# The use of in vivo binding methods to study the pharmacology of receptors in the rat central nervous system

by

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Abstract

The use of in vivo models to study the biochemistry of receptors in the central nervous system

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These studies examine the use of *in vivo* receptor binding methodologies to determine the pharmacology of antagonists at receptors in the central nervous system. The utility of *in vivo* binding methods as a tool to measure receptor occupancy within an intact biological system was demonstrated. Specifically, these studies describe the use of *in vivo* binding methods to determine the penetrability of radiolabeled ligands specific for dopaminergic and muscarinic receptors into the CNS, as well as their dose-dependent inhibition by selective antagonists. The unique contribution of these studies is that by determining the percentage of receptors occupied by various antagonists, a correlation between receptor occupancy and specific behavioral effects can be made.

In the dopaminergic studies, $^{3}$H-SCH 39166, a D1 selective antagonist, was administered to rats s.c. with competing antagonists, including D1, D2 and non-selective dopamine antagonists. The $EC_{50}$ values were obtained and compared with their $EC_{50}$ values against the D2 antagonist $^{3}$H-raclopride. It was found that D1 antagonists displaced $^{3}$H-SCH 39166 with the highest affinity, D2 antagonists displaced $^{3}$H-raclopride with the greatest affinity, and that the mixed antagonists had activity at both receptors. Significant correlations between both D1 and D2 antagonist

affinity and minimal effective doses for the conditioned avoidance response (CAR) test suggest that this behavioral effect is mediated through dopamine receptors. In addition, *ex vivo* studies were performed to confirm the results found using *in vivo* methodologies.

Similar *in vivo* studies were performed using $^{3}$H-scopolamine to examine muscarinic cholinergic receptors in the CNS and periphery. Results indicate that antagonists which cross the blood-brain barrier had the highest affinity, while those that do not cross had low affinity. Regional differences in binding were seen, which were further studied by examining differences in kinetics and the affinity of $^{3}$H-scopolamine for the different muscarinic receptor subtypes m1-m5. A significant correlation between $EC_{50}$ values for *in vivo* binding and MED values for passive avoidance responding, a behavioral test associated with learning and memory, and muscarinic receptors, was found.

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# CHAPTER 1

## INTRODUCTION: OVERVIEW OF IN VIVO METHODOLGIES

In the early 1970's, the synthesis of radiolabeled compounds with high specific activity which bound to neurotransmitter receptors led to the advancement of the study of the biochemistry of receptor systems using both *in vitro* and *in vivo* receptor binding techniques. *In vitro* binding studies generally involved providing a physiological buffer system in which the labeled ligand could optimally interact with its membrane-bound receptor. *In vitro* binding studies were vital for the characterization of membrane-bound receptors in both the brain and periphery, and allowed determinations of the affinity of various compounds for these receptors. As a consequence of these affinity determinations, some compounds were found to possess affinity for a select portion of a receptor population, and thus came to define types, or subtypes, within a family of receptors. Such was the case for muscarinic receptors, that were found to have at least two subtypes based on differences in the affinity of the antagonist pirenzepine for a subpopulation of muscarinic receptors in brain. *In vitro* receptor binding assays have since become tools to screen for compounds that could display selectivity for given receptor subtypes. They have also become important tools in drug discovery and in chemical synthesis of drugs selective for subtypes that may be involved in disease states. Other *in vitro* techniques were later developed to characterize the second messenger properties of receptor subtypes, leading to further classification of receptor subtypes based not only on differences in affinity for standard compounds, but on their functional responses as well.

