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MODULATION OF $[^3H]$NA UPTAKE BY PHENCYCLIDINE, SIGMA RECEPTOR LIGANDS AND HISTOGRANIN

BY

CHERYL ROGERS

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Department of Physiology, University of Ottawa.

April, 1993

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LIST OF ABBREVIATIONS:

AMPA	= α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ACh	= acetylcholine
ACN	= acetonitrile
AP5	= 2-amino-5-phosphonovaleric acid
ATP	= adenosine triphosphate
arcaine	= (1,4-diguanidinobutane)
B_{max}	= maximal number of binding sites
BMY-14802	= α-(4-fluophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine butanol
BTCP	= benzothienylcyclohexylpiperidine
BW 234U	= cis-9[3,5-dimethyl-1-piperazinyl)propyl]-9H-carbazole
CA	= catecholamine
cadaverine	= (1,5-diaminopentane)
CNS	= central nervous system
CPP	= (+)3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid
DA	= dopamine
DA10	= diaminodecane
DA8	= diaminooctane
DAGO	= [D-Ala^{2}, MePhe^{4},Gly-ol^{5}]-enkephalin
DET	= diethyltriamine
DMEM	= Dulbecco's modified Eagle medium
DMI	= desmethylimipramine
DSLET	= [D-Ser^{2}, Leu^{5}]enkephalinyl-Thr
DXM	= dextromethorphan
DTG	= 1,3 di-(2-tolyl)guanidine
EAA	= excitatory amino acid
FCS	= fetal calf serum
glu	= glutamate
gly	= glycine
HN	= histogranin
HPLC	= high performance liquid chromatography
IC_{50}	= 50% inhibitory concentration
K_d	= equilibrium dissociation constant
K_i	= inhibitory constant
K_m	= Michaelis-Menton constant
LTP	= long-term potentiation
MK-801	= 5-methyl-10,11 dihydro 5H-dibenzo [a,d] cyclohepan-5,10-imine maleate
NA	= noradrenaline
NMDA	= N-methyl-D-aspartate
NPY	= neuropeptide Y
PCP	= phencyclidine
3(+)PPP	= 3-[3-hydroxyphenyl]-N-(1-propyl)piperidine

putrescine = (1,4-diaminobutane)
σ = sigma
SKF-10047 = N-allylnormetazocine
spermine = N,N'-bis(3-aminopropyl)-1,4-butanediamine
spermidine = N-(3-aminopropyl)-1,4-butanediamine
TCP = 1-N-(2-thienyl)cyclohexylpiperidine
TFA = trifluoroacetic acid
$Uptake_1$ = high affinity uptake of catecholamines
$Uptake_2$ = low affinity uptake of catecholamines
U-69593 = (5-α,7α,8B,)(+)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro (4,5) dec 8yl) benzeneacetamide
V_{max} = maximal velocity

MODULATION OF [^{3}H]NA UPTAKE BY PHENCYCLIDINE, SIGMA RECEPTOR LIGANDS AND HISTOGRANIN.

Cheryl Rogers

Department of Physiology, University of Ottawa

ABSTRACT

The effects of PCP and sigma ligands on NA uptake were studied in rat brain synaptosomes and adrenal chromaffin cells. The noradrenaline re-uptake system plays an important role in the termination of synaptic transmission in the CNS, and in the periphery this system lowers circulating levels of catecholamines. Adrenal medullary PCP and sigma binding sites were previously characterized although their function remains unknown. The main focus of the thesis was to determine if PCP and sigma ligands modulated NA uptake and if so, whether this mechanism could be the site of action of the endogenous "PCP-like" peptide histogranin. Thus, the following objectives were examined, 1) the effects of PCP and sigma ligands on noradrenaline uptake in adrenal chromaffin cells and rat brain, 2) the interaction of PCP and sigma ligands with the substrate recognition site on the transporter 3) the effects of the endogenous "PCP-like peptide" histogranin, on noradrenaline uptake 4) the identification and characterization the central binding site(s) for histogranin.

The characteristics of NA uptake process in both rat brain synaptosomes and adrenal chromaffin cells was first evaluated. It was observed that

noradrenaline uptake was dependent on time and temperature, and both tissues displayed similar kinetics and capacity to transport NA. The presence of phencyclidine or sigma ligands was found to inhibit the uptake of NA. A correlation was drawn between the inhibitory potencies of PCP and sigma ligands in rat brain synaptosomes and bovine adrenal chromaffin cells. However, the rank order of potency of these ligands in NA uptake assays did not match that obtained previously in PCP and sigma receptor binding assays.

The antidepressant [^{3}H]desmethylimipramine labelled adrenal medullary sites with similar characteristics to those found in CNS. The sites displayed high affinity, exhibited inverse-temperature and Na^+ dependency and were potently inhibited by nisoxetine. [^{3}H]Desmethylimipramine binding revealed that PCP and sigma ligands interacted in a negative allosteric manner with the substrate recognition site on the NA transporter. The effects of PCP and sigma ligands on [^{3}H]DMI binding did not match that obtained in high affinity PCP and sigma receptor assays. The potency of sigma ligands in NA uptake assays correlated with that observed in [^{3}H]DMI binding assays (r= 0.84). In contrast, PCP ligands did not correlate, inhibiting [^{3}H]desmethylimipramine binding with a different order of potency than that observed in [^{3}H]noradrenaline uptake assays. Scatchard plot analysis of the effects of PCP and sigma ligands on [^{3}H]desmethylimipramine binding revealed the non-competitive nature of both PCP and sigma ligands at the [^{3}H]DMI binding site, suggesting that these ligands bound to sites distinct from that of substrates on the transporter.

The comparison between the potencies of PCP and sigma ligands in both $[^3H]$noradrenaline uptake and $[^3H]$desmethylimipramine binding assays led to the following two conclusions. First, due to the low degree of affinity and selectivity of PCP and sigma ligands in these assays, the involvement of high affinity sigma and PCP receptors is ruled out. Second, the correlation found for sigma ligands in both $[^3H]$NA uptake and $[^3H]$DMI binding assays suggested that sigma ligands may mediate NA uptake blockade by binding to an allosteric domain on the NA transporter. The lack of correlation found for PCP ligands in both assays suggests that PCP blocks uptake at sites that are distinct from the substrate recognition site on the transporter. The rank order of sigma ligand potencies in these assays differed from previously characterized $sigma_1$ and $sigma_2$ receptors, but has been observed in other tissues and therefore may represent the existence of a low affinity $sigma_3$ subtype.

The endogenous peptide histogranin, (HN) and its analog $[Ser^1]$HN were ineffective inhibitors in both $[^3H]$noradrenaline uptake and $[^3H]$DMI binding assays. This result suggested that the "PCP-like" behavioral effects of histogranin were not due to blockade of NA transport. The characterization of histogranin binding sites was carried out using an iodinated analog of the peptide ($[^{125}I][Ser^1]$HN). The specific binding of $[^{125}I][Ser^1]$HN demonstrated typical receptor characteristics of dependency on protein, temperature and time. $[^{125}I][Ser^1]$HN labelled high affinity sites in rat brain and this binding was inhibited by structurally similar fragments of the 15 amino acid peptide. The selectivity of $[^{125}I][Ser^1]$HN binding was

demonstrated in competition studies with un-related peptides such as enkephalins, NPY, beta-endorphin, dynorphin and Substance P which were ineffective inhibitors of [^{125}I][Ser^1]HN binding. PCP, sigma and dopamine D_2 receptor ligands were also inactive in competing for binding sites labelled by [^{125}I][Ser^1]HN. However, inhibition of [^{125}I][Ser^1]HN binding did occur in the presence of polyamine site agonists and antagonists.

Polyamines are known to act on the NMDA receptor complex, a receptor which possesses the high affinity PCP_1 binding site. The nature of the interaction of polyamine site ligands was found to be non-competitive with that of [^{125}I][Ser^1]HN. Additionally, HN and [Ser^1]HN were not able to inhibit the binding of the polyamine [^{3}H]spermidine from its site on rat brain membranes, providing further evidence to support the theory that HN occupies a distinct domain on the membrane. Like polyamine agonist [^{3}H]spermidine, [^{125}I][Ser^1]HN binding was inhibited by micromolar concentrations of Zn^{2+} and Mg^{2+}.

A comparison between the density of sites labelled by [^{125}I][Ser^1]HN and the selective PCP_1 receptor ligand [^{3}H]MK-801 in various brain regions revealed that HN and NMDA receptors were concentrated in the hippocampus and cortex but not in striatum. The results strongly suggest that [^{125}I][Ser^1]HN labels high affinity sites in rat brain membranes. Whether or not histogranin directly interacts with the NMDA receptor in various brain regions requires further study, and is presently being investigated through the use of autoradiographic and immunohistochemical techniques.

RELATED PUBLICATIONS

The data presented in this thesis have been previously reviewed and accepted by the following journals.

1. Rogers, C. A., and Lemaire, S. (1991) Role of the σ receptor in the inhibition of [^{3}H]noradrenaline uptake in brain synaptosomes and adrenal chromaffin cells. British Journal of Pharmacology, **103**: 1917-1922.

2. Rogers, C. A. and Lemaire, S. (1992) Characterization of [^{3}H]desmethylimipramine binding sites in bovine adrenal medulla: interaction with σ and/or PCP-receptor ligands. Canadian Journal of Physiology and Pharmacology, **70**: 1508-1514.

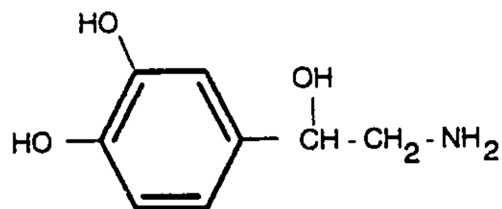
3. Rogers, C. A. and Lemaire, S. (1993) Binding of [^{125}I][Ser^{1}]histogranin to high affinity sites in rat brain membranes. Journal of Pharmacology and Experimental Therapeutics. **In Press.**

1.0. INTRODUCTION

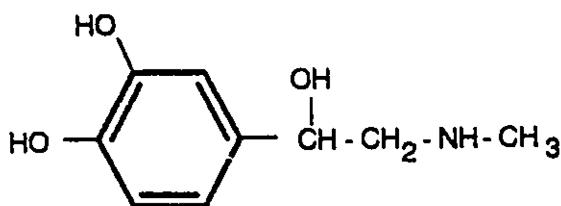
1.1. PREAMBLE

Catecholamines (CA), noradrenaline, adrenaline and dopamine are structurally similar compounds (Fig 1.). In the brain they are released from neurons as neurotransmitters; in the periphery they act as hormones once released from sympathetic nerve endings and chromaffin cells of the adrenal medulla. The catecholamines, adrenaline and noradrenaline (NA), bind to adrenergic receptors producing mainly excitatory responses in the central nervous system (CNS), and exert powerful vasopressive effects in the periphery. Thus catecholamines play key roles in important physiological responses.

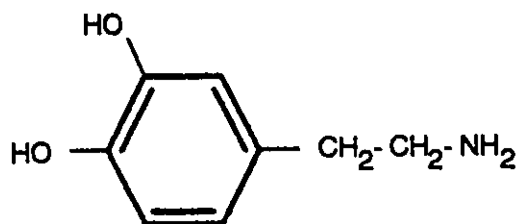
In the periphery, circulating adrenaline and noradrenaline, reach receptor sites in the heart producing positive chronotropic and inotropic effects within seconds while other hormones require several hours to simply initiate a response. In the CNS however, successful neurotransmission requires that signals are rapidly initiated and terminated. This requirement is satisfied by the fact that the neuronal membrane, once depolarized, allows Ca^{2+} channels to open and Ca^{2+} acts within 0.2 msec to achieve transmitter release (Llinas and Heuser, 1977). The signal is partially terminated by enzymes and diffusion into surrounding tissue, but 95 per cent of the catecholamine is recycled pre-synaptically by a Na^{+}-dependent uptake process (Fig 2.). How that recycling occurs has been the subject of much study since the 1960s.



Noradrenaline



Adrenaline



Dopamine

FIGURE 1. The Structure of Catecholamines

The chemical structure of noradrenaline, adrenaline and dopamine all contain a "catechol" plus an amine group. In vivo, dopamine can be metabolized to form noradrenaline by the action of the enzyme dopamine-B-hydroxylase, and similarly, noradrenaline can be converted to adrenaline by the enzyme phenylethanolamine-N-methyltransferase.

Axelrod and Strombland were the first to discover that nerve terminals accumulate catecholamines. Axelrod and colleagues (1959) demonstrated that peripheral injection of CA resulted in its sequestration into specific tissue sites. Strombland (1961) found that after in vivo destruction of sympathetic nerve terminals the concentration of circulating CA decreased.

Thirty years hence, our knowledge of catecholamine re-uptake has advanced greatly. Today, it is known that CA are transported by a saturable, sodium-dependent, temperature-sensitive mechanism and both the transport protein and its cDNA have been identified (Graefe and Bonish, 1988; Kilty et al., 1991; Pacholezyk et al., 1991). The noradrenaline transporter consists of a 617 amino acid protein which forms 12-13 transmembrane domains each of 18-25 amino acids and both the amino and carboxyl termini of the protein are located intracellularly (Pacholezyk et al., 1991). Three potential regulatory sites are located in the large extracellular loop. The cloned sequence suggests a molecular weight of 69 kDa which differs slightly from the purified transporter protein (53 to 54 Kda; Howard et al. 1990; Bonish, 1991).

The uptake process has received great attention over the years due to the implication of biogenic amines in mental disease. According to the "monoamine hypothesis of depression" put forth by Schildkraut and Kety (1967), "depressive illness is associated with a decrease in synaptic activity of connections that employ the transmitters norepinephrine and serotonin." In order to facilitate neurotransmission, drugs have been developed that inhibit transmitter re-uptake

and presumably permit a longer sojourn of neurotransmitter at the receptor site.

The knowledge gained from studies on noradrenaline uptake has been put to use in the development of the potent tricyclic anti-depressant drug desmethylimipramine, (Norpramin, Pertofrane). The tricyclic antidepressants have become the most popularly prescribed mood elevator with close to 25 million prescriptions written per year in the United States (Cox et al., 1983). Desmethylimipramine is known to bind to the NA binding site (or substrate recognition site) and thus the radiolabelled form of this drug has been used to localize and characterize the pharmacology of the NA transporter in brain (Lee et al., 1982). Whether or not the body produces its own uptake blocker is unknown, but several studies have suggested the presence of such endogenous factors in brain and periphery (Rehavi et al., 1985; Hole et al., 1979).

Other drugs, such as phencyclidine (PCP), are known to block re-uptake of catecholamines (Garey and Heath, 1976; Smith et al., 1977). But the use of PCP has led to psychosis in humans (Meltzer, 1991). Whether or not the psychosis is related to this blocking function is unknown. By the same token, drugs acting at sigma receptors have also been reported to induce psychotomimetic experiences and are known to enhance the effects of noradrenaline in periphery (Haertzen, 1970; Massamiri et al., 1989). These observations suggest that sigma ligands could act in a similar fashion to PCP and block catecholamine uptake.

Noradrenaline uptake has been frequently measured in preparations of rat

brain synaptosomes, however this preparation lacks the stability and homogeneity that one can obtain in cell culture. The mRNA coding for the brain NA transporter possesses the same characteristics as that isolated from the adrenal medulla (Pacholezyk et al., 1991). Chromaffin cells of the adrenal medulla have also demonstrated their usefulness as a model for the study of central neuronal function by their ability to store, release and efficiently re-uptake NA (Kenigsberg and Trifaro, 1980; Role and Perlman, 1983).

In previous studies we have demonstrated that the adrenal medulla contains two high affinity sites for PCP ([^{3}H]TCP) and sigma ([^{3}H]3(+)PPP) receptor-specific ligands (Rogers and Lemaire, 1990). PCP and sigma ligands have been shown to block the release of catecholamines from the adrenal medulla (Purifoy and Holtz, 1984; Paul et al., 1992). However, the concentration range at which this effect occurs is 1000 fold higher than the measured Kd of these ligands in binding studies. Thus the blockade of adrenal catecholamine secretion is presently attributed to PCP and sigma ligand interaction with the nicotine ACh receptor (Malave et al., 1983; Purifoy and Holz, 1984; Paul et al., 1992) and the function of the high affinity PCP and sigma-like binding sites remains unknown.

The purpose of the present study was to verify whether the PCP and sigma-like receptors present in the adrenal medulla are involved in the modulation of noradrenaline uptake, and if so, to compare this effect with that in the CNS. The observation that an adrenomedullary peptide "histogranin", (HN), produced

a PCP-like behavioral profile (ataxia, locomotion and stereotypy; Lemaire et al., 1993) when injected (i.c.v.) into rats, raised the possibility that histogranin may also bind to PCP and sigma sites and modulate NA uptake. Thus, in parallel with studies on the effects of PCP and sigma ligands on NA uptake, it was of interest to extent this study to examine the binding site for HN and its possible effect on NA uptake.

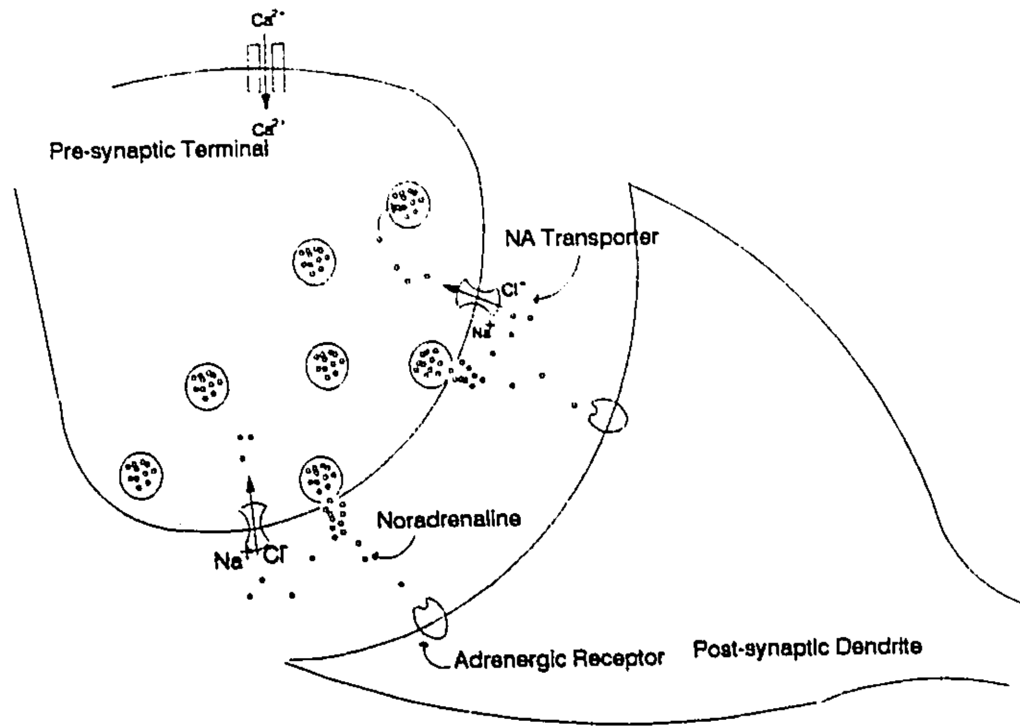


FIGURE 2. Neural Transmission at a Typical Noradrenergic Synapse Depolarization of the presynaptic nerve terminal allows voltage-sensitive calcium channels to open. The resulting influx of Ca^{2+} results in the release of vesicles containing noradrenaline. Once released, noradrenaline binds to postsynaptic adrenergic receptor sites. During the repolarization phase, noradrenaline is transported back into the presynaptic terminal by the action of the NA transporter and the signal is terminated.

1.2. NORADRENALINE UPTAKE

Noradrenergic neurons project diffusely to most parts of the mammalian brain from cell bodies in various brain stem nuclei, particularly the locus coeruleus (latin for "blue place"). These neurons are responsible for excitatory modulation in regions where they terminate. After its release from nerve terminals, NA is rapidly transported back into the presynaptic neuron by an active uptake process ($uptake_1$).

The $uptake_1$ process is considered to be the main mechanism of inactivation of noradrenergic transmission, but there also exists an $uptake_2$ process that refers to the uptake of NA by post-synaptic effector cells and other extraneuronal cells (Salt, 1972). In light of the widespread distribution of NA terminals in the brain, any alteration of $uptake_1$ would greatly affect normal brain function.

The basic properties of neurotransmitter transporters were revealed in studies of the uptake of radiolabelled monoamines in brain slices and preparations of pinched-off nerve endings, referred to as "synaptosomes" (Iversen, 1965; Iversen, 1967; Coyle and Snyder, 1969; Horn et al., 1971; Gray and Whittaker, 1962). Numerous studies have characterized the uptake of various catecholamines by nerve terminals and have shown that the initial uptake process is governed by saturation kinetics of the Michaelis-Menten type (Iversen, 1963). The results reported in most studies showed that the apparent K_m of the system for a number of substrates was in the micromolar range. The value of K_m is

defined as the concentration of substrate at which the initial rate of uptake has reached half its maximum value (V_{max}).

In the periphery, circulating NA is accumulated by many tissues, however, the heart, spleen, and adrenal glands accumulate the greatest amounts (Axelrod et al., 1959). The chromaffin cells of the adrenal medulla are derived embryologically from neural crest tissue and are functionally homologous to post-ganglionic sympathetic neurones (Livett, 1984). The capacity to take up NA has been extensively used to load chromaffin cells with radiolabelled NA to study uptake mechanisms and to simplify measurement of secretion (Kenigsberg and Trifaro, 1980; Banerjee et al., 1987; Role and Perlman, 1983). The uptake of tritiated (-)noradrenaline into chromaffin cells is substrate specific, saturable, and dependent upon external Na^+ (Role and Perlman, 1983). In this context, it is not surprising that these cells exhibit a catecholamine uptake system essentially identical to neuronal uptake$_1$ (Kenigsberg and Trifaro, 1980; Role and Perlman, 1983; Banerjee et al., 1987; McKay, 1989).

The physiological significance of adrenal medullary catecholamine uptake is less clear. It would seem unlikely that the NA transport process in adrenal chromaffin cells plays a role as in neurons by providing control of catecholamine concentration in the vicinity of the release site. This statement is supported by the fact that catecholamines would rapidly diffuse away from the gland and even low levels of efflux would far exceed the calculated maximal rate of accumulation (Bunn et al., 1992). Thus uptake$_1$ in this tissue may have a more important role

in scavenging catecholamines from the circulation.

Isolated chromaffin cells provide a more homogenous physiologically relevant preparation when compared to synaptosomes which are prepared by harsh treatment of brain tissue and isolation of a mixture of neuronal vesicles (Gray and Whittaker, 1962). Thus, the robustness of bovine adrenal medullary chromaffin cells provides a convenient model system in which to investigate the NA uptake process.

Recently, transporters for dopamine, GABA, noradrenaline, and 5HT have been cloned and the adrenal medulla has been shown to possess mRNA identical to that found in the locus coeruleus for the NA transporter (Shimada et al., 1991; Guastella et al., 1990; Kilty et al., 1991; Blakely et al, 1991; Pacholezyk et al., 1991). Thus the adrenal medullary NA transporter may be part of a novel gene family encoding proteins that could play key roles in both the CNS and peripheral sympathetic nervous system.

1.2.1. Dependence on Ions and Temperature

Detailed investigation revealed that NA uptake was Na^+-dependent, since it was completely inhibited if Na^+ was removed from the extracellular medium (Bogdanski and Brodie, 1969; Iversen and Kravitz, 1966). Depolarization of the membrane and subsequent influx of Na^+, disrupts the ion gradient and thus inhibits the transport of catecholamines (Role and Perlman, 1983). Interestingly, increasing intracellular Na^+, relative to extracellular Na^+ has been found to reverse

the direction of transport and results in NA efflux (Graefe et al., 1971: Trendelenberg, 1991). This Ca^{2+}-independent release is not due to passive diffusion but rather carrier-mediated, and commonly referred to as outward transport. Transport of noradrenaline also depends upon external Cl^- and internal K^+ (Sanchez-Armass and Orrego, 1977; Harder and Bonisch, 1985; Friedrich and Bonisch, 1986). Although virtually no other cation can effectively substitute for Na^+ to bring about transport, the action of external Cl^- is partially mimicked by Br^- (Friedrich and Bonisch, 1986; Sanchez and Orrego, 1977).

Neuronal accumulation of amines is highly dependent on temperature (Harris & Baldessarini, 1973). Thus noradrenaline uptake observed at 0-4° C is often used as a background or control value. This dependence on temperature suggests that free energy derived from cell metabolism is a requirement for operation of the NA transporter (Wakade and Furchgott, 1968; Paton, 1968). Studies with membrane vesicles (ghosts) derived from plasma membrane of cultured PC-12 cells clearly show that ATP per se is not required for the carrier-mediated transport (Harder and Bonisch, 1984). Additionally, NA transport into synaptosomes can be driven by ion gradients artificially generated across the vesicle membrane (Harder and Bonisch, 1984). It was shown that Na^+,K^+ ATP-ase inhibitors (eg. ouabain) inhibited CA uptake probably as a consequence of inhibiting the Na^+,K^+ ATP-ase driven pump (Bogdanski et al., 1970). As ATP is required to maintain these ion gradients in intact neurones, the carrier system responsible for neuronal uptake is classified as a secondary active transport

(Graefe et al., 1976).

1.2.2 The Transport Process

The effectiveness of the neuron as a whole in inactivating released NA depends upon the activity of the transporter and is greatly strengthened by vesicular uptake and the enzyme MAO (monoamine oxidase), which both constitute intra-neuronal "sink" mechanisms (Youdim et al., 1988). These processes are highly effective in keeping the synaptic and cytoplasmic concentration of transmitter low, thus enabling the neuron to function as a compartment which can rapidly terminate synaptic transmission.

Although it is generally agreed that neuronal NA uptake is Na^+-dependent, the kinetics of the interaction between catecholamine substrates and Na^+ at the uptake site has been debated for several years (Surgue and Shore, 1969; Sanchez-Armass and Orrego, 1978). A re-examination of the effects of Na^+ on the neuronal uptake of [^{3}H]NA by Sammet and Graefe, (1979) and Friedrich and Bonish, (1986) showed that the NA carrier system exhibits the kinetic properties of a two-substrate sequential enzyme reaction in which both noradrenaline and Na^+ (1:1) must bind the carrier for transport to occur.

The current model of neuronal NA uptake, as reported by Graefe and Bonisch, (1988) demonstrates that the transport of NA involves a four step process of (a) association of the substrate with the carrier protein (b) translocation of the amine across the membrane to the intracellular face (c) dissociation of the

amine into the intracellular medium (d) recycling of the free carrier to the extracellular face (see Fig 3). It should be mentioned that this scheme is similar to that proposed for the platelet serotonin transporter (Nelson and Rudnick, 1979).

Of the three substrates (Na^+, Cl^-, and NA), Na^+ is the leading substrate which forms a non-transported Na^+-carrier complex at the side of the membrane that contains the highest concentration of Na^+, (and lowest K^+). Thus under normal (non-depolarizing) physiological conditions, this complex is formed on the extracellular face of the membrane. Both extracellular concentrations of Na^+, and Cl^- facilitate the binding of NA to the carrier, and increase the total number of sites available for inward NA transport. Noradrenaline, is highly hydrophillic, and thus does not easily permeate the lipid barrier unless a carrier mechanism such as the one described exists. At physiological pH, NA is predominantly ionized and is bound to the carrier as NA^+ (Mack and Bonisch, 1979). The final bound complex of NA^+-Na^+-Cl^- is transported in the direction of the chemical gradient for Na^+. Thus the membrane potential (inside negative) provides an additional driving force by increasing the rate of translocation of this positively charged complex (NA^+-Na^+-Cl^-). Due to its high intracellular concentration, K^+ successfully competes with Na^+ for a common binding site and causes a dissociation of the Na^+-NA^+-Cl^- complex. The newly formed K^+-carrier complex is mobile and moves in the direction of the chemical gradient of K^+ (outward). Thus the inward transport of one positive charge carried on the complex (NA^+-Na^+-Cl^-) is balanced by the

outward transport of one positive charge (K^+), resulting in the transport of NA by an electro-neutral mechanism.

At the extracellular face where the concentration of Na^+ is high, Na^+ blocks the binding of K^+ to the carrier and forms a non-transported (Na^+-carrier) 'dead end' complex that results in the pooling of substrate binding sites on this side of the membrane.

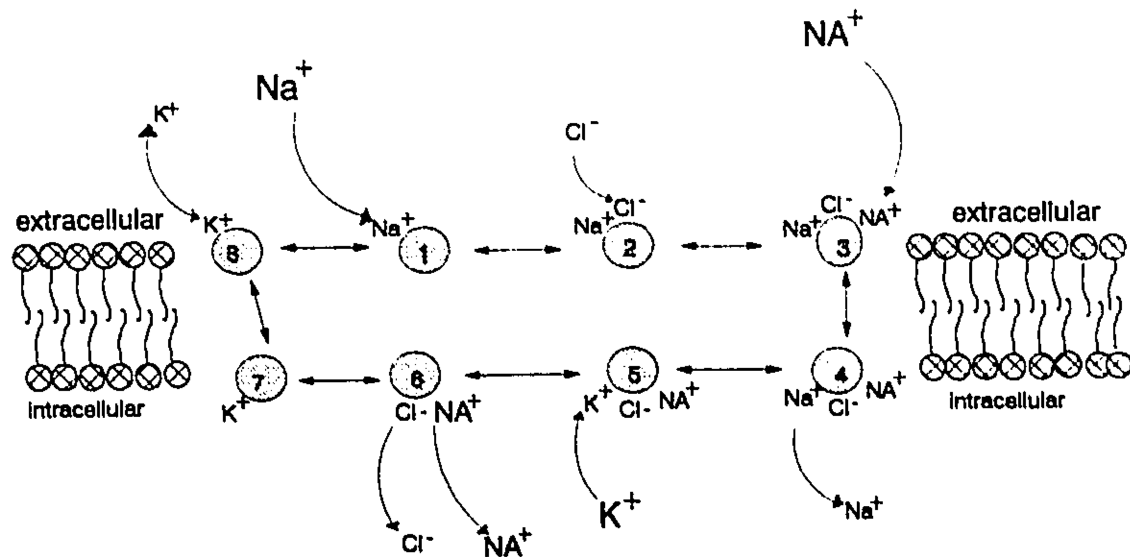


FIGURE 3. The Noradrenaline Transport Cycle

The steps involved in the transport of NA are diagrammed sequentially. The free carrier, located on the extracellular face of the membrane binds Na^+, (step 1), and Cl^- (step 2). This causes an allosteric change in the conformation of the protein increasing its affinity for NA (in its ionized form, NA^+). This binding of NA^+ (3) mobilizes the carrier which translocates to the intracellular face of the membrane (4). The high concentration of intracellular K^+ competes for binding sites occupied by Na^+ on the carrier (4,5). This alters the conformation of the carrier protein again, causing a release of both Cl^- (5) and NA^+ (6) into the cytoplasm. The transporter, when bound to K^+ is mobile and translocates back to the extracellular face (8). Here, the high concentration of Na^+ competes for the site bound by K^+ and the cycle repeats.

1.2.4. Inhibitors of Uptake

1.2.4.1. Cocaine and the Antidepressants

Many antidepressant agents are powerful inhibitors of noradrenaline uptake, these include the derivatives of the tricyclic antidepressants (e.g. desmethylimipramine, nortriptyline, and protriptyline) and some newer drugs which are structurally unrelated to the tricyclics (e.g. nomifensine, nisoxetine, oxaprotiline; Tyrer and Marsden, 1985). It is generally agreed that the uptake blocker, desipramine binds competitively to the amine recognition site of the noradrenaline transporter (Schomig and Bonisch, 1985; Schomig and Bonisch, 1988). By contrast, amine uptake by dopaminergic neurones appears to be non-competitively antagonized by these drugs (Horn et al., 1971).

Radioligand binding studies have proven to be valuable in the identification and characterization of neuronal transporters for biogenic amines (Andersen, 1989). The tritiated forms of desmethylimipramine and cocaine have been used as ligands to label sites associated with the re-uptake of NA and dopamine in the CNS (Hrdina et al., 1981; Langer et al., 1981; Raisman et al., 1982; Kennedy and Hanbauer, 1983).

The following lines of evidence support the view that after correction for non-specific binding, the binding of [^{3}H]DMI is highly selective for the substrate site on the NA transporter of noradrenergic neurons. Firstly, sympathectomy results in a pronounced decrease in the number of [^{3}H]DMI sites (Hrdina et al., 1981b). Secondly, the density of noradrenergic innervation parallels that of

$[^3H]$DMI binding sites and thirdly, the potencies of various drugs for inhibition of NA transport closely correlate with the potency values of these drugs for inhibition of $[^3H]$DMI binding (with exception of substrates for NA carrier which are transported themselves; Langer et al., 1984; Graefe and Bonisch, 1988). Additionally, both $[^3H]$NA uptake and the binding of $[^3H]$DMI are Na^+-dependant (Bogdanski and Brodie, 1966; Hrdina, 1981; Lee and Snyder, 1981).

The observation that substrates of the NA carrier are relatively weak inhibitors of $[^3H]$DMI binding was first thought to indicate that the $[^3H]$DMI binding site and the substrate site were not identical (Raisman et al, 1982). It was thought that $[^3H]$DMI bound to a site that modulated NA uptake through an allosteric mechanism (Langer et al., 1984). However, the reason for the lack of correlation between the inhibitory potency of substrates in $[^3H]$NA uptake assays and their potency in $[^3H]$DMI binding is explained by the fact that substrates act at two stages in the cycle of NA uptake. This dual action is discussed below.

Assuming that the uptake system was dependent entirely on the rate at which a compound binds the substrate site and thus its K_d, all inhibitors would block uptake with the same potency as they inhibit binding of a radiolabel to the substrate site (e.g. $[^3H]$DMI). This statement is based on the erroneous assumption that the binding of the substrate to the carrier site is rate-limiting in the transport cycle. This is simply not the case.

The model of catecholamine transport described in section 1.3.2., (Fig. 3) provides the clue to explain the potency differences between transported

substrates and 'dead end' inhibitors such as cocaine and desmethylimipramine which 'lock' the carrier at the extracellular membrane face. Substrates which bind to the carrier and travel to the intracellular face of the membrane block catecholamine uptake mainly by two mechanisms. Firstly, substrates compete with noradrenaline for sites on the carrier ([^{3}H]DMI sites) but more importantly, they slow the dissociation of the substrate-carrier complex at the intracellular membrane face (see step 6, Fig. 3). This latter step is rate limiting to the uptake of NA and thus these compounds are more potent in blocking [^{3}H]NA uptake than in [^{3}H]DMI binding assays due their ability to slow the cycling of the transporter (Graefe and Bonish, 1988).

[^{3}H]Desmethylimipramine is known to be selective for the NA transporter, possessing very little affinity for dopamine and serotonin transporters (Lee et al. 1982; Lee and Snyder, 1981). Cocaine, on the other hand exhibits a small degree of selectivity for the NA transporter when compared to its affinity at the DA transporter (Horn, 1990). Of the two enantiomers of cocaine, the (-)form is much more potent than the (+)form (Koe, 1976). [^{3}H]DMI binds only to the carrier after the initial binding of Na^+ (as NA does before being translocated) and arrests the transporter at the extracellular face of the membrane. Cocaine, on the other hand, can bind to site on the carrier in absence or presence of Na^+ and halt transport (Reith et al.,1980; Kennedy and Hanbauer, 1983). In contrast to cocaine, desipramine inhibits uptake to a greater extent at high rather than low Na^+ concentrations (Graefe and Bonish, 1988). A recent study has shown that

desipramine, like the substrate noradrenaline, was proposed to interact with a form of the transporter in which chloride ions are already bound at the extracellular membrane, whereas cocaine was suggested to interact with a chloride ion free state of the transporter molecule (Ungell et al., 1989). The above differences in the binding of [^{3}H]DMI and [^{3}H]cocaine to the NA transporter suggest that they occupy slightly different domains on the transporter protein.

1.2.4.2. PCP and Sigma ligands

Hormones, stress and circadian rhythms have been reported to influence catecholamine transport, raising the possibility that transport systems might be modulated by specific regulatory mechanisms (Horn, 1990). Noradrenaline uptake has been reported to be regulated by adenine nucleotides, divalent cations (Hendley et al., 1988) and by insulin (Boyd et al., 1986), and by an 'imipramine-like endogenous peptide' (Rehavi et al., 1985).

Several synthetic compounds have been examined for their effects on catecholamine uptake in vitro, however, of all the substances studied, phencyclidine and cocaine have generated the most interest due to the resulting addicting euphoria which has led to their illicit street use (Byck and Vandyke, 1977). It is now well established that cocaine and related analogs induce feelings of euphoria by blocking dopamine uptake since the potency of cocaine analogs correlates well with the behavioral effects (Ritz et al.,1987). In contrast, the mechanism involved in PCP-induced psychosis remains a mystery. Specific

receptor sites for [^{3}H]PCP have been identified in rat brain (Vincent et al., 1979). Today, it is well known that PCP interacts with PCP_1, PCP_2 sites and sigma receptors, however due to the lack of an in vivo animal model for human psychosis, it is not clear which one, if any of these sites mediates the PCP-induced psychotomimetic effects. The PCP_2 site is known to block the uptake of dopamine, however, involvement of the PCP_2 in the blockade of NA uptake has not been established. Such action of PCP on NA uptake may also play a role in the generation of psychosis (Garey and Heath, 1976; Smith et al., 1977, Ary and Komiskey, 1980; Snell et al., 1988).

Psychotic effects are not only produced by phencyclidine-like compounds but also by sigma ligands such as the benzomorphan opiates i.e. N-allylnormetazocine (SKF-10047), (±)cyclazocine, and (±)pentazocine (Forrest et al., 1969; Haertzen, 1970; Sonders et al., 1988). The sigma receptor is presently thought to be responsible for mediating the behavioral effects of these ligands (Sonders et al., 1988), although this theory is presently the subject of debate (Musacchio, 1990). Sigma receptor ligands have demonstrated potency in the potentiation of NA-induced contractions of the rat vas deferens and rat tail artery, a response that may result from the blockade of NA uptake (Kennedy et al., 1987; Massamiri and PiperDuckles, 1989). However, at the time of this investigation, no report of the effects of sigma ligands on NA uptake had been published.

1.3. PHENCYCLIDINE

Phencyclidine (PCP), originally introduced into clinical practice in 1958 as a general anaesthetic (Chen et al., 1959), was later withdrawn from human use because it was found to produce postanesthetic hallucinations (Meyer et al., 1959; Luby et al., 1959). This drug has now become one of the most popular illicit drugs of abuse in the United States, and has gained the nickname "angel dust" (Cox et al., 1983). The remarkable similarities between the behavioral effects induced by PCP and those observed in the disease state schizophrenia, have led to the study of PCP as a model of this illness (Domino, and Luby, 1981). PCP intoxication induces a prolonged psychosis typically lasting 4-6 weeks. The symptoms of PCP intoxication (Showalter et al., 1977; Allen and Young, 1978) include memory impairment, anxiety, a general disorganization of thought processes, and unusual thought content, delusions and hallucinations which are prominent: they can be visual, auditory or affect perceptions of body image. The general effect or behaviour may range from extreme agitation, paranoia, and violent hallucinations to a quiet, cataleptic state that has been defined as "dissociative anaesthesia". In these extreme manifestations PCP closely mimics both the positive and negative symptoms of schizophrenia (Clouet, 1986; Allen and Young, 1978; Luby et al., 1959).

1.3.1. PCP Receptors

Specific [^{3}H]PCP binding sites were identified in 1979 in rat brain, (Vincent et al, 1979; Zukin and Zukin, 1979; Quirion et al., 1981). Since this time, several laboratories have been successful in the autoradiographic identification of PCP binding sites using [^{3}H]MK-801, [^{3}H]TCP, and [^{3}H]PCP (Maragos et al., 1988; Rothman et al., 1989; Reid et al., 1990). The binding of [^{3}H]TCP is preferentially distributed in the nonpyramidal layers of the hippocampus, cerebral cortex, and in certain regions of the thalamus. The potency with which many analogs of PCP inhibit [^{3}H]PCP or its analog [^{3}H]TCP from PCP binding sites is highly correlated with their potencies in behavioral, and drug discrimination assays (Brady and Balster, 1982; Shannon, 1981), strengthening the hypothesis that the in vitro binding sites might function as receptors in vivo. Thus, the PCP-induced behavioural alterations are thought to be mediated by one of the specific receptors to which PCP binds, namely PCP receptor subtypes PCP_1 and PCP_2, and at higher doses, sigma receptors. Recent evidence has shown that the neuronal systems involved in mediating the effects of PCP (glutamatergic, dopaminergic, and sigma) may be implicated in the pathogenesis of schizophrenia (for review see Meltzer, 1991).

1.3.1.1. PCP_1 Receptors

The high affinity PCP binding site (PCP_1), has been located within the ion channel of the N-methyl-D-aspartate (NMDA) receptor where PCP sterically blocks the passage of ions into the cell (Anis et al., 1983; MacDonald and Nowak, 1990).

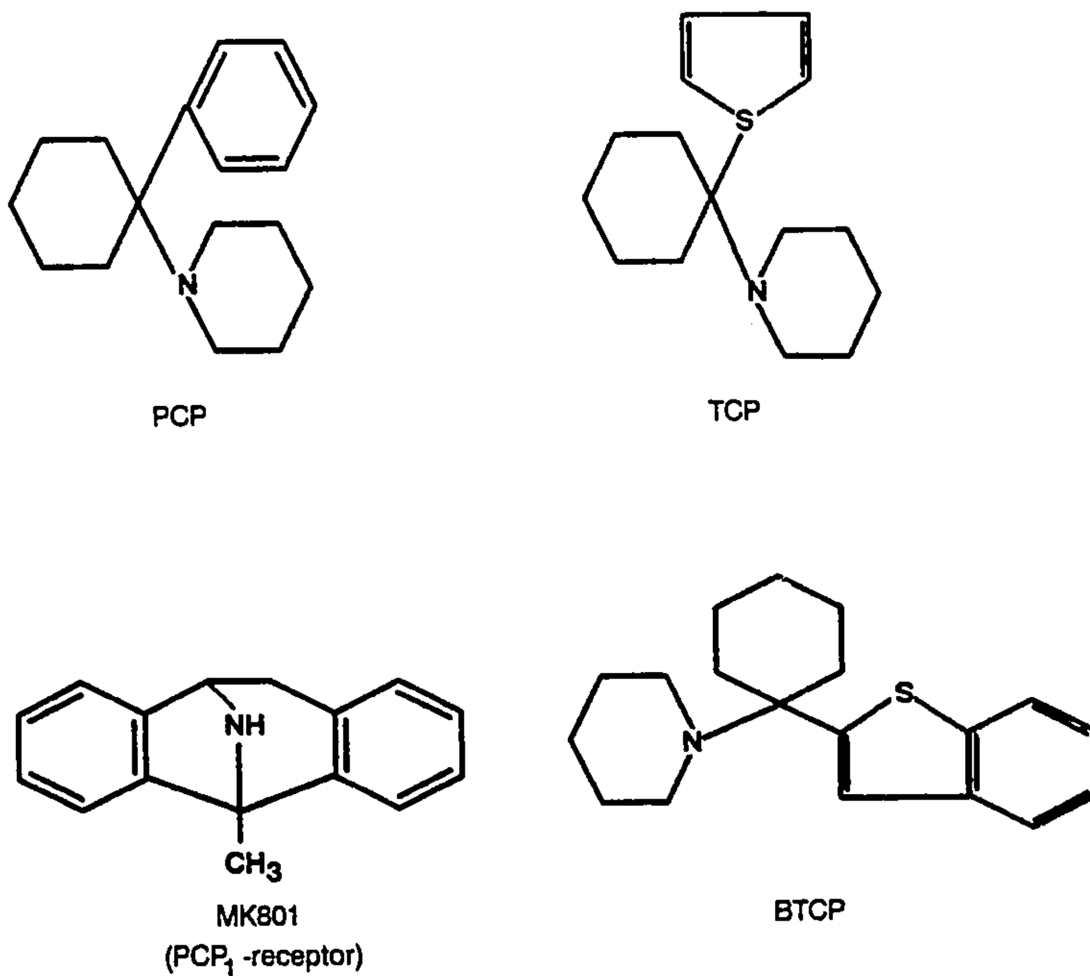


FIGURE 4. Structure of PCP ligands

Phencyclidine and its analog TCP label both PCP_1 and PCP_2 receptors. The drug MK-801 is selective for the PCP_1 receptor site on the NMDA receptor whereas the PCP analog BTCP binds PCP_2 receptors with high affinity and selectivity.

Activation of NMDA ion channels has been implicated in the processes of learning and memory, activities that rely upon a type of neuronal synaptic plasticity termed long-term potentiation, (LTP; Morris et al., 1986;Cotman et al., 1988). However, excessive stimulation of NMDA receptors leads to the excessive influx of Ca^{2+} through these channels and results in the generation of seizure activity (Fig 5; Piredda and Gale, 1986; Herron et al., 1985; Turski et al., 1986). NMDA-receptor over-activation has also been proposed to play a role in the neurodegeneration caused by anoxia and stroke (Simon et al., 1984; Rothman and Olney, 1987; Choi, 1988).

The initial finding that PCP and the related dissociative anaesthetic ketamine acted as noncompetitive antagonists of the excitatory amino acid neurotransmitter glutamate (Anis et al., 1983), triggered investigation into the possibility that PCP_1 receptor analogs may be of therapeutic value. Radioligand binding and electrophysiological experiments provided the first evidence that the NMDA receptor agonist site, the glycine binding domain and PCP sites were physically coupled (Martin and Lodge, 1985; Harrison and Simmonds, 1985; Loo et al., 1986). Of the key observations supporting this hypothesis, the two most relevant were that; 1) agonists and competitive antagonists of the NMDA receptor modulate the PCP binding site in vitro (Loo et al., 1987; Javitt et al., 1987) and ; 2) high affinity PCP receptor ligands act as potent noncompetitive antagonists of the NMDA receptor ion channel complex (Cotman and Iversen, 1987). Therefore the binding of the selective PCP_1 receptor ligand [^{3}H]TCP is inhibited by MK-801,

and regulated by NMDA modulators (glutamate, glycine, polyamines, Zn^{2+}, and Mg^{2+}) and antagonists (AP-5, CPP) which control the open and closed state of this receptor-linked ion channel (Foster and Fagg, 1987). Since PCP and ketamine were some of the first antagonists of this ion channel discovered, the pharmacology of the PCP_1 site has been well characterized (MacDonald and Nowak, 1990).

Several groups have successfully identified and purified the proteins bound by non-competitive NMDA antagonists MK-801, and PCP (Haring et al., 1987; Ambar et al., 1988; Haring et al., 1990). The purified subunits (59 kDa and 90 kDa) photolabelled by azido-[^{3}H]PCP are believed to be part of the NMDA complex, (Haring et al., 1990) although PCP is known to interact with other receptors, ie PCP_2, and sigma sites.

Recently, the NMDA receptor cDNA was isolated and to date, several isoforms of NMDA receptor have been cloned (Moriyoshi et al., 1991). The recent identification of the Mg^{2+} binding site on the NMDA receptor complex (Mori et al., 1992) has cleared the way for future studies to determine the cDNA sequence encoding the PCP_1 binding domain.

Function of PCP_1 Receptors

Evidence from animal studies has indicated that non-competitive NMDA receptor antagonists such as PCP and MK-801 may prevent neuronal degeneration caused by excessive stimulation of NMDA receptors. PCP selectively antagonizes NMDA mediated neuronal responses in several different

assays, including NMDA-mediated depolarization of spinal cord neurons (Anis et al., 1983; Martin and Lodge, 1985) and cortical cells (Thompson et al., 1985). Others have found that PCP inhibits NMDA-induced acetylcholine and dopamine release from slices of rat striatum and nucleus accumbens (Jones et al., 1987; Snell and Johnson, 1986) and non-competitively inhibits NMDA-induced release of [^{3}H]NA

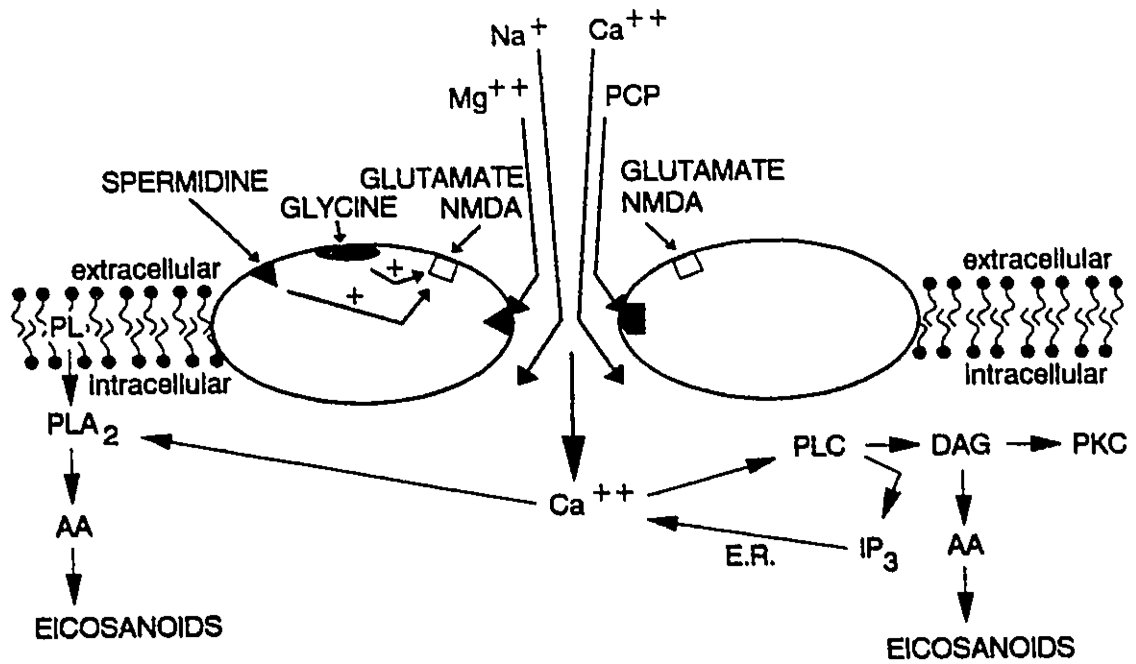


FIGURE 5. Interaction of PCP with the N-Methyl-D-Aspartate Receptor The binding site for PCP and MK-801 is thought to exist within the channel pore of the receptor. The binding of PCP and MK-801 is favoured when the NMDA ion channel is open, thus when NMDA agonists such as glutamate are present. The binding of glycine to its strychnine-insensitive site permits the interaction of glutamate with the agonist site causing channel activation. NMDA receptor activation can be modulated by polyamines such as spermidine which bind to extracellular sites on the receptor complex. Mg^{2+} also binds to a site deep within the channel pore, but this block is removed upon depolarization of the membrane.

from rat hippocampal slices (Jones et al., 1987). PCP was also found to modulate NMDA-induced signal transduction (Wroblewski and Danysz, 1989), including the increase in cGMP induced by NMDA in rat cerebellum, (Danysz et al., 1989). Therefore the role of PCP in the physiology of NMDA receptor activation has been well elucidated (Lodge and Johnson, 1990).

Unfortunately, PCP analogs are not useful in the treatment of NMDA-associated disorders due to their possible psychotomimetic properties and abuse potential in man. A crucial question concerning whether the adverse effects of PCP analogs are a result of NMDA receptor blockade, or are due to a separate action of these compounds remains to be answered. If the latter is true, it should be possible to develop molecules that retain NMDA-receptor antagonism, but are devoid of the undesirable psychotomimetic effects. It is worth mentioning that MK-801 does not produce behavioural effects identical to that of PCP (Koek and Woods, 1988), and the work of Vignon and Lazdunski (1984) has suggested that activation of non-NMDA associated PCP receptors may be responsible for the psychotomimetic effects.

1.3.1.2. PCP_2 Receptors

There are PCP receptors which are not linked to NMDA receptors (Vignon et al., 1986; Snell et al., 1988; Rothman et al., 1989), these sites have been termed PCP_2 sites. Membranes of human, rabbit, mouse and guinea pig were found to contain two high affinity binding sites for [^{3}H]TCP, one being the NMDA

receptor (k_d= 14 nM; B_{max}= 631 fmol/mg protein) and the other associated with the DA re-uptake carrier (K_d= 46.5 nM; B_{max}= 829 fmol/mg protein; Akunne et al, 1991; Rothman et al., 1989; Vignon et al., 1986).

Pharmacological characterization of the second high affinity [^{3}H]TCP binding site (PCP_2) has revealed that it is selective for PCP analogs bearing an unsubstituted phenyl ring (Chaudieu et al, 1989; Vignon and Lazdunski, 1984). Thus the replacement of the phenyl ring by a 2-thienyl ring leads to a molecule (TCP) which is more selective for the PCP_1 receptor while the replacement of the phenyl ring by a benzothiophenyl one (BTCP) leads to a compound that is selective for the PCP_2 receptor, (Fig. 4).

The rank order of potency of PCP ligands at the PCP_1 site is the reverse of that observed at the PCP_2 site and therefore differentiation between PCP_1 and PCP_2 receptors according to their respective drug potencies is possible. The PCP_1 site binds MK-801 > TCP > PCP > BTCP whereas the PCP_2 site binds PCP ligands with the affinity BTCP > PCP > TCP > MK-801 (Vignon et al., 1988). The PCP_2 ligand BTCP, potently blocks DA uptake and is devoid of affinity for the PCP_1 receptor (Chaudieu et al, 1989; Vignon et al, 1988).

The binding of [^{3}H]BTCP in human brain is elevated in both caudate and putamen, two areas which are rich in dopamine containing nerve terminals (Allaoua et al., 1992). The [^{3}H]BTCP binding site on the dopamine transporter is not identical to the substrate recognition site and thus differs from that of cocaine (Maurice et al., 1991). Most of the studies on BTCP were completed in striatal

tissue and substantia nigra, where PCP analogs were not potent inhibitors of its binding (Maurice et al., 1989). BTCP in vitro has 70 fold greater potency than PCP in blocking DA uptake (IC_{50}= 8 Nm), and requires Na^+ for high affinity binding, whereas [^{3}H]PCP binding is inhibited in the presence of Na^+. Thus, although BTCP is chemically related to PCP, its neurochemical (Vignon et al., 1988) and behavioral actions (Koek et al., 1989) indicate that it is best described as a cocaine-like drug, which like [^{3}H]cocaine, binds to the DA transporter in a Na^+-dependent manner.

It is interesting to note that PCP has also been reported to block the reuptake of NA (Garey and Heath, 1976; Smith et al., 1977), and this suggests that a similar 'PCP_2-like' binding domain may exist on other macromolecules in the family of biogenic amine transporters. Initial studies by Smith et al., (1977) showed that PCP was a competitive inhibitor of [^{3}H]NA reuptake, in contrast to the non-competitive effects of BTCP on dopamine uptake. Although the receptor responsible for the PCP-induced inhibition of [^{3}H]NA uptake remains uncharacterized, the reported inhibitory potency of various PCP analogs tested in [^{3}H]NA uptake assays differs from those values reported in PCP_2 binding and [^{3}H]dopamine uptake assays (Snell et al., 1988). It is possible that the PCP site responsible for the blockade of [^{3}H]NA uptake resembles a site distinct from the PCP_2 receptor and may resemble a σ receptor subtype. Thus it may be such that the behavioural alterations observed in response to PCP (Meyer et al., 1959), are due to stimulation of a sigma receptor subtype (Deutsch et al., 1988).

1.4. THE SIGMA RECEPTOR

After reviewing the enormous amount of literature on the sigma (σ) receptor, one can safely state that no other receptor in the history of pharmacology has been this confusing. Since its first classification as an opiate receptor subtype in 1976, the sigma receptor has led investigators in circles searching for its function (Martin et al., 1976; Walker et al., 1990). Today, it is believed that two subtypes of sigma receptors exist and the primary function of these sites is still not clear.

1.4.1. The History of Sigma Receptors

The original observation of head weaving and "mania" in the chronic spinal dog produced by the administration of the benzomorphan (±)SKF-10047, (N-allylnormetazocine), was attributed to the stimulation of an opiate receptor subtype designated sigma (Martin et al., 1976). The receptor classification was changed after the discovery that the sigma effects induced by (±)SKF-10047 in the chronic spinal dog were due to the dextro-isomer of this compound, rather than its opiate or levo-isomer and not blocked by naloxone (Martin et al., 1984). Thus the sigma site was separated from the opiate receptor class (Su, 1982, Tam and Cook, 1984), and is defined today on the basis of its unique pharmacology (Quirion et al., 1992).

The fact that racemic σ drugs were found to produce a variety of psychotomimetic symptoms in man (Telford et al., 1961; Keats and Telford, 1964; Forrest et al., 1969; Haertzen, 1970) generated increasing interest in sigma

ligands as tools to study psychosis (Domino and Luby, 1981; Snyder, 1989). Based on the ability of (+)SKF-10047 to inhibit [^{3}H]PCP binding (Zukin et.al., 1979) and induce a PCP-like behavioural pattern, (Brady et al., 1982), investigators speculated that a common receptor for both phencyclidine and sigma ligands could exist, thus the receptor was named σ/PCP (Quirion et al., 1981; Mendelsohn et al., 1985). Later, radioligand binding studies and detailed autoradiography through many brain regions demonstrated the distinction between sigma and PCP receptors (Gundlach et al, 1986).

Extensive pharmacological characterization of sigma receptors in CNS and periphery continued through the later half of the 1980's, however no one investigator was successful in obtaining a correlation between psychotomimetic effects of these ligands and their potency in sigma receptor assays. To date, there are a few investigators that argue against tne idea that sigma receptors are involved in psychosis (Mussacchio, 1990) although this view is not easily strengthened without additional human studies.

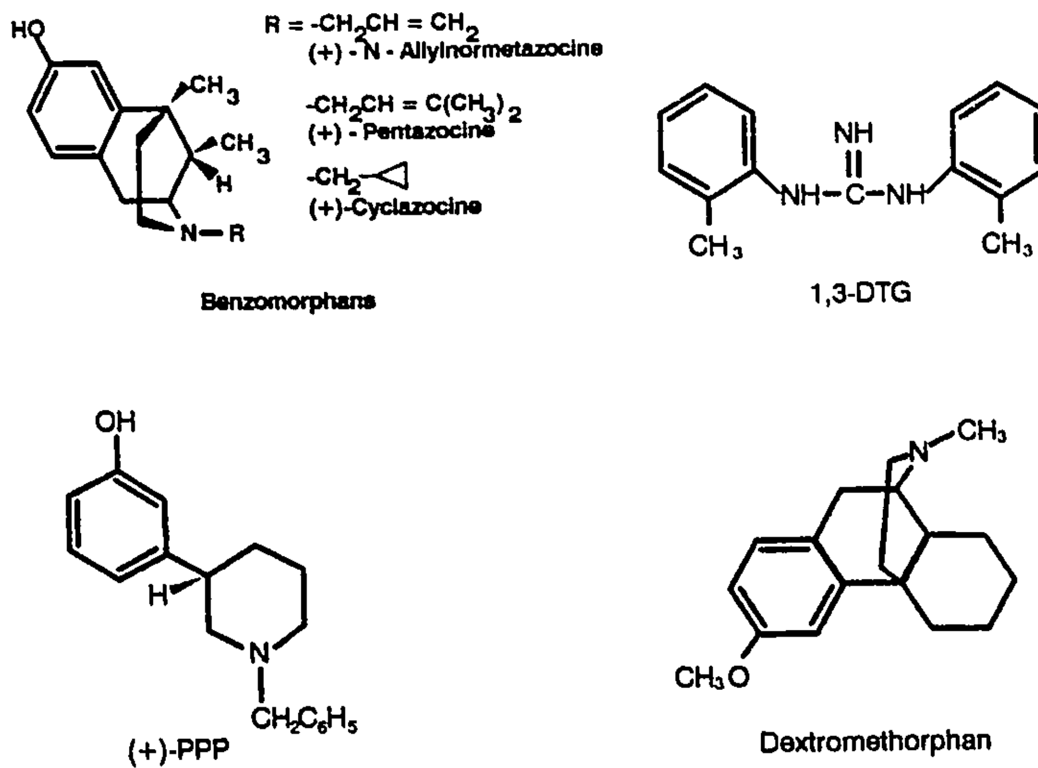


FIGURE 6. Structure of Some Sigma Receptor Ligands

Sigma ligands with high affinity for sigma receptors are shown. The dextro-isomers of the benzomorphan series are selective for the $sigma_1$ receptor, whereas the guanidine compound DTG binds both $sigma_1$ and $sigma_2$ sites with high affinity. The piperidine, 3(+)PPP and the antitussive/anticonvulsant dextromethorphan interact with both sigma subtypes however, binding of these two ligands to the $sigma_1$ receptor can be up-regulated in the presence of ropizine or phenytoin.

1.4.2. Sigma Receptor Subtypes

Sigma receptors have been shown to bind compounds from diverse chemical classes. Among these are butyrophenones such as haloperidol (Su, 1982; Tam and Cook, 1984), piperidines such as 3(+)PPP, (Largent et al., 1984; Wikstrom et al., 1987) symmetric guanidines such as DTG (Weber et al., 1986) and benzomorphans such as (+)pentazocine and (+)SKF-10047 (Largent et al., 1987). These ligands exhibit varying degrees of selectivity for sigma receptors in ligand binding assays, see Table 1. Whereas DTG appears to be relatively selective for sigma sites (Weber et al., 1986), haloperidol exhibits nearly equal affinity for sigma and dopamine-D_2 receptors (Bowen et al., 1989). Although (+)3-PPP has high affinity for sigma sites (Koe et al., 1989), it exhibits some residual affinity for dopamine D_2 sites (Wikstrom, 1987). Of the opiate-related benzomorphans, (+)pentazocine exhibits high selectivity for sigma receptors (DeCosta et al., 1989; Bowen et al., 1990) whereas (+)SKF-10047 shows significant cross-binding activity with phencyclidine receptors (Largent et al., 1986).

In an attempt to straighten out the classification of sigma receptors, several investigators in the field met and arrived at a consensus for the nomenclature of these sites (Quirion et al., 1987; Quirion et al., 1992). It is now agreed that sigma receptors exhibit high affinity for (+)pentazocine, DTG, haloperidol; (+)3-PPP; dextromethorphan and some neuroleptics and have been classified into subtypes, σ_1 and σ_2 (Table 2).

The σ_1 subtype binds dextromethorphan and (+)pentazocine and can be

pharmacologically differentiated from the σ_2 site by its stereoselectivity for (+)benzomorphans (Walker et al., 1990). In addition, the binding of the sigma ligands [^{3}H]dextromethorphan and [^{3}H]3(+)PPP is allosterically up-regulated in the presence of an anticonvulsant such as phenytoin or ropizine (Musacchio et al., 1987; Musacchio et al., 1988). It is also clear that σ_1 sites are modulated by GTP while the σ_2 sites are unaffected (Itzhak and Khouri, 1988; Beart et al., 1989). The σ_2 receptor may be identified by the binding of [^{3}H]DTG in presence of the σ_1 blocker, (+)pentazocine (Quirion et al., 1992). The σ_2 site binds the (-)isomers of benzomorphans with higher affinity than (+)benzomorphans. At present, a high affinity and selective σ_2 radiolabel is not available. Compounds with slightly higher affinity at σ_2 sites include DTG, BMY-14802, rimcazole, and ifenprodil (Walker et al., 1990). In contrast to the σ_1 receptor, the σ_2 receptor has been reported to interact with Ca^{2+} channel blockers and Zn^{2+} (Rothman et al., 1991; Connor and Chavkin, 1992). Additional evidence for the differentiation of σ receptor subtypes came from photoaffinity labelling experiments using azido-[^{3}H]DTG. This probe labels a polypeptide of 29 kDal (thought to be the σ_1 subtype) in membranes from guinea pig brain (Kavanaugh et al., 1989) and a polypeptide of 18-21 Kdal in PC-12 cells (the σ_2 receptor; Hellewell et al.,1990a).

TABLE 1. **Sigma Ligand Specificity**

Sigma Ligand	Interaction with Other Receptors Classes
Haloperidol	high affinity as a D_2 receptor antagonist
3(+)PPP	Low affinity as a D_2 autoreceptor agonist
(+)pentazocine	low affinity for kappa opiate receptors
DTG	none
(+)SKF-10047	high affinity for PCP receptors
dextromethorphan	high affinity at anti-tussive sites (DM sites)
rimcazole	10 fold lower affinity for D_2 receptors
BMY-14802	15 fold lower affinity for D_2 receptors
BW-234U	low affinity for 5HT receptors
(-)butaclamol	very low affinity at D_2 receptors
Ifenprodil	polyamine site antagonist at the NMDA receptor

TABLE 2. Binding potencies of various compounds at $sigma_1$ and $sigma_2$ receptor subtypes[1]

ligand	$sigma_1$	$sigma_2$
(+)benzomorphans	high	low
stereoselectivity for benzomorphans	+ > -	- > +
DTG	high	high
haloperidol	high	high
(+)3-PPP	high	high
dextromethorphan	high	very low
PCP	low	moderate

[1] $Sigma_1$ and $sigma_2$ receptors also differ in molecular weight (25 kDa and 18 kDa, respectively) and the allosteric modulation of $sigma_1$ sites by both U.V. light and the presence of anticonvulsants such as phenytoin and ropizine (Walker et al., 1990).

1.4.3. Localization

1.4.3.1. Central Sites

In hopes that the clarification of σ receptor function might come from receptor localization, the autoradiographic studies of Largent et al., (1986) and McLean and Weber, (1988) showed that sigma receptors were concentrated mainly in brainstem areas that involve motor functions, certain limbic structures, some sensory areas, and brain areas associated with endocrine function. Highest σ receptor densities by far occur in neuronal cell bodies of motor circuits, the red nucleus, inferior olive, locus coeruleus, and also sensory processing areas such as dorsal root ganglia, lateral geniculate and anterior pretectal areas. In the spinal cord, receptors are also highly concentrated in motor cells of the ventral horn (Aaonsen and Seybold, 1989).

1.4.3.2 Peripheral Sites

The discovery of high density, but low affinity σ-like sites in peripheral tissues may suggest the existence of peripheral and central-type sigma sites, analogous to the benzodiazepine receptor (Braestrup et al., 1977). To date, sigma binding sites have been reported in many peripheral tissues. These include, rat liver (Samilova et al., 1988; Hellewell et al., 1990b), guinea pig spleen (Su et al., 1988) bovine adrenal medulla (Rogers et al., 1989), guinea pig myenteric plexus (Roman et al., 1988) human peripheral blood leukocytes (Wolfe et al., 1988) and rat ovary and testis (Wolfe et al., 1989). Recently, $sigma_1$ and $sigma_2$ sites have also been reported in kidney (Bowen et al., 1992).

The putative physiological role of high affinity PCP and σ binding sites in the adrenal medulla has not been investigated although it is known that high concentrations of these ligands interact with the nicotinic ACh receptor complex (Albuquerque et al., 1980; Purifoy and Holz, 1984; Paul et al., 1992).

Both the sigma ligand [^{3}H]3(+)PPP and the PCP receptor ligand [^{3}H]TCP labelled one homogenous population of high affinity receptors in bovine adrenal medulla (Rogers et al., 1989; Rogers and Lemaire, 1990). The binding of [^{3}H]TCP was found to be potently inhibited by haloperidol, (K_i: 19 μM; Rogers and Lemaire, 1990), suggesting that PCP ligands were binding to a site distinct from the haloperidol-insensitive PCP_1 and PCP_2 sites defined in brain (Vignon et al.,1986). These studies also revealed that [^{3}H]TCP binding displayed a σ receptor profile in its rank order of inhibition by σ ligands. The results suggest that the adrenal medulla lacks the defined high affinity PCP_1 and PCP_2 receptors, and [^{3}H]TCP binding here represents interaction with a sigma subtype, possibly $sigma_2$. In addition, the PCP_1- receptor ligand [^{3}H]MK-801 (5 nM) was unable to label specific sites in this tissue adding support to the hypothesis that the bovine adrenal medulla lacks PCP_1 receptors.

The high affinity σ ligand [^{3}H]3(+)PPP, bound to high affinity sites in the adrenal which were stereospecifically inhibited by the dextroisomer of SKF-10047, and inhibited in appropriate rank order by other sigma ligands, indicative of σ_1 binding sites in this tissue.

1.4.4. Function

Although the roles of PCP receptors with respect to NMDA antagonism (PCP_1) and dopamine uptake (PCP_2) appear to be well understood, the role of the sigma receptor has not been elucidated.

The strongest support for a biologically relevant sigma receptor is seen in the high correlations (>0.70) between sigma binding affinity and potency in the following biological assays systems. $Sigma_1$ receptor profile correlated with; (a) in vivo and in vitro gastrointestinal effects (Riviere et al., 1990; Pascaud et al., 1990); (b) potentiation of guinea pig ileum contraction in response to 5HT and electrical stimulus (Walker et al, 1990; Campbell et al., 1987); and (c) inhibition of muscarinic receptor-induced increases in IP_3 (Bowen et al., 1988).

On the other hand, $sigma_2$ sites correlated better with motor function. DTG, when microinjected into the red nucleus of the rat produced movements resembling dystonia (disordered tonicity of muscle; Walker et al., 1990; Walker et al., 1988; Matsumoto et al., 1990). Sigma receptors are also found in other motor areas such as the substantia nigra (McLean and Weber, 1988). $Sigma_2$ receptor affinity was also found to correlate with the potency of some sigma ligands in the blockade of K^+ channels (Barshat and Blaustein, 1988; Kennedy and Henderson, 1990).

The role of sigma receptors present in tissues such as the testis, ovary and adrenal medulla is unknown. Many sigma ligands produce functional effects in both CNS and periphery which do not correlate with high affinity sigma receptor

subtypes. This is demonstrated in several tissues; for example, in rat hippocampal slices sigma ligands were shown to modulate NMDA-induced electrophysiological responses (Monnet et al., 1990); in guinea pig and rat liver, sigma receptors were postulated to be involved in the P450-2D1 enzyme system (McCann et al., 1991); while in peripheral blood leukocytes sigma ligands were found to alter the release of lymphokines (Wolfe et al.,1989). Finally, in preparations of rat tail artery, sigma ligands potentiated the NA-induced contractions of vascular smooth muscle (Massamiri and PiperDuckles, 1990). Whether these effects are due to the stimulation of a $sigma_3$ subtype or simply due to cross-binding activity at other receptors has not been investigated.

1.5. ENDOGENOUS LIGANDS FOR PCP AND σ RECEPTORS

The existence of specific PCP and σ receptors raises the question as to whether endogenous ligands for these binding sites exist. In the past, several laboratories have described the presence of endogenous compounds in brain extracts which compete for PCP and σ receptors (Contreras et al.,1987; Dimaggio et al., 1988; Quirion et al., 1984; Su et al., 1986; Zhang et al., 1988; Zukin et al.,1987). Roman et al., (1989) suggested that NPY could bind to sigma receptors, although these results could not be replicated (Quirion et al., 1991; Tam and Mitchell, 1991). More recently, Bouchard et al., (1992) have suggested that a metabolite of NPY could be responsible for the observed in vivo interaction between NPY and the sigma receptor.

Chavkin and co-workers, have provided evidence for the stimulated release of an endogenous sigma ligand from nerve terminals, suggesting that the endogenous ligand may be processed, packaged and released in a similar fashion to prodynorphin and proenkephalin derived peptides (Neumaier and Chavkin, 1989). Later, this same group suggested that Zn^{2+} may act as a true endogenous ligand for sigma receptors (Connor and Chavkin, 1992) although this hypothesis requires further study.

The eventual purification of possible endogenous σ ligand from brain extracts will satisfy the final criteria necessary to prove that the σ binding site is a truly biologically significant receptor. In light of the knowledge that stimulation of PCP-receptors induces a psychotic state resembling schizophrenia, it is logical

to postulate that excessive production and release of endogenous PCP ligands may play a role in the mechanisms behind this disease. A biologically significant endogenous ligand should possess not only the receptor binding properties, but also the biological effects of PCP and σ-like drugs. The lack of a suitable bioassay for the testing of endogenous σ receptor ligands prevents rapid progress in this area of research and therefore many investigators rely upon radioligand binding data to analyze endogenous PCP and σ ligands. Until a suitable animal model for psychosis is developed, one can only postulate the possible implications of endogenous σ factors in the central nervous system. Another question concerns the possibility that endogenous ligands for the allosteric modulatory sites of the σ receptor could also exist and play an important part in the regulation of sigma receptor function. Increased production and release of endogenous allosteric regulators of σ binding could lead to chronic up-regulation of σ receptor activity.

The adrenal medulla is known to contain a vast array of neurotransmitters, neuromodulators and receptors (Lemaire et al., 1984; Kondo et al., 1985; Lemaire et al., 1993;, Rogers et al., 1989; Rogers and Lemaire, 1993). It is possible that endogenous peptides for PCP and/or σ receptors may exist in the adrenal medulla. Thus the bovine adrenal medulla represents an interesting model for the study of the effects of PCP, sigma ligands and endogenous adrenomedullary peptides on catecholamine synthesis, storage, secretion and uptake. The present study was focussed on the effects of PCP, sigma and endogenous histogranin on

[^{3}H]NA uptake by isolated adrenomedullary cells.

1.6. HISTOGRANIN: A NOVEL PEPTIDE POSSESSING PCP-LIKE BINDING AND BEHAVIOURAL ACTIVITIES.

Histogranin (HN), a pentadecapeptide (H-Met-Asn-Tyr-Ala-Leu-Lys-Gly-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-COOH), was isolated as a co-fraction of neuromedin C during the extraction of bombesin-like peptides from bovine adrenal medulla (Lemaire et al., 1993). The ability of HN to cross-react with an antibody developed against bombesin was pivotal in the successful identification and subsequent purification of the peptide. Details concerning the initial purification, structural identification, and distribution of histogranin are detailed in the original paper (Lemaire et al., 1993). The name 'histogranin' was derived from the fact that the peptide possesses 80% homology with a fragment of histone H_4 and was first identified from extracts rich in adrenal chromaffin granules. The theory that HN is derived from or a possible metabolite, or processed peptide from histone H_4 is ruled out by the following evidence. First, the synthetic fragment corresponding to the homologous portion in histone H4 ([Val^1, Val^2, Arg^7]HN) possesses HPLC retention times that differ from those of the natural HN in three different systems. Second, the cDNA encoding the histone H4 fragment-(86-100) has not suffered a single change in amino acid sequence through evolution from the simple pea to homo sapiens (Wells, 1986). Finally, bombesin antisera used to detect the endogenous histogranin in bovine adrenal extracts, was unable to recognize the corresponding synthetic fragment of histone H4. Therefore, histogranin more likely possesses its own precursor that can be processed in

chromaffin granules along with other peptides.

The peptide is mainly present in the pituitary, adrenal medulla and lymphoid tissues (spleen, lung) but significant levels of the peptide are also found in the brain, although its regional localization in the brain has not yet been investigated (Lemaire et al., 1993). In the adrenal medulla, the peptide is more concentrated in the secretory granules of adrenal chromaffin cells and stimulation of perfused adrenal glands with carbamylcholine leads to an important release of the peptide in the perfusate. Significant levels of the peptide (20 fmol/ml) are also observed in plasma.

Initial investigation of histogranin revealed that it inhibited the binding of [^{3}H]PCP from a preparation of rat brain membranes. This activity was variable and represented only partial competition (25-30%) of specific [^{3}H]PCP binding. Later, HN was shown to affect the binding of the PCP_1 receptor ligand [^{3}H]MK-801 at relatively high doses (Lemaire et al., 1993). These initial observations derived from radioligand binding led to further investigation of possible PCP-like effects in vivo. Behavioural assays were set up to verify if histogranin had PCP-like effects in the rat. The injection of HN (i.c.v) produced dose-dependent stereotypy, locomotion and ataxia, three effects that are also observed with PCP (Lemaire et al., 1993b). Although initial studies of HN did not demonstrate interaction with high affinity binding sites labelled with [^{3}H]3(+)PPP (sigma sites), later studies (Lemaire et al., 1993) revealed its ability potentiate the binding of [^{3}H]dextromethorphan, a σ receptor ligand to rat brain membranes. Since

dextromethorphan was known to act at many sites including sigma sites, anti-tussive sites, and those mediating its neuroprotective action against the excitatory effects of NMDA (Musacchio et al., 1989) the observed potentiation by HN of [^{3}H]dextromethorphan binding was difficult to interpret. One could postulate that HN may act as a regulator of sigma, NMDA or antitussive sites. The original "PCP-like" binding and behavioural activities of histogranin raised our interest in its mechanism of action. The following questions were then posed:

is histogranin acting on,

1) The PCP_1 receptor (modulating NMDA receptor activity)
2) The PCP_2 receptor (blocking catecholamine uptake)
3) The σ receptor (psychotic effects, motor effects)
4) Its own receptor and allosterically modulating the sites involved in producing PCP-like activity.

Results by Lemaire et al., (1993b) revealed that the potentiation of [^{3}H]dextromethorphan binding was reversed in the presence of NMDA and PCP_1 receptor ligands such as CPP, NMDA and MK-801, but not by sigma ligands such as DTG or (+)pentazocine. This clue led to the investigation of a putative role for HN in excitatory amino acid transmission. Radioligand binding studies of the effects of HN on NMDA receptors revealed that HN could inhibit the binding of [^{3}H]CGP-39653, a selective NMDA receptor antagonist to its sites in rat brain membrane preparations (Lemaire et al., 1993b), and this effect was non-competitive (Shukla et al., 1993).

NMDA responses measured in vivo were subsequently shown to be blocked by HN (Lemaire et al., 1993). Injection (i.c.v.) of the peptide increased the NMDA-induced seizure threshold in convulsant assay carried out in mice. The peptide was also found to produce potent blockade of extracellular recorded NMDA-induced cation flux in slices prepared from locus coeruleus (Shukla et al., 1993). The blockade of NMDA-induced convulsions in mice was specific, not being observed when convulsion or cell depolarization was induced by kainate, AMPA, bicuculline and pentylenetetrazole. In vitro, HN also blocks the NMDA-induced potentiation of [^{3}H]TCP binding to rat brain membranes (Lemaire and Shukla, 1993). Thus, the present results support the notion that HN may act as a putative endogenous NMDA receptor antagonist. It is therefore tempting to postulate that the release of HN in the CNS may act to protect neurons from glutamatergic over-excitation observed in cases of ischemia and neuronal cell death.

The aims of this thesis were established before it was known that HN inhibited NMDA-induced responses. At that time, all that was known was that HN produced "PCP-like" binding and behavioural activities and therefore, the second and fourth questions listed above have been addressed by determining the primary characteristics of the binding of [^{125}I][Ser^{1}]HN and its possible interaction with PCP and sigma receptors.

2.0. THESIS AIMS AND OBJECTIVES

Previous studies involving the identification of PCP and sigma binding sites in bovine adrenal medulla (Rogers et al., 1989), and the isolation of an adrenomedullary peptide which produced a "PCP-like" behavioural profile in rats (Lemaire et al., 1993), triggered the present investigation of the physiological role(s) of PCP, sigma ligands and HN in this tissue. The following thesis objectives were tested to examine the hypothesis that, PCP and sigma ligands modulate the re-uptake of noradrenaline in bovine adrenal medulla. The hypothesis was extended to include examination of the effects of the endogenous PCP-like peptide on NA uptake. In order to better understand the mechanism involved in histogranin's "PCP-like" effects, characterization of its binding site was also completed in rat brain.

1. To verify whether PCP and sigma receptor ligands affect the uptake of noradrenaline in isolated adrenal chromaffin cells and to compare the potencies of these drugs with those obtained from similar studies in rat brain synaptosomes.
2. To examine the possible interaction of PCP and sigma ligands with the substrate recognition site on the NA transporter.
3. To verify that the "PCP-like" peptide, histogranin, alters [^{3}H]NA uptake, or modulates the action of PCP and sigma ligands in this system.
4. To characterize the binding site for histogranin in rat brain.

3.0. MATERIALS AND METHODS

3.1. Preparation of Chromaffin Cells

Bovine adrenal glands obtained from a local slaughterhouse were separated from their cortices and perfused in vitro for 10 min at 37°C with Ca^{2+}-free, Mg^{2+}-free Locke's solution as described by (Trifaro and Lee, 1980). Perfusion was continued for 60 min with fresh solution to which 0.05% collagenase (Sigma Chemical Co., St. Louis) had been added and chromaffin cells were isolated and cultured as described previously (Fenwick et al., 1976). Cells were cultured for one day and then centrifuged at 900 rpm in a Beckman centrifuge for 10 min and resuspended in buffer A ($NaHCO_3$, 16.2 mM; KCl, 4.7 mM; NaCl 133 mM; $CaCl_2$, 2.5 mM; KH_2PO_4, 1.2 mM; ascorbic acid, 1.14 mM, and nialamide, 0.0124 mM and equilibrated at pH 7.4 with O_2/CO_2, 95%/5%) at a concentration of 5×10^6 cells/ml. Cell viability was 95% or greater by trypan blue exclusion. At this stage, cells were ready to be included in the uptake assay. The properties of [^{3}H]NA uptake did not vary significantly from day 2 to 7 of culture (Kenigsberg and Trifaro, 1980); therefore, all assays were performed at day 2.

3.2. Preparation of Rat Brain Synaptosomes

Synaptosomes were prepared according to the method of Smith et al., (1977). This technique is based on the original method of synaptosome preparation first described by Gray and Whittaker, (1962) who first visualized and described the difference between synaptosomes and other membrane vesicles using electron

microscopy. Briefly, male Wistar rats of the same age and weight (250-275 g) were decapitated, the brains weighed and homogenized in a glass-teflon homogenizer with a clearance of 0.025 cm, containing 0.32 M sucrose (40 ml/g wet weight). During tissue homogenization, the pestle was rotated at 800 rpm with 12 up and down strokes. The homogenate was centrifuged at 1000 x g at 4 °C in a Sorvall SS34 rotor to remove nuclei and cellular debris. The resulting supernatant was centrifuged at 30,000 x g at 4°C for 30 min. The pellets (P_2) containing crude synaptosomes were used without further purification. The synaptosomes were resuspended in 30 ml of 0.32 M sucrose and diluted with buffer A to yield a concentration equivalent to 1.3 mg of protein per ml (Lowry et al., 1951).

3.3. Measurement of [^{3}H]NA uptake

The uptake assay was performed by prewarming tubes containing 600 µl of buffer A containing 20 mM Hepes, 100 µl of fixed (0.1 µM) or increasing concentrations of [^{3}H]NA, as indicated, and 100 µl of non-labelled σ, PCP or other receptor ligands (10^{-9}-10^{-4}M) for 5 min before the addition of 200 µl aliquot of either synaptosomes at a final concentration of 0.26 mg protein (Lowry et al., 1951) or 10^6 chromaffin cells. This mixture was vortexed and incubated for 10 min (or at the indicated time) at either 0°C (control), 30°C or 37°C as indicated. Control experiments were also performed in the absence of Na^+ by replacing NaCl and $NaHCO_3$ (buffer A) with equimolar LiCl and $LiHCO_3$, respectively.

Samples were then placed on ice for ten minutes prior to filtration by reduced pressure through Whatman GF/B filter circles (Snell et al., 1988). Filters were subsequently washed with 4 x 3 ml of ice-cold buffer A, transferred to vials containing 8 ml of ACS scintillation cocktail (Amersham) and allowed to equilibrate overnight. Radioactivity was measured in a Beckman LS 7800 Beta counter at 37% efficiency. The total uptake at a given time point or concentration was determined by correction, subtracting the 0°C uptake (or uptake in Na^+-free medium) from that obtained at 30°C or 37°C. The complex kinetics, composed of saturable and non-saturable components were analyzed by the method of Jaques et al. (1984). In the analysis of the data, the non-linear curve-fitting program, BDATA (EMF Softwares, Knoxsville, TN., USA) was utilized to determine the values of the K_m and V_{max} of the high affinity uptake. The concentration of PCP and σ receptor ligands that produced 50% inhibition of [^{3}H]NA uptake (IC_{50} values) were obtained from log-logit plots. Values are means ± s.e.m. of three separate preparations performed in duplicate.

3.4. Preparation of Adrenal Medullary Membranes

Bovine adrenal medulla were obtained from a local slaughterhouse (St. Albert, Ontario) within five minutes after death and immediately transported on ice to the laboratory. The boundary between the adrenal medulla and cortex may be quite irregular and thus during dissection of the medulla, a distinct ring of medulla is left behind in order to avoid contamination by non-medullary tissues. The

dissected adrenal medullary tissue was then minced with razor blades and processed (2 x 20 s) in a Waring blender with 10 vols of ice-cold Tris-HCl, (50 mM, pH 7.4 supplemented with 120 mM NaCl and 5 mM KCl; buffer B). The tissue suspension was then homogenized with a 4 strokes of a glass teflon homogenizer (2000 rpm), and particulate matter was removed by centrifugation at 1000 x g for 30 min at 4°C in a Sorvall SS34 rotor. The pellets were discarded and the resulting supernatant fraction was centrifuged at 30,000 x g for 30 min at 4°C. The membrane pellets were resuspended, homogenized and incubated on ice for 1 hr, with intermittent mixing, in Buffer B supplemented with 300 mM KCl. At the end of the incubation, the membranes were centrifuged at 30,000 X g for 30 min and resuspended in buffer B. This last washing procedure was repeated twice and the final membrane pellets were resuspended in buffer B at a concentration of 2 mg/ml (Lowry et al., 1951) and kept frozen at -80°C.

3.5. [^{3}H]DMI Binding Studies

Unless otherwise stated, the binding of [^{3}H]DMI was routinely measured in duplicate at 4°C for 1 hr using 0.4 mg of adrenomedullary membrane protein and the indicated concentration of [^{3}H]DMI in the presence and absence of test drugs in a total volume of 1 ml (buffer B). At the end of the incubation period, tubes were filtered by reduced pressure through GF 934AH filter circles. Each filter was then washed 4 times with 3 ml of buffer B per wash, and the filters were placed in vials containing 10 ml of scintillation cocktail overnight. Radioactivity was

measured in a Beckman LS7800 Beta counter. The non-specific binding was defined as the residual binding measured in the presence of 1 μM nisoxetine; this concentration of nisoxetine inhibits [^{3}H]DMI from its high affinity binding sites, as determined in competition binding studies. Saturation data was analyzed by Ligand, (Munson and Rodbard, 1980). Competition binding data were analyzed by the CDATA (EMF Softwares, Knoxsville, TN) and inhibitory constants (K_i values) were calculated according to the method of Cheung and Prusoff (1973). In order to study the effect of Na^+ on [^{3}H]DMI binding, the membranes were washed an additional 3 times and resuspended in Tris-HCl (50 mM, pH 7.4), before incubation with increasing concentrations of NaCl as described by Keller and Graefe (1979).

3.6. Iodination of [^{125}I][Ser^1]HN

[Ser^1]HN was iodinated by the chloramine-T procedure (Hunter and Greenwood, 1962) and purified by HPLC using a reverse phase μ-Bondapak C18 column (Waters). The peptide was eluted from the column at 32-35 min, using a gradient of 15-60% ACN in 0.1% TFA and 5 μl aliquots of the eluent were monitored for radioactivity as described for the preparation of iodinated bombesin (Lemaire et al., 1989). Specific radioactivity of the [^{125}I][Ser^1]HN peptide was determined by radioimmunoassay (Chiang, 1987). Aliquots of [^{125}I][Ser^1]HN were stored at -90°C until use.

3.7. Preparation of Rat Brain Membranes

Six male Wistar rats were decapitated and the brains were rapidly removed and homogenized in ice-cold Tris-HCl (50 mM, pH 7.4; buffer C) with a glass-teflon homogenizer. The homogenate was centrifuged at 27,000 x g for 30 min. The pellet was resuspended in buffer C and centrifuged at 27,000 x g for 30 min. The resulting pellet was homogenized and incubated on ice in a total volume of 1.0 L of buffer C supplemented with 0.3 M KCl for 60 min (Lee et al., 1982). The suspension was centrifuged at 27,000 x g for 30 min and the pellet was resuspended in buffer C. This washing procedure was repeated an additional 3 times and the final membrane pellet was resuspended in buffer C at a concentration of 2.0 mg protein /ml (Lowry et al., 1951) and frozen until used at -80°C.

3.8. [^{125}I][Ser^1]HN Binding Assay

Rat brain membranes (0.6 mg protein) that had been pre-treated with 0.3 M KCl were incubated in buffer C at a final volume of 0.5 ml. All binding reactions, unless indicated, were carried out at equilibrium (45 min) at 4°C in the presence of protease inhibitors bacitracin (25 μM), bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM). Competition experiments, were carried out in duplicate, with [^{125}I][Ser^1]HN (1.0 nM) in presence of increasing concentrations of competing ligand. In saturation binding experiments, rat brain membrane protein (0.6 mg) was incubated in duplicate with increasing concentrations of [^{125}I][Ser^1]HN (0.25-

50 nM) in buffer C in the absence and presence of 20 μM unlabelled [Ser^1]HN. Binding reactions were terminated by the rapid addition of 3 ml of ice-cold Tris-HCl and vacuum filtration using GF-934AH filters. Glass-fiber filters were pretreated with 0.1% polyethylenimine for 1 hr at room temperature. Tubes were rinsed once with 3 ml of buffer C and the filters were rinsed with 4 times with 3 ml of buffer C supplemented with 6.6 % polyethyleneglycol to lower non-specific binding. Total rinse volume passing over each filter was 15 ml and this took approximately 8.5 seconds. All filters were transferred into polypropylene tubes and radioactivity was monitored in a gamma counter at 75% efficiency. In all binding experiments, non-specific [^{125}I][Ser^1]HN binding was defined as the residual binding measured in the presence of 20 μM of HN or [Ser^1]HN. This value was taken from competition curves where 20 μM unlabelled HN and [Ser^1]HN maximally inhibited [^{125}I][Ser^1]HN binding. Brain regions were dissected on ice and membranes were prepared as above. Results were calculated using the computer programs Ligand (Munson and Rodbard, 1980) and CDATA (EMF Softwares, Knoxville, TN). K_i values were calculated according to the method of Cheung and Prusoff, (1973) using the equation $Ki = IC_{50} / (1 + [L]/K_d)$. Data represent the mean ± s.e.m. of three experiments completed in duplicate.

3.9. Materials

[^{3}H]NA (43.7 Ci/mmol), [^{3}H]desmethylimiprimine (64.9 Ci/mmol;[^{3}H]DMI), carrier-free NaI^{125} and [^{3}H]spermidine (20.0 Ci/mmol) were purchased from New England Nuclear, Boston, MA, USA. PCP (N-(1-phenyl-cyclohexyl) piperidine) and TCP (N-[2-thienyl] cyclohexyl-3,4-piperidine) were obtained from Dr. H. Avdovich, Health and Welfare, Ottawa. Metaphit (1-(1-(3-isothiocyanato)-phenyl) cyclohexyl)-piperidine methanesulfonate), (+)pentazocine, (-)pentazocine, butaclamol, putrescine, spermidine, spermine, DET, DA10 and 3(+)PPP (3-(3-hydroxyphenyl)N-(1-propyl)piperidine) were obtained from Research Biochemicals Inc., Natick, MA., USA. DTG was generously donated by Dr. E. Weber, Portland, OR., USA. (+)-SKF-10047 ((+)-N-allynormetazocine) and (-)SKF-10047 were obtained from Natl. Inst. of Drug Abuse, Baltimore, MD., USA. DAGO ([D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin), DSLET ([D-Ser^2, Leu^5] enkephalinyl-Thr), bestatin and thiorphan were purchased from Penninsula Laboratories, CA, USA. Desipramine, nisoxetine, imipramine, dextromethorphan, haloperidol, nialamide, dextromethorphan, trypsin, soybean trypsin inhibitor, cadaverine, arcaine, NMDA, polyethylenimine, bacitracin, captopril and spiperone were purchased from Sigma Chemical Co., St. Louis, MO., USA. BMY-14802 (α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine butanol) was obtained from Bristol Myers Co. Wallingford, CT., USA. Rimcazole (BW 234U : cis-9[3-(3,5-dimethyl-1-piperazinyl) propyl]carbazole dihydrochloride) was supplied by R.M. Ferris, Burroughs Wellcome, Research Triangle Park, NC., USA. U-69593 ((5α,

7α, 8β,)-(+)-N-methyl-N-(7-(1 pyrrolidinyl)-1-oxaspiro (4,5) dec 8yl) benzeneacetamide) was obtained from the Upjohn Co., Kalamazoo, MI., USA. MK-801, ((+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate), and AP-5 (D (-)-2-amino -7-phosphonoheptanoic acid) were purchased from Tocris Neuramin, Buckhurst Hill, Essex, U.K. BTCP (N-[1-(2-benzo(b)thiophenyl)cyclohexyl] piperidine) was a generous gift from Dr. J.-M. Kamenka, Montpelier, France. Analogs and fragments of HN were synthesized in our laboratory as previously described (Lemaire et al., 1993).

4.0. RESULTS

4.1. [^{3}H]NA Uptake Studies

4.1.1. Dependance of [^{3}H]NA uptake on Time and Temperature

Incubation of rat brain synaptosomes or isolated bovine adrenal chromaffin cells in the presence of [^{3}H]NA (0.1 μM) for differing time periods at different temperatures (30° C or 37° C) induced a time and temperature dependent uptake of [^{3}H]NA in both preparations (Fig. 8). At 37° C, the synaptosomal uptake rapidly reached a maximum value (15 min) and decreased thereafter, while the chromaffin cell uptake was directly proportional to the time of incubation, up to 45 min. At 30° C, the linearity of the initial uptake lasted longer in both preparations reaching 15 min in synaptosomes and exceeding 60 min in chromaffin cells. The time of incubation was set the same (10 min) for the subsequent assays with both preparations. The linearity of the kinetics of uptake was preserved by setting the temperatures at 37° C and 30° C for chromaffin cells and synaptosomes, respectively. Under these conditions, the initial rates of [^{3}H]NA uptake were 0.65 pmol/min/mg synaptosomal protein and 0.08 pmol/min/10^6 chromaffin cells.

4.1.2. The kinetic constants of [^{3}H]NA uptake

Cultured adrenal chromaffin cells and isolated synaptosomes were incubated for 10 min in presence of increasing concentrations of [^{3}H]NA at 37° C and 30° C, respectively. Both preparations displayed high and low affinity uptake processes, (Figs. 9 & 10). The high affinity displayed the following

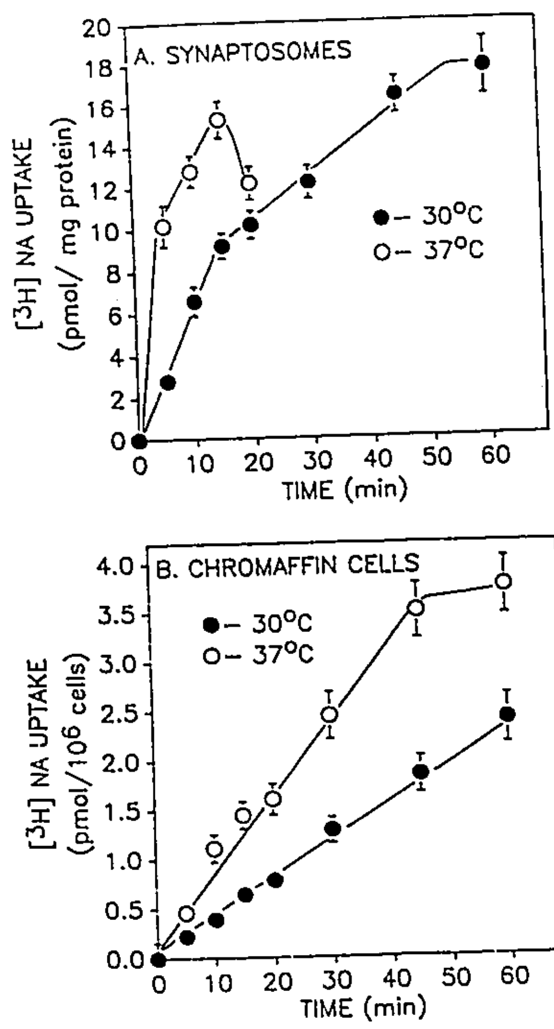


FIGURE 7 Time and temperature dependence of apparent [^{3}H]NA uptake into rat brain synaptosomes and cultured bovine adrenal chromaffin cells. Synaptosomes and chromaffin cells were incubated at 30°C or 37°C as indicated in the presence of [^{3}H]NA (0.1 μM). Uptake was terminated at various times as described in "Materials and Methods". Each point represents mean uptake ± s.e.m. of three sets of duplicates.

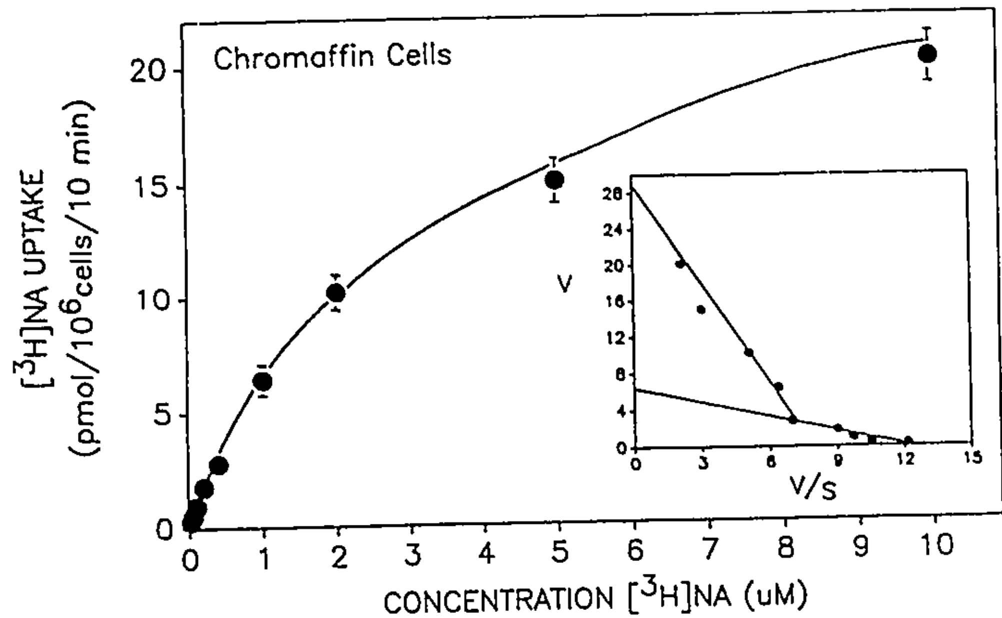


FIGURE 8 Uptake of [^{3}H]NA in adrenal chromaffin cells measured over a concentration range of 0.3 to 10 μM [^{3}H]NA uptake. Cells were isolated as described in Materials and Methods. The isolated cells were incubated at 37° C for 10 min in the presence of increasing concentrations of [^{3}H]NA. Evaluation of the kinetic constants for uptake was evaluated by linearization of the data in the Eadie-Hofstee plot (inset). Each point represents the mean uptake ± s.e.m. of three experiments performed in duplicate.

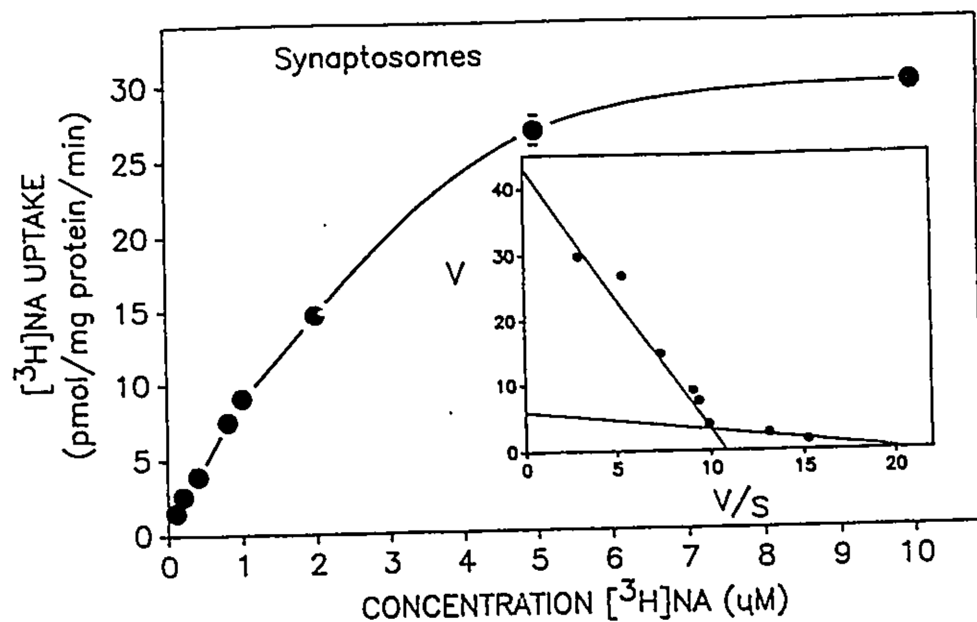


FIGURE 9 Uptake of [^{3}H]NA in rat brain synaptosomes measured over a concentration range from 0.3 to 10 µM [^{3}H]NA. Rat brain synaptosomes were prepared as described in Materials and Methods. The synaptosomes were incubated for 10 min at 30° C in the presence of increasing concentrations of [^{3}H]NA. Evaluation of kinetic constants for uptake was completed in the Eadie-Hofstee plot (inset). Each point represents the mean uptake ± s.e.m. of three experiments performed in duplicate.

characteristics: K_m of 0.56 and 0.22 μM for chromaffin cells and synaptosomes, respectively, and V_{max} of 0.7 pmol/min/10^6 chromaffin cells and 2.5 pmol/min/mg synaptosomal protein. The low affinity uptake was not characterized. All experiments were performed at a concentration (0.1 μM) of [^{3}H]NA where uptake occurred mainly through the Na^+-dependent high affinity site (0.8 pmol/mg protein as compared with 0.1 pmol/mg protein measured in the absence of Na^+).

4.1.3. Inhibition of [^{3}H]NA uptake by PCP and sigma receptor ligands

The uptake of [^{3}H]NA was examined in the presence of increasing concentrations of PCP and σ receptor ligands (Fig. 10, Table 3). PCP and haloperidol caused a dose-dependent inhibition of [^{3}H]NA uptake with total inhibition occurring at a concentration of 10-100 μM. The concentration of PCP or haloperidol producing 50% inhibition was quite similar in both systems, PCP (0.17 - 0.24 μM) and haloperidol (0.42 - 0.47 μM). In order to better characterize the receptor involved in the inhibition of catecholamine uptake, the inhibitory potencies of various specific ligands for PCP and σ receptors were compared in both uptake systems (Table 3). Among the various PCP receptor ligands tested, PCP and TCP were the most potent (IC_{50} of 0.17-0.42 μM) while MK-801, a specific ligand for the PCP_1 receptor, was less active (IC_{50}: 1.92 and 4.90 μM in chromaffin cells and synaptosomes, respectively). The σ receptor ligands haloperidol, 3(+)PPP, dextromethorphan and rimcazole displayed intermediate potency between PCP and MK-801 with IC_{50} ranging between 0.42 and 4.03 μM.

However, other selective σ receptor ligands such as (+)pentazocine, (+)SKF - 10047, BMY - 14802 and DTG were less potent (IC_{50} range of 7.24 to 29.7 μM). The σ receptor ligand (+)SKF-10047, displayed stereoselectivity for the inhibition of [^{3}H]NA uptake, the (+)-isomer being approximately 3 times more potent than the (-)-isomer in both systems. However, the stereoselective preference for butaclamol was less evident (Table 3). Several concentrations of the endogenous peptide HN and its analog [Ser^1]HN (1 nM - 20 μM) did not inhibit more than 8% of the measured [^{3}H]NA uptake. Likewise, the D_2 receptor ligand, spiperone, selective opioid agonists, DAGO (μ), DSLET (δ) and U-69593 (κ), and the NMDA receptor antagonist AP-5 were ineffective at reaching an IC_{50} value, inhibiting no more than 20% of the [^{3}H]NA uptake at 20 μM.

4.1.4. Effect of HN and [Ser^1]HN on [^{3}H]NA uptake in rat brain synaptosomes

HN and its closely related analog, [Ser^1]HN at concentrations from 1 nM to 10 μM maximally inhibited only 6 ± 4 and 8 ± 3 percent of specific [^{3}H]NA uptake in rat brain synaptosomes, respectively. HN was also tested for its ability to modulate the inhibitory effects of sigma and PCP ligands on [^{3}H]NA uptake. However, the addition of 10 μM HN, to experiments measuring the inhibitory potency of either PCP or DXM revealed that the IC_{50} values for these uptake inhibitors were not significantly altered (data not shown).

4.1.5. Correlation between brain and adrenal medulla

The uptake of [^{3}H]NA measured in both rat brain synaptosomes and adrenal chromaffin cells was inhibited by similar concentrations of PCP and sigma ligands (Fig 11). Linear regression of this plot revealed a Spearman Rank correlation coefficient of $r= 0.96.$, $p< 0.002$.

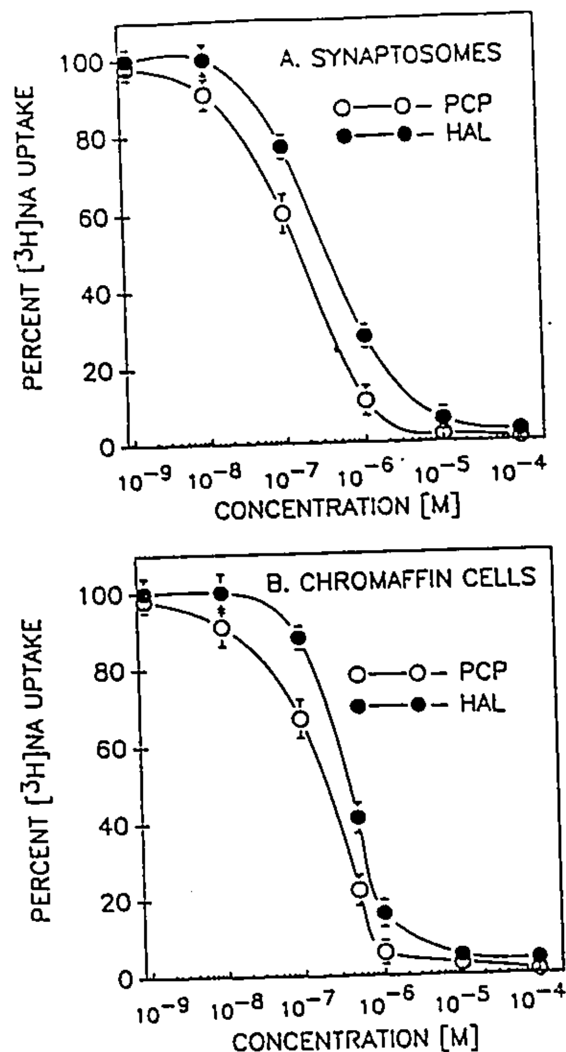


FIGURE 10 Effects of PCP and haloperidol on the uptake of [^{3}H]NA into rat brain synaptosomes and cultured bovine adrenal chromaffin cells. Synaptosomes and chromaffin cells were incubated at 30°C and 37°C respectively, for 10 min in presence of [^{3}H]NA (0.1 μM) and increasing concentrations of PCP or haloperidol. Uptake was terminated as described in "Materials and Methods". Each point represents mean uptake ± s.e. of three sets of duplicates.

TABLE 3. **Inhibition of [^{3}H]NA uptake by PCP and σ receptor ligands in rat brain synaptosomes and cultured bovine adrenal chromaffin cells.**

Drug	Synaptosomes $IC_{50}(\mu M)^a$	Chromaffin Cells $IC_{50}(\mu M)^a$
Desmethylimipramine	0.03 ± 0.009	0.01 ± 0.002
PCP ligands		
PCP	0.17 ± 0.09	0.24 ± 0.07
TCP	0.34 ± 0.08	0.42 ± 0.08
Metaphit	0.75 ± 0.11	0.68 ± 0.12
MK-801	4.90 ± 1.34	1.92 ± 0.18
BTCP	0.02 ± 0.003	-
Sigma ligands		
Haloperidol	0.42 ± 0.11	0.47 ± 0.09
3(+)PPP	0.82 ± 0.07	0.50 ± 0.08
Dextromethorphan	1.73 ± 0.31	0.51 ± 0.02
Rimcazole	1.96 ± 0.78	4.03 ± 0.28
(-)Butaclamol	4.06 ± 0.89	5.11 ± 0.12
(+)Butaclamol	6.06 ± 0.97	8.25 ± 0.15
(+)SKF-10047	7.24 ± 1.45	14.0 ± 2.87
BMY-14802	9.45 ± 1.16	10.5 ± 2.45
(-)SKF-10047	20.5 ± 2.46	38.3 ± 6.04
DTG	29.7 ± 2.37	20.0 ± 4.24
Peptides		
HN	NA	
[Ser^1]HN	NA	

^{a}The ligands HN, [Ser^1]HN, spiperone (D_2), DAGO (μ) DSLET (δ) and U-69593 (κ), AP-5 (NMDA) at concentrations up to 20 μM did not inhibit more than 20% of [^{3}H]NA uptake in both systems. HN and [Ser^1]HN did not inhibit more than 8% of NA uptake.

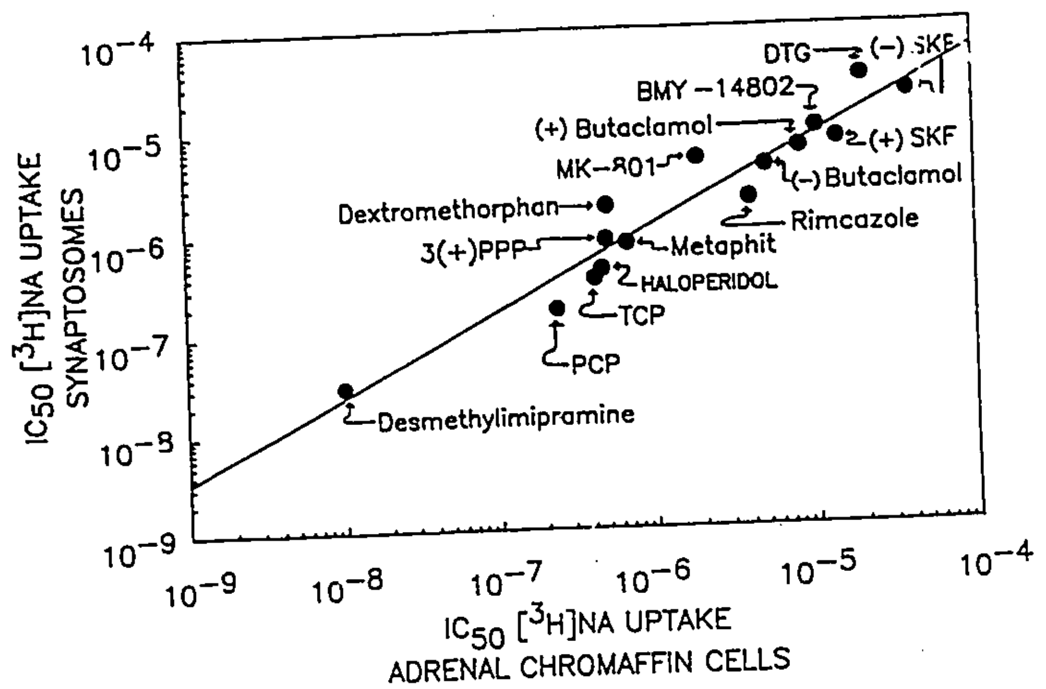


FIGURE 11 Correlation between the potencies of various PCP and σ receptor ligands in inhibiting the uptake of [^{3}H]NA in rat brain synaptosomes and cultured bovine adrenal chromaffin cells. The values were taken from Table 3. ($r = 0.96$; (+) or (-) SKF = (+) or (-) SKF-10047).

4.2. [^{3}H]Desmethylimipramine Binding Studies

4.2.1. Effects of KCl-pretreatment of bovine adrenomedullary membranes on [^{3}H]DMI binding.

Low affinity and non-specific binding of [^{3}H]DMI to rat brain membranes has been shown to be reduced by pretreatment of the membranes with 0.3 M KCl (Lee et al.,1982). Pretreatment of adrenal medullary membranes with 0.3 M KCl enhanced both the specific signal, (cpm bound, specific/total bound x 100; from 30% to 60%) and the specific binding of [^{3}H]DMI (1.0 nM) at equilibrium from 14 $\pm$ 2 to 34 $\pm$ 3 fmol/mg protein. Therefore, all binding assays were carried out on bovine adrenal membranes that had been pretreated with 0.3 M KCl as described in Methods, in order to reduce background [^{3}H]DMI binding.

4.2.2. Protein dependence of [^{3}H]DMI binding

The dependence of [^{3}H]DMI binding on the concentration of membrane protein was investigated (Fig. 12 A). [^{3}H]DMI (1.0 nM) was incubated in the presence of increasing concentrations of membrane protein. Specific binding of [^{3}H]DMI increased linearly with increasing protein concentration from 0.1 mg/ml to 0.50 mg/ml. At higher protein concentrations, the specific binding plateaued due to increases in non-specific binding. Therefore, in all subsequent experiments, [^{3}H]DMI binding was measured at 4°C, for 60 min in KCl washed membranes with 0.4 mg/ml protein.

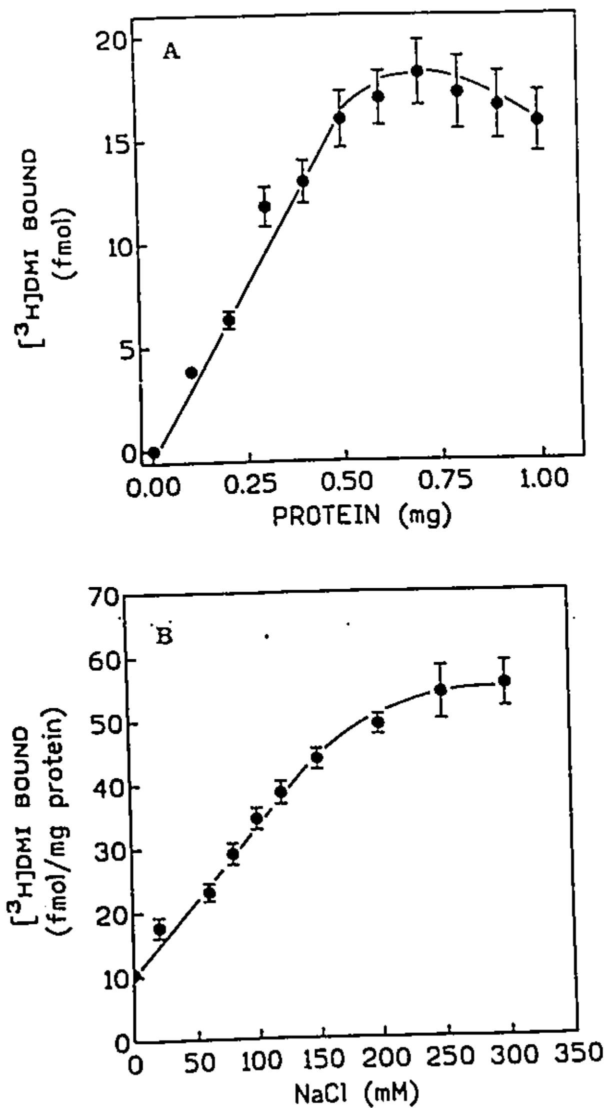


FIGURE 12 Dependence of [³H]DMI binding on protein and sodium concentration.

(A) Bovine adrenal medulla membranes pretreated with 0.3 M KCl were incubated (0.1-1.0 mg/ml) with 1.0 nM [³H]DMI for 60 min at 4°C in the absence and presence of 1 μM nisoxetine to determine non-specific binding. (B) Bovine adrenal membranes pretreated with 0.3 M KCl were washed with Na^+-free Tris-HCl (50 mM, pH 7.4,) and incubated in the presence of [³H]DMI (1 nM) at the indicated NaCl concentrations. Binding was terminated as described in Materials and Methods, and non-specific binding was subtracted from total binding to yield specific binding. Results are the mean ± s.e.m.

4.2.3. Dependence of [^{3}H]DMI binding on Na^+

Previous reports indicate that the [^{3}H]DMI binding associated with neuronal norepinephrine uptake sites is dependent upon Na^+ (Lee and Snyder, 1981; Rehavi et al., 1982). The high affinity uptake of [^{3}H]NA (uptake$_1$), measured in rat brain synaptosomes and bovine adrenal chromaffin cells, also requires Na^+ (Rogers and Lemaire, 1991). A similar sodium dependency was found for [^{3}H]DMI binding to adrenal medullary membranes (Fig. 12 B). At 100 mM NaCl, specific basal binding of [^{3}H]DMI was increased 3.5-fold, and a maximal enhancement of 5.5-fold occurred at 300 mM NaCl. This effect was specific to Na^+ since LiCl (150 mM) did not enhance basal binding activity.

4.2.4. Time and temperature dependence of [^{3}H]DMI binding

The time course of high affinity specific [^{3}H]DMI binding at three different temperatures is shown in Fig. 13. At 4°C, binding increases gradually to reach half-maximal binding at 6 min, and attains equilibrium by 30 min, after which binding remains stable for over 70 min. At 25°C, the binding occurred more rapidly, reaching half-maximal levels at 4 min and maximal levels at 15 min, although a decline in specific [^{3}H]DMI binding is observed in incubations longer than 15 min. At 37°C, binding plateaued at 10 min and represented only 58% of maximal binding observed at 4°C. Based on the data described above, all subsequent binding experiments were carried out at 4° C for 1 hr in the presence of 0.4 mg of adrenomedullary membrane protein.

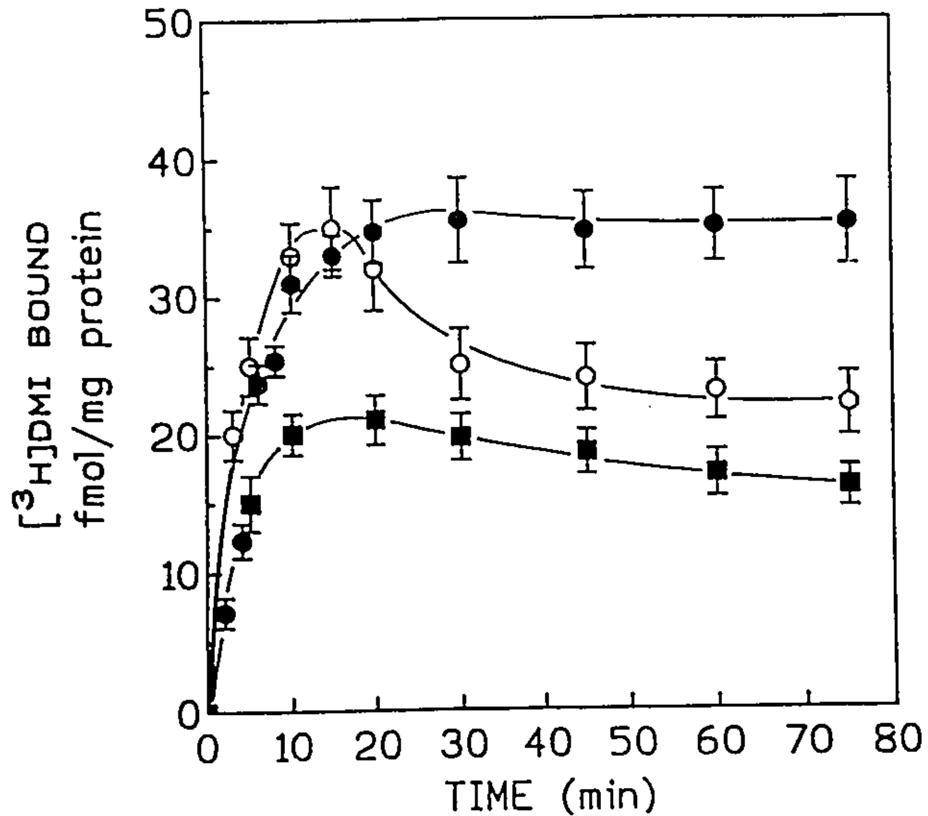


FIGURE 13 Time and temperature dependence of [^{3}H]DMI binding

Bovine adrenomedullary membranes (0.4 mg/ml) pretreated with 0.3 M KCl were incubated with [^{3}H]DMI (1.0 nM) at 4°C (●), 25°C (o), and 37°C(▪). The incubation was terminated at various time intervals by rapid vacuum filtration, as described in Materials and Methods. Specific [^{3}H]DMI binding was determined as the difference between the [^{3}H]DMI bound in the absence and presence of 1 μM nisoxetine. Results are the means of three experiments carried out in duplicate ± s.e.m.

4.2.5. Saturation Studies

Saturable binding of [^{3}H]DMI to adrenal medullary membranes is shown in Fig. 14. Specific binding was dependent upon the concentration of [^{3}H]DMI until the concentration of 10 nM was reached and binding began to plateau. Transformation of the curve in the form of a Scatchard plot (Fig. 14, inset) generated a linear curve, implying that [^{3}H]DMI bound to a homogenous population of sites. Analysis of the data indicated an equilibrium dissociation constant (k_d) of 2.87 ± .36 nM and a maximal number of binding sites (B_{max}) of 216 ± 18 fmol/mg protein.

4.2.6. Effect of haloperidol, 3(+)PPP and MK-801 on [^{3}H]DMI binding.

The binding of [^{3}H]DMI to membrane preparations of bovine adrenal medulla was assessed in the presence of 0.5 µM haloperidol, (Fig. 14). The presence of haloperidol did not affect the affinity of [^{3}H]DMI for its specific binding site but reduced its maximal binding to the membrane preparation. Scatchard analysis (Fig 14, inset) revealed a k_d = 3.98 ± 0.47 nM, and a B_{max}= 101 ± 13 fmol/mg protein in presence of haloperidol. Similar results were seen in the presence of the sigma ligand 3(+)PPP (10 µM; k_d = 3.18 ± 0.71 and a B_{max} = 111 ± 15 fmol/mg protein). The specific PCP_1 receptor ligand MK-801 (10 µM) also reduced the B_{max} to a value of 117 ± 18 fmol/mg protein while the k_D was unchanged at 2.91 ± 0.62 nM.

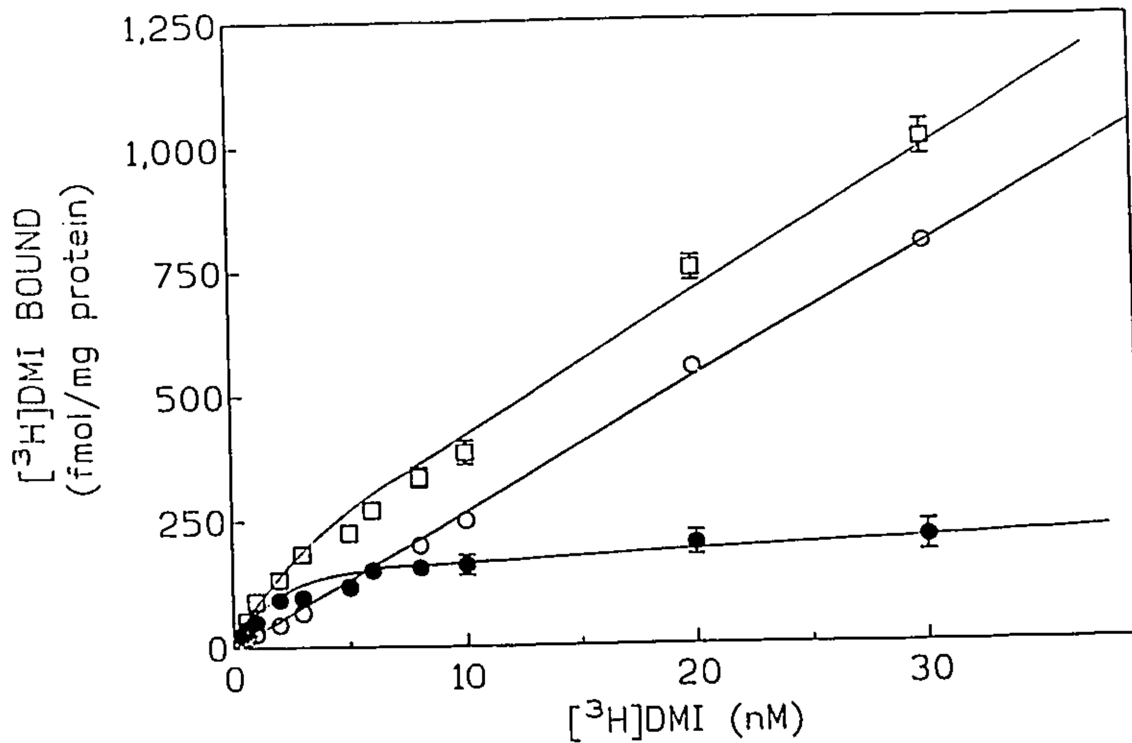


FIGURE 14 Representative plot of total (□), non-specific (o) and specific (●) binding of [^{3}H]DMI to membrane preparations of bovine adrenal medulla. Non-specific binding was defined in the presence of 1 μM nisoxetine. Specific binding was obtained by subtracting non-specific binding from total binding. The apparent K_d and B_{max} values were 3.1 nM and 210 fmol/mg protein.

4.2.7. Effects of uptake inhibitors, PCP and sigma ligands on [^{3}H]DMI binding.

Table 4 shows the relative potency of various monoamine uptake blockers, PCP ligands, and σ receptor ligands in inhibiting the binding of [^{3}H]DMI to membrane preparations of bovine adrenal medulla. The binding of [^{3}H]DMI was potently inhibited by NA uptake blockers, desipramine (IC_{50}: 20 nM), and nisoxetine (IC_{50}: 40 nM) and moderately sensitive to other inhibitors of monoamine uptake imipramine (IC_{50}: 100 nM) and cocaine (IC_{50}: 20 μM). [^{3}H]DMI binding was also inhibited by the presence of σ and PCP receptor ligands. Haloperidol was the most potent σ ligand followed by rimcazole > (-)butaclamol > dextromethorphan > MK-801 > 3(+)PPP > PCP > TCP > (+)SKF-10047 > (-)SKF-10047. In contrast to the effects of the other compounds, BTCP, an analog of PCP acting specifically at the PCP_2 site in brain, did not completely inhibit [^{3}H]DMI binding, decreasing the binding activity maximally by only 44% at a concentration of 10 μM. The drugs, DTG, (+)pentazocine, (-)pentazocine, metaphit and (+)butaclamol did not inhibit 50% of [^{3}H]DMI binding. DTG inhibited only 17% of [^{3}H]DMI binding at a concentration of 100 μM, whereas 10 μM (+)butaclamol inhibited 38% of [^{3}H]DMI binding. Both isomers of pentazocine were ineffective, inhibiting only 15% of [^{3}H]DMI binding at 100 μM. Histogranin and [Ser^1]HN were ineffective inhibitors of [^{3}H]DMI binding. When tested at concentrations as high as 10 μM, neither HN nor [Ser^1]HN inhibited more than 5% of [^{3}H]DMI binding.

TABLE 4. **Inhibition of [^{3}H]DMI Binding to membrane preparations of bovine adrenal medulla by monoamine uptake inhibitors and various σ and PCP receptor ligands.**

Drug	IC_{50} (µM)[1] ± s.e.m.	Relative Potency[2]
Uptake Inhibitors		
Desipramine	0.020 ± .0003	100
Nisoxetine	0.040 ± .0004	50
Imipramine	0.100 ± .017	20
cocaine	20.0 ± 1.66	0.1
Sigma Ligands		
Haloperidol	0.82 ± .09	100
Rimcazole	1.20 ± .067	68
(-)butaclamol	6.41 ± 0.83	12.8
Dextromethorphan	12.0 ± 1.11	6.8
3(+)PPP	19.9 ± 2.04	4.1
(+)SKF-10047	64.0 ± 4.95	1.3
(-)SKF-10047	92.0 ± 5.25	0.9
DTG	NA	
(+)pentazocine	NA	
(-)pentazocine	NA	
PCP Ligands		
MK-801	15.0 ± 1.3	5.5
PCP	41.9 ± 2.67	1.9
TCP	50.9 ± 1.63	1.6
BTCP	NA	
metaphit	NA	
Peptides		
HN	NA	
[Ser[1]]HN	NA	

[1] Experiments were carried out as described in Materials and Methods. Results are the mean ± s.e.m. of three experiments carried out in duplicate. Percent inhibition produced by ligands which are labelled " inactive " are detailed in the text. [2]Relative to desipramine for monoamine uptake inhibitors and relative to haloperidol for σ and PCP receptor ligands. HN: histogranin; [Ser[1]]HN: [Ser[1]]histogranin.

4.3. [^{125}I][Ser^1]Histogranin Binding Studies

One of the aims of this study was to verify if the PCP-like effects of the adrenomedullary peptide histogranin (Lemaire et al., 1993) were mediated by the modulation of NA uptake systems. Based on the previous results, the PCP-like behavioural effects of histogranin were obviously not related to the noradrenaline uptake system. In order to determine what site was involved in the PCP-like effects of histogranin, characterization of the binding of [^{125}I][Ser^1]HN to rat brain membranes was carried out.

4.3.1. Dependence of [^{125}I][Ser^1]HN binding on protein

Binding of [^{125}I][Ser^1]HN increased linearly with respect to protein concentration from 0.1 mg to 1 mg per 0.5 ml (Fig 15A). Based on these results, a concentration of 0.6 mg per ml was chosen for subsequent experiments. The protein nature of the binding site was confirmed by experiments measuring binding of [^{125}I][Ser^1]HN to rat brain membrane preparations pretreated with trypsin (Fig 15B). Approximately, 95% of the specific binding of [^{125}I][Ser^1]HN was abolished by trypsin pretreatment (100 μg). Forty nine percent of the binding was recovered by blockade of the enzyme activity by co-treatment of the membrane preparation with soybean trypsin inhibitor (10 μg).

4.3.2. Time and temperature dependencies of [^{125}I][Ser^1]HN binding

The binding of [^{125}I][Ser^1]HN was found to depend upon the time and temperature of incubation (Fig. 16). At 4°C, maximum binding was reached within 30 min and remained stable up to 90 min. Increasing the temperature from 4° C

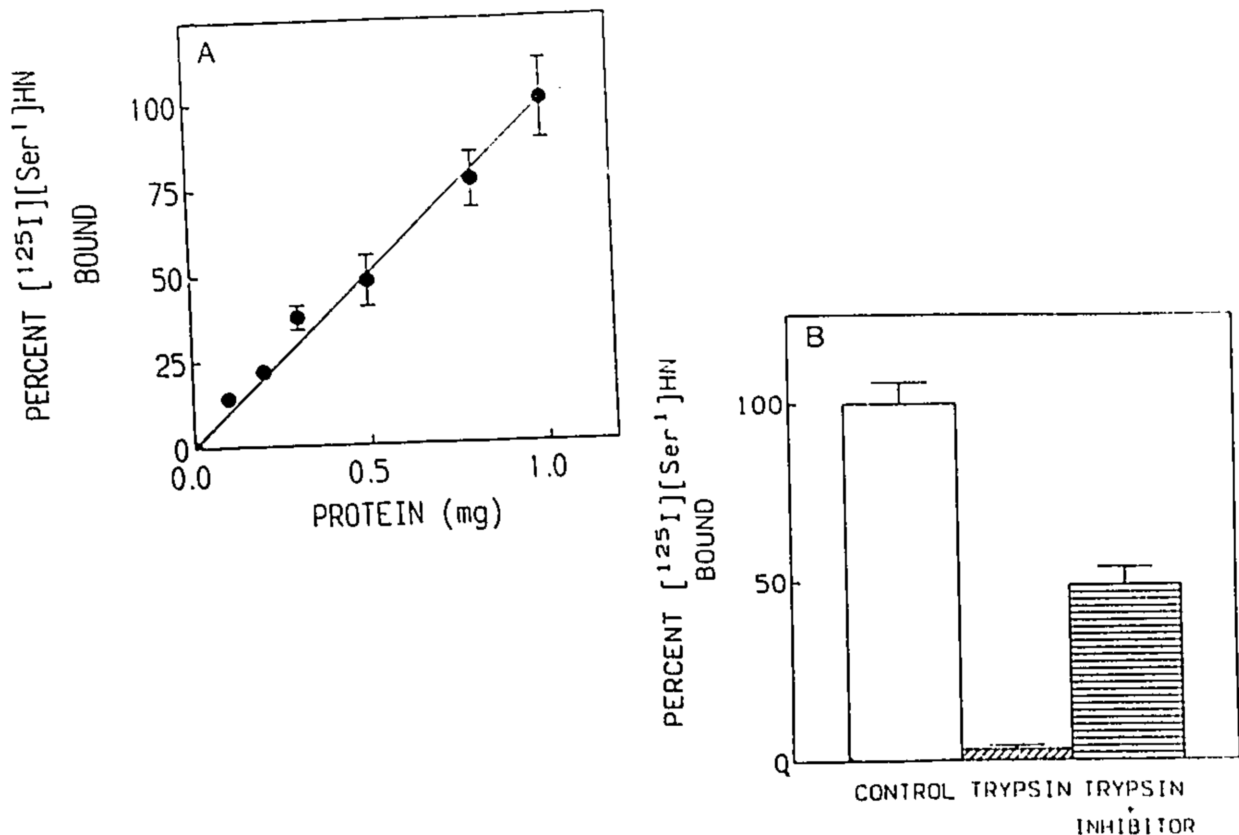


FIGURE 15 Effects of increasing protein concentration and trypsin pretreatment on [^{125}I][Ser^1]HN binding to rat brain membranes.

(A) Binding of [^{125}I][Ser^1]HN (1 nM) to rat brain membranes (0.1-1.0 mg) was conducted for 45 min as described in Materials and Methods. Specific binding was determined by subtraction of the non-specific binding obtained in the presence of 20 µM unlabelled [Ser^1]HN. Data represent the mean of three experiments performed in duplicate ± s.e.m. (B) Binding was carried out with untreated membranes (control) or membranes pretreated with trypsin (100 µg;diagonal bars) for 30 min at room temperature and subsequently washed by centrifugation prior to the binding assay. Membranes were also treated with a mixture of trypsin (100 µg) and trypsin inhibitor (10 µg; horizontal bar). Data represents the mean ± s.e.m. of three experiments carried out in duplicate.

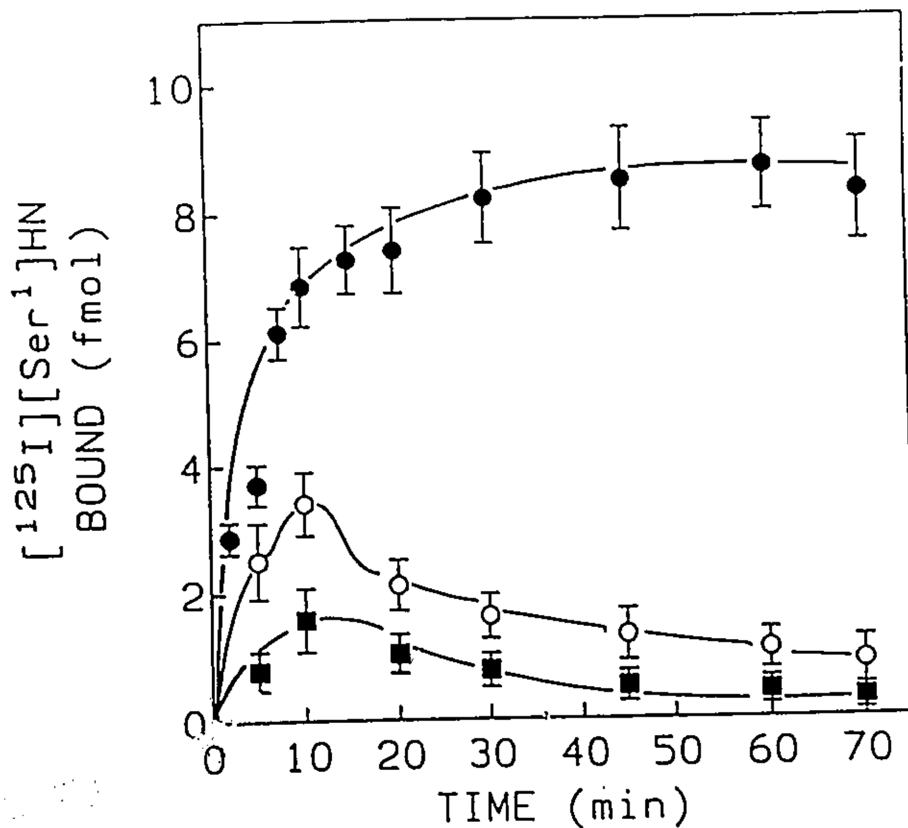


FIGURE 16 Time and temperature dependence of [^{125}I][Ser^{1}]HN binding. Rat brain membranes (0.6 mg) were incubated with [^{125}I][Ser^{1}]HN (1.0 nM) at 4°C (●), 22°C (o), and 37°C (■), for 45 min and the incubation was terminated at various time intervals as described in Materials and Methods. Specific binding of [^{125}I][Ser^{1}]HN was determined as the difference between [^{125}I][Ser^{1}]HN bound in the absence and presence of 20 μM [Ser^{1}]HN. Data represent the mean ± s.e.m. of three independent experiments carried out in duplicate.

to 25° C decreased specific binding of [^{125}I][Ser^1]HN. Binding of [^{125}I][Ser^1]HN measured at 37° C was further reduced, and at 45 min represented only 6.2% of the maximal bound [^{125}I][Ser^1]HN at 4° C. All subsequent binding studies were conducted at 4° C and an incubation time of 45 min.

4.3.3. Saturation binding of [^{125}I][Ser^1]HN

[^{125}I][Ser^1]HN binding displayed dependency upon the concentration of the radiolabelled ligand (Fig 17). Transformation of the binding data in the form of a Scatchard plot (Fig 17 inset) revealed a K_d of 25.0 ± 2.0 nM and a B_{max} of 410 ± 18 fmol/mg protein, implying that [^{125}I][Ser^1]HN bound to a single class of high affinity binding sites.

4.3.4. Association and dissociation of [^{125}I][Ser^1]HN binding

[^{125}I][Ser^1]HN rapidly associated to its receptor in rat brain membranes reaching half-maximal binding levels at 10 min and equilibrium by 30 min (Fig 18). Analysis of the association kinetics revealed a K_{obs} = 0.200 min^{-1} and a k_{+1} = 5.63 X 10^6 M^{-1}min^{-1}. The binding of [^{125}I][Ser^1]HN was reversible upon the addition of 20 μM unlabelled HN. The analysis of the dissociation kinetics revealed a $t_{1/2}$= 6 min and a K_{-1}= 0.1155. Calculation of the apparent affinity from these values revealed a K_d (20.5 ± 4.7 nM) which is close to that obtained in equilibrium saturation analysis (Fig 17; K_d= 25 nM).

4.3.5. Structure-activity relationships of [^{125}I][Ser^1]HN binding.

HN and HN fragments were compared for their ability to inhibit the bound radiolabelled peptide (Table 5). The binding of [^{125}I][Ser^1]HN (1 nM) was inhibited

by HN analogs possessing close homology to the full length peptide. Reduction of only one amino acid from either the N- or C-terminal of the molecule resulted in 20 and 17 fold decreases in potency in binding assays, respectively.

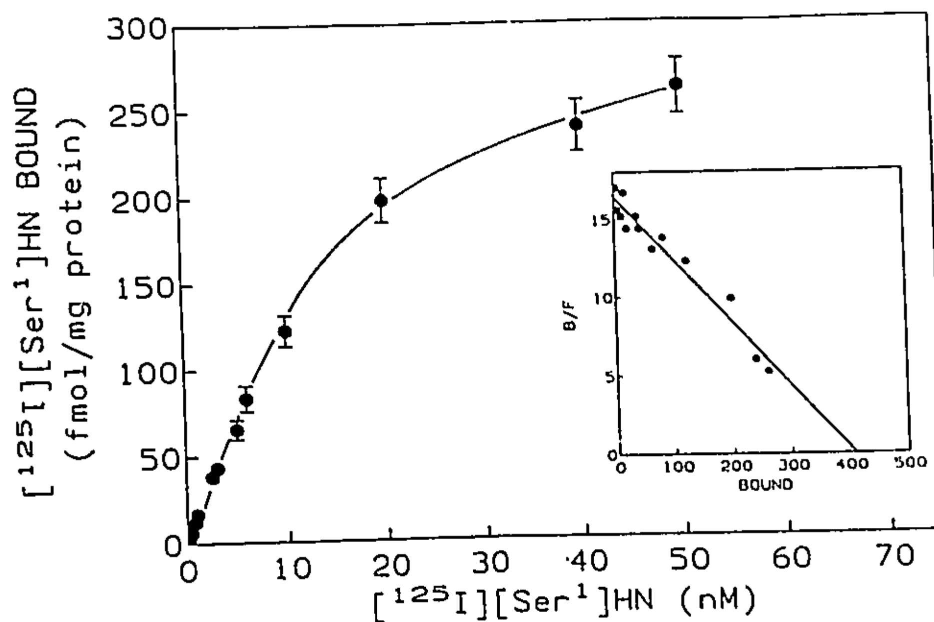


FIGURE 17 Saturation analysis of [^{125}I][Ser^{1}]HN binding to rat brain membranes. Specific binding was assessed with increasing concentrations of [^{125}I][Ser^{1}]HN (0.5 - 50 nM), in presence or absence of unlabelled HN (20 μM). The data represent the mean ± s.e.m. of three independent experiments carried out in duplicate. Inset: Scatchard plot analysis of the data. K_d and B_{max} were calculated by the computer program 'Ligand' (Munson and Rodbard, 1980).

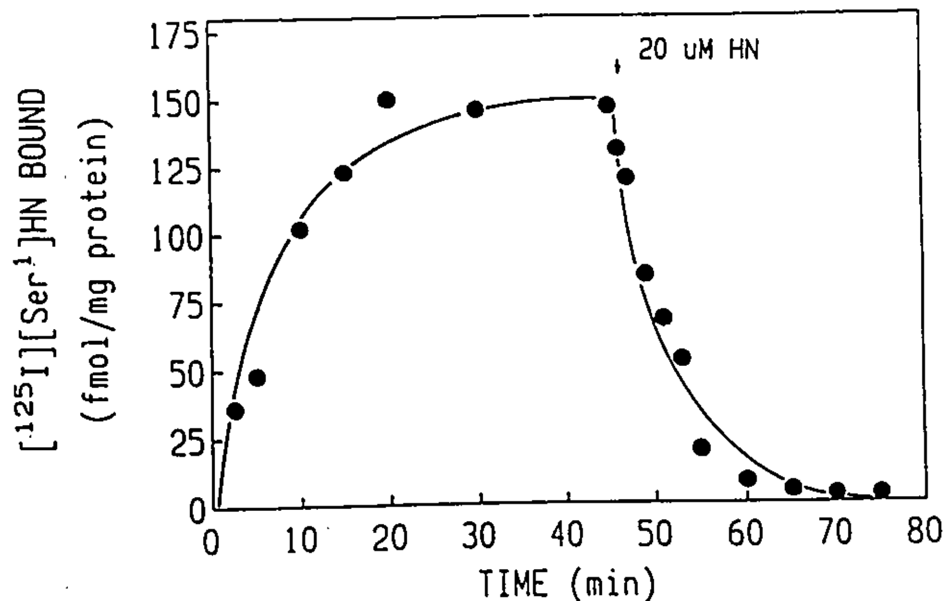


FIGURE 18 Time course of association and dissociation of specific [^{125}I][Ser^1]HN binding. Binding of [^{125}I][Ser^1]HN (1 nM) was measured at various time intervals. After 45 min incubation, an excess of HN (20 μM) was added and binding was assessed at different time intervals up to 75 min. The kinetic values calculated in this typical experiment revealed a k_{+1}= 2.18 X 10^7 mol min^{-1}, and k_{-1}= 0.87. The calculated affinity (K_d) in this experiment was 25.1 nM. Binding was terminated at the indicated intervals as described in Materials and Methods.

All N-terminal and C-terminal fragments of HN showed some potency in competing for [^{125}I][Ser^1]HN binding sites. The C-terminal fragment HN-(4-15) was 5 fold less potent than HN-(3-15) and the N-terminal fragment [Ser^1]HN-(1-10) was 4 fold less potent than fragment [Ser^1]HN-(1-12). These data suggest that the full 15 amino acid structure is required for high affinity interaction with the HN receptor. The N-terminal of the molecule, particularly the 3rd amino acid, and the C-terminal of the molecule, amino acids 11 and 12, are also important for binding activity. The central peptide fragment HN-(6-10) possessed very little potency (0.04%) as compared to HN but displayed complete inhibition of [^{125}I][Ser^1]HN binding at higher doses (10^{-4} M). Ligands specific for PCP, sigma and NMDA receptors, were unable to inhibit [^{125}I][Ser^1]HN binding (Table 5). Peptides such as substance P, ß-endorphin, neuropeptide Y, [Leu^5]enkephalin, [Met^5]enkephalin, dynorphin A(1-13) and neuromedin C at a concentration of 10 μM did not inhibit more than 10% of [^{125}I][Ser^1]HN binding.

4.3.6. Interaction with polyamine site-specific ligands

Various compounds selective for the spermidine binding domain on the NMDA receptor complex were potent inhibitors of [^{125}I][Ser^1]HN binding (Table 6). These ligands inhibited [^{125}I][Ser^1]HN binding with IC_{50} values in the same range as that reported for the inhibition of [^{3}H]spermidine binding, with the exception of diethylenetriamine (DET). DET inhibited [^{125}I][Ser^1]HN binding with an IC_{50} of 175 ± 20 nM, which is within the same range as that obtained with HN (75 nM). Saturation binding studies in presence of DET (0.1 μM) caused a minor change

TABLE 5. **Relative potencies of HN and various fragments in displacing the binding of [^{125}I][Ser^1]HN from rat brain membranes.**

Peptide	K_i (nM)[a]	Relative potency (%)[b]
HN	72 ± 6.5	100
[Ser^1]HN	28 ± 8.3	257
HN-(2-15)	1,385 ± 395	5
HN-(3-15)	1,495 ± 301	5
HN-(4-15)	6,641 ± 594	1
HN-(5-15)	6,749 ± 487	1
HN-(6-15)	6,864 ± 533	1
HN-(7-15)	96,300 ± 6,480	0.07
HN-(8-15)	97,251 ± 4,312	0.07
[Ser^1]HN-(1-14)	1,162 ± 289	6
[Ser^1]HN-(1-13)	1,278 ± 272	6
[Ser^1]HN-(1-12)	1,821 ± 315	4
[Ser^1]HN-(1-10)	5,977 ± 486	1
HN-(6-10)	164,400 ± 12,000	0.04

[a]Results are the means of three experiments performed in duplicate ± s.e.m. Ligands specific for, sigma receptors ((+)pentazocine, DTG, 3(+)PPP), NMDA receptors (CPP, AP5), PCP sites (MK-801, TCP), Glycine sites (Gly) and Glutamate sites (Glu) were ineffective at 100 μM. Dynorphin A-(1-13), Met-enkephalin and Leu-enkephalin were also inactive at 10 μM. [b]Relative to HN.

in the K_d (from 25 ± 2 to 33 ± 4 nM) but an important reduction in the B_{max} (from 410 ± 18 to 213 ± 23 fmol/mg protein; Fig 19). The spermidine precursor putrescine was ineffective in reducing specific [^{125}I][Ser^1]HN binding.

In order to determine in HN interacts directly with the spermidine binding domain, unlabelled HN was monitored for its ability to compete with the binding of [^{3}H]spermidine (20 nM). The synthetic peptide HN (10^{-9} -10^{-5} M) was unable to compete with [^{3}H]spermidine for its binding sites in rat brain membrane preparations (9.6 pmol/mg protein in both absence and presence of 10^{-5} M HN). Spermidine and spermine were effective in reducing [^{3}H]spermidine binding from rat brain

membranes with IC_{50} of 10.8 µM ± 0.9 and 10.2 ± 1.1 µM , respectively.

4.3.7. Effect of cations

The ability of exogenously added divalent cations to modulate the binding of [^{125}I][Ser^1]HN is illustrated in Fig 20. The addition of Mg^{2+} or Zn^{2+} ions inhibited the binding in a monophasic fashion. The IC_{50} values were 240 ± 22 µM and 18 ± 3 µM for Mg^{2+} and Zn^{2+}, respectively.

4.3.8. Regional distribution of [^{125}I][Ser^1]HN binding sites

In order to determine if the HN receptor had the same distribution as the NMDA receptor in rat brain, we compared the binding activities of [^{3}H]MK-801, a specific marker of the PCP binding domain on the NMDA receptor complex (Wong et al., 1988) and [^{125}I][Ser^1]HN in isolated brain regions. The hippocampus and cerebral cortex contained the highest density of [^{125}I][Ser^1]HN binding sites, when

compared to cerebellum and striatum (Table 7). This profile was similar to that of [^{3}H]MK-801, although the cerebellum possessed a greater percentage of [^{125}I][Ser^{1}]HN binding sites.

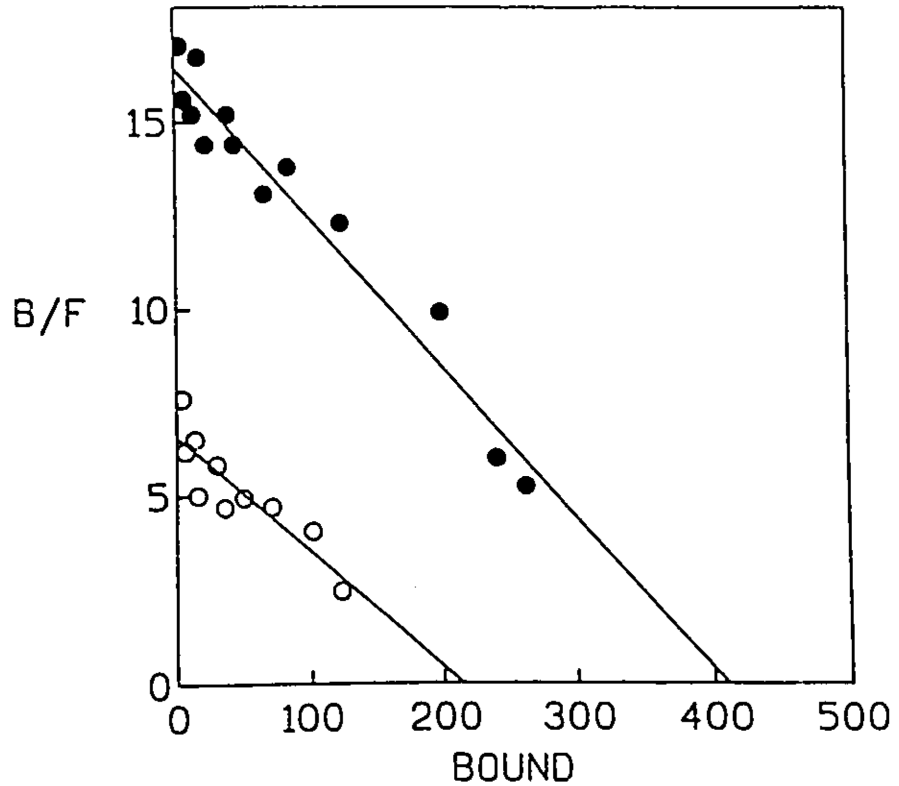


FIGURE 19 Effect of the polyamine antagonist DET on the binding of [^{125}I][Ser^{1}]HN to rat brain membranes. Saturation binding curves in the presence (o) and absence (●) of DET (0.1 μM) were analyzed by Scatchard plot (as shown). Binding parameters were calculated using the computer program 'Ligand'. The data represent the mean of three independent experiments carried out in duplicate.

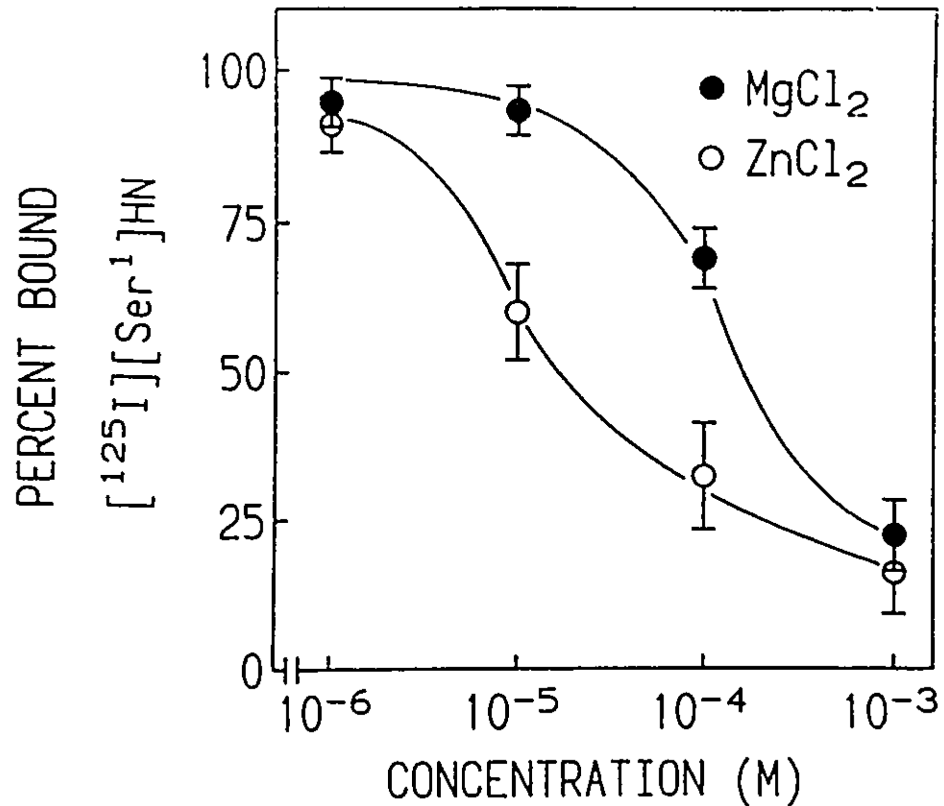


FIGURE 20 Effect of divalent cations Mg^{2+} and Zn^{2+} on $[^{125}I][Ser^1]$HN binding to rat brain membranes. Rat brain membranes (0.6 mg) were incubated with 1.0 nM $[^{125}I][Ser^1]$HN for 45 min at 4°C in presence of increasing concentrations of $MgCl_2$ or $ZnCl_2$. Reactions were terminated as described in Materials and Methods. Non-specific binding was determined in the presence of 20 μM unlabelled $[Ser^1]$HN. Points are means ± s.e.m. of three experiments carried out in duplicate.

TABLE 6. Relative potencies of polyamines in inhibiting [^{125}I][Ser^1]HN binding to rat brain membranes.

Compound	IC_{50} (nM)[a]	Relative potency (%)[b]
HN	75 ± 7	100
DET	176 ± 18	41
spermine	20,000 ± 1400	0.4
spermidine	20,000 ± 900	0.4
DA10	81,570 ± 7380	0.09
arcaine	93,120 ± 9840	0.08
cadaverine	>100,000	<0.07
putrescine	>100,000	<0.07

[a]Results are the means of three experiments performed in duplicate ± s.e.m.

[b]Relative to HN.

TABLE 7. **Binding of [^{125}I][Ser^1]HN (0.5 nM) and [^{3}H]MK-801 (5 nM) to membrane preparations of whole rat brain and various brain areas.**

Membrane	[^{125}I][Ser^1]histogranin		[^{3}H]MK-801	
Preparation	fmol/mg protein[a]	(%)[b]	fmol/mg protein[a]	(%)[b]
Whole Brain	15.8 ± 2.3	71	283 ± 27	39
Cortex	20.5 ± 4.1	93	635 ± 4	88
Striatum	10.9 ± 1.1	49	340 ± 21	47
Hippocampus	22.1 ± 4.2	100	718 ± 84	100
Cerebellum	12.9 ± 2.6	58	18.4 ± 3	3

[a]Results are expressed as the mean ± s.e.m. of three experiments performed in duplicate.

[b]Relative to hippocampus

4.4. Summary of Results

Part I. [^{3}H]Noradrenaline Uptake studies: Effects of PCP and sigma ligands.

1. Both adrenal chromaffin cells and rat brain synaptosomes display similar properties in the uptake of [^{3}H]NA.
2. Sigma and PCP ligands affect [^{3}H]NA uptake with similar potency and rank order in both tissues.
3. The inhibitory profile produced by sigma and PCP ligands does not correlate with either sigma or PCP receptor binding characteristics.
4. HN does not affect [^{3}H]NA uptake in rat brain synaptosomes and adrenal chromaffin cells.

Part II. [^{3}H]desmethylimipramine binding to adrenal medulla: interaction with PCP and sigma receptor ligands.

1. [^{3}H]desmethylimipramine binds to high affinity sites in bovine adrenal medulla, that resemble the antidepressant binding sites previously characterized in brain.
2. The sigma ligands haloperidol, 3(+)PPP and the PCP ligand (MK-801) modulate the binding of [^{3}H]DMI to these sites in a non-competitive manner.
3. The rank order profile of inhibition by PCP and sigma ligands did not resemble a known receptor, but a correlation could be drawn for the effect of sigma ligands on [^{3}H]DMI binding and [^{3}H]NA reuptake in adrenal.

PART III Characterization of the HN receptor.

1. HN binds to a unique trypsin-sensitive, saturable, reversible, site in rat brain membranes.

2. The binding of HN is selective for the entire 15 a.a. sequence of the peptide, and a reduction of amino acid constituents from either terminal reduces its potency considerably.

3. [^{125}I][Ser^{1}]HN is selectively inhibited by structurally related analogs but not by un-related peptides such as B-endorphin, NPY, dynorphin A, nor by sigma and PCP receptor ligands.

4. The binding of [^{125}I][Ser^{1}]HN is sensitive to polyamines and cations that interact with the NMDA receptor.

5. The polyamine DET interacts non-competitively with the HN binding site.

6. [^{125}I][Ser^{1}]HN binding sites are distributed in brain regions that are known to contain NMDA receptors.

5.0. DISCUSSION

General Overview

PCP and σ-like receptors were first identified and characterized in bovine adrenal medulla using [^{3}H](+)SKF-10047, a ligand that is known to bind to both PCP and sigma receptors (Rogers, MSc Thesis, 1989; Rogers et al., 1989). Later, it was found that that sigma receptor ligand [^{3}H]3(+)PPP and the PCP receptor ligand [^{3}H]TCP bound to distinct high affinity sites in this tissue (Rogers and Lemaire, 1990). Adrenomedullary [^{3}H]3(+)PPP binding was inhibited by several sigma ligands and displayed a stereoselective preference for the dextro-isomer of N-allylmormetazocine (SKF-10047). These results provided support for the idea that in adrenal medulla [^{3}H]3(+)PPP labelled a sigma$_1$ receptor subtype (Rogers, 1989). Surprisingly however, the site labelled by [^{3}H]TCP did not resemble a typical PCP receptor. The binding of [^{3}H]TCP was inhibited more potently by sigma ligands such as DTG and haloperidol than PCP receptor ligands and even TCP itself. The PCP$_1$ receptor ligand MK-801 which demonstrates nanomolar affinity for PCP sites associated with the NMDA receptor complex in CNS was also ineffective in competing for adrenal medulla [^{3}H]TCP sites. From these studies, it was concluded that [^{3}H]TCP (5 nM) labelled "sigma-like" sites in this tissue (Rogers, MSc Thesis, 1989).

Due to lack of information available on peripheral PCP and σ sites, it seemed important to verify whether a function could be found for these sites in the adrenal medulla. The main functions performed by adrenal medullary

chromaffin cells involves the uptake, storage and release of catecholamines (Stjarne, 1972). Thus receptors on the surface of the chromaffin cell may play a role in the modulation of one of these processes. Malave et al. (1983) first reported that PCP blocked the release of catecholamines from the perfused adrenal gland, and this report was later confirmed in cultured chromaffin cells by Purifoy and Holz (1984). However, the action of PCP was very weak, requiring high concentrations (micromolar) to block catecholamine release (Malave et al., 1983; Purifoy and Holz, 1984). The fact that PCP did not block catecholamine release in the nanomolar range suggested that high affinity adrenal medullary [^{3}H]TCP binding sites were not involved. It is presently thought that PCP inhibits adrenal medullary catecholamine release by sterically hindering the passage of cations through the nicotinic ACh receptor (Purifoy and Holz, 1984). Recently, the inhibition of catecholamine release from isolated adrenal chromaffin cells by sigma ligands was demonstrated and this mechanism is also thought to involve blockade of nicotinic ACh receptors (Paul et al., 1992).

The question concerning the function of the characterized high affinity [^{3}H]3(+)PPP and [^{3}H]TCP binding sites in bovine adrenal medulla remains largely speculative. PCP, at submicromolar concentrations, has been shown to block the re-uptake of catecholamines in rat brain (Garey and Heath, 1976; Smith et al, 1977; Snell et al., 1988). Since adrenal medullary [^{3}H]TCP binding was inhibited by sigma ligands, a role for PCP and possibly sigma ligands in the blockade of NA uptake seemed plausible. Thus the first objective of this study involved

investigation of the effects of PCP and sigma ligands on [^{3}H]NA re-uptake in bovine adrenal chromaffin cells.

5.1. Effects of PCP and Sigma ligands in [^{3}H]NA uptake assays in bovine adrenal chromaffin cells

The properties of high affinity [^{3}H]NA uptake in the adrenal medulla resembled that measured in rat brain synaptosomes. In both tissues, the uptake process was dependent on Na^+ and temperature. Uptake measured at 4°C was equal to that measured in Na^+-free buffer, and was used as a control to establish low affinity uptake. The optimal conditions for measuring the uptake of NA were found to consist of an incubation period of 10 min at 30°C for synaptosomes and 10 min at 37°C for chromaffin cells. The difference in temperature may be related to a greater stability of adrenal chromaffin cells when compared with brain synaptosomes. Both tissues displayed similar affinity for [^{3}H]NA transport (K_m of 0.22 and 0.56 µM for synaptosomes and chromaffin cells, respectively) that was saturable and potently blocked by desmethylimipramine, and cocaine (Fig 7 & Table 3).

PCP-induced blockade of NA uptake has been described previously (Smith et al., 1976; Snell et al., 1988), however this is the first report of direct evidence for sigma ligand-induced blockade of NA uptake. PCP and sigma ligands were found to inhibit NA uptake (Table 3; Fig 10) and the potencies of these drugs in both the adrenal medulla and rat brain assays correlated well ($r = 0.96$, $p < 0.001$),

suggesting that similar receptor(s) may mediate the effects of these ligands on NA uptake in both the CNS and periphery. Several other ligands selective for opiate receptor subtypes, and dopamine D_2 receptors were inactive (Table 3). Thus [^{3}H]NA uptake was specifically inhibited by antidepressants, PCP analogs and sigma ligands tested.

The fact that PCP and sigma receptor ligands blocked [^{3}H]NA uptake in both adrenal medulla and rat brain led to a series of experiments examining the receptor site involved in mediating this effect. However, the potencies of PCP and sigma ligands in [^{3}H]NA uptake assays (Table 3), was different from that measured in [^{3}H]TCP and [^{3}H]3(+)PPP receptor binding assays in both the brain and adrenal medulla (Walker et al., 1990; Rogers and Lemaire, 1990). Thus it was not possible to conclude that high affinity PCP, or sigma receptors were directly involved in the blockade of [^{3}H]NA uptake. Despite this result, the inhibitory effects of PCP and sigma ligands on [^{3}H]NA uptake suggested that these compounds may both interact with the substrate recognition site of the NA transporter. Therefore the potency of PCP and sigma ligands was monitored in competition binding assays with [^{3}H]DMI, a potent uptake blocker.

5.2 Interaction between PCP and sigma ligands and the [^{3}H]DMI binding site on the NA transporter.

[^{3}H]DMI is known to bind to the substrate recognition site of the NA transporter and has been widely used to label these sites in the CNS (Hrdina et

al., 1981; Lee et al., 1982). The binding of [^{3}H]DMI to both adrenal medulla and rat brain was saturable and of high affinity (Fig 14), enhanced by pretreatment of the membrane with KCl, inversely-dependent upon temperature (Fig 13), and sensitive to monoamine uptake inhibitors such as nisoxetine and imipramine (Table 4; Lee et al., 1982; Lee and Snyder, 1981; Rehavi et al., 1982; Raisman et al., 1982; Hrdina et al., 1981; Graefe and Bonish, 1988).

The effect of PCP analogs in [^{3}H]DMI binding assays in adrenal medulla revealed a different order of potency than that measured in [^{3}H]NA uptake assays. This difference was exemplified by the drug BTCP which was a potent blocker of [^{3}H]NA uptake in rat brain synaptosomes, but ineffective in [^{3}H]DMI binding assays in adrenal medulla (Table 3; Table 4). The fact that the potency of PCP ligands in [^{3}H]NA uptake assays differed from that in [^{3}H]DMI binding assays suggests that PCP ligands inhibit uptake by acting through a site which is distinct from the [^{3}H]DMI binding site and may be similar to the PCP_2 site described for the blockade of dopamine uptake. Stimulation of the PCP_2 site however is not thought to be responsible for the effects of sigma ligands on [^{3}H]NA uptake observed in this study due to the fact that Vignon and colleagues, (1988), described the PCP_2 receptor as 'haloperidol-insensitive'.

The effect of sigma ligands on [^{3}H]NA uptake and [^{3}H]DMI binding did correlate, although this was not a strong one (Spearman rank correlation value; $r=0.84$, $p < 0.002$). In both [^{3}H]DMI binding and [^{3}H]NA uptake assays, haloperidol, dextromethorphan, rimcazole and 3(+)PPP displayed a relatively high

degree of potency. (-)Butaclamol and (+)SKF-10047 had a medium rank or potency, whereas DTG and (+)pentazocine displayed little effect. Although several sigma ligands were active in both assays, the pharmacological potency did not match that of a $sigma_1$ or $sigma_2$ receptor. High affinity sigma receptors bind sigma ligands in the nanomolar range (Largent et al., 1987), unlike the observed micromolar concentrations which are necessary to block [^{3}H]NA uptake and inhibit [^{3}H]DMI binding (Table 3;Table 4).

5.3 Receptor sites for PCP and sigma ligands: are they relevant to the effects of these ligands on NA uptake?

The following discussion evaluates the potential involvement of receptor sites in the action of PCP and sigma ligands on NA uptake in bovine adrenal medulla. The various receptors to which these compounds are known to bind include, PCP_1 sites on the NMDA receptor; PCP_2 sites which are known to block dopamine uptake; $sigma_1$ sites whose function is not clear; and $sigma_2$ sites which have been implicated in dystonia (Loo et al., 1987; Vignon et al., 1984; Walker et al., 1990).

5.3.1 PCP Receptor Involvement

In the CNS, high affinity PCP_1 receptors which exist within the ion channel pore of the NMDA receptor demonstrate nanomolar affinity for drugs such as MK-801 and TCP (Wong et al., 1987). If PCP ligands were stimulating an adrenal

medullary PCP_1 site, then the expected rank order of inhibition should be: MK-801 > TCP > PCP > BTCP. However, in [^{3}H]NA uptake assays this was not the case. In addition, the adrenal medulla was shown to lack high affinity PCP_1 receptors (Rogers and Lemaire, 1990). The results indicate that PCP was the most effective inhibitor of [^{3}H]NA uptake followed by TCP and MK-801. This result alone suggests that PCP_1 receptors are not involved.

PCP_2 receptors bind BTCP with high affinity resulting in the blockade of dopamine uptake (Vignon et al., 1988; Maurice et al., 1990). The fact that the CNS PCP_2 binding site is defined as 'haloperidol-insensitive' (Vignon et al., 1984), ruled out the possibility that the haloperidol-sensitive [^{3}H]TCP binding site in bovine adrenal medulla represented a PCP_2 receptor. Regardless of this, if one postulates that a PCP_2 subtype is involved, then the profile of inhibition according to the defined PCP_2 subtype should be BTCP > PCP > TCP > MK-801. Although this rank order of potency was obtained for PCP analogs in the adrenal medulla and rat brain, it is in contrast to the report that BTCP was a poor inhibitor of NA uptake (Vignon et al., 1988). The potency of PCP and MK-801 reported in this NA uptake study match those of Snell et al., (1989) who found that MK-801 was 3 fold less potent than PCP in inhibition of [^{3}H]NA uptake in rat hippocampal synaptosomes (Snell et al., 1988). However, it is difficult to attribute this effect to PCP_2 receptor stimulation, especially due to the fact that the adrenal medulla PCP binding sites are haloperidol sensitive. Since the characterization of the PCP_2 site was originally carried out in the striatum with respect to dopamine uptake, further

studies are needed to characterize the PCP_2 site in other brain regions that involve NA uptake, such as hypothalamus. Until an adrenal medullary [^{3}H]BTCP binding site is characterized, firm statements concerning the involvement of PCP_2 receptors in the observed effects of PCP on NA uptake can not be made.

The lack of correlation obtained between the potencies of PCP ligands in adrenal medullary NA uptake assays and [^{3}H]TCP binding assays may be explained by the different buffers used. Adrenal medullary [^{3}H]TCP binding was carried out in the absence of Na^+, while uptake assays included physiological concentrations of this cation. The presence of Na^+ increases the labelling of high affinity PCP_2 receptors by the ligand [^{3}H]BTCP (Vignon et al., 1988). Earlier studies on PCP sites using [^{3}H]TCP in the adrenal medulla were carried out in a Na^+-free buffer (Rogers and Lemaire, 1990) due to the fact that the presence of cations was shown to decrease the binding of ligands to PCP and sigma receptors (Schwarcz et al., 1990; Basile et al., 1992). The question as to whether the absence of Na^+ in these binding studies precluded the identification of PCP_2 sites is answered by a recent study by Ackunne et al., (1991) who reported that [^{3}H]TCP is a good label for the identification of both NMDA (PCP_1) and dopamine uptake sites (PCP_2) in the absence of Na^+.

5.3.2 Sigma Receptor Involvement

According to the agreed definition of sigma receptors (Quirion et al., 1992), implication of $sigma_1$ or $sigma_2$ sites may not be made here due to the

following discrepancies. $Sigma_1$ receptors have high affinity for (+)pentazocine, and are frequently separated from $sigma_2$ receptors by their stereoselectivity for the dextro-isomer of SKF-10047. In both NA uptake and $[^3H]$DMI binding assays, (+)pentazocine was one of the least potent drugs tested, and little stereoselectivity for SKF-10047 was found. If the inhibition of uptake was due to $sigma_2$ receptor stimulation, then the high affinity $sigma_2$ receptor ligand DTG should have been one of the most potent inhibitors, however this ligand was inactive. Due to the fact that sigma ligands interact with $[^3H]$DMI sites, one could postulate that sigma ligands merely cross-bind at high concentrations to these sites. However, the possibility of modulation of $[^3H]$DMI binding by a low affinity sigma receptor has not been ruled out and is supported by the non-competitive nature of the effect of sigma ligands on $[^3H]$DMI binding (Fig 14).

Other studies have recently found low affinity sites for sigma ligands in other tissues, and more importantly, these sites possess distinct pharmacological profiles that do not resemble the $sigma_1$ and $sigma_2$ sites. This has raised the question as to whether these peripheral sites represent a $sigma_3$ site. Massamiri and PiperDuckles (1991), showed that PCP and sigma ligands potentiated NA-induced contractions of the rat tail artery with the following rank order of potency; PCP > haloperidol > 3(+)PPP > rimcazole. Interestingly, this order of potency is similar to that found in $[^3H]$NA uptake assays in this study (PCP > TCP > haloperidol > 3(+)PPP > MK-801 > rimcazole; Rogers and Lemaire, 1991). In addition, DTG, the least potent sigma ligand tested in this study was also inactive

in potentiating the contractile effect of NA in rat tail artery leading to the conclusion that high affinity $sigma_1$ and $sigma_2$ sites were not involved. More recently, Vilner and Bowen, (1993) found that high concentrations of sigma receptor ligands (100 μM) applied to C6 glioma cells in culture leads to a cessation of cell division and eventually cell death, implicating sigma receptors in the role of cell growth or development. However, caution should be used in defining these sites as sigma receptors since DTG and (+)pentazocine were inactive in this study as well.

The observed inhibition of [^{3}H]NA uptake produced by sigma ligands (with the exception of DTG and (+)pentazocine) may represent an indirect effect resulting from the interaction of sigma ligands with a fundamental enzyme involved in cell metabolism. Alteration of cell metabolism by sigma ligands could interfere with the ability of the cell to maintain the Na^+ gradient which is essential for NA uptake. However, the non-competitive inhibitory effect of sigma ligands on [^{3}H]DMI binding suggests that more likely, sigma ligands bind to a low affinity site on the protein composing the NA transporter and allosterically regulate NA uptake.

5.4. Effects of Histogranin on [^{3}H]NA uptake and PCP-induced Inhibition of [^{3}H]NA uptake.

During the initial phases of this study, others in the laboratory had sucessfully identified and isolated an adrenomedullary peptide that produced

"PCP-like" behaviours in rat (Lemaire et al., 1993). The mechanism involved in the production of these "PCP-like" behavioural effects were unknown and therefore, it was of interest to examine whether the endogenous peptide, namely histogranin (HN), was capable of mimicking the action of PCP on [^{3}H]NA uptake.

HN and its analog [Ser^1]HN were ineffective inhibitors of [^{3}H]NA uptake and [^{3}H]DMI binding (Table 3; Table 4). Therefore additional studies attempted to determine if HN potentiated the inhibitory action of PCP or σ ligands in [^{3}H]NA uptake assays in order to produce its PCP-like behavioural activity. However, HN was ineffective in altering the potency of either PCP or sigma ligands in [^{3}H]NA uptake assays. Incubation of HN (10 μM) in [^{3}H]NA uptake assays with either PCP or DXM did not significantly change the IC_{50} values for these ligands. The lack of activity of HN in these assays suggested the following two possibilities. Firstly, HN may act on other receptors to which PCP and sigma ligands bind, or secondly, HN may bind to its own distinct receptor site on brain membranes and initiate a similar intracellular signal. Therefore the next objective was to characterize the binding site for this novel peptide in rat brain membranes, and assess the interaction of PCP and sigma ligands with this site as discussed below.

5.5. Characterization of [^{125}I][Ser^1]Histogranin Binding Sites in Rat Brain

In this study, we have characterized the binding site for the iodinated analog of histogranin, [^{125}I][Ser^1]HN and investigated its interaction with various

PCP, sigma ligands and modulators of the NMDA receptor complex using a membrane preparation of rat brain. [^{125}I][Ser^1]HN bound to high affinity sites (K_d= 25.0 nM) that exhibit trypsin-sensitivity, specificity, reversibility, and saturability (Fig 15; Fig 17; Fig 18; Table 5). In addition, [^{125}I][Ser^1]HN binding sites were found to be differentially distributed in specific brain regions (Table 7). These results suggest that the site bound by [^{125}I][Ser^1]HN possesses the basic characteristics commonly found for membrane bound receptors (Bennett and Yamamura, 1985).

The inverse temperature dependency of the binding may be due to rapid degradation of the peptide at 22°C and 37°C as compared with 4°C. However, the presence of peptidase inhibitors in the binding assay should have prevented the degradation of the peptide. Interestingly, reduction of the binding of characterized ligands for distinct binding domains on the NMDA receptor complex such as [^{3}H]Glu and [^{3}H]spermidine is also found when the incubation temperature is increased in the range from 4° C to 30° C (Yoneda and Ogita, 1991; Mantione et al., 1990), suggesting that the NMDA receptor may possess temperature-sensitive binding domains. The binding of [^{125}I][Ser^1]HN proved to be selective for HN and structurally related analogs and was unaffected by the presence of substance P, ß-endorphin, neuropeptide Y, enkephalins, dynorphin A(1-13) and neuromedin C (Table 5). High affinity binding of [^{125}I][Ser^1]HN requires the intact 15 amino acid peptide. In this regard, removal of N- or C-terminal amino acids of HN greatly affected its binding potency. Future

investigation will confirm whether the stepwise reductions in potency of HN fragments measured in binding assays correlates with similar potency reductions in <u>in vivo</u> bioassays.

5.6 Effects of PCP, sigma receptor ligands and modulators of the NMDA receptor complex on [^{125}I][Ser^1]HN binding

In competitive binding assays, the sigma receptor ligands DTG, (+)pentazocine, haloperidol, (+)SKF-10047, 3(+)PPP, and rimcazole did not inhibit [^{125}I][Ser^1]HN binding. Similarily, PCP receptor ligands such as PCP, TCP, MK-801 and BTCP were equally ineffective in reducing the binding of [^{125}][Ser^1]HN to rat brain membranes. The fact that polyamines (spermidine, spermine, DA10, DET) but not other NMDA receptor modulators or ligands (CPP, AP5, MK-801, TCP, Gly and Glu) inhibited specifically bound [^{125}I][Ser^1]HN suggests that the HN binding domain may be situated near the polyamine domain on the NMDA receptor complex. In support of this hypothesis, others in the laboratory have shown that the binding of the NMDA antagonist [^{3}H]CGP-39653 and the NMDA-induced potentiation of [^{3}H]TCP binding to rat brain membranes are inhibited in the presence of HN (Lemaire et al. 1993; Lemaire and Shukla, 1993). Furthermore, in vivo studies have found that HN blocks NMDA-induced seizure activity in mice (Lemaire et al., 1993).

Polyamine agonists are characterized by their ability to enhance NMDA responses (Brackley et al., 1990; Sprosen and Woodruff, 1990) and potentiate the

binding of the non-competitive NMDA antagonist, [^{3}H]MK-801 (Ransom and Stec, 1988). Both polyamine site-specific agonists and antagonists were effective in reducing [^{125}I][Ser^{1}]HN binding (Table 6). The spermidine antagonist, DET, was most potent, displacing 50% of the bound [^{125}I][Ser^{1}]HN at a concentration (180 nM) well below that needed to inhibit bound [^{3}H]spermidine (Williams et al., 1991). Spermine, spermidine and DA10 inhibited [^{125}I][Ser^{1}]HN at concentration ranges similar to their affinity for the [^{3}H]spermidine binding domain on the NMDA receptor complex (London et al, 1991), indicating that the high affinity polyamine site linked to the NMDA receptor complex may allosterically regulate HN binding. Support for the selectivity of the interaction between polyamines and HN sites is derived from the fact that a structurally similar compound, putrescine, does not inhibit [^{125}I][Ser^{1}]HN nor [^{3}H]spermidine binding (Table 6; London et al., 1991).

Available evidence suggests that spermine, spermidine, DET and the inverse agonist, DA10, all act competitively at a common recognition site on the NMDA receptor complex (Williams et al, 1991). While agonists such as spermine and spermidine potentiate both the binding of [^{3}H]MK-801 and the actions of NMDA, the inverse agonist DA10 inhibits [^{3}H]MK-801 binding and blocks NMDA responses. The antagonist DET has been found to block the effects of both polyamine agonists and inverse agonists (Williams et al., 1991; Romano et al., 1992). The interaction of the polyamine antagonist DET with the binding of [^{125}I][Ser^{1}]HN was shown to be non-competitive (Fig 19). In addition, neither HN

nor [Ser^1]HN was able to inhibit the binding of [^3H]spermidine. Thus, the non-competitive inhibition of [^{125}I][Ser^1]HN binding by DET and the lack of potency of HN in [^3H]spermidine binding assays indicate that the HN binding site is distinct from the polyamine binding domain and interactions between the two sites must be allosteric.

Physiological concentrations of Mg^{2+} (Nowak et al., 1984; Mayer et al., 1984), and Zn^{2+} (Ascher and Nowak, 1988; Peters et al, 1987; Westbrook and Mayer, 1987) have been shown to decrease the current carried by the NMDA-gated ion channel. NMDA activity is antagonized by the binding of Mg^{2+} to the NMDA ion channel pore, and Zn^{2+} to the extracellular surface of the receptor (Reynolds and Miller, 1988; Barnes and Henley, 1992). These cations were found to inhibit the binding of [^{125}I][Ser^1]HN with IC_{50} values that were similar to their ability to inhibit [^3H]spermidine, [^3H]ifenprodil and [^3H]MK-801 binding (Fig. 20; Reynolds and Miller, 1988; Schoemaker et al. 1990). Therefore, Zn^{2+} and Mg^{2+} may bind to specific sites on the NMDA receptor complex and affect the binding of specific ligands for NMDA binding domains and the HN binding site in a similar fashion.

The co-localization of the NMDA receptor with HN sites was supported by the study of the regional distribution of [^3H]MK-801 and [^{125}I][Ser^1]HN binding (Table 7). The profile of binding site density for HN in three brain regions was similar to that measured with the specific non-competitive NMDA antagonist [^3H]MK-801. However, the cerebellum contained a greater proportion of

$[^{125}I][Ser^1]$HN binding sites. This discrepancy may be explained by the fact that several lines of evidence are suggestive of heterogeneity of the NMDA receptors in the rodent brain (Monaghan, 1991; Monaghan and Beaton, 1991). For example, the rat cerebellar membrane preparations bind $[^3H]$TCP and $[^3H]$MK-801 with lower affinity and density than cerebral cortical preparations (Yi et al., 1988; Ebert et al., 1991). Moreover, Xenopus oocytes injected with cerebellar mRNA respond to NMDA in a Gly insensitive manner (Sekiguchi et al., 1990). The aforementioned polyamines markedly potentiate $[^3H]$MK-801 binding in hippocampal synaptic membranes, but do not affect the binding to cerebellar membranes (Yoneda et al., 1991). These findings suggest that the relatively high density of $[^{125}I][Ser^1]$HN binding sites in the cerebellum may result from an interaction with an NMDA receptor subtype that is not recognized by the PCP_1 receptor ligand $[^3H]$MK-801.

6.0 CONCLUSIONS

The results herein strongly suggest that sigma and PCP ligands play a role in the blockade of NA re-uptake. However the site(s) involved in mediating the effects of these compounds differ from high affinity PCP and sigma receptors previously characterized in both CNS and adrenal medulla. Both PCP and sigma ligands alter the binding of [^{3}H]DMI to the substrate recognition site of the NA transporter, however this effect may only be relevant to the action of sigma ligands on NA uptake since the potency of PCP ligands in [^{3}H]DMI binding and NA uptake assays did not correlate.

The "PCP-like" behavioural effects produced by the endogenous peptide histogranin do not seem to be due to blockade of NA re-uptake, or potentiation of PCP-induced NA re-uptake blockade. Furthermore, no interaction between sigma and PCP analogs and [^{125}I][Ser^1]HN binding sites has been found. Receptor sites for [^{125}I][Ser^1]HN, have been identified and characterized in rat brain. The binding of [^{125}I][Ser^1]HN was inhibited by known modulators of the NMDA receptor complex and displayed an NMDA-like distribution profile in rat brain. In light of the finding that HN blocks NMDA-induced seizure activity, and modulates the binding of the specific NMDA antagonist [^{3}H]CGP-39653 (Lemaire et al., 1993), the results suggest that the HN binding site may be located on, or in close proximity to, the NMDA receptor complex. Further investigation of the hypothesis that HN interacts with brain NMDA receptors is presently being carried out using autoradiographic and immunohistochemical techniques.

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