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Evaluation of the Antagonism of Nicotine by Mecamylamine and Pempidine in the Brain

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Dedication

I dedicate this thesis to my wife, Ronda, whose capacity for love, support and devotion never ceases to amaze me. Her tremendous support and sacrifice has allowed me to pursue this research and my doctoral degree with a single-minded effort, and I will always be in her debt for this opportunity.

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List of Abbreviations

Å	angstrom
ACh	acetylcholine
AD_{50}	dose producing 50 % antagonism
BC_9	octamethylenebis(carbamylcholine)
B_{max}	density of binding sites
BTX	bungarotoxin
℃	degrees Celsius
C_6	ganglionic nicotinic receptors
C_{10}	neuromuscular nicotinic receptors
CA	anterior commissure
CFD	dorsal commissure of the hippocampus
Ci	Curies
C. L.	95 % confidence limits
cm	centimeter
CNS	central nervous system
COR	cortex
COTX	cobratoxin
cp	caudate putamen
DA	dopamine
dcgl	dorsolateral geniculate bodies
ED_{50}	dose that produces a 50 % effect

ED_{84}	dose that produces an 84 % effect
EEG	electroencephalogram
EPC	end-plate current
F	fornix
FH	hippocampal funiculus
fmol	femptomoles
FOR	reticular formation
FSH	follicle-stimulating hormone
g	gram
GP	globus pallidus
^3H	tritium
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HI	hippocampus
hr	hour
5-HT	5-hydroxytryptamine, serotonin
H_{12}-HTX	perhydrohistrionicotoxin
IC_{50}	concentration resulting in 50 % inhibition of binding or an enzymatic reaction
i.c.v.	intracerebroventricular
i.e.	id est, L. that is
IPN	interpeduncular nucleus
i.v.	intravenous
K_d	binding affinity constant
kg	kilogram
K_i	inhibitory constant
LD_{50}	dose resulting in death of 50 % of the animals tested
LH	leutinizing hormone

mcgm	medial geniculate bodies
mg	milligram
mh	medial habenula
min	minute
ml	milliliter
mmol	millimole
% MPE	% maximum possible effect
N	normal units/liter
nAChR	nicotinic cholinergic receptor
nCi	nanocurie
NE	norepinephrine
nM	nanomoles/liter
pH	negative logarithm of the hydrogen ion concentration
pmole	picamoles
p.o.	orally
r	correlation coefficient
RCC	rostral part of the corpus callosum
s.c.	subcutaneous
s.e.m.	standard error of the mean
SGS	stratum griseum (superficial gray layer) of the superior colliculus
sl	septal nucleus
SNR	substantia nigra pars reticulata
TCC	tegmentum of the corpus callosum
tl	lateroventral thalamic nuclei
tv	ventrolateral thalamic nuclei
tvd	ventrodorsal thalamic nuclei
ts	triangular septal nuclei

TSH	thyroid stimulating hormone
µl	microliter
µm	micrometer
µM	micromoles/liter
w/v	weight/volume, 1 g/100 ml

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ABSTRACT

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Antagonists have been crucial in the characterization of nicotine's pharmacology. Initial evidence for the existence of central nicotinic receptors was based on the fact that nicotine produced a number of behavioral effects that were antagonized by ganglionic blockers that crossed the blood-brain barrier, such as mecamylamine and pempidine. Although the mechanism of action of these compounds has been studied extensively in the periphery, little is known about their mechanisms of action in the brain. These compounds are thought to be noncompetitive antagonists due to the fact that they do not compete for agonist binding to brain homogenate *in vitro*. However, pharmacological evidence in support of noncompetitive antagonism is lacking.

Dose-response curves for nicotine were determined in the presence of various doses of pempidine for depression of spontaneous activity and antinociception in mice. Pempidine was found to shift the dose-response curves for these effects of nicotine in a manner consistent with noncompetitive antagonism. A number of mecamylamine analogs were investigated for antagonism of these central effects of nicotine as well. These studies revealed that the N-, 2-, and 3-methyls were crucial for optimal efficacy and potency and suggests that these compounds possess a specific mechanism of action, possibly involving a receptor. Furthermore, the structure-activity relationships for the mecamylamine analogs

were found to be different than that previously reported for the agonists, suggesting that they do not act at the same site.

The binding of [^{3}H]-L-nicotine and [^{3}H]-pempidine was studied *in vitro* to mouse brain homogenate and *in situ* to rat brain slices. The *in situ* binding of [^{3}H]-L-nicotine to rat brain slices was quantitated autoradiographically to discrete brain areas in the presence and absence of 1, 10 and 100 μM nicotine and pempidine. Pempidine did not effectively displace [^{3}H]-L-nicotine binding. The studies with [^{3}H]-pempidine failed to demonstrate saturable binding.

The evaluation of the antagonism of nicotine by mecamylamine and pempidine presented in this thesis supports a noncompetitive action of these compounds in the brain. The shift in the dose-response curves for nicotine, the structure-activity relationship for mecamylamine analogs and the binding studies are consistent with this hypothesis. The noncompetitive nature of these compounds suggests that they do not compete for the binding site of the agonist, and that endogenous nicotinic antagonists may exist in the brain.

I. General Introduction

A. Preface

Interest in the pharmacology of nicotine arose due to the prevalence of tobacco use following the commercialization and production of finished products from tobacco. Following its isolation from tobacco and subsequent synthesis, nicotine was shown to produce a myriad of pharmacological effects both centrally and peripherally. Investigations of nicotine's pharmacology resulted in the characterization of a major neuronal system that came to be known as the nicotinic cholinergic system. Nicotine was also one of the first compounds that was thought to interact with a specific endogenous component of neurons and contributed to the development of receptor theory. Indeed, more is known about the molecular morphology of the nicotinic cholinergic receptor than any other receptor. The antagonists of nicotine have been crucial for the development of nicotine's pharmacology and in the classification of peripheral and central nicotinic receptors. The effects of nicotine that are attenuated by nicotinic antagonists are attributed to nicotinic cholinergic receptors, however evidence is being accumulated that suggests that the antagonists may not be acting directly at the receptor. Little is known regarding the events that lead to the pharmacological effects of nicotine subsequent to receptor binding, and even less is known regarding the mechanism of action of the antagonists in the CNS. A greater appreciation of the mechanism of action of nicotine in the CNS can be obtained by investigation of the antagonists.

B. Tobacco Use and Nicotine

The first record of tobacco use is a Mayan stone carving dated at 600 to 900 A. D. (U.S. Dept. of Health and Human Services, 1988). The use of nicotine through the smoking or chewing of leaves from the *Nicotania tabacum* and *N. rustica* plants was introduced into various European countries by sailors returning from voyages in the New World in the mid 1500's. The genus *Nicotania* was named after the French ambassador to Lisbon, Jean Nicot, who is said to have sent the seed of *N. tabacum* to the queen consort and regent of France, Catherine de Medicis (Encyclo. Britannica, 1985). Nicot also touted tobacco as a cure-all throughout Europe in the late 1500's (U.S. Dept. of Health and Human Services, 1988). Before its commercial cultivation began in Jamestown, Virginia in 1612, tobacco had been introduced into all parts of the known world, including Russia and Japan. In 1619, the production of tobacco had become so commercially successful that the crop was used not only to barter for goods, but also to pay taxes and for the salaries of public officials. Furthermore, the profit margin of tobacco production became so great that in 1621, production limitations had to be imposed in order to increase the production of food crops in the English colonies (Encyclo. Americana, 1987).

Tobacco use increased greatly in the following years. Following the development of the cigarette shortly after the Civil War and of blended tobacco in the 1910's, the processing and production of finished products from tobacco became a tremendously successful industrial enterprise (U.S. Dept. of Health and Human Services, 1988). From 1930 to 1940, an average of 347 billion pounds of manufactured tobacco and 148 billion cigarettes were produced per annum by U. S. companies. Despite growing concerns over the adverse health effects of tobacco use, per capita consumption of tobacco in the United States increased from 9.62 pounds in 1940 to 11.82 pounds in 1960 (U. S. Bureau of the Census, 1967). Although per capita consumption of tobacco products in the U. S. was down to 6.9 pounds in 1984, the tobacco industry produced 657 billion cigarettes and 4.5

billion cigars in the United States alone. The value of all tobacco products produced in the United States in 1984 was over $17 billion (U. S. Bureau of the Census, 1987).

Due to the prevalence of tobacco use in the world, research began in the early 1800's to identify the constituents of tobacco that contribute to its habitual use. An oily substance was isolated from tobacco by Cerioli and Vanquelin in the early 1800's and was named "Nicotianine". This substance was further purified and renamed "Nikotin" by Posselt and Reiman in 1828. The empirical formula of this substance was elucidated in the 1840's and nicotine was first synthesized in the 1890's (U.S. Dept. of Health and Human Services, 1988). Since then, numerous investigators have sought to determine the role of nicotine in tobacco use.

The evidence in support of nicotine being the principal component of tobacco that contributes to its habitual use is a culmination of chemical and biological data on the constituents of tobacco smoke, their absorption into the blood and distribution into the brain, and their effects on humans and laboratory animals. An extensive review of this subject is provided in The Health Consequences of Smoking: Nicotine Addiction, a report of the Surgeon General (U.S. Dept. of Health and Human Services, 1988). Nicotine has been found to be the primary alkaloid present in tobacco smoke (Piade and Hoffman, 1980) and Benowitz et al. (1987) have shown that nicotine enters the bloodstream following tobacco use. Furthermore, peak blood concentrations are similar following the use of a variety of tobacco products (Benowitz, 1987). Given free access to tobacco, smokers have been shown to rapidly achieve a blood level of nicotine in the morning hours that remains steady throughout the day (Benowitz et al., 1982). Earlier, Schmiterlow et al. (1967) had shown that nicotine was readily distributed into the brains of mice and cats following i.v. administration. Other researchers have found this to be true for mice (Stålhandske, 1970, Maziere, et al., 1976), rats (Oldendorf, 1974), and rabbits (Maziere, et al.,1976). Therefore, based on these studies and others, nicotine has been shown to enter the bloodstream and, consequently, the brain following tobacco use.

Nicotine possesses pharmacological properties that are consistent with other drugs of abuse. Drugs of abuse have been shown to be positive reinforcers in humans and animals (Thompson and Unna, 1977; Thompson and Schuster, 1968). Nicotine has been shown to be a positive reinforcer in beagles (Risner and Goldberg, 1983), squirrel monkeys (Goldberg and Spealman, 1983), rhesus monkeys (Yanagita, 1977), and humans (Goldberg and Henningfield, 1983; Henningfield et al., 1983). Nicotine has been shown to be an even more effective secondary reinforcer, where drug administration is associated with an external cue (Goldberg et al., 1981; Spealman and Goldberg, 1982). Mecamylamine antagonized both the primary (Goldberg and Spealman, 1982) and secondary (Goldberg et al., 1981; Spealman and Goldberg, 1982) reinforcing properties of nicotine. Furthermore, nicotine administration decreases smoking in human subjects whether administered i.v. (Lucchesi et al., 1967; Henningfield et al., 1983) or p.o. in the form of capsules (Jarvik et al., 1970) or gum (Nemeth-Coslett, et al., 1987; Russell, et al., 1976). Conversely, mecamylamine increases cigarette smoking (Stolerman et al., 1973a; Nemeth-Coslett et al., 1986; Pomerleau et al., 1987). Pentolinium, which does not cross the blood-brain barrier, has no effect on cigarette smoking, suggesting that nicotine acts centrally to reinforce smoking behavior , and that mecamylamine's antagonism is centrally mediated (Stolerman et al., 1973a). Cessation of chronic nicotine intake has also been shown to produce a characteristic withdrawal syndrome (American Psyhciatric Association, 1987). These data suggest that smoking is a means of obtaining nicotine in order to experience its reinforcing properties or prevent withdrawal effects.

C. Pharmacology of Nicotine

1. Physiological Responses and Behavioral Effects

Investigations of nicotine's pharmacology have sought to answer other questions which are not related to tobacco use, but rather to characterize the nicotinic cholinergic system. Nicotine produces a myriad of effects when administered to animals which are peripherally mediated, centrally mediated, or a composite of both. Nicotine was first demonstrated to

act at autonomic ganglia by Langley and Dickinson in 1889. Dale (1905) demonstrated that acetylcholine had nicotinic and muscarinic components that could be separated.

Peripherally, nicotine's effects can be largely explained by its actions on the sympathetic and parasympathetic ganglia, where transient stimulation by nicotine is followed by a more prolonged blockade. Nicotine has a biphasic effect on the release of catecholamines from the adrenal medulla and on stimulation of skeletal muscle. In the latter tissue however, the stimulant effect is largely masked by a more prolonged relaxation. Nicotine also stimulates a number of sensory receptors that include mechanoreceptors of the skin, tongue, lung, mesentery, and stomach; as well as chemoreceptors in the carotid body, thermal receptors of the skin and tongue, and pain receptors. Hexamethonium has been found to antagonize these actions. Composites of the ganglionic effects lead to tachycardia and hypertension, increased gastrointestinal motility and tone in the gut, and increased salivary and bronchial secretions. Nicotine causes nausea and vomiting as a result of stimulation of sensory nerves in vagal and spinal afferents in combination with stimulation of chemoreceptors in the area postrema of the medulla (Taylor, 1985). Nicotine also stimulates respiration due to activation of chemoreceptors in the carotid bodies as well as direct stimulation of central respiratory centers at higher doses. Stimulation is followed by respiratory depression due to desensitization of the central respiratory centers of the brainstem and neuromuscular blockade of respiratory muscles (Westphal, 1982).

In the CNS, nicotine has an intriguing profile of pharmacological effects which has been reviewed in detail (Martin, 1986). As in the periphery, both stimulation and depression occur. As mentioned above, nicotine can cause nausea and vomiting due to its stimulatory actions on the area postrema of the medulla oblongata. Other stimulatory effects include tremors, convulsions, and release of antidiuretic hormone from the pituitary (Westphal, 1982). Nicotine has been found to cause EEG activation (Longo et al., 1954; Rinaldi and Himwich, 1955) which may be mediated in part by peripheral afferent C fibers

(Ginzel, 1987; Murphree et al., 1967). In addition to the effects mentioned above, nicotine has been shown to cause antinociception (Phan, et al., 1973; Sahley and Berntson, 1979; Tripathi, et al., 1982) and alterations in behavior and learning (Larson, et al., 1961; Iwamoto, et al., 1987). Nicotine has been shown to depress activity in rodents (Morrison, 1969; Martin, et al., 1983). A review by Hall (1984) describes temperature regulation, sleep and arousal, release of coricosteroids, and water intake as effects of intracerebrally administered nicotine. Nicotine has also been found to possess discriminative stimulus effects in the CNS (Stolerman, et al., 1987; Morrison and Stephenson, 1969; Rosecrans and Chance, 1978), some of which may not be cholinergic (Rosecrans and Chance, 1977; Rosecrans, 1987). Ganglionic blockers that penetrate the CNS, such as mecamylamine, have been shown to antagonize most, if not all, of nicotine's central effects (Martin, 1986; Stolerman et al., 1983).

2. Effects of Nicotine on Neurotransmitter Release

Numerous investigators have attempted to attribute the behavioral effects of nicotine to its actions on the release of neurotransmitters in the CNS. Extensive reviews on this subject are available (Aceto and Martin, 1982; Balfour, 1982; Rowell, 1987; U. S. Dept. of Health and Human Services, 1988). Nicotine has been shown to stimulate the release of norepinephrine (NE), dopamine (DA), serotonin (5-HT) and acetylcholine (ACh) (Rowell, 1987). The specific behavioral effects associated with each of these various neurotransmitters remains unclear, however.

Nicotine stimulates the release of catecholamines in various brain regions by two distinct mechanisms; one that is calcium dependent and occurs at lower doses, and one that is not calcium dependent and is similar to the release seen by tyramine. The first process occurs with adrenergic nerve terminals in the hypothalamus as shown with both slices and synaptosomes. Norepinephrine (NE) released in response to nicotine in other brain areas appears to be due to displacement from storage vessicles in that it is calcium-independent and occurs only at doses greater than 10 μM (Balfour, 1982). Nicotine indirectly affects

NE release in the locus coeruleus, an important NE-containing nucleus. Peripherally administered nicotine stimulates the locus coeruleus, whereas microiontophoresis of nicotine onto this structure has no effect (Svensson and Engberg, 1980). Mecamylamine has been shown to be an effective antagonist of nicotine's actions on NE release in the brain (Balfour, 1982).

The effects of nicotine on brain DA release and turnover has been studied less extensively. Nicotine also increases DA release in striatal slices that is abolished by pempidine or the omission of calcium (Giorgiueff-Chesselet et al., 1979). Nicotine has also been shown to increase DA release *in vivo* in the striatum (Giorgiueff-Chesselet et al., 1976) and in the nucleus accumbens (Misfud et al., 1989). Nicotine has been shown to increase DA release in the mesolimbic and mesostriatal dopaminergic neurons, and it has been postulated that some of the euphoric effects of nicotine may be mediated by these systems. Nicotine appears to modulate neuroendocrine effects through both NE and DA release, with noradrenergic neurons being involved in the control of the hypothalamic-pituitary-adrenal-axis, and dopaminergic neurons inhibiting the release of prolactin, LH, FSH, and TSH (Fuxe et al., 1987).

Nicotine appears to have little effect on the serotonergic system in the CNS. This aspect of nicotine's pharmacology has not been studied extensively, however. Nicotine has been shown to reduce the turnover of 5-HT in the hippocampus and reduces the capacity of hippocampal synaptosomes to accumulate L-typtophan. These changes ocurred only after chronic administration of nicotine and were produced by cotinine, nicotine's major metabolite, as well. Furthermore, these effects were not antagonized by mecamylamine (Aceto and Martin, 1982; Balfour, 1982).

Nicotine has been shown to affect ACh release as well. Armitage et al. (1968) demonstrated that nicotine would increase the release of ACh from the parietal cortex, whereas higher doses decreased release. Cortical EEG activation and inhibition followed the stimulated and reduced ACh release, respectively. Morrison (1968) postulated that

ACh release was involved in the depressant effects of nicotine on bar-pressing for water reward in that physostigmine, at doses that had no effect by themselves, poteniated this effect of nicotine. Neostigmine, which does not penetrate the CNS, had no effect. Armitage et al. (1966) have likewise shown that nicotine would induce an ear-twitching response in cats that was associated with central ACh release, in that physostigmine potentiated this response as well.