Whereas *in vitro* binding techniques and second messenger assays have provided a wealth of information as to the characteristics of receptors in the peripheral and central nervous systems, these techniques are unable to establish the physiological role of a receptor in behavior. To answer questions concerning the functional significance of receptor systems in behavior, *in vivo* methodologies were utilized. *In vitro* methodologies generally use cell membranes prepared from either extracted tissue or cultured cells, whereas *in vivo* methods use intact animal that are treated with radiolabeled compounds; then the tissues of interest are then analyzed directly. The advantage of *in vivo* methods is that the interactions between receptor systems remain intact in the whole animal. In addition, *in vivo* binding methodologies allow investigators to determine how a ligand is distributed in receptor populations, especially across the blood-brain barrier. *In vitro*, drug concentration is constant, whereas *in vivo* the concentration of drug at the receptor at any one time is always changing. Therefore the ratio of selectivity of a compound for two different receptor subtypes *in vivo* may be different than what is found *in vitro* . *In vivo* methods can also assess the pharmacokinetic effects that may alter the availability of a drug for its' receptor, and therefore may more accurately determine the effects of physiology on the binding event. Finally, *in vivo* methods provide a means to determine a correlation between receptor occupancy and behavioral effects. Methods that utilize *in vivo* receptor binding may therefore provide the link between methods that are strictly *in vitro* and those that are purely physiological. Thus, they may allow for a better understanding of the pharmacology and biochemistry of receptor systems in whole animals.

For the purposes of this thesis, a distinction must be made between *in vivo* and *ex vivo* studies. Generally, in *ex vivo* studies the animal is treated,

the tissue removed, processed and the effects of the treatment determined by post hoc testing using *in vitro* procedures. With *ex vivo* studies, the outcome of the drug treatment is determined. With *in vivo* binding assays the entire procedure is performed in the intact animal, after which the tissues of interest are removed and drug effects determined directly. *In vivo* binding studies measure the interaction of the drug with its receptor directly, rather than indirectly as with *ex vivo* studies. Although the usefullness of *ex vivo* methods cannot be ignored, this review will concentrate on *in vivo* techniques using direct infusion of radiolabeled ligands.

Over the years methods have been employed that use direct infusion of radiolabeled compounds to study the physiology and biochemistry of receptor systems *in vivo* in the central nervous system (CNS). This section will review both early *in vivo* studies and more recent studies utilizing non-invasive techniques to study neurotransmitter function *in vivo* within the CNS. This review will concentrate on studies in which radiolabeled compounds are delivered directly *in vivo* via systemic routes of administration.

**Previous research using in vivo binding methods**

Much of the early work using radiolabeled compounds to examine receptor binding *in vivo* concentrated either on determinations of specific binding of compounds to brain homogenates or to the localization of various receptors in the brain and periphery using autoradiographic techniques (Murrin, 1981). Very few studies concentrated on the use of these procedures to suggest what functional role these receptors played in physiological and behavioral events. When *in vitro* autoradiographic techniques were perfected that were less costly and could be more easily controlled, a decline in the use of *in vivo* methods to determine receptor localization was seen. By the early 1980's, the number of studies using *in*

*vivo* binding methods had decreased dramatically (Kuhar et al., 1986). But the need for methods that could help answer how receptor occupation is correlated with physiological and behavioral responses has led to a new interest in the use of *in vivo* binding methodologies and an increase in the number of publications utilizing these methods, particularly in examining dopaminergic and muscarinic receptors.

Generally, early *in vivo* studies used the same types of procedures. A large concentrations of radiolabel was injected systemically followed by a large concentration of competing cold compound to determine non-specific binding. The brains were later removed. For procedures using brain homogenates, the tissues of interest were dissected and homogenized. The bound radioactivity in the homogenates was then determined using standard scintillation counting. In those studies using *in vivo* autoradiographic techniques, the brain was removed and thin slices were affixed to emulsion covered slides. Following many weeks of incubation, the slides were developed and the density of grains deposited in various regions was calculated (Murrin, 1981).

The earliest studies used *in vivo* autoradiographic methods to describe the localization of various hormonal receptors within the brain. For example, one early study used tritiated corticosterone injected systemically into adrenalectomized rats to demonstrate binding sites for this hormone in the hippocampus (Gerlach and McEwen, 1972). As with other hormones, corticosterone binds far from its site of release, the adrenal glands. Earlier autoradiographic studies using $^{3}$H-estradiol had shown low concentrations of ligand in hippocampus, but high concentrations in the preoptic area of the hypothalamus, an area associated with sexual behavior in rats (Stumpf, 1968). These two studies, when taken together, demonstated the existence

of binding sites for hormones within the brain in regions exclusive from one another and, in the case of the $^{3}$H-estradiol study, to a region known to be linked to a behavior influenced by that hormone.

Whereas a large number of early studies examined hormone receptors within the brain, other researchers were concentrating on using these same techniques to examine the exsistence of proposed receptors for neurotransmitters. Many of these early *in vivo* studies helped determine that neurotransmitter receptors are present in the brain and bind ligands that were later found to be endogenous neurotransmitters. The number of receptors examined using *in vivo* binding methods was limited only by the availability of radiolabeled compounds with high specific activity that did not rapidly dissociate from the receptor (Murrin, 1981).

Cholinergic receptors

One of the more extensively studied neurotransmitter receptors was the nicotinic cholinergic receptor, largely because of the availability of an irreversible ligand, $^{125}$I-α-bungarotoxin (α-BGT). Many of these studies examined the binding of $^{125}$I-α-BGT to motor end plates and found extremely high concentrations of ligand bound post-synaptically at neuromuscular junctions. This distribution was distinct from the distribution of acetylcholinesterase, determined by other investigators using immunocytochemisty, and provided morphological evidence of the distinction between the neurotransmitter receptors binding $^{125}$I-α-BGT, localized postsynaptically, and the enzyme acetylcholinesterase, localized in the synaptic cleft ( Murrin, 1981).

Studies examining nicotinic receptors in brain were hampered due to the inability of α-BGT to cross the blood-brain barrier. Hunt and Schmidt (1978a) found that $^{125}$I-α-BGT, administered intracerebroventricularly, bound to the

outer layers of the hippocampus, the stratum oriens, that receives input from the medial septum. They also reported that lesions of the medial septum did not change receptor number in the stratum oriens, suggesting that the nicotinic receptors in this region are postsynaptic. In addition, Hunt and Schmidt (1978b) found large amounts of $^{125}$I-α-BGT binding in other limbic structures, including other portions of the hippocampus and amygdala, as well as structures receiving sensory input, such as the olfactory bulb, superior and inferior colliculus, mammillary bodies and some cranial nuclei. Finally, they reported labelling in layers I, IV and VI of the cortex.

Just recently, a new ligand to examine nicotinic receptors in the brain has been developed. When $^{3}$H-epibatidine was administered to mice, it was found to bind specifically to nicotinic receptors in whole brain homogenates and to be selectively displaced by nicotinic antagonists (London et al., 1995).

Other early studies examined muscarinic cholinergic receptors in the CNS (Yamamura et al.,1974). In the initial studies, large quantities of $^{3}$H-quinuclidinyl benzilate (QNB) were injected i.v. into rats and the amount of specific binding to homogenates of various brain regions was assessed by scintillation counting. They found the greatest amount of binding in cortex, hippocampus and striatum, and the least amount in cerebellum. They also found the time course of binding was different for cortical structures than for cerebellum. Where specific binding in cerebellum declined rapidly, the binding in cortex did not decrease over time, out to twenty four hours post-injection. Later, an *in vivo* autoradiographic procedure was used in which $^{3}$H-QNB was administered i.v. to rats, after which the brain was removed and slices affixed to slides that were subsequently analyzed for grain density (Kuhar and Yamamura, 1975). A similar pattern of $^{3}$H-QNB binding was

found in these studies, with high levels in cortex, striatum and hippocampus and low levels in cerebellum.

These studies suggested that there is not considerable overlap in the localization of nicotinic and muscarinic cholinergic receptors. Hunt and Schmidt (1978a) reported that the pattern of binding of $^{125}$I-α-BGT in hippocampus suggests that the nicotinic receptors in this region are located primarily on cell bodies in the outer layer of the stratum oriens. Kuhar and Yamamura (1975) interpreted the pattern of binding of $^{3}$H-QNB in hippocampus as indicating that muscarinic receptors are located mainly on dendrites in the deeper regions of the stratum oriens, with little in the outer layer. In other regions, such as the sensory nuclei in the brain stem, nicotinic receptors dominate. Still others, such as the striatum, contain virtually no nicotinic receptors but large numbers of muscarinic receptors. These studies suggest that even when muscarinic and nicotinic cholinergic receptors are found postsynaptically within the same general region, they have a distinct localization on neurons and therefore probably receive input from different cells and have different roles.

More recent studies examining muscarinic receptor binding using *in vivo* procedures have concentrated on examining their theoretical kinetics. One such study examined, using mathematical models, how the washout rate of $^{3}$H-QNB, that has been found to vary by tissue, can be standardized by accounting for the differences in overall muscarinic receptor concentration in each tissue (Gibson et al., 1991). Other studies compared the binding of $^{125}$I-QNB in rat parotid gland and brain and applied various compartmental models of metabolism to determine which theoretical model best fit the data (Hiramatsu et al., 1993; Hiramatsu et al., 1994). Most recently, this group

reported that adding an iodine to the 4 position of $^{125}$I-QNB confers m2 selectivity to the molecule (Gitler et al., 1994; Cohen et al., 1995).

Dopaminergic receptors

Similar procedures to those used for cholinergic receptors were used to examine dopamine receptors, *in vivo* . One of the most widely used ligands for these studies was $^{3}$H-spiperone, or spiroperidol. The earliest studies determined the binding of $^{3}$H-spiperone in rat (Laduron and Leysen, 1977) and mouse (Hollt et al, 1977) brain homogenates. In rat brain, $^{3}$H-spiperone was found to bind specifically to areas associated with the presence of dopamine receptors, including the striatum and nucleus accumbens, but not in areas that have a low number of dopamine receptors, such as the cerebellum. The $^{3}$H-spiperone was displaced by unlabeled spiperone in striatum, but not cerebellum, again indicating the specificity of the binding of $^{3}$H-spiperone to rat brain (Laduron and Leysen, 1977). A later study (Pedigo et al., 1979) demonstrated that the neuroleptic haloperidol inhibited $^{3}$H-spiperone binding in rat striatal homogenates. Similarly, studies of the *in vivo* binding of $^{3}$H-spiperone in mouse brain found receptor densities to be highest in the striatum and lowest in the cerebellum. In addition, a stereoselective effect of the ability of butaclamol to displace 3H-spiperone from striatal homogenates was reported (Hollt et al.,1977). The l-butaclamol configuration produced no displacement, whereas the d-butaclamol isomer displaced $^{3}$H-spiperone from striatal homogenates. Hollt's group reported other neuroleptics displaced $^{3}$H-spiperone, including haloperidol and chlorpromazine.

Later, autoradiographic procedures were used to examine the binding of $^{3}$H-spiperone to dopamine receptors *in vivo* in rat brain (Kuhar et al., 1978). In these studies, Kuhar's group was able to demonstrate that $^{3}$H-spiperone

bound specifically in the striatum, nucleus accumbens and olfactory tubercle, structures known to have dopamine receptor localization. This group then used a similar *in vivo* autoradiographic procedure to confirm and extend their earlier findings with $^{3}$H-spiperone to structures in the CNS (Klemm et al., 1979). In these studies, their more detailed light microscopic analysis of grain densities in various brain regions confirmed the highest densities were seen in regions receiving dopaminergic input. A later study (Murrin and Kuhar, 1979) also reported binding of $^{3}$H-spiperone in layers V and VI of the frontal cortex in rats. Similarly, autoradiographic analysis of *in vivo* binding of $^{3}$H-spiperone in mice found the highest grain densities in striatum and nucleus accumbens (Hollt and Schubert, 1978). Again, Hollt showed a stereoselective effect on the displacement of $^{3}$H-spiperone by competing cold compounds. Cis-flupenthixol was found to displace $^{3}$H-spiperone binding in mouse striatum using this procedure, whereas trans-flupenthixol did not.

*In vivo* autoradiographic procedures were later used to examine the *in vivo* binding of $^{3}$H-spiperone following lesions to various areas sending input to stuctures containing dopaminergic receptors (Murrin et al., 1979). They found a significant decrease in receptor number in the striatum following both administration of kainic acid land decortication. No effect on the substantia nigra compacta was found following intrastriatal kainic acid lesions. When 6-hydroxydopamine (6-OHDA) was administered intrastriatally, however, receptor number in the caudate-putamen significantly increased, indicating denervation supersensitivity. This same treatment produced a significant decrease in substantia nigra receptor number. These results were interpreted as indicating that the receptors which bind $^{3}$H-spiperone in the substantia nigra compacta are on cell bodies of dopamine neurons and are distinct from other dopamine receptors in this region.

Spiperone was later found to be a D2 selective antagonist, and therefore many of these early studies need to be reinterpreted in light of the discovery of the heterogeneity of dopaminergic receptors. One recent study (Coppens et al., 1995) examined the effect of chronic haloperidol treatment on the *in vivo* binding of $^{3}$H-*N* - methyl spiperone (MSPIP). They found that treatment of rats with haloperidol for 12 weeks produced a significant increase in MSPIP binding in the striatum and nucleus accumbens, indicating an up-regulation of receptors resulting from prolonged blockade by haloperidol. A challenge dose of either the D1 antagonist SCH 23390 or the serotonin antagonist ritanserin did not alter the degree of up-regulation in these regions seen with chronic haloperidol treatment, indicating that the effect was specific to D2 receptors.

Other recent studies have used *in vivo* binding methods to determine the occupancy of dopaminergic receptor subtypes within the CNS by selective compounds. Chapter 2 of this thesis concentrates on these more recent studies and how the availability of radiolabeled ligands selective for dopaminergic receptor subtypes has led to the ability to form correlations between receptor occupancy and behavioral effects.

Opiate receptors

Another receptor that had been the subject of intense research using *in vivo* binding studies was the opiate receptor. A highly specific ligand, $^{3}$H-diprenorphine, became available early on and allowed investigators to determine the localization of opiate receptors in rats using *in vivo* autoradiographic techniques. High densities were found in the caudate putamen, locus coeruleus, substantia nigra and substantia gelatinosa (Pert et al., 1975). A more detailed study by this group (Pert et al., 1976) further characterized the binding of $^{3}$H-diprenorphine *in vivo* to discrete areas of the thalamus, amygdala, reticular formation and medulla. A later study (Atweh

and Kuhar, 1977) concentrated on the binding of $^{3}$H-diprenorphine to telencephalic regions in rats. A high grain density was found in regions of the hippocampus, particularly the dentate gyrus and entorhinal cortex, and deeper layers of the cortex. Lesion studies in which dopaminergic input to the striatum was removed led to no change in $^{3}$H-diprenorphine binding, whereas kainate lesions directly into the striatum resulted in dramatic losses of $^{3}$H-diprenorphine binding, suggesting that in the caudate opiate receptors are located postsynaptically (Murrin et al., 1980).

It is in the study of opiate receptors using *in vivo* methodologies that the first studies were performed that examined the functional role of receptors in behavior. Early work by Hollt (1975) compared the binding of $^{3}$H-naltrexone in naive mice with that seen in morphine dependent mice. They found no changes in the ability of cold naltrexone to displace $^{3}$H-naltrexone and concluded that tolerance to morphine was not due to changes in receptor number or affinity of the receptor. They also correlated $^{3}$H-naltrexone binding *in vivo* with withdrawal jumping in mice produced by cold naltrexone administration. They found that displacement of $^{3}$H-naltrexone by naltrexone occurred within the same dose range as the increase in withdrawal jumping following naltrexone administration, suggesting a correlation between opiate receptor occupation and this behavior.

A more recent study used an *in vivo* autoradiographic procedure to examine the effects of stress on opiate receptors in the CNS using $^{3}$H-diprenorphine (Seeger et al., 1984). They found that exposing rats to both prolonged intermittent footshock and forced swims in cold water produced significant decreases in the binding of $^{3}$H-diprenorphine in the periaquaductal gray and reticular formation, areas associated with nocioception.

Finally, a newly published report (Vanderschuren et al., 1995) examined the binding of $^{3}$H-diprenorphine in the brains of juvenile rats in which varying periods of social isolation were followed by social play. These authors report increases in the binding of $^{3}$H-diprenorphine in the paraventricular nucleus of the hypothalamus after social play, regardless of the amount of isolation the animal received beforehand. This would suggest a decrease in the amount of endogenous endorphins following social play, which they further suggest indicates that social interaction may decrease stress.

Other receptors studied using in vivo methods

Other receptors within the CNS have also been studied less extensively than those described above, largely due to the lack of radiolabeled ligands with sufficient specificity and affinity for the receptor of interest. Many of these studies rely on more involved methods to prevent the dissociation of the ligand from the receptor, such as covalent modification following irradiation by ultraviolet light (Murrin, 1979).

One example of an attempt to examine other receptors in brain using *in vivo* methods was the *in vivo* binding of $^{3}$H-flunitrazepam to benzodiazepine receptors. Chang and Snyder (1978) examined the the binding of $^{3}$H-flunitrazepam to mouse whole brain homogenates. Despite high levels of non-specific binding, they found that the benzodiazepines clonazepam, diazepam, and chlordiazepoxide produced a dose-dependent decrease in $^{3}$H-flunitrazepam binding in mouse brain. Further, they reported that the calculated doses at which 50 percent of the receptors were occupied with competing drug were similar to the doses that produced anticonvulsant effects in mice. Another early study (Williamson and Paul, 1978) examined the binding of $^{3}$H-diazepam to homogenates of different regions of rat brain. They found high amounts of specific binding in cortex, hypothalamus and

midbrain homogenates, intermediate amounts in striatum and septal regions and low amounts in cerebellum and pons.

## Non-invasive in vivo binding methods

More recently, advances in technology have allowed for the development of more sophisticated techniques to examine receptor biochemistry *in vivo* using radiolabeled ligands. This group of *in vivo* techniques that has been used more extensively in recent years are non-invasive techniques such as nuclear magnetic resonance (NMR) and the closely-related magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), as well as positron emission tomography (PET). These non-invasive techniques allow investigators to determine, in humans and larger non-human primates, the metabolic activity and regional blood flow to structures within the brain, as well as direct determinations of receptor binding using radiolabeled compounds.

In PET, radioisotopes with short half-lives are synthesized in a local cyclotron and attached to compounds of biological interest, that are then injected into the subject. Following an incubation period, the subject is placed in a scanner which detects the breakdown of the label. The label emits protons as it breaks down, that collide with an electron, leading to an annihilation that produces two positrons $180^0$ apart, that are then detected by the scanner. PET can be used to detect cerebral metabolism, including blood flow and oxygen use, as well as neurotransmitter synthesis and receptor binding (Mazziotta, 1994). PET has been used to examine the functional neuroanatomy of sensory and motor systems in normal subjects and in disease states such as epilepsy and stroke (Mayberg, 1994). PET has also become an aide in the diagnosis of dementias such as Alzheimer's disease

(Jobst et al., 1994) and a tool to understand the effects of disease on the brain in other illnesses such as Huntington's chorea (Jagust, 1994). The major disadvantage to the use of PET is the cost of the scanner and the need for an on-site cyclotron to synthesize the radioisotopes. In addition, inconsistencies in results between similar studies have repeatedly emerged, partly due to the fact that functional studies rely on the subtraction of the image achieved in the "inactive" state from the "active" state image, that can result in a number of confounding variables when interpreting the data (Sergent, 1994).

Other studies have used single-photon emission computed tomography (SPECT), that is similar to PET except that the radioisotopes used emit single photons and have longer half-lives, that eliminates the need for an on-site cyclotron (Mazziotta, 1994). Both PET and SPECT have good resolution, with PET being superior to SPECT.

In MRI, the scanner emits radio waves that change the orientation of the spin of the nuclei of the label as it breaks down. When the pulse of radio waves is turned off, the nuclei return to their original orientation, and emit radio waves in the process, that resonate at a characteristic frequency and rate that can then be measured (Martin et al., 1991). Whereas initially MRI was used only for structural mapping of the brain, functional MRI studies have been done more recently that require no radioisotopes or contrast media, but simply detect differences in the oxygenation of hemoglobin in the venous blood of various tissues (Mazziotta, 1994). One advantage to these functional MRI and MRS studies is the speed at which images can be recorded, in as little as 30 msec (Shulman et al., 1994). In the closely related MRS, isotopes such as carbon-13 and sodium-23 have been used to make functional determinations such as the use of lactate in various brain regions during

behavioral tasks. The spatial resolution of MRI and MRS is considered excellent, superior to both PET and SPECT, with detection of quantities in the millimolar range (Mazziotta, 1994).

The major disadvantages to the use of non-invasive techniques is their cost and the lack of availability of different types of specific highly energetic isotopes. In addition, these types of studies can be performed only in larger species of animals. Therefore correlating *in vivo* imaging results with the literature using the more standard *in vivo* binding methods described above is possible only for those studies using non-human primates, which are very few. All of the literature on *in vivo* binding of radiolabeled compounds to rat and mouse brain cannot be correlated, but only used as between species comparisons.

Despite some shortcomings, the methods described above illustrate the major advantages to the use of *in vivo* methods to examine the biochemistry of receptor systems in the CNS. These advantages include: the ability to determine CNS penetrability across the blood-brain barrier; the determination of a dose-dependent displacement of the ligand by selected drugs; the correlation between a dose that produces a given percent of occupied receptors *in vivo* with that which produces behavioral effects; and the localization of receptors using *in vivo* autoradiographic methods. In addition, *in vivo* methods may allow for the determination of selectivity of compounds for specific receptor subtypes.

As this review points out, however, the amount of information that can be gained using *in vivo* methodologies is dependent upon the selectivity and sensitivity of the tools that are available. The availability of highly energetic isotopes that can be attached to compounds of biological interest is crucial to any PET study. For the studies to be reported here, the availability of labeled

compounds found to be specific for receptor subtypes, as well as the specificity of the effects of lesions on a given receptor system, play a large role in determining the amount of information that can be gained using an *in vivo* methodology.

This thesis will examine two different examples of the use of *in vivo* biochemical methodologies to examine the biochemistry and pharmacology of receptor systems in the CNS. These studies will demonstrate how the use of these methods can answer questions ranging from the CNS penetrability of compounds to their localization and selectivity for specific receptor subtypes within the CNS. In addition, the use of *in vivo* methodologies to suggest correlations between receptor occupancy and behavioral effects will be demonstrated, with the aim of presenting possible functional roles for specific receptors in behavior. The goal of presenting this body of work is to demonstrate the usefulness of *in vivo* binding methods to understanding the biochemistry and pharmacology of the intact central nervous system. The two receptor systems that this thesis will focus on are the dopaminergic and muscarinic cholinergic systems. Various methods used to examine the occupancy of dopamine receptors *in vivo* will be presented to demonstrate how they may determine CNS penetrability, receptor localization and receptor subtype selectivity. In addition, a behavioral correlate of dopamine receptor occupancy in the striatum will be presented in order to suggest a functional role of the dopamine receptor in behavior. Similarly, an *in vivo* method to examine muscarinic receptor occupancy will also be presented, which illustrates the same principles of determinations of CNS penetrability and receptor localization. These studies will also serve to demonstrate the limitations of *in vivo* methods as a result of the lack of specific tools available for the study of muscarinic receptor subtypes.

# CHAPTER 2

# OVERVIEW OF DOPAMINERGIC PHARMACOLOGY IN THE CNS AND THE USE OF IN VIVO MODELS TO ASSESS SELECTIVITY OF COMPOUNDS FOR DOPAMINE RECEPTOR SUBTYPES

## I. INTRODUCTION

Dopamine is a catecholamine that acts both as a neurotransmitter in its own right and as the precursor for norepinephrine and epinephrine. It is synthesized by a series of steps culminating in the decarboxylation of L-dopa. Once synthesized, dopamine is released presynaptically from neurons located in distinct pathways within the CNS. These pathways include: those projecting rostrally from the dorsal portion of the substantia nigra to the caudate-putamen and globus pallidus (the nigro-striatal pathway); another rostral pathway from the ventral tegmental area to the olfactory tubercle and nucleus accumbens, that continues to the frontal cortex (the mesolimbocortical pathway); a pathway from the posterior hypothalamus to the dorsomedial thalamus (the incertohypothalamic tract), with fibers from the paraventricular nucleus of the hypothalamus to the suprachiasmatic nucleus and septal nuclei and finally; a pathway that connects the arcuate nucleus, through the tuberoinfundibular bundle, to the median eminence and pituitary gland (Role and Kelly, 1991).

Dopamine neurotransmission can be affected pharmacologically both pre- and post-synaptically through a variety of mechanisms. Dopamine neurotransmission can be increased through presynaptic mechanisms, for example, by increasing synthesis with dopa plus carbidopa, a peripheral

decarboxylase inhibitor, blocking reuptake with cocaine or, increasing release with amphetamine. Dopamine activity can also be decreased presynaptically by interfering with its storage into vesicles in the presynaptic terminal with reserpine administration. In addition, dopamine neurotransmission can be increased by blocking its breakdown to DOPAC and homovanillic acid with MAO inