). Therefore, the high-affinity, spiperone-sensitive binding of HAL apparently was

to dopaminergic or noradrenergic sites or both, and the lower affinity population was the σ receptor.

Similarities in the binding properties (for reviews, see Zukin 1984; Quirion 1986; Johnson 1987) and behavioral effects of NANM and PCP (Keats and Telford 1964; Snyder 1980; Iwamoto and Martin 1981; Brady et al. 1982; Shannon 1982) led us to explore whether σ and PCP receptors showed similar phylogenetic or development profiles. We studied σ and PCP receptor binding in a wide variety of animal species, using [^{3}H]HAL and [^{3}H]TCP as the respective ligands (Vu et al. 1990).

We performed saturation and single-concentration studies of both binding sites on whole brains of various species, and the cerebellum [^{3}H]HAL) and frontal cortex ([^{3}H]TCP) of the squirrel monkey. K_d values for σ sites varied about five-fold, with the highest densities in the frog and guinea-pig brains. [^{3}H]TCP binding affinities at PCP receptors varied about four-fold, and maximum densities of PCP receptors varied four- to five-fold. Binding at a single concentration of ligand showed no orderly relationship between σ and PCP receptor binding across the species tested (figure 1). However, the fact that HAL and TCP binding were detected in all of the species tested indicates that σ and PCP binding sites are phylogenetically old. The early appearance and ubiquitous distribution of these sites suggest that they have a role in some basic neurobiological function in the animal kingdom.

We observed marked similarities in the affinities of the various inhibitors for σ receptors in guinea-pig and frog brains and PCP receptors in guinea-pig and chicken brains, suggesting conservation of the pharmacological properties of these receptors throughout vertebrate evolution. The results with σ receptors showed relatively high affinity for d-NANM, d-pentazocine, l-butaclamol, and HAL, which have been reported to be potent σ ligands. Pharmacological characteristics of PCP receptor binding in the guinea-pig brain agreed with published data on the guinea-pig and other rodent species.

Studies on the postnatal ontogeny of σ and PCP receptors yielded surprising results (figure 2) (Majewska et al. 1989). Whereas the PCP receptor showed development increase postnatally, there was no age-dependent change in the σ receptor. The lack of postnatal development of σ receptors in the central nervous system, as compared with postnatal changes in other classical neurotransmitter receptors, and the fact that σ sites are much denser in peripheral organs, such as the liver (Samovilova et al. 1989) and immune and

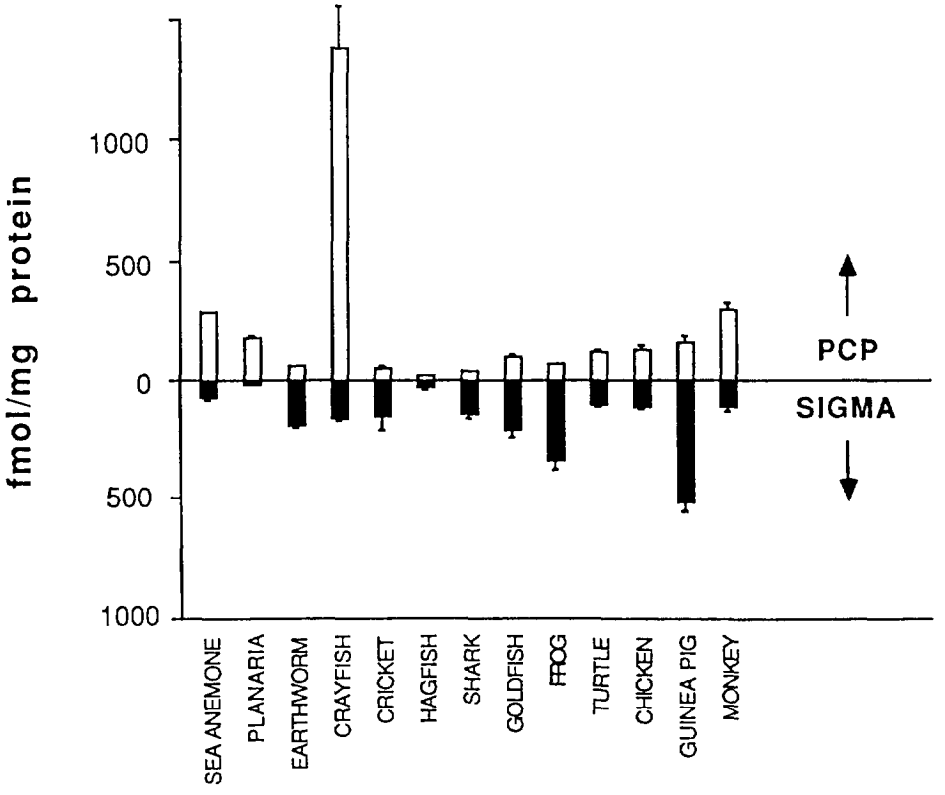

FIGURE 1. *Phylogenetic distribution of [^{3}H]HAL and [^{3}H]TCP binding sites. Assays of σ receptor binding (■) were performed at a sing/e concentration of [^{3}H]HAL (1 nM) in the presence of 75 to 100 nM spiperone. Data for sea anemone, planaria, earthworm, crayfish, cricket, and hagfish are mean + SEM (bars) values from nine samples. Data for other organisms are mean + SEM (bars) values for at least three experiments, each done at least in duplicate. PCP receptor binding (□) was assayed with 2.5 nM [^{3}H]TCP. From Vu et al. (1990).*

endocrine tissues (Wolfe et al. 1988, 1989), suggest a universal role for these sites in cellular function.

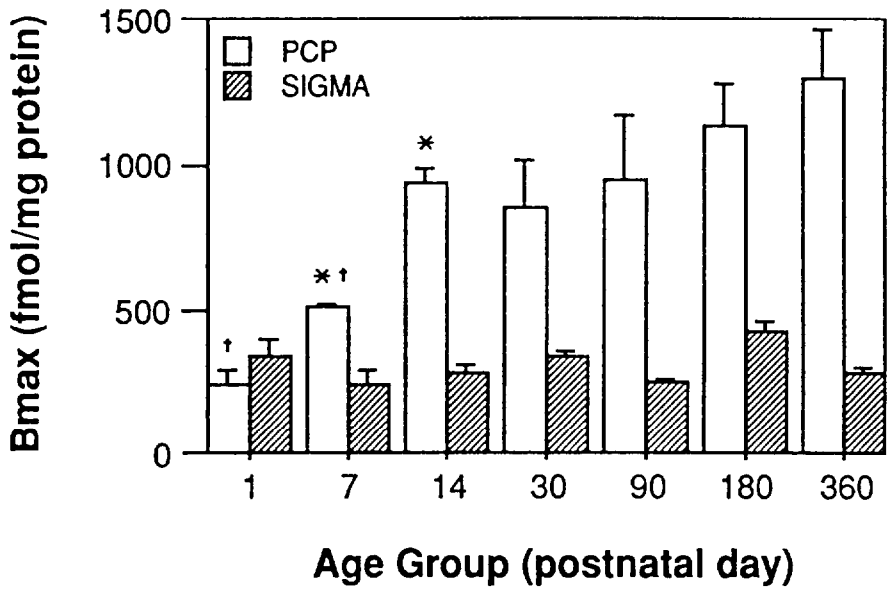

FIGURE 2. *Postnatal development of σ ¿and PCP binding. Equilibrium binding data of [^{3}H]HAL and [^{3}H]TCP were analyzed by the LIGAND programs (Munson and Rodbard 1980). Data represent mean values ± SEM from 3 to 6 individual experiments. * = statistically different from previous age group, p < 0.05 by Bonferroni t statistics. † = statistically different from 180-day-old rats; p < 0.05 by Dunnett's test. From Majewska et al. (7989).*

Despite such a possible universal role, several lines of evidence suggested that σ receptors might be part of a system involved in psychotomimetic or antipsychotic actions of drugs or endogenous substances in the brain. The evidence included the following: (1) that HAL, a clinically useful antipsychotic drug, has high affinity for σ receptors (Su 1982, Tam and Cook 1984; Largent et al. 1988); (2) that several new, structurally unrelated potential antipsychotic drug candidates have in common the ability to bind to σ receptors (Largent et al. 1988); and (3) that canine delirium, produced by NANM, appeared to be mediated through σ receptors (Vaupel 1983). We therefore studied σ receptors in postmortem material from human brain, including autopsy material from schizophrenics (Weissman et al. 1988*a,* 1988*b).*

Saturation and inhibition studies, performed in control cerebellar membranes, revealed a single population of sites, with an average K_d of 1.4 nM, a B_{max} of about 350 fmol/mg protein, and pharmacological characteristics consistent with the view that under our experimental conditions, [^{3}H]HAL bound to a receptors in the human brain. Inhibition studies (table 1) indicated that HAL was the most potent drug tested, followed by d-pentazocine, di-o-tolylguanidine, and then PPP. The d-isomers at NANM and pentazocine were more potent, as was the l-isomer of butaclamol, as compared with the corresponding stereoisomers. TCP and PCP were not potent, and apomorphine had negligible affinity.

An analysis of thirteen brain regions at a single ligand concentration showed the highest level of binding in the cerebellar cortex, accumbens nucleus, and cortical regions (see figure 3). There was little variation in the cortical distribution of σ ıreceptors. Pontine nuclei, thalamus, and spinal cord had the

TABLE 1. *Inhibition of 1 nM [^{3}H]haloperidol binding in human cerebellum*

Drug	K_i (nM)	Relative potency
HAL	1.8±1[a]	1.000
d-pentazocine	26±8	0.236
DTG	32 ± 9	0.196
d-PPP	72±8	0.084
l-butaclaol	263 ± 66	0.024
l-pentazocine	366±106	0.017
spiperone	647 ± 244[b]	0.010
d-NANM	975 ± 381	0.006
d-butaclamol	2270 ± 240	0.003
TCP	3740 ± 290	0.002
PCP	5500 ± 430	0.001
l-NANM	7570 ± 229	0.001
apomorphine	>10,000	Negligible

NOTE: K_i values are means ± SEM from single experiments performed In triplicate on three brains, using 13 to 15 concentrations of competing drug. K_i values were obtained with the LIGAND programs (Munson and Rodbard 1980).

[a] K_i was obtained using an estimate of nonspecific binding determined by adding d-NANM because high concentrations of HAL reduced estimated nonspecific binding below that obtained with other a ligands, suggesting that [^{3}H]HAL In the presence of 5.0 nM spiperone bound specifically to non-σ sites with an affinity lower than for σ sites. For other inhibitor assays, nonspecific binding was determined with an excess of unlabeled HAL.

[b] K_i Is for the lower affinity (presumed a) spiperone-sensitive site. From Weissman et al. (1988*b*).

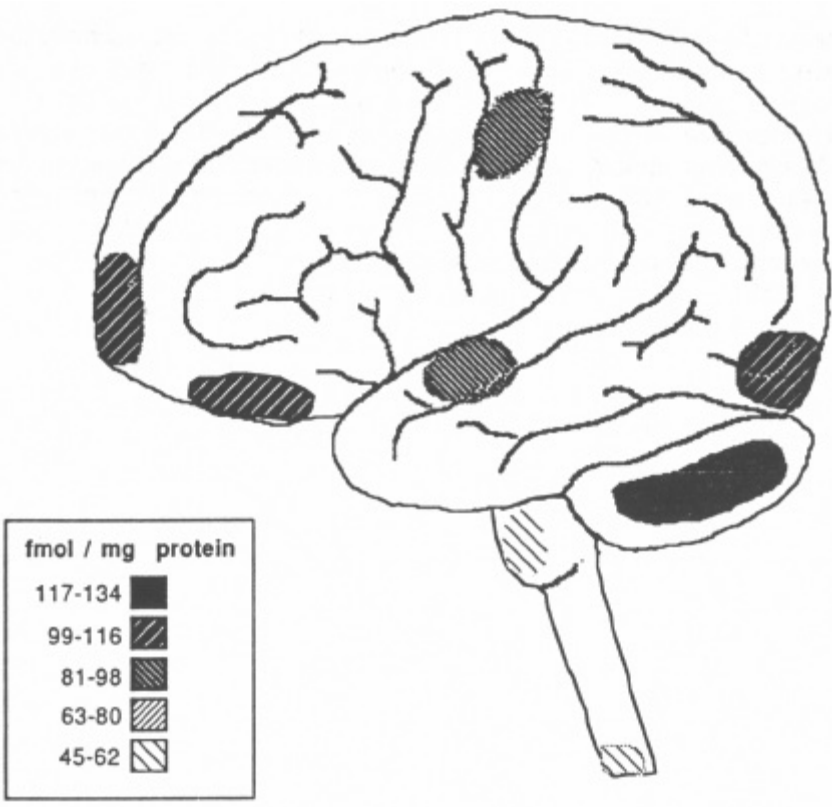

FIGURE 3. *Regional distribution of specific binding (fmoles/mg protein) of 1 nM [^{3}H]HAL to specific human brain regions in the presence of 50 nM spiperone. Receptor densities are coded as different patterns. From Weissman et al. (1988b).*

lowest levels of binding. Other areas, including limbic areas and components of the extrapyramidal motor system, had intermediate levels.

Following the characterization of σ receptors in control brain, we assayed the binding sites in postmortem brains from age-matched controls, nonschizophrenic suicide victims, and schizophrenics. We observed significantly lower densities of σ receptors in the temporal cortex in schizophrenic brains (Weissman et al. 1988b). The possible explanations of the decrement in σ receptors in the temporal cortex include an alteration in the level

of a putative endogenous ligand that may regulate the σ receptor. Alternatively, schizophrenia may involve focal lesions of σ receptor-bearing neurons.

The demonstration of a specific alteration of σ receptors in schizophrenics suggests that in vivo studies of schizophrenics using positron emission tomography (PET) might be useful in the characterization and diagnosis of this disorder. Therefore, we tested both radiolabeled HAL and d-NANM as potential ligands for in vivo labelling of σ receptors in the brain (Weissman et al. 1987*a).* We gave mice tracer quantities of [^{3}H]HAL or [^{3}H]d-NANM. In some mice which received HAL, 0.5 mg/kg spiperone was given to block binding to catecholaminergic sites. Other mice received 1 mg/kg unlabelled HAL to block binding to catecholaminergic as well as σ sites, defining nonspecific binding. Mice that were given injections of d-NANM received a pretreatment with 1 mg/kg TCP to block binding to PCP receptors, or 1 mg/kg each of HAL and TCP to block binding to σ and PCP receptors (nonspecific binding). Both radioligands showed high estimates of specific σ binding in the cerebellum, cranial nerve nuclei of the medulla-pons, and midbrain, and low levels in the hippocampus. The distribution of binding generally agreed with in vitro data (figure 4).

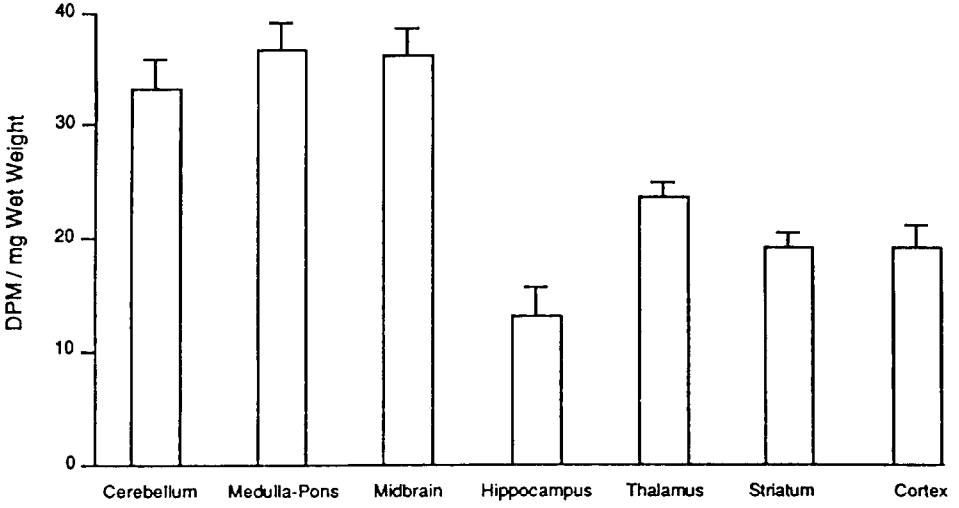

FIGURE 4. *Regional distribution in the mouse brain of in vivo σ binding of [^{3}H]HAL in the presence of spiperone. From Weissman et al. (1987a).*

In addition to receptor mapping and quantitation, metabolic mapping with deoxyglucose is a valuable approach to delineating the distribution of drug action in the brain of an intact animal (McCulloch et al. 1982; London et al. 1986; London 1989). While receptor maps provide information about the primary sites of action of drugs in the brain, deoxyglucose mapping provides information about primary as well as secondary effects of drugs in the brain of an intact animal. Radiolabeled deoxyglucose (2-deoxy-D-[1-^{14}C]glucose (DG) is used as a tracer for glucose metabolism (Sokoloff et al. 1977). Because glucose is the primary substrate of the adult brain for energy metabolism, the deoxyglucose method provides information about local brain function.

Recent interest in σ receptors has led to the introduction of several drugs that bind to σ receptors and have been proposed as candidate antipsychotic agents (Largent et al. 1988; Su et al. 1988). These drugs include BW 234U (rimcazole), BMY 14802, and HR 375. We were interested in whether the cerebral metabolic responses to these drugs relate to the distribution of σ receptors and if the drugs produce a characteristic "σ" profile, which might provide information on a mechanism for their potential antipsychotic action. Therefore, we tested the acute effects of 30 mg/kg i.p. BW 234U and BMY 14802 on local cerebral glucose utilization (LCGU) in rats by the DG method (della Puppa and London 1989).

Both BW 234U and BMY 14802 produced effects on LCGU in epithalamic, metathalamic, hypothalamic, and mesencephalic regions, as well as in cranial nerve nuclei and the cerebellum (table 2). However, BMY 14802 increased LCGU, whereas BW 234U reduced LCGU at the doses tested. It appears that most of the changes in LCGU were driven by direct interactions with σ receptors because the distribution of the effects on LCGU roughly paralleled the distribution of σ receptors visualized autoradiographically using [^{3}H]PPP as a ligand (Largent et al. 1986). In this regard, affected regions such as the paraventricular hypothalamic, medial mammillary and supramammillary nuclei; cerebellum; granular layer of the dentate gyrus; locus ceruleus; and cranial nerve nuclei contain high densities of σ receptors. However, no changes in LCGU were observed in areas, such as the molecular layer of the dentate gyrus and superficial layers of the cerebral cortex, which reportedly are devoid of σ receptors.

We also tested the LCGU effects of d-NANM (della Puppa and London 1989; Hill et al. 1989; della Puppa, Vu, Kimes, and London, unpublished findings), which might be thought of as an agonist by virtue of its activity in behavioral

TABLE 2. *Effects of σ ligands on glucose utilization in the rat*[a]

Brain regions	Vehicle control	BMY 14802 (30 mg/kg)	Vehicle control	BW 234U (30 mg/kg)
Frontal cortex layers 1-3	83 ± 5.0	95 ± 2.4	85 ± 8.3	96 ± 10.6
Layer 4	101 ± 4.9	102±3.5	104±12.1	108 ± 12.1
Anterior cingulate cortex	103±3.1	112±2.7*	111 ± 10.5	103 ± 7.7
Dentate gyrus granular layer	71 ± 4.7	60 ± 3.1*	56 ± 4.6	52 ± 4.2
Medial geniculate nucleus	123±5.0	142 ± 5.3*	113±10.8	84 ± 3.5*
Lateral habenula	127±9.8	229 ± 2.5*	113±14.1	82 ± 8.4*
Paraventricular nucleus	77 ± 3.5	104±4.1*	65 ± 9.6	81 ±12.6
Medial mammillary nucleus	122±8.3	149 ± 6.6*	107±11.4	84 ± 8.8
Supramammillary nucleus	82 ± 5.5	97 ± 4.9*	72 ± 7.8	56 ± 9.7
Inferior colliculus	188±7.2	196 ±5.2	146 ± 10.7	97 ± 6.8*
Superior colliculus	90 ± 4.6	100 ±4.2	72 ± 5.3	52 ± 4.9*
Substantia nigra reticulata	62 ± 3.2	80 ± 2.8*	54 ± 3.8	44 ± 3.8
Compacta	84 ± 3.8	105 ± 2.3*	76 ± 7.7	53 ± 5.6*
Locus ceruleus	68 ±4.8	90 ± 4.6*	60 ± 4.8	47 ± 5.7
Cochlear nucleus	100 ± 8.8	138 ± 12.2*	96 ± 6.9	94 ± 16.6
Facial nucleus	61 ± 3.7	83 ± 3.2*	50 ± 1.9	41 ±7.9
Abducens nucleus	61 ± 3.3	66 ± 4.9	49 ± 2.1	39 ± 4.4*
Lateral vestibular nucleus	82 ± 5.2	100 ± 3.4*	78 ± 4.0	56 ± 6.1*
Cerebellar vermis	69 ± 5.5	85 ± 1.9*	58 ± 5.9	49 ± 6.5

[a]LCGU values (μmol/100 g/min) are expressed as means ± SEM for *n* = 4 to 6 rats.
*Significant from control P < 0.05 according to one-way ANOVA.
From della Puppa and London (1969).

systems (Vaupel 1983; Iwamoto and Martin 1981; Brady et al. 1982; Shannon 1982). The effect of d-NANM was dose-dependent. Although 0.5 mg/kg d-NANM reduced LCGU in several brain regions, including the cerebellum and cranial nerve nuclei (which are rich in σ I receptors), higher doses were without effect in these regions. In other regions, including the accumbens nucleus, the globus pallidus, thalamic nuclei, the dentate gyrus, and the medial cortex, d-NANM increased LCGU in a dose-dependent manner. These effects of d-NANM resembled the acute effects of PCP on LCGU (Weissman et al. 19876). Therefore, it appears that low doses of d-NANM reduce LCGU by interactions with σ I receptors. The biphasic actions of d-NANM on LCGU is in

keeping with the relative affinities of the drug for σ and PCP receptors (Su 1982; Tam 1983; Largent et al. 1986).

The demonstration that cerebral metabolic effects of σ ligands are localized to brain regions that show high densities of σ receptors is an important demonstration that σ binding sites are coupled to functional activity. Nonetheless, our metabolic studies did not assign agonist or antagonist functions to the drugs tested on the basis of the direction of effects on LCGU. A similar lack of separation of possible agonist from antagonist functions was noted in the effect of various σ ligands, including HAL, to antagonize carbachol-stimulated phosphoinositide turnover in vitro (Bowen et al. 1988). In addition, whole-cell voltage-clamp studies in our laboratory demonstrated that various σ ligands caused an apparent inward current, which was due to blockade of a tonic outward K^+ current (Bell et al. 1988). Although HAL blocked the effect of d-pentazocine, consistent with an antagonist action of HAL, it was also the most potent drug in blocking the outward K^+ current. In fact, all σ ligands tested produced the same effect. Therefore, studies in several systems have failed to distinguish among agonist, reverse agonist, and antagonist properties of σ ligands.

CONCLUSIONS

Receptor binding studies indicate that σ receptors are present, with conserved pharmacologic properties, throughout vertebrate evolution. They differ from classical neurotransmitter receptors in that they show no postnatal ontogeny in the rat. σ Receptors are present in the human brain and are lost selectively from the temporal cortex of schizophrenic brains. In vivo studies suggest that σ receptors can be studied in the living human brain by PET imaging, possibly by using HAL or other ligands labeled with positron-emitting radionuclides.

Metabolic studies support the view that σ receptors have functional significance, for glucose utilization is affected by σ ligands in areas of brain that show high densities of σ receptors. Therefore, metabolic mapping with DG provides a functional index of σ-receptor activation. Both metabolic mapping studies and other approaches have not yet provided us with a means of delineating agonist or antagonist roles for various σ ligands.

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σ Receptors and Signal Transduction: Negative Modulation of Signaling Through Phosphoinositide-Linked Receptor Systems

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INTRODUCTION

σ Receptors were first proposed by Martin and colleagues (1976) as a class of opiate receptor. However, recent studies show that σ receptors are distinct from any known drug or neurotransmitter receptor (Sonders et al. 1988). Several drugs with central nervous system actions exhibit high to moderate affinity for these sites. These drugs include dextrorotary opiates such as (+)-pentazocine and dextromethorphan, antipsychotic dopamine antagonists such as haloperidol and fluphenazine, and certain phencyclidine-related compounds.

Although the functions of these sites are still largely unknown, σ receptors are likely to mediate at least some of the physiological or behavioral effects of these compounds or both. Various studies have indicated that the σ binding site is a functional entity. σ Ligands modulate electrically induced contractions of the guinea pig ileum (Campbell et al. 1989) and contractions of other smooth-muscle tissue (Campbell et al. 1987; Vaupel and Su 1987). The rate of firing of rubral neurons is slowed by selective σ ligands (Matsumoto and Walker 1988). Microinjection of σ ligands into the rat substantia nigra or red nucleus produces marked alterations in motor behavior and posture (Walker et al. 1988; Matsumoto et al. 1990). This latter finding suggests a role of σ receptors in neural regulation of movement and the possibility that these sites mediate some of the adverse motor side effects of antipyschotic drugs. An involvement of σ receptors in the motor disorder dystonia has also been suggested (Bowen et al. 1988*a;* Walker et al. 1988).

A step toward elucidating the role of σ receptors is to establish the transduction mechanisms utilized or modulated by σ compounds. Several neurotransmitters act as agonists at receptors that are coupled to phosphoinositide (PPI) turnover (Berridge and Irvine 1984; Chuang 1989; Sladeczek 1987). The binding of ligands to these receptors results in activation of phospholipase C, with the resultant cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) and production of diacylglycerol (DG) and inositol trisphosphate (IP_3). IP_3 causes release of calcium from the endoplasmic reticulum. This effect results ultimately in activation of calcium/calmodulin-dependent protein kinase. DG activates protein kinase C. By activation of these kinases, a myriad of cellular responses can be produced. Two transmitters known to signal through this pathway are acetylcholine, via M_1- (and possibly M_3) muscarinic cholinergic receptors, and norepinephrine, via α_1-adrenergic receptors (Chuang 1989). We have previously reported that σ receptors attenuated PPI turnover stimulated by the cholinergic agonist carbachol (Bowen et al. 1988*b).* Here we further investigate the relationship between σ receptors and PPI-linked receptor system.

MATERIALS AND METHODS

PPI Turnover

Synaptone urosomes were prepared and PPI turnover assayed as described previously (Bowen et al. 1988*b),* using the same procedure originally described by Gusovksy and Daly (1988) with minor modifications. Brains (minus cerebellum) of 150 to 200 male Sprague-Dawley rats were homogenized in 10 volumes of oxygenated (95 percent O_2/5 percent CO_2) Krebs-Henseleit buffer, pH 7.4 (118.5 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.18 mM KH_2PO_4; 24.9 mM $NaHCO_3$, 10.0 mM glucose) using five hand-driven strokes in a Kontes Duall-24 glass-glass homogenizer. The homogenate was centrifuged at 1,000 x g for 10 minutes and the pellet resuspended in 20 volumes Krebs-Henseleit supplemented with 50 mM Hepes, pH 7.4.

To a volume of the suspension equivalent to 320 µL/assay tube was added [^{3}H]myo-inositol (23 Ci/mmol) to a final concentration of 0.8 to 1.0 µM (about 5 µCi per assay tube). Aliquots of 320 µL were removed and incubated for 60 minutes at 37°C to label the inositol lipid pool. Twenty microliters of 200 mM LiCl were then added (10 mM final concentration) and incubation was allowed to proceed for 10 more minutes before drugs were added. Test compound and cholinergic or adrenergic agonist were then added. Test compound was always added just before PPI agonist was added. Labeled synaptoneurosomes and

drugs were then incubated for 90 minutes in a final volume of 400 µL. All incubations were carried out in capped 14.5-mm by 84-mm polypropylene tubes under an atmosphere Of 95 percent O_2/5 percent CO_2.

After transferral to microfuge tubes and centrifugation for 5 minutes, the synaptoneurosome pellet was washed once with 1 mL fresh buffer and then extracted by resuspension in 1 mL 6 percent trichloroacetic acid. Lysed synaptoneurosomes were removed by centrifugations, and the extract was mixed with 1 mL of a slurry of BioRad anion exchange resin AG 1-X8, 100 to 200 mesh, formate form (1:1 , w:v resin:water). After incubation for 5 minutes at 25°C the slurry was placed into small polypropylene columns and the resin washed four times with 1 mL of water to remove excess [^{3}H]myo-inositol. [^{3}H]Inositol-1-phosphate ([^{3}H]IP_1) was then eluted directly into scintillation vials by washing the column twice with 1 mL of 200-mM ammonium formate/100 mM formic acid. After addition of 2 mL of Hydrofluor (National Diagnostics, Manville, N.J.) and vigorous mixing, vials were counted 2 to 4 hours later to avoid chemiluminescence. Previous studies have shown that measurement of [^{3}H]IP_1 gives sufficient indication of agonist-stimulated PPI turnover, for [^{3}H]inositol trisphosphate is rapidly degraded to [^{3}H]IP_1 under the conditions of this assay.

Receptor Binding

Muscarinic receptor binding was performed using either the lysed, frozen, crude P_2 membrane fraction ($LFCP_2$) or synaptoneurosome fraction of male Sprague-Dawley rat brain (minus cerebellum). $LFCP_2$ was prepared as described previously (Bowen et al. *19896)*. Synaptoneurosomes were prepared as described above. [^{3}H]Oxotremorine-M ([^{3}H]Oxo-M, 87 Ci/mmol, 5 nM) was incubated with about 500 µg membrane protein in 0.5 mL of Krebs-Henseleit/ Hepes, pH 7.4 for 60 minutes at 37°C. Nonspecific binding was determined in the presence of 10 µM unlabeled oxotremorine-M. Assays were terminated by dilution with 5 mL ice-cold phosphate-buffered saline (PBS), pH 7.4, and vacuum filtration through glass-fiber filters. Filters were then washed twice with ice-cold PBS.

σ Receptors were labeled using 3 nM [^{3}H] (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ([^{3}H] (+)-3-PPP, 98.9 Ci/mmol) and the crude P_2 fraction ($LFCP_2$) of guinea pig brain. Incubations were carried out for 120 minutes in 0.5 mL 50 mM Tris-HCl, pH 8.0, at 25°C using 500 µg membrane protein. Nonspecific binding was determined in the presence of 1 µM haloperidol. Assays were terminated by dilution with 5 mL ice-cold 10 mM Tris, pH 8.0, and

vacuum filtration through glass-fiber filters. Filters were then washed twice with 5 mL buffer. Filters were soaked in 0.5 percent polyethyleneimine for at least 30 minutes at 25°C prior to use. Some σ assays were carried out, as specified, with 5 nM [^{3}H]1,3-di-stolylguanidine ([^{3}H]DTG, 52.3 Ci/mmol) using rat brain $LFCP_2$ and Krebs-Henseleit/Hepes buffer. Protein concentration was determined by the method of Lowry et al. (1951).

Materials

All scintillation counting was performed with a Packard model 4450 scintillation spectrometer. [^{3}H]Myo-inositol was obtained from American Radiochemicals, Inc. (St. Louis, MO.). [^{3}H] (+)3-PPP, [^{3}H]DTG, and [^{3}H]Oxo-M were obtained from Dupont/New England Nuclear (Boston, Mass.). Carbachol, norepinephrine, haloperidol, polyethyleneimine, Tris, and Hepes were obtained from Sigma Chemicals (St. Louis, MO.). (+)-3-PPP and oxotremorine-M (Oxo-M) were obtained from Research Biochemicals, Inc. (Natick, Mass.). DTG was from Aldrich Chemical Co. (Milwaukee, Wis.). Levallorphan was obtained from the National Institute on Drug Abuse (NIDA) (Rockville, Md.). (+)-Benzomorphans and (+)-morphinans were synthesized in the laboratory of Dr. Kenner C. Rice. BD614 and BD737 are, respectively, (±)-*cis*- and 1*S,* 2*R*(-)-cis-*N*-[2-(3,4-dichlorophenyl)ethyl]- *N*-methyl-2-(l-pyrrolidinyl)cyclohexylamine. Synthesis and characterization of these highly selective σ ligands is described elsewhere (Rice et al., in press). The following abbreviations are used for σ compounds: DEX (dextrallorphan), DTG (1,3-di-*o+tolylguanidine),* FLU (fluphenazine), HAL (haloperidol), KCR 12-83.1 ((+)-*N*-cyclopropylmethyl-nordihydrocodeinone), LEV (levallorphan), MDEX (3-methoxydextrallorphan), (+)-PENT ((+)-pentazocine), (+)-3-PPP ((+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine), and Red. HAL (reduced haloperidol).

RESULTS

Characterization of Effect and Determination of σ Specificity

The muscarinic cholinergic agonists carbachol and oxotremorine-M (Oxo-M) and the α_1-adrenergic agonist norepinephrine stimulated the basal rate of PPI turnover in rat brain synaptoneurosomes in which the PPI pool had been radiolabeled by preincubation with [^{3}H]myo-inositol. At a concentration of 100 μM, carbachol and norepinephrine produced approximately a doubling of the basal activity. Oxo-M at 10 μM produced 150 percent to 170 percent stimulation of basal activity. The carbachol stimulation was completely blocked

by the competitive cholinergic antagonist atropine (100 µM). The norepinephrine stimulation was completely blocked by the adrenergic antagonist phenoxybenzamine (30 µM). These results are similar to those observed with brain slices and confirm that synaptoneurosomes are a valid system for the study of PPI turnover.

The effect of σ ligands on basal PPI metabolism is investigated in figure 1. HAL, DTG, (+)-PENT, and (+)-3-PPP had a negligible effect on PPI turnover at concentrations up to 100 *µM.* Above 100 µM, DTG and (+)-PENT produced a slight inhibition of basal activity and reached only 20 percent inhibition at 1,000 µM. HAL, however, produced marked inhibition of basal activity at concentrations above 100 µM. (+)-3-PPP produced no significant effect on

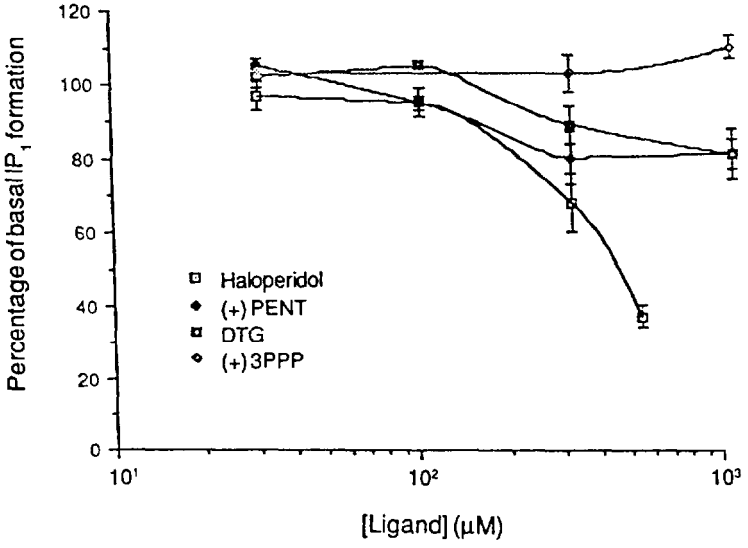

FIGURE 1. *Effect of σ ligands on phosphoinositide turnover. Various concentrations of the indicated ligands were incubated with prelabeled rat brain synaptoneurosomes and production of [^{3}H]IP$_1$ measured as described in the methods section. Values are expressed as percentage of basal activity and are the mean of two to four experiments ± SEM. Each experiment was performed in duplicate.*

basal activity at concentrations up to 1,000 μM. (+)-Morphine, which lacks affinity for σ I receptors, was without effect on basal activity (not shown).

Figure 2 shows the effect of four representative σ ligands on the ability of two cholinergic agonists, carbachol (panel A) and Oxo-M (panel B), to stimulate PPI metabolism in rat brain synaptoneurosomes. (+)-PENT, HAL, DTG, and dextrallorphan all produced concentration-dependent attenuation of the PPI response. Dose curves were linear with least-squares-derived coefficients of greater than 0.95. ED_{50} values were calculated from the least squares fits and are shown in table 1 along with those of other σ ligands. The compounds are from five different chemical classes, and all produced linear dose curves with least squares coefficients of greater than 0.95. It is noteworthy that inhibition of both carbachol- and Oxo-M-stimulated PPI turnover occurred at concentrations of σ ligand below those required to produce significant effects on basal activity.

TABLE 1. *Comparison of σ binding affinity and potency at inhibition of cholinergic phosphoinositide turnover*

Ligand	σ K_i (nM) $[^3H]$ (+)-3-PPP	ED_{50} (μM) Oxo-M	Carbachol
BD 737	1.3± 0.3	3.1 ± 0.2	ND
BD 614	2.0± 0.4	4.7 ± 0.8	ND
(+)-Pentazocine	3.1 ± 0.3	7.2 ± 0.9	8.3
Haloperidol	3.8 ± 0.6	9.6 ± 0.8	35.4
Reduced haloperidol	5.1 ± 2.3	15.6 ± 0.6	ND
Dextrallorphan	16.1 ± 1.8	22.5 ± 1.0	27.0
DTG	20.5 ± 3.6	12.0 ± 2	25.0
Fluphenazine	26.3 ± 0.6	36.0 ± 1	ND
Methoxy-dextrallorphan	32.1 ± 8.9	15.4 ± 0.3	11.9
KCR 12-83.1	138.4 ± 15	147.0 ± 24	81.5
Levallorphan	7,540	ND	155.0

NOTE: Binding affinities of σ ligands were determined using the $LFCP_2$ fraction of guinea pig brain by competition of 0.05 to 10,000 nM test ligand with 3 nM $[^3H]$(+)-3-PPP. Assay conditions were as described in the methods section. IC_{50} values were determined using the iterative curve-fitting program CDATA (EMF Software, Baltimore, Md.), and K_i values were calculated using the Cheng-Prusoff equation and a K_D of 27.4 nM (Cheng and Prusoff 1973). ED_{50} values in the PPI assays were determined from linear least squares fits of the dose curves for each compound. Values for Oxo-M (10 μM) were determined from the individual least squares fits of data from two to four experiments and are expressed as averages, μ SEM. Values for carbachol (100 μM) were determined from a least squares fit of the averaged data from two or three experiments that varied by less than 10 percent. All experiments were carried out in duplicate. ND = not determined.

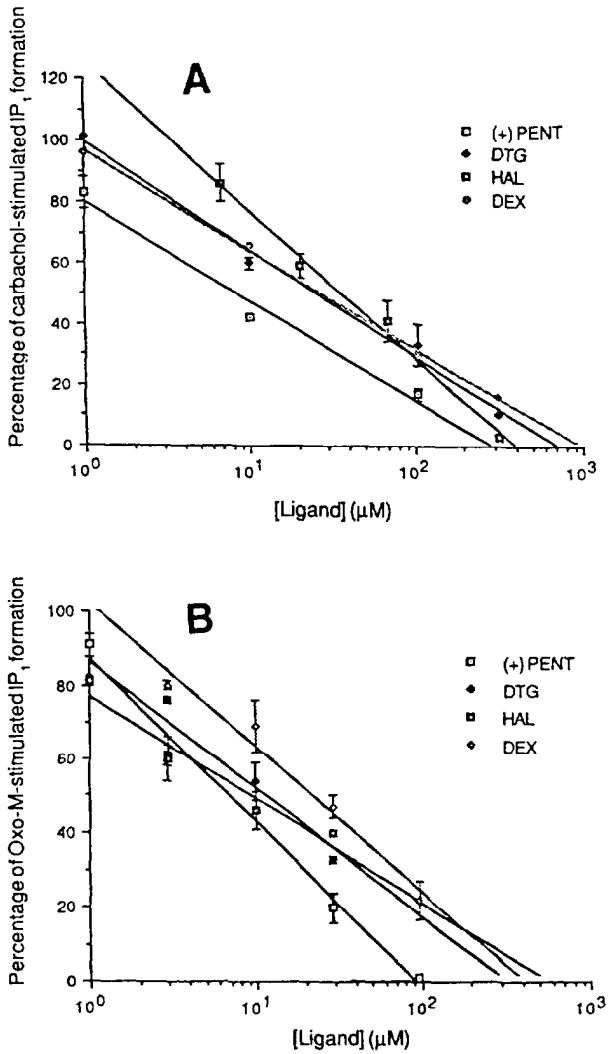

FIGURE 2. *Effect of representative σ ligands on the cholinergic phosphoinositide response. Various concentrations of σ ligands were incubated for 90 minutes with prelabeled synaptoneurosomes in the presence of 100 µM carbachol (panel A) or 10 µM Oxo-M (panel B). Values are expressed as the percentage of the stimulation produced by cholinergic agonist in the absence of a ligand and are the averages of two to four experiments ± SEM. Each experiment was carried out in duplicate.*

Binding affinities of various ligands to σ sites of guinea pig brain, determined by competition with [^{3}H](+)-3-PPP, are shown in table 1. Figure 3 shows the correlation between σ binding affinities (K_i) and ED_{50} values at blocking carbachol-stimulated (panel A) or Oxo-M-stimulated (panel B) PPI turnover in rat brain synaptoneurosomes. Although the ED_{50} values for PPI inhibition fall in the micromolar concentration range, whereas σ K_i values are in the nanomolar range, the rank order of the compounds in the two assays is very similar. The correlation coefficient for carbachol (7 compounds) was 0.81, while that for Oxo-M (10 compounds) was 0.92. The high correlation between rank order of affinities at σ sites and ED_{50}, rank order suggests that attenuation of the cholinergic PPI response is mediated by σ receptors.