D. The Nicotinic Cholinergic Receptor

1. Peripheral Nicotinic Receptors

The concept of a nicotinic receptor evolved over the past century. In 1889, Langley and Dickinson demonstrated that nicotine exerted a direct effect on the ganglion. Langley also postulated the existence of a "receptive substance" for nicotine and, in 1914 reported that curare would block nicotine's actions on skeletal muscle (Langley, 1905; Langley, 1914). Dale (1914) found that acetylcholine had effects that could be separated into two components, one mimicked by muscarine and the other by nicotine. The existence of muscarinic receptors at parasympathetic effector sites and of nicotinic receptors at the neuromuscular junction, adrenal medulla, and at both the sympathetic and parasympathetic ganglia is now fully appreciated. The use of specific antagonists has resulted in the classification of ganglionic and neuromuscular nicotinic receptors as distinct subtypes based upon the number of carbon atoms in the chain that attaches the two amino groups that results in optimum antagonistic activity. Attempts to isolate this receptor from peripheral tissues met with little success until the discovery of α-bungarotoxin and toxins from other elapid snakes that bind with high affinity to the peripheral nicotinic receptor (Aceto and Martin, 1982). A glycosidic protein has been isolated from electric eel and fish, as well as from mammalian muscle, that binds nicotine and acetylcholine (Conti-Tronconi and Raftery, 1982). The extensive biochemistry that has been done on this protein will be reviewed later.

2. Central Nicotinic Receptors

a. Pharmacological Evidence

The existence of central nicotinic receptors was postulated due to the fact that nicotine produced behavioral effects that were antagonized by ganglionic blockers. As mentioned previously, nicotine produces a myriad of central effects that are antagonized by mecamylamine, a ganglionic antagonist that penetrates the CNS. Antagonism of the effects of nicotine has also been seen by a number of investigators following central administration of hexamethonium. These effects include hypotension (Armitage and Hall, 1967; Feldberg and Guertzenstein, 1976), hypothermia (Hall, 1972), salivation (Hall and Reit, 1966) and motor reflexes (Hall and Reit, 1966). These observations led investigators to postulate the existence of a central nicotinic receptor of the C_6 type. However, other investigators have shown that dihydro-ß-erythroidine will antagonize nicotine centrally, suggesting the existence of C_{10} nicotinic receptors in the CNS as well (Bradley et al., 1699; Bradley and Dray, 1976; Bradley and Lucy, 1979). Aceto et al. (1969) demonstrated that nicotine-induced convulsions were blocked by the ganglionic blockers chlorisondamine, pentamethonium, mecamylamine, and hexamethonium, but not by atropine, chlorpromazine, morphine, or phenobarbitone. It has been found by a number of investigators that the only compounds that block the nicotine cue in drug discrimination are those that block nicotine's effects at autonomic ganglia and penetrate the CNS (Stolerman et al., 1987). It is interesting to note that chlorisondamine, a quaternary ganglionic-blocking agent, antagonizes the nicotine cue when given centrally (Garcha et al., 1985) but not peripherally (Romano et al., 1981). Therefore, these antagonists have proven invaluable for the characterization of central nicotinic recepors pharmacologically.

Other pharmacological evidence for the existence of a nicotinic receptor in the CNS comes from studies on the stereoselectivity of nicotine. It has been shown that the naturally occuring isomer, (-)-nicotine, has a similar pharmacological profile as the

unnatural (+)-nicotine. Studies of the central activity of these compounds by a number of investigators has revealed that (-)-nicotine is only 1 to 25 times more potent than (+)-nicotine in most assays for central nicotinic activity (Martin,1986). It has been postulated that this low degree of stereoselectivity is due to the flexible nature of nicotine and that the nitrogen atoms of (+)- and (-)-nicotine can be superimposed using molecular models (Aceto et al., 1984). Attempts to construct conformationally restricted analogs that retain pharmacological activity has been unsuccessful thus far (Martin, 1986).

Using drug discrimination, several investigators have demonstrated structural requirements for nicotinic activity. Chance et al. (1978) found that of a number of analogs tested, only 3-pyridylmethylpyrrolidine generalized to nicotine. An intact pyrrolidine ring appeared to be necessary for activity and increasing the distance between the pyridine and pyrrolidine rings decreased potency. Rosecrans et al. (1978) showed that nornicotine and cotinine were less potent than nicotine. Furthermore, Garcha et al. (1982) demonstrated that cytisine produced nicotine-like responding to a nicotine cue. Stolerman et al. (1987) showed that cytisine and anabasine generalized to the nicotine cue, although they were less potent than nicotine. Cytisine given i.c.v. has also been found to induce changes in locomotor activity in rats, an effect that was antagonized by mecamylamine (Pert and Clarke, 1987). Rosecrans et al. (1978) showed that nicotine-trained animals did not generalize to a quaternary analog of nicotine, supposedly due to its inability to enter the brain. The nicotine cue has been found to be selective in that neither arecoline (Meltzer and Rosecrans, 1981), picrotoxin, nor chlordiazepoxide (Stolerman et al., 1987) generalize from nicotine in nicotine-trained animals. Therefore, there appears to be structural requirements for nicotine's central effects.

b. Radioligand Binding Studies

Early attempts to characterize nicotinic receptors *in vitro* utilized a number of radiolabelled cholinergic ligands other than nicotine due to the lack of availability of radiolabelled nicotine of high specific activity (Martin, 1986). Eldefrawi et al. (1970)

studied ^{3}H-muscarone binding to housefly brain. These investigators were not able to elucidate a high affinity binding site and interpretation of the binding regarding its relevance to nicotine was difficult due to the mixed nicotinic and muscarinic properties of muscarone. Early studies with radiolabelled tubocurarine and decamethonium likewise met with little success as binding to rat brain tissue was found to be of low affinity (Eldefrawi et al., 1970). High affinity binding sites have been found for ^{3}H-tubocurarine in rat hippocampus, suggesting the existence of C_{10} type receptors in the CNS (Nordberg and Larsson, 1980). Two sites with Kd's of 1.5 and14 nM were reported and nicotine was found to be 50 times more effective than tubocurarine in competing for the high-affinity binding. Schwartz et al. (1982) studied ^{3}H-acetylcholine binding to rat brain and was able to elucidate a high affinity site in the presence of diisopropylfluorophosphate and atropine. This binding was found to have a Kd of 12 nM and a Bmax of 4.6 pmoles/g tissue. Nicotinic agonists were found to compete effectively for this binding, whereas decamethonium and mecamylamine were 3 and 5 orders of magnitude less potent than (-)-nicotine, respectively. It has been subsequently shown that the autoradiographic distribution of ^{3}H-nicotine and ^{3}H-ACh in the presence of atropine to mammalian brain are vitually identical (Clarke et al., 1985a).

The use of α–neurotoxins to label central nicotinic sites met with popularity in the 1970's and early 1980's due to their high affinity for peripheral nicotinic receptors and the belief that peripheral and central nicotinic receptors were similar. It has been shown that the affinity of α–neurotoxins for peripheral and central binding sites are similar (Oswald and Freeeman, 1981). Nicotinic receptors partially purified from rat brain and electroplaque tissue using affinity chromatography with α–neurotoxins display similar chromatographic properties as well (Salvaterra and Mahler, 1976). However, investigators have found that α–bungarotoxin is without activity at central and autonomic synapses (Chou and Lee, 1969; Duggan et al., 1976; Ko et al., 1976; Brown and

Fumagalli, 1977; Patrick and Stallcup, 1977; Misgeld et al., 1980). Furthermore, nicotinic agonists and antagonists do not compete for this binding with high affinity (Salvaterra and Mahler, 1976; McQuarrie et al., 1976; Schmidt, 1977; Moore and Brady, 1977; Morley et al., 1977; Ben-Barak and Dudai, 1979; Block and Billiar, 1981). Autoradiograms of ^{3}H-nicotine and ^{3}H-α–bungarotoxin binding to brain reveal unique distributions of binding sites for these ligands as well (Clarke et al., 1985a). It has been shown that α-bungarotoxin binding sites can be physically separated from high-affinity nicotine binding sites using affinity chromatography with α-bungarotoxin as the affinity reagent (Wonnacott, 1986). Nicotine was found to compete for α-bungarotoxin binding to the protein retained on the column only at micromolar concentrations (Wonnacott, 1986). Whiting and Lindstrom (1988) have proposed the existence of a nicotinic receptor family based upon the purification and cloning of nicotinic receptors from a variety of tissues, including mammalian brain. It has been shown that the peripheral and central nicotinic receptors are quite different in several salient structural features (Whiting et al., 1987). Therefore, the relevance of central α–neurotoxin binding sites to nicotine's pharmacology is questionable.

The availability of radiolabelled nicotine with high specific activity in the late 1970's and 1980's greatly enhanced investigations of central nicotinic binding sites. The binding of ^{3}H-nicotine to rat brain has been found to be saturable and dependent upon temperature, time, and pH of the incubation medium (Martin and Aceto, 1981). Due to variations in the techniques used, there is a discrepancy in the literature for affinity constants for ^{3}H-nicotine binding to brain, ranging from 0.2 to 590 nM (Martin, 1986). However, binding sites for nicotine with a Kd in the low nM range have been demonstrated in the brains of mice (Marks and Collins, 1982), rats (Romano and Goldstein, 1980), monkeys (Friedman et al., 1985), and humans (Shimohama et al., 1985; Flynn and Mash, 1986; Whitehouse et al., 1986).

There is also a lack of consensus regarding the existence of multiple binding sites and

the stereoselectivity of ^{3}H-nicotine binding to brain (Martin, 1986). The number of distinct nicotinic binding sites in the CNS varies from one (Marks and Collins, 1982) to four (Sloan et al., 1984). It has been suggested that the low-affinity sites arise from proteolytic degradation of the high affinity site (Lippiello and Fernandes, 1986). The ratio of the IC_{50} of (+)- to (-)-nicotine in displacing ^{3}H-(±)-nicotine binding to brain tissue varies from 3 to 63 (Martin, 1986). A direct comparison of the binding properties of the separate enantiomers of ^{3}H-nicotine by the same laboratory revealed that the binding characteristics of ^{3}H-(+)- and (-)-nicotine were similar, differing only in their affinity constants. The dextrorotatory antipode was found to posses three-fold less affinity for the binding site than its levorotatory counterpart (Vincek et al., 1981). Subsequent binding studies of the displacement of ^{3}H-(-)-nicotine by (+)- and (-)-nicotine were consistent with this study (Vincek et al., 1980; Sershen et al., 1981; Sloan et al., 1983; Abood et al., 1983). These findings are consistent with the low stereoselectivity of nicotine's pharmacological effects, as mentioned previously.The existence of noncholinergic nicotinic binding sites in brain has been supported by the work of Abood et al.(1980). They have been able to elucidate saturable nicotine binding with high affinity that is not displaced by cholinergic ligands. Other investigators have postulated noncholinergic effects of nicotine in the brain, supporting the notion of nicotine binding sites that may be noncholinergic. Regardless of the cholinergic or noncholinergic nature of the nicotine binding sites that have been elucidated to date, it has been shown that none of the nicotinic antagonists compete for *in vitro* ^{3}H-nicotine binding to brain (Martin, 1986). Romano and Goldstein (1980) have suggested that the long incubation used causes an agonist-induced shift of the conformation of the receptor to an antagonist-insensitive form. Marks and Collins (1982) have postulated that the agonists and antagonists bind to distinct receptors. Binding studies with radiolabelled antagonists that address these issues have not been documented to date.

3. Biochemical Characterization of the Nicotinic Receptor

The molecular structure of the nicotinic acetylcholine receptor-ionophore complex (nAChR) from electric eel and fish organs has been studied extensively. An excellent review of the literature has been provided by Conti-Tronconi and Raftery (1982). The nAChR has been purified from extracts of the electric organs of a number of species of fish and eel as well as from mammalian muscle using affinity chromatography with either cholinergic ligands (Schmidt and Raftery, 1972; Olsen et al., 1972) or α-neurotoxins (Raftery, 1973; Karlsson et al., 1972). This molecule represents the first neurotransmitter receptor to be isolated, purified, and reconstituted into membranes with retention of its physiological properties. This macromolecule is a glycosidic protein consisting of four distinct subunits, termed α, ß, γ, and δ, with a final stoichiometry of $\alpha_2\beta\gamma\delta$. The biochemical characterization of these subunits is beyond the scope of this discussion, but it suffices to note that there is considerable homology between the subunits from different fish and eel, as well as mammalian muscle. These five subunits form a rosette-like macromolecule with a central pore that is 15 to 25 Å in diameter. Each of the subunits traverse the membrane several times. Upon depolarization of the membrane, either by receptor agonists or electrical stimulation, the pore opens and the ionophore allows cations to pass through the pore. Anions do not flow through the pore due to a large number of negatively charged moieties at the pore entrance (Conti-Tronconi and Raftery, 1982).

Using monoclonal antibodies generated from electroplax, Conti-Tronconi et al. (1985) isolated a nicotinic receptor from the chick optic lobe and muscle. The central receptor was found to possess some homology to the muscle receptor, but displayed significant differences in subunit molecular weights as well. Barnard et al. (1980) found that nicotinic receptors isolated from the chick optic tectum in this manner possessed only two distinct subunits, whereas the muscle and electroplacque receptors are found to possess five.

Whiting et al. (1987) have used immuno-affinity techniques to purify and clone putative

nicotinic receptors from chick and rat brain. These proteins bind nicotine and acetylacholine with high affinity, but not α-bungarotoxin. They have found that only two distinct subunits exist for this protein and the α subunit is nearly identical for the brain and muscle receptors. Conversely, the ß subunit of the central receptor appears to be quite unique and they have concluded that the central receptor is a distant relative of the electroplacque and muscle nicotinic receptors. They have also provided evidence that the stoichiometric ratio of the subunits in the brain nicotinic receptor is $\alpha_3\beta_2$. The autoradiographic pattern of localization in rat brain of the monoclonal antibodies that these investigators used to purify and clone this receptor is nearly identical to that of nicotine and acetylcholine (Swanson et al., 1987). Therefore, it is apparent that the neuromuscular and central nicotinic receptors are quite different, but their relationship to the ganglionic receptor is unknown.

4. Functional Significance of Central Nicotinic Binding Sites

Evidence that the binding sites elucidated in brain for nicotine have pharmacological and physiological relevance consists primarily of studies comparing the selectivity, structure-activity relationships, and localization of these binding sites in the brain to nicotine's central pharmacological effects. The selectivity and structure-activity relationships for the discriminative effects of nicotine has been discussed earlier. The ability of nicotine, cytisine, and anabasine to generalize to the nicotine cue in rats is well correlated with their ability to displace ^{3}H-nicotine binding to brain tissue (Marks and Collins, 1982; Romano and Goldstein, 1980; Scimeca and Martin, 1988). As mentioned above, the stereoselectivity of nicotine in displacing ^{3}H-(-)-nicotine binding to brain is consistent with its low degree of stereoselectivity in producing central pharmacological effects. Muscarinic compounds have not been found to compete effectively for ^{3}H-nicotine binding to brain tissue, demonstrating that nicotine binding to brain is selective for nicotinic compounds (Marks and Collins, 1982; Romano and Goldstein,

1980; Scimeca and Martin, 1988).

The central distribution of ^{3}H-nicotine binding sites in brain has been studied extensively using quantitative receptor autoradiography. These studies have revealed that ^{3}H-nicotine binds to brain slices with a discrete pattern of localization. The greatest number of nicotinic receptors have been found in the thalamus, interpeduncular nucleus, cortex, superficial layer of the superior colliculus, and medial habenula, whereas the hippocampus, hypothalamus, and reticular formation possess relatively few nicotinic receptors (Clarke et al., 1985a; Segal et al., 1978; Duggan et al., 1976). This pattern has been found to be strikingly similar to the autoradiographic localization of nicotine's metabolic effects in brain through 2-deoxyglucose utilization studies (London et al., 1985). These investigators have found that mecamylamine would completely antagonize nicotine's effects on the metabolic activity of central neurons. Therefore, the binding sites for nicotine are not uniformly distributed in brain and nicotine's ability to increase the metabolism of neurons in a particular brain area is well correlated with the presence of nicotine binding sites. This suggests that these binding sites are relevant to the pharmacological effects of nicotine.

E. Nicotinic Antagonists

1. History

Nicotinic antagonists have served to define and delineate the various nicotinic cholinergic systems in mammals. Their role can be readily appreciated by the previous discussion of nicotine's pharmacology and of nicotinic binding sites found in a variety of mammalian tissues, as well as electric organs of fish and eel. The remainder of the discussion will focus on the antagonists themselves with respect to their mechanism of action on the various effects of nicotine and their relevance to nicotinic binding sites. In 1956, Stone et al. reported that a tertiary amine, 3-methylaminoisocamphane, termed mecamylamine, was found to possess potent ganglion-blocking activity. Prior to this, most potent ganglion-blocking agents were quaternary amines of quick onset and short

duration of action. Most tertiary amines that had demonstrated ganglion-blocking properties at that time either possessed other pharmacological properties or had low potency (Stone et al., 1956). However, these investigators found that mecamylamine was potent and had a longer duration of action that was slower in onset than the classical quaternary amino compounds. They concluded that such a compound had therapeutic potential as an antihypertensive. Spinks and Young first described the ganglion-blocking properties of a series of polyalkylpiperidines in 1958 and found that 1,2,2,6,6-pentamethylpiperidine had the greatest potency of all tested and termed the compound pempidine. They demonstrated that pempidine was approximately twice as potent as mecamylamine and yet had an LD_{50} four- to seven-fold greater than mecamylamine, depending upon the route of administration. These investigators concluded that these compounds had therapeutic potential as antihypertensives, however the adrenergic antagonists have largely replaced them for this purpose due to their greater efficacy and therapeutic index (Taylor, 1987).

2. Antagonism of Nicotine

a. Neuromuscular Junction and Electroplax Tissue

The mechanism of action of nicotinic antagonists at the neuromuscular junction and electroplax tissue has been studied extensively using a variety of depolarizing blockers and neurotoxins. This discussion will focus on the studies that have attempted to discern the relationship between such compounds and the central nicotinic antagonists, such as mecamylamine and pempidine. Mecamylamine was shown to antagonize contractions of the frog rectus abdominus muscle induced by octamethylenebis(carbamylcholine) (BC_9) by van Rossum and Ariëns (1959) in a noncompetitive manner. They found that mecamylamine and chlorisondamine shifted the dose-response curves of BC_9 downward, unlike a number of bis(quaternaryamino) derivatives that shifted dose-response curves to the right in a parallel manner, consistent with competitive antagonism. Blackman and Ray

(1964) demonstrated that blockade of muscle twitch in the rat phrenic nerve-diaphragm preparation by mecamylamine and pempidine was extracellularly mediated in that quaternary derivatives of each compound had nearly identical potencies. Using microelectrode recordings of the potential of cell body membranes of cockroach motoneurones, David and Sattelle (1984) demonstrated that mecamylamine blocked ACh-induced depolarization in a voltage-independent manner. This suggests that mecamylamine is acting on a closed-channel state of the receptor-ionophore complex, in that increasing the applied voltage (i.e., less negative) increases the number of open channels. In contrast, blockade by d-tubocurarine decreased with increasing membrane potential, suggesting open-channel blockade. Pretreatment with α-cobrotoxin (α-COTX), a reversible neurotoxin, was found to prevent irreversible blockade of the motoneurones by α-BTX, whereas mecamylamine pretreatment had no effect on the reversibility of this blockade, suggesting that its site of action is distinct from that of α-BTX.

The histrionicotoxins have proven to be useful compounds for the characterization of the channel of the nAChR found in muscle and electroplax tissue. Histrionicotoxin is an alkaloid isolated from the skin of *Dendrobates histrionicus* , a frog native to Colombia. This compound, as well as its saturated derivative perhydrohistrionicotoxin (H_{12}-HTX) has been shown to produce voltage- and time-dependent blockade of neuromuscular transmission through the nicotinic receptor at the motor end-plate (Albuquerque and Oliveira, 1979; Masukawa and Albuquerque, 1978). Aronstam et al. (1981) demonstrated that ^{3}H- H_{12}-HTX binding to electroplax tissue was affected by the conformational state of the receptor ionophore complex. They found that cholinergic agonists increased the initial rate and affinity of ^{3}H-H_{12}-HTX binding but had no effect on the Bmax. It has been shown that cholinergic agonists increase the potency of H_{12}-HTX in blocking end-plate currents in frog and mammalian muscle (Lapa et al.,1975). This

suggests that H_{12}-HTX binds to a site within the open channel of the nAChR to block ion flow. However, the binding kinetics of ^3H-H_{12}-HTX suggests that there are multiple binding sites. Further evidence for the existence of multiple binding sites for this compound is the difference in the ability of carbamylcholine to stimulate the displacement of ^3H-H_{12}-HTX binding by other compounds. Compounds that interact directly with ionic channels, such as phencyclidine, compete more effectively for ^3H-H_{12}-HTX binding in the presence of carbamylcholine, suggesting that ^3H-H_{12}-HTX binds to an open-channel form of the receptor. Conversely, carbamylcholine has no effect on compounds that do not bind to sites within the channel, such as tetraethylammonium, suggesting that ^3H-H_{12}-HTX binds to sites outside of the channel as well (Aronstam et al.,1981).

The relationship of mecamylamine to perhistrionicotoxin antagonism at the neuromuscular junction and binding to electroplax has also been investigated. Varanda et al. (1985) studied the effect of mecamylamine on ^3H-H_{12}-HTX binding to electroplax nAChR. They found that mecamylamine competed effectively for ^3H-H_{12}-HTX binding to electroplax tissue, but that this interaction was not enhanced by carbamylcholine. This suggests that mecamylamine may be binding to one of the sites for ^3H-H_{12}-HTX outside of the ionic channel. Mecamylamine was not found to compete effectively for ^3H-ACh binding to this tissue. These investigators also studied the ability of mecamylamine to block end-plate currents of frog sartorius muscle. They found that mecamylamine produced a concentration- and voltage-dependent shortening of mean channel open time and the end-plate current (EPC) peak amplitude. A linear relationship between the reciprocal of the decay time constant of the EPC and the concentration of mecamylamine was demonstrated, suggesting open-channel blockade. Single-channel recordings from rat myoballs demonstrated that mecamylamine did not alter channel conductance or reversal

potential, but decreased mean channel-open time in a concentration-dependent manner. Therefore, these studies collectively indicate that mecamylamine noncompetitively inhibits neuromuscular transmission by binding to an open-channel form of the receptor to decrease open-channel time and that this binding site is not located within the channel itself.

b. Ganglia

The ganglionic-blocking properties of mecamylamine was first described by Stone et al. (1956). These investigators found that mecamylamine was a specific antagonist for nicotinic stimulation of the ganglia. They found that vascular responses in the dog attributed to autonomic ganglia, such as carotid occlusion and peripheral vagal stimulation, were antagonized in a dose-dependent manner by mecamylamine. Both acetylcholine-induced hypotension and epinephrine-induced hypertension were potentiated by mecamylamine, suggesting blockade of compensatory mechanisms through the ganglia. They further demonstrated mecamylamine's ganglion-blocking effects in the nictitating membrane of the cat superior cervical ganglion, where mecamylamine blocked contractions induced by preganglionic electrical stimulation, but not by direct administration of epinephrine. Mecamylamine was also shown to produce decreases in mean arterial pressure and heart rate in anesthetized dogs and cats that were qualitatively similar to quaternary ganglionic-blockers, although slower in onset and longer in duration. These investigators found that mecamylamine had no atropine-, curare-, or antihistaminic-like activity.

Other ganglion-blocking effects have been documented for this compound, as well as pempidine. Bentley and Sabine (1963) found that mecamylamine blocked electrically-stimulated contractions of the guinea-pig vas deferens at the ganglion and found no evidence of a bretylium-like action. This finding was corroborated by Clarke and Capps (1972) in the rabbit ileum. Likewise, mecamylamine and pempidine were found to decrease gastrointestinal motility in dogs, rabbits, and guinea pigs (Garg, 1966).

Gokhale et al. (1967) found that mecamylamine and pempidine potentiated the actions of acetylcholine and epinephrine on the iolated rat ileum but totally blocked the actions of nicotine. Therefore, these investigations have provided considerable evidence that mecamylamine and pempidine act directly at autonomic ganglia.

Due to the availability of a variety of isolated tissue preparations, the mechanism of action of these compounds has been studied more intensely at autonomic ganglia than at any other site. A scheme for the classification of ganglionic blockers was proposed by van Rossum et al. (1962) following the analysis of the mechanism of a number of ganglionic-blockers. By analyzing the patterns of the shift of dose-response curves for nicotinic agonists by these compounds, three distinct categories were found to exist. The depolarizing blockers are termed class I compounds, and the nondepolarizing blockers are termed class II and III. Class II compounds are the competitive blocking agents, such as hexamethonium, and class III compounds are the noncompetitive blocking agents, such as chlorisondamine. The nicotine dose-response curves for contractions of guines pig jejunum were shifted downward and to the right by mecamylamine and pempidine. These compounds were therefore termed class II-III due to the fact that they exhibited the characteristics of both types of antagonism, with pempidine showing a greater degree of competitive antagonism than mecamylamine.

Lees and Nishi (1972) investigated the mechanism of action of mecamylamine on the rabbit superior cervical ganglion using intracellular recording techniques. They found that mecamylamine, but not hexamethonium or d-tubocurarine, inhibited post-tetanic potentiation. Post-tetanic potentiation arises predominately as a result of presynaptic facilitation of neurotransmitter release, and therefore suggests that mecamylamine possesses presynaptic actions. By measuring the excitatory postsynaptic potentials of the neurones in response to a train of stimuli, they found that the fractional release of readily available ACh was actually increased by mecamylamine, but the amount of ACh readily available for release was decreased. The net effect of these opposing actions was an

overall decrease in the release of ACh by a presynaptic stimulus, and the time course of this effect followed that of inhibition of post-tetanic potentiation. Interestingly, mecmaylamine has been shown to have no effect on post-tetanic potentiation of the neuromuscular junction (Bennett et al., 1957). Lees and Nishi (1972) also demonstrated that mecamylamine's only effect on the postganglionic fiber was to decrease the excitatory postsynaptic potentials in response to ACh as it had no effect on membraine excitability or resting membrane potential. This was found to be true for hexamethonium and d-tubocurarine as well. Their conclusions were therefore that mecamylamine had both presynaptic and postsynaptic effects at autonomic ganglia.

Ascher et al. (1979) studied mecamylamine's antagonism of the parasympathetic neurones at the rat submandibular ganglion by a two microelectrode voltage-clamp technique. They found that tubocurarine and hexamethonium produced greater blockade of agonist-induced currents in the presence of increasing concentrations of agonists. This blockade was voltage-dependent as well, and the data supported a sequential scheme whereby these agents bind to the receptor only in an open-channel form. Conversely, mecamylamine and trimetaphan were found to produce a blockade that was voltage-independent and decreased with increasing agonist concentration, suggesting that mecamylamine acts with the closed-channel form of the receptor.

These studies suggest that mecamylamine antagonism at autonomic ganglia occurs by at least two mechanisms. Presynaptically, mecamylamine decreases the amount of acetylcholine release in response to a depolarizing stimulus. Postsynaptically, mecamylamine alters the response of the nuerone to acetylcholine. It appears to do this in a competitive manner, but is clearly different from hexamethonium in its actions in that mecamylamine favors a closed-channel form of the receptor.

c. Central Nervous System

The mechanism of action of nicotinic antagonists in the brain has not been characterized to the same extent as in the periphery largely due to the lack of available models and tissue

preparations for measuring nicotine's central effects. As mentioned previously, nicotine produces a myriad of central behavioral and biochemical effects that are antagonized by mecamylamine and pempidine, but neither of these compounds displace *in vitro* acetylcholine or nicotine binding to brain tissue. Evidence that mecamylamine is acting centrally include administration into the brain directly to antagonize nicotine's effects (Wu and Martin,1983; Hall, 1972; Armitage et al., 1966). Hall (1972) also demonstrated that hexamethonium would mimic mecamylamine in antagonizing nicotine-induced hypothermia, salivation, and motor reflexes only when administered centrally, as this quaternary compound does not cross the blood-brain barrier. Furthermore, Stolerman et al. (1987) using drug discrimination have reported that peripheral nicotinic antagonists that do not cross the blood-brain barrier do not block the nicotine cue, whereas mecamylamine is a quite effective antagonist. Mecamylamine has also been shown to antagonize the hyperpolarization of cultured astrocytes from rat brainstem and spinal cord induced by nicotine and acetylcholine (Hösli et al., 1988).

Stolerman et al. (1987) studied mecamylamine's ability to block nicotine's discriminative stimulus effects in an attempt to determine whether mecamylamine acted competitively or noncompetitively. The pattern of shift for percent correct lever responding is similar to the findings of van Rossum et al. (1962) in the guinea pig jejunum in that the dose-response curves for nicotine were shifted downward and to the right. However, the pattern of shift for depression of response rate was markedly different in that the curves were shifted to the right in a parallel manner, suggesting competitive antagonism. Several aspects of this study make interpretation of the data difficult. Specific ED_{50} values with corresponding confidence intervals for nicotine at each dose of mecamylamine were not reported for either effect. There appears to be a lowering of the maximum effect of nicotine in producing correct lever responding by mecamylamine, but the statistical significance apparently was not determined. As mentioned previously, mecamylamine's antagonism of nicotine-induced depression of response rates was

overcome by increasing the dose of nicotine. This was a confounding factor in that the maximum dose of nicotine that could be tested to overcome the antagonism of correct lever responding by mecamylamine was limited by the suppression of response rate, and evaluation of the data at the higher doses of nicotine is difficult to interpret.

3. Structure-activity Relationships

The inability of mecamylamine and pempidine to displace the binding of ^{3}H-nicotine from brain tissue suggests that they act at a different site than the agonist. However, binding studies have not been conducted with the antagonists themselves. If these compounds act at receptors, they should possess structural requirements for activity. Furthermore, a comparison of the structure-activity relationships of the agonists and antagonists should provide insights as to the likelihood that they are acting at the same site.

Mecamylamine (N,2,3,3-tetramethyl-2-norbornamine) possesses three chiral centers, making eight stereoisomers theoretically possible (Figure 1). However, two of the chiral centers (carbons 1 and 4) are connected by a methylene bridge, limiting the possibilities to four. The isomers are divided into two groups, namely the exo- and endo-isomers. When

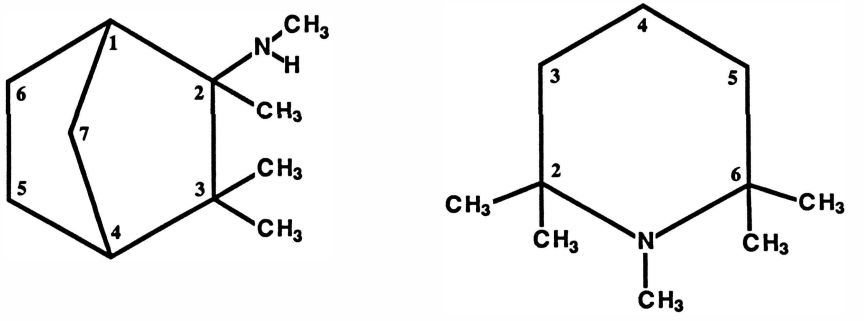


Figure 1. Structures of mecamylamine and pempidine.

the nitrogen extends away from the carbon cage, the compound is referred to as (±)-exo-mecamylamine. When the nitrogen is beneath the carbon cage, the compound is termed (±)-endo-mecamylamine. The structure-activity relationships of mecamylamine analogues in antagonizing nicotine-induced convulsions and pupil dilitation in mice was studied by Stone et al. (1962). Optical isomerism was found to have a minor role in the activity of these compounds, and mecamylamine was found to be the most potent of all compounds tested. The methyl groups at the N, 2 and 3 positions were found to be necessary for activity. The structural requirements for antagonism of nicotine-induced convulsions were correlated with ganglionic blockade (r= 0.95), suggesting a similar mechanism may be involved. These findings led Stone et al. (1962) to conclude that mecamylamine has a specific mechanism, possibly involving a receptor. Unfortunately, confidence intervals for the estimated ED_{50}'s of each compound were not given and it is not apparent which changes produce a statistically significant alteration in the activity of mecamylamine. Furthermore, convulsions induced by nicotine occur at relatively high doses compared to other central effects and may therefore be a less sensitive assay for antinicotinic activity.

Bretherick et al. (1959) examined a number of pempidine derivatives in the cat nictitating membrane. It was found that the nature of the N-substitution was important for activity. The N-ethyl homologue of pempidine was found to be slightly more potent that the parent compound. Substitutions that decreased the base strength of the nitrogen resulted in greatly diminished activity, whereas electron-donating groups in this position enhanced the activity somewhat. Three of the four methyls in the 2- and 6-positions were required for activity, however substitutions in the 4-position had no significant effect. The pyrollidine counterpart of pempidine (N,2,2,5,5-pentamethyl pyrollidine) was found to be less potent than pempidine, and all four methyls in the 2- and 5-positions were necessary for activity. Double bonds in the 2:3 or 3:4 positions of pempidine, as well as in the 3:4 position of its pyrollidine counterpart did not significantly alter activity. A noncyclic

analogue, di-t-butylamine was found to be approximately equipotent with pempidine. These findings suggest that the base strength of the nitrogen and the substituents of the adjacent carbons are important for the pharmacological activity of pempidine at the ganglion.

F. Objectives

The objective of the research presented in this thesis is to more clearly define the antagonism of nicotine's effects in the brain by the reversible ganglionic blockers mecamylamine and pempidine. More specifically, the competitive and noncompetitive nature of this antagonism is explored. If the antagonists act at the same site as nicotine, they may be a valuable tool for the determination of the conformational states of the nicotinic receptor necessary for neuronal activation. If these compounds are found to be noncompetitive however, they may be useful tools for understanding the biochemical and metabolic events that lead to the pharmacological effects of nicotine subsequent to receptor binding. Therefore, in order to obtain a better understanding of nicotine's central pharmacology, it is necessary to characterize the interaction of nicotine and central nicotinic antagonists with the nicotinic receptor.

To determine if the antagonists act at the same site as the agonists, behavioral assays and radioligand binding techniques were utilized. The competitive or noncompetitive nature of the antagonism of nicotine by pempidine was assessed using *in vivo* assays for nicotine's central effects. The structure-activity relationship for mecamylamine's antagonism of nicotine's effects was determined in order to address the possibility that the antagonist interacts with a receptor and to determine whether these compounds act at the same site. The binding of [^{3}H]-L-nicotine and [^{3}H]-pempidine was characterized *in vitro* and *in situ* to brain tissue. Quantitative autoradiography was also utilized to determine the interaction between these compounds. By studying this interaction *in vivo* and *in vitro* one may be able to better delineate between competitive and noncompetitive anagonism of nicotine by these compounds.

II. Pharmacological Characterization of the Interaction *in vivo* Between Nicotinic Agonists and Antagonists

Introduction

Central nicotinic antagonists have yet to be fully characterized regarding the competitive or noncompetitive nature of their antagonism of nicotine using *in vivo* assays for nicotine's behavioral effects. The study by Stolerman et al. (1987) on mecamylamine's antagonism of the nicotine cue in rats is difficult to interpret for the reasons mentioned previously. The characterization of the antagonism of other behavioral effects of nicotine by central nicotinic antagonists may provide insights into this interaction *in vivo* and determine the relevance of the observation that these compounds do not compete *in vitro* for agonist binding sites. Nicotine produces a myriad of other central effects, including depression of spontaneous activity and antinociception, that are antagonized by central nicotinic antagonists. However, the competitive or noncompetitive nature of these central nicotinic antagonists for these central effects of nicotine has yet to be documented.

Nicotine has been shown to produce depression of spontaneous activity in a number of species. Morrison and Armitage (1967) demonstrated that nicotine produced depression of spontaneous activity in mice in a dose-dependent manner. Marks et al. (1986) demonstrated a similar effect in four different mouse strains. Morrison and Stephenson (1972) demonstrated that rats became tolerant to this effect of nicotine when administered daily (0.8 mg/kg) for 4 days and that locomotor activity actually increased after 7 days. Using a Y-shaped runway, Stolerman et al. (1973) demostrated that acute and chronic

tolerance developed to nicotine's depressant effects in rats. It has been shown that acute administration of nicotine will produce an initial depression of spontaneous activity which is followed by stimulation (Clarke and Kumar, 1983; Clarke and Kumar, 1983a; Morrison et al., 1969). Chronic administration of nicotine has been shown to produce a stimulation of locomotor activity (Clarke and Kumar, 1983; Clarke and Kumar, 1983a; Morrison et al., 1969). Both acute and chronic effects of nicotine on locomotor activity are dose-dependent and are antagonized by mecamylamine, but not hexamethonium, suggesting that this effect is centrally mediated.

Nicotine has been shown to produce antinociception centrally as well. Mattila et al. (1968) showed that nicotine had antinociceptive activity in mice and rabbits, and that this effect was antagonized by mecamylamine but not altered by physostigmine, atropine, or by reserpine pretreatment. Phan et al. (1973) showed that mecamylamine would antagonize nicotine-induced antinociception in the hot-plate test in mice and rats. Sahley and Berntson (1979) found that mecamylamine, but not hexamethonium, would antagonize nicotine-induced antinociception in mice using the tail-flick assay, and that nicotine's effect was greatly enhanced by central administration, suggesting a central site of action. Kamerling et al. (1982) found that nicotine given i.v. produced antinociception in the dog as measured by a skin-twitch response to a heat stimulus that was not antagonized by naltrexone. The potency of nicotine was enhanced by central administration. Using the tail-flick assay, Tripathi et al. (1982) found that 3 mg/kg of nicotine s.c. produced antinociception in mice that was maximal after 5 min. Furthermore, the time course for this effect was well correlated with nicotine brain levels. Aceto et al. (1983) demonstrated that nicotine, but not its quaternary methiodide derivatives, produced antinociception in rats and mice. Mecamylamine was found to antagonize nicotine, but hexamethonium was without effect. These investigations therefore suggest that nicotine produces antinociception centrally that is antagonized by central nicotinic antagonists.

Since nicotine-induced alterations of locomotor activity and antinociception are

selectively antagonized by central nicotinic antagonists, these compounds are thought to have a specific mechanism of action. Although this antagonism is thought to involve nicotinic receptors, direct evidence is lacking. Pharmacological criteria for a receptor-mediated mechanism of action includes structure-activity relationships. Although extensive studies of this nature have been conducted in the periphery, the structural requirements for antagonism of the behavioral effects of nicotine have yet to be thoroughly documented (Stone et al., 1962; Bretherick et al., 1966). Studies addressing the structure-activity relationships for nicotine in producing its behavioral effects suggests that stereoisomerism plays a minor role and that an intact pyrollidine ring is necessary (Martin, 1986). Comparisons of the structural requirements for nicotine agonists and antagonists, as well as analysis of the structural overlap of these compounds will yield insights as to the plausability that an antagonist binding site exists and that the agonists share this binding site *in vivo* .

In order to determine the nature of the antagonism of nicotine by pempidine, the ability of pempidine to alter the dose-response relationships of nicotine in producing depression of spontaneous activity and antinociception in mice was determined. Dose-response curves for nicotine were determined in the presence of increasing doses of the antagonist and evaluated for differences in the ED_{50}'s and the maximum effect produced by nicotine at each dose of pempidine. In addition the structural requirements for mecamylamine's antagonism of these effects of nicotine were determined by comparing the potency of several mecamylamine analogs to the parent compound.

Materials and Methods

Subjects

Male ICR mice (Dominion Laboratories, Dublin, VA) weighing 24-30 g were used for all test procedures, and a minimum of 12 mice were utilized for each dose and time point. Mice were maintained on a 12-hr light/dark cycle and had free access to Purina Rodent

Chow (Ralston Purina, St. Louis, MO) and water.

Drugs

L-Nicotine bitartrate was synthesized by Dr. Everette L. May (Virginia Commonwealth University). Pempidine was purchased from Sigma Chemical Co. (St. Louis, MO) and was converted to its tartrate salt. Mecamylamine HCl was a gift from Merck, Sharp and Dohme & Co. The mecamylamine analogs were synthesized by Drs. Everette L. May and John Suchoki (Virginia Commonwealth University). All drugs were administered in 0.9% NaCl, pH=7.4 as mg free base/kg body weight in a volume of 10 ml/kg body weight.

Experimental Procedures

Mice were placed into individual photocell activity cages (28 x 16.5 cm) immediately after sc administration of either 0.9% saline, pH=7.4 or nicotine bitartrate. They were allowed to acclimate for 10 min, and then interruptions of the photocell beams were recorded for the next 10 min. Data were expressed as % depression where:

% depression =

(counts from nicotine-treated animals/ counts from saline-treated animals) x 100.

Antagonists or saline were administered sc 10 min prior to saline or nicotine bitartrate. Dose-response curves were determined for nicotine in the presence of 0, 0.3, 1.0, and 3.0 mg/kg of pempidine. For the structure-activity studies mecamylamine, pempidine and mecamylamine analogs were administered sc 10 min prior to an ED_{84} dose of nicotine. The data were expressed as % antagonism where:

% antagonism =

[1-(% effect with antagonist pretreatment/% effect with nicotine alone)] X 100.

The potency ratios for each compound compared to (±)exo-mecmaylamine were determined where:

potency ratio = AD_{50} of compound/ AD_{50} of (±)exo-mecamylamine.

Tail-flick reaction time to a heat stimulus was determined following drug or saline administration using the method of D'Amour and Smith (1941) as modified by Dewey et al. (1970). Pre-injection control values (2-4 sec) were determined for each animal. Mice were retested 5 min after sc administration of nicotine bitartrate or saline and the latency to the tail-flick response was recorded. A 10-sec maximum latency was set to prevent tissue damage. Data were recorded as change in latency between pre- and post-injection testing for each animal. Data were expressed as % maximum possible effect (% MPE) where:

% MPE =[(test latency - control latency)/(10 sec- control latency)] x 100.

Antagonists or saline was administered sc 10 min prior to saline or nicotine bitartrate. Dose-response curves were determined for nicotine in the presence of 0, 0.03, 0.1, 0.3, and 1.0 mg/kg of pempidine. Mecamylamine analogs were evaluated for antagonistic potency as described above.

Data Analysis

ED_{50} and AD_{50} values with 95% confidence limits (C.L.) and ED_{84} values were determined by the method of Litchfield and Wilcoxon (1949). Dunnett's t-test was used to make comparisons between the maximum effects produced by nicotine in the presence of saline and each dose of antagonist (Dunnett, 1964).

Results

Pempidine antagonism

Nicotine produced a dose-responsive depression in spontaneous activity with an ED_{50} of 0.73 (0.49-1.10) mg/kg as can be seen in Figure 2. Pempidine shifted the dose-response curve for nicotine to the right in a dose-related manner. The ED_{50} of nicotine was increased to 1.46 (0.95 - 2.25), 3.01 (2.19 - 4.14), and 3.44 (2.06 - 5.74) mg/kg by 0.3,

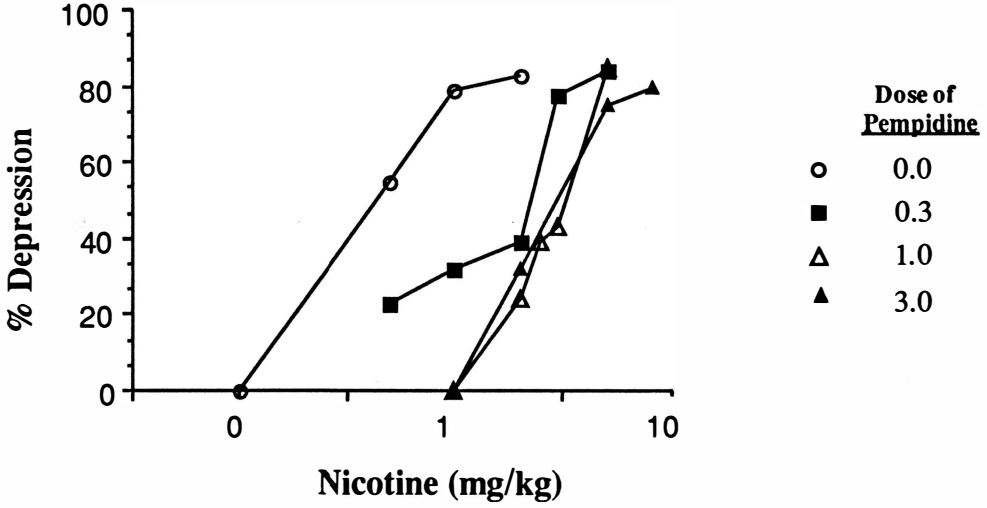


Figure 2. Antagonism of nicotine-induced depression of spontaneous activity by pempidine

1.0, and 3.0 mg/kg of pempidine, respectively. The increase in the ED_{50} of nicotine was statistically significant from control at 1.0 and 3.0 mg/kg of pempidine, as determined by a lack of overlap in the 95% confidence intervals of the ED_{50}'s (figure 2). The maximum effect of nicotine was not decreased significantly by pempidine. Pempidine alone had slight effects on spontaneous activity at doses the doses studied in that 0.1, 0.3, 1 and 3 mg/kg produced 21 (14), 23 (12), 13 (7) and 22(22) % depression, respectively. A 10 mg/kg dose of pempidine alone produced 63 (4) % depression of spontaneous activity.

The pattern of shift for nicotine dose-response curves in producing antinociception by pempidine was different than for depression of spontaneous activity, as can be seen in figure 3. The dose-response curves for nicotine were shifted downward and to the right by pempidine. The ED_{50} of nicotine was increased from 1.94 (1.22 - 3.07) mg/kg to 3.72 (2.03 - 6.81), 4.67 (2.67 - 8.13), and 26.7 (15.25 - 46.54) mg/kg by 0.03, 0.01, and0.30 mg/kg of pempidine, respectively. The increase in the ED_{50} of nicotine was significant with 0.3 mg/kg of pempidine, as can be seen from the lack of overlap in the 95% confidence interval of the ED_{50} compared to the saline-pretreated values. The dose-response curve for nicotine was shifted even farther to the right by 1.0 mg/kg of pempidine and the highest %MPE that could be obtained was 54% at 30 mg/kg of nicotine. Maximum antinociception (100 % MPE) following saline pretreatment was produced by 3 mg/kg of nicotine. The maximum antinociception that was found in the presence of 0.3 and 1.0 mg/kg of pempidine was produced by 30 mg/kg of nicotine and was 75 and 54 %MPE, respectively. The %MPE for these groups were found to be lower than the group given 3 mg/kg of nicotine following saline-pretreatment at the 0.01 level of significance. Doses of nicotine greater than 40 mg/kg with 0.3 and 1.0 mg/kg of pempidine pretreatment resulted in convulsions and death. Pempidine alone had no effect on tail flick latency at doses up to 10 mg/kg.

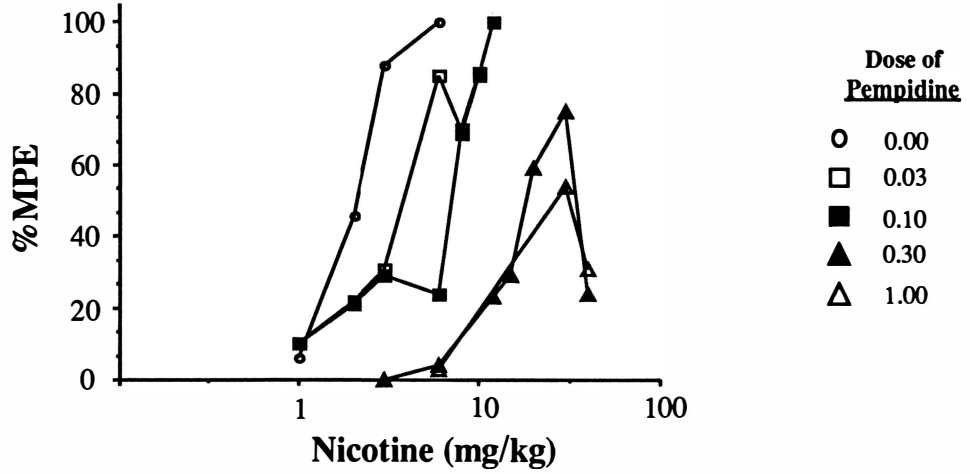


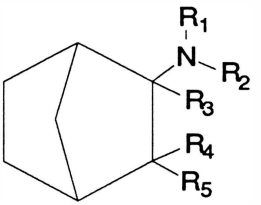
Figure 3. Antagonism of nicotine-induced antinociception by pempidine

Structure-activity relationship

Nicotine produced depression of spontaneous activity and antinociception with ED_{84}'s of 1.71 and 2.56 mg/kg, respectively. The (±)-exo-isomer of mecamylamine antagonized the effect of nicotine at these ED_{84} doses with AD_{50}'s of 0.24 (0.14-0.42) mg/kg for depression of spontaneous activity and 0.08 (0.02-0.29) mg/kg for antinociception. The potency of pempidine was not found to be significantly different from that of mecamylamine in that the AD_{50}'s for depression of spontaneous activity and antinociception were 0.10 (0.06-0.17) and 0.13 (0.05-0.29) mg/kg, respectively. The potency ratios for pempidine in antagonizing these effects can be seen in Table 1. For depression of spontaneous activity, (-)-exo-mecamylamine (IIA, Table 1) was approximately equipotent with its racemic counterpart in that it has an AD_{50} of 0.42(0.22-0.78) mg/kg. The (+)-isomer (IIB) produced a maximum of 40% antagonism of the hypoactivity at doses from 0.2 to 1.0 mg/kg. No antagonism was found at 3.0 mg/kg of the (+)-isomer due to the fact that this dose produced 55% depression of spontaneous activity when given 10 min prior to saline. Therefore, an AD_{50} could not be calculated for this compound in this assay. However, both the (-)- and (+)-antipodes of exo-mecamylamine were found to have similar potency to their racemic counterpart for antagonism of nicotine-induced antinociception in that their AD_{50}'s were found to be 0.09 (0.04-0.23) and 0.24 (0.10-0.57) mg/kg, respectively.

The N-methylated derivative of (±)-exo-mecamylamine (III, table 1) was found to be equipotent with the parent compound in that AD_{50}'s of 0.33 (0.14-0.75) and 0.17 (0.11-0.26) mg/kg were found for depression of spontaneous activity and antinociception, respectively. None of these compounds other than (+)exo-mecamylamine were found tohave agonist effects in either assay. The importance of the methyl groups in positions adjacent to the nitrogen atom in mecamylamine is illustrated in table 1. The racemic

Table 1. Influence of isomerism and omission of methyl groups from mecamylamine.



COMPOUND	+/-	endo/exo	R_1	R_2	R_3	R_4	R_5	Rel. Potency [a] Spont. Act.	Antinocicep.
mecamylamine	±	exo	H	CH_3	CH_3	CH_3	CH_3	1.00	1.00
pempidine	--	--	--	--	--	--	--	0.40	1.60
IIA	-	exo	H	CH_3	CH_3	CH_3	CH_3	1.75	1.13
IIB	+	exo	H	CH_3	CH_3	CH_3	CH_3	- [b]	3.00
III	±	exo	CH_3	CH_3	CH_3	CH_3	CH_3	1.40	2.13
IVE	±	exo	H	H	H	H	H	>40	>125
IVD	±	endo	H	H	H	H	H	>40	>125
VE	±	exo	CH_3	H	H	H	H	>40	>125
VD	±	endo	CH_3	H	H	H	H	>40	>125
VI	±	exo	CH_3	H	H	CH_3	H	4.92	31.25

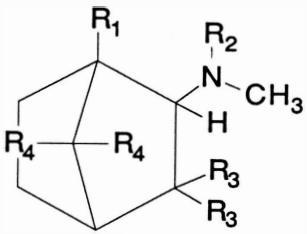
[a] Relative potency compared to mecamylamine as described in text.

[b] AD_{50} could not be determined for reasons given in Results.

isomers of exo- and endo-2-norbornamine (IVE and IVD, respectively) were found to be inactive up to 10 mg/kg in both assays. The maximum antagonism produced for depression of spontaneous activity was 10% for the exo-isomer at 10 mg/kg. No antagonism was seen with the endo-isomer up to the same dose. For antinociception, the maximum antagonism that could be produced at 10 mg/kg was 25 and 18% for the exo- and endo-isomers, respectively. The N-methylated counterparts of these compounds (VE and VD, table 1) were found to be equally inactive. The maximum antagonism of nicotine-induced depression of spontaneous activity by the N-methylated derivatives of exo- and endo-2-norbornamine was found to be 37 and 17%, respectively. For antinociception, these values were 32 and 66% at 10 mg/kg of the exo- and endo-isomers, respectively. None of these compounds had agonistic effects in either assay at 10 mg/kg. The addition of one methyl group to the 3-position of VE was found to restore efficacy in that compound VI antagonized nicotine-induced hypoactivity and antinociception with AD50's of 1.18 (0.74-1.89) and 2.50 (1.22-4.86) mg/kg, respectively (table 1).

The effect of translocating the methyl groups adjacent to the nitrogen to various positions on the norbornane ring is demonstrated in table 2. The 2-desmethyl derivative of (±)-endo-mecamylamine (compound VII, Table 2) possesses similar potency to (±)-exo-mecamylamine, with AD_{50}'s of 0.20 (0.13-0.31) and 0.13 (0.07-0.27) mg/kg for antagonism of nicotine-induced depression of spontaneous activity and antinociception, respectively. Translocation of the 2-methyl group to the 1-position has little effect on the activity of mecamylamine, as can be seen with compounds VIIIA and VIIIB. The (-)- and (+)-exo-isomers were found to have AD50's of 0.29 (0.12-0.44) and 0.25 (0.15-0.42) mg/kg for depression of spontaneous activity, respectively. For antagonism of nicotine-induced antinociception, the (-)- and (+)-exo-isomers were found to have respective AD_{50}'s of 0.89 (0.43-1.84) and 0.22 (0.09-0.56) mg/kg. Their (-)- (IXA) and (+)-endo (IXB) counterparts had similar potency in that their respective AD_{50}'s for

Table 2. Influence of transposition of methyl groups of mecamylamine.



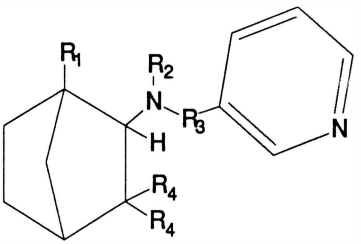
COMPOUND	+/-	endo/exo	R_1	R_2	R_3	R_4	Rel. Potency [a] Spont. Act.	Antinocicep.
VII	±	endo	H	H	CH_3	H	0.80	1.60
VIIIA	-	exo	CH_3	H	CH_3	H	1.08	11.10
VIIIB	+	exo	CH_3	H	CH_3	H	1.04	2.75
IXA	-	endo	CH_3	H	CH_3	H	0.83	1.50
IXB	+	endo	CH_3	H	CH_3	H	1.08	2.38
X	±	exo	CH_3	H	H	CH_3	4.50	12.63
XM	±	exo	CH_3	CH_3	H	CH_3	8.88	12.13

[a] Relative potency compared to mecamylamine as described in text.

depression of spontaneous activity are 0.20 (0.14-0.30) and 0.26 (0.20-0.34) mg/kg. They had similar potency in antagonizing nicotine's antinociceptive effects in that the AD_{50}'s of the (-)- and (+)-isomers were found to be 0.12 (0.002-9.66) and 0.19 (0.02-1.60) mg/kg, respectively. Translocation of the 3-gem-dimethyl groups to the 7-position (compound X, table 2) was found to reduce the potency of mecamylamine in that the AD_{50}'s for depression of spontaneous activity and antinociception were 1.08 (0.50-2.29) and 1.01 (0.58-1.76) mg/kg, respectively. The N-methylated counterpart (XM, table 2) was found to have similar potency in that respective AD_{50}'s of 2.13 (1.10-4.11) and 0.97 (0.76-1.24) mg/kg were found for depression of spontaneous activity and antinociception. No evidence of agonistic activity was found for any of these compounds at the highest dose tested for antagonism.

The effect of adding a pyridinyl group to the nitrogen atom of mecamylamine can be seen in the series of compounds in table 3. These compounds were not found to possess full activity up to 10 mg/kg, and therefore the maximum % antagonism found is reported since AD_{50} values could not be calculated. None of these compounds were found to possess agonistic activity in either assay up to 10 mg/kg. Addition of a pyridinyl group to the N-methyl of compound VA (table 1) does not increase the activity (XI, table 3) in that the antagonism produced by 10 mg/kg of this compound was not significantly greater than VA. Furthermore, the antagonism was not dose-responsive at lower doses. Addition of an N-methyl (compound XII) to XI or alteration of the methylene group connecting the nitrogen to the pyridine ring (compounds XIII and XIV) did not affect the activity as well. The antagonism seen with these compounds was variable and not statistically significant. Addition of methyl groups to the positions adjacent to the nitrogen was not found to result in an increase in antagonism. Addition of a pyridinyl group to the nitrogen of VIIIA (table 2) through a methylene group (XVA) abolishes the activity of this compound in that no significant antagonism was seen in either assay. The (+)-isomer (XVB) as well as the

Table 3. N-pyridinyl-substituted derivatives of mecamylamine



$X_1 =$ 

$X_2 =$ $=CH_2-$

COMPOUND	+/-	endo/exo	R_1	R_2	R_3	R_4	Max. Antag. [a] Spont. Act.	Antinocicep.
XI	±	exo	H	H	CH_2	H	6	47
XII	±	exo	H	CH_3	CH_2	H	9	40
XIII	±	exo	H	H	X_1	H	0	17
XIV	±	exo	H	H	X_2	H	21	48
XVA	-	exo	CH_3	H	CH_2	CH_3	12	28
XVB	+	exo	CH_3	H	CH_2	CH_3	30	14
XVIA	-	endo	CH_3	H	CH_2	CH_3	0	10
XVIB	+	endo	CH_3	H	CH_2	CH_3	15	29
XVIIA	-	exo	CH_3	CH_3	CH_2	CH_3	28	0
XVIIB	+	exo	CH_3	CH_3	CH_2	CH_3	44	27

[a] Maximum percent antagonism found at doses up to 10 mg/kg.

respective (-)- and (+)-endo isomers (XVIA and XVIB, respectively) were found to be inactive up to 10 mg/kg. Addition of an N-methyl group to the (-)- and (+)-exo-isomers (XVIIA and XVIIB, respectively) had no effect on their activity.

4. Discussion

It has been inferred that ganglionic blockers that penetrate the blood-brain barrier antagonize nicotine centrally in a purely noncompetitive fashion based upon the fact that they do not displace ^{3}H-nicotine binding *in vitro* from brain tissue (Martin, 1986). However, the correlation between the *in vivo* pharmacology of compounds and their ability to displace radiolabelled ligands from tissue determines the relevance of binding data. It is therefore necessary to evaluate the effects of these compounds in the intact animal with regard to their competitive or noncompetitive nature. Pempidine shifts the dose-response curves for nicotine in producing depression of spontaneous activity in a manner consistent with competitive antagonism, since the ED_{50}'s of nicotine are increased by doses of pempidine that do not alter its maximum effect. These data are consistent with the findings of Stolerman et al. (1982), in that mecamylamine's antagonism of nicotine in decreasing rate of responding in a drug-discrimination paradigm was completely overcome by increasing the dose of nicotine. Questions have arisen as to whether the effects of nicotine on spontaneous activity are receptor mediated. Stolerman et al. (1982) suggested that depressant effects are a nonspecific measure of nicotine's central activity. On the other hand, the ability of central, but not peripheral, antagonists to block this effect coupled with the fact that tolerance develops to the depressant effects of nicotine suggests that specific central receptor mechanisms are involved in nicotine's alteration of spontaneous activity. However it is possible that nicotine's ability to overcome pempidine's antagonism of depression of spontaneous activity may be due to non-receptor mechanisms that are not blocked by pempidine.

The pattern of shift of nicotine dose-response curves by pempidine for the production of

antinociception is different from what was found for depression of spontaneous activity and is strikingly similar to that reported by van Rossum et al. (1962) in the guinea pig jejunum. This type of antagonism was termed by van Rossum as "dualistic" antagonism, in that both the characteristics of competitive and noncompetitive antagonism are displayed. It is not clear from van Rossum's data if there is a dose of pempidine that alters the ED_{50} of nicotine while failing to alter its maximum effect, as statistics were not reported for these measures. In this study, it is clear that at doses of pempidine where the ED_{50} of nicotine is significantly increased, the maximum effect that can be obtained with nicotine is decreased. This suggests that pempidine acts noncompetitively to antagonize nicotine-induced antinociception in the mouse. The mouse tail-flick assay has an advantage in that the range of doses of nicotine that can be studied is not as limited as it is for some behavioral tests. The maximum dose of nicotine that can be used in drug-discrimination studies with nicotine is limited due to rate-suppressive effects at higher doses that are not antagonized by mecamylamine. This is not a trivial point when one is atttempting to determine if the antagonism can be completely reversed by increasing the dose of the agonist. However, *in vivo* assays possess limitations in predicting the mechanism of action of compounds. Obviously, many events occur following the administration of a compound other than receptor activation or blockade. The researcher has less control over the metabolism and distribution of compounds *in vivo* than *in vitro*. Nicotine produces changes in many neurotransmitter systems and changes in behavior following nicotine administration undoubtedly occur as a result of effects on more than one system. An example of one such limitation in this study is the range of pempidine doses that could be tested in the spontaneous activity assay. The range of doses of pempidine was limited due to its depressant effects. This may account for the inability to find a decrease in the maximum depression produced by nicotine following pempidine pretreatment.

The difference in the nature of pempidine's antagonism of these effects may also be due

to multiple mechanisms of either nicotine or pempidine. Pempidine may compete for a select subset of nicotine binding sites that are responsible for nicotine's depressant effects and that have not been elucidated under the conditions that have been studied for agonist binding *in vitro* to brain tissue. Another explanation that may attribute for the discrepancy between pempidine's ability to antagonize nicotine's depressant and antinociceptive effects is noncompetitive antagonism of spare receptors. This theory, first proposed by Stephenson (1956), assumes that the maximum possible effect that can be achieved with a compound depends upon both the number of available receptors and the physiological limits of the responding tissue. A spare receptor pool exists when the tissue responds maximally while only a fraction of the total receptor pool is occupied. A decrease in the maximum response is seen only when the receptor pool is depleted to a critical level by a noncompetitive antagonist. This theory has been supported by the studies of Furschgott (1955) and Nickerson (1956). The pattern of the shift of the nicotine dose-response curves in producing depression of activity by pempidine is consistent with this model. The lack of pempidine's ability to decrease the maximum possible effect of nicotine-induced hypoactivity may indicate that the spare receptor pool for this effect is not sufficiently depleted by doses of pempidine up to 3 mg/kg. Studying the binding of the agonists as well as antagonists under *in situ* or *in vivo* conditions may provide insights as to which of these explanations best accounts for the antagonism of nicotine-induced depression of spontaneous activity and antinociception by pempidine.

The structural alterations made in the mecamylamine molecule reveal the positions within the molecule that are important for its antagonistic activity. The compounds in table 1 clearly demonstrate that the methyl groups in the N and 3 positions are crucial for activity. It is further illustrated in table 2 that at least 3 methyl groups must be present at the N, 1, 2 and 3 positions for optimal potency. Furthermore compounds X and XM in table 2 demonstrate that the presence of methyl groups in the 7- as opposed to the 3-position significantly reduces the potency, possibly due to increased steric hindrance of the

nitrogen. Table 3 clearly shows that addition of bulky substituents to the nitrogen reduces the activity of these compounds. One finding that was consistent with all compounds tested was the lack of effect of stereoisomerism on their activity and potency. This can be seen by comparing all compounds that differ in designation by A or B, with A consistently denoting the (-)-isomer and B denoting its (+)antipode. The data show that exo/endo isomerism is without effect on the activity and potency of these compounds as well. These findings are in agreement with those of Stone et al. (1956) for antagonism of nicotine-induced convulsions and pupil dilitation in mice. These investigators found that of the N, 2 and 3 methyls and the methylene bridge, at least three must be present for optimal activity and potency. Furthermore, increasing the bulk of the N-substitution decreased the potency of the antagonists in these assays. They likewise found that stereoisomerism had little effect on the potency of all analogs tested. Therefore, the activity and potency of mecamylamine seems to depend only upon the presence and position of the methyl groups adjacent to the nitrogen atom in both the CNS and at the ganglia.

The methyl groups may serve two possible roles in the activity and potency of mecamylamine. They may serve to provide steric bulk to block a binding site or ion channel, or as electron-donating groups that increase the base strength of the nitrogen. Bretherick et al. (1962) examined the structure-activity relationships of a number of pempidine analogs for antagonism of nicotine-induced contractions of guinea-pig ileum. They found that attachment of electron-donating groups to the nitrogen atom in pempidine increased potency, whereas electron-withdrawing groups in this position decreased potency. Therefore the base strength of the nitrogen atom in these compounds appears to be important for their potency.

Such meager structural requirements for the activity of mecamylamine suggests that, if a binding site exists for these compounds, it is not likely to be the agonist binding site. The nitrogen of mecamylamine is not contained within a ring, which was found to be necessary for agonistic activity (Chance et al., 1978). Furthermore, the pyrrolidyl nitrogen of

nicotine is in close proximity to the pyridine ring, and increasing the distance between the two has been shown to decrease the activity of nicotinic agonists (Chance et al., 1978). Addition of such a moiety to active structural isomers of mecamylamine was found to abolish activity suggesting that the nitrogen cannot gain access to its site of action with such bulky substituents in place. These findings lend support for noncompetitive antagonism of nicotine by these compounds, as it does not appear that the agonists and antagonists possess similar structural requirements for their activity.

III. Receptor Binding Interactions of Nicotinic Agonists and Antagonists

Introduction

The existence of "receptive substances" for the action of nicotine was first proposed by Langley (1905) as a result of observations regarding nicotine's ability to contract skeletal muscle. Since that time, nicotinic receptors have been postulated at the autonomic ganglia and in the brain as well. Originally, evidence for the existence of central nicotinic receptors was that nicotine produced a myriad of behavioral effects that were selectively antagonized by ganglionic-blocking agents that penetrated the blood-brain barrier. Since the early 1970's numerous attempts have been made to characterize a nicotinic binding site to brain tissue. A variety of cholinergic agonists and antagonists, as well as α–neurotoxins and nicotine itself have been used. This subject has been reviewed in detail by Martin (1986).

The relevance of the nicotine binding sites that have been characterized in brain remain in question with regard to nicotine's pharmacology. One of the most consistent findings is that none of the central nicotinic antagonists, such as mecamylamine and pempidine, displace ^{3}H-nicotine binding to brain. Although this suggests that these compounds act as noncompetitive antagonists, pharmacological studies that address this issue have led to ambiguous findings. Mecamylamine has been shown to possess both competitive and noncompetitive characteristics in its antagonism of nicotine at the ganglia (van Rossum et al.,1962) and in the CNS (Stolerman et al.,1987). It has also been shown that the nicotinic receptor can exist in a variety of states. Some of these states are termed "desensitized" states due to the fact that agonist binding does not lead to receptor activation. Many investigators have postulated that the nicotine binding sites characterized to date *in vitro* are

in fact desensitized receptors. ^{3}H-Nicotine binding *in situ* to brain slices has been characterized by a number of investigators (Marks et al., 1986; Clarke et al., 1984). However, the sensitivity of these binding sites to displacement by the central nicotinic antagonists has not been documented. Binding studies with radiolabelled reversible ganglionic blockers such as mecamylamine and pempidine *in vitro* and *in situ* have not been documented as well.

Over the last decade, quantitative receptor autoradiography has proven to be a valuable tool for the determination of radioligand binding to discrete brain areas. It has been applied to the study of many receptor-ligand interactions, including nicotine. An excellent review on the methods, advantages and possible pitfalls of this technique has been provided by Kuhar (1985). Other pertinent methodological considerations regarding tritium quantitation by the use of tritium-sensitive films has been provided by Geary et al. (1985) and Geary and Wooten (1985). Quantitative autoradiography allows the investigator to study radioligand localization patterns *in situ* using tissue slices. This method offers advantages over classical *in vitro* methods in that the architecture of the tissue remains intact and quantitation is more sensitive in that discrete brain areas may be studied. The advent of computerized densitometry has rendered the technique even more amenable to standardization and quantitation (Goochee et al., 1980). Nicotinic receptors have been studied autoradiographically using these techniques by a number of investigators (Clarke et al., 1985a; Segal et al., 1978; Duggan et al., 1976). The pattern of localization in brain that has been found for ^{3}H-nicotine is consistent for all of these studies. Furthermore, the localization pattern of ^{3}H-nicotine binding sites is identical to that of ^{3}H-acetylcholine in the presence of a muscarinic antagonist (Clarke et al., 1985a). The effect of the antagonists on ^{3}H-nicotine binding *in situ* to any of these brain areas remains to be documented autoradiographically.

Therefore *in situ* studies were carried out to determine the sensitivity of ^{3}H-nicotine binding to displacement by nicotinic antagonists using rat brain. The characteristics of

^{3}H-nicotine binding were compared *in situ* and *in vitro*. The *in situ* ^{3}H-nicotine binding in the presence and absence of nicotinic antagonists was further quantitated autoradiographically in discrete brain areas. The binding characteristics of ^{3}H-pempidine were determined *in vitro* and *in situ* to rat brain for comparison with those of the agonists and to test the hypothesis that the agonists and antagonists may share binding sites that have not been elucidated to date.

Materials and Methods

Animals

Male ICR mice (Dominion Laboratories, Dublin, VA) weighing 18-25 g were used for the *in vitro* binding experiments. Male Sprague-Dawley rats (Dominion Laboratories, Dublin, VA) weighing 200-250 g were used for *in situ* binding and autoradiography experiments. All animals were kept on a 12 hr light/dark cycle and given Purina Rodent Chow (Purina Inc., St. Louis, MO) and water *ad libitum*.

Materials

[^{3}H]-L-Nicotine (80 Ci/mmol) was purchased from New England Nuclear (Boston, MA), and purity was determined by thin-layer chromatography (methanol:chloroform: ammonium hydroxide, 70:30:0.2). [^{3}H]-Pempidine (80 Ci/mmol) was a gift from Dr. Richard Young, New England Nuclear (Boston, MA), and purity was determined by thin-layer chromatography as described for [^{3}H]-L-nicotine and by HPLC using a Flo-one\Beta radioactive flow detector (Radiomatic Instr. and Chem. Co., Inc., Tampa, FL). (-)-Nicotine and (+)-nicotine were kindly donated by Dr. Everette L. May of Virginia Commonwealth University (Richmond, VA) as their bitartrate salts. (-)-Cotinine was a gift from Dr. Edward Bowman of Virginia Commonwealth University. (±)-Atropine sulfate, bethanechol hydrochloride, hexamethonium, (±)-anabasine, (-)-cytisine, mecamylamine and pempidine were purchased from Sigma Chemical Co. (St. Louis, MO).

[^{3}H]-L-Nicotine binding *in vitro*

[^{3}H]-L-Nicotine binding was performed *in vitro* according to the method of Scimeca and Martin (1988) with minor modifications. Tissue homogenate was prepared from whole mouse brain (minus cerebellum) in 10 volumes of ice-cold 0.05 M Na-K phosphate buffer (pH 7.4) and centrifuged (17500 g, 4 °C) for 30 min. The pellet was then

resuspended in 20 volumes of ice-cold glass-distilled water and allowed to remain on ice for 60 min before being centrifuged as before. The resulting pellet was then resuspended to a final tissue concentration of 40 mg/ml of buffer. To various concentrations of [^{3}H]-L-nicotine was added 0.5 ml of tissue homogenate for a final incubation volume of 1 ml. Specific binding was defined as the difference in the amount of binding in the presence and absence of 100 μM L-nicotine tartrate and determinations were made in triplicate. The tissue was incubated for 2 hr at 4 °C and then rapidly filtered through Whatman GF/C filter discs (previously soaked overnight in 0.1 % poly-l-lysine) using a vacuum manifold (Millipore Corp., Bedford, MA). Following three consecutive washes with ice-cold buffer, the filter discs were allowed to air dry and then placed in scintillation vials with 10 ml of Budget-Solve (Research Products International Corp., Mt. Prospect, IL). The vials were vigorously shaken for 1 hr and then counted by scintillation spectrometry. Correction for quench was by external standardization. Following transformation of the data by the method of Scatchard (1949) the K_d and B_{max} values were determined using the LIGAND program of Munson and Rodbard (1980) supplied by NIH.

Displacement of [^{3}H]-L-nicotine binding at 1 nM was determined in the presence of increasing concentrations of various ligands and converted to % displacement where:

% displacement = binding displaced by ligand concentration ÷ binding displaced by 100 μM nicotine tartrate.

The IC_{50}'s were determined from a plot of the log concentration vs. %displacement and converted to K_i values by the method of Cheng and Prusoff (1973).

[^{3}H]-L-Nicotine binding *in situ*

Rats were decapitated and their brains quickly removed and frozen in isopentane at -60

°C. The brains were then mounted onto cryostat chucks and stored at -70 °C until sectioned. Following equilibration of the mounted brain at -20 °C for 1 hr, 20 μm sections were taken and thaw-mounted onto slides coated with 0.5 % gelatin (w/v) and 0.05% chromium potassium sulfate (w/v). The sections were stored overnight under vacuum in a dessicator at 4 °C and used for binding studies the following day. Sections containing primarily frontal cortex and caudate putamen (A8900-A6300, König and Klippell, 1963) were used for the binding isotherms and Scatchard analysis, as these sections have no longitudinal gradient of binding for [^{3}H]-L-nicotine (Clarke and Pert, 1985). The slides were placed in slide mailers (Thomas Scientific, Swedesboro, NJ) and incubated as described for the *in vitro* assay. Following incubation, the slides were removed, placed in staining racks and carried through four consecutive 500 ml washes of ice-cold buffer. The sections were then removed by wiping the slides twice with Whatman GF/C filters. The duplicate filters were then placed in the same scintillation vial, solubilized overnight with 1 ml of TS-2 (Research Products International Corp., Mt. Prospect, IL). The samples were then acidified with 1 ml of 1N HCl and counted by scintillation spectroscopy in 10 ml of Budget-Solve (Research Products International Corp., Mt. Prospect, IL). Correction for quench was by external standardization. The data were analyzed as described above. The amount of protein per section was determined by the method of Bradford (1976) by collecting 5 adjacent sections and homogenizing in 1 ml of buffer.

Displacement studies were performed as described for the *in vitro* assay using 2 nM of [^{3}H]-L-nicotine. The data were analyzed in the same manner as well.

[^{3}H]-L-Nicotine Autoradiography

Autoradiograms of the [^{3}H]-L-nicotine binding to various sections were obtained by using conditions identical to the *in situ* assay and a 10 nM radioligand concentration. Nonspecific binding was assessed in the presence of 100 μM nicotine tartrate. Displacement of this binding was also assessed in all areas in the presence of 100 μM pempidine tartrate. Instead of wiping the sections from the slides following the washes,

the slides were rapidly dried under a stream of cool, dry air and placed in a dessicator under vacuum at 4 °C overnight to assure uniform hydration. The sections were then apposed in duplicate to tritium-sensitive film (Hyperfilm-^{3}H, Amersham Corp., Arlington Hts., IL) along with a range of tritium standards (0.114-9.468 nCi/mg) (^{3}H-microscales, Amersham Corp., Arlington Hts., IL) for 10 weeks. All studies were conducted in triplicate. Section designations were made according to the nomenclature of König and Klippel (1963), where the number of microns rostral to an F0 plane is given, so that larger numbers represent more rostral sections. The F0 plane is defined as the plane dividing the diencephalon and the mesencephalon. The autoradiograms were quantitated using a computerized densitometric system (MCID System, Imaging Research Inc., Toronto, Canada) and an IBM PC. The % displacement was calculated as described above.

Calibration of ^{3}H-microscales against brain paste standards

The ^{3}H-microscales were calibrated against brain paste standards to correct for tritium quench. Brain paste was obtained by homogenizing 3 rat brains in 1 ml of deionized water. The paste was then centrifuged for 30 min to remove air bubbles. Aliquots were weighed into microfuge tubes and 50 µl of various concentrations of [^{3}H]-L-nicotine were added for final standard values of nCi/mg tissue, wet weight. The paste standards were then vortexed for 5 min and centrifuged for 10 min to remove any air bubbles. Actual standard values were then obtained by weighing aliquots of the radioactive brain paste standards on Whatman GF/C filters, which were then solubilized and counted as described for the tissue sections. The standards were then immersed in liquid nitrogen, mounted onto cryostat chucks. Triplicate 20 µm sections were taken of each brain paste standard, thaw-mounted onto gelatin-coated slides and stored as described for the autoradiographic sections. The brain paste standards and ^{3}H-microscales were then apposed to the tritium-sensitive film for 10 weeks. Values for the ^{3}H-microscales were obtained by fitting a third degree polynomial equation to the brain paste standards and these calibrated values

were used for quantitation of the radioactivity in each brain area.

[^{3}H]-Pempidine binding *in vitro* and *in situ*

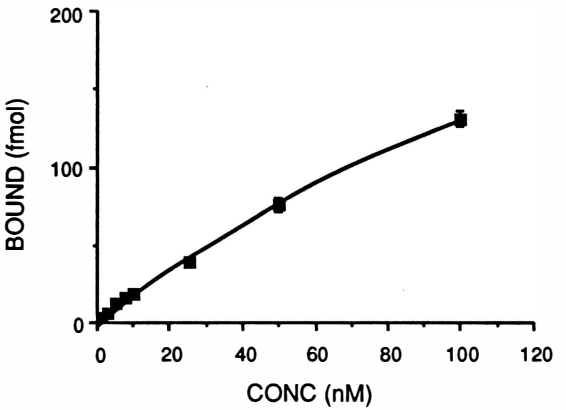
[^{3}H]-Pempidine binding to mouse brain was assessed *in vitro* using the assay conditions described for [^{3}H]-nicotine. This binding was further assessed with alterations of time, temperature, buffer and protein concentration. Displacement of 1 nM of [^{3}H]-pempidine by various concentrations of nicotine and pempidine was assessed as described for [^{3}H]-nicotine *in vitro*. [^{3}H]-Pempidine binding to rat brain was assessed *in situ* as described for [^{3}H]-nicotine. The time course of [^{3}H]-pempidine binding was also assesed *in situ* at 4, 25 and 37 °C. Using assay conditions for [^{3}H]-nicotine binding, [^{3}H]-pempidine binding was assessed to sections from A10,000 to A1200 (König and Klipell, 1963). [^{3}H]-pempidine binding was assesed *in situ* as described for [^{3}H]-nicotine binding using two alternate buffers of 10 mM HEPES or 50 mM Tris. Displacement of 1 nM of [^{3}H]-pempidine by various concentrations of nicotine and pempidine was assessed as described for [^{3}H]-nicotine *in situ*.

Results

[^{3}H]-L-Nicotine binding *in vitro* and *in situ*

[^{3}H]-L-Nicotine was found to label two binding sites in mouse brain *in vitro* . As can be seen in figure 4, [^{3}H]-L-nicotine bound to brain homogenate *in vitro* in a concentration dependent manner and displayed the characteristics of two binding sites, as can be seen by the Scatchard transformation of the isotherm. The specific binding ranged from 45 to 85% of the total binding. The binding appears to approach saturation at 10 nM, however the binding increases linearly at concentrations higher than 20 nM. Resolution of the Scatchard plot yields K_d and B_{max} values of 0.9 nM and 12 fmol/mg protein, respectively for the high affinity site. The low affinity site was found to have a K_d of 194 nM and B_{max} of 1265

A.



B.

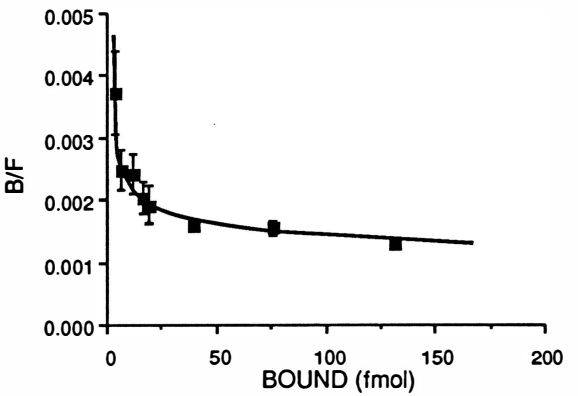


Figure 4. ^{3}H-L-Nicotine binding to mouse brain homogenate *in vitro* .

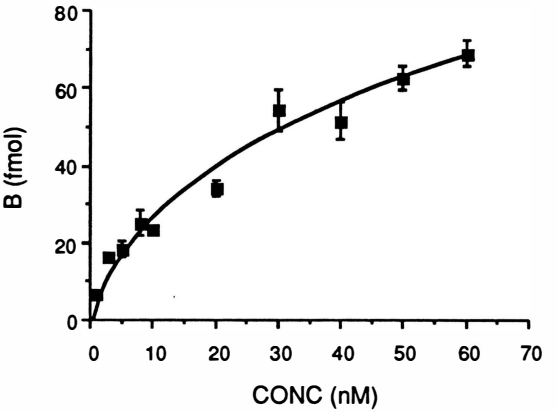
fmol/mg protein.

[^{3}H]-L-Nicotine binding was found to display similar characteristics *in situ* to rat brain slices as can be seen in figure 5. The binding was saturable and the specific binding was found to be 65 to 90% of the total. Transformation of the isotherm gave yield to a curvilinear Scatchard plot as with the *in vitro* binding. Resolution of the Scatchard plot resulted in K_d and B_{max} values of 2 nM and 67 fmol/mg protein, respectively, for the high affinity site. These values for the low affinity counterpart were found to be 99 nM and 482 fmol/mg protein, respectively. The *in situ* binding of [^{3}H]-L-nicotine was selective to displacement by nicotinic agonists, as can be seen in figure 6. Displacement of this binding by the nicotinic agonists (-)-cytisine, (-)-nicotine, (+)-nicotine and (±)-anabasine gave K_i values of 0.5, 2.5, 42 and 94 nM, respectively. Displacement with the muscarinic compounds (±)-atropine and bethanechol resulted in K_i values of 14 and 6.4 μM, respectively. The K_i for hexamethonium was 76 μM, whereas (-)-cotinine, pempidine and mecamylamine did not compete for this binding effectively as their K_i values were greater than 1000 μM.

Autoradiography of [^{3}H]-L-nicotine binding

Quantitation of autoradiograms of *in situ* [^{3}H]-L-nicotine binding to various sections resulted in the data presented in tables 4, 5 and 6. The data presented in table 4 demonstrates the localization pattern of nicotinic receptors in sections A10,000 to A6200, consisting primarily of frontal cortex and caudate putamen (figure 7). The largest number of nicotinic receptors were found in the septal nucleus (sl), cortex (COR) and caudate putamen (cp) and the specific binding ranged from 65 to 82% of the total for these regions. Although some displaceable binding was found in the fornix (F), it represented only 43% of the total. The specific binding found in the corpus callosum (TCC and RCC), anterior commisure (CA), globus pallidus (GP) and triangular septal nucleus (ts) was less than 14%

A.



B.

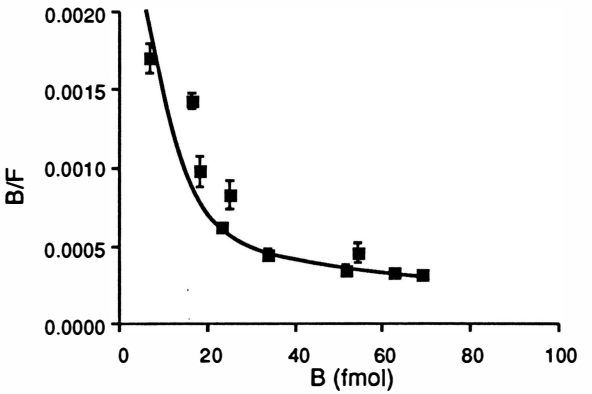


Figure 5. ^{3}H-L-Nicotine binding to rat brain slices *in situ*

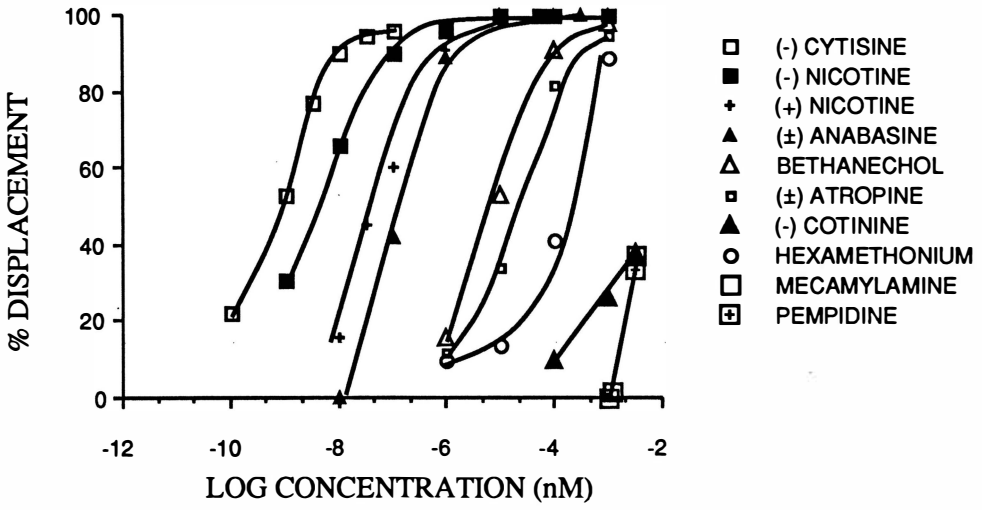


Figure 6. Displacement of ^{3}H-L-Nicotine binding *in situ*

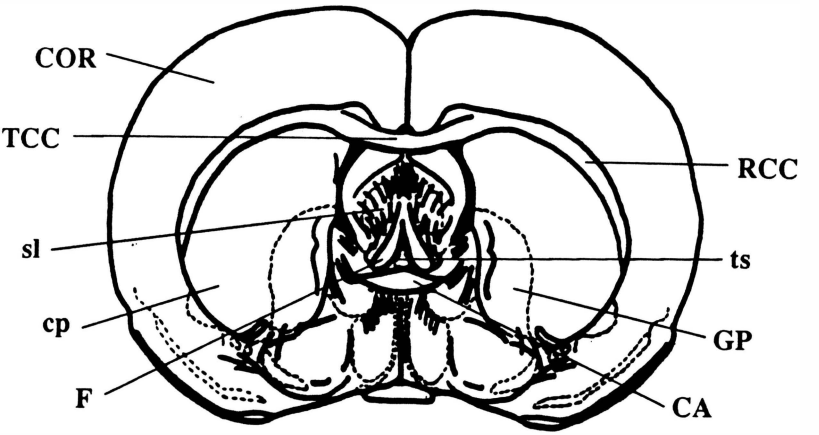


Figure 7. Anatomical map of section A7000 (König and Klippell, 1963)

Table 4. Distribution of ^{3}H-L-Nicotine binding and displacement by pempidine to sections A10,000 - A6200

Brain area	Displaced [a] Nicotine	Pempidine	% Displacement [b]
COR	5.344 (2.139)	3.165 (1.244)	59
cp	4.271 (1.670)	2.621 (0.800)	61
sl	7.809 (3.299)	6.046 (3.000)	77
F	2.956 (1.114)	1.315 (0.783)	44
TCC	0.534 (0.310)	0.366 (0.354)	69
RCC	0.713 (0.358)	0.053 (0.053)	7
CA	1.473 (0.385)	0.998 (0.513)	68
GP	0.619 (0.536)	0.384 (0.259)	62
ts	0.909 (0.909)	0.819 (0.819)	90

[a] Mean (s.e.m.) fmol/mg wet weight tissue

[b] % displacement with 100 μM of pempidine as defined in text

of the total binding. Pempidine was found to compete for the [^{3}H]-L-nicotine binding most effectively in the septal nucleus, cortex and caudate putamen, with 77, 59 and 61 % displacement, respectively. There was little displacement by pempidine in the other areas of these brain sections. An anatomical map of these areas is shown in figure 7.

The localization pattern of nicotinic receptors in sections A5700 to A4000, which are at the level of the thalamus and hippocampus is shown in table 5. The largest number of nicotinic receptors were found in the medial habenula (mh), dorsolateral geniculate bodies (dcgl) and lateroventral and ventrodorsal thalamic nuclei (tl and tvd), followed by the ventrolateral thalamic nuclei (tv) and the cortex (COR). Little specific binding was found in the hippocampus (HI) and hippocampal funiculus (FH). Displacement of this binding by 100 μM of pempidine was greatest in the medial habenula, dcgl, thalamic nuclei and cortex, and least in the hippocampus and hippocampal funiculus. An anatomical map of these regions is demonstrated in figure 8 and representative autoradiograms are shown in figure 9.

Table 5. Distribution of ^{3}H-L-Nicotine binding and displacement by pempidine to sections A5700 - A4000

Brain area	Displaced [a] Nicotine	Pempidine	% Displacement[b]
COR	11.369 (4.288)	9.681 (5.536)	85
HI	6.448 (3.925)	6.009 (4.346)	93
FH	0.285 (0.146)	0.173 (0.158)	61
mh	56.854 (10.136)	41.325 (19.566)	73
dcgl	31.559 (5.416)	23.066 (12.268)	73
tl	22.573 (3.129)	11.951 (8.168)	53
tvd	16.259 (2.624)	8.916 (5.760)	55
tv	10.469 (1.410)	5.136 (2.761)	49

[a] Mean (s.e.m.) fmol/mg wet weight tissue

[b] % displacement with 100 μM of pempidine as defined in text

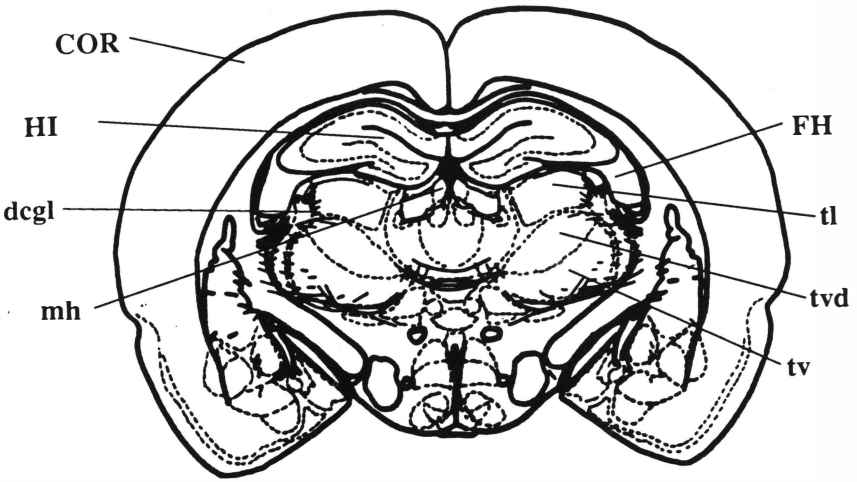


Figure 8. Anatomical map of section A4400 (König and Klippell, 1963)

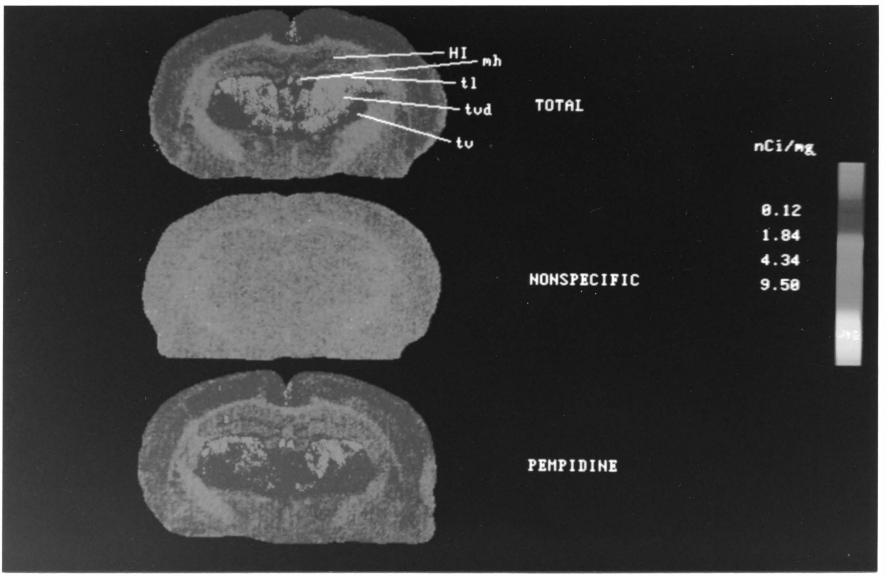


Figure 9. Representative autoradiograms of ^{3}H-L-nicotine binding to section A4400

The localization pattern of nicotinic receptors in sections A2400 to A1200 is demonstrated in table 6. The greatest amount of [^{3}H]-L-nicotine binding was found in the interpeduncular nucleus (IPN), superficial gray layer of the superior colliculus (SGS), medial geniculate bodies (mcgm), substantia nigra pars reticulata (SNR) and cortex (COR). The specific binding in these regions represented 66 to 96% of the total binding. Less binding was found in the hippocampus (HI), reticular formation (FOR) and the dorsal commissure of the hippocampus (CFD) and the displaceable binding was only 52, 47 and 32% of the total for these areas, respectively. The greatest degree of pempidine displacement of ^{3}H-L-nicotine binding was in the interpeduncular nucleus, superficial gray of the superior colliculus, medial geniculate bodies, substantia nigra pars reticulata and cortex. Pempidine did not displace a significant amount of binding in the hippocampus and dorsal hippocampal commissure. An anatomical map of these regions is demonstrated in figure 10 and representative autoradiograms are shown in figure 11.

Table 6. Distribution of ^{3}H-L-Nicotine binding and displacement by pempidine to sections A2400 - A1200

Brain area	Displaced[a] Nicotine	Pempidine	% Displacement[b]
COR	5.838 (2.190)	3.594 (1.184)	62
HI	2.946 (1.928)	2.231 (1.416)	76
CFD	0.973 (0.396)	1.050 (0.620)	108
SGS	20.053 (4.878)	12.273 (7.689)	62
mcgm	11.254 (4.196)	8.509 (3.828)	76
SNR	6.325 (2.744)	4.500 (1.874)	71
FOR	2.156 (1.806)	1.628 (1.386)	76
IPN	75.865 (12.214)	47.116 (25.878)	62

[a] Mean (s.e.m.) fmol/mg wet weight tissue

[b] % displacement with 100 μM of pempidine as defined in text

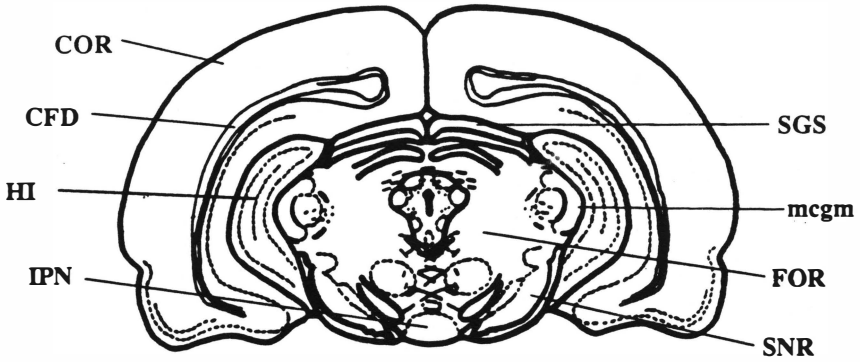


Figure 10. Anatomical map of section A1800 (König and Klippell, 1963)

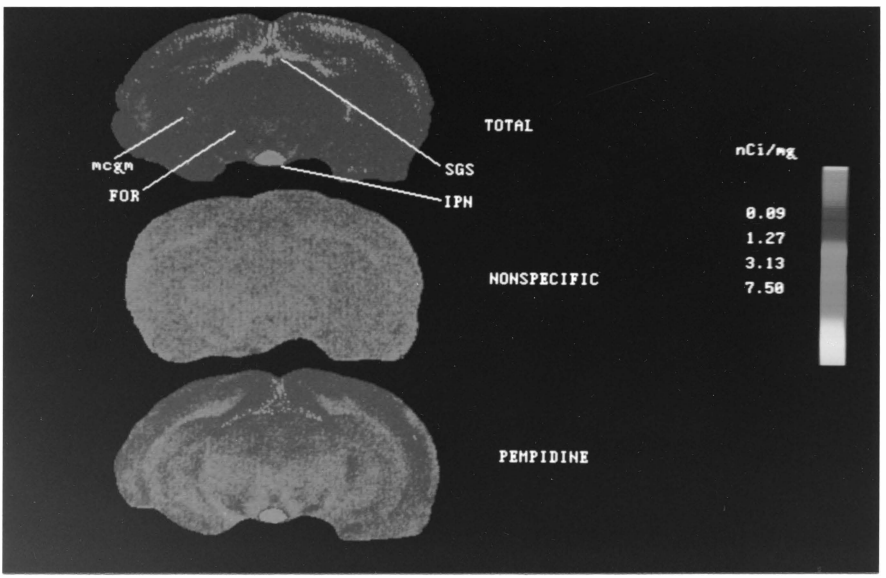


Figure 11. Representative autoradiograms of ^{3}H-L-nicotine binding to section A1800

The displacement of [^{3}H]-L-nicotine binding was also assessed in the presence of a range of nicotine and pempidine concentrations in those areas containing the largest number of nicotinic receptors. The % displacement of ^{3}H-L-nicotine binding to various areas in section A7000 by 1, 10, and 100 μM of nicotine and pempidine can be seen in figure 12. Nicotine competed effectively for this binding at all concentrations and displaced 78 ± 6, 77 ± 6, and 49 ± 10 % of the total binding in the cortex, caudate putamen, and septal nucleus, respectively, at a concentration of 100 μM. The respective values for pempidine at this concentration were 12.2 ± 4.3, 8.2 ± 3.1, and 4 ± 4. At 1 μM, the amount of ^{3}H-L-nicotine binding displaced by pempidine was 7 ± 4, 8 ± 6, and 1 ± 1 % of the total in the cortex, caudate putamen, and septal nucleus, respectively. These values for 1 μM of nicotine were not different from those found with 100 μM.

A similar pattern is seen with the other two remaining sections, as can be seen with section A4400 in figure 13 and with section A1800 in figure 14. Nicotine competed for ^{3}H-L-nicotine binding effectively in the cortex, various thalamic nuclei, and medial habenula, as can be seen in figure 13. Pempidine displaced only 7 ± 3, 8 ± 5, 4 ± 2, 14 ± 4 and 6 ± 1 % of the total ^{3}H-L-nicotine binding in the cortex, lateroventral thalamic nucleus, ventrodorsal thalamic nucleus, ventrolateral thalamic nucleus and medial habenula, respectively, at a concentration of 100 μM. Figure 14 also demonstrates the inability of pempidine to compete effectively for ^{3}H-L-nicotine binding to the cortex, hippocampus, superior colliculus, medial geniculate body, interpeduncular nucleus and reticular formation at this relatively high concentration. As with the two preceding sections, nicotine was found to compete effectively for this binding at all concentrations studied.

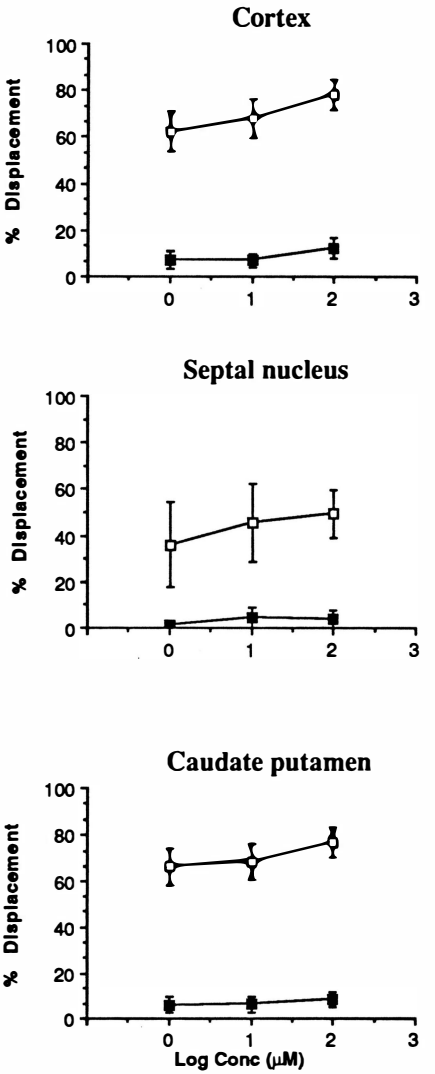


Figure 12. Displacement of ^{3}H-L-nicotine binding to regions in section A7000 by nicotine (□) and pempidine (■).

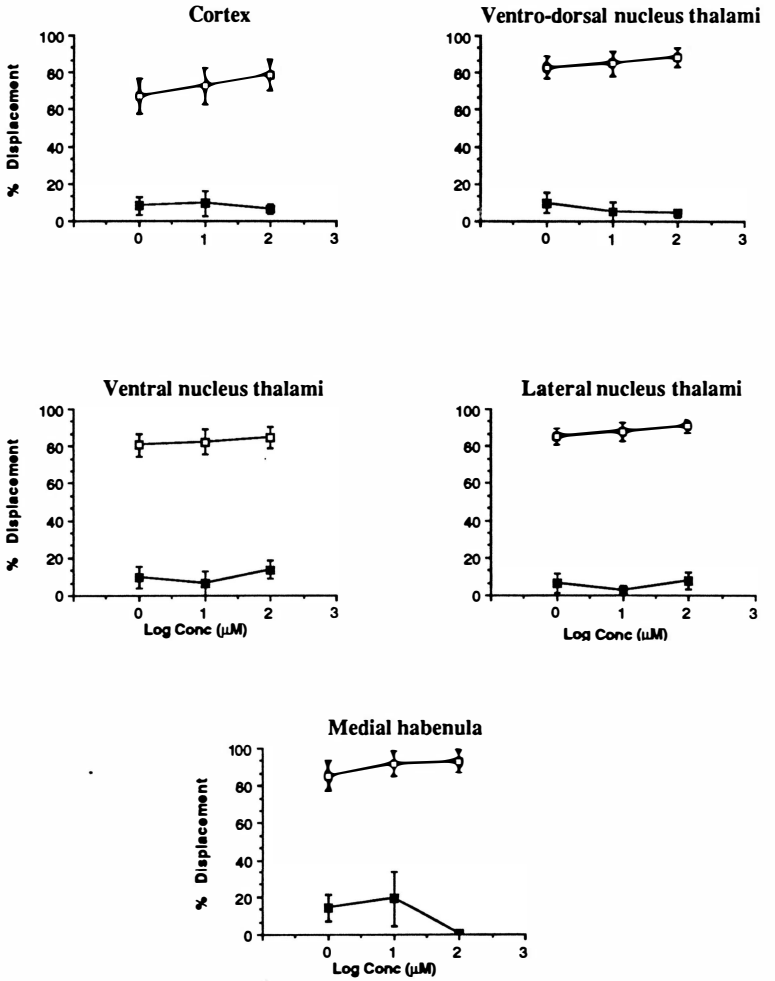


Figure 13. Displacement of ^{3}H-L-nicotine binding to regions in section A4400 by nicotine (□) and pempidine (■).

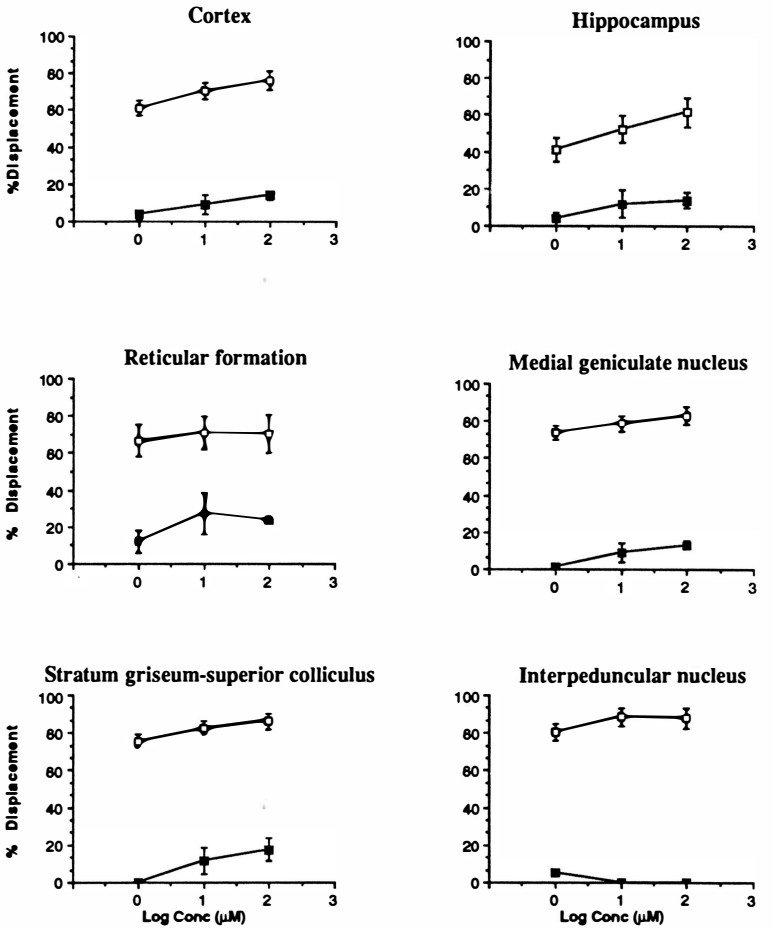


Figure 14. Displacement of ^{3}H-L-nicotine binding to regions in section A1800 by nicotine (□) and pempidine (■).

[^{3}H]-Pempidine binding to rat brain *in vitro* and *in situ*

The binding of ^{3}H-pempidine to mouse brain homogenate was studied under a number of conditions *in vitro*. Under the same conditions used for ^{3}H-L-nicotine binding, ^{3}H-pempidine was not found to bind in a manner consistent with receptor binding, as can be seen in figure 15. Pempidine did not displace 1 nM of ^{3}H-pempidine from brain homogenate in a saturable manner as the greatest amount of displaceable binding was only 5% of the total binding at 10 μM. The time course of ^{3}H-pempidine binding was assessed at 37 °C. The greatest amount of displaceable ^{3}H-pempidine binding in the presence of 10 μM pempidine was found at 90 min and represented only 30 ± 3 % of the total binding. Increasing the number of washes of the filters with ice-cold buffer did not significantly increase the % specific binding.

Investigation of ^{3}H-pempidine binding *in situ* to rat brain slices under a number of conditions led to similar results as *in vitro*. ^{3}H-Pempidine binding under the same conditions as ^{3}H-nicotine produced an isotherm and Scatchard plot inconsistent with receptor binding, as can be seen in figure 16. The time course of ^{3}H-pempidine binding was also assessed at 4, 25 and 37 °C. At no time or temperature was the displaceable binding found to be greater than 40 % of the total binding. No significant difference was found in the ability of ^{3}H-pempidine to bind to various brain sections from A10,000 to A1200 as well. Athough the greatest amount of specific binding was found in sections rich in thalamic nuclei, it represented less than 35 % of the total binding. Varying the time of wash from 2 sec to 2 min did not significantly increase the percentage of specific binding. Nicotine was found to increase the amount of displaceable ^{3}H-pempidine binding, which was neither concentration nor temperature dependent. The greatest amount of displaceable ^{3}H-pempidine binding was found with 50 nM nicotine at 4 °C and represented only 25 % of the total binding. Utilizing a 10 mM HEPES buffer or a 50 mM Tris buffer, both at pH=7.4, did not result in an increase in the amount of ^{3}H-pempidine binding.

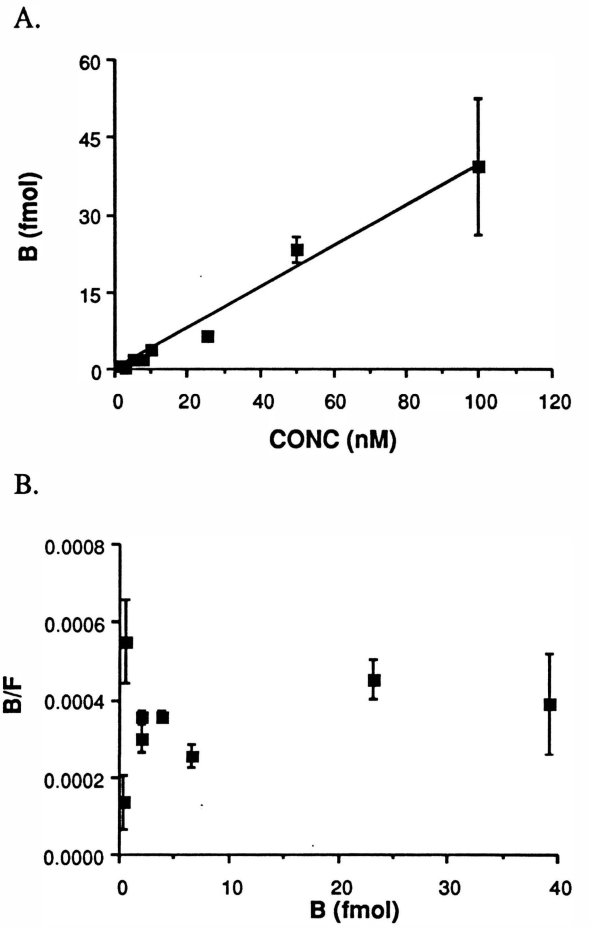


Figure 15. ^{3}H-Pempidine binding to mouse brain *in vitro*

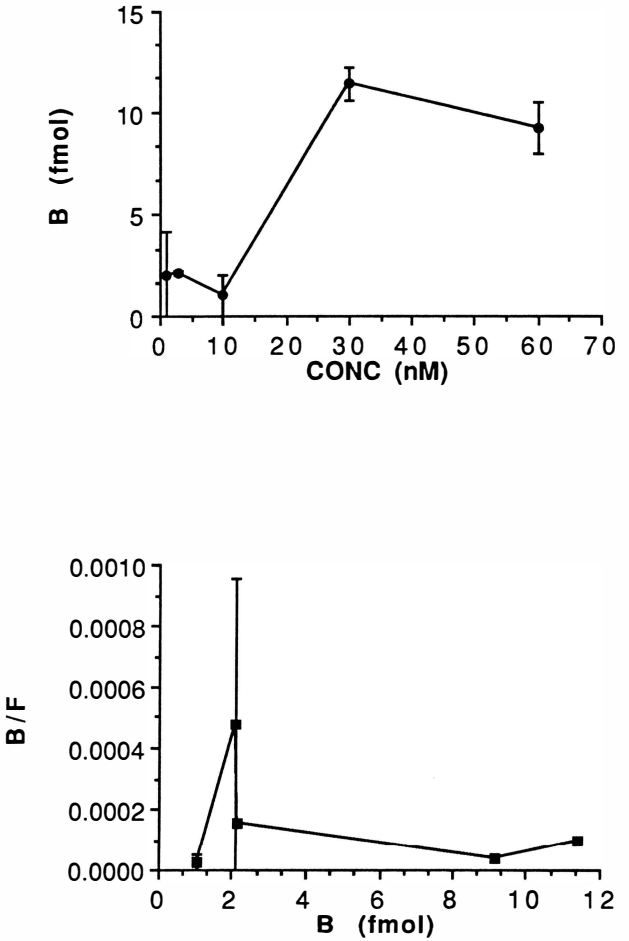


Figure 16. ^{3}H-Pempidine binding to rat brain *in situ*

Discussion

The characterization of central ^{3}H-L-nicotine binding sites *in vitro* and *in situ* revealed similar findings. The K_d and Bmax values for the two ^{3}H-L-nicotine binding sites *in vitro* and *in situ* were found to be similar. Also, the displacement of ^{3}H-L-nicotine binding to the high-affinity binding site by various cholinergic compounds was similar to what has been found *in vitro* (Marks and Collins, 1982; Scimeca and Martin, 1988). Neither cholinergic agonists nor antagonists have been found to displace ^{3}H-L-nicotine binding to a low-affinity binding site (Scimeca and Martin, 1988). The nicotinic antagonists hexamethonium, mecamylamine, and pempidine were not found to compete for this binding *in situ* with high affinity. Similar findings *in vitro* have led some investigators to conclude that these compounds act as noncompetitive antagonists in the CNS (Marks and Collins, 1982), although pharmacological evidence is lacking. Romano and Goldstein (1980) have suggested that the long incubation times used for agonist binding may shift the receptor into an agonist-selective state, and that binding studies may be a poor indicator of mechanism of action. Another possibility is that the homogenization of the brain tissue alters the ^{3}H-L-nicotine high-affinity binding site in a manner that renders it antagonist-insensitive. The inability of the antagonists to displace ^{3}H-L-nicotine binding to brain slices suggests that homogenization alone does not render the high-affinity binding site antagonist-insensitive.

Autoradiographic localization of ^{3}H-L-nicotine binding to brain slices revealed a pattern consistent with that reported previously (Clarke et al., 1984; Marks et al., 1986a). ^{3}H-L-Nicotine binding sites were found to be most plentiful in regions containing cholinergic innervation (Hoover et al., 1978; Armstrong et al., 1983; Mesulam et al., 1983). The most dense labelling was found in the interpenduclar nucleus, and several investigators have found evidence for cholinergic innervation of this area (Lake, 1973;

Cuello et al., 1978; Sastry,1978; Ogata, 1979; Sastry et al., 1979; Vincent et al., 1980). An increase in firing rate of neurons has been found following ionophoretic application of acetycholine and carbachol into the interpeduncular nucleus of rats (Brown et al., 1983). The action of both of these compounds was antagonized by hexamethonium, d-tubocurarine, and mecamylamine. Similar results were found in rat brain slices with carbachol. This study suggests that functional nicotinic receptors are contained in the interpeduncular nucleus and that mecamylamine acts to antagonize the effects of agonists on neurons in this area. Pempidine, however, was not found to compete *in situ* for ^{3}H-L-nicotine binding in this region with relatively high affinity, as can be seen in figure 14.

Dense labelling of the medial habenula and striatum was also found with ^{3}H-L-nicotine. The medial habenula has been shown to project cholinergic efferents to the interpeduncular nucleus and receive afferent innervation from the nucleus triangularis septi as well as other nuclei from the postcommissural septum (Herkenham and Nauta, 1977). Specific interactions between nicotine and nicotinic antagonists have not been documented in this region. The effects of nicotine on neurons in the striatum have been documented by a number of investigators. Giorguieff et al. (1975) found that acetylcholine and carbachol increased the release of newly synthesized dopamine *in vitro* from rat striatal slices and *in vivo* from cat caudate nucleus and that both agonists were antagonized by mecamylamine. Giorguieff et al. (1976) likewise demonstrated that pempidine would antagonize acetylcholine's stimulation of dopamine release from rat striatal slices. As can be seen in figures 12 and 13, pempidine did not compete effectively for ^{3}H-L-nicotine binding to either of these sites.

Collinridge and Davies (1980) have demonstrated that acetylcholine increases the firing rate of neurons in the substantia nigra pars reticulata. Nicotine has also been shown to increase the firing rate of neurons in the substantia nigra pars reticulata in rats when administered by either iontophoresis or subcutaneous injection (Lichtensteiger et al., 1982).

Iontophoresis of dihydro-β-erythroidine into this region antagonized nicotine's effect. Clarke et al. (1985) have likewise shown that nicotine given intravenously stimulates the firing rate of single units in the substantia nigra pars reticulata and that this effect is completely antagonized by mecamylamine. These investigators suggested that peripheral mechanisms are also involved in this action of nicotine in that the bisquaternary ganglionic blocker chlorisondamine antagonized most of nicotine's effects when given intravenously. The effects of nicotine and nicotinic antagonists on the other areas found to contain large numbers of ^{3}H-L-nicotine binding sites has not been documented.

^{3}H-L-Nicotine binding was found to be dense in those areas in which nicotine has been shown to possess effects on neurotransmitter release and neuronal firing rate which are antagonized by mecamylamine and pempidine. The experiments cited above unfortunately do not address the issue of competitive or noncompetitive antagonism of nicotine by these compounds. The inability of pempidine to effectively displace ^{3}H-L-nicotine binding to all of these areas clearly demonstrates that these compounds do not compete for the agonist binding sites that have been elucidated to date. These findings show that the inability to detect displacement of agonist binding by the antagonists is not due to the inability to measure the binding in areas that are specifically rich in nicotine binding sites by *in vitro* techniques. Therefore, nicotinic antagonists do not compete effectively for ^{3}H-L-nicotine *in situ* binding even in specific brain regions containing a large density of nicotine binding sites, suggesting that pempidine is a noncompetitive antagonist of nicotine.

Evidence for the noncompetitive nature of pempidine's antagonism of nicotine is further strengthened by the lack of ^{3}H-pempidine binding under conditions that result in high-affinity agonist binding. Since nicotine did not affect the binding of ^{3}H-pempidine to brain tissue, it is unlikely that the inability of these compounds to displace agonist binding can be explained by an agonist-induced shift of the receptor to an antagonist-insensitive state, as suggested by Romano and Goldstein (1980). The inability of ^{3}H-pempidine to

bind to brain tissue is surprising due to its relatively potent antagonistic properties. However, it is clear that if a binding site exists for the antagonists, its characteristics are distinct from those of the agonist binding sites that have been elucidated to date. The results of these binding studies with both nicotine and pempidine suggest that these compounds act at mutually exclusive sites.

The elusive issue that these studies do not address is the functional significance of these ^{3}H-L-nicotine binding sites and their relevance to nicotine's pharmacology. Although it is clear that pempidine neither competes for ^{3}H-L-nicotine binding to specific nuclei rich in agonist binding sites nor binds to brain tissue itself under conditions maximized for agonist binding, one cannot conclude that pempidine is a noncompetitive antagonist of all of nicotine's effects based on binding studies alone. Several investigators have argued that these high-affinity binding sites have functional significance based on the fact that upregulation occurs following chronic treatment (Marks et al., 1983; Schwartz and Kellar, 1983). This may suggest however, that the high-affinity states are associated with tolerance and receptor desensitization as opposed to behavioral effects and receptor activation. Such a model has been proposed for nicotinic receptors in electric eel (Conti-Tranconi et al., 1982).

Other strong evidence in favor of the functional significance of these high-affinity binding sites is that the localization patterns for nicotine's metabolic effects and high-affinity binding sites are well correlated (London, 1985). However, if the sites that lead to receptor desensitization and activation are present on the same macrocolecule, such a finding would not be unexpected. Nicotine has been shown to cause both activation and desensitization of purified, reconstituted nicotinic receptors from electroplaque (Conti-Tranconi et al., 1982). These investigators suggest that distinct agonist binding sites modulate these effects that are present on the same macromolecule. The fact that mecamylamine completely antgonizes nicotine's metabolic effects and yet does not displace agonist binding even at micromolar concentrations suggests that these binding sites may not

mediate the behavioral and metabolic effects of nicotine. It is interesting that nicotine's effects on neurotransmitter release and neuronal excitation cited above occur only at micromolar concentrations. This brings the relevance of agonist binding sites of nanomolar affinity into question. Of course, it is possible that the preparations used can only detect effects that are reasonably large compared to those necessary for nicotine to exert behavioral effects in the whole animal. However, nicotine has been shown to produce antinociception only at doses that result in micromolar concentrations in plasma (Tripathi et al., 1982). Therefore, central nicotinic antagonists do not compete for high-affinity ^{3}H-L-nicotine binding to relevant brain areas, suggesting that either these compounds are noncompetitive antagonists, or that these high-affinity agonist binding sites do not mediate nicotine's behavioral, biochemical, or electrophysiological effects in brain.

IV. General Discussion

The research presented in this thesis was undertaken to address the ambiguity of the mechanism of action of nicotinic antagonists in the brain. The assumption of noncompetitive antagonism of nicotine by these compounds has been based largely upon the fact that they do not displace ^{3}H-nicotine or ^{3}H-acetylcholine binding to brain tissue *in vitro*. Pharmacological studies to corroborate this assumption are lacking, however. The study by Stolerman et al. (1987) using drug discrimination was difficult to interpret due to confounding behavioral effects of nicotine. Although the mechanims of action of these compounds has been studied extensively in the periphery, there is some discrepancies regarding their competitive or noncompetitive nature at the ganglion as well (van Rossum et al., 1962). The recent findings that peripheral and central nicotinic cholinergic receptors display distinct biochemical characteristics makes extrapolation of these findings to the brain even more difficult (Whiting and Lindstrom, 1988).

Two assays for nicotine's central effects, namely depression of spontaneous activity and antinociception, were chosen for the evaluation of pempidine's antagonism of nicotine. The range of doses of nicotine that could be studied was greater than for drug discrimination due to fewer confounding behavioral effects. Pempidine displayed different characteristics with regard to antagonism of these effects of nicotine. The antagonism of nicotine-induced hypoactivity displayed the characteristics of competitive antagonism, wheras antinociception produced by nicotine was antagonized by pempidine in a noncompetitive manner. It is possible that two mechanisms for the antagonists exist, one

that is competitive and another being noncompetitive. However, the structure-activity relationship of analogs of mecamylamine's antagonism of these effects of nicotine does not suggest two distinct mechanisms. The structural requirements for antagonism of nicotine's depression of spontaneous activity and production of antinociception were found to be identical. If the agonists and antagonists shared common binding sites for modulation of motor activity, but not for pain modulation, then the structural requirements for antagonism of these effects should be different. The structure-activity relationship that was found for the antagonists was different than what has been previously reported for nicotinic agonists in the brain (Martin, 1986).

There are other possible explanations for this discrepancy. Stolerman (1987) has postulated that central depression is a nonspecific effect of nicotine, although antagonism of this effect by pempidine and mecamylamine suggests that this is a receptor-mediated effect of nicotine. Higher doses of nicotine may cause central depression through non-receptor mediated mechanisms that are not antagonized by pempidine. Noncompetitive antagonism of spare nicotine receptors for depression of spontaneous activity by pempidine is another possible explanation for the inability of pempidine to decrease the maximum effect of nicotine in this assay. The pattern of shift of nicotine's dose-response curves for these central effects, coupled with the structure-activity studies, do not support a competitive action of these antagonists.

These data therefore suggest that mecamylamine and pempidine act noncompetitively in the brain. However, the structural requirements for antagonism of these effects were found to be quite minimal. It is possible that the structural changes made in the molecule are not sufficient to delineate between multiple mechanisms for these compounds. Arguments against a competitive mechanism of action for these compounds have been based largely on *in vitro* binding assays in which the antagonists have not been shown to compete effectively for agonist binding (Marks et al., 1986). However, such assays have limited sensitivity for discrete brain regions and destroy the integrity of the tissue by

homogenization. Therefore, *in situ* studies were performed to determine if the lack of sensitivity of the agonist binding sites to displacement by the antagonists is due to homogenization of the tissue. Autoradiographic localization of ^{3}H-L-nicotine binding and its displacement by pempidine was performed to test the hypothesis that the antagonists may compete for a subset of agonist binding sites that cannot be chartacterized due to the lack of sensitivity of *in vitro* assays.

The *in situ* binding and autoradiography studies strongly suggest that neither of these issues are responsible for the lack of ability to find displacement of ^{3}H-L-nicotine binding to brain tissue by the antagonists. Pempidine did not effectively displace agonist binding to any of the areas studied. However, these studies did not address the other explanations that have been offered with regard to the antagonist's lack of ability to displace agonist binding, namely that the agonists are capable of inducing an agonist-selective state of the receptor and thereby preventing antagonist binding (Romano and Goldstein, 1980). Therefore, binding studies with ^{3}H-pempidine were conducted to address these issues and to determine if the chartacteristics of agonist and antagonist binding to brain are similar. The binding studies with ^{3}H-pempidine *in vitro* and *in situ* clearly demonstrate that, if an antagonist binding site exists, its characteristics are undoubtedly different from the agonist binding sites that have been elucidated to date. The inability of ^{3}H-pempidine to bind to brain tissue in a saturable manner under the myriad of conditions studied suggest quite strongly that the antagonists do not interact with the agonist binding sites that are labelled by these methods. These studies, taken together with the behavioral studies, demonstrate that pempidine and mecamylamine are noncompetitve antagonists of nicotine in the brain.

The noncompetitive nature of these compounds introduces several important questions pertaining to nicotine's pharmacology. Since these compounds do not appear to share a binding site with acetylcholine, then there may be some other endogenous compound that serves as a nicotinic agonist or antagonist centrally. The identification of such a compound would greatly enhance the knowledge of nicotine's pharmacology and of the nicotinic

cholinergic system. This compound may serve to modulate cholinergic function in the CNS and therefore have a role in nicotine dependence as well as pathological states associated with cholinergic disfunction. Mecamylamine has been shown to increase smoking in humans (Stolerman et al., 1973). Comparisons of the levels of a putative endogenous nicotinic antagonist in smokers and nonsmokers may yield insights into the mechanism by which nicotine reinforces the use of tobacco. Similar studies in animals might yield insights into the fundamental reinforcing properties of the nicotinic cholinergic system. Loss of cortical nicotinic receptors and basal forebrain cholinergic function has been shown to be the most consistent findings associated with Alzheimer's disease (Whitehouse and Kellar, 1987). Furthermore, the degree of senile dementia and other symptoms of this disorder have been shown to be correlated with the degree of neuronal loss (Whitehouse and Kellar, 1987). Similar findings have also been reported for dementia associated with Parkinson's disease (Whitehouse et al., 1986). It is therefore feasible that an endogenous antagonist of the nicotinic cholinergic system may have a role in this disorder, as well as senile dementia that is not due to Alzheimer's disease. Nicotine has been shown to increase learning and memory in humans and animals (Wesnes and Warburton, 1984; Iwamoto et al., 1987). Therefore, the noncompetitive nature of nicotinic antagonists in the CNS poses interesting questions pertaining to nicotine's pharmacology.

The inability of these antagonists to bind to brain tissue is puzzling due to the potency and specificity of their action. It is possible that these compounds interact with a conformation of the nicotinic receptor that exists only *in vivo*. These compounds antagonize practically all of nicotine's electrophysiological, biochemical, and behavioral effects regardless of the type of tissue and species studied, suggesting that the aspects of the nicotinic cholinergic receptor involving the antagonists have been preserved throughout the evolution of this system. The study of the relationship between the agonists and antagonists would be greatly enhanced if radioligand binding could be studied with functional nicotinic receptors. Correlations of the affinity of the receptor for the agonist

with receptor desensitization may provide insights into the issue of the functional significance of the high-affinity binding sites. Such a preparation may also be necessary for the antagonists to bind to their receptor, if such a receptor exists. These studies could be performed *in vivo* in awake animals or possibly *ex vivo* to superfused brain slices. Nicotine has been shown to release dopamine from striatal slices in such a preparation (Giorguieff et al., 1975). This effect is antagonized by mecamylamine (Giorguieff et al., 1975) and pempidine (Giorguieff et al., 1976). Although this research clearly demonstrates that mecamylamine and pempidine do not act at the same binding site as nicotinic agonists, the mechanism by which this antagonism occurs is unknown. Noncompetitve antagonism could occur if the antagonists bind to an allosteric site on the nicotinic receptor-ionophore complex that results in channel blockade or inactivation. Alternatively, this binding could result in accelerated dissociation of the agonists from their binding site. The most appropriate way to address these issues is by correlating electrophysiological, biochemical, or behavioral effects of the agonists and antagonists with receptor binding in the same preparation.

The research presented in this thesis demonstrates that mecamylamine and pempidine do not display the characteristics of competitive antagonism of nicotine in the brain. The differences in the structural requirements for agonistic and antagonistic potency suggest that nicotine and mecamylamine act at different sites in the brain. The binding studies with [^{3}H]-nicotine and [^{3}H]-pempidine support this conclusion. Therefore, mecamylamine and pempidine antagonize nicotine in the brain through a noncompetitive mechanism of action.

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Vita