Of particular note is the stereoselectivity between dextrallorphan and levallorphan. Also, several (+)-opiates are structurally related to (+)-PENT and dextrallorphan but lack affinity for σ receptors, exhibiting IC_{50} values versus [^{3}H](+)-3-PPP of greater than 10,000 nM in guinea pig brain homogenates. Compounds in this class are (+)-morphine, (+)-oxymorphone, (+)-naltrexone, (+)-dihydrocodeinone, and (+)-nordihydrocodeinone (unpublished observations). These compounds lacked ability to attenuate carbachol-stimulated PPI turnover at concentrations up to 300 μM (Bowen et al. 1988*a).* Sulpiride, like HAL, is an antipsychotic dopamine-D_2 receptor antagonist. However, unlike HAL, sulpiride lacks affinity for σ receptors and failed to produce effects on carbachol-stimulated PPI metabolism at concentrations up to 300 μM (Bowen et al. 1988a).

The only σ compound tested that gave anomalous results was (+)-3-PPP. As shown in figure 4, this compound produced an effect that was markedly different from that of the other σ ligands. (+)-3-PPP produced concentration-dependent attenuation of the carbachol effect between 10 and 100 μM. However, at concentrations above 100 μM, no additional inhibition was observed. A plateau was reached at about a 40 percent inhibition of the carbachol stimulation. Little additional inhibition was observed at concentrations as high as 1,000 μM. Other σ ligands, even those with lower binding affinity, were able to produce complete inhibition of carbachol-stimulated PPI turnover. The inability to produce a maximal effect suggests that (+)-3-PPP may have partial agonist or antagonist properties in this system.

Figure 5 shows the effects of DTG and (+)-pentazocine, two highly selective σ ligands (Weber et al. 1986; de Costa et al. 1989), on the ability of norepinephrine to stimulate PPI hydrolysis. Like observations with cholinergic

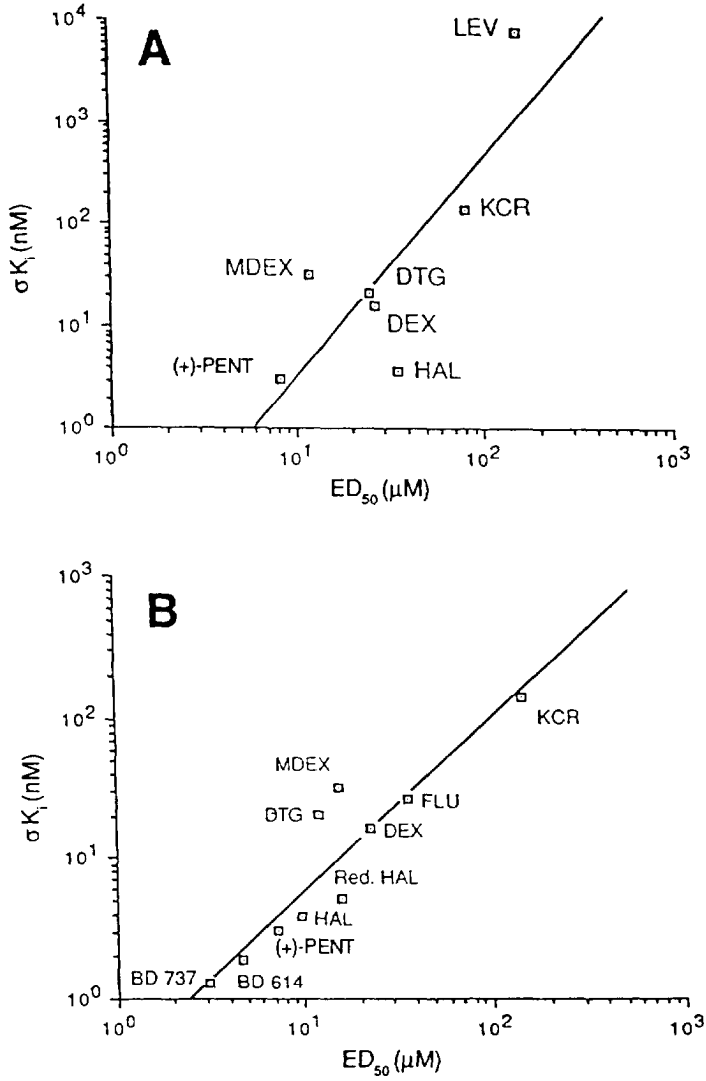

FIGURE 3. *Correlation of σ binding affinity to potency at inhibiting the cholinergic PPI response. Data from table 7 were used to construct correlation plots for σ binding K_i vs. inhibitory ED_{50} against carbachol- (panel A) or Oxo-M- (panel B) stimulated PPI turnover. Correlation coefficients were r = 0.81 for carbachol and r = 0.92 for Oxo-M.*

agonists, the ability of norepinephrine to stimulate PPI metabolism was attenuated in a concentration-dependent manner by these σ ligands. Consistent with σ I binding affinity, (+) PENT was more potent than DTG, with ED_{50} values of 129 μM and 354 μM, respectively. However, compared with cholinergic stimulation, adrenergic stimulation was less sensitive to inhibition by σ ligands. The ED_{50} values for (+)-PENT and DTG were about 15 times higher for norepinephrine stimulation than for carbachol stimulation. ED_{50} values for (+)-PENT and DTG were, respectively, 18 and 30 times higher for norepinephrine than for Oxo-M. (+)-Morphine and (+)-naltrexone, (+)-opiates that lack affinity for σ receptors, failed to inhibit norepinephtine-stimulated PPI turnover at concentrations up to 1,000 μM (not shown). These results suggest that this effect on the adrenergic phosphositide response is also mediated by σ receptors.

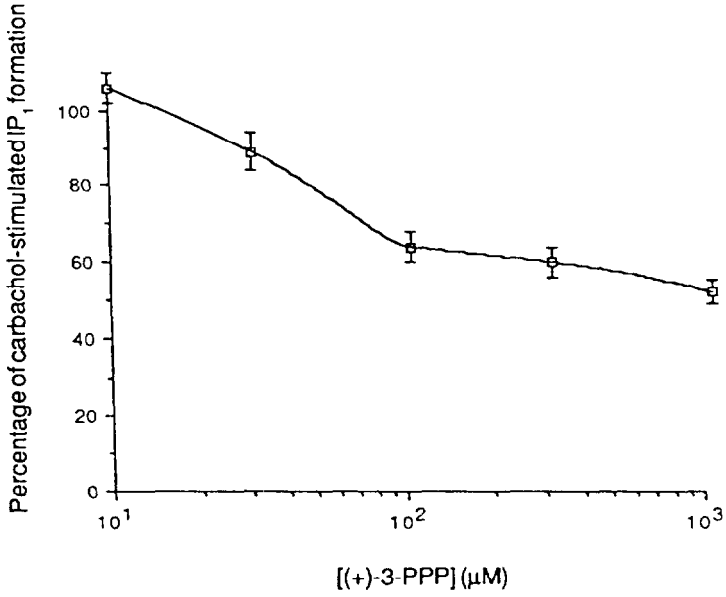

FIGURE 4. *Dose curve for (+)-3-PPP inhibition of carbachol-stimulated PPI metabolism. The indicated concentrations of (+)-3-PPP were incubated with prelabeled synaptoneurosomes and 100 μM carbachol. Values are expressed as the percentage of stimulation observed with carbachol alone. Results are the average of two experiments, ± SEM, each carried out in duplicate.*

We investigated whether the inhibition by σ ligands of agonist-stimulated PPI metabolism was competitive or noncompetitive by determining the effect of a fixed concentration of σ ligand on the dose response of Oxo-M or norepinephrine. These results are shown in figure 6. In panel A, the concentration of Oxo-M was varied up to 1,000 μM in the presence and absence of fixed concentrations of (+)-PENT. Oxo-M alone produced dose-dependent stimulation of [^{3}H]IP$_1$ production; stimulation was greatest at about 315 percent above basal. At 10 μM, (+)-PENT had little effect on the concentration of Oxo-M required to produce half-maximal stimulation of PPI turnover but reduced the maximal stimulation, even at large excess of agonist. A significant rightward shift was observed with 100 μM (+)-PENT. However, the largest effect was a decrease in maximal activity.

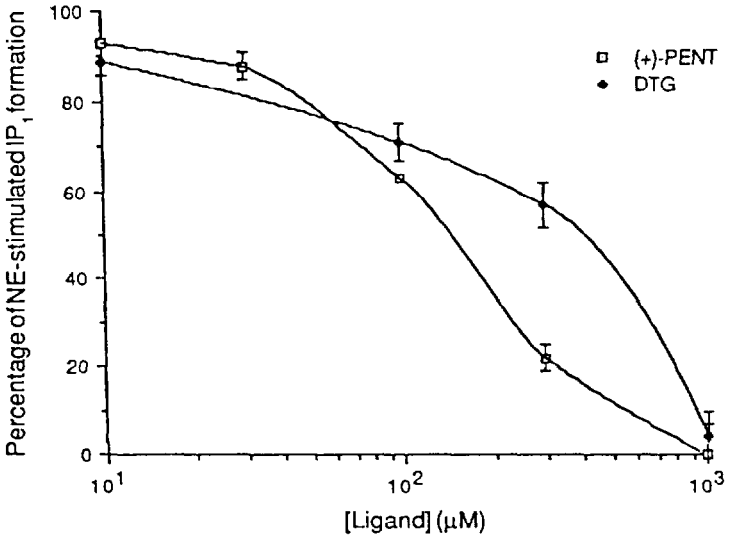

FIGURE 5. *Effect of σ ligands on norepinephrine-stimulated PPI turnover. Various concentrations of (+)-PENT or DTG were added to prelabeled synaptoneurosomes prior to addition of 100 μM norepinephrine as described in the methods section. Values are expressed as a percentage of the stimulation of [^{3}H]IP$_1$ production produced by norepinephrine alone. Results are the mean of two to three experiments ± SEM. Each experiment was carried out in duplicate.*

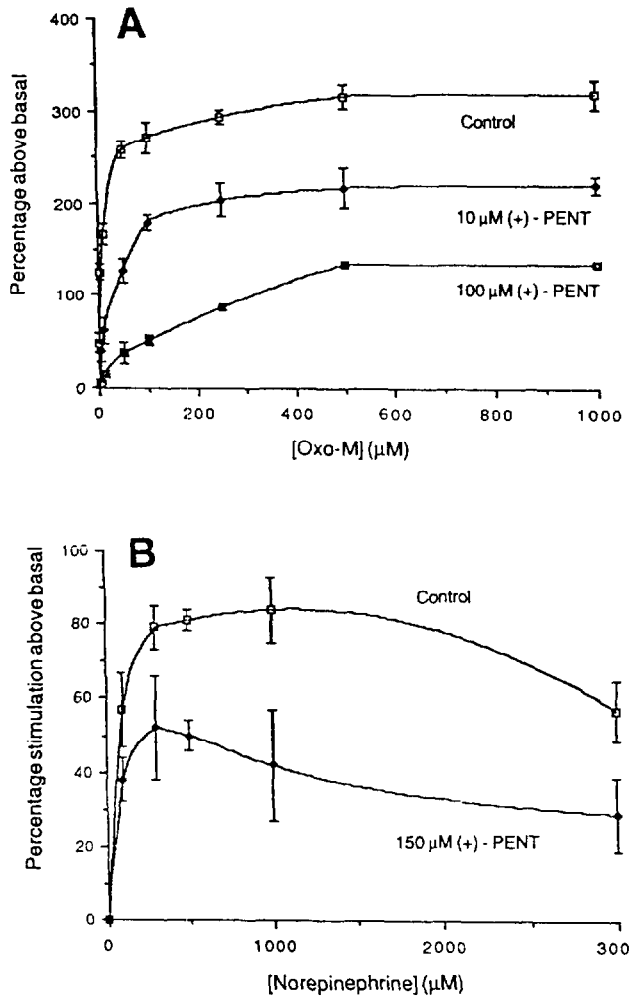

FIGURE 6. *Dose curves for oxotremorine-M- and norepinephrine-stimulated PPI turnover in the absence and presence of (+)-PENT Various concentrations of Oxo-M (panel A) or norepinephrine (panel B) were incubated with prelabeled synaptoneurosomes in the absence and presence of the indicated concentration of (+)-PENT. Values are expressed as the percentage of stimulation of [^{3}H]IP_1 production above basal activity and are the averages of two to four experiments ± SEM. Each experiment was carried out in duplicate.*

Panel B shows results obtained with norepinephrine-stimulated PPI turnover and (+)-PENT. With norepinephrine alone, maximal stimulation was 84 percent above basal [^{3}H]IP$_1$ production, with half-maximal stimulation occurring at approximately 60 μM norepinephrine. At high norepinephrine concentrations, the amount of stimulation decreased, producing a bell-shaped curve. The presence of 150 μM (+)-PENT had no effect on the concentration of norepinephrine required to produce half-maximal stimulation but reduced the maximal stimulation to 52 percent above basal. The bell-shaped nature of the dose curve was still evident in the presence of (+)-PENT, and the inhibition produced by 150 μM (+)-PENT was not overcome by up to 3,000 μM norepinephrine. Thus, σ ligands appear to possess the ability to noncompetitively inhibit the PPI response produced by muscarinic cholinergic and α_1-adrenergic agonists, reducing the maximal effect while not affecting the ED$_{50}$ of the agonists. That σ ligands produce noncompetitive inhibition of cholinergic and adrenergic PPI turnover is consistent with mediation of the effect through specific interaction with σ receptors.

Investigation of Mechanism

Because of the differential sensitivity of cholinergic and adrenergic PPI turnover to σ inhibition and lack of effect at others points in the inositol lipid pathway (Bowen et al. 1989*b),* we investigated the possible involvement of cholinergic receptors in the mechanism of the response.

Using lysed, frozen crude P$_2$ fraction of rat brain (LFCP$_2$), we investigated the ability of various σ ligands to compete for muscarinic binding sites labeled by [^{3}H]Oxo-M. These results are shown in table 2. All of the active σ ligands were found to exhibit weak affinity for muscarinic receptors as measured by inhibition of [^{3}H]Oxo-M binding. These affinities were greater than an order of magnitude lower than affinities at σ receptors (table 1). Surprisingly, as shown in figure 7, the potency to inhibit Oxo-M-stimulated PPI turnover correlated very well with ability to inhibit [^{3}H]Oxo-M binding (r = 0.92). This correlation was equal to the correlation of rank order of potency with rank order in σ receptor affinity (figure 3, panel B). These correlations suggested the possibility that the effect could be mediated by either σ receptor activation or by a direct antagonist action of σ ligands at the cholinergic receptor. Therefore, a series of experiments was carried out to eliminate one or the other receptor as the mediator of the effect.

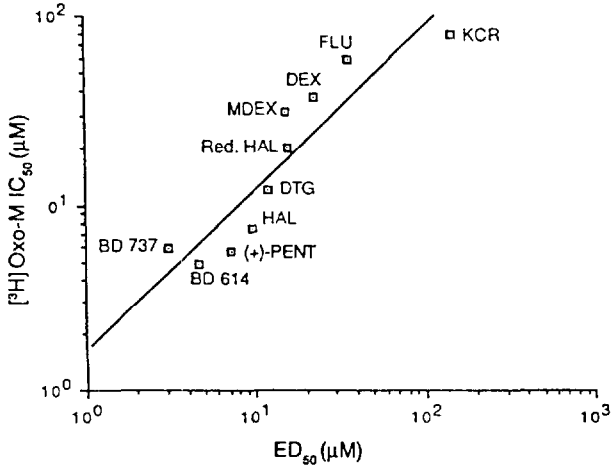

FIGURE 7. *Correlation of muscarinic binding affinity to potency a? inhibiting oxotremorine-M-stimulated phosphoinositide turnover. The ability of various σ ligands to displace [^{3}H]Oxo-M was correlated with potency at inhibiting Oxo-M-stimulated PPI turnover (table 7). The correlation coefficient was r = 0.92.*

The first experiments were aimed at determining whether the σ effect would be evident under conditions where there is no occupation of muscarinic receptors by σ ligand. The rationale is as follows. If synaptoneurosomes were pretreated with (+)-PENT and then free σ ligands were removed, the σ receptor should remain occupied, but any (+)-PENT bound to muscarinic receptors should rapidly dissociate because of its low binding affinity. Upon subsequent challenge with Oxo-M, stimulation would be inhibited if the effect is via σ sites but not if (+)-PENT acts as a direct antagonist at cholinergic sites.

Intact synaptoneurosomes were labeled with [^{3}H]myo-inositol and then pretreated for 30 minutes with 50 μM (+)-PENT under the normal assay conditions. The preparation was then centrifuged and the synaptoneurosome pellet resuspended in the same medium except without (+)-PENT. The preparation was then challenged with 10 μM Oxo-M to assess ability to stimulate PPI turnover. Table 3 shows that, compared with synaptoneurosomes pretreated with no ligand, the PPI response was inhibited in the (+)-PENT pretreated samples. Stimulation was inhibited by 46 percent and 55 percent during 15 and 90 minutes of incubation with Oxo-M, respectively.

TABLE 2. *Affinity of various σ ligands for muscarinic receptors*

Ligand	Muscarinic IC_{50} (μM) [^{3}H]Oxo-M
BD 737	5.9 ± 0.3
BD 614	4.8 ± 0.1
(+)-Pentazocine	5.6 ± 0.4
Haloperidol	7.5 ± 0.9
Reduced haloperidol	20 ± 2
Dextrallorphan	37 ± 2
DTG	12 ± 2
Fluphenazine	58 ± 5
Methoxy-dextrallorphan	31 ± 6
KCR 12-83.1	78 ± 14

NOTE: Concentrations of test iigand ranging from 0.1 to 500 μM were incubated with 5 nM [^{3}H]Oxo-M and $LFCP_2$ from rat brain as described in the methods section. IC_{50} values were determined as in the caption to table 1. Values are averages of two to four experiments ± SEM. Each experiment was carried out in duplicate.

TABLE 3. *Effect of (+)-pentazocine pretreatment on stimulation of PPI turnover by oxotremorine-M*

	Percent above basal activity with 10 μM Oxo-M	
Incubation time	Control	50 μM (+)-PENT pretreated
15 minutes	69 ± 6	37 ± 1
90 minutes	188 ± 8	83 ± 10

NOTE: Intact synaptoneurosomes were labeled with [^{3}H]myo-inositol and then pretreated for 30 minutes with 50 μM (+)-pentazocine under the normal assay conditions described in the methods section. The preparation was then centrifuged and the synaptoneurosome pellet resuspended in the same medium, except without (+)-PENT. The preparation was then challenged for 15 or 90 minutes with 10 μM Oxo-M to assess ability to stimulate PPI turnover. Values are expressed as percentage increases above basal activity. Results are averages of 2 experiments, ± SEM. Each experiment was carried out in duplicate.

In order to confirm that (+)-PENT did not occupy muscarinic receptors under the conditions of this experiment, rat brain $LFCP_2$ was again employed. $LFCP_2$ was incubated with 50 μM (+)-PENT. After centrifugation and resuspension in fresh

buffer, the degree of occupation of both muscarinic (5 nM [^{3}H]Oxo-M) and σ (5 nM [^{3}H]DTG) was determined. Results are shown in table 4. Compared with controls pretreated with no σ ligand, only 41 percent of the control [^{3}H]DTG binding remained after pretreatment, whereas all of the control [^{3}H]Oxo-M binding was recovered. Taken with the results described above, these data demonstrate that (+)-PENT can inhibit Oxo-M-stimulated PPI turnover without occupying the muscarinic receptor. The σ effect is therefore mediated solely by occupation of σ receptors.

TABLE 4. *Effect of (+)-pentazocine pretreatment on occupation of muscarinic and σ receptors*

	[^{3}H] Ligand bound (fmol/mg protein)	
Pretreatment condition	Muscarinic 5 nM [^{3}H]Oxo-M	5 nM [^{3}H]DTG
Control	47.1 ±8.5	*58.9 ± 6.8*
50 μM (+)-PENT	45.6 ± 5.6	23.9 ± 1.5
	(96.8 ± 11.9 percent)	(40.6 ± 2.5 percent)

NOTE: $LFCP_2$ of rat brain was incubated with 50 μM (+)-PENT for 30 minutes at 37°C in Krebs-Henselet/Hepes buffer. After centrifugation and resuspension In fresh buffer without (+)-PENT, the degree of occupation of both muscarinic and σ receptors was determined. Muscarinic and σ receptors were labeled with 5 nM [^{3}H]Oxo-M and [^{3}H]DTG, respectively, using Krebs-Henseleit/Hepes buffer as described In the methods section. Values for bound ligand (fmol/mg protein) are the averages of three experiments, ± SEM. Each experiment was carried out in triplicate. Numbers in parentheses are the percentages of recovery of control binding for the respective [^{3}H] ligands.

Other data also serve to rule out direct binding to muscarinic receptors as the mechanism for σ action. Scatchard analysis of [^{3}H]Oxo-M binding to synaptoneurosomes revealed labeling of an apparent single site with K_D = 39 nM (see figure 8). The apparent K_i of (+)-PENT at this muscarinic binding site is 5.6 μM (table 2). In the normal protocol for measuring the effect of σ ligands on muscarinic PPI turnover, Oxo-M and (+)-PENT are present simultaneously. Oxo-M was routinely used at a concentration of 10 μM (250 times higher than its observed K_D), and (+)-PENT was effective at concentrations approximating its muscarinic K_i (ED_{50} = 7.2 μM). Taking into account the concentrations of (+)-PENT and Oxo-M present, their respective affinities at the muscarinic binding site, and the expected levels of receptor saturation under these conditions, it is highly unlikely that there would be significant occupation of muscarinic receptors by effective levels of (+)-PENT.

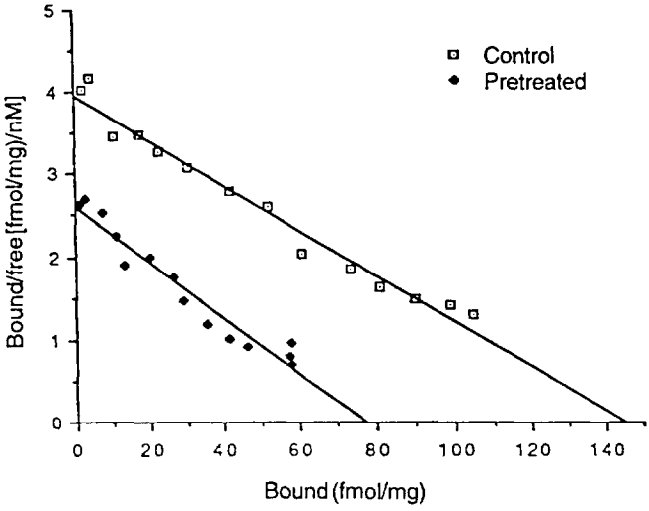

FIGURE 8. *Effect of (+)-PENT pretreatment on muscarinic receptors of synaptoneurosomes. Intact synaptoneurosomes were pretreated with 50 µM (+)-PENT under the identical conditions of the PPI inhibition assay except that [^{3}H]myo-inositol was replaced by unlabeled myo-inositol. The tissue was then centrifuged and the synaptoneurosome pellet was resuspended in fresh medium containing no (+)-PENT Scatchard analysis of [^{3}H]Oxo-M binding was performed using increasing concentrations of radioligand and the conditions described in the methods section. Results were compared to synaptoneurosomes pretreated with no σ ligand. Data were analyzed using the iterative curve fitting program BDATA (EMF Software, Baltimore, Md.). Values are the averages of five experiments, ± SEM. Each experiment was carried out in duplicate. Control: K_D = 39 ± 4 nM, B_{max} = 151 ± 10 fmol/mg protein; (+)-PENT-pretreated: K_D = 32± 6 nM, B_{max} = 78 ± 8 fmol/mg protein.*

Oxo-M should effectively compete away (+)-PENT and preferentially occupy the receptor. This supposition is even more true in view of the inability of even 1,000 µM Oxo-M to overcome the inhibition by 10 µM (+)-PENT (figure 6). Also, when Oxo-M was allowed to prebind to the muscarinic receptor before addition of (+)-PENT, inhibition of stimulation was still observed (not shown). Taken

together, these results strongly suggest that σ ligands exert their effects through occupation of σ receptors and not by direct interaction with the muscarinic receptor.

To further investigate the possible involvement of muscarinic receptors in the mechanism of the σ effect, we again used a pretreatment protocol coupled with Scatchard analysis. Intact synaptoneurosomes were pretreated with 50 μM (+)-PENT under the identical conditions of the usual assay except that [^{3}H]myo-inositol was replaced by unlabeled myo-inositol. The tissue was centrifuged and the synaptoneurosome pellet was resuspended in fresh medium containing no (+)-PENT. Scatchard analysis of [^{3}H]Oxo-M binding was then performed. Results were compared with synaptoneurosomes pretreated with no σ ligand and are shown in figure 8. In untreated synaptoneurosomes [^{3}H]Oxo-M bound to an apparent single class of sites with K_D = 39 ± 4 nM and B_{max} = 151 ± 10 fmol/mg protein. Pretreatment of synaptoneurosomes with 50 μM (+)-PENT resulted in a 50 percent reduction in the B_{max} of [^{3}H]Oxo-M with no effect on the K_D.

This effect cannot be due to the presence of residual (+)-PENT occupying the muscarinic receptor, because the experiments described above with $LFCP_2$ show that there is negligible muscarinic binding of (+)-PENT after pretreatment under similar conditions. Furthermore, Scatchard analysis of [^{3}H]Oxo-M binding to $LFCP_2$ in the simultaneous presence of 5.6 μM (+)-PENT showed that (+)-PENT was a *competitive* inhibitor, increasing K_D and having no effect on the number of sites (not shown). Unlike the direct effect of (+)-PENT on [^{3}H]Oxo-M binding to $LFCP_2$, the effect observed upon σ pretreatment of synaptoneurosomes is solely a B_{max} effect.

DISCUSSION AND SUMMARY

Functional roles of σ receptors are now beginning to be demonstrated (Campbell et al. 1987; Vaupel and Su 1987; Bowen et al. 1988*a,* 1988*b;* Matsumoto and Walker 1988; Walker et al. 1988; Campbell et al. 1989; Matsumoto et al. 1990). The finding that σ sites attenuate the cholinergic and adrenergic PPI response may suggest this as an underlying biochemical mechanism by which σ ligands produce their effects.

σ Ligands from various structural classes inhibited the stimulation of PPI turnover by cholinergic agonists in our study. Despite disparity between the actual values for σ binding affinity and ED_{50} for PPI inhibition, there was excellent correlation in the rank order of potencies of compounds in the two

assays. There is apparent specificity of this effect for the cholinergic PPI response. Adrenergic signaling through α_1 receptors was less sensitive than was cholinergic signaling through muscarinic (presumably M_1) receptors. Adams and Weber (1989) have reported σ inhibition of the carbachol-stimulated PPI response but not the response to bradykinin in NCB-20 cells. In addition, our preliminary data suggest that histamine stimulation is much more sensitive to σ inhibition than is serotonin stimulation (not shown). It remains to be seen what other PPI-linked systems can be modulated by σ receptors.

Mechanism of Action

The differential sensitivity of cholinergic and adrenergic stimulation, coupled with lack of effect on either inositol phosphate hydrolysis or synthesis of inositol lipids (Bowen et al. 1989*b),* led us to suspect the cholinergic receptor as the target of σ action. One possibility was competitive or noncompetitive antagonism by direct binding of σ ligands to cholinergic receptors. All the active σ ligands did exhibit weak binding affinity to cholinergic receptors. Cholinergic binding affinities were in the same concentration range as the ED_{50} concentration, and the correlation of inhibitory potency with cholinergic affinity was the same as that with σ binding affinity (though it should be noted that poor correlation was observed when [^{3}H]quinuclidinyl benzilate was used as cholinergic ligand instead of [^{3}H]Oxo-M (Bowen et al. 1989*b)).*

However, several experiments ruled out direct antagonism of the cholinergic response (i.e., a direct anticholinergic effect). The strongest evidence is that (+)-PENT inhibition of Oxo-M-stimulated PPI turnover could be observed under conditions where there was no occupation of [^{3}H]Oxo-M binding sites. Also, based on the high binding affinity of Oxo-M to muscarinic receptors and the high concentration used to stimulate the response, there should be little occupation of muscarinic receptors by σ ligands under the normal conditions of the assay (both ligands present together). The noncompetitive nature of the effect also argues against competitive antagonism at the receptor.

However, the cholinergic receptor does appear to be involved in the mechanism of the σ effect. When intact synaptoneurosomes were pretreated with (+)-PENT, a marked decrease in the number of [^{3}H]Oxo-M binding sites was observed. There was no change in the binding affinity. Thus, activation of σ receptors causes a down-regulation of cholinergic receptors. This down-regulation occurred in as little as 15 minutes. A mechanism involving decreased receptor number is consistent with the observed o-induced decrease in maximal

response without change in the ED_{50} of the cholinergic agonist (figure 6). Also, the decrease in Oxo-M-induced stimulation of PPI turnover as shown in table 3 was equivalent to the magnitude of the B_{max} decrease shown in figure 8. These observations are consistent with results of studies of the effect of cholinergic receptor loss due to site-directed alkylation. In slices from cerebral cortex and hippocampus, propylbenzilylcholine mustard was shown to cause a decrease in carbachol-stimulated PPI metabolism that was equivalent to the loss of receptors, without a change in the ED_{50} (Fisher and Snider 1987).

Figure 9 shows schematically the a-induced heterologous desensitization of the cholinergic PPI response. Activation of σ receptors produces a "σ signal," which causes a subsequent decrease in the number of cholinergic receptors and a resultant desensitization of the cholinergic response. Preincubation with (+)-PENT produced no effect on cholinergic binding when a preparation of membrane fragments ($LFCP^2$) was used instead of synaptoneurosomes (table 4). The difference between results observed with membrane fragments versus synaptoneurosomes shows that an intact system is required in order to produce the σ effect. This might suggest the involvement of ion gradients, soluble enzymes or factors, intracellular compartments, or components sensitive to freezing and thawing in the "σ signal" that mediates the inhibitory effect. In addition, because binding of some σ ligands is inhibited by guanine nucleotides (Itzhak and Khouri 1988) it is possible that G-proteins are also involved in the mechanism.

Though the σ effect does not appear to involve direct interaction of σ ligands with the [^{3}H]Oxo-M binding site, the high correlation of potency with the weak muscarinic binding affinity cannot be ignored. This correlation is most likely a consequence of the close relationship of rank order of affinity for compounds at σ receptors and rank order at cholinergic receptors. For the 10 compounds shown in tables 1 and 2, the correlation of σ K_i versus [^{3}H]Oxo-M IC_{50} gave a coefficient of r = 0.89 (graph not shown). Thus, although these compounds bind to muscarinic receptors with much lower affinity than to σ sites, the rank order of affinity is nearly the same. The explanation for this is not clear, and this phenomenon deserves further investigation. However, it may suggest that the topography of the muscarinic receptor resembles that of the σ receptor in some way. Whether this effect has anything to do with the mechanism of action of σ ligands remains to be demonstrated.

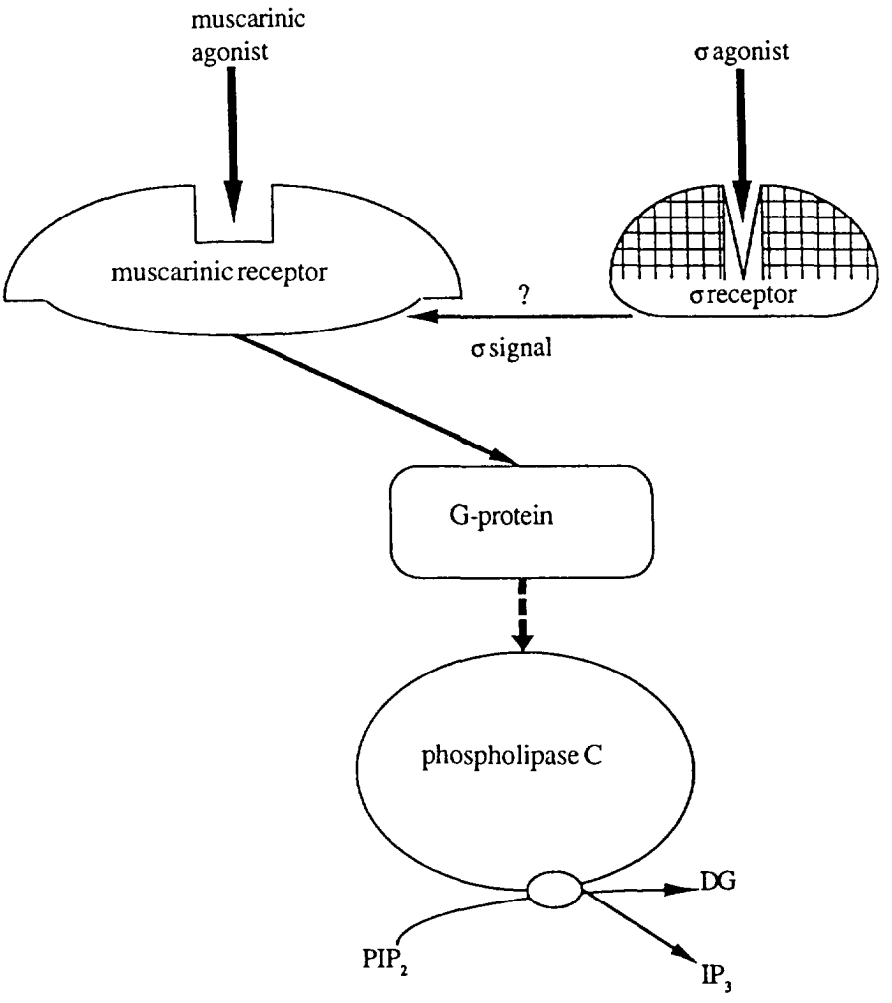

FIGURE 9. *Schematic diagram of the effect of σ ligands on the cholinergic PPI response.*

Agonist vs. Antagonist Actions

Due to the current lack of functional assays for σ sites and lack of identified endogenous ligands, it has been difficult to determine which compounds are

agonists and which compounds are antagonists at σ sites. An important observation from this study is that all of the σ ligands tested produced effects in the same direction. All produced inhibition of the PPI response. Neuroleptics such as HAL and FLU produced the same effect as dextrorotary opiates such as (+)-PENT and dextrallorphan. Similar observations were made in other functional assays (Walker et al. 1988; Campbell et al. 1989; Matsumoto et al. 1990). Thus, there does not appear to be a clear agonist-antagonist relationship between these classes of compounds.

Chronic HAL has been shown to cause down regulation of a receptors (Itzhak and Alerhand 1989; Matsumoto et al. 1989), suggesting that HAL is an agonist at σ sites. Because the other compounds behaved like HAL in the current study, it is suggested that inhibition of the PPI response represents an agonist action at σ receptors. Also, it is difficult to visualize how antagonism at σ sites would cause inhibition of the PPI response, for one would then have to invoke a requirement of σ agonism for efficient PPI signaling.

If other σ compounds are in fact agonists, then (+)-3-PPP could be characterized as a partial agonist. It was the only σ ligand that failed to produce complete inhibition of agonist-stimulated PPI turnover. (+)-3-PPP has been shown to exhibit anomalous activity or lack of efficacy in other systems that show biological functions of σ receptors (Campbell et al. 1989; Matsumoto et al. 1990). Further studies will be necessary to determine the agonist and antagonist nature of σ compounds.

CONCLUSION

Attenuation of signaling via the PPI pathway is a potential mechanism by which σ ligands could modulate the functions of neurotransmitters in vivo. σ Receptors appear to be one of a growing number of receptors postulated to utilize a variety of mechanisms to effect negative modulation of PPI turnover (Linden and Delahunty 1989). Further studies will be aimed at the in vivo interactions of neurotransmitters and σ ligands as well as further elucidation of the mechanism of the σ effect. Furthermore, inhibition of the cholinergic PPI response provides a convenient bioassay system for use in development and characterization of novel σ agonists and antagonists.

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σ and Phencyclidine Receptors in the Brain-Endocrine-Immune Axis

Seth A. Wolfe, Jr., and Errol B. De Souza

INTRODUCTION

Recently there has been growing awareness of the existence of functional links among the nervous, endocrine, and immune systems. Clinical studies have associated affective disorders and psychological states such as bereavement, depression, and anxiety with alterations in immune function in humans (reviewed in Stein et al. 1985; Tecoma and Huey 1985). In animal studies, immune modulation has been brought about by experimentally induced stress (reviewed in Croiset et al. 1987; Stein et al. 1985) neural lesions (reviewed in Rozman et al. 1985), and classical Pavlovian conditioning (Ader et al. 1983). Candidates for mediators of these immunologic effects include a growing list of neurotransmitters, neuroendocrine peptides, neurotropic factors, and their receptors in lymphoid cells (reviewed in Blalock 1989). These substances may be delivered via the bloodstream or secreted by nerves within target tissues. Primary sensory afferents in lymphoid tissues have the potential to release transmitter substances upon antidromic stimulation (Payan and Goetzl 1985) and these tissues also contain sympathetic nerve fibers that establish synaptic-like contacts with lymphocytes and macrophages (D.L. Felten 1987; S.Y. Felten and Olschowka 1987).

There is also compelling evidence that the immune system modulates central nervous system (CNS) and endocrine functions. Immune responses and immune-cell products have been reported to produce changes in behavior, hormone release, neurotransmitter turnover, and neuronal electrical activity (reviewed in Besedovsky et al. 1986; Blalock 1989). Lymphokines and monokines, such as interferon, interieukin-1 (IL-1), and thymosin, have neurotropic effects (reviewed in Besedovsky et al. 1985) and immune-cell products have been identified in the CNS (Breder et al. 1988). Leukocyte-cell surface markers and specific membrane receptors for immune-cell products

have also been identified in the CNS and endocrine tissues (reviewed in Blalock 1989). Finally, neuroendocrine peptides are produced by cells of the immune system (reviewed in Blalock 1989). Thus, the nervous, endocrine, and immune systems share common transmitter/communication molecules, are physically and chemically interconnected, and they interact extensively.

Our interest, and the subject of this chapter, is receptors mediating the action of σ/phencyclidine drugs on the CNS-endocrine-immune axis. In humans and mice, phencyclidine (PCP) and PCP analogs have been reported to depress a variety of in vitro indices of immune function, such as [^{3}H]thymidine and [^{3}H]-2-deoxy-*d*-glucose uptake, mitogen-driven antibody production, and lipopolysaccharide-driven IL-l production (Dornand et al. 1987; Khansari et al. 1984). Preliminary data suggest the presence of [^{3}H]PCP binding sites on human lymphocytes (Fudenberg et al. 1984), and PCP and Nallylnormetazocine (SKF 10,047) have also been reported to alter neuroendocrine function. Both of these compounds stimulate hypothalamic-pituitary-adrenocortical secretion (Boggan et al. 1982; Pechnick et al. 1985) and suppress luteinizing hormone (LH) (Pechnick et al. 1985) and prolactin (Pechnick et al. 1985; Saller et al. 1982) secretion in rats. *d*-[^{3}H]SKF 10,047 binding sites have been demonstrated in the anterior pituitary (Tam 1983) and PCP and SKF 10,047 also bind to cultured pituitary cells and alter LH release in vitro (Stojilkovic et al. 1987).

σ and PCP receptors in brain have been well characterized by others, most notably those participating in this workshop. Because PCP and SKF 10,047 bind with high affinity to both PCP and σ receptors in brain (Largent et al. 1986; Tam 1985) the identity of the receptor(s) mediating the immunologic and endocrine effects of these compounds is unknown. Furthermore, it is unclear whether the endocrine and immune effects of PCP and SKF 10,047 are mediated in brain or through direct actions at the target endocrine organs or lymphoid tissue. This chapter will focus on data from our recent studies on the identification, characterization, and localization of these receptors in immune and endocrine tissues. The characteristics of receptors in human peripheral blood leukocytes (HPBLs) and rat pituitary, adrenal, testis, and ovary will be compared with those of the well-characterized σ and PCP receptors in rat brain. In these studies, these selective ligands were used in order to identify and characterize σ and PCP receptors in peripheral tissues: [^{3}H]haloperidol (Tam and Cook 1984) 1,3-di(2-[5-^{3}H]tolyl)guanidine ([^{3}H]DTG) (Weber et al. 1986) and *d*-3-(3-hydroxyphenyl)- *N*-(1-propyl)-2,3-[^{3}H]piperidine ([^{3}H]-3-PPP) (Largent et al. 1986) to label σ receptors; and 3,4-[^{3}H]-(N)-[1-(2-thienyl)cyclohexyl]-

piperidine ([^{3}H]TCP) (Vignon et al. 1983) and *d*-[^{3}H]5-methyl-10,11-dihydro-5H-dibenzo-[a,d]+cyclohepten-5,10-imine ([^{3}H]MK-801) (Bowery et al. 1988) to label PCP receptors.

ABSENCE OF PCP RECEPTORS IN ENDOCRINE AND LYMPHOID TISSUES

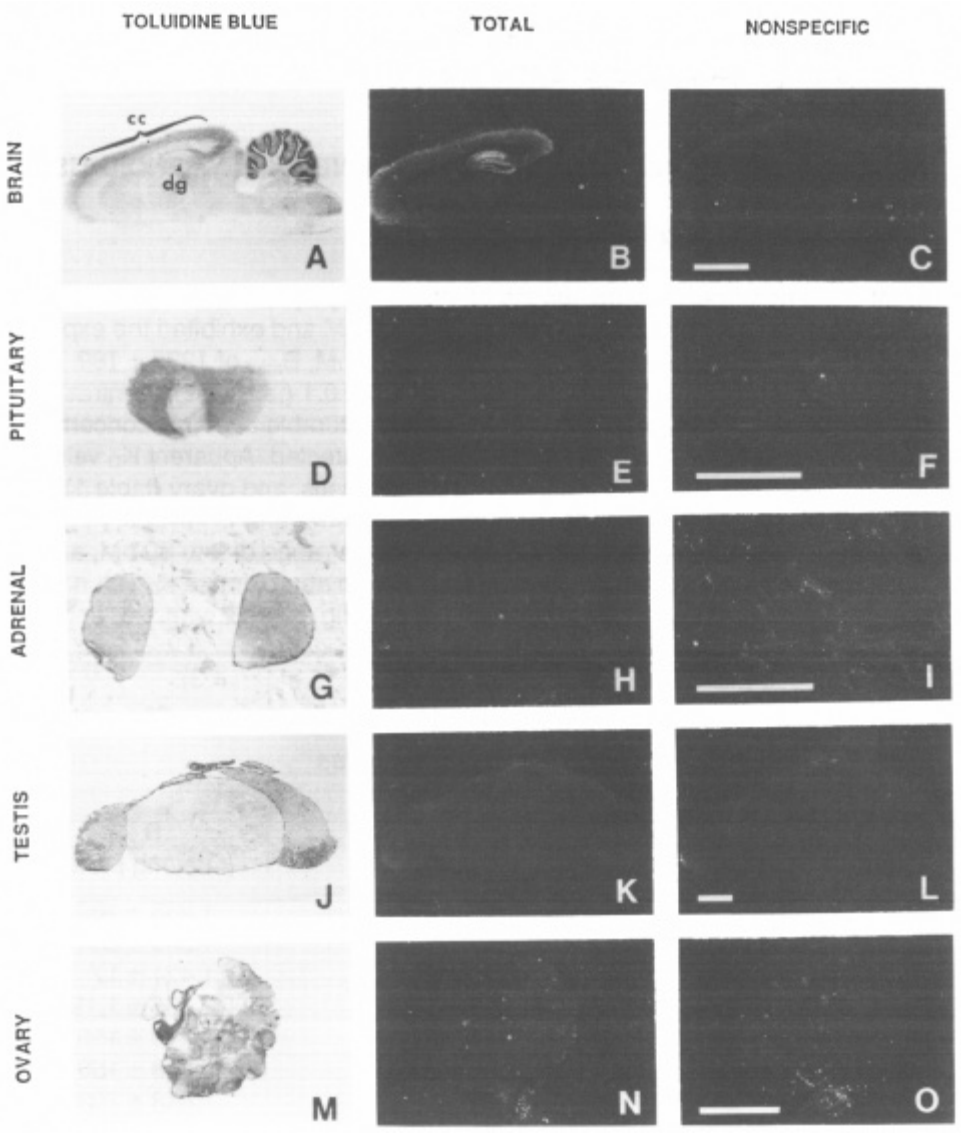
Homogenate Binding Studies

In rat brain homogenates (minus the cerebellum), [^{3}H]TCP binding was saturable over the concentration range 2 to 100 nM and exhibited the expected high affinity K_D (mean ± SEM, *n = 5)* of 6.7 ± 0.9 nM, B_{max} of 1233 ± 189 fmol/mg protein, and Hill coefficient value of 1.0 ± 0.1 (table 1). In contrast, in the endocrine glands [^{3}H]TCP binding was not saturable over this concentration range, nor was high-affinity binding of [^{3}H]TCP detected. Apparent K_D values ranged from 28 to 211 nM for pituitary, adrenal, testis, and ovary (table 1). HPBLs were similar to rat endocrine tissues in this regard. In HPBLs, [^{3}H]TCP binding was not saturable within the concentration range of 2 to 80 nM, and Scatchard analysis revealed a binding site with an approximate K_D value (mean ± SEM, *n* = 3) of 62 ± 19 nM. This affinity of binding in HPBLs and rat endocrine organs was not consistent with high-affinity PCP receptors and was kinetically comparable to that seen in cerebellum (K_D = 57 ± 10 nM, *n = 3).*

TABLE 1. *[^{3}H]TCP binding in tissue homogenates*

Tissue	K_D (nM)	B_{max} (fmol/mg-protein)
Rat brain	6.7 ± 0.9	1,233 ± 189
Rat cerebellum	57±10	1,399 ± 354
Rat pituitary	110±31	1,421 ± 17
Rat adrenal	211 ± 106	2,606 ± 1 ,192
Rat testis	111 ±4	7,010 ± 155
Rat ovary	28±5	978±158
Human PBLs	62±-19	1,818 ±293

NOTE: There was an absence of high-affinity PCP receptors in human PBL and rat endocrine tissues. [^{3}H]TCP saturation binding (2 to 80 nM for human PBL and 2 to 100 nM for other tissues) was carried out in tissue homogenates as previously described. Nonspecific binding was defined in parallel incubations containing 20 mM nonradioactive PCP. Data from Wolfe et al. 1988, 1989.


TOLUIDINE BLUE
TOTAL
NONSPECIFIC
BRAIN
PITUITARY
ADRENAL
TESTIS
OVARY
cc
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A
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Autoradiography

In homogenate binding studies, a small number of high-affinity receptors would not be detectable in the presence of a high density of lower affinity binding sites, such as were seen in the above experiments. However, if high-affinity sites were anatomically clustered, they might be revealed by in vitro receptor autoradiography. Therefore, autoradiographic experiments were carried out in the rat endocrine system using [^{3}H]TCP to label PCP receptors. The most notable feature of the resultant autoradiograms of pituitary (figure 1 D-F), adrenal (figure 1G-I), testis (figure 1 J-L), and ovary (figure 1M-O) was the absence of [^{3}H]TCP-labeled PCP receptors under conditions that caused appropriate, specific labeling of PCP receptors in the cerebral cortex and Ammon's horn-dentate gyrus regions of brain (figure 1A-C). Furthermore, the pattern and intensity of nonspecific binding were identical to total binding, indicating that no specific labeling of PCP receptors occurred in endocrine tissues. Therefore, these autoradiographic studies confirmed the results of the preceding homogenate binding experiments and demonstrated the absence of high-affinity PCP receptors in pituitary, adrenal, testis, and ovary.

FIGURE 1. *Autoradiographic localization of [^{3}H]TCP-labeled PCP receptors in rat brain and endocrine organs. Each horizontal row shows: left (TOLLUIDINE BLUE) - brightfieldphotograph of toluidine blue-stained section showing tissue histology; center (TOTAL) - negative enlargement (similar appearance to darkfield photograph) of [^{3}H]Ultrofilm autoradiographic image of the same section, showing the "total" distribution of silver grains (light areas) indicating regions of [^{3}H]TCP binding; and, right (NONSPECIFIC) - autoradiogram of adjacent tissue section, under identical incubation, exposure and photographic conditions, in which specific [^{3}H]TCP binding was blocked by the addition of 200 μM PCP. In saggital sections of brain (B), the cerebral cortex (cc) and Ammon's horn - dentate gyrus (dg) regions had the highest densities of [^{3}H]TCP binding sites; this binding was blocked by PCP (C), indicating the presence of specific PCP receptors in these areas. In contrast, little or no [^{3}H]TCP binding was seen in pituitary (E), adrenal (H), testis (K) or ovary (N), and an identical pattern was seen in the presence of excess PCP (F, I, L, and O, respectively), indicating the absence of specific PCP receptors in these sections. Bars = 4 mm. From Wolfe et al. 7989.*

We sought to corroborate these findings using the novel PCP-receptor-selective radioligand [^{3}H]MK-801 (Bowery et al. 1988). [^{3}H]MK-801 did produce a high level of background labeling in endocrine tissues, but it was nonspecific, because it was not blocked by the presence of 200 µM PCP (data not shown). In all other respects, [^{3}H]MK-801 autoradiograms were in agreement with those produced by [^{3}H]TCP. Specific binding of [^{3}H]MK-801 was not seen in rat pituitary, adrenal, testis, or ovary, although the conditions employed were adequate to produce a pattern of specific labeling in the brain that was comparable in intensity and distribution with the pattern that was obtained by using [^{3}H]TCP (data not shown).

Discussion

In these studies, we have used selective probes to label PCP receptors in immunologic and endocrine tissues. In binding studies using washed membranes from tissue homogenates, the PCP receptor that binds with high affinity to [^{3}H]TCP (Vignon et al. 1986) appeared to be either absent or present at undetectably low levels in HPBLs and in rat pituitary, adrenal, testis, and ovary. The nonsaturable binding of [^{3}H]TCP that we observed in HPBLs, rat cerebellum, and rat endocrine tissues had an affinity that was similar to the lower affinity [^{3}H]TCP binding site (K_D value 50 to 80 nM) that has been described in rat hindbrain and spinal cord (Vignon et al. 1986) and in synaptosomal membranes of rat hippocampus (Haring et al. 1987). This lower affinity [^{3}H]TCP binding site remains to be characterized, but it is not thought to be the traditional PCP receptor. The absence of PCP receptors in the endocrine organs was confirmed by autoradiographic studies in slide-mounted sections using the selective PCP receptor ligands [^{3}H]TCP and [^{3}H]MK-801.

PRESENCE OF σ RECEPTORS IN HUMAN PBL AND RAT ENDOCRINE ORGANS

Homogenate Binding Studies

In homogenate binding studies, [^{3}H]haloperidol was used to label σ receptors. In these experiments [^{3}H]haloperidol was prevented from binding to D_2 dopamine and $5HT_2$ serotonin receptors by inclusion of excess nonradioactive spiperone in all incubations. Under the conditions employed (Wolfe et al. 1988, 1989) [^{3}H]haloperidol bound to membrane homogenates of HPBLs and rat pituitary, adrenal, testis, and ovary in a specific, concentration-dependent manner (representative experiment shown in figure 2). Specific binding was

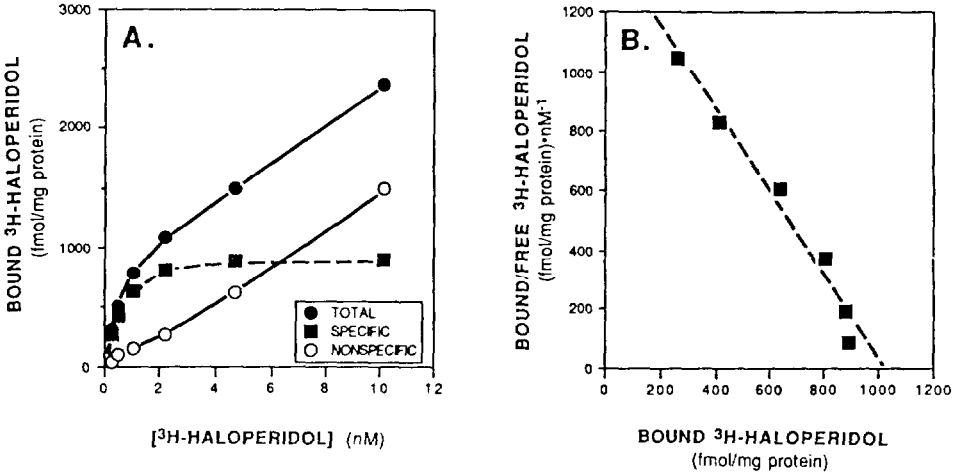

FIGURE 2. *Saturability and high affinity of [^{3}H]haloperidol binding to human PBL membranes. A. Representative saturation experiment showing: (—●—),total binding of [^{3}H]haloperidol in the presence of 25 nM spiperone;'—○—), nonspecific binding in the presence of 25 nM spiperone plus 30 mM I-butaclamol; and specific (- - ■ - -) binding (the difference between total and nonspecific) over a concentration range of 0.2 - 10 nM [^{3}H]haloperidol. B. Scatchard plot of specific [^{3}H]haloperidol binding in the same experiment. Saturability of specific [^{3}H]haloperidol binding is demonstrated in A, while the affinity of binding (K_D = 0.44 ± 0.10 nM), density of binding sites (B_{max} = 752 ± 737 fmol/mg protein), and fit of the points to a straight line (corr. coef. = 0.99) is demonstrated in B. From Wolfe et al. 1988.*

saturable, and Scatchard analysis revealed high-affinity binding, with similar K_D values of 0.4 nM in HPBLs, and 1.9 to 2.8 nM in endocrine tissues. These [^{3}H]haloperidol binding sites were comparable to [^{3}H]haloperidol-labeled sites in rat cerebellum (K_D = 0.5 to 1.02 nM), where a high density of σ receptors is known to be present (Largent et al. 1986). In all of the tissues tested, the best fit was obtained with a single-site model of [^{3}H]haloperidol binding, and Hill coefficients approached a value of unity (data not shown). The most striking feature of these experiments was that the density of [^{3}H]haloperidol binding sites in HPBLs and rat endocrine organs was consistently greater than that found in brain. HPBLs had a B_{max} 1.8 times greater than that of rat cerebellum,

and the relative amounts for rat endocrine tissues were pituitary, 2.0; adrenal, 1.6; testis, 2.6; and ovary, 4.1 times greater than cerebellum (see figure 3).

The pharmacological profile of [^{3}H]haloperidol binding was examined in competition experiments by incubating rat cerebellar, HPBLs, and rat endocrine tissue homogenates with [^{3}H]haloperidol (in the presence of 25 nM spiperone) plus increasing concentrations of drugs representing a wide range of potencies for σ and PCP receptors. These results are summarized in table 2 and figures 4 and 5.

The pharmacology and stereospecificity of [^{3}H]haloperidol-labeled sites in human PBLs and rat pituitary, adrenal, testis, and ovary were consistent with those previously reported using a variety of radioligands for σ receptors in brain (Largent et al. 1984; Martin et al. 1984; Tam 1985; Tam and Cook 1984; Weber

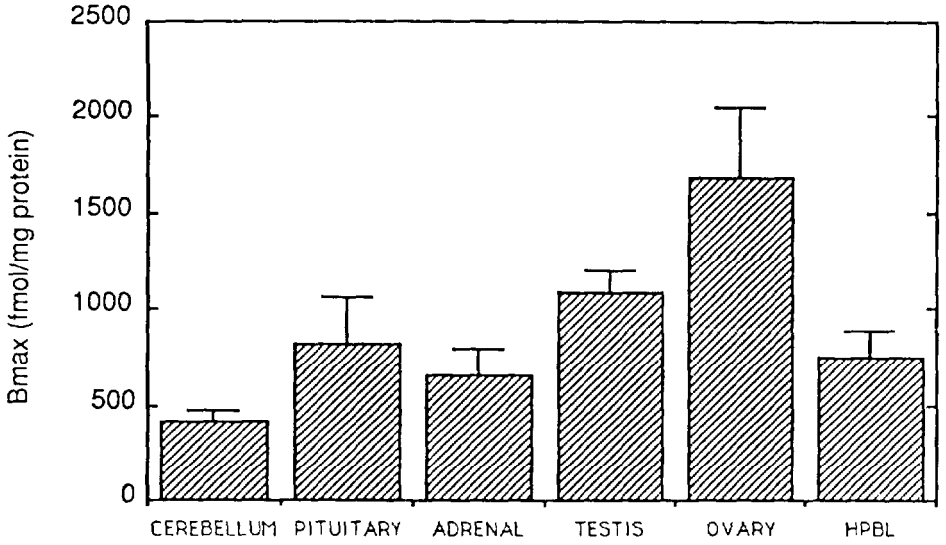

FIGURE 3. *Densities of [^{3}H]haloperidol-labeled σ receptors in endocrine and lymphoid tissues. Saturation binding experiments were carried out with [^{3}H]haloperidol as previously described (0.2 -10 nM in human peripheral blood leukocytes (HPBL), and 0.2 -15 nM in rat brain and endocrine tissues). Spiperone (25 nM) was included in all incubations, and nonspecific binding was defined in the presence of 30 mM l-butaclamol. Data from Wolfe et al. 1988, 1989.*

TABLE 2. *Pharmacologic* specificity *of [^{3}H]haloperidol-labeled sigma receptors*

Drug	Rat cerebellum	Human PBL
Haloperidol	0.42 ± 0.05 (3)	0.30 ± 0.05 (3)
l-Butaclamol	21.8 ± 6.0 (3)	14.7 ± 5.9 (3)
Pentazocine	28.9 ± 16.8 (2)	19.7 ± 5.4 (2)
d-3-PPP	167 ± 64.6 (5)	283 ± 62.5 (5)
DTG	307 ± 97.0 (2)	1,386 ± 348 (2)
d-SKF 10,047	461 ±110(5)	1,666 ± 416 (5)
d-Butaclamol	1,851 ± 351 (3)	1,040 ± 251 (3)
l-SKF 10,047	3,850 ± 595 (5)	28,173 ± 5,260 (5)
*d,l*PCP	7,410 ± 6,986 (2)	19,628 ± 13,432 (2)
Levallorphan	9,624 ± 1,684 (4)	12,502 ± 4,984 (4)
TCP	10,41 1 ± 3,865 (3)	42,656 ± 11,406 (3)
MK-801	94,619 ± 23,489 (2)	212,968 ± 52,353 (2)

NOTE: The K_i values (nM, mean ± SEM) of drugs were determined at [^{3}H]haloperidol (1 nM) -labeled σ receptors in rat cerebellum and human PBL in the presence of 25 nM spiperone. Nonspecific binding was defined in parallel incubations by the inclusion of 30 mM *l*-butaclamol. From Wolfe et al. 1988.

et al. 1986). *l*-Butaclamol was 11 to 44 times as potent as *d*-butaclamol in competing for [^{3}H]haloperidol binding sites in rat endocrine tissues, and 70 to 80 times as potent in HPBL (representative experiment shown in figure 4). Since *d*-butaclamol is selective for D_2 dopamine receptors (Tam and Cook 1984), this pattern suggests that [^{3}H]haloperidol is not binding to D_2 dopamine receptors under these experimental conditions. On the other hand, the dstereoisomers of 3-PPP, pentazocine, and SKF 10,047 were more potent than their /-forms in all of the tissues tested (figure 4 and table 2), which is consistent with the stereospecificity of σ receptors in brain. The recently described σ drug DTG and the PCP receptor ligands PCP, TCP, and MK-801 were also tested. DTG was 14 to 34 times as potent as PCP and TCP, and 154 to 308 times as potent as MK-801 (figure 4B and table 2) which confirms that [^{3}H]haloperidol was labeling σ and not PCP receptors under the conditions employed. These observations were further confirmed using [^{3}H]DTG, which bound HPBL in a specific, saturable manner and had kinetic properties (K_D = 26.4 ± 3.6 nM) comparable with rat cerebellum (K_D = 29.5 ± 3.5 nM) (data not shown).

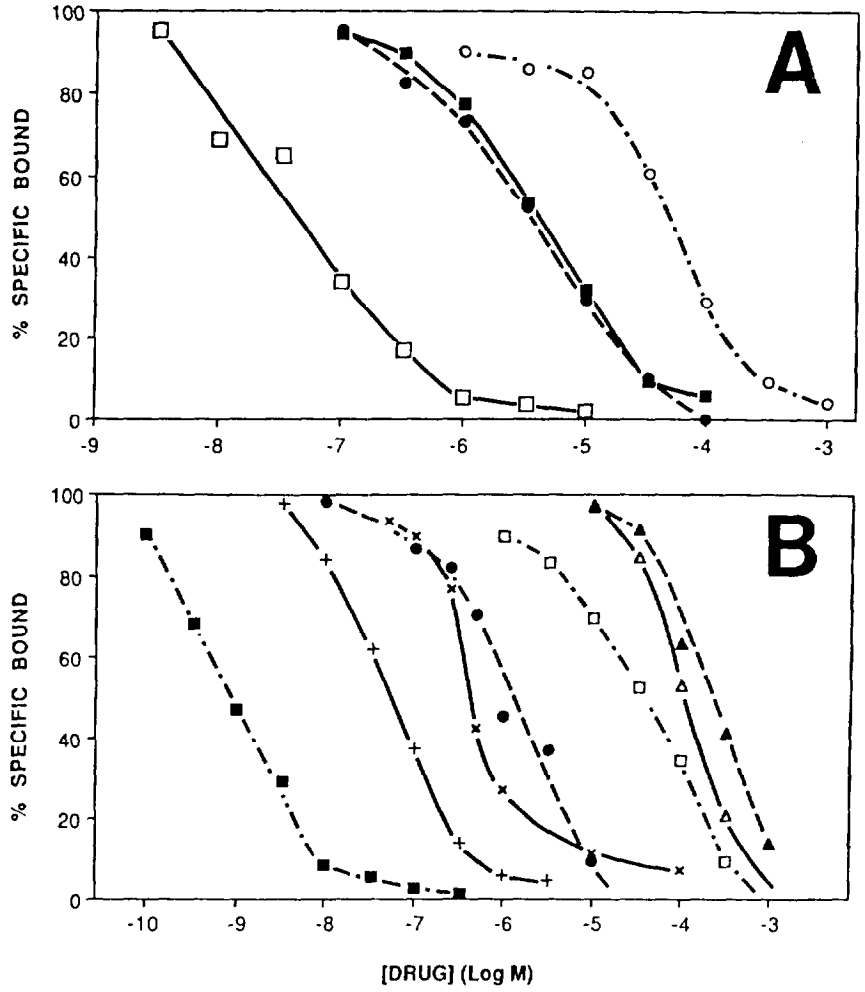

FIGURE 4. *Pharmacologic profile of [^{3}H]haloperido-labeled σ receptors in HPBL. A. Competition by d- and l-stereoisomers of butaclamol and SKF 10,047 with 1 nM [^{3}H]haloperidol in the presence of 25 nM spiperone. l-Butaclamol (—□—)is more potent than the d-form (—■—), and d-SKF 10,047 (- -●- -) is more potent than l-SKF 10,047 (— -○- —) at this site. B. Competition by haloperidol (– ·■– ·), d-3-PPP (—x—), pentazocine (—+—), DTG (- -●- -), levallorphan (– ·□– ·), PCP (—Δ—), and TCP (- -▲- -) under identical conditions. From Wolfe et al. 1988.*

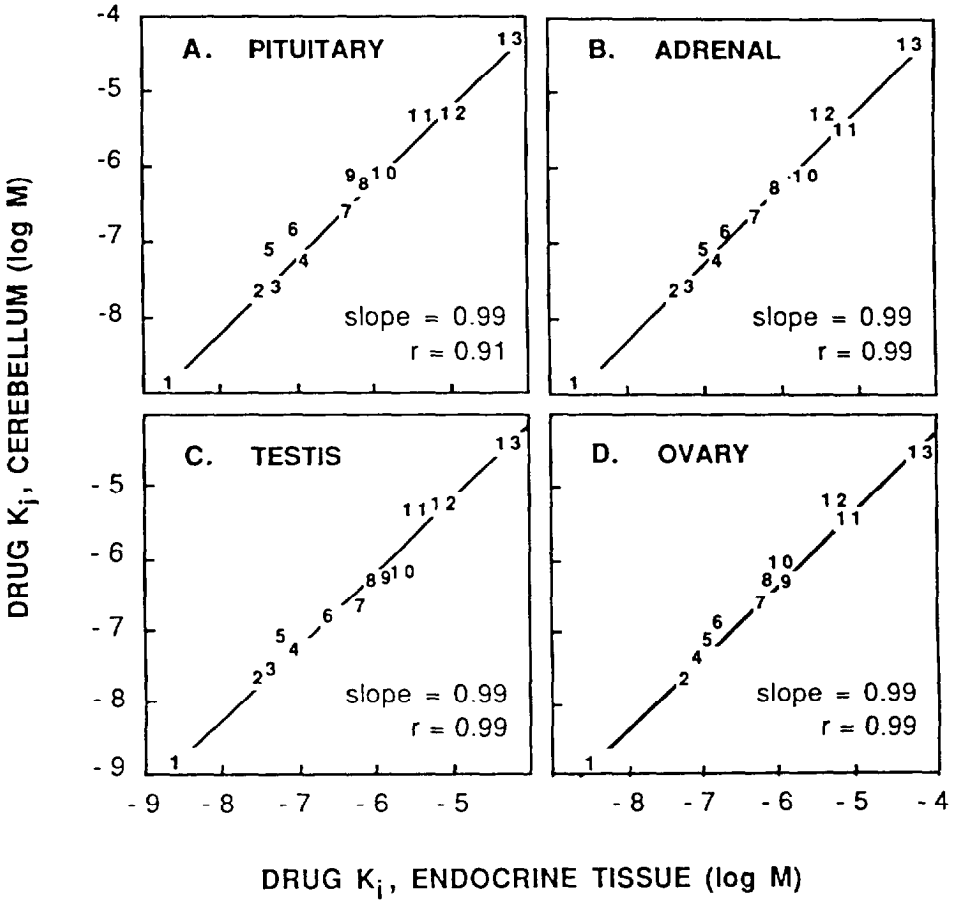

FIGURE 5. *Correlation of drug potencies of at [^{3}H]haloperidol-labeled σ receptors in rat endocrine tissues with their respective potencies in rat cerebellum. Numbers refer to the following compounds: 1, haloperidol; 2, d-pentazocine; 3, l-butaclamol; 4, l-pentazocine; 5, DTG; 6, d-3-PPP; 7, d-SKF 10,047; 8, l-3-PPP; 9, l-SKF 10,047; 10, d-butaclamol; 11, PCP; 12, TCP; 13, MK-801. From Wolfe et al. 1989.*

The rank orders of potency of the various drugs tested in HPBL and rat endocrine tissues were similar to that of the rat cerebellum, and there was a highly significant positive correlation ($p < 0.001$ in all tissues) of the K_i values of the drugs in blood leukocytes and endocrine tissues with their K_i values in the cerebellum. This relation is illustrated for the endocrine system in figure 5, in which the K_i values of the drugs in the various tissues are plotted versus their K_i values in the cerebellum. All such plots resulted in straight lines with slopes closely approaching unity (0.99 for endocrine tissues and 0.87 for human PBLs) and correlation coefficients (r values) of 0.91 for pituitary, 0.96 for HPBL, and 0.99 for rat adrenal, testis, and ovary.

Autoradiography

The drug [^{3}H]-3-PPP gave a better signal-to-noise ratio than [^{3}H]haloperidol in binding to slide-mounted tissue sections and was therefore used instead of [^{3}H]haloperidol to label σ receptors for in vitro receptor autoradiography. Autoradiograms of rat pituitary, adrenal, testis, and ovary showed specific, localized binding of [^{3}H]-3-PPP (figure 6). In the pituitary, specific binding was seen in all three lobes (figure 6D-F). Labeling was homogeneous within each lobe, with highest densities of [^{3}H]-3-PPP binding sites being present in the anterior lobe and lower concentrations seen in the neural (posterior) and intermediate lobes.

In the adrenal gland, [^{3}H]-3-PPP binding was localized primarily in the outer cortical regions (figure 6G-I). There was a slightly higher density of receptors visualized in the capsule and the outer (zona glomerulosa and zona fasciculata) than in the inner (zona reticularis) regions of the cortex. A low level of specific binding was present in the adrenal medulla.

The testis displayed a high density of specific [^{3}H]-3-PPP binding sites (figure 6J-L). Specific [^{3}H]-3-PPP labeling in the seminiferous tubules was uniform and comparable in intensity with the cerebellum of the brain (figure 6B). Interstitial areas and the contents of the tubule lumens did not show visible radioligand binding. The highest densities of σ receptors were found in the epididymis in the two regions of the ductuli efferentes, and in the corpus and the initial segment of the caput of the ductus epididymis. Lower intensity labeling was seen in the rest of the caput, the proximal and distal cauda, and the luminal surfaces of the pars epididymica and pars libera of the ductus deferens. The muscular layers of the ductus deferens contained still-lower densities of σ receptors.

The ovary contained the greatest concentration of [^{3}H]-3-PPP binding sites found in the endocrine tissues examined, and the highest density of σ receptors in the ovary was localized in the maturing follicles (figure 6M-O). A uniform, lower density of σ receptors was seen throughout the remainder of the ovary and in follicles not undergoing active development.

In addition to using [^{3}H]-3-.PPP, we also carried out autoradiography using [^{3}H]DTG to localize sigma receptors. [^{3}H]DTG-generated autoradiograms were in agreement with those presented above for [^{3}H]-3-PPP. Specific labeling of σ receptors was demonstrated in the anterior lobes of the pituitary, the adrenal cortex, the noninterstitial areas of the testis, and ovarian follicles (data not shown).

Effect of Hypophysectomy

In order to determine whether sigma receptors in endocrine organs require trophic maintenance by pituitary hormones, hypophysectomized rats were also examined in autoradiographic studies. Two to three weeks after hypophysectomy, the adrenals, testes, and ovaries were visibly atrophied relative to those of sham-operated animals. However, [^{3}H]-3-PPP autoradiography revealed that there was no apparent loss of σ receptors in the adrenals or testes. In the adrenals, shrinkage of receptor-poor interstitial areas caused a relative increase in receptor density of the spared tissues (figure 7A and B). Consequently, the concentration of receptors in the adrenal cortex, paucity of receptors in the medulla, and the gradation in density from outer (zona glomerulosa and zona fasciculata) to inner (zona reticularis) regions of the cortex was easier to visualize in the adrenals from the hypophysectomized rats than it was in intact animals (figure 7B).

In the testis, the pattern of change was similar to that of the adrenal. As is evident from the autoradiogram, labeling did not occur in interstitial areas of testes from normal animals (figure 7C). While atrophy of interstitial areas and shrinkage of the organ was evident, hypophysectomy had no effect on the localization or density of σ receptors in the testis (figure 7D).

Because the estrus cycle ceased in hypophysectomized rats, no maturing follicles were present in their ovaries. This resulted in loss of the high-density [^{3}H]-3-PPP binding sites seen in the maturing follicles of sham-operated rats (figure 7E and F). The remainder of the ovary, although reduced in size, contained distribution and density of σ receptors comparable to that of ovaries

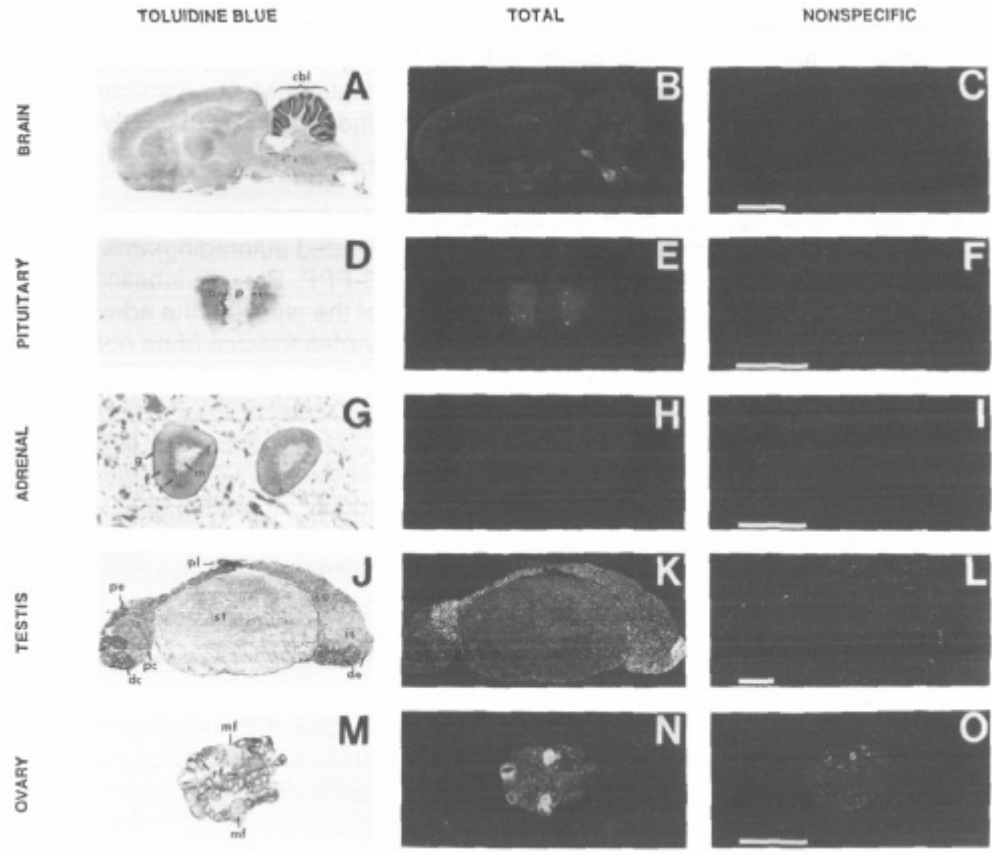

FIGURE 6. *Autoradiographic localization of σ receptors in rat brain and endocrine organs. Left column (TOLUIDINE BLUE) - brightfield photographs of toluidine blue-stained, slide-mounted sections showing histology of brain (A), pituitary (D), adrenal (G), testis (J) and ovary (M). Center column (TOTAL) - negative enlargements of [^{3}H]Ultrofilm autoradiograms showing binding of [^{3}H]-3-PPP (light areas) to the sections shown in A, D, G, J and M. Right column (NONSPECIFIC) - autoradiograms of adjacent sections in which specific [^{3}H]-3-PPP binding was blocked by 10 mM haloperidol. Specific binding was widely distributed in the brain (A, B, C), and present in high densities in the cerebellum (cbl). In the pituitary (D, E, F), the highest concentrations off [^{3}H]-3-PPP*

of sham-operated controls. Significant concentrations of [^{3}H]-3-PPP binding sites were present in quiescent follicles of normal and hypophysectomized animals alike.

Discussion

The high-affinity, specific [^{3}H]haloperidol and [^{3}H]DTG binding that we demonstrated in HPBLs and rat pituitary, adrenal, testis, and ovary exhibited a pharmacology consistent with that of the σ receptor. The kinetic and pharmacologic characteristics of [^{3}H]haloperidol-labeled binding sites in these tissues were similar to those of σ receptors in rat cerebellum with respect to their affinity (K_D values), stereospecificity, and rank orders of drug potencies. [^{3}H]Haloperidol binding was inhibited by nanomolar concentrations of the σ receptor ligands *l*-butaclamol, *d*-pentazocine, DTG, *d*-3-PPP, and *d*-SKF 10,047. Competition binding studies, using drugs representing a wide range of potencies for σ and PCP receptors, demonstrated similar pharmacologic profiles of [^{3}H]haloperidol binding in all of the tissues examined. In addition, there were strong positive correlations between drug potencies at the [^{3}H]haloperidol binding sites in lymphoid and endocrine tissues and their respective potencies at [^{3}H]haloperidol binding sites in rat cerebellum. The K_i

binding sites were found in the anterior lobes (a), with lower densities present in the intermediate (i) and posterior (p, neural) lobes. The adrenal gland (G, H, I) contained slightly higher densities of σ binding sites in the outer (zona glomerulosa (g) and zona fasciculata (f)) regions of the cortex than in the inner (zona reticularis (r)) cortical areas. Binding sites were very sparse in the adrenal medulla (m). The highest density of σ receptors in the testis (J, K, L) was present in the epididymis in the ductuli efferentes (de) and the initial segment (is) of the caput (ca). Slightly lower concentrations were seen in the rest of the caput, proximal cauda (pc) and distal cauda (dc), and luminal surfaces of the pars epididymica (pe) and pars libera (pl) of the ductus deferens. The seminiferous tubules (st) of the testis proper were comparable in σ receptor density to the cerebellum of brain. [^{3}H]-3-PPP binding sites were not apparent in the interstitial areas of the testis or epididymis. The ovary (M, N, 0) contained very high concentrations of σ receptors in maturing follicles (mf), and lower, but significant, densities in the resting follicles (rf). Bars = 4 mm. From Wolfe et al. 1989.

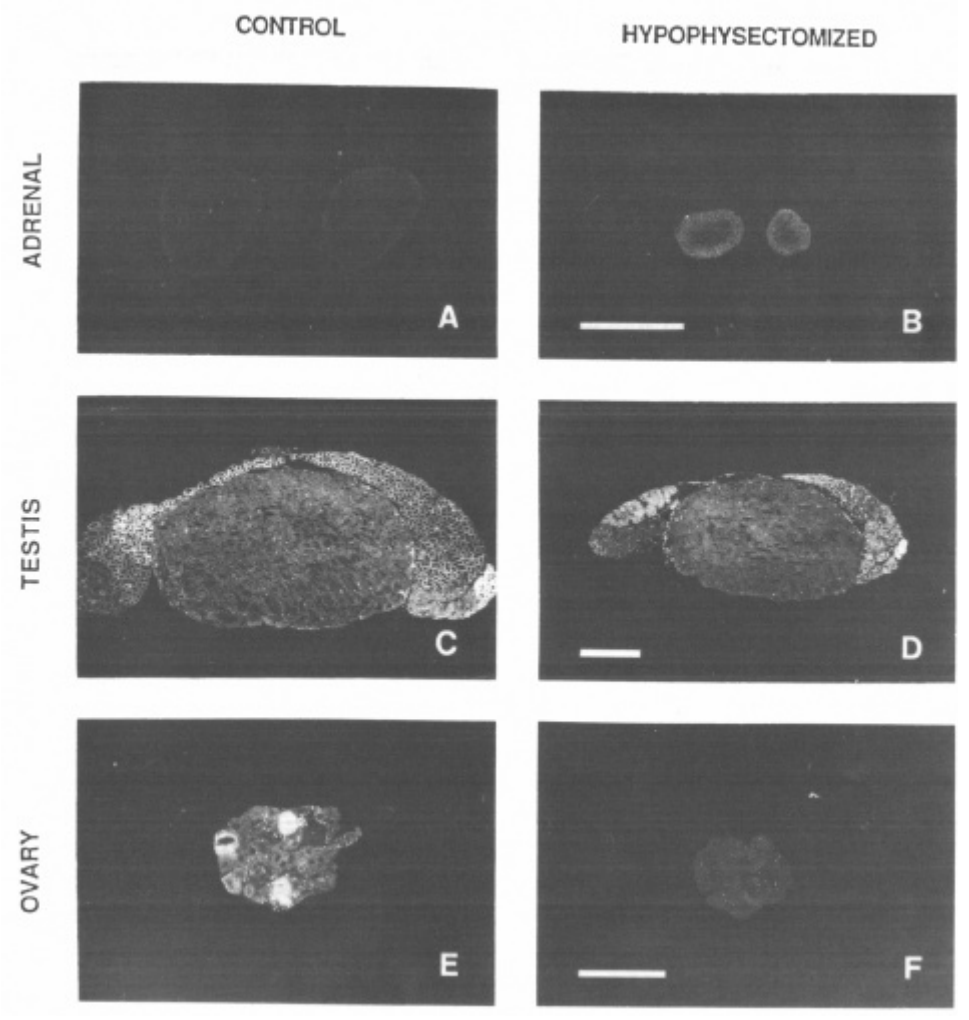

FIGURE 7. *Effect of hypophysectomy on σ binding sites in endocrine organs. Negative enlargements of autoradiograms of adrenals (B), testis (D) and ovary (F) from hypophysectomized rats compared to corresponding organs from sham-operated controls (A, C, and E, respectively). Autoradiograms generated under identical conditions as in Fig. 6. Bars = 4 mm. From Wolfe et al. 1989.*

values that we obtained for competing drugs are similar to those reported by others in rat (Largent et al. 1984, 1986) and in guinea pig brain (Tam and Cook 1984). Clearly, σ binding sites in lymphoid and endocrine organs have kinetic and pharmacologic characteristics identical to those of the well-characterized σ receptors present in brain.

Thus, the present studies suggest that the immunologic effects of PCP (Dornand et al. 1987; Khansari et al. 1984) are mediated through actions on σ rather than on PCP receptors. The immunosuppressive effects of PCP were observed at relatively high concentrations (10 μM) of drug. This may relate in part to the low affinity of PCP for σ receptors in PBLs (K_i = 20 μM; see table 2). Although the relevance and characteristics of the low-affinity [^{3}H]TCP binding site in PBLs remains to be determined, actions of PCP at this binding site resulting in immune modulation cannot be ruled out.

The mechanism by which PCP produces its immunosuppressant effects is unknown, as the mechanism of action of the σ receptor has yet to be elucidated. The work of Itzhak and Khouri (Itzhak and Khouri 1988; Itzhak 1989) suggests that guanine nucleotides can modulate the binding affinity of the σ receptor. Elsewhere in this volume, Bowen et al. present evidence that σ receptors may be involved in negative modulation of phosphoinositide-linked receptor signaling. Lymphocyte activation is well known to be modulated by guanine nucleotide regulatory proteins and changes in phosphoinositide turnover (reviewed in De Franco et al. 1987; Isakov et al. 1987). Another putative mechanism of action may involve potassium channels. Potassium channels are present on T lymphocytes and are activated during delivery of "lethal hits" by cytotoxic T lymphocytes (Russell and Dobos 1983) and during stimulation with mitogens such as phytohemagglutinin and succinyl concanavalin A (DeCoursey et al. 1984; Matteson and Deutsch 1984). Because PCP blocks potassium channels in the CNS (Albuquerque et al. 1981; Blaustein and Ickowicz 1983), and because blockade of these channels can suppress lymphocyte proliferative responses (Decoursey et al. 1984) one can speculate that σ receptors on HPBLs may play a role in regulating immune responses through potassium channels.

The absence of PCP receptors and the localization of σ receptors to specific areas within endocrine tissues suggest probable sites of action at which σ agonists and antagonists may affect endocrine physiology. Using membrane-homogenate binding techniques, other investigators have previously reported *d*-[^{3}H]SKF 10,047 binding sites localized primarily to the anterior lobe

of the pituitary (Tam 1983). In the present autoradiographic study, σ binding sites were evident in all three lobes of the pituitary, but the highest densities were found in the anterior lobe. The anterior lobe of the pituitary contains several distinct cell types that secrete a variety of hormones. The homogeneous distribution of σ binding sites within the anterior lobe probably reflects a scattered distribution of the target cells and suggests a generalized action of σ drugs on anterior pituitary function. Such action is evident from studies in the literature reporting effects of σ receptor agonists including PCP and SKF 10,047 to stimulate adrenocorticotropic hormone (ACTH) secretion (Boggan et al. 1982; Pechnick et al. 1985) and to suppress LH (Pechnick et al. 1985) and prolactin (Pechnick et al. 1985; Saller et al. 1982) secretion. Recently, Pechnick et al. (1987) reported that the PCP-receptor-specific ligand MK-801 stimulated the release of ACTH but did not affect the release of prolactin in the rat.

These data, in conjunction with reported effects of PCP and SKF 10,047 to stimulate ACTH and to suppress prolactin secretion (see above), suggest that the effects of these compounds on ACTH secretion may be mediated through both PCP and σ receptors, while the effects on prolactin secretion appear to be primarily mediated through actions on σ receptors. The absence of high-affinity PCP receptors in the anterior pituitary suggests that these effects may be mediated through high-affinity PCP receptors in the hypothalamus; moderate densities of [^{3}H]TCP-(figure 1B) and [^{3}H]MK-801 -labeled PCP receptors were present in the hypothalamus.

Both σ and PCP receptors appear to be involved in the ability of PCP and SKF 10,047 to suppress LH release. Evidence in support of σ receptors comes from recent studies demonstrating direct actions of PCP and SKF 10,047 to inhibit LH release in anterior pituitary cells (Stojilkovic et al. 1987). The in vivo effects of PCP and SKF 10,047 on LH release may also be mediated through indirect actions on hypothalamic gonadotropin-releasing hormone (GnRH) release. Progesterone, at physiologically relevant concentrations, has been reported to have a relatively high affinity for σ receptors and may represent an endogenous ligand ford receptors (Su et al. 1988). If one assumes that progesterone acts as an agonist at σreceptors, then σ binding sites in the pituitary and hypothalamus may play important roles in the negative feedback effects of progesterone to suppress GnRH and LH release. Recent studies have established that PCP receptors in brain are coupled to glutamate receptors and that PCP acts as an antagonist at this complex (Fagg 1987). Administration of *N*-methyl-D-aspartic acid (NMDA), a glutamate receptor agonist, stimulates LH secretion in rodents and monkeys (Gay and Plant 1987; Price et al. 1978;

Schainker and Cicero 1980) and this secretion can be blocked by administration of the NMDA antagonist *(d,l)*-2-amino-5-phosphonopentanoic acid (AP5) (Arslan et al. 1988; Gay and Plant 1987). It is conceivable that PCP and SKF 10,047 antagonistic actions at the NMDA-PCP receptor complex in hypothalamus may inhibit GnRH and LH release.

A variety of reports in the literature have documented potent effects of haloperidol to alter anterior pituitary hormone secretion. Specifically, haloperidol has been reported to stimulate prolactin, proopiomelanocortin-derived peptide, and corticosterone secretion in rodents and in human subjects (Dickerman et al. 1974; Frantz and Sachar 1976; Höllt et al. 1982; Mueller et al. 1976). Although haloperidol has a high affinity for D_2 dopamine receptors, it has an equally high affinity for σ binding sites. This suggests that some of the effects of haloperidol on anterior pituitary hormone secretion may in fact be modulated through actions at σ receptors. Consistent with this interpretation, σ agonists including PCP and SKF 10,047 have been reported to suppress prolactin secretion (Pechnick et al. 1985; Saller et al. 1982) whereas haloperidol (presumably through antagonistic actions at σ or D_2 dopamine receptors or both) stimulates secretion (Dickerman et al. 1974). In contrast, the ability of haloperidol to stimulate pituitary-adrenocortical secretion (Aimoto et al. 1980) does not appear to be mediated through o-receptor antagonism, because haloperidol produces the same effects as σ agonists, such as PCP and SKF 10,047 (Boggan et al. 1982; Pechnick et al. 1985).

In the adrenal glands, σ receptors were localized primarily in the adrenal cortex, with significantly lower concentrations in the medulla. Because the densities of σ binding sites were not decreased following hypophysectomy, these receptors in the adrenal cortex are probably not present on cells that are dependent on maintenance by trophic pituitary hormones. Therefore, σ binding sites in the adrenal gland do not appear to be localized to glucocorticoid-producing cells. Although o-receptor agonists including PCP and SKF 10,047 have been reported to stimulate corticosterone secretion in mice and in rats (Boggan et al. 1982; Pechnick et al. 1985) these effects appear to be primarily mediated via pituitary ACTH regulation, rather than through direct actions at the adrenal gland, because they can be blocked by dexamethasone administration (Boggan et al. 1982). Chronic PCP administration has been reported to increase adrenal weight in rats (Mielke and Gallant 1982) presumably through sustained ACTH secretion. The role of the σ binding sites in the adrenal gland in modulating other adrenal functions remains to be elucidated.

There was a high density of σ binding sites in the testis. σ Binding sites were uniformly distributed in the walls of the seminiferous tubules and were notably absent in the interstitial areas and the contents of the tubule lumens. Like those in the adrenal cortex, σ receptors in the testis were not altered after hypophysectomy, suggesting that they were not dependent on trophic maintenance from the pituitary gland. The paucity of σ receptors in interstitial areas suggests that σ ligands may not have any direct action on the testis to alter androgen secretion. The high densities of σ receptors in the epididymis suggest that they may serve an important biologic function, possibly in transit of sperm. The marked differences in the density of σ receptors within zones of the epididymis appear to correspond to the tubule size and wall thickness in the different regions of the ductuli efferentes, ductus epididymis, and ductus deferens. On the other hand, the differences in the density of σ receptors in the different areas of the epididymis may correspond to changes in cellular composition of the duct epithelium in the different regions. At present, the distinction between these two possibilities cannot be made and the role of σ receptors in the epididymis remains to be determined. Fairly low densities of σ binding sites were observed in the muscular areas of the ductus deferens. Nevertheless, σ receptors in the vas deferens appear to be functional, as evidenced by potentiation of the vas deferens muscle twitch after exposure to σ agonists (Vaupel and Su 1987).

Of the various endocrine organs examined, the highest density of σ receptors was present in the ovary. Within the ovary, the highest density of σ receptors appeared to be localized to maturing follicles, which were lost in hypophysectomized rats. It is of note that low to moderate concentrations of σ binding sites were also present in quiescent follicles in both normal and hypophysectomized animals, Although the role of σ receptors in the ovary is unclear at present, the high density in this organ is consistent with a recent report by Su et al. (1988) that progesterone has a relatively high affinity for σ receptors.

Given the widespread distribution of σ receptors in the CNS, a significant question is whether the σ binding sites are on elements of neural tissue within endocrine organs or are, in fact, a component of the endocrine cells. Although the resolution of the autoradiographic technique is not adequate to visualize neuronal elements in the endocrine tissues, the distribution pattern of σ binding sites is not consistent with the known location of neurons in the organs studied. For example, σ receptors are present in high density in the anterior lobe of the pituitary, with much lower concentrations present in the neurointermediate lobe.

On the other hand, the neuronal elements of the pituitary gland are primarily localized in the intermediate and posterior lobes. Similarly, in the adrenals, σ receptors are present in higher density in the cortex than in the medulla; the medulla is derived from neural crest. The identity of the receptor-bearing cell(s) within the testis and ovary is not known. However, the high density of σ binding sites in maturing follicles, which decrease significantly after hypophysectomy, suggests localization of these receptors to pituitary-hormone-regulated endocrine cells. The case of PBLs is more clear-cut, because σ receptors were characterized in single-cell suspensions of circulating leukocytes. These suspensions contained no neural cells.

RELEVANCE OF σ RECEPTORS IN THE ENDOCRINE-IMMUNE AXIS

A striking feature of the pattern of receptor distribution that we have observed is that PCP receptors are found only in brain, whereas σ receptors exist both in brain and peripheral tissues. We are unaware of any exceptions to this rule. This pattern is reminiscent of benzodiazepine receptors, which have central and peripheral subtypes that are also differentially distributed between the CNS and peripheral tissues (reviewed in Anholt et al. 1985*a,* 1985*b;* De Souza et al. 1985). However, unlike peripheral benzodiazepine receptors (Anholt et al. 1985*b),* σ receptors in the adrenal and testis are not dependent on trophic maintenance by pituitary hormones.

Another striking feature of these results is the very high densities of σ receptors in HPBLs and rat endocrine tissues. This observation suggests that endogenous σ ligands may play an important physiological role in modulating and integrating immune and endocrine functions. Because isolation and characterization of endogenous PCP (Quirion et al. 1984) and σ (Contreras et al. 1987; Su et al. 1986) ligands is only in preliminary stages at present (see also chapter by Contreras et al. in this volume), it remains to be seen where these endogenous substances are present in the body and by what means they are delivered to target tissues. Furthermore, because lymphokines and endocrine peptides can modulate CNS activity, alteration of the release of hormones and lymphokines/cytokines could be an additional mechanism for the central actions of σ agonists.

PCP induces a schizophrenia-like psychosis (Allen and Young 1978) and the hypothesis has been put forward that in many cases schizophrenia may be caused by genetic defects in PCP receptors, endogenous ligand structure, regulation and expression of receptors and ligands, or alternatively to

autoimmune reactions against these receptors or ligands (Fudenberg et al. 1984). Because we now know that PCP acts at σ as well as at PCP receptors, this hypothesis may be extended to include the σ system. If the hypothesis is true, then some schizophrenics may also have endocrine and immunologic abnormalities caused by the same disease process.

Lymphocytes of schizophrenic patients have been noted to have altered morphology (Erban 1986; Fessel and Hirata-Hibi 1963; Kamp 1962); reduced viability in culture (Erban 1965); and depressed functional activity as measured by such in vitro assays as mitogen-driven proliferation, mixed lymphocyte culture, generation of cytotoxic lymphocytes, and interferon production (Liedeman and Prilipko 1978; Moises et al. 1985; Vartanian et al. 1978). However, these phenomena may reflect both organic disease and drug effects, because haloperidol is widely prescribed as a neuroleptic for these patients. Haloperidol has extensive endocrine effects, which have been discussed. Haloperidol's effect on the immune system appears to be complex. Moises et al. (1985) found that haloperidol in vitro had no effect on proliferation or interferon production by normal human lymphocytes; Goldstein et al. (1980) observed that proliferative responses of schizophrenic patients rebounded to above-normal levels 4 weeks after withdrawal of neuroleptic therapy. In mice, haloperidol suppressed in vivo antibody responses but not contact hypersensitivity (Lovett et al. 1978). The receptor mechanisms involved in the effects of haloperidol are unclear, because haloperidol binds to D_2 dopamine receptors and to α_1-adrenergic receptors (U'Prichard et al. 1977) as well as to σ receptors; [^{3}H]dopamine binding sites were recently detected in rat lymphocytes (Ovadia and Abramsky 1987). Further study is therefore needed to determine what immunologic and endocrine anomalies are an organic part of the schizophrenic disease process and whether haloperidol's effects on these systems are modulated primarily by σ or dopamine receptors.

SUMMARY AND CONCLUSIONS

We have demonstrated the absence of PCP receptors and the presence of σ receptors in PBLs and rat pituitary, adrenal, testis, and ovary that have kinetic and pharmacologic characteristics comparable with the σ receptor found in the CNS. The physiologic significance of the σ receptors in the various lymphoid and endocrine tissues remains to be determined, but their presence in such high densities suggests that endogenous σ ligands may play an important role in regulating and integrating endocrine and immune responses. Thus, in addition to their central actions, σ agonists including PCP and SKF 10,047 may

exert their immunosuppressive and endocrine effects directly through actions in the pituitary and target organs. On the other hand, the effects of selective PCP agonists on endocrine function appear to be mediated primarily through actions in brain. Furthermore, the immunologic and endocrine effects of neuroleptics such as haloperidol, which have previously been attributed primarily to actions at D_2 dopamine receptors, may also be mediated via σ receptors in brain, lymphoid, and endocrine organs. Finally, the immune and neuroendocrine systems may represent useful "windows to the brain" to assess the role of σ and PCP receptors in the CNS.

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A Role for σBinding in the Antipsychotic Profile of BMY 14802?

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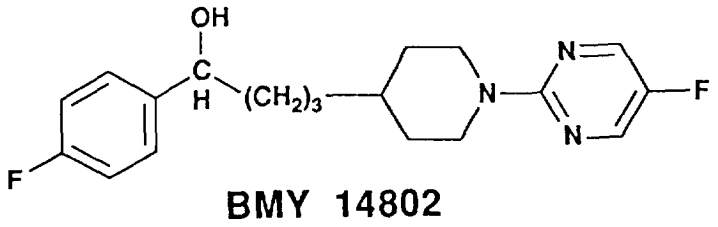
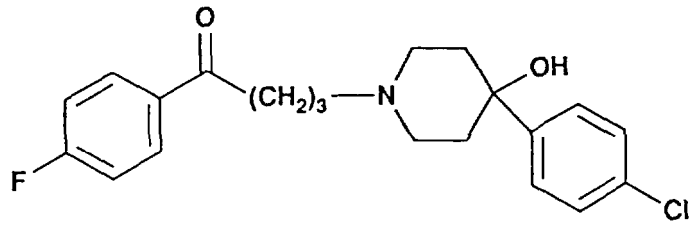
INTRODUCTION

Although the dopamine D-2 antagonists (such as the phenothiazines, thioxanthenes, and butyrophenones) have proven effective in managing of the positive symptoms of schizophrenic disorders, the long-term outlook for patients is not always hopeful. Only 25 percent of patients achieve full recovery with existing drugs, while long-term care is required for another 25 percent of this population. The relapse rate for those for whom drugs are effective averages 40 percent in the first year after an acute illness. Should antipsychotic medication be discontinued at any time in the first 4 years after an acute illness, the relapse rate rises to 65 percent (see Tantam and McGrath 1989 for a review). Moreover, side effects of D-2 dopamine antagonist antipsychotic agents range from antihistaminic and antimuscarinic properties, which are inconvenient and interfere with patient compliance; to tardive dyskinesia, which is debilitating for patients and may put physicians at risk for legal liabilities; to potentially life-threatening effects, such as neuroleptic malignant syndrome, cardiotoxicity, and agranulocytosis.

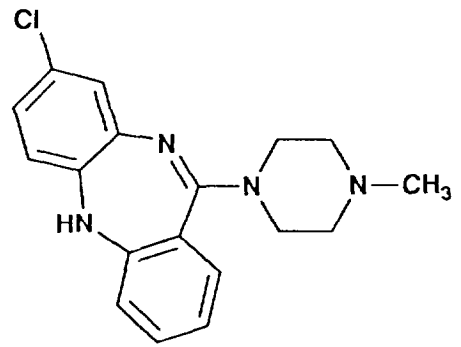
As a consequence of these kinds of drawbacks of existing drugs, many pharmaceutical companies have been engaging in a long-term search for more effective, safer antipsychotic drugs. BMY 14802 is one compound to emerge from this effort (figure 1; Yevich and Lobeck 1986) and it is our purpose to profile this agent pharmacologically in order to explore the potential role of the σ binding site in the treatment of schizophrenia.



BMY 14802



Haloperidol



Clozapine

FIGURE 1. *Chemical structures of current or potential antipsychotics.*

PRECLINICAL PREDICTIONS OF ANTIPSYCHOTIC EFFICACY

BMY 14802 was originally identified as an antipsychotic candidate by its activities in classical neuropharmacologic tests. Among these were the abilities to block the conditioned avoidance response in rats, to inhibit apomorphine-induced stereotypy in rats, and to inhibit apomorphine-induced pole climbing in mice (see table 1; Taylor et al. 1985; Bremer et al. 1989). In general the potency of BMY 14802 in these tests was less than that of haloperidol but equivalent to that of clozapine. In the somewhat more refined discriminated (Sidman) avoidance test, BMY 14802 produced a profile similar to that seen with clozapine in that avoidance was suppressed without a concomitant inhibition of escape performance (see figure 2; Taylor et al. 1985). With the more conventional antipsychotic agent chlorpromazine, a decrement in both of these performance parameters is seen. In addition, BMY 14802 blocks the induction of hyperlocomotion by the dopamine-mimetic agent amfonelic acid (Matthews et al. 1986).

Further persuasive evidence supporting the potential for antipsychotic efficacy comes from work on amphetamine-induced “psychotic” behavior in a primate social colony (Schlemmer and Davis 1986; see table 2). Chronic

TABLE 1. *Activity of selected agents in models that predict antipsychotic efficacy, side effects, or other properties*

Test	BMY 14802	Clozapine	Haloperidol
Inhibition of conditioned avoidance response (ED,, mg/kg, p.o., in rat)	26	27	2.8
Inhibition of apomorphine-induced stereotypy (ED,, mg/kg, p.o., in rat)	33	49	0.5
Inhibition of apomorphine-induced pole climbing (MED, mg/kg, p.o., in mouse}	60	10	1
Induction of catalepsy (ED,,, mg/kg, p.o., in rat)	IA(100)*	IA(200)*	0.6
Reversal of trifluoperazine-induced catalepsy (ED,, mg/kg, p.o., in rat)	17	>20*	-

NOTE: Data from Taylor et al. (1991). MED = minimally effective dose.
*IA = inactive at dose shown in parentheses.

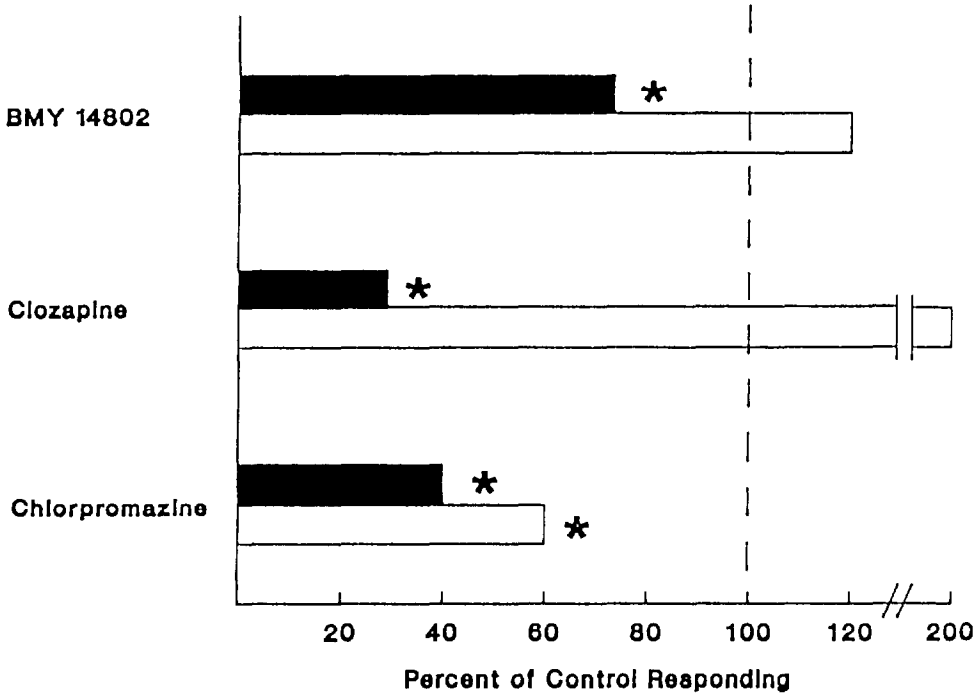


FIGURE 2. *Effects of current and potential antipsychotics on rats in a discriminated avoidance task after dosing at 100 mg/kg, p.o. Solid bars represent avoidance responding and open bars represent escape responding, both as a percent of vehicle-treated controls. Asterisks denote levels of responding that were significantly different from controls at the $p < 0.05$ level. Data from Taylor et al. (1991).*

dextroamphetamine administration (1.6 mg/kg, n.g., b.i.d., 12 days) produces intense scratching, inappropriate submissiveness, increased distancing, decreased initiated social grooming, increased checking or vigilance, and stereotyped behavior in stumptail macaques. Currently available antipsychotics can block all of these manifestations, although the atypical agent clozapine does not alter stereotyped behavior. When BMY 14802 is given concomitantly (5 mg/kg, n.g., b.i.d., 12 days) with amphetamine, it attenuates or significantly reverses all of these behaviors with the exception of stereotyped movements in a profile resembling clozapine's (see figure 3).

TABLE 2. *Possible human behavioral correlates of signs exhibited by macaques in social colonies following chronic amphetamine intoxication*

Behavioral sign	Human correlate
Increase in submissive gestures	Paranoia: perception of aggression or hostility in a nonthreatening situation
Increase in scratching	Scratching in response to tactile hallucinations (parasitosis)
Increase in distancing or spatial isolation	Social withdrawal
Increase in checking or visual scanning	Hypervigilance

Another persuasive line of evidence in predicting the potential antipsychotic efficacy of BMY 14802 comes from the work of Wachtel and White (1987), who showed that chronic administration of BMY 14802 at relatively low doses (5 mg/kg, s.c., 28 days) depressed the number of neurons that fire in the A10 (ventral tegmental) region of the brain in a manner similar to the effect of all clinically effective antipsychotic drugs. However, chronic administration of BMY 14802 does not depress the number of neurons that fire in the A9 (substantia nigra) region, a profile similar to clozapine's and one that is dissimilar to the "neuroleptic" antipsychotics that produce movement disorders as side effects (see next section).

PRECLINICAL PREDICTIONS OF MINIMAL SIDE EFFECT LIABILITY

Unlike the typical antipsychotics, BMY 14802 does not induce catalepsy in rats, a test frequently employed to predict liability for the acute production of extrapyramidal symptoms and the production of tardive dyskinesia after chronic administration (Taylor et al. 1985; see table 1). Clozapine also fails to produce catalepsy. In contrast to clozapine and all conventional antipsychotics, BMY 14802 actually reverses catalepsy induced in rats by the neuroleptic antipsychotics haloperidol and trifluoperazine (Taylor et al. 1985; Matthews et al. 1986; Grimes et al. 1990, and submitted; see table 1). In addition, BMY 14802 produces no movement abnormalities in macaque monkeys (see table 3). This failure to produce abnormal movements cannot be attributed to sedative effects of the drug, as haloperidol, which does produce abnormal

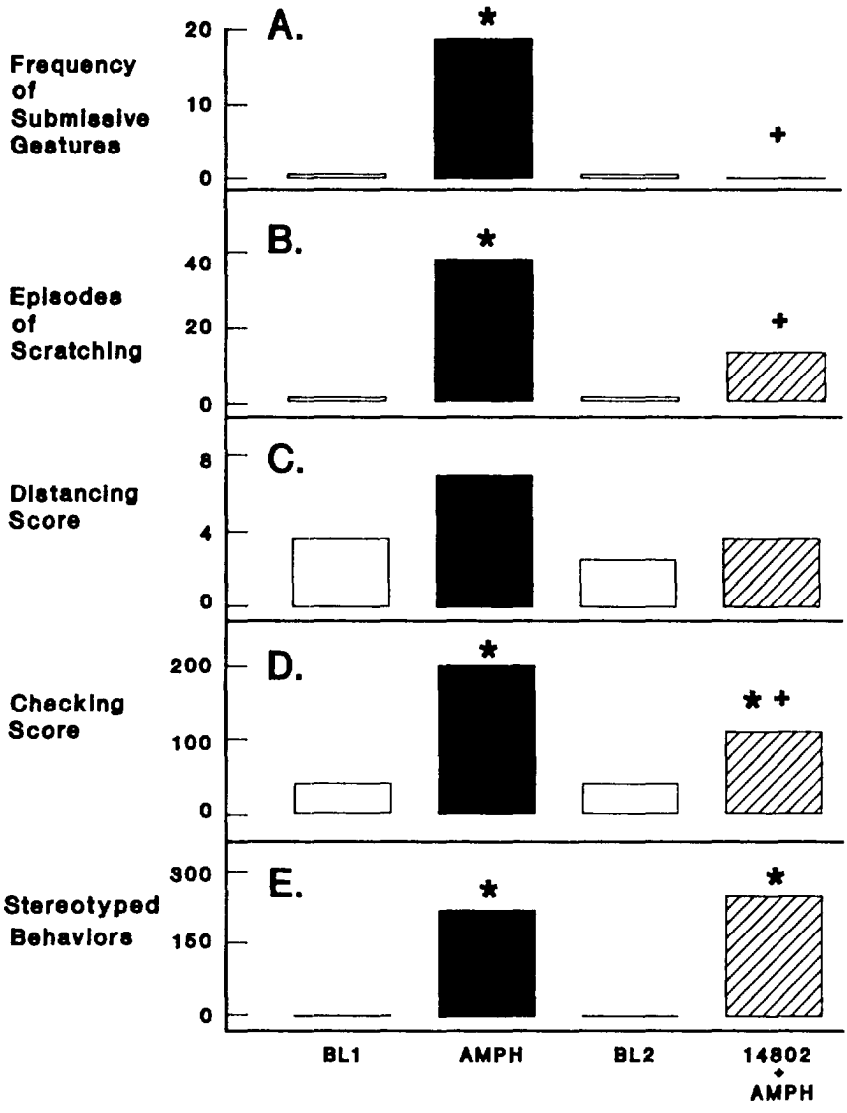

A.
B.
C.
D.
E.
Frequency of Submissive Gestures
Episodes of Scratching
Distancing Score
Checking Score
Stereotyped Behaviors
20
10
0
40
20
0
8
4
0
200
100
0
300
150
0
*
+
BL1
AMPH
BL2
14802 + AMPH

FIGURE 3. *Effects of BMY 14802 on selected behaviors in social colonies of macaque monkeys. Baseline (BL 1) measurements were taken by a blinded observer during two l-hour observation periods. Animals then received 7.6 mg/kg, n.g., (+)amphetamine, b.i.d. for 12 days (AMPH) and were rated for various behaviors each day. After a 4-month washout (minimum), a second set of baseline (BL2) observations was recorded. Following this, BMY 74802, 5 mg/kg, n.g., was administered b.i.d. for 72 days concomitantly with amphetamine by previous regimen (74802 + AMPH). A. Number of submissive gestures displayed by a nondominant female. Data shown are mean values. For AMPH and BMY 74802 + AMPH, data represent means of all observations on days 5 through 8. There was no increase in aggressive gestures received by this animal (not shown). B. Incidence of scratching, distinct from normal se/f-grooming, exhibited by a nondominant female. Data shown are mean values throughout all observation periods. C. Spatial isolation (>3 feet from other monkeys) by a nondominant female. Data shown are mean values. For AMPH and BMY 74802 + AMPH, data represent means of all observations on days 5 through 8. D. Incidence of checking or visual scanning by nondominant females. Data are means of total incidence averaged over four animals. Data for AMPH and BMY 74802 + AMPH represent means of all observations on days 5 through 8. E. Induction of stereotyped behaviors in nondominant females. Data are means of total incidence averaged over four animals. I significantly different from baseline, $p < 0.05$. $^{+}$ significantly different from AMPH alone, $p < 0.05$.*

movements, also increases time spent resting. It is significant that clinical trials have revealed no signs of extrapyramidal symptoms at single doses of BMY 14802 of 800 mg or multiple doses of 500 mg, q.i.d., for 21 days (Shukla et al. 1990).

The ability of conventional D-2 dopamine antagonists to produce D-2 receptor supersensitivity after chronic administration may be correlated with their ability to provoke the disabling and sometimes irreversible syndrome of tardive dyskinesia. Consistent with its atypical profile of effects, chronic administration of BMY 14802 at behaviorally relevant doses produced no change in D-2

TABLE 3. *Effects of agents on time spent in abnormal movements or resting in stumptail macaque monkeys*

Treatment (dose, mg/kg, i.m.)	Abnormal movements (sec)	Resting (sec)	Locomotion (sec)	Checking (number of scans)
Saline	0	17±5	84±26	40 ± 1
Haloperidol (0.1)	68±22*	16±30*	54±28	24±2*
BMY 14802 (5)	0	30±17	103±49	43 ± 1

NOTE: Data are mean ± SEM for observations of four animals. Abnormal movements included bizarre posturing, tremor, rigidity, and slow movements.
*$p < 0.01$ vs saline (three-way partially crossed analysis of variance followed by least significant differences method).

receptor binding while chronic administration of haloperidol did (see table 4; Taylor et al. 1985, 1991).

Also, neuronal inactivation of A9 (substantia nigra) cells after chronic administration of conventional D-2 dopamine antagonist drugs may contribute to the production of tardive dyskinesia. Unlike typical agents such as haloperidol, chlorpromazine, and metoclopramide (an antiemetic that produces extrapyramidal side effects), which produce such inactivation, chronic treatment of rats with BMY 14802 (2.5, 5, or 10 mg/kg/day, s.c., 28 days) produced no change in the firing rate of A9 neurons, resulting in a pattern similar to that seen with the atypical antipsychotic clozapine (Wachtel and White 1987; see above).

TABLE 4. *Effect of chronic drug administration on D-2 dopamine receptor binding in rat striata*

Treatment (dose, mg/kg, p.o.)	B_{max} (fmole/mg protein)
Vehicle	228 ± 5 (5)
BMY 14802 (15)	233 ± 7 (4)
BMY 14802 (30)	212±7(5)
Haloperidol (3)	290 ± 9 (5)*

NOTE: Data are mean ± SEM for number of pooled preparations shown in parentheses. Each pool contained striatal membranes from three rats, B_{mex} was determined by saturation analysis employing [^{3}H]spiperone according to accepted methods (Taylor and Hyslop 1991).
*$p < 0.05$ *vs* vehicle (Student's *t*-test, two-tailed).

ATYPICAL NEUROCHEMICAL PROFILE

Conventional D-2 dopamine antagonists such as haloperidol produce changes in levels of dopamine and its metabolites in both mesolimbic and nigrostriatal dopaminergic pathways (see figure 4; Taylor et al. 1991). The ability of clozapine to selectively affect levels of dopamine and its metabolites in mesolimbic brain regions has been hypothesized to underlie its atypical clinical profile. BMY 14802 produces a clozapine-like profile in its regional effects on levels of the dopamine metabolite dihydroxyphenylacetic acid (DOPAC; see figure 4) in that effects are seen in a mesolimbic region, the nucleus accumbens, at lower doses than those required to produce comparable effects in the striatum. An additional distinction of BMY 14802 over haloperidol is the ability of BMY 14802 to produce significant decreases in levels of dopamine in both brain regions, whereas haloperidol lacks this effect (see figure 4). These data demonstrate a preferential effect of BMY 14802 in mesolimbic brain regions and support predictions of antipsychotic efficacy. The differential effects of BMY 14802 relative to haloperidol on the mesolimbic and nigrostriatal dopamine systems are consistent with the predictions and observations of diminished side-effect liability noted above.

The atypical neurochemical profile of BMY 14802 is consistent with its in vitro interactions with neurotransmitter receptors. Unlike conventional D-2 dopamine antagonist antipsychotics such as haloperidol, BMY 14802 has very low affinity for the D-2 receptor site (table 5; Taylor et al. 1985, 1991; Taylor and Dekleva 1988; Largent et al. 1988). Moreover, in vivo binding studies of serum neuroleptic radioreceptor binding levels and ex vivo studies of [^{3}H]spiperone binding in the striata of rats receiving BMY 14802 suggest that biologically relevant levels of metabolites of the drug, which may interact at D-2 dopamine binding sites, are not generated (Taylor et al. 1985). These data are consistent with the inability of chronic BMY 14802 administration to induce the proliferation of D-2 dopamine receptor binding sites as noted above. Furthermore, BMY 14802 does not bind to D-l dopamine receptors nor inhibit dopamine-stimulated adenylate cyclase in the rat striatum (Yocca et al. 1986; Taylor et al. 1991). Finally, BMY 14802 displays low affinity for a variety of other receptor sites: α_1-, α_2-, and β--adrenergic sites; benzodiazepine and γ- aminobutyric acid sites; glutamate (including *N*-methyl-D-aspartate, kainate, and quisqualate types) and glycine (including both strychnine-sensitive and insensitive types) sites; μ and κ opiate sites, the dihydropyridine calcium channel antagonist site; and 5-HT$_2$ serotonergic sites (Taylor et al. 1985).

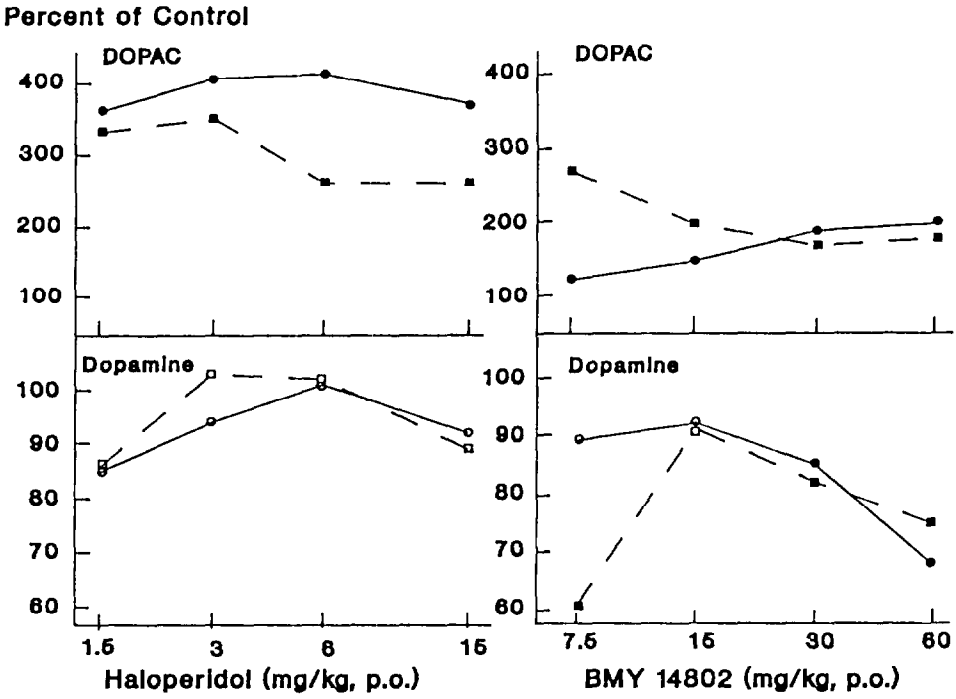


FIGURE 4. *Effects of BMY 14802 and haloperidol on levels of DOPAC and dopamine in selected brain regions. Tissues were dissected one hour after drug administration, and DOPAC and dopamine levels were determined by high-pressure liquid chromatography with electrochemical detection. Regions investigated were striatum (circles, solid lines) and nucleus accumbens (squares, broken lines). Filled figures denote values that were significantly different from vehicle-treated animals ($p < 0.05$, Student's t-test, two-tailed). All values for DOPAC produced by all doses of either drug were significant/y different from vehicle. Adapted from Taylor et al. (1991).*

TABLE 5. *Inhibition of in vitro radioligand binding by selected agents*

[^{3}H] Ligand:	spiperone	SCH 23390	spiperone	dizocilpine	glycine
Site labeled:	D-2 dopamine	D-1 dopamine	5-HT$_2$ serotonin	phencyclidine	glycine
Tissue used:	striatum	striatum	cortex	cortex	cortex
Compound					
BMY 14802	6,400	>100,000	1,700	>100,000	>100,000
Haloperidol	1.1	860	61	18,000	-
Clozapine	570	1,100	21	9,100	-

NOTE: Data are IC_{50} values in nM. Adapted from Taylor et al. (1965, 1991).

INTERACTIONS OF BMY 14802 AT THE σ BINDING SITE

Although the mechanism by which BMY 14802 achieves its various biologic effects is unclear, a large body of evidence suggests that it may have an action at the so-called σ binding site (see table 6; Taylor and Dekleva 1987; Taylor et al. 1991). BMY 14802 exhibits high affinity in vitro for σ binding sites in guinea pig brain (Taylor and Dekleva 1987) as well as rat brain (McCann et al. 1989; Koe et al. 1989; Largent et al. 1988; Schmidt et al. 1989; Steinfels and Tam 1989). [^{3}H]BMY 14802 appears to label σ sites specifically in guinea pig brain "sausage patties" (slices of frozen, minced tissues) with high affinity (K_D = 73 nM, specific binding = 86 percent at 20 nM radioligand). The rank order of potencies of various drugs to inhibit [^{3}H]BMY 14802 binding is consistent with the ranking of these drugs in inhibition of binding employing other σ radioligands (see table 6; Taylor et al. 1991).

In addition, in vitro labeling of brain slices with [^{3}H]BMY 14802 revealed a distribution of binding with many similarities to the localization of (+)-[^{3}H]-3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)-[^{3}H]3-PPP) labeling, especially in cortical regions, hippocampus, cerebellar cortex, hypothalamus, bed nucleus of the stria terminalis, periaqueductal grey, and superficial layers of the superior colliculus (Taylor et al. 1991; Moon 1987; Gundlach et al. 1986). Furthermore, BMY 14802 exhibits stereospecificity at the σ site, with the R-dextrorotatory enantiomer being more potent than the S-levorotatory one (Taylor and Dekleva 1987; Behling et al. 1990; Taylor et al. 1989, 1991).

TABLE 6. *Inhibition of radioligand binding by selected agents*

	In vitro assays, IC_{50} (nM)				In vivo assays, ED, (µmole/kg)		
[^{3}H]Ligand: Compound	NAN[a]	(+)3-PPP[b]	DTG[C]	BMY 14802	NAN	(+)3-PPP	DTG
Haloperidol	2.0	1.4	2	9.4	2	0.13	0.3
DTG	5.9	19	22	8.6	-	6	150
(+)3-PPP	28	24	91	-	2	-	74
R(+)BMY 14802	43	28	32	-	-	-	-
(±)BMY 14802	83	112	-	33	9	13	59
S(-)BMY 14802	420	310	140	-	-	-	-
(+)NAN	42	79	620	-	0.1	-	63
(-)NAN	600	2000	3900	8600	6.1	-	310
Rimcazole	1100	1000	-	-	19	24	-

NOTE: In vitro data are for binding to membranes from whole guinea pig brains (adapted from Taylor and Dekleva 1987, 1988; Taylor et al. 1989, 1991). In vivo data are for binding to membranes from mouse brains and are adapted from Ferris et al. (1988) and R.M. Ferris (personal communication) for NAN, from Koe et al. (1989) for (+)3-PPP, and binding to rat brain membranes for DTG, from Singh et al. (1990). The data for in vivo binding of DTG were derived assuming inhibition occurred in a linear fashion according to the law of mass action for a single class of noninteracting sites.

[a] *N*-allyl-*N*-normetazocine (SKF 10,047).

[b](+)-3-[3-hydroxyphenyl]- *N*-(l-propyl)piperidine.

[C]1,3-di-o-tolylguanidine.

The inhibition of in vitro binding of (+)-[^{3}H]-*N*-allylnormetazocine((+)-[^{3}H]NAN, (+)-SKF 10,047) and (+)-[^{3}H]3-PPP by (+)BMY 14802 is competitive, although its interaction at the site labeled by [^{3}H]-1,3-di-o-tolylguanidine ([^{3}H]DTG) is more complex (see table 7). These data suggested that [^{3}H]DTG labeled more sites than (+)-[^{3}H]3-PPP or (+)-[^{3}H]NAN, but the membrane preparations used in these studies were different. When a purified P_2 membrane (Weber et al. 1986) preparation of guinea pig brain was studied in a pairwise fashion in saturation analyses, we found that a significantly greater number of sites were labeled by [^{3}H]DTG than by (+)-[^{3}H]3-PPP (B_{max} values of 53 ± 12 and 20 ± 3 pmol/g wet weight, respectively, for three preparations; $p < 0.05$, Student's t-test for paired observations, two-tailed). These findings are consistent with preliminary observations from a number of laboratories that suggest the existence of multiple types and/or states of the σ site (Naper et al. 1989; Arnold

TABLE 7. *Effect of R(+)BMY 14802 on in vitro radioligand equilibrium binding parameters at σ sites in guinea pig brain membranes*

		Addition	
Radioligand	Parameter	None	R(+)BMY 14802
(+)NAN	K_D	43	110*
	B_{max}	17	20
(+)3-PPP	K_D	23	82*
	B_{max}	29	35
DTG	K_D	51	120*
	B_{max}	40	28*

NOTE: For equilibrium analyses using (+)-[^{3}H]NAN, the concentration of R(+)BMY 14802 was 50 nM (Taylor and Dekleva 1987). For studies using (+)-[^{3}H]3-PPP and [^{3}H]DTG, the concentration of R(+)BMY 14802 was 30 nM.
*p < 0.05 vs no addition (Students t-test for paired observations, two-tailed).

TABLE 8. *Characteristics of types of σ receptors*

	Receptor type	
Property	σ_1	σ_2
Present in		
Guinea pig CNS?	Yes	Yes
Rat CNS?	No	Yes
PC-12 cells?[a]	No	Yes
Labeled by		
(+)-[^{3}H]3-PPP?	Yes	Yes
[^{3}H]DTG?	Yes	Yes
(+)-[^{3}H]benzomorphans?	Yes	No
Estimated molecular weights (kD)[b]	28 to 29	63,65

NOTE: This nomenclature is for discussion purposes only (taken from Reid et al. 1988).
[a]Pharmacologic studies are discussed by Bowen et al. (1988).
[b]Molecular weights are from target size analyses presented by Arnold et al. (1988).

et al. 1988; Bowen and Hellewell 1988; Hellewell and Bowen 1988; Reid et al. 1988; Wu et al. 1990; see table 8).

Evidence from in vivo binding studies supports an interaction of BMY 14802 with σ sites. Ferris et al. (1988) used (+)-[^{3}H]NAN to label these sites in mouse brain, and the position of BMY 14802 in the rank order of potency after systemic administration is consistent with its potency in in vitro binding studies (R.M. Ferris, personal communication; see table 6). A similar result was obtained by Koe et al. (1989) using (+)-[^{3}H]3-PPP to label sites in mouse brain (see table 6). Recently, Singh et al. (1990) were able to inhibit the in vivo binding of [^{3}H]DTG to σ sites in rat brain with BMY 14802 (see table 6).

Identifying a physiological function or behavior that is mediated by the σ binding site has been problematic. However, several model systems have recently emerged, and data from them are consistent with an interaction of BMY 14802 at σ sites. For instance, Bremer et al. (1989) noted that chronic administration of haloperidol decreased the maximal number of binding sites for (+)-[^{3}H]3-PPP in rat brain while chronic administration of BMY 14802 increased this equilibrium parameter. These opposite effects suggest that in some systems one may be a σ "agonist" and the other an "antagonist" (see below). Recently, BMY 14802 was shown to block the NMDA-evoked release of acetylcholine from rat striatal slices in vitro, and this property was shared by the σ ligands sabeluzole, haloperidol, (+)3-PPP, and DTG (Ryan et al. 1990).

Steinfels and Tam (1989) showed in physiological studies that the dose-dependent inhibition of dopaminergic neurons in the substantia nigra evoked by (+)-3-PPP could be completely reversed by BMY 14802. In addition, pretreatment with BMY 14802 shifted the (+)-3-PPP dose-response curve to the right, suggesting an agonist-antagonist relationship between the two compounds. Monitoring extracellular recording in the CA_3 region of dorsal hippocampal pyramidal neurons in rats, Monnet and coworkers determined that DTG and JO-1784, another σ ligand, could enhance the excitatory effect of NMDA, and this enhancement was blocked by the σ ligands haloperidol, BMY 14802, and rimcazole but not the D-2 dopamine antagonists spiperone or sulpiride (Monnet et al. 1988, 1989; Debonnel et al. 1990). In the guinea pig ileal longitudinal muscle/myenteric plexus BMY 14802, DTG and haloperidol were found to inhibit serotonin- or electrically evoked muscle twitching, whereas (+)-NAN and (+)cyclazocine potentiated such twitching (Campbell et al. 1989). These authors suggest that the inhibitory σ ligands may act by inhibiting acetylcholine release in this preparation. This explanation would be consistent with the observations of Ryan et al. (1990; see above). In the perfused rat tail artery Massamiri and Duckles (1990) observed that the order of potency for σ

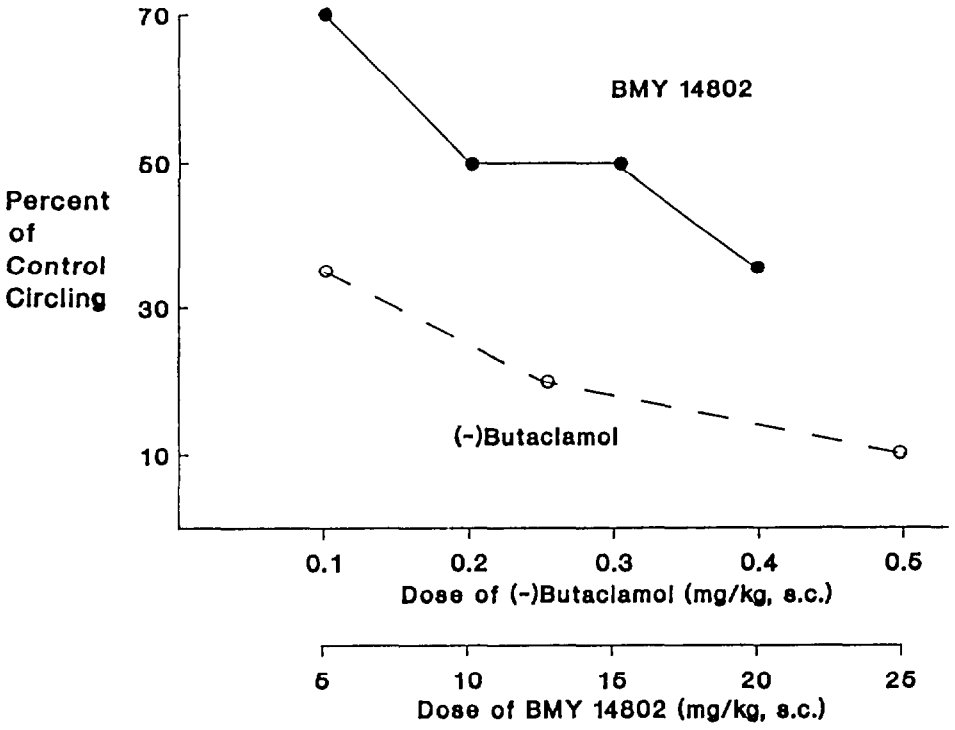


FIGURE 5. *Effect of drugs on (+)NAN-induced stereotyped circling in mice. Saline or drug was administered 30 (BMY 14802) or 40 minutes [(-)butaclamol] prior to testing. A 15 mg/kg, s.c., dose of (+)NAN was administered 10 minutes prior to testing. Testing consisted of a 10-minute observation period during which time circling activity was assessed. Vehicle circling rates were 47±4 movements/10 min for (-)butaclamol (n = 5 at all doses) and 29 ± 3 movements/10 min for BMY 14802 (n = 8 at all doses). Further details are found in Gallant (1988).*

ligands, including BMY 14802, for the inhibition of contractile responses to norepinephrine parallels their order of potency for the inhibition of in vitro σbinding.

Grimes et al. (1990) have noted In intact animals that the σ ligands rimcazole and (+)-3-PPP as well as BMY 14802 reversed catalepsy induced by haloperidol or trifluoperazine in rats, while DTG facilitated the effects of

TABLE 9. *Is BMY 14802 a σ '"antagonist"?*

System	Effect of BMY 14802 is mimicked by:	BMY 14802 blocks, reverses, or its effects are opposite to effects of:
σ Binding after chronic treatment (Bremer et al. 1989)	-	haloperidol
Attenuation of dopaminergic cell firing (Steinfels and Tam 1989)	-	(+)3-PPP
NMDA-induced increase in CA^3 pyramidal cell firing (Monnet et al. 1988, 1989; Debonnel et al. 1990)	rimcazole haloperidol	DTG JO-1784
Muscle twitching in guinea pig ileum (Campbell et al. 1989)	haloperidol DTG	(+)NAN (+)cyclazocine
Reversal of neuroleptic-induced catalepsy (Grimes et al. 1990)	rimcazole (+)3-PPP	DTG
Blockade of stereotyped behavior (Iyengar et al. 1989)	-	(+)NAN rimcazole
Blockade of circling	(-)butaclamol	(+)NAN

subthreshold doses of the neuroleptics. In addition, we have recently observed that BMY 14802 attenuated circling induced by (+)NAN in mice, an effect mimicked by (-)butaclamol (see figure 5). Likewise, Iyengar et al. (1989) observed that BMY 14802 antagonized (+)NAN-evoked behaviors, such as stereotypy and ataxia, in rats. Finally, Iwamoto (1989) observed that BMY 14802, as well as haloperidol and rimcazole, antagonized the so-called "σ syndrome," particularly retropulsion and sideways circling, in rats. Data such as these surely beg the question concerning the role of BMY 14802 as a potential σ ' "antagonist." A consideration of these data (collected in table 9) leads to the realization that no other agent uniformly mimics the effects of BMY 14802, although (+)NAN appears to consistently produce opposite effects. It would appear to be premature at this time to term BMY 14802 a σ '"antagonist," especially in view of the poorly characterized physiologic actions of σ ligands.

ADDITIONAL PROPERTIES OF BMY 14802

As part of our ongoing effort to identify compounds active in models of cerebral ischemia and stroke, we routinely tested experimental agents in clinical development for their ability to prevent nitrogen anoxia-induced lethality in rats, to antagonize *N*-methyl-D-aspartic acid-(NMDA) induced convulsions in mice, and to attenuate hippocampal neuronal damage after bilateral carotid occlusion in the gerbil.

BMY 14802 exhibited activity in these models. Specifically, when given 15 minutes prior to a 1-minute exposure to a 100 percent nitrogen atmosphere, a treatment that normally kills all rats so exposed, a 50 mg/kg, i.p., dose of BMY 14802 protected all animals receiving the drug (see figure 6). Other compounds, (e.g., haloperidol, sabeluzole, and ifenprodil) that bind to the σ site (Taylor et al. 1990; Koe et al. 1990; Karbon et al. 1990; Contreras et al. 1990) were also active in this model. In addition, 20 mg/kg, s.c., BMY 14802 significantly increased the dose of NMDA required to produce convulsions in mice (see figure 7, table 10). The σ ligand sabeluzole was also active in this model, although haloperidol was not. Moreover, Moon et al. (1990) have shown that 25 mg/kg, i.p., BMY 14802 significantly attenuated the extent of hippocampal lesions in gerbils subjected to bilateral carotid occlusion/ reperfusion (see table 11). Haloperidol also was active in this model, but ifenprodil and sabeluzole were not. However, when given subacutely for 3 additional days after surgery, a significant attenuation in damage was seen with sabeluzole. Further evidence of neuroprotective properties for BMY 14802 derived from the observation that this agent attenuated the NMDA-evoked release of acetylcholine from rat striatal slices in vitro in a noncompetitive fashion (Ryan et al. 1990, see above). Finally, Rao et al. (1990) have noted that BMY 14802 reversed harmaline-induced elevations in cerebellar cyclic GMP levels.

That σ ligands could modulate NMDA-mediated events was suggested by Monnet et al. (1988, 1989) who demonstrated that BMY 14802 blocked enhancement by DTG of the excitatory effect of NMDA on CA_3 hippocampal pyramidal neurons (Debonnel et al. 1990). Unfortunately, further review of the neuroprotective properties of other σ ligands, such as dextromethorphan, (+)NAN, (+)3-PPP, DTG and its analogs, and haloperidol, lies outside the scope of this article. However, the "σ enigma" that these agents may play a role in other physiologic responses in addition to schizophrenia is under active investigation by many laboratories in addition to those referenced here.

TABLE 10. *Effect of drug treatment on convulsant threshold (CD_{50}) of NMDA in mice*

Drug	Dose (mg/kg, s.c.)	CD_{50}, controls	CD_{50}, drug animals
Dizocilpine	1	117(103 to 131)	Complete protection[a]
BMY 14802	20	98 (88 to 109)	143(114 to 360)
Haloperidol	0.5	121 (100 to 139)	133(117 to 150)
Sabeluzole	5	113 (94 to 128)	179[b]

NOTE: CD_{50} values are for NMDA (mg/kg, i.p.) with 95 percent fiducial limits given in parentheses. Values represent data obtained from 24 to 46 animals per group (from Taylor et al. 1990; Moon et al. 1990).

[a]Animals receiving dizocilpine exhibited no thrashing convulsions, and no deaths occurred after receiving NMDA. However, those animals did engage in 'popcorn" convulsions immediately following administration of dizocilpine.

TABLE 11. *Effect of drug pretreatment on extent of lesions of the hippocampus of gerbils following bilateral carotid occlusion and reperfusion*

Drug	Dose (mg/kg, i.p.)	Control score	Drug score
Dizocilpine	1	1.86 ± 0.09 (7)	0.78 ± 0.32 (6)[a]
BMY 14802	10	1.86 ± 0.09 (7)	1.50 ± 0.22 (5)
	25	1.86 ± 0.09 (7)	0.69 ± 0.25 (7)[a]
Haloperidol	0.1	3.55 ± 0.45 (7)	2.28 ± 0.32 (6)[a]
Ifenprodil	25	1.66 ± 0.24 (8)	1.19 ± 0.33 (5)[a]
Sabeluzole	5	3.10 ± 0.47 (7)	2.56 ± 0.43 (6)[a]
	5[b]	1.85 ± 0.30 (9)	0.81 ± 0.24 (9)[a]

NOTE: Data are mean score ± SEM for the number of animals given in parentheses. Drugs were given at the indicated doses 1 hour before and after surgery. Artery clamps, used to achieve occlusion, were released after a 15-minute period, and reperfusion/recovery proceeded for 4 subsequent days. Slices through the hippocampal level were evaluated on a scale of integers denoting no damage (0) to total damage (4). Scores from slices for each animal were averaged, and the mean of these average scores is reported here. Statistical comparisons were conducted using the Mann-Whitney U-test, two-tailed (from Moon et al. 1990).

[a]$p < 0.05$ *vs* respective control score.

[b]Dose also given q.d. for three additional days.

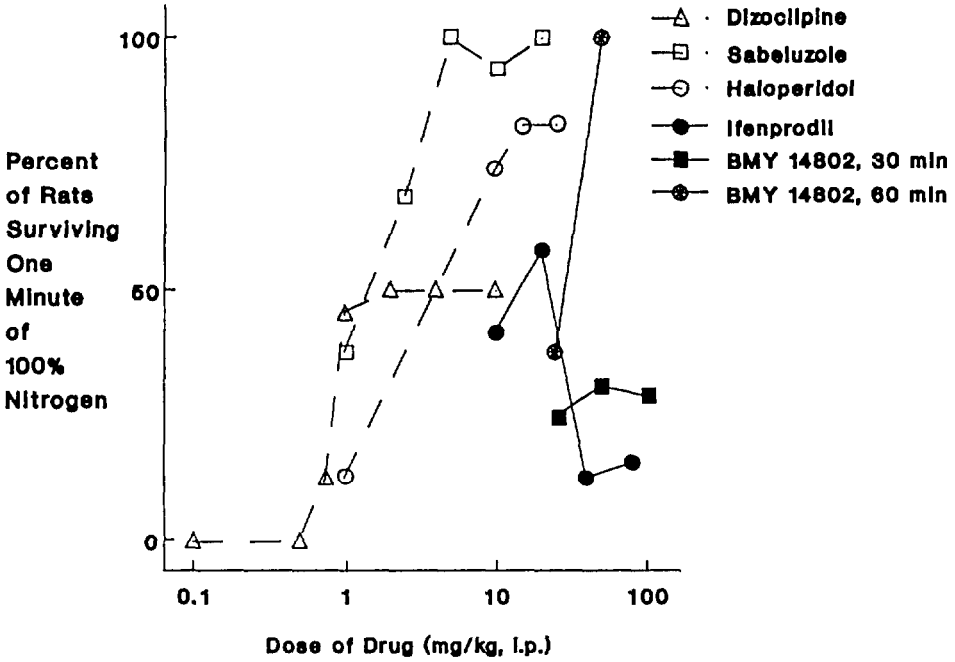


FIGURE 6. *Effect of selected agents on nitrogen-anoxia-induced lethality. Groups of rats (8 to 72) were administered a drug, i.p., 30 or 15 minutes (BMY 14802 only, as indicated) prior to exposure to a 100 percent nitrogen atmosphere for 1 minute. This exposure kills 100 percent of untreated animals. The percent of treated animals surviving was evaluated at 2 hours post-anoxia (from Taylor et al. 1990; Moon et al. 7990).*

CAVEATS

Before reaching the conclusion that the many interesting, even exciting, effects of BMY 14802 are solely due to its action at a sites, it is important to note two additional properties of the agent. These are its ability to act as a partial agonist at 5-HT_{1A} receptors and the potential for its metabolism to a molecule with D-2 antagonist properties.

Like another pyrimidinylpiperazine, the anxiolytic drug buspirone (Taylor 1988) BMY 14802 binds to 5-HT_{1A} receptors (tables 12 and 13). That BMY 14802 may

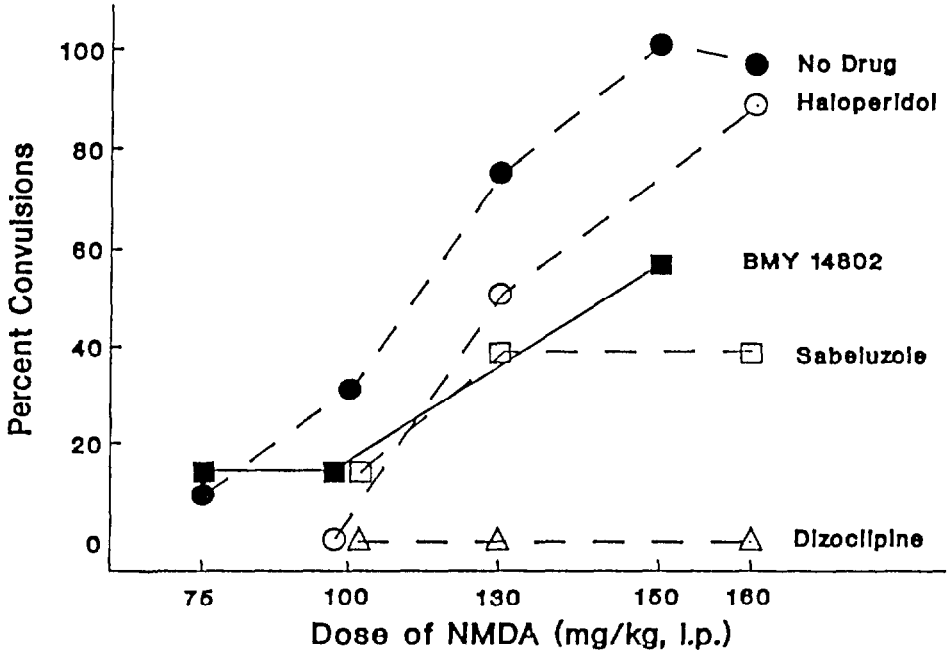


FIGURE 7. *Effect of selected agents on NMDA-induced convulsions. Groups of mice (8 to 42) were administered a drug, s.c., 15 minutes prior to doses of NMDA and observed for 60 minutes for clonic or tonic convulsions. The dose producing convulsions in 50 percent of animals, the CD_{50}, and its 95 percent fiducial limits were calculated using a log-logit transform and linear regression. See table 10 for doses of drugs (from Taylor et al. 1990; Moon et al. 1990).*

act as a 5-HT_{1A} agonist has been suggested by the work of VanderMaelen and Braselton (1990), who showed that the compound can inhibit the firing of serotonergic cells in the dorsal raphe nucleus with a potency ratio compared to buspirone that was close to that for binding to the 5-HT_{1A} receptor (see table 12). In addition, like buspirone, BMY 14802 can produce signs of the serotonin syndrome in reserpinized rats.

TABLE 12. *Comparison of activities of BMY 14802 and buspirone in selected biological tests*

Test	Buspirone	BMY 14802	Ratio
Inhibition of [^{3}H]DPAT binding to 5-HT_{1A} sites in vitro, IC_{50} (nM)	26	320	12
Inhibition of serotonergic cell firing in rat dorsal raphe nucleus, ED, (mg/kg, i.v.)	0.011 [a]	0.19[b]	17
Production of 5-HT syndrome in reserpinized rat, agonist index[C] (dose, mg/kg, p.o.)	0.29 (10)	0.65 (50)	—
Inhibition of 5-MeODMT-induced 5-HT syndrome in reserpinized rat, antagonist index[d] (dose, mg/kg, p.o.)	0.85 (10)	0.40 (50)	—
Discriminable stimulus in pigeon (mg/kg, i.m.)[e]	0.056	5.6	100
Inhibition of intraspecies ag ression in mice, ED_{50} (µmol/kg, i.p.)[f]	9	64	7
Reversal of neuroleptic-induced catalepsy:			
Trifluoperazine, ED_{50} (mg/kg, p.o.)	3.6[g]	17	4.7
Haloperidol, drug dose x score[h]	0.87	16.9	19.4
Inhibition of (+)-[^{3}H]-3-PPP binding to σ sites in vitro, IC_{50} (nM)	61	110	1.8
Inhibition of conditioned avoidance responding, ED, (mg/kg, p.o.)	48[i]	26	0.5
Inhibition of apomorphine-induced stereotypy in rats, ED, (mgkg, p.o.)	28[j]	26	0.93

[a]From VanderMaelen et al. 1986.
[b]From VanderMaelen and Braselton 1990.
[c]Agonist index: a measure from 0 to 1.0, where 0 is no forepaw treading and 1.0 is forepaw treading equivalent to that produced by 5 mg/kg, s.c., 5-MeODMT.
[d]Antagonist index: a measure ranging from 0 to 1.0, where 1.0 is complete antagonism of the forepaw treading produced by 5 mg/kg, s.c., 5-MeODMT, and 0 is no antagonism of the forepaw treading.
[e]From Vanecek et al. 1989.
[f]From McMillen et al. 1988*a*.
[g]From New et al. 1986.
[h]From McMillen et al. 1988*b*.
[i]From Yevich et al. 1983.

TABLE 13. *Inhibition of in vitro radioligand binding by BMY 14802 and BMY 14786*

Binding site	R(+)BMY 14802	(±)BMY 14802	S(-)BMY 14802	BMY 14786
σ	28	110	310	140
5-HT_{1A}	210	320	340	>1000
α_1	610	460	570	29
D-2	>1000	6400	>1000	490

NOTE: Data are mean K_i or IC_{50} (nM).σ binding assays were performed using (+)-[^{3}H]3-PPP and membranes from whole guinea pig brain. 5-HT_{1A}, α_1, and D-2 binding assays were performed using membranes from rat brain hippocampus, cortex, and striatum, respectively (from Behling et al. 1990).

Based on its ability to partially block the serotonin syndrome induced by 5-methoxydimethyltryptamine (5MeODMT) in reserpinized rats, BMY 14802 appears to behave as a partial agonist at 5-HT_{1A} receptors. Moreover, in pigeons, BMY 14802 can serve as a discriminative stimulus training cue to which buspirone will generalize (Vanecek et al. 1989). McMillen et al. (1988*a, b)* have suggested that the ability of buspirone and BMY 14802 to inhibit intraspecies aggression in mice and to reverse haloperidol-induced catalepsy in rats is a result of the affinity of these agents for 5-HT_{1A} receptors and probable partial agonist action at those sites (see table 12). Moreover, buspirone and BMY 14802 also reverse trifluoperazine-induced catalepsy. For many of these actions, the ratio of effective doses of buspirone and BMY 14802 is similar to the ratio of their affinities for the 5-HT_{1A} receptor (see table 12).

While these results suggest that at least some actions of BMY 14802 are produced by its partial agonism at 5-HT_{1A} receptors, they do not obviate a role for action at the σ"receptor" in the potential antipsychotic effect of the drug. For instance, Grimes et al. (1990 and submitted) recently showed that σ ligands, such as (+)3-PPP and rimcazole, which do not possess affinity for 5-HT_{1A} receptors, can also reverse catalepsy induced by both trifluoperazine and haloperidol. While buspirone is slightly more potent than (±)BMY 14802 in its affinity for σ binding sites in vitro, it is less active in the traditional animal tests that identified BMY 14802 as an antipsychotic candidate, the inhibition of the conditioned avoidance response and apomorphine-induced stereotypy (see table 12). In this regard it is useful to recall that in a single trial in schizophrenics the efficacy of the 5-HT_{1A} partial agonist buspirone was doubtful (Sathananthan et al. 1975).

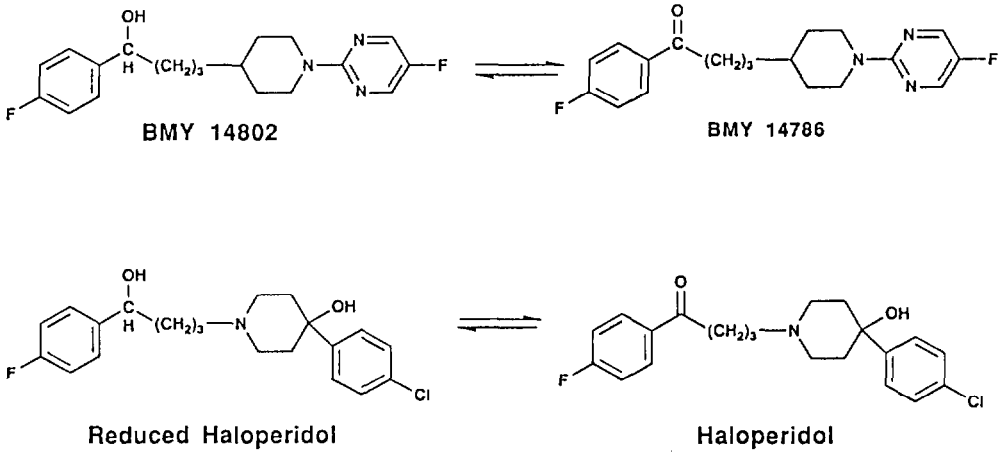


FIGURE 8. *Structures of BMY 14802 and its precursor, BMY 74786, and of haloperidol and its metabolite, reduced haloperidol.*

BMY 14802 was originally prepared by reduction of a ketone precursor, BMY 14786 (figure 8). This reaction is analogous to the reduction of haloperidol to the carbinol metabolite formed in humans (figure 8; Forsman and Larsson 1978). Since reduced haloperidol may be oxidized to haloperidol in humans (Midha et al. 1987) it was of interest to us to characterize the pharmacologic profile of BMY 14786 and to determine if significant levels are formed in vivo after administration of BMY 14802 in order to evaluate if this compound might contribute to the in vivo effects seen after administration of the parent compound.

The receptor binding profile of BMY 14786 is compared to that for the resolved enantiomers of BMY 14802 as well as the racemic mixture in table 13. Like BMY 14802, BMY 14786 exhibits affinity for the σ binding site. This observation is in parallel with the recent finding of Bowen et al. (1990) that both haloperidol and reduced haloperidol exhibit affinity for σ binding sites. Unlike BMY 14802, BMY 14786 has low affinity for 5-HT_{1A} receptors but high affinity for α_1 adrenergic receptors. Finally, BMY 14786 exhibits modest affinity for D-2 dopamine receptors.

The behavioral consequences of this different profile for BMY 14786 are shown in table 14. As previously noted, the R dextrorotatory enantiomer of BMY 14802 is more potent than S(-)BMY 14802 in inhibition of the conditioned avoidance

response and reversal of trifluoperazine-induced catalepsy, whereas S(-)BMY 14802 is more potent in inhibition of apomorphine-induced stereotypy (Taylor et al. 1989). BMY 14786 is about as potent as S4(-)BMY 14802 in inhibition of the conditioned avoidance response and much less potent than this enantiomer in inhibition of apomorphine-induced stereotypy. At high doses BMY 14786 produces catalepsy in rats. Because such a finding might be of concern if BMY 14786 were formed in vivo, we undertook an investigation on the formation of bioactive metabolites.

After acute administration of BMY 14862, a serum radioreceptor assay for D-2 dopamine binding activity as well as ex vivo binding studies of D-2 dopamine binding levels in the striata of rats at behaviorally relevant doses and time points failed to support the hypothesis that significant levels of such metabolites were formed (Taylor et al. 1985). Moreover, chronic administration of BMY 14802 failed to alter D-2 dopamine receptor binding (table 4).

TABLE 14. *Effects of BMY 14802 and BMY 14786 in selected behavioral tests*

Test	R(+)BMY 14802	(±)BMY 14802	S(-)BMY 14802	BMY 14786
Inhibition of conditioned avoidance responding	22[a]	26 (20 to 35)	39 (30 to 50)	36 (27 to 49)
Inhibition of apomorphine-induced stereotypy	44 (38 to 50)	33 (23 to 48)	25 (17 to 37)	59 (48 to 73)
Induction of catalepsy	>50[b]	>100[b]	>50[b]	144[a]
Reversal of trifluoperazine-induced catalepsy	11 (7 to 19)	17 (11 to 26)	38 (27 to 54)	not tested

NOTE: Data are ED_{50} values (mg/kg. p.o.), with 95 percent fiducial limits given in parentheses, for tests in rats (from Behling et al. 1990).
[a]Distribution of data not amenable to calculation of fiducial limits
[b]No catalepsy observed at these doses.

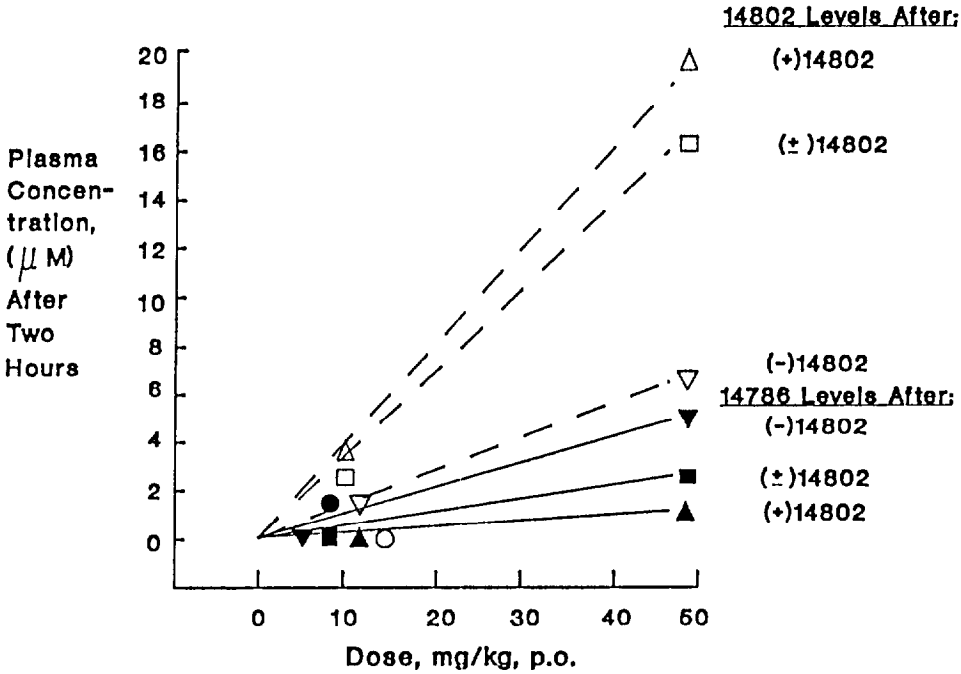


FIGURE 9. *Effect of administration of BMY 14802 or BMY 14786 on levels of either agent in rat plasma. Groups of 4 to 6 rats received doses of drug orally. Plasma fractions were prepared from blood samples taken from jugular vein cannulae (50 mg/kg doses) or by cardiac puncture (10 mg/kg doses). BMY 74802 and BMY 14786 were assayed by reverse phase HPLC with fluorescence detection. Levels of BMY 14802 are depicted as open symbols, while levels of BMY 14786 are depicted as filled symbols. Dosing occurred with R(+)BMY 14802 (upright triangles), racemic BMY 14802 (squares), S(-)BMY 14802 (inverted triangles), or BMY 14786 (circles).*

More recently we have directly assayed the plasma of rats for levels of BMY 14786 and BMY 14802 after administration of either agent (figure 9). These studies have revealed that levels of BMY 14802 and BMY 14786 found after dosing with the resolved enantiomers or the racemate appear to be dose proportional. In addition BMY 14802 was found in plasma after administration of

the following drugs (descending order): R(+)BMY 14802, (±)BMY 14802, S(-)BMY 14802, and BMY 14786. Furthemore, BMY 14786 was found in plasma after administration of the same drugs, but there was a reversal in the order of these drugs with respect to how much BMY 14786 was detected. These observations permitted us to conclude that BMY 14802 and BMY 14786 are reversibly metabolized in the rat.

Recent phase 1 safety, tolerance, and pharmacokinetic studies in humans demonstrated that quantifiable plasma concentrations of BMY 14786 were observed only after higher doses of (±)BMY 14802 (500 mg or more, in single doses), and these levels were 5 percent to 8 percent of the corresponding BMY 14802 concentrations (Shukla et al. 1990). Levels of BMY 14786 below 10 percent of those of BMY 14802 were not detected in radioreceptor assays (Taylor and Behling, unpublished findings). In toto, we conclude that formation of biologically relevant amounts of an active metabolite of BMY 14802 does not occur and that the pharmacologic profile observed to date can only be attributed only to the properties intrinsic to the parent compound.

SUMMARY

BMY 14802 was identified as a potential antipsychotic drug in traditional model systems, and this identification was confirmed in modem behavioral and electrophysiological systems. The drug appears to be atypical as an antipsychotic in its lack of activity in models predictive of the potential to produce extrapyramidal side effects and tardive dyskinesia. Indeed, this suggestion is corroborated by clinical findings to date.

The atypical profile of BMY 14802 extends to its neurochemical actions and appears to find its basis in regionally selective, indirect modulation of the dopamine system. Furthermore, BMY 14802 exhibits interactions with σ binding sites in vitro and in vivo, a notion supported by data from neurophysiological, behavioral, and biochemical investigations. BMY 14802 also appears to be neuroprotective in some model systems and may have utility in the treatment of stroke (Boissard et al. 1991). BMY 14802 appears to interact with 5-HT_{1A} receptors, but this interaction does not seem to contribute significantly to the potential antipsychotic actions of the drug. Moreover, the formation of active metabolites of BMY 14802 does not appear to occur in animals or humans to an extent of physiological or behavioral relevance. If clinically efficacious, BMY 14802 may treat the symptoms of schizophrenia by a mechanism novel

for antipsychotic drugs: regionally selective, indirect modulation of dopaminergic systems by specific interaction at σ sites.

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Molecular Biology of PCP and NMDA Receptors

Leslie Kushner, Michael V.L. Bennett, and R. Suzanne Zukin

L-Glutamate, the major excitatory neurotransmitter of the central nervous system, activates at least five receptors (Foster and Fagg 1984; Cotman and Iversen 1987; Mayer and Westbrook 1987; Monaghan et al. 1989), which can be distinguished by the selective agonists *N*-methyl-D-aspartate (NMDA), kainate, α- -amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), 2-amino-4-phosphonobutyrate (L-AP4), and trans-1-amino-cyclopentyl-1,3-dicarboxylate (ACPD) (table 1). Although most of the excitatory amino acid (EAA) receptors are coupled to ligand-gated cation channels, the ACPD-selective receptor is apparently G-protein linked.

Of the channel-forming EAA receptors, the NMDA receptor is unique in that it is permeable to Ca^{2+} and is blocked by physiologic concentrations of Mg^{2+} in a voltage-dependent manner (MacDermott et al. 1986; Mayer et al. 1987). This Ca^{2+}-gating property of the NMDA receptor is thought to be responsible for its participation in long-term potentiation (Lynch et al. 1983; Harris et al. 1984; Wigström and Gustafson 1984; Collingridge and Bliss 1987) memory formation (Smith 1987), excitotoxicity (Schwartz et al. 1983; Jansco et al. 1984; Choi 1985; Wieloch 1985; Rothman and Olney 1987), and ischemic neuronal damage (Simon et al. 1984). In addition, the NMDA-activated channel has been shown to contain the pharmacologic receptor for phencyclidine (PCP) and has been implicated in endogenous psychosis.

Until recently, little was known about the molecular properties of the EAA receptors. A putative kainate receptor cDNA has been cloned and sequenced and shown to direct the translation of kainate-gated channels in *Xenopus* oocytes (Hollmann et al. 1989). Based on its predicted amino acid sequence, the kainate receptor subunit has a relative molecular mass (M_r) of 99,800. This mass is much larger than the sizes predicted for subunits of other cloned ligand-gated channel receptors (the largest reported is 67 kD (Boulter et al.

TABLE 1. *Excitatory amino acid receptors*

Receptor	Agonist	Competitive antagonist	Channel blocker	Allosteric inhibitor	Allosteric potentiator	Antagonist at potentiation site
NMDA	NMDA	CPP	$Mg2^{+}$	Zn^{2+}	glycine	7-chlorokynurenate
	L-glutamate	CGS-1975	PCP		D-serine	cydoleuclne
	L-aspartate	D-AP5	MK-801			HA-966
	Ibotenate	D-AP7				
Kainate	domoate	CNQX				
	kainate	DNQX				
	L-glutamate					
AMPA	AMPA	GDEE				
	quisqualate	CNQX				
	L-glutamate	DNQX				
L-AP4	L-AP4)					
	qulsqualate					
	L-glutamate					
ACPD	*trans*-ACPD					
	qulsqualate					
	L-glutamate					

NOTE: NMDA=N-methyl-D-aspartate; AMPA=a-amino-3-hydroxy-5-methyl-4-isoxazpionate; L-AP4=L-2-amino-7-phosphonoheptanoate; ACPD=1-amino-cyclopentyl-1,3-dicarboxylate; CPP=3-3(2-carboxypiperazine-4-yl)propyl-1-phosphonate; CGS-1975=cis-4-phosphonomethyl-2-piperidine carboxylic acid; D-AP5=D-2-amino-5-phosphonovalerate; D-AP7=D-2-amino-7-phosphonoheptanoate; CNQX=6-cyano-7-nitroquinoxaline-2,3dione; DNQX=6,7-dinitroquinoxaline-2,3-dione; GDEE=glutamate diethyl ester; PCP=phencyclidine; MK-801=dibenzocycloheptenelmine; HA-966=3-amino-1-hydroxypyrrolid-2-one.

1987)), although it agrees quite well with the estimated size of the largest protein component of the NMDA receptor, as determined by photoaffinity-labeling experiments in brain (Haring et al. 1987) and NCB-20 cells (Haring et al. 1990*a).* Like other ligand-gated channels, this putative kainate receptor is predicted to have four membrane-spanning domains and has a large N-terminal extracellular domain that contains several putative glycosylation sites, a "cys-cys loop" with partial homology to other ligand-gated channels, and a cytoplasmic C-terminal domain (Barnard et al. 1987; Greeningloh et al. 1987).

Although direct comparison of the putative kainate receptor cDNA sequence with the sequences of the subunits of other ligand-gated channel receptors revealed no significant homology (Hollmann et al. 1989), allowance for conservative substitutions and moderate length deletions give an alignment with considerable homology (E. Barnard, personal communication). Thus, it probably belongs to the family of other ligand-gated channel receptors. The

findings for the mammalian brain kainate receptor may provide important clues to the structural features of the NMDA receptor.

The kainate-selective EAA receptor can be distinguished pharmacologically from NMDA and quisqualate receptors. The kainate receptor is insensitive to selective antagonists of the NMDA receptor. Although it is activated by quisqualate (Evans et al. 1987) it can be distinguished from quisqualate-activated receptor subtypes by its sensitivity to domoate and lack of sensitivity to the quisqualate-receptor subtype-specific agonists (see below) or the antagonist glutamate diethyl ester (GDEE) (Haldeman and McLennan 1972; London and Coyle 1979; McLennan and Liu 1982; Slevin et al. 1983). The kainate receptor is a ligand-gated cation channel that is permeable to Na^+ and K^+. Autoradiography of radioligand binding sites (Monaghan et al. 1983; Greenamyre et al. 1985; Monaghan et al. 1985) patch clamp (MacDonald et al. 1987; Cull-Candy et al. 1988; Huettner and Bean 1988), and oocyte expression studies (Lerma et al. 1989*b)* have shown that this receptor is distinct from other excitatory amino acid receptors.

Quisqualate activates three distinct receptors or transducing mechanisms, which can be distinguished by specific ligands as well as by functional properties. GDEE (Krogsgaard-Larsen et al. 1980) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Honoré et al. 1988) inhibit the AMPA type of quisqualate receptor. (CNQX and DNQX also block the kainate receptor but are more specific than GDEE in *not* blocking the NMDA receptor (Blake et al. 1988; Drejer and Honor 1988; Honoré et al. 1988).) This receptor is a ligand-gated cation channel that gates Na^+ and K^+. L-AP4 acts at a site distinct from those activated by NMDA, kainate, or AMPA (Davies and Watkins 1982; Yamamoto et al. 1983; Lanthom et al. 1984). However, complete characterization of this site has not been reported. A third receptor, activated by quisqualate, is coupled to inositol phosphate metabolism and has been referred to as a metabolotropic receptor (Nicoletti et al. 1986; Sugiyama et al. 1987). The glutamate analog ACPD is a specific agonist for this receptor.

The NMDA receptor is unique among ion-channel-forming excitatory amino acid receptors in that it gates Ca^{2+} as well as Na^+ and K^+ (MacDermott et al. 1986; Mayer et al. 1987). It contains several pharmacologically distinct binding sites, which may have regulatory functions. These functions include a binding site within the channel for Mg^{2+}, which blocks the channel in a voltage-dependent manner (Mayer et al. 1984; Nowak et al. 1984; Ascher and Nowak 1988); an inhibitory binding site for Zn^{2+} and other divalent cations, which shows no

voltage dependence (Peters et al. 1987; Westbrook and Mayer 1987; Christine and Choi 1990); an inhibitory binding site for Zn^{2+}, which blocks the channel in a voltage-dependent manner (Christine and Choi 1990); a site at which glycine and several other small amino acids bind and act as coagonists (i.e., this site must be occupied for glutamate or NMDA to cause channel opening) (Johnson and Ascher 1987; Kleckner and Dingledine 1988; Lerma et al. 1990); and at least one site for polyamines, which potentiate the maximum response and increase the affinity of ligands for the glycine binding site (Sacaan and Johnson 1989; McGurk et al. 1991).

Considerable evidence suggests that the PCP receptor, which mediates the psychotomimetic effects of PCP derivatives, σ opioids, and the dioxalanes (see Zukin and Zukin 1988), is a site within the NMDA receptor channel complex. PCP receptor ligands block NMDA-induced currents in a voltage- and use-dependent manner (Honey et al. 1985; MacDonald et al. 1987; Bennett et al. 1988; Huettner and Bean 1988; Kushner et al. 1988*a;* Lerma et al. 1989*a,* 1991) and specifically antagonize NMDA-induced neurotransmitter release (Snell and Johnson 1985,1986; Drejer and Honoré 1987; Jones et al. 1987). The antagonism of NMDA receptors by PCP receptor ligands is noncompetitive with respect to NMDA (Honey et al. 1985; Martin and Lodge 1986; Javitt et al. 1987; Lerma et al. 1991). The drug potencies for blocking activity correlate with potencies in receptor binding (Anis et al. 1983; Berry et al. 1984; Vincent et al. 1979; Zukin and Zukin 1979; Sircar et al. 1987) and behavioral assays (Holtzman 1980; Hampton et al. 1982; Hayes and Balster 1985). NMDA and glycine enhance binding of radiolabeled PCP receptor ligands, whereas competitive antagonists of the NMDA binding site inhibit binding to the PCP receptor in rat brain membranes (Javitt et al. 1987; Reynolds et al. 1987; Benavides et al. 1988; Johnson et al. 1988; Kloog et al. 1988) or to solubilized rat brain PCP receptors (Ambar et al. 1988; Haring et al. 19906). NMDA receptors (labeled by [^{3}H]3-(2-carboxypiperazin-4-yl)propyl-1-phosphate (CPP) or [^{3}H]glutamate) and PCP receptors (labeled by [^{3}H]1-[1-(2-thienyl)cyclohexyl]-piperidine (TCP)) are colocalized throughout rat forebrain and midbrain (Maragos et al. 1986; Jarvis et al. 1987; Maragos et al. 1988). [^{3}H]Azido-PCP photoaffinity labels five distinct polypeptides in NCBQO cell membranes; binding to two of the polypeptides is inhibited by D-amino-5-phosphonovaleric acid (D-AP5), a competitive inhibitor of the NMDA site (Haring et al. 1990*a).* NMDA and PCP receptors are always coexpressed in Xenopus oocytes into which rat brain mRNA, rat hippocampus or frontal cortex mRNA, NCB-20 cell mRNA, or size-fractionated mRNA is injected (see below). Thus, it is apparent

that the two receptors (NMDA and PCP) exist as part of the same supramolecular complex, with the PCP binding site located within the channel.

The NMDA receptor has attracted considerable attention because of its proposed roles in long-term potentiation (Lynch et al. 1976; Stringer et al. 1983; Harris et al. 1984; Wigström and Gustafson 1984; Morris et al. 1986; Collingridge and Bliss 1987) memory formation (Morris et al. 1986; Morris 1988; Lincoln et al. 1988) and developmental structuring (Singer et al. 1986; Stryker and Harris 1986; Cline and Debski 1987; Rauschecker and Hahn 1987). In addition, it is involved in excitotoxicity (Schwartz et al. 1983; Simon et al. 1984; Wieloch 1985; Olney et al. 1987) and epilepsy (Herron et al. 1985; Meldrum 1985; Dingledine et al. 1986; Mody and Heinemann 1987) and may play a role in Alzheimer's disease (Greenamyre et al. 1985, 1987; Geddes et al. 1986; Maragos et al. 1987) Huntington's disease (Schwartz et al. 1983; Greenamyre et al. 1985; Young et al. 1988), and schizophrenia (Kim and Kornhuber 1982; Contreras et al. 1987; Zukin and Javitt, this volume). The functional significance of the NMDA receptor has prompted several laboratories to investigate the molecular nature of this receptor. This chapter will review recent studies that used molecular biological approaches to gain a greater understanding of the NMDA receptor. In particular, use of the *Xenopus laevis* oocyte expression system and clonal cell lines for the study of this receptor will be described.

EXPRESSION OF NMDA RECEPTORS IN *XENOPUS* OOCYTES INTO WHICH RAT BRAIN mRNA HAS BEEN INJECTED

The *Xenopus* oocyte is a self-contained expression system that correctly translates exogenous mRNAs encoding membrane proteins and is capable of eukaryotic posttranslational modifications, including processing of precursor molecules (Ghysdael et al. 1977) phosphorylation (Gedamu et al. 1978) glycosylation (Colman et al. 1981; Lund et al. 1986) and assembly and insertion of subunits into the plasma membrane with the correct orientation (Sumikawa et al. 1981; Parker et al. 1985). Moreover, the oocyte provides a precisely controlled environment for the study of receptor structure and function. In the absence of protein purification and sequencing information, the *Xenopus* oocyte system can be used for expression cloning (Masu et al. 1987; Julius et al. 1988; Hollmann et al. 1989).

Recently two groups reported the expression of NMDA-activated channels in *Xenopus* oocytes following injection of exogenous mRNA isolated from adult rat

brain (Verdoorn et al. 1987; Kushner et al. 1988*a),* primary cell cultures derived from fetal rat brain (Verdoom et al. 1987) and a clonal cell line of neuronal origin (Lerma et al. 1989*a).* NMDA-activated channels were distinguished from other excitatory amino acid receptors by their electrophysiologic and pharmacologic characteristics (Kushner et al. 1988*a;* Verdoorn and Dingledine 1988; Lerma et al. 1989*b).* NMDA application (with glycine) evoked an inward current after short latency that rapidly desensitized to a steady level (Kushner et al. 1988*a;* Lerma et al. 1989*b).* Kainate produced a more slowly rising, nondesensitizing current also of short latency. Quisqualate induced both a small, early, inward current and a much larger current characterized by long latency and large oscillations, indicative of second-messenger-mediated activation of endogenous Ca^{2+}-activated Cl- channels (Sugiyama et al. 1987; Kushner et al. 1988*a;* Verdoom and Dingledine 1988). These findings indicate that rat brain mRNA directs the expression of NMDA-, kainate-, and two kinds of quisqualate-activated excitatory amino acid receptors in the *Xenopus* oocyte.

The pharmacologic characteristics of the NMDA receptor expressed in the oocyte were similar to the characteristics of the neuronal receptor. At a holding potential of -60 mV, the half-maximal response to NMDA (in the presence of 10 μM glycine) was elicited at 16 μM (figure 1A). Oocytes that were not subjected to injections or received injections of water did not respond to NMDA. Glycine markedly potentiated the effect of NMDA (Verdoorn et al. 1987; Kushner et al. 1988*a)* with a half-maximal effective concentration (EC_{50}) of 0.19 μM (figure 1 B). There was essentially no response to 50 μM NMDA alone, and 100 μM glycine alone had negligible effect. Glycine (or other agonists at the glycine site) appeared to be an absolute requirement for the activation of the NMDA receptor in *Xenopus* oocytes (Kleckner and Dingledine 1988; Lerma et al. 1990), and rapid desensitization could not account for the failure of NMDA responses in the absence of glycine (Lerma et al. 1990). The NMDA-activated current could be blocked by the selective NMDA antagonist D-AP5 (Verdoorn et al. 1987; Kushner et al. 1988a) (concentration for half-inhibition, IC_{50}=3.3 μM) (figure 1C) but not by its inactive enantiomer L-AP5. D-AP5 acted as a competitive inhibitor in that it shifted the NMDA dose-response curve rightward (i.e., the potency was decreased) without changing the slope or maximum response (i.e., Hill coefficient [n] and I_{max} are constant) (Lerma et al. 1991); competitive inhibition was also demonstrated by Schild analysis (Verdoorn et al. 1988). Inhibition by Zn^{2+} was noncompetitive (i.e., I_{max} was decreased but EC_{50} of NMDA was unchanged) (Lerma et al. 1991). The NMDA responses were blocked by Mg^{2+} in a voltage-dependent manner (Verdoorn et al. 1987; Kushner et al. 1988*a).* When 0.1 μM Mg^{2+} was included in the perfusion medium, the

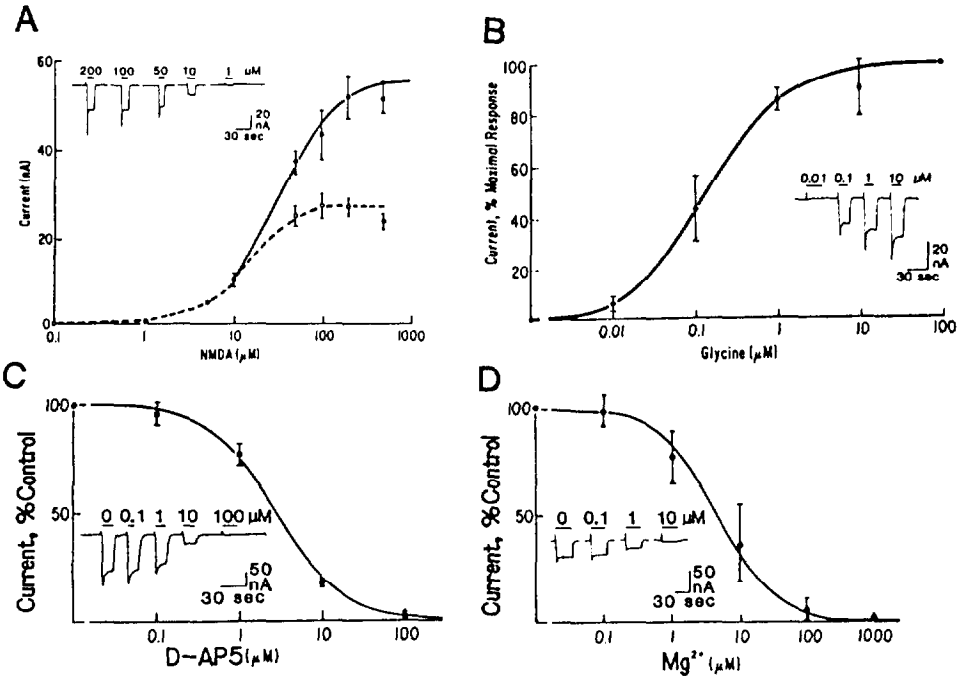

FIGURE 1. *Dose-response relations for N-methyl-D-aspartate- (NMDA) induced currents in Xenopus oocytes subjected to injections of rat brain mRNA (Kushner et al. 1988a). Cells were voltage clamped at a holding potential of -60 mV. Drugs were applied in the bath. Error bars indicate SEMs from three oocytes; several applications were made to each oocyte at each concentration.*
(A) NMDA-induced current in the presence of 10 μM glycine. Responses (sample records inset) are graphed as both peak (—) and steady-state (- - -) values. (B) Glycine potentiation of the response to 50 μM NMDA normalized with respect to the maximum response at 100 μM glycine. (C) D-AP5 inhibition of the response to 50 μM NMDA with 10 μM glycine. (D) Mg^{2+} inhibition of the response to 50 μM NMDA with 10 μM glycine.

relationship between the NMDA-induced current and voltage exhibited a negative slope between holding potentials of -80 and -30 mV after which the current decreased linearly with voltage to a reversal potential of approximately -10 mV. When Mg^{2+} was omitted from the medium, the NMDA-induced current

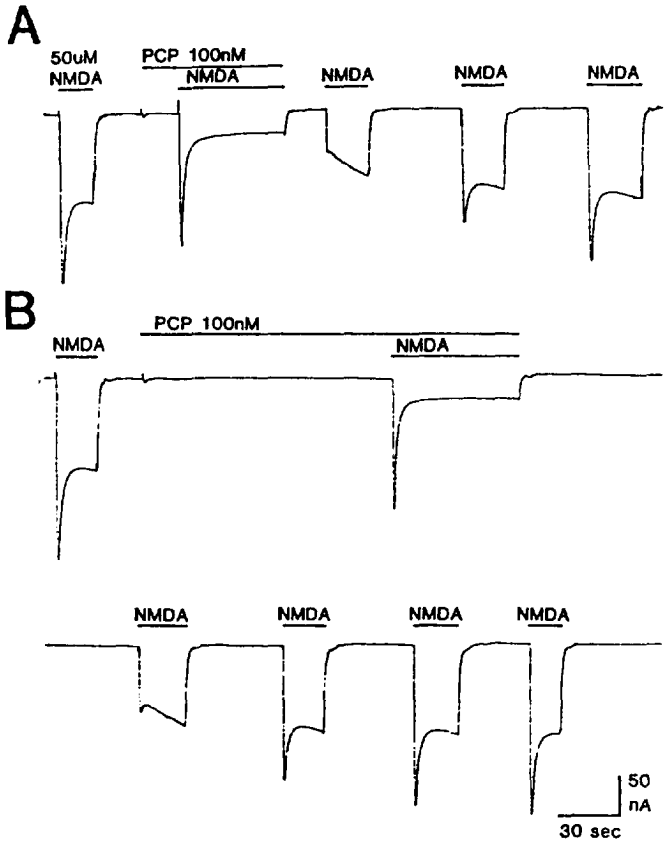

FIGURE 2. *Use dependence of PCP block and recovery from block of NMDA-induced currents in Xenopus oocytes subjected to injections of rat brain mRNA (unpublished data, with J. Lerma). (A) PCP was applied 30 seconds before NMDA, and the response was partially blocked (the plateau more than the peak, consistent with use-dependent block). A test application of NMDA at 20 seconds showed the degree of block; further applications showed recovery. (B) After full recovery PCP was applied for 2 minutes before NMDA. The initial response was a/most identical to that in A, indicating that block in the absence of NMDA developed very slowly. The continuous trace in the lower record shows that after 2 minutes recovery was only slightly greater than after 20 seconds as in A and markedly less than after 2 minutes in A, when there had been two prior NMDA applications.*

was a nearly linear function of voltage. The IC_{50} for Mg^{2+} was 8.3 μM at a holding potential of -60 mV (figure 1 D). Inhibition by Mg^{2+} was noncompetitive in that I_{max} was decreased, but EC_{50} of NMDA was unchanged (Lerma et al. 1991).

These results show that the distinguishing features of neuronal NMDA channels are reproduced by NMDA receptors expressed in *Xenopus* oocytes following injection of rat brain mRNA. In this system, as in spinal, striatal, cortical, and hippocampal neurons, NMDA receptors are activated by NMDA, potentiated by glycine, and blocked competitively by the antagonist D-AP5 and noncompetitively by Mg^{2+}, which blocks in a voltage-dependent manner, and by Zn^{2+}.

To determine whether the NMDA channel expressed in *Xenopus* oocytes is functionally associated with the PCP receptor, PCP receptor ligands were tested for their ability to modulate NMDA-activated currents (Kushner et al. 1988*a;* Lerma et al. 1991). Application of PCP blocked the NMDA-evoked current in a use-dependent manner (figure 2). When PCP was applied together with NMDA, the initial peak of inward current was either essentially unchanged or slightly reduced from the response to NMDA alone. Although the rate of onset of block at a given concentration of PCP was faster at higher NMDA concentrations, the degree of block was unaffected by the NMDA concentration (Lerma et al. 1991). PCP blocked very slowly in the absence of NMDA; responses to NMDA after a 20-second and a 2-minute exposure to PCP were nearly identical and only slightly smaller than when NMDA and PCP were applied together (Lerma et al. 1991). Recovery from PCP block also occurred very slowly in the absence of NMDA; responses after a 2-minute saline wash were only slightly greater than after a 20-second wash (Lerma et al. 1991). Thus, both PCP block of the NMDA-induced current and relief of block were use dependent, indicating that the PCP receptor is a site within the channel of the NMDA receptor, which permits entry or egress of PCP only if the channel is open. This use dependence is consistent with the action of PCP receptor ligands on neurons (MacDonald et al. 1987; Huettner and Sean 1988).

The PCP receptor ligands tested were able to block, in a use-dependent manner, the NMDA-evoked response in a rank order of potency that closely matched the rank order of potency in binding to rat brain membranes (Vincent et al. 1979; Zukin and Zukin 1979; Sircar et al. 1987) in electrophysiological studies of neural tissues (Anis et al. 1983; Berry et al. 1984; Honey et al. 1985) and in behavioral studies (Holtzman 1980; Hampton et al. 1982; Hayes and Bolster 1985). The PCP receptor was stereoselective in that NMDA responses

were reduced by the active ligand dexoxadrol but hardly affected by its inactive stereoisomer levoxadrol at the same concentration (Kushner et al. 1988*a)*.

In addition, both PCP and dibenzocycloheptenеimine (MK-801), an extremely potent PCP receptor ligand, inhibited NMDA-evoked currents without effect on quisqualate- or kainate-evoked currents (Kushner et al. 1988*a;* Lerma et al. 1989*b).* These data indicate that many of the pharmacologic properties of PCP receptors are reproduced in the oocyte system. Most important, in the large number of oocytes expressing NMDA receptors that were tested, including oocytes that received injections of mRNA from specific brain regions, PCP receptor ligands reduced or blocked NMDA-induced current, demonstrating coexpression of these receptors. Moreover, in size fractions prepared from rat brain mRNA, the NMDA receptors always cofractionated with PCP receptors.

These results demonstrate that in this system PCP receptor ligands are acting at the receptor implicated in PCP's behavioral effects and that PCP and NMDA receptors are always coexpressed in *Xenopus* oocytes. This study provides important direct evidence in support of the hypothesis that the PCP receptor is a binding site within the channel of the NMDA receptor protein.

DEMONSTRATION OF PCP RECEPTORS IN THE NEUROBLASTOMA-BRAIN HYBRID CELL LINE NCB-20

Clonal cell lines are particularly useful for purification and gene-cloning studies of neurotransmitter receptors in that they provide a homogeneous population of cells that can be grown in large quantity and subjected to precise physico-chemical manipulation. In the search for a clonal cell line that possesses NMDA receptors, we examined the receptors of the neuroblastoma-brain hybrid cell line NCB-20. NCB-20 cells had been reported to have δ opioid receptors and a benzomorphan-specific binding site on their membranes (McLawhon et al. 1981; West et al. 1983). Benzomotphans bind to κ opioid receptors and σ receptors as well as to PCP receptors. Largent et al. (1986) demonstrated the presence of σ receptors on the NCB-20 cell membrane. An important unanswered question was whether NCB-20 cell membranes also have the PCP receptor site associated with NMDA-activated channels, as in neuronal tissue. Thus, highly specific radioligands were used to measure the density of opioid, σ, and PCP receptors on NCB-20 cells in quantitative receptor binding assays (Kushner et al. 1988*b).*

NCB-20 cells were shown to possess high-affinity δ opioid receptors, but not μ or κ opioid receptors, on their cell membrane (Kushner et al. 19886). In addition, these cells possessed distinct sites for σ opioids and PCP derivatives. One site was labeled by [^{3}H](+)*N*-allylnormetazocine ([^{3}H](+)SKF 10,047). Scatchard analysis of [^{3}H](+)SKF 10,047 binding to NCBQO cell membranes revealed an apparent single class of sites with a binding affinity of 69 nM and receptor density of 4,100 fmol/mg protein (figure 3A). The rank order of potency of drugs at that site was

(+)3-(3-hydroxyphenyl)- *N*-(1-propyl)piperidine ((+)3-PPP) > haloperidol > (+)SKF 10,047 > (±)ethylketocyclazocine (EKC) > (μ)bremazocine > *N*-[1-(2-thienyl)cyclohexyl]piperidine (TCP) > dexoxadrol.

This site was similar in its ligand selectivity to the haloperidol-sensitive σ receptor of rat brain, confirming the previous report of Largent et al. (1986).

The other site was labeled by the potent phencyclidine derivative [^{3}H]TCP. Saturation curves and Scatchard analysis of equilibrium binding of the PCP receptor-specific ligand [^{3}H]TCP to NCB-20 cell membranes revealed binding to be monophasic, saturable, and of high affinity (K_D=335 nM, B_{max}=9,300 fmol/mg protein) (figure 3B). This density was approximately five times the density found in rat brain and is equivalent to about 60,000 sites per cell. The rank order of potency of drugs at this site was

TCP > (+)3-PPP > PCP > dexoxadrol > haloperidol > cyclazocine > levoxadrol > (+)SKF 10,047;

μ and δ ligands were inactive. Stereoselectivity at this site was demonstrated in that dexoxadrol was more potent than levoxadrol. The drug potency profiles characterized by displacement of [^{3}H](+)SKF 10,047 and [^{3}H]TCP binding fit very well to a two-site model. Collectively, these data suggest that NCB-20 cell membranes have a PCP receptor-like site and a haloperidol-sensitive o-like site.

Further analysis of [^{3}H]TCP binding to NCB-20 cell membranes suggested the presence of two distinct PCP sites that can be distinguished by their affinities for MK-801 (Hating et al. 1990*a).* This finding is consistent with the finding that PCP receptors in hindbrain exhibit an approximately tenfold lower affinity for PCP than do forebrain PCP receptors (Haring et al. 1987), suggesting the presence of different PCP receptor subtypes in brain. The NCB-20 cell line is derived from a neoplasm of mouse spinal cord (Dawson et al. 1983) and would be expected to have receptors that resemble more closely those of spinal cord and hindbrain.

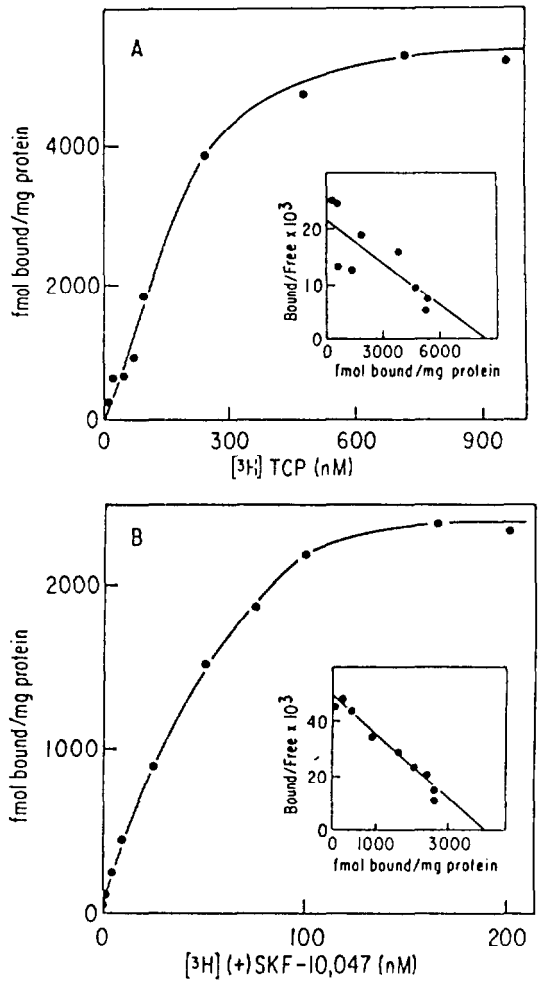

FIGURE 3. *Saturation plots and Scatchard plots (insets) of specific binding to PCP and σ ıreceptors of NCB-20 cell membranes (Kushner et al. 1988b). Membranes (0.6 mg of protein/ml) were incubated with [^{3}H]TCP (0.01 to 1 μM) (A) or (+)-[^{3}H]SKF 10,047 (1 to 300 nM) (B) in 5 mM Tris buffer, pH 7.4 at 4 °C for 45 minutes in the presence or absence of unlabeled ligand. The Scatchard plots were fit by a straight line using linear regression analysis. These data are from a representative experiment that was performed in triplicate and replica ted a minimum of three times.*

To investigate the possibility that the [^{3}H]TCP binding site of NCB-20 cell membranes is the PCP receptor site of the NMDA-gated channel, NCB-20 cells were tested electrophysiologically for their responses to NMDA. The majority of tested passages failed to respond to NMDA (see Kushner et al. 1988*b).* However, high-affinity, stereoselective binding to PCP receptors was observed in all cell passages tested, and mRNA isolated from NCB-20 cells lacking functional NMDA channels did cause expression of functional NMDA channels in oocytes (Lerma et al. 1989*a).* In addition, [^{3}H]azido-PCP photoaffinity labeled five distinct polypeptides in NCB-20 cell membranes, and such binding to two specific polypeptides (M_r 68K and 90K) was inhibited by inclusion of D-AP5 or MK-801 in the binding reaction (Haring et al. 1990*a).* These data suggest that expression of functional NMDA receptors in NCB-20 cells may be defective or subject to regulation. The NCB-20 cell line is the only cultured cell line that has been shown to have PCP receptors, although evidently not functional NMDA-activated channels.

EXPRESSION OF THE NMDA RECEPTOR IN *XENOPUS* OOCYTES INTO WHICH mRNA FROM NCB-20 CELLS WAS INJECTED

To further characterize the excitatory amino acid receptors of NCB-20 cells, we injected poly(A^+)RNA prepared from these cells into *Xenopus oocytes.* After several days, inward currents were evoked by applying glutamate and NMDA but not by kainate, quisqualate, or γ-aminobutyric acid (GABA) (Lerma et al. 1989*a).* At a holding potential of -60 mV, NMDA (with 10 μM glycine) application evoked an inward current at short latency that partially desensitized, a response identical to that obtained after injection of rat brain mRNA (Kushner et al. 1988*a).* No responses were observed for kainate (500 μM), quisqualate (10 μM), or GABA (100 μM), compounds that evoke pronounced currents in oocytes into which rat brain mRNA had been injected, or for glycine (100 μM) in the absence of NMDA (Lerma et al. 1989*a)* (figure 4). Glutamate (100 μM) activated an early current that resembled the response to NMDA. Of four mRNA preparations, all encoded NMDA receptors. These results indicate that the NCB-20 cells contain the precursor mRNA necessary to encode NMDA receptors.

Properties of NCB-20 cell NMDA receptors expressed were typical of neuronal NMDA receptors. The relation between the steady level of inward current and NMDA concentration indicated a single-component response with an apparent affinity (K_D) of 22 μM and Hill coefficient of 1.3. As in neurons, glycine markedly potentiated the response to NMDA (EC_{50} about 0.2 μM with 50 μM NMDA). The

NMDA-activated current was blocked by the competitive antagonist D-AP5 (at 50 μM NMDA, IC_{50} 2.5 μM). NMDA currents were also blocked by Mg^{2+} at inside negative potentials and by Zn^{2+}.

PCP and MK-801 blocked NMDA-activated currents in a use-dependent manner. Application of PCP together with NMDA resulted in an initial peak of inward current that was little different from the response to NMDA alone. Application of PCP alone for up to several minutes had no effect on subsequent NMDA-evoked responses. Recovery from PCP block occurred rapidly in the presence of agonist but very slowly in the absence of agonist. Consistent with the results for rat brain NMDA receptors expressed in oocytes, these data indicate that the channel of the NCB-20 cell NMDA receptors expressed must be open in order for PCP receptor ligands to block or exit from it. Thus, the PCP receptor encoded by NCB-20 mRNA appears to be within the NMDA channel. In summary, the NMDA receptor, but not the kainate or quisqualate receptor, is expressed in the oocyte after injection of NCB-20 cell mRNA. The NMDA receptors expressed possess the salient pharmacological features of the

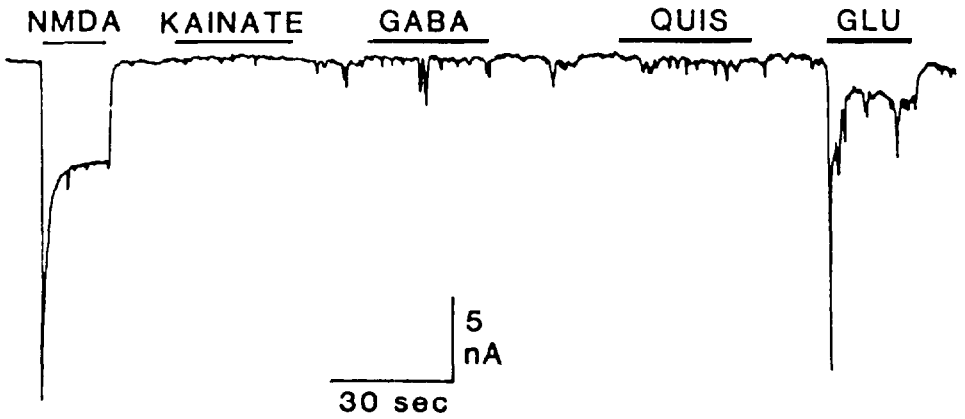

FIGURE 4. *Expression of neurotransmitter receptors in Xenopus oocytes injected with NCB-20 cell mRNA (Lerma et al. 1989a). Records from an oocyte clamped at -60 mV, and subjected to injection of mRNA 6 days previously. Horizontal bars indicate duration of drug application. The small, brief, inward currents were due to endogenous Ca^{2+}-activated chloride channels; these currents disappeared in these oocytes after injection of EGTA.*

neuronal NMDA receptor, including the apparent location of the PCP receptor at a site within the channel.

The absence of expression of kainate and quisqualate receptors in the oocyte suggests the absence of the encoding mRNA from NCB-20 cells. If NCB-20 cells do not have mRNA encoding these excitatory amino acid (EAA) receptors, they would be a relatively pure source of mRNA encoding the NMDA type of EAA receptor.

SUMMARY

The studies described demonstrate that rat brain mRNA directs the synthesis of at least four types of functional EAA receptors in the *Xenopus oocyte* system, whereas in this system NCB-20 cell mRNA directs the synthesis of only the NMDA type of EAA receptor. The NMDA channel expressed in the oocyte, using either rat brain mRNA or NCB-20 cell mRNA, exhibits the pharmacologic properties of the neuronal receptor, including the functional association with the PCP receptor located within the NMDA-gated channel. The demonstration that mRNA isolated from NCB-20 cells lacking functional NMDA-activated channels, but bearing PCP binding sites, can encode functional NMDA-activated channels in the oocyte indicates some defect or regulating step in posttranslational processing or insertion of the receptors into the plasma membrane in the cell of origin. This is the only cell line known to (1) have PCP receptors that appear to be associated with NMDA receptors and (2) provide a homogeneous, self-replicating population of cells that can be manipulated genetically and by changing the extracellular environment. Consequently, the NCB-20 cell line will be useful for the study of the NMDA receptor and its expression.

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Excitatory Amino Acid Neurotoxicity in the Developing Brain

John W. McDonald and Michael V. Johnston

INTRODUCTION

Excitatory neurotransmission in the mammalian central nervous system is mediated principally by glutamate and structurally related compounds. Excitatory effects of glutamate are mediated via activation of at least three subtypes of glutamate receptors named by their preferential agonists, *N*-methyl-D-aspartate (NMDA), quisqualate, and kainate. In addition to the role of excitatory amino acids (EAAs) in transynaptic information coding, recent evidence suggests that EAAs are involved in a variety of other physiologic actions, including regulation and maintenance of neuronal cytoarchitecture as well as regulation of certain forms of learning and memory (long-term potentiation [LTP]). EAAs also have several physiologic roles in development, including the regulation of neuronal survival, dendritic and axonal structure, synaptogenesis, and activity-dependent synaptic plasticity. However, excessive activation of EAA receptors and subsequent overactivation of some of the same molecular mechanisms that mediate the structural changes associated with synaptic plasticity can lead to neuronal destruction and death. Our current understanding of the molecular mechanisms that mediate the structural changes associated with synaptic plasticity can lead to neuronal destruction and death. Our current understanding of the molecular mechanisms that contribute to synaptic plasticity and to excitotoxicity are discussed along with work from our laboratory related to the pathophysiologic characteristics of excitotoxicity during development. We also reviewed this area in a longer recent article (McDonald and Johnston 1990) and several figures from that review are reproduced here with permission.

PHYSIOLOGIC ROLES OF EXCITATORY AMINO ACIDS DURING DEVELOPMENT

EAAs may serve several physiologic functions during development and in the adult brain, such as promotion of neuronal survival, differentiation, and regulation of activity-dependent synaptic plasticity (Ascher and Nowak 1987; Tsumoto et al. 1987). EAAs may also act as neurotrophic factors during development (Balazs et al. 1988; McDonald and Johnston 1990).

MECHANISMS OF EXCITATORY AMINO ACID NEUROTOXICITY

Excessive activation of the cellular mechanisms that participate in neuronal signaling and neural plasticity can initiate a series of intracellular biochemical events that cause neuronal damage (referred to as excitotoxicity). Overstimulation of each of the EAA receptor subtypes can initiate a cascade of events resulting in neuronal injury in the developing and adult brain. The mechanism underlying EAA neurotoxicity has been divided into two component processes based on in vitro studies (figure 1) (Choi 1987, 1988; Rothman and Olney 1987). The first involves depolarization of neuronal membranes and an influx of sodium, chloride, and water. The second phase is characterized by excessive calcium influx, primarily via NMDA receptor/channels, and activation of secondary toxic events (Choi et al. 1988). This sequence of events appears to be a major pathway leading to neuronal injury resulting from a variety of acute brain insults. Under normal conditions excessive activation of EAA receptors is prevented by the presynaptic high-affinity glutamate uptake system that reduces the glutamate concentration in the synaptic cleft. When cellular energy is depleted, high-affinity uptake of EAA is compromised (Silverstein et al. 1986*a).* Excessive neuronal activation exacerbates any imbalance between cellular energy requirements and energy availability. These mechanisms cause metabolic stress and energy depletion and may ultimately produce excitotoxicity (Novelli et al. 1988).

Overactivation of EAA receptors, particularly NMDA receptors, has been implicated in the pathogenesis of neuronal injury in a variety of acute neurologic disorders (Choi 1988; Rothman and Olney 1987) (figure 2).

ONTOGENY OF EXCITATORY AMINO ACID RECEPTORS

Synaptic markers of EAA neurotransmitter pathways change markedly during the early postnatal period (Monaghan et al. 1989). The regional distribution of

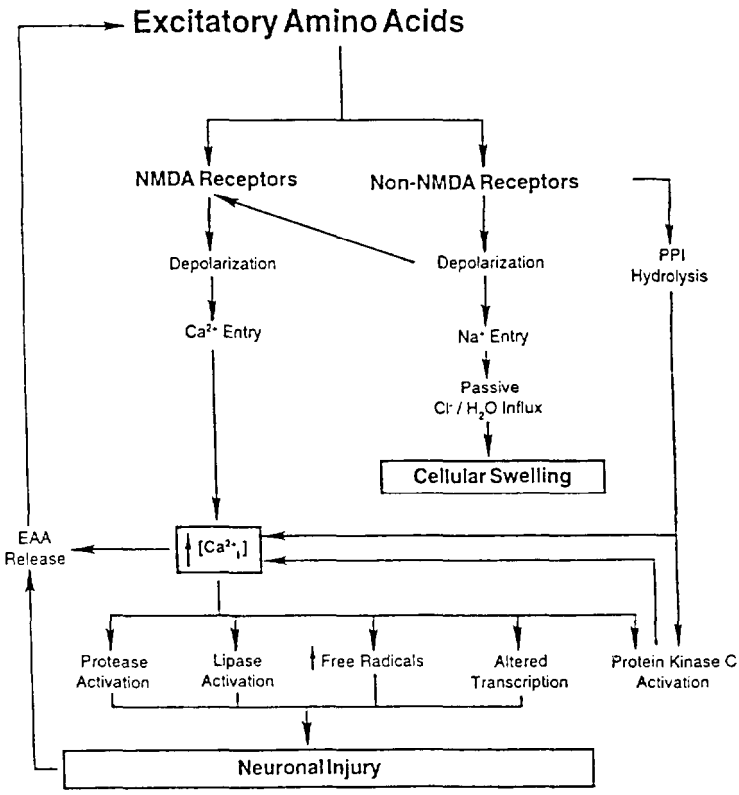

FIGURE 1. *This schematic diagram summarizes some of the mechanisms that may contribute to EAA neurotoxicity. In vitro experiments suggest that EAA neurotoxicity may have two components. The first component, mediated by excessive activation of non-NMDA receptors, is characterized by influx of Na^+ followed by passive influx of Cl^- and H_2O that may produce osmotic neuronal swelling. These are acute events occurring within hours of exposure to EAA agonists. The second, more prominent, component is produced by overactivation of NMDA receptors, which leads to a rise in the intracellular concentration of $Ca2^+$. A sustained rise in intracellular Ca^{2+} may trigger a biochemical cascade of events that leads to neuronal injury and death. Furthermore, activation of a subset of non-NMDA receptors coupled to polyphosphoinositide hydrolysis may also elevate intracellular Ca^{2+}. EAAs released from synaptic terminals would further propagate neuronal injury. Adapted from Choi 1988.*

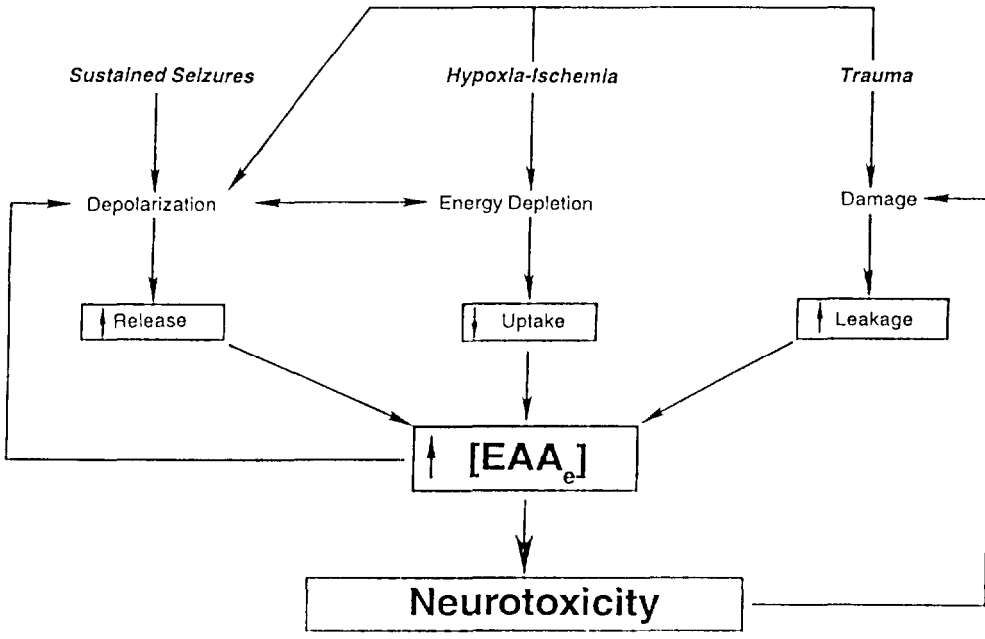

FIGURE 2. *This diagram illustrates the possible role of EAA neurotoxicity in acute brain injury. EAA neurotoxicity may be a common mechanism in neuronal injury resulting from sustained seizures, hypoxia-ischemia, and physical brain trauma. All these conditions tend to elevate the extracellular concentration of EAAs via different mechanisms. As detailed in figure 1, excessive activation of EAA receptors, due to elevated concentrations of EAAs in the synaptic cleft, can result in neuronal injury and death. Two recurrent pathways could make these mechanisms self-propagating. Adapted from Choi 1988.*

NMDA- and quisqualate-sensitive glutamate binding is illustrated in figure 3. High binding densities are present within the hippocampus and the outer layers of the cerebral cortex followed by intermediate binding densities in the basal ganglia. Lower binding densities are present in the brain stem and cerebellum. In contrast to the high density of NMDA- and quisqualate-type glutamate receptors in the postnatal day (PND)-7 brain, the density of kainate receptors is relatively low compared to values in adults. High-affinity [^{3}H]-kainate binding in rat brain is low during the first postnatal week and then rises rapidly to achieve

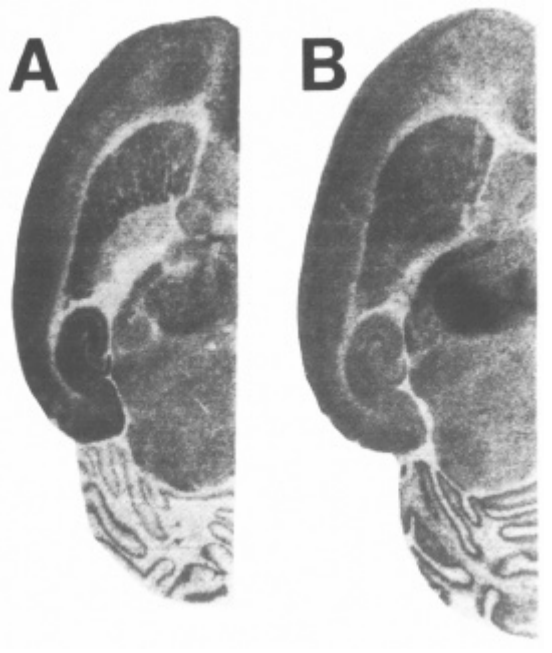

FIGURE 3. *Comparison of the distribution of (A) NMDA and (B) quisqualate-sensitive [^{3}H]glutamate binding in horizontal brain sections from PND 7 rats. NMDA receptors were labeled with 40 nM [^{3}H]glutamate in 50 mM Tris-acetate (pH 7.2 at 4°C) containing 2.5 μM quisqualate. Quisqualate receptors were labeled with 40 μM [^{3}H]glutamate in 50 mM Tris-HCl (pH 7.2 at 4°C) containing 2.5 mM $CaCl_2$ and 100 μm NMDA.*

adult levels by postnatal week 3. In the striatum, [^{3}H]-kainate binding densities rise tenfold from 2 percent to 6 percent of adult values at PND 4 and to 70 percent at PND 21 (Campochiaro and Coyle 1978). By PND 28, receptor densities are 80 percent of adult levels. A similar developmental profile of kainate binding is present in the hippocampus, where binding remains relatively low between PND 3 and 12 and then rises rapidly (twofold) to PND 17, when adult levels are achieved (Ben-Ari et al. 1984). In contrast, quisqualate-sensitive glutamate binding increases approximately threefold from PND 1 to maximal adult levels at PND 7 in the rat striatum. The transient expression of quisqualate-sensitive glutamate binding sites has been described in the globus pallidus with peak binding densities present by PND 7 and thereafter declining over 2 weeks to the very low levels present in adults (Greenamyre et al. 1987).

Using quantitative receptor autoradiography, we have recently described the postnatal development of three receptors that constitute the NMDA receptor/channel complex in the rat hippocampus (McDonald et al. 1989*e).* NMDA-sensitive [^{3}H-glutamate binding, strychnine-insensitive [^{3}H]-glycine binding, and [^{3}H]-TCP binding were measured to examine the ontogeny of NMDA recognition sites, glycine modulatory sites, and PCP receptors, respectively (McDonald and Johnston 1990; Wong et al. 1988).

Each receptor component of the NMDA receptor/channel complex has a unique developmental profile in the hippocampal formation (figure 4). Furthermore, each receptor component is transiently overexpressed during development. NMDA-sensitive [^{3}H]-glutamate binding exceeds adult levels by 50 percent to 120 percent in all regions examined, with peak densities occurring between PND 10 through 28. In stratum radiatum CA_1, binding increased slowly from 49 percent to 61 percent of adult value between PND 1 and PND 7, after which binding rapidly rose to 151 percent of adult values at PND 14, remained elevated through PND 28, and then decreased to adult levels. The ontogenic profile of NMDA recognition site binding was similar in other hippocampal regions.

The developmental changes we found in patterns of binding to each of the receptor components of the NMDA receptor/channel complex are consistent with postnatal changes in synaptogenesis. However, the relative overexpression of the NMDA recognition sites with respect to glycine and PCP receptor between PND 7 and PND 21 suggest preferential regulation of the expression of these receptor components during development. These findings are consistent with the hypothesis that multiple genetic forms of NMDA receptor complexes exist (Stone and Burton 1988).

The physiologic relevance of the overexpression of the NMDA recognition sites compared with the other components of the NMDA receptor/complex is unclear. However, these changes parallel the maximal rate of change in stratum radiatum pyramidal dendrites (Loy 1980; Pokorny and Yamamoto 1981). The development of NMDA sites in stratum molecular dentate gyrus (SMDG) parallels SMDG synaptogenesis (Crain et al. 1973). Dendritic growth and afferent lamination of the entorhinal terminal zone (stratum moleculare) of the dentate gyrus increase more rapidly between PND 4 and PND 10 than at later times (Loy et al. 1977).

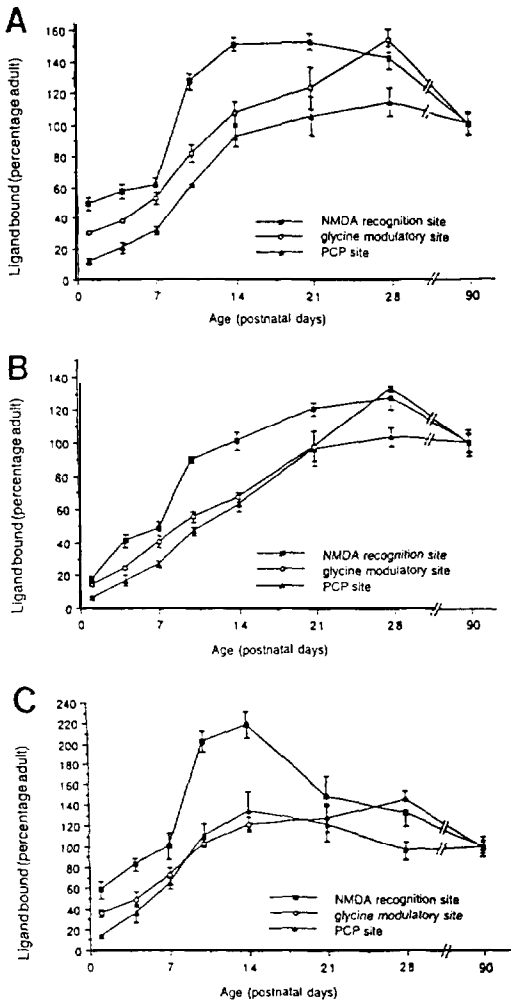

FIGURE 4. *Comparison of postnatal developmental profiles of NMDA-sensitive [^{3}H]glutamate binding to NMDA recognition sites, strychnine-insensitive [^{3}H]giycine binding to glycine modulatory sites, and [^{3}H]TCP binding to PCP receptors in stratum radiatum of the CA1 hippocampal subfield. Each data point represents the relative binding densities (mean ± S. E. M.) compared with binding densities in corresponding areas in adult rats (adult = PND 90). Four animals were used at each age (except PND 21, n = 3). Vertical error bars represent S. E. M.*

The ontogeny of NMDA receptor binding could be related to several developmental changes in the physiology of EAA receptors. In area CA_3, superfusion of NMDA elicits recurrent synchronized burst activity; the epileptogenic effect of NMDA increases from PND 1 to PND 10 (King et al. 1989). Also, the susceptibility to convulsant-induced epileptiform activity increases from PNDs 4 through 6 to PNDs 14 through 16 (Swann and Brady 1984; Chapman and Meldrum 1989). Specific NMDA receptor antagonists block the seizure activity (Brady and Swann 1986). Developmental changes also occur in the chemosensitivity of CA_1 perinatal neurons to NMDA (Hamon and Heinemann 1988). Maximal responses to NMDA occur between PND 12 and PND 30 in stratum radiatum and correspond closely with the period of overproduction of NMDA receptors in this area. The ontogeny of area CA_1 NMDA-sensitive [^{3}H]-glutamate binding parallels the development of LTP, an experimental model of learning and memory, in area CA_1 (Harris and Teyler 1984; Duffy and Teyler 1978). Nevertheless, the ontogeny of the absolute number of hippocampal NMDA receptors does not correlate well with the ontogeny of NMDA-induced brain injury that transiently peaks on PND 7 (McDonald et al. 1988*b).*

ROLE OF EXCITATORY AMINO ACIDS IN HYPOXIC-ISCHEMIC BRAIN INJURY

Overactivation of NMDA-type glutamate receptors contributes to the pathophysiology of hypoxic-ischemic neuronal injury in the adult brain, as shown in figure 5. This diagram demonstrates a proposed schematic representing the interrelationships among mechanisms that mediate hypoxic-ischemic neuronal injury in the developing brain (Johnston et al. 1988).

Intracellular calcium overload probably plays an important role in causing cell death. Calcium-activated release of free fatty acids and free radicals following lipid degradation are also important steps producing toxic injury. Calcium-activated proteases may also contribute to neuronal dysfunction and destruction. These events can create a cycle of seizures and brain edema that could worsen damage.

Our laboratory has examined the role of EAA in the pathogenesis of hypoxic-ischemic brain injury by using a perinatal model of hypoxia-ischemia. In this model, focal cerebral hypoxia-ischemia is produced by ligating the right common carotid artery of PND 7 rats and exposing them to moderate hypoxia (8 percent oxygen balance nitrogen) for 3 hours (Johnston 1983; McDonald et

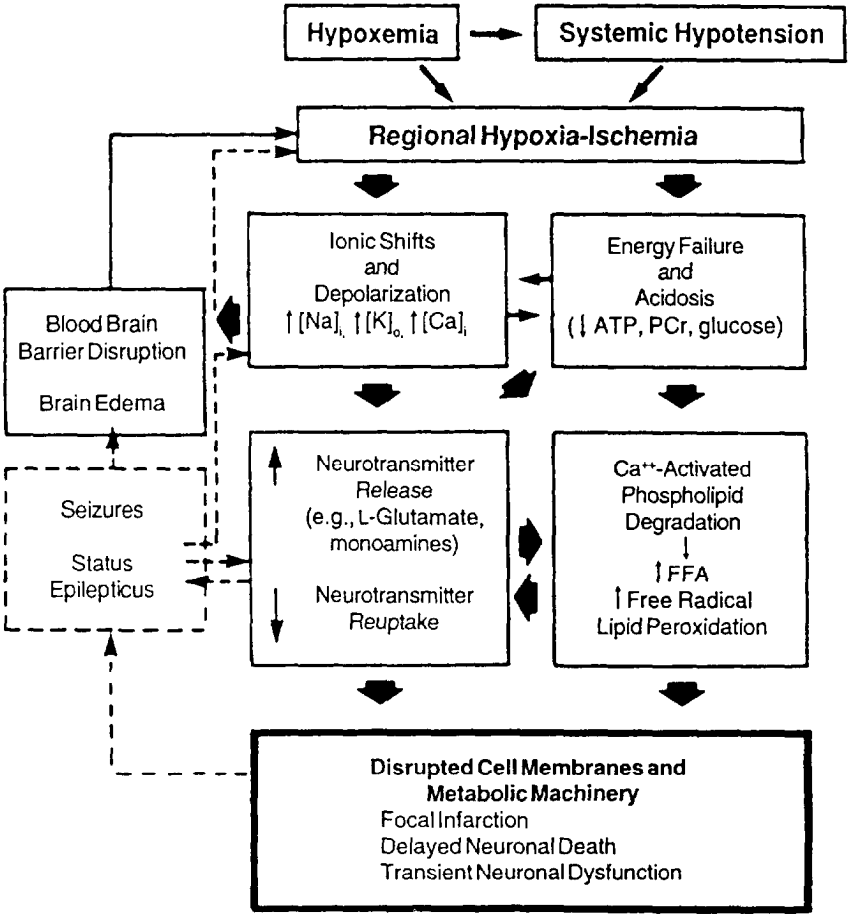

FIGURE 5. *Schematic representation of events that contribute to altered neurologic function and neuronal cell death in perinatal hypoxic-ischemic brain injury.*

al. 1987). This procedure reliably produces focal ischemia and hypoxia in the cerebral forebrain ipsilateral to the ligation while the brainstem and opposite hemisphere are exposed primarily to hypoxia. Unilateral hypoxia-ischemia in this model causes permanent neuronal injury in the corpus striatum, overlying cortex, the hippocampus, globus pallidus, and thalamus.

To examine whether enhanced release and inhibition of uptake of EAA contribute to the pathogenesis of hypoxic-ischemic brain injury in the immature brain, we administered the noncompetitive NMDA antagonist MK-801 at various times before or after injury in the model (Wong et al. 1986). In initial experiments 1 mg/kg of MK-801 was administered intraperitoneally (i.p.) after carotid ligation and just before onset of hypoxia, along with another dose 1.25 hours into hypoxia. This treatment protocol reduced hypoxic-ischemic brain injury by 85 percent relative to saline-injected controls as assessed by comparison of cerebral hemisphere weights 5 days following hypoxia-ischemia (McDonald et al. 1987). A single dose of MK-801 at the onset or after 1.25 hours of hypoxia was similarly effective, while single injections at 1.5 or 2.5 hours into hypoxia were less effective. These data suggest that there is a critical time threshold for drug intervention. Glycine antagonists are also effective in this model (Uckele et al. 1989).

DEVELOPMENTAL ALTERATIONS IN EXCITATORY AMINO ACID NEUROTOXICITY

Our studies indicate that the susceptibility of the brain to EAA neurotoxicity changes considerably during development, and different brain regions and neuronal types exhibit their own developmental profile of susceptibility to selective EAA receptor agonists. The 7-day-old rat brain is most susceptible to NMDA-induced brain injury, whereas quisqualate is less toxic and kainate is relatively nontoxic on an equimolar basis (figure 6). In contrast, the adult brain is most susceptible to kainate-induced brain injury, followed by injury from NMDA and then from quisqualate.

Kainate is a potent neurotoxin in the adult brain, but it is much less toxic in the immature brain. Nevertheless, kainate does produce prolonged tonic-clonic seizures in immature animals. The susceptibility of striatal neurons to kainate neurotoxicity initially appears at PND 7 and gradually rises to adult levels by PND 21 (Campochiaro and Coyle 1978). The sensitivity to kainate neurotoxicity parallels the development of glutamatergic innervation into the striatum as measured by synaptosomal uptake of [^{3}H]-glutamate. Similar developmental trends of kainate toxicity have been reported in the hippocampus (Wolf and Keilhoff 1984).

The susceptibility of the developing brain to NMDA toxicity transiently peaks near PND 7 in rats (figure 7). The severity of brain injury produced by direct intrastriatal infusion of NMDA is approximately 60 times greater at PND 7

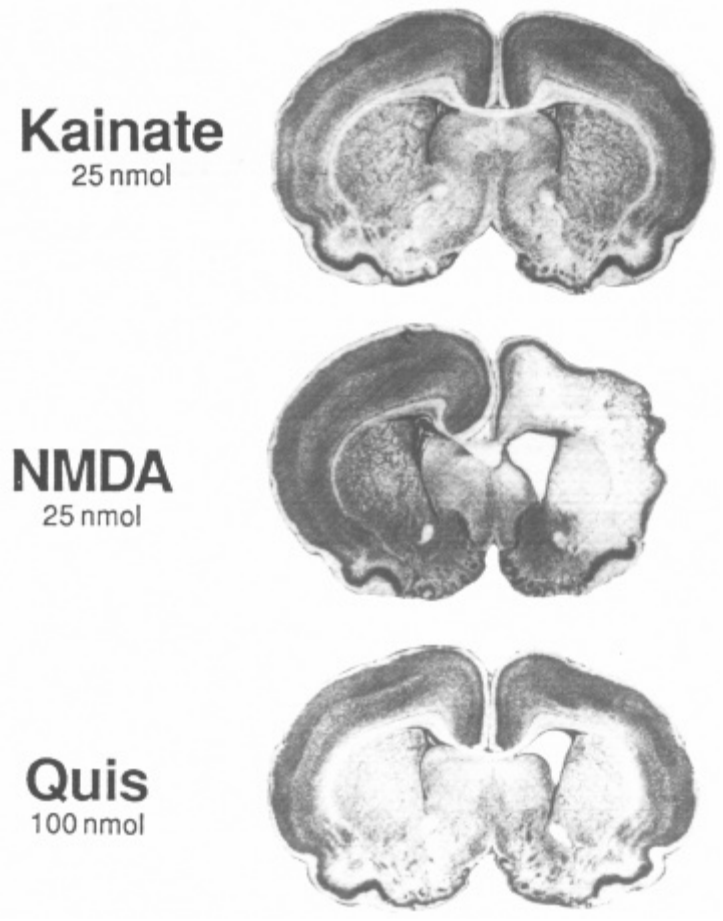

FIGURE 6. *Comparison of the severity of excitotoxic brain injury produced by the selective EAA receptor agonists kainate, NMDA, and quisqualate. PND 7 rats received unilateral intrastriatal injections (right hemisphere) of either kainate (25 nmol/0.5 µL), NMDA (25 nmol/0.5 µL), or quisqualate (100 nmol/0.5 mL). Animals were sacrificed on PND 12. All three agonists produced prolonged tonic-clonic seizures. Injections of NMDA produced the greatest degree of brain injury whereas quisqualate injections produced less. In contrast, kainate injections produced few signs of neuronal injury.*

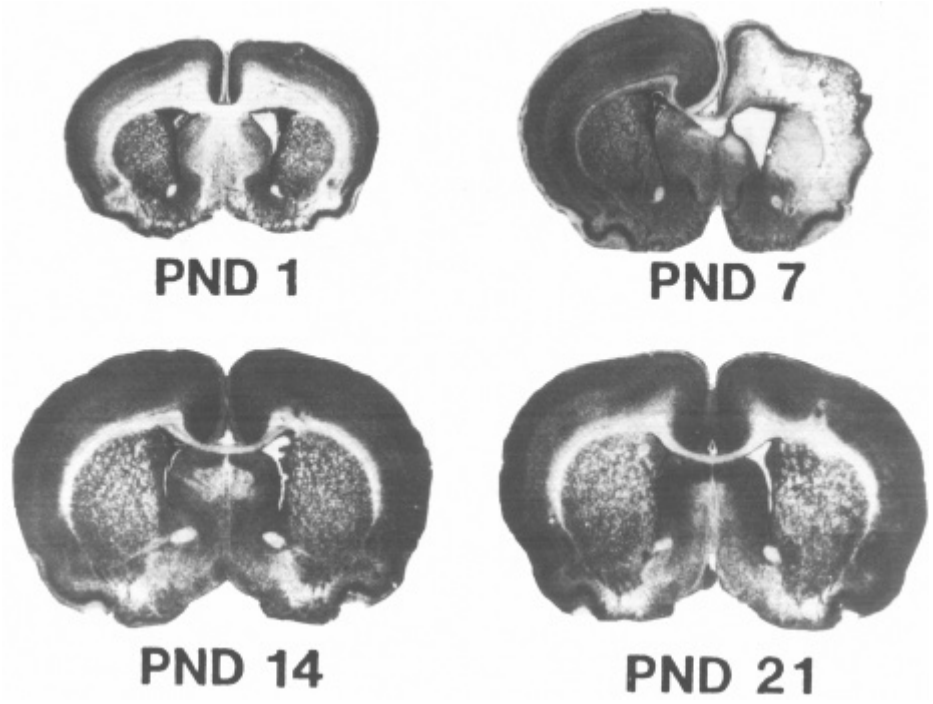

FIGURE 7. *Ontogeny of NMDA-mediated brain injury. Comparison of Nissl-stained coronal brain sections at the level of the corpus striatum from PND 1, 7, 14, and 21 rats that received unilateral intrastriatal injections of 25 nmol(0.5 μL injection volume) NMDA. Animals were sacrificed 5 days later. The severity of brain injury is maximal at PND 7 the lesion is characterized by confluent neuronal necrosis involving the striatum and extending into the thalamus, dorsal hippocampus, and overlying neocortex. Compared with PND 7, the severity of NMDA-mediated brain injury is substantially reduced at PND 1, 14, and 21.*

(McDonald et al. 1988*a*). NMDA toxicity peaks at different development time points in various brain regions. It is noteworthy that the susceptibility of the developing brain to NMDA parallels its susceptibility to hypoxic-ischemic brain injury (Ikonomidou et al. 1989).

Susceptibility of striatal and hippocampal neurons to quisqualate-mediated brain injury during postnatal development does not change as markedly, although toxicity appears to be somewhat enhanced in younger animals (Hastings et al. 1985; Silverstein et al. 1986*b).* However, the quisqualate agonist AMPA (which binds to a subtype of quisqualate receptors) is approximately 10 to 15 times more potent on an equimolar basis than quisqualate as a neurotoxin when injected intercerebrally in PND 7 rats (data not shown).

The noncompetitive NMDA antagonist MK-801 is approximately tenfold more potent as a neuroprotective against NMDA- and hypoxia-ischemia-mediated neuronal injury in immature rodents than in adults (McDonald et al. 1987; McDonald et al. 1989*c;* Foster et al. 1987; Gill et al. 1987). Virtually complete neuroprotection can be achieved with NMDA antagonists in the perinatal brain, in contrast to only partial protection in adults.

CHARACTERIZATION AND PHARMACOLOGY OF NMDA-INDUCED BRAIN INJURY

We have developed a model of NMDA-induced brain injury to characterize the mechanism of EAA neurotoxicity and the effectiveness of neuroprotective compounds against NMDA neurotoxicity in this model may relate to the pathogenesis of hypoxia-ischemia brain injury (McDonald et al. 1987, 1989*c,* and 1990).

In this model, unilateral intrastriatal injection of 25 nmol NMDA on PND 7 typically produces tonic-clonic seizure activity and results in ipsilateral hemispheric necrosis 5 days later (McDonald et al. 1989*c,* 1990). Due to the enhanced susceptibility of the PND 7 brain to NMDA toxicity and also to the rapid phase of brain growth, the severity of brain injury can be quantified by comparison of the weights of the injected and contralateral cerebral hemispheres (McDonald et al. 1988*b,* 1989*c,f).* This method appears to provide a reliable index of neuronal injury (McDonald et al. 1989*c,f).* There is a linear relationship between the amount of NMDA (up to 25 nmol) injected into the striatum and the severity of resulting brain injury (R^2 = 0.97) and measurements of regional cross-sectional areas (R^2 = 0.98). Because of the sensitivity and rapidity of this model, detailed pharmacologic dose-response relations of neuroprotective compounds against NMDA-induced brain injury can be examined.

We have systematically examined the neuroprotective characteristics of four classes of NMDA receptor antagonists, anticonvulsants, σ receptor ligands, as well as additional compounds that theoretically could protect against excitotoxicity (McDonald et al. 1988*a,* 1989*a,c,e,f).* Only competitive and noncompetitive NMDA antagonists were completely protective. Injury was partially reduced by the σ ligands +PPP and haloperidol (36 percent reduction). Of five anticonvulsants, only carbamazepine afforded partial protection (36 percent).

TEMPORAL CHARACTERISTICS OF NMDA-MEDIATED BRAIN INJURY

The temporal progression of NMDA-mediated brain injury was examined by varying the MK-801 posttreatment interval after intrastriatal NMDA injection. One mg/kg MK-801 was administered (i.p.) to PND 7 rats at seven time intervals after intrastriatal injection of 25 nmol NMDA. The severity of resulting brain injury was assessed 5 days later on PND 12 by comparison of cerebral hemisphere weight disparities. Administration of MK-801 up to 8 hours after the NMDA injection reduced the severity of brain injury compared to saline-treated controls *(p* < 0.001, ANOVA). The MK-801 posttreatment time interval was linearly related to the severity of resulting brain injury between l-hour and 10-hour posttreatment intervals (R^2 = 0.99). For every hour treatment was delayed (between 2 and 10 hours) the severity of brain injury increased by approximately 6.5 percent. Extension of the posttreatment interval to greater than 8 hours did not attenuate NMDA-mediated brain injury. The ability of MK-801 to reduce NMDA-mediated brain injury when treatment is delayed suggests that the biochemical events initiated by overactivation of NMDA receptors are, in part, reversible for up to one third of a day after the initial insult.

MK-801 ENHANCES NMDA-MEDIATED BRAIN INJURY AND INCREASES NMDA RECOGNITION SITES

Another observation that may be clinically important is that administration of MK-801 24 hours prior to intrastriatal injection of NMDA paradoxically enhances NMDA-induced brain injury (McDonald et al. 1988*c).* One mg/kg MK-801 was administered interperitoneally 0.5, 2, 6, 12, and 24 hours prior to intrastriatal injection of NMDA. Animals were sacrificed 5 days later on PND 12, and the severity of brain injury was assessed by comparison of the weights of injected and contralateral cerebral hemispheres.

In these studies, MK-801 protected against NMDA toxicity when administered 0.5, 2, 6, and 12 hours prior to intrastriatal injection of NMDA in comparison to saline-treated NMDA-injected controls *($p < 0.001$* vs. saline-treated, NMDA-injected controls, ANOVA). In contrast, administration of MK-801 24 hours prior to intrastriatal injection of NMDA paradoxically enhanced the severity of NMDA-mediated brain injury *($p < 0.05$* vs. saline-treated, NMDA-injected controls, ANOVA). The severity of brain injury was greater in groups that received MK-801 24 hours earlier than in saline-treated controls (regression analysis, $p < 0.001$). The mortality rate did not differ between saline- and MK-801 -treated groups.

The enhanced toxicity of NMDA produced by 24-hour MK-801 pretreatment was blocked by a second injection of MK-801 15 minutes after intrastriatal injection of 25 nmol NMDA. This observation suggests that the enhanced toxicity produced by MK-801 pretreatment is mediated by NMDA receptor channel activation. The effects of MK-801 pretreatment were selective for enhancement of NMDA-mediated brain injury, since quisqualate-mediated brain injury was not increased by a similar MK-801 pretreatment.

Quantitative receptor autoradiography was used to determine if alterations in EAA receptor binding characteristics are associated with enhanced toxicity of NMDA produced by MK-801 pretreatment. NMDA-sensitive [^{3}H]-glutamate binding was increased by up to 30 percent in comparison with saline-treated controls in the four brain regions examined: CA_1 and CA_3 hippocampal subfields corpus striatum and cingulate cortex (McDonald et al. 1989*b)*.

The mechanisms by which MK-801 treatment alters NMDA-sensitive [^{3}H]-glutamate and [^{3}H]-TCP binding to NMDA receptor channels and quisqualate-sensitive [^{3}H]-glutamate and [^{3}H]-TCP binding are unclear (McDonald et al. 1990; Young et al. 1988). The increase in NMDA-sensitive treated glutamate binding produced by MK-801 may reflect a compensatory response to transient channel blockade.

The heightened sensitivity to NMDA neurotoxicity found 24 hours after MK-801 administration could relate to a persistent elevation in NMDA-sensitive [^{3}H]-glutamate receptors after the neuroprotective effect of MK-801 has diminished.

The pharmacology of MK-801- and PCP-like compounds in vivo in the developing brain appears to be complex. In addition to blocking NMDA-

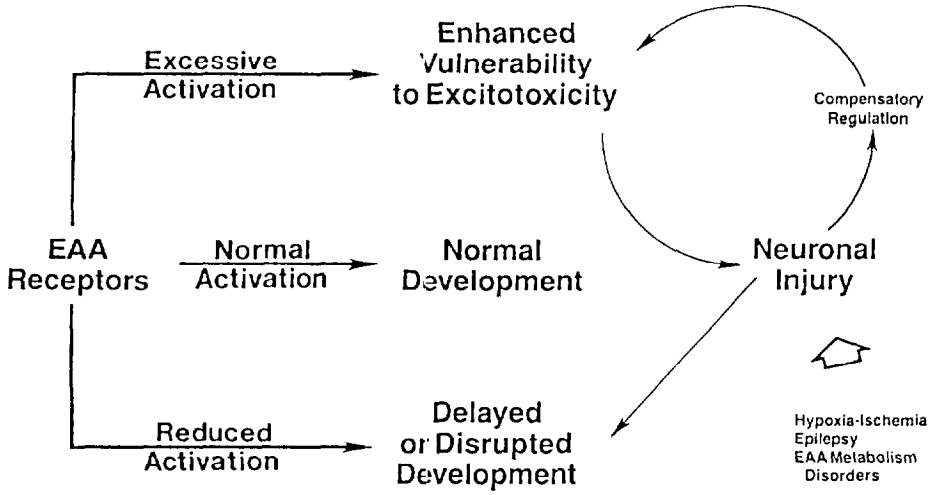

FIGURE 8. *Schematic diagram relating the level of EAA receptor activation and the balance between normal and delayed or disrupted development and excitotoxicity. Evidence presented in this review suggests there is an optimum level of EAA activation required for normal development. However, excessive receptor activation can produce neuronal injury, while under activation may delay or disrupt normal development. Acute brain injury (e.g., hypoxia-ischemia, etc.) can directly disrupt the normal course of development. following brain injury, neuronal adaptive regulatory mechanisms are activated. These compensatory processes, while promoting neuronal plasticity and repair, may paradoxically enhance the susceptibility of the brain to excitotoxic injury. A corollary to this phenomenon is that the developing brain would be predicted to be most vulnerable to excitotoxic injury during periods when the capacity for synaptic plasticity is greatest.*

responses and NMDA-mediated brain injury, they also regulate NMDA recognition site binding in a complex manner.

SUMMARY AND CONCLUSIONS

EAA neurotransmitters participate in a variety of physiologic processes during central nervous system development. EAAs function as neurotransmitters but also regulate development of neuronal cytoarchitecture and neuronal

connectivity. EAAs play a major role in several forms of activity-dependent synaptic plasticity including learning and memory, and stabilization and elimination of synaptic connections during development. Similar molecular mechanisms may underlie plastic changes during development and neuronal destruction by overactivation of EAA receptors. A critical level of EAA neurotransmitter activity is required for normal development. However, there appears to be a continuum of physiologic reactions to EAA receptor activation: underactivation can retard or disrupt normal development, whereas overactivation can lead to neuronal injury and destruction (figure 8).

The susceptibility of the brain to excitotoxicity is dramatically altered during postnatal development. As a result of these changes, the contribution of NMDA receptor activation in excitotoxic brain injury may be greater during early periods of postnatal development, whereas non-NMDA receptors may make a greater contribution to excitotoxic injury in the adult brain.

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Isolation and Characterization of an Endogenous Ligand for the PCP and σ Receptors From Porcine, Rat, and Human Tissue

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Phencyclidine (PCP) is a dissociative anesthetic that alters sensory perception, impairs memory, and induces a psychosis resembling schizophrenia (Luby et al. 1959). Two different receptors have been characterized that mediate some of the effects of PCP. One is the PCP receptor, which is distinguished by its interaction with *N*-methyl-D-aspartic (NMDA) receptors (Lodge and Anis 1982; Loo et al. 1986) and the inability of σ ligands, such as haloperidol and (-)butaclamol, to label this site. The second receptor is the σ receptor, which is distinguished by the finding that many neuroleptics label this site (Tam and Cook 1984). Also, the distribution of PCP and σ receptors in the central nervous system (CNS) is different (Largent et al. 1984; Contreras et al. 1987*a;* Gundlach et al. 1985).

Several studies have examined the roles of PCP and σ receptors in the normal functioning of endocrine (Su et al. 1988), immune (Su et al. 1988; Wolfe et al. 1988), and central nervous systems (Maragos et al. 1987; Mouradian et al. 1988), but very few have studied the role or presence of endogenous ligands for PCP or σ receptors. Lack of a difference in PCP or σ receptors between normal and disease states does not eliminate any possible role of PCP or σ system because there could instead be changes in synthesis, metabolism, or release of endogenous ligands, which have been identified by several groups.

Endogenous PCP-like compounds have been identified by extracting material from porcine (Quirion et al. 1984) or bovine brains (Zukin et al. 1987) that inhibits binding to PCP receptors. The material isolated from porcine brains has been shown to bind selectively to PCP receptors, to have a specific distribution,

to induce PCP-like effects, and to be peptidergic in nature (Quirion et al. 1984; DiMaggio et al. 1988).

Endogenous σ like ligands have also been identified. One possible ligand is progesterone (Su et al. 1988), which interacts with σ receptors with moderate affinity. Several peptidergic components isolated from brain have also been found to inhibit binding to σ receptors (Su et al. 1986; Contreras et al. 1987*b).* These peptidergic σ like compounds have been shown to be different from endogenous PCP-like compounds (Contreras et al. 1987*b)* and to bind selectively to σ receptors (Su et al. 1986; DiMaggio et al. 1988).

The purpose of this study was to further characterize endogenous ligands isolated from porcine brain and to determine whether endogenous PCP-like material is also present in human tissue.

METHODS

Animals and Drugs

Sprague-Dawley rats weighing 200 to 250 g (Sasco Farms) were used in all experiments. Each animal was used only once.

Phencyclidine HCl (PCP) and NMDA were obtained from Sigma Chemical Company (St. Louis, MO). (+)-*N*-allylnormetazocine (NANM) was obtained from Research Biochemicals Inc. (Wayland, Mass.). 1,3-Di-*o*-tolyl-guanidine (DTG) was obtained from Aldrich Chemical Company (Milwaukee, Wis.). [^{3}H]-TCP (1-(2-thienyl)-cyclohexylpiperidine), [^{3}H]-(+) 3-PPP (3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine), and [^{3}H]-(+)-*N*-allylnormetazocine were obtained from New England Nuclear (Boston, Mass.).

Isolation of Endogenous PCP- and σ-Like Activity

PCP- and a-like activity was purified from porcine tissue as described by Contreras and colleagues (1987*b)* and DiMaggio and coworkers (1988).

PCP- and o-like activity was isolated from rat brains, samples of human cortex, and human cerebrospinal fluid (CSF) by homogenizing the tissue or mixing the CSF with a solution of trifluoroacetic acid (TFA), hydrochloric acid, formic acid, and sodium chloride. The mixture was centrifuged at 5,000 x g for 15 minutes. The supernatant was extracted with ether and the aqueous phase titrated to a

pH of 4.0 with sodium hydroxide. Particulate matter was removed by filtering the solution through a Millex-HV, 0.45 µm, durapore filter. Next, the solution was chromatographed by analytical reverse-phase high-pressure liquid chromatography (HPLC) using a C-18 column. Salts in the solution were first eluted by washing the column (Vydac C-18 or Waters µBondapak C-18) for 20 minutes with 0.1 percent TFA, and then a linear gradient of acetonitrile (0 to 60 percent in 0.1 percent TFA) was used to fractionate the sample. Aliquots from the fractions, collected at l-minute intervals, were assayed in a PCP or σ radioreceptor assay.

Radioreceptor Assays

The PCP and σ radioreceptor assays were carried out as described by Contreras and colleagues (1988). Briefly, the aliquots from the column were lyophilized to dryness and then tested for the ability to inhibit the binding of 2 nM of [^{3}H]-TCP, 6 nM of [^{3}H]-(+)3-PPP, or 6 nM of (+)NANM. The assay was incubated at room temperature for 60 minutes, then filtered through glass filters (Schleicher & Schuell, #32), which were presoaked for 1 hour in 0.05 percent polyethylenimine. Nonspecific binding was determined using 30 µM of PCP, (+)NANM, or DTG.

NMDA-Induced Dopamine Efflux

The method used to measure PCP-like antagonism of NMDA-induced dopamine release was modified from a super-fusion method described by Middlemiss and coworkers (1988). Male Sprague-Dawley rats (200 to 250 g) were decapitated and the brains removed. The nucleus accumbens was removed and sliced to a thickness of 350 µm with a McIlwain tissue chopper. The tissue slices were preincubated for 15 minutes at 37°C in a modified Krebs buffer (NaCl, 135 mM; KCl, 5 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.25 mM; $MgS0_4$, 1 mM; glucose, 10 mM; ascorbate, 0.11 mM; pargyline, 1 nM), pH 7.4, containing 25 nM of [^{3}H]-dopamine. The tissue slices were rinsed twice with the Krebs buffer before three to four slices were placed on a mesh support and incubated sequentially in the modified Krebs buffer containing 1 µM of GBR-12909 and 100 µM of sulpiride at pH 7.4 for 30 minutes at 37°C and then incubated in the modified Krebs buffer minus $MgSO_4$ for 5 minutes at 37°C.

The effect of NMDA on dopamine release was determined by incubating tissue sequentially for 1 minute in vials containing 0.5 mL of Krebs buffer minus $MgSO_4$, in the presence or absence of PCP, pig, or rat extracts or in

combination with 100 μM of NMDA. The amount of radioactivity remaining in the tissue slices and the amount released into the incubation medium was determined by liquid scintillation spectrometry. The percent of radioactivity release into the incubation medium was calculated as the amount of radioactivity in the incubate relative to the total amount in the slices at that time. The amount of radioactivity released in response to NMDA, which is added in fraction 5, is expressed as the following fractional release:

$$\text{fractional release} = \frac{\text{percent radioactivity in fraction 5}}{\text{percent radioactivity in fraction 4}}$$

The amount of radioactivity in the incubation medium was used as an index of dopamine release. The effect of PCP and tissue extracts on NMDA-stimulated dopamine release was compared using a Dunnett's test subsequent to a one-way analysis of variance.

Synthesis of ß-Endopsychosin (1-12)

The peptide was prepared using an Applied Biosystems Peptide Synthesizer and Boc-protected amino acids on an amine resin. Coupling was achieved using dicyclohexylcarbodiimide activation, and deprotection of the intermediate Boc-peptide was achieved using trifluoroacetic acid in methylene chloride. The peptide was cleaved and deprotected using anhydrous hydrogen fluoride and purified on a C-l 8 reversed-phase column using an acetonitrile:water gradient containing 0.05 percent trifluoroacetic acid.

Behavioral Assay

Rats were placed individually in rat cages for at least 1 hour in advance of the experiment. DTG or synthetic peptide was administered by i.c.v. injection. The behavior of each rat was scored using PCP rating scales developed by Sturgeon and coworkers (1979) as described by Contreras and colleagues (1986). Briefly, the rating scale for stereotyped behavior is (1) sniffing, grooming, rearing, and chewing behavior; (2) undirected head movements or reciprocal forepaw treading; (3) some circling, running backward, or head-weaving behavior; (4) continuous circling, weaving, or running backward; and (5) dyskinetic extension and flexion of head and limbs. The rating scale for ataxia is (1) awkward movement, may fall when rears; (2) stumbles; (3) falls; (4) cannot move beyond a small area, may support its weight on stomach or haunches; and (5) unable to move. A rating of 5 was considered a 100-percent response. ED_{50} values were determined using a Finney assay.

RESULTS

Endogenous PCP-Like Activity

In previous reports the purification of endogenous PCP-like activity was described (DiMaggio et al. 1988). To determine the distribution of this PCP-like activity (inhibition of the binding of [^{3}H]-TCP), areas of porcine brain were extracted and fractionated over a C-18 column. The greatest concentration of PCP-like activity in the brain was isolated from cortex, with smaller amounts found in thalamus and hypothalamus and even smaller amounts in hippocampus and cerebellum (figure 1). No PCP-like activity was detected in pituitary or brainstem of porcine brains.

Endogenous PCP-like activity has also been isolated from sources other than porcine brains, such as from the gastrointestinal tract (figure 1). The amount of PCP-like activity found in the stomach is about sevenfold greater than that found in cortex. PCP-like activity isolated from pancreas and small intestine was greater than that found in CNS but less than that found in stomach. PCP-like activity has also been found in the rat brain. The distribution of PCP-like activity paralleled the distribution found in the porcine brain (figure 1). As in porcine brain, there was little or no PCP-like activity detected in cerebellum or pituitary. Unlike porcine brains, a small amount of PCP-like activity was detected in rat brainstem.

To assess whether the endogenous PCP-like activity, like PCP, also antagonized NMDA, the effect of endogenous PCP-like material on dopamine basal and NMDA-stimulated efflux was determined (figure 2). In this paradigm, PCP had no significant effect on the basal release of dopamine but inhibited NMDA-stimulated release of dopamine in a dose-dependent manner. Like PCP, an aliquot of endogenous PCP-like activity isolated from porcine or rat brain that inhibited about 75 percent of the binding of [^{3}H]-TCP had no significant effect on basal release of dopamine. Addition of NMDA produced a stimulation of dopamine release that was antagonized by both PCP and the endogenous PCP-like activity isolated from rat and porcine brain (figure 3). However, an equivalent volume from an HPLC fraction that contained a similar amount of solvent as the active fractions did not antagonize NMDA-stimulated dopamine release.

To assess whether there was endogenous PCP-like activity in human tissue, pieces of human cortex were extracted and fractionated using a C-18 column

and reverse phase-HPLC (figure 4). Four samples of human cortex were extracted and tested in the same manner with similar results. Inhibition of binding of [^{3}H]-TCP by aliquots from fractions 1 through 20 probably reflects nonspecific inhibition due to salt because changes in PCP-like activity paralleled changes in conductivity. There was not enough material to purify the PCP-like activity, but it was clear that aliquots containing PCP-like activity were present in three groups of fractions, a pattern also seen during the purification of endogenous PCP-like material from porcine brain (Quirion et al. 1984).

Endogenous PCP-like activity was also detected in human CSF (5 mL) (figure 5). The inhibition of the binding of [^{3}H]-TCP detected in fractions 1 through 20 was probably nonspecific inhibition of the binding of [^{3}H]-TCP largely due to salt. Like porcine and rat brain, the endogenous PCP-like

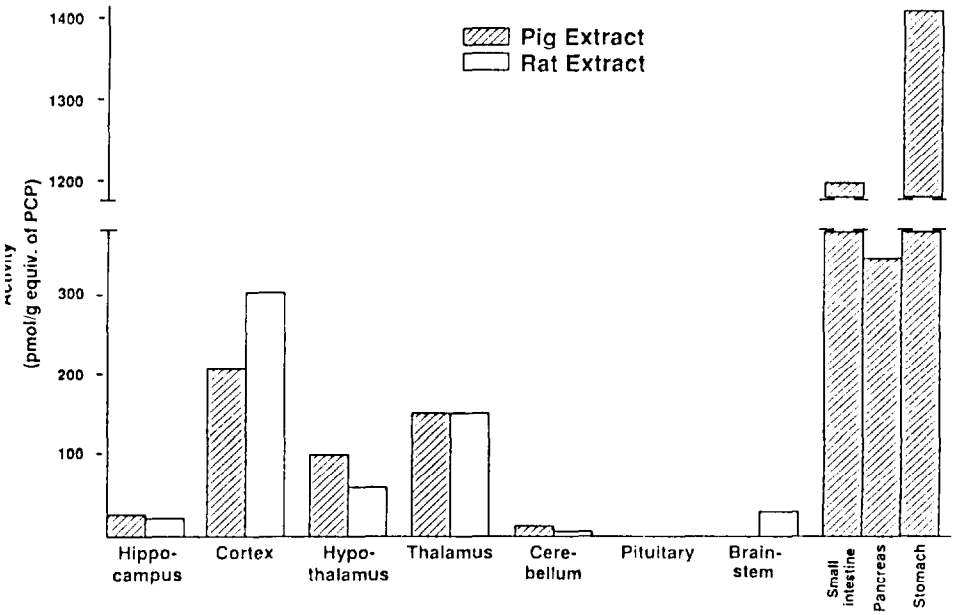

FIGURE 1. *Distribution of PCP-like activity from porcine and rat tissue. Inhibition of the binding of [^{3}H]-TCP by fractions extracted from porcine or rat tissue was expressed as the equivalent concentration of PCP that inhibits the same amount of binding of [^{3}H]-TCP per gram of tissue.*

material also eluted in three groups of fractions. The total amount of PCP-like activity in CSF (32 pmol equiv. PCP/mL) is about 10 to 15 times less than that detected in human or rat cortex.

Endogenous σ-Like Activity

The isolation and purification of endogenous σ-like activity has already been described (Contreras et al. 1987*b*). The distribution of this activity in porcine brain is shown in figure 6. Unlike the distribution of endogenous PCP-like material, the greatest amount of σ-like activity (inhibition of the binding of [^{3}H]-(+)NANM) was found in the pituitary, with smaller amounts detected in caudate and hypothalamus and even smaller amounts in frontal cortex, hippocampus, and thalamus. Similarly, endogenous o-like activity was detected in brainstem but not in cerebellum. The amount of endogenous o-like activity

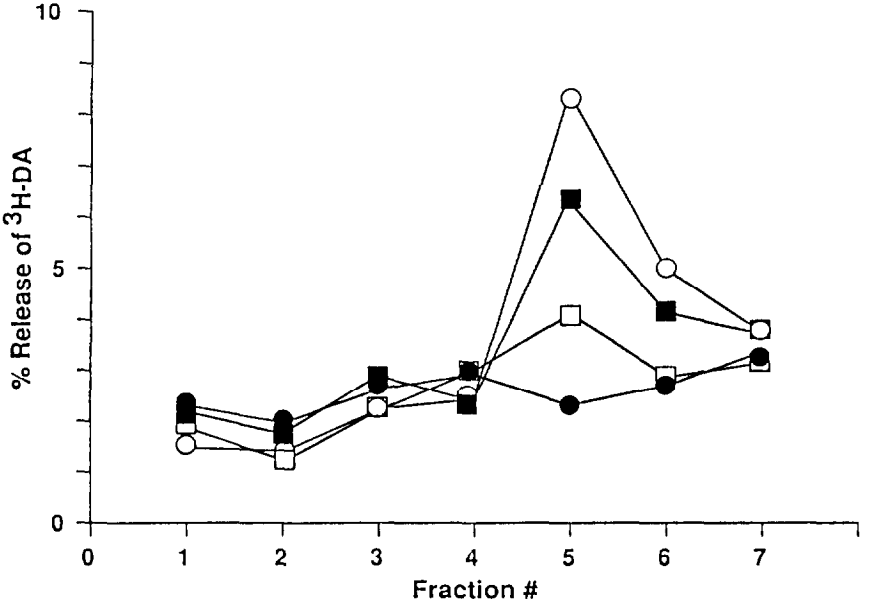

FIGURE 2. *Inhibition of NMDA-induced dopamine efflux by PCP. The percent release of [^{3}H]-dopamine from slices of the nucleus accumbens was determined at 1-minute intervals. Vehicle (○) or PCP at a concentration of 0.1 μM (■) or 1 μM (□) was added to the incubation buffer in fractions 3 through 7. NMDA (100 μM) was added in fraction 5 of all groups except the control group (●).*

(nmol/g) was about 1,000 times greater than that of PCP-like activity (pmol/g) in areas of the brain that contained both PCP- and o-like compounds.

Like the endogenous PCP-like material, the structure of the o-like compound is at least in part peptidergic (DiMaggio et al. 1988). A small peptide consisting of 12 amino acids was synthesized. This peptide, ß endopsychosin (1-12) inhibited the binding of [^{3}H]-(+)NANM but not that of [^{3}H]-TCP (figure 7).

This peptide also possessed o-like bioactivity. Like DTG, the peptide induced stereotyped behavior and ataxia in a dose-dependent manner (figure 8).

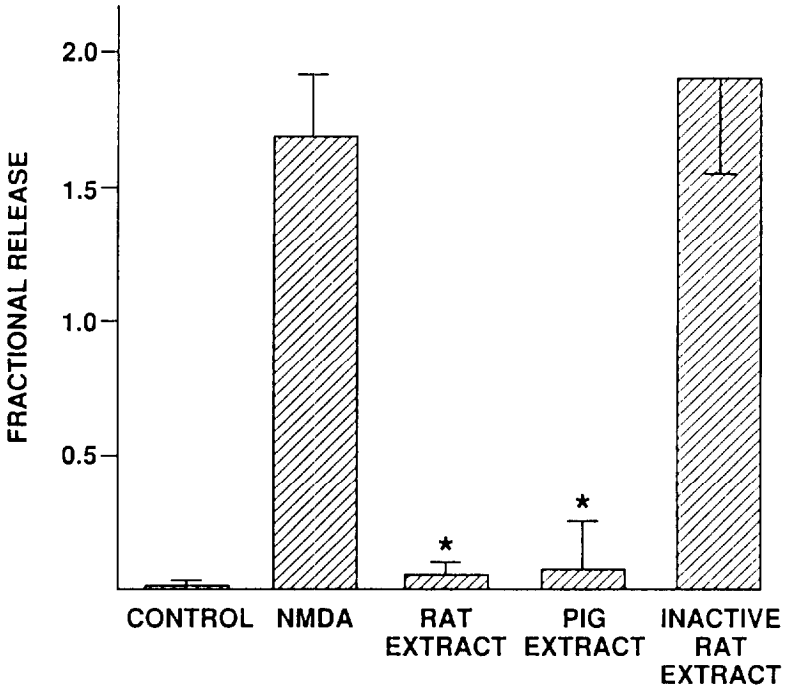

FIGURE 3. *Antagonism of NMDA-stimulated dopamine release by endogenous PCP-like activity. The fractional release of [^{3}H]-DA was determined in fraction 5. When slices of the nucleus accumbens were incubated with PCP (1 µM) or endogenous PCP-like activity isolated from porcine or rat brains, NMDA-stimulated dopamine release was significantly attenuated ($P<0.05$).*

DISCUSSION

This study extends earlier findings that endogenous PCP- and o-like ligands are present in the CNS. The endogenous PCP-like ligand had been found to specifically bind to PCP receptors (DiMaggio et al. 1988) and to have a specific distribution in porcine brains. Since no PCP-like activity was detected in pituitary or cerebellum, it is unlikely that the endogenous PCP-like ligand is an artifact of the extraction or purification procedure. Endogenous PCP-like activity was not limited to porcine brains but was detected in human CSF and tissue from rat and human CNS, and porcine gastrointestinal tract. Since many neuroactive peptides were first isolated from the gastrointestinal tract, it was not

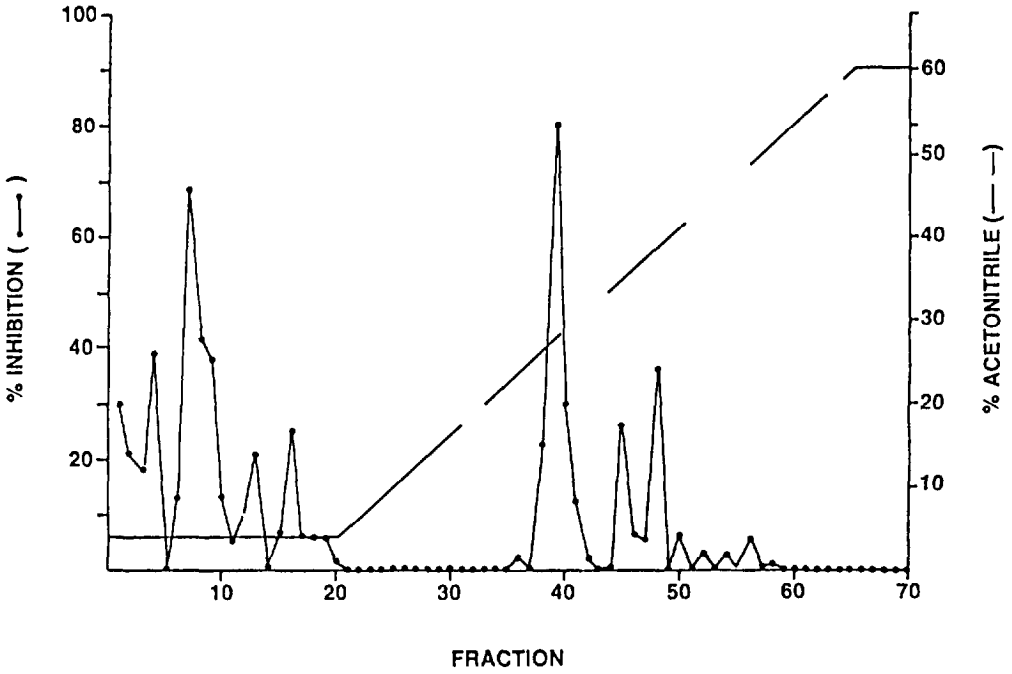

FIGURE 4. *An example of the isolation of endogenous PCP-like activity from human cortex. A comparison showing the gradient of acetonitrile used to fractionate a sample of human cortex and PCP-like activity.*

surprising that endogenous PCP-like activity was isolated from pancreas, stomach, or small intestine, but it was surprising that the activity was greater than that found in the CNS.

The finding that PCP antagonizes NMDA-stimulated dopamine release was used to further explore the biological activity of the endogenous PCP-like ligand. Like PCP, the PCP-like activity isolated from rat or porcine brain inhibited NMDA-stimulated dopamine release, which was not mimicked by HPLC fractions that were inactive in PCP radioreceptor assays. This result is consistent with earlier reports that the endogenous PCP-like ligand has PCP-like bioactivity in two other paradigms, inhibition of spontaneous firing of cortical and hippocampal neurons and contralateral turning after intranigral injection (Quirion et al. 1984; DiMaggio et al. 1988).

An endogenous o-like ligand has also been identified in porcine brains. The endogenous σ-like ligand interacts selectively with σ receptors (DiMaggio et al. 1988) and has a specific distribution in CNS. Unlike the endogenous PCP-like

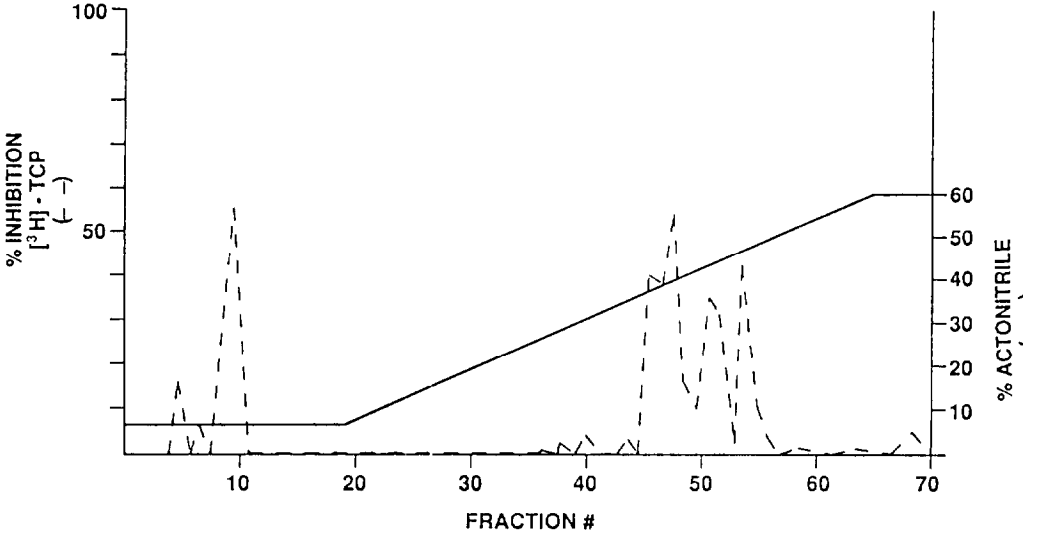

FIGURE 5. *Isolation of endogenous PCP-like activity from human CSF. The figure shows a comparison of the acetonitrile gradient used to fractionate human CSF and PCP-like activity in the HPLC fractions.*

ligand, the largest concentration of o-like activity was isolated from pituitary. This finding is consistent with the hypothesis that σ receptors may be involved in modulation of endocrine function (Su et al. 1988).

The result of the authors' studies to isolate and purify the endogenous σ ligand has been the synthesis of a small peptide, ß endopsychosin (1-12). Like DTG, this peptide produced o-like behaviors, which supports studies showing that

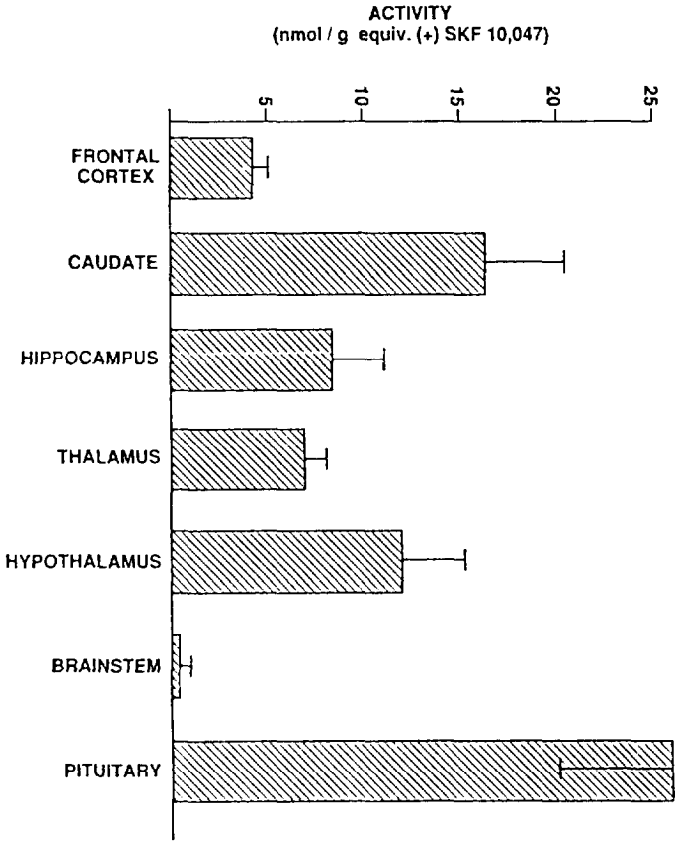

FIGURE 6. *Distribution of endogenous σ-like activity in porcine brain. σ-like activity is expressed as the equivalent amount of (+)NANM required to produce the same degree of inhibition of the binding of [^{3}H]-(+)NANM ([^{3}H]-(+)SKF 10,047).*

endogenous σ-like activity isolated from porcine brain represents an endogenous ligand for σ receptors and is not an artifact.

In summary, an endogenous PCP-like ligand can be detected in several types of tissue, including human cortex and CSF; its distribution is different from that of o-like activity; and it has PCP-like activity. The endogenous σ-like ligand also has a unique distribution, and a synthetic peptide derived from these studies has o-like bioactivity.

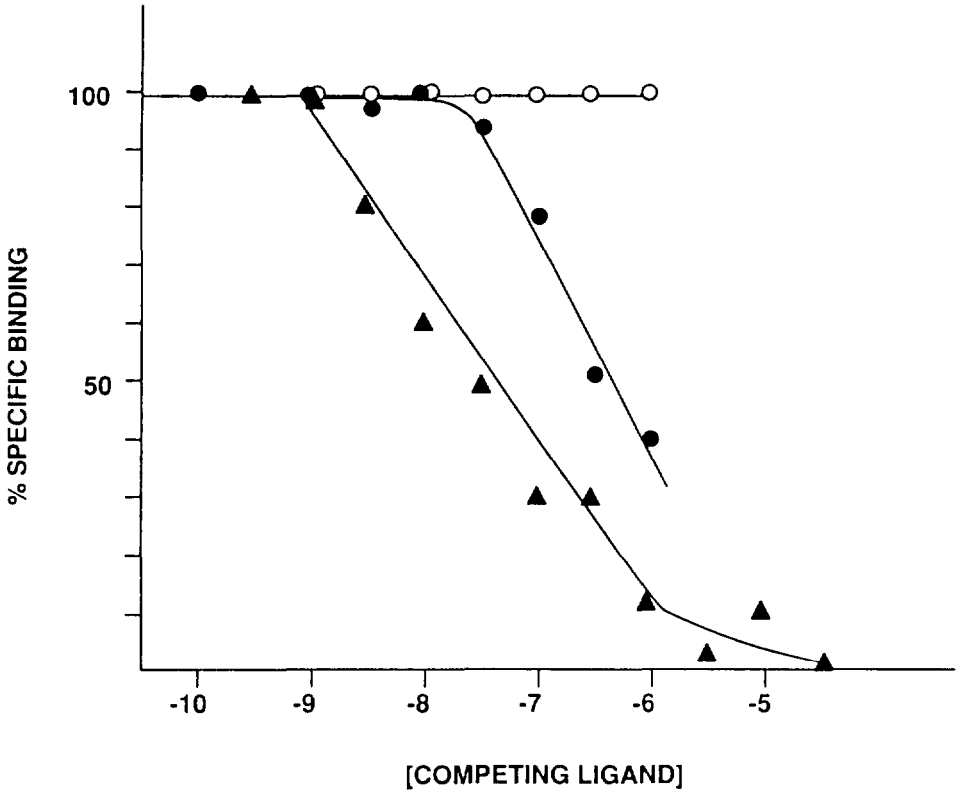

FIGURE 7. *Inhibition of σ-binding and PCP binding by (+)NANM and ß-endopsychosin (1-12). Inhibition of the binding of [^{3}H]-(+)NANM by (+)NANM (A) or ß-endopsychosin (1-12) (●) was determined. The inhibition of the binding of [^{3}H]-TCP by ß-endopsychosin (1-12) (O) was also determined.*

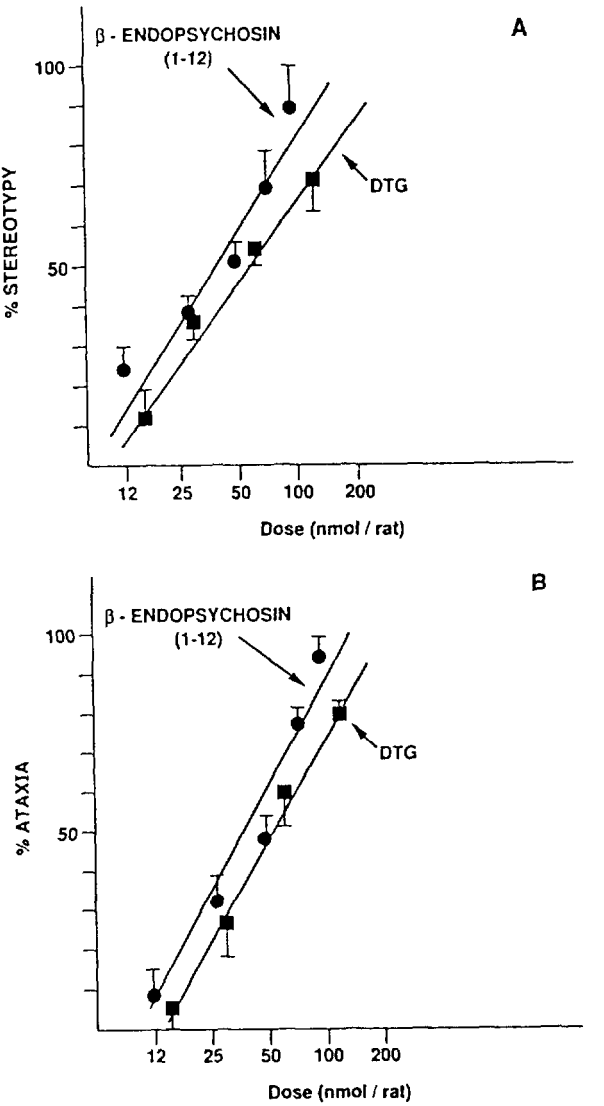

FIGURE 8. *Stereotyped behavior and ataxia induced by DTG and ß-endopsychosin 1-12. DTG (■)or ß-endopsychosin (1-12) (●) was administered by i.c.v. injection, and the ratings determined at the time of peak effect (5 minutes) were used to determine the dose-response curve for (A) stereotyped behavior and (B) ataxia.*

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Summary and Future Directions

Edward F. Domino

The following generalizations can be made as a result of this technical review: First, much more research should be done in this area. Second, many research areas have been clarified, although some aspects are still quite confused. Third, the σ receptor story remains, in many respects, an enigma because most drugs and chemicals, including σ ligands, have several different biological activities. Hence, most are "dirty" in that their multiple pharmacologic actions tend to confound interpretation of research findings. Obviously, new σ chemicals are needed that are very selective and specific in their actions. To a large extent, many current ligands are not "pure" enough in their mechanisms and sites of action. Fourth, the researchers who made presentations are to be congratulated on their contributions. Some aspects of this scientific field are very frustrating because of their complexity. Nature tends to reveal its secrets slowly, which means that these researchers worked very hard, sometimes under very frustrating circumstances, to extract nature's secrets; but that is what science is all about: The end is worth the effort. Fifth, use of a more precise terminology would result in better communication. During this 2-day review, there was difficulty with the use of the terms "receptor" and "binding site." Some speakers referred to the "σ receptor," while others hedged and talked about a "σ binding site." Most speakers did not hedge when they referred to the "PCP receptor," yet it is clear that the high-affinity PCP binding site is part of the glutamic acid NMDA receptor subtype.

Is "PCP receptor" the appropriate term or the "PCP site in the NMDA receptor complex"? Historically, the work of many scientists led to the concept of chemicals acting in a biological system on "receptive substances." Hence, the term "drug receptor" or simply "receptor" was used to define the specific site at which a drug or chemical acts in a functional molecular component in an organism. A second definition of receptor has evolved that endogenous hormones, neurotransmitters, and autocoids act on certain macromolecules, usually in cell membranes as the first chemical messengers to trigger a series of second, third, etc., chemical messenger events intracellularly. The targets of

drug action can include various extracellular and intracellular enzymes, transport proteins, lipoproteins, and endogenous first messenger receptors, among others. To define a drug receptor, many criteria must be met, including:

1. Saturability
2. Specificity
3. Correlation of binding affinity to biologic response
4. Reversibility
5. Reconstitution in an in vitro system
6. Presence of endogenous ligand

Readers are encouraged to extend this list and communicate their criteria to the author. Clearly, the PCP binding site meets most of the criteria of a PCP drug receptor. Although some researchers have promoted the σ binding site to the status of the σ receptor, others still hedge because some of the above-mentioned criteria have not been met.

At the beginning of the technical review, Dr. Doris H. Clouet stated that this is a hot area of research but raised the issue as to whether the meeting would be more illuminating or more confounding. The author believes it has been very illuminating, although there are still many areas of confusion; that is the challenge for future research.

Dr. Stephen R. Zukin stated that PCP abuse is still a problem, especially in large cities, and predicts that it will become an even bigger problem as the war on drugs reduces the availability of cocaine and amphetamine. Why some people ingest PCP, which produces a global psychotic reaction with productive, deficit, and hebephrenic symptoms of schizophrenia, is a mystery that further research must solve. He described the historical controversy that took place 10 years ago concerning the existence of a PCP receptor in the brain and its present relationship to the NMDA receptor complex. His chart illustrating the many actions of PCP in relation to plasma PCP concentrations is very important. Only the action of PCP on the NMDA receptor complex and its

actions on the NE, DA, and 5-HT amine uptake systems, as well as possibly its σ ligand properties, seem to be pertinent in vivo.

The issue of precisely defining a σ versus a PCP receptor action still remains: Are the σ or PCP sites true receptors? As described above, a drug receptor must be distinguished from an endogenous neurotransmitter, hormone, or autocoid as the molecular site of action of the drug.

Dr. Zukin emphasized an important analogy between the glutamic acid NMDA receptor complex and the nicotinic ACh receptor complex. Both are members of a receptor channel superfamily. He discussed the evidence for a model of receptor ligand interactions requiring two molecules of ligand for both the ACh and NMDA receptor. Research is clearly needed to isolate, clone, and define the molecular details of the NMDA receptor complex, as is currently the case for the nicotinic ACh receptor.

Dr. Kenneth M. Johnson provided us with evidence of the complexity of the NMDA receptor, particularly its glycine modulating center. Clearly, the schematic drawings of the NMDA receptor complex as published, for example, by Reynolds and Miller (1988*a),* Yoneda et al. (1988), and Costa (1989) are too incomplete to explain the facts that Dr. Johnson's research team and others have generated. Dr. Johnson promised us his view of the NMDA receptor "cartoon" soon. To get an overview of what must be included, the schematic drawing in figure 1 may be helpful. It is derived from other published schemes and/or publications (Monaghan et al. 1989; Reynolds and Miller 1986*b)* but includes additional crucial sites. For example, possible site(s) must also be included for the role of tricyclic antidepressants (Reynolds and Miller 1988*c;* Sernagor et al. 1989) a nonpsychotropic cannabinoid HU-211 (Feigenbaum et al. 1989), as well as polyamines on the glycine modulating center with opposite actions for spermine and spermidine. In view of their important intracellular synthesis from ornithine, Dr. Johnson has suggested an intracellular site(s) for polyamines, although an extracellular site(s) also may exist.

The glycine allosteric center is very complex, in which 7-Cl kynurenic acid (7-Cl KYN) is a selective glycine antagonist and HA-966 is a negative allosteric modulator. Dr. Johnson presented evidence that glycine causes a remarkable facilitation of the NMDA receptor channel and thus enhances TCP binding to the PCP site, The effects of glycine are antagonized by the competitive NMDA antagonist APV. The stimulant effects of Mg^{++} are also blocked by APV. The complexities of these relationships need much more research, as does the

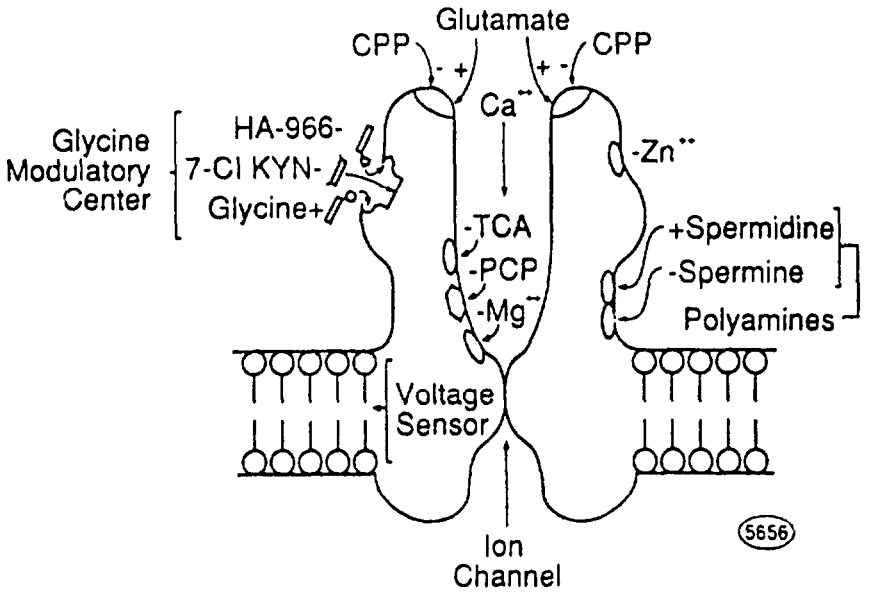

FIGURE 1. *Diagram of an NMDA glutamatergic receptor. This schematic is based on an outline of the molecular configuration of the nicotinic cholinergic receptor. Various binding sites are shown, including the active site for flutamic acid (glutamate) and allosteric sites of polyamines (spermine and spermidine), the glycine modulator center for glycine, HA-966, 7-Cl KYN, and in the ion channel for Mg^{++}, TCA, and PGP. The symbols are as follows: CPP: 3-(carboxypiperazin-4-yl)-propyl-1-phosphate; PCP: phencyclidine; TCA: tricyclic antidepressants; HA-966: 3-amino-1-hydroxypyrrolidone-2; and 7-Cl KYN: 7-chlorokynurenate. It is not known as yet if the polyamine site is extracellular or intracellular. It is drawn as extracellular in this diagram.*

modulatory role of Zn^{++}, tricyclic antidepressants, and others. Wroblewski and colleagues (1989) and Wroblewski and Danysz (1989) have reviewed the known molecular mechanisms of the modulation of glutamic-acid receptors, especially the NMDA receptor, and their functional implications. One subcategory of NMDA receptors in the cerebellum (where high-affinity PCP binding sites are normally minimal but low-affinity σ sites are maximal) increases c-*fos* proto-oncogene expression (Szekely et al. 1989*a,* 1989*b).*

Whether high-affinity PCP binding sites are in low density or low-affinity PCP (σ) sites are in high density in primary cultures of neonatal rat cerebellar granule cells, as in adult cerebellum, needs to be studied further.

As knowledge of the precise role of PCP and σ receptors increases, it may be possible in the future to devise some ways to antagonize the actions of PCP in vivo. Since the high-affinity PCP binding site is within an ion channel, it seems unlikely that compounds like metaphit that acylate the PCP site will ever be useful in vivo (Domino et al. 1987). Clearly, these are important directions for future research that are of great interest to the mission of NIDA.

In contrast to the solid base of facts now accumulated regarding the PCP receptor as the binding site within the channel of the NMDA glutamic acid receptor complex, our knowledge of the σ receptor or binding site is very spotty. The original hypothesis of Martin and his colleagues (1976) of μ and κ opioid receptors is now well accepted. However, the dysphoric and psychotomimetic effects of nalorphine, which led them to study (±) SKF 10,047 as the prototypic σ opioid receptor agonist, have been subdivided into κ opioid agonist and PCP agonist-like effects.

A recommendation was made by the Committee on Terminology (Quirion et al. 1988) to drop the word "opioid" when referring to the σ receptor. This action was approved by the participants of the second Joint U.S.-French Seminar on Sigma and Phencyclidine-Like Compounds as Molecular Probes in Biology meeting held at the University of Michigan in summer 1987. Musacchio (1990) has been especially critical of the confusion among investigators and in the literature regarding the term "σ receptor." He has pointed out that the psychotomimetic and dysphoric effects of opioids are produced by the (-) and not the (+) isomers. The psychotomimetic effects of the (-) opioids are antagonized by naloxone. Thus, Martin and colleagues' (1976) idea of a σ receptor has evolved to take on an entirely new meaning in that ligand stereospecificity is completely reversed and now ascribed to the (+) isomers, which are nonopioid. Compounds such as (+) SKF 10,047 have high- as well as low-affinity binding sites; the high-affinity site is called a "σ receptor" and the low-affinity site a "PCP receptor." The reverse is true of PCP. The σ site resembles the high-affinity dextromethorphan binding site. Whether the dysphoric and psychotomimetic effects of (-) opioids can be explained by κ receptor interactions or a still unknown opioid receptor is a subject for further research.

The term "σ receptor" in 1991 refers to a binding site shared by a variety of chemicals (haloperidol through progesterone), with an order of potency opposite to their binding affinity to the PCP receptor. To date, it is not possible to distinguish between σ agonists and antagonists. Furthermore, their biological and behavioral mechanisms of action are relatively unknown. However, this Technical Review has been very helpful in shedding some light on the σ receptor story.

Dr. Tsung-Ping Su provided us with a historical and chemical perspective of the σ receptor involving a hydrophobic pocket, a side chain, and a nitrogen or a 20-keto group that acts as a nitrogen. He showed us a complex overlay of molecular models of σ drugs, involving both bond length as well as molecular orbital cloud structures with different distributions of charges. His scholarly analysis reminded me of the hypothetical central nervous system receptor of Lloyd and Andrews (1986) as modified for PCP by Manallack and Beart (1987)

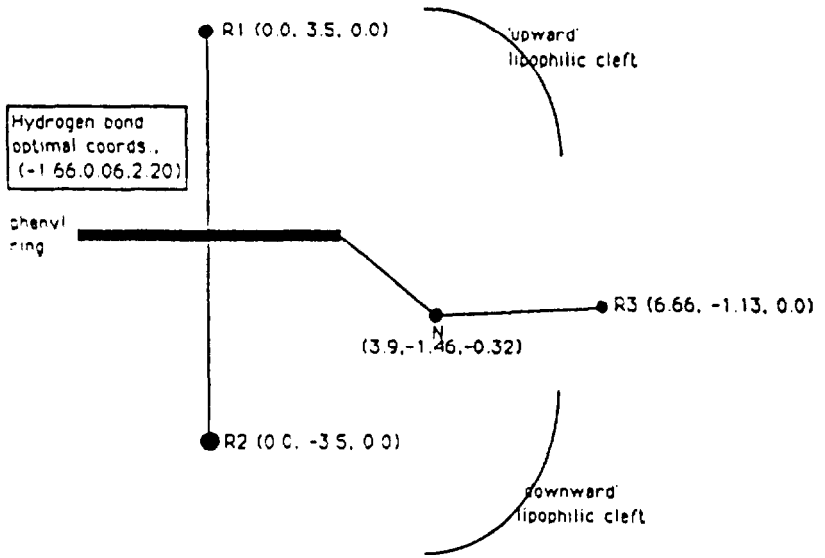

FIGURE 2. *Primary (R1, R2, R3, and N) and secondary (lipophilic clefts and hydrogen bonding of phenyl substituents) binding sites of PCP-like ligands as defined by Manallack et al. (1988). The distance between the center of the phenyl ring and the nitrogen is 4.18 A.*

and Manallack and co-workers (1986). Figure 2 summarizes their views, which need to be integrated and contrasted with those of Dr. Su and his colleagues regarding the molecular requirements for a σ receptor ligand. Especially important will be an analysis of the role of stereoisomers to further define the σ and PCP drug receptor complexes. Dr. Su's molecular analysis led to the synthesis of a methylene derivative of PCP that reversed the usual PCP versus σ spectrum of activity based on binding assays. Future research will require a description of the pharmacology of this compound and especially challenge medicinal chemists to make more selective rigid stereoisomers. Clearly, the σ site resides in membranes, and σ ligands carry no charge. Their K_d almost triples with an increase of two pH units.

What is the functional role of σ receptors, especially in relation to major organ systems, including the brain? What is their status in human psychoses? Dr. Edythe London's presentation was clearly a step in the direction of answering these questions, specifically the role of σ receptors in schizophrenia. Several crucial points were made:

1. Regional brain glucose utilization is unique following administration of σ ligands. σ Ligands in the brain produce functional changes in glucose utilization.

2. It is not possible to distinguish between σ agonists and antagonists.

3. Temporal lobe σ receptors are reduced in autopsy material from the brains of paranoid schizophrenic patients. Is this due to down-regulation following treatment with those neuroleptics (e.g., haloperidol) that have σ effects? Obviously we need more research. We need to pursue PET studies in drug-free patients, etc.

4. There is no postnatal increase in σ binding in contrast to PCP binding sites in the brain. This is contrary to all we know about neurotransmitter receptors, so there is much future work to be done.

Dr. Wayne D. Bowen tackled the crucial problem of σ receptors and signal transduction. He reminded us that σ ligands have (1) smooth muscle, (2) electrophysiologic, (3) motor dystonic, and (4) PI turnover effects. The latter are new and exciting developments. Dr. Bowen pointed out that M1 ACh receptor stimulated PI turnover by carbachol is decreased by σ drugs in a noncompetitive manner. He systematically reviewed their possible mechanisms

of action. Although there is good correlation between MI and σ receptor-ligand effects, σ ligands act at the σ site to decrease B_{max}. Their linkage to the MI cholinergic receptor to decrease PI turnover raises interest and all kinds of exciting possible experiments.

Dr. Errol B. De Souza stressed the relationship between the nervous, endocrine, and immune systems. He pointed out that some first messengers of the nervous system (neurotransmitters) are also first messengers (hormones) in the endocrine system and first messengers (autocoids) in the immune system and vice versa. Although there are primarily high-affinity PCP receptor sites in the cerebrum and spinal cord, there are many low-affinity PCP binding sites in diverse tissues, including cerebellum, spleen, and lymphocytes. Some of the pharmacological effects of PCP may be mediated by actions on low-affinity σ receptors. The σ sites seem to be very important in leucocytes. Much future research is needed to identify the function of σ receptors in T and B lymphocytes.

In addition to the role of σ receptors in the immune system, it appears that such receptors are of importance in the endocrine system. Why are σ receptors present in highest concentrations in the ovary? Why do σ ligands suppress luteinizing hormone (LH) and prolactin (PRL) hormone release? Will they inhibit ovulation or alter the menstrual cycle? Dr. De Souza reminded us of the analogy between high- and low-affinity benzodiazepine receptors in which the high-affinity sites are in high density in the brain and the low-affinity sites are in high density in the periphery. It appears that σ receptor function will be of importance in the endocrine and immune systems as additional future research is undertaken.

Dr. Brian de Costa provided hope that new σ ligands are being synthesized that have more selective actions. He pointed out that the diastereoisomers of U50,488 tend to separate κ opioid and σ effects. He and his colleagues synthesized a series of benzeneacetamines in an attempt to obtain a highly selective σ ligand. BD-737 is an especially promising compound whose pharmacology needs to be studied extensively. Especially important is the development of pairs of compounds, one of which is highly stereospecific for σ effects.

Ketamine and other PCP-like drugs produce analgesia through a minor opioid (Smith et al. 1987) and a major nonopioid (France et al. 1989*a;* Maurset et al. 1988, 1989, 1990) mechanism. Dr. Woods reported that the μ opioid antagonist

quadazocine antagonized alfentanil (μ)-induced analgesia more than U50,488 (κ)-induced analgesia but had no significant effect on PCP-like drug-induced analgesia. The latter drugs included MK-801, PCP, ketamine, (+)SKF 20,047, and dextrorphan. Interestingly, the competitive NMDA antagonist CGS 19755 also induced analgesia and anesthesia in a dose of 100 mg/kg i.v. with a very slow onset (30 minutes to 2 hours) and long duration (24 to 48 hours) of action. France and colleagues (19896) have shown that much smaller doses of CGS 19755 attenuated the rate-decreasing effects of NMDA in monkeys, but did not produce ketamine-like discriminative stimulus effects. These observations will stimulate much more research and the development of better competitive NMDA antagonists. Especially important is the fact that CGS 19755 does not appear to be self-administered, although this observation may be due to its very slow onset of action.

One of the disappointing observations of both Dr. Balster and Dr. Woods was that selective σ ligands do not produce unique and specific behavioral effects. This strong conclusion is important in terms of what future direction should be taken. Although specific σ ligands produce a large variety of animal behaviors, including piano playing, sideways walking or jumping, convulsions, etc., these behavioral effects are elicited by other drugs.

According to Dr. Woods, BMY 14802 appears to act behaviorally through the 5-HT_{1A} rather than σ or DA receptors. Dr. Duncan P. Taylor reviewed the antipsychotic profile of BMY 14802 in relation to other antipsychotic agents. The former agent clearly has a most complex pharmacologic profile, including binding to the σ receptor. The pharmacological actions of BMY 14802 are so diverse that it will be a challenge to researchers to find the mechanism of its antipsychotic effect should it prove to be useful in human therapy.

Another complexity of the in vivo pharmacology of BMY 14802 is its conversion in the liver to BMY 14786. Thus, the analogy is that of reduced haloperidol conversion to haloperidol and vice versa. Dr. Taylor reported that Umesch and colleagues have observed that (+)BMY 14802 is not converted to the ketone but (-)BMY 14802 is. He emphasized that better antipsychotic drugs should be selective and stereospecific for the σ sites on postsynaptic dopaminergic neurons. He also stressed the σ enigma as fertile territory for research.

The group of researchers represented by Dr. Leslie Kushner presented some of their exciting findings on the molecular biology of NMDA receptors and the PCP binding site. Of special importance are the voltage and use dependence of PCP

ligands. By using the *Xenopus* oocyte injected with mRNA, they have been able to express NMDA, kainate, and quisqualate receptors. The NMDA receptor complex behaves as expected with glycine potentiation (Kleckner and Dingledine 1988), voltage dependency of Mg^{++}, etc. Unique cell lines with σ receptors are useful sources of mRNA for oocyte expression. Furthermore, unique NMDA receptor forms (such as in the cerebellum) can be expressed. This area of research shows much promise for improving the understanding of the molecular mechanisms involved.

Dr. Michael V. Johnston presented a remarkable overview of NMDA receptor-mediated neurotoxicity in the developing brain. He contrasted the important differences between the neonate and the adult with regard to glutamate toxicity and selective vulnerability. The neonate brain is much more sensitive to NMDA than to kainic acid compared with the adult brain. Especially important is the observation that, of all of the PCP-like ligands, MK-801 is the most potent in brain protection, and that this effect is independent of hypothermia. PCP itself is not as potent and not as effective as MK-801. An important observation is that MK-801, given 24 hours prior to NMDA, causes enhanced neurotoxicity in contrast to its brain-protection actions when given after NMDA administration. It appears that NMDA receptors play a crucial role in neuronal plasticity of the newborn.

Dr. Patricia C. Contreras gave a status report of her group's efforts in isolating endogenous σ and PCP-like peptides. There has been steady progress (DiMaggio et al. 1986, 1988; Contreras et al. 1987) but the problems of isolation are enormous. One can only compliment Dr. Contreras for her efforts in spite of innumerable impediments. Especially interesting and consistent with an endocrine role, endogenous σ ligand activity is highest in the pituitary. This is related to Dr. De Souza's observation that the ovary and testes have a very high density of σ receptors.

CONCLUSION

There is much work to be done. This Technical Review has shed new light on the stranger-than-fiction developments that PCP- and o-related research has revealed. We are still a long way from solving the problem of PCP abuse, but only through more research is there any hope of doing so. In the process, some useful therapeutic agents may emerge.

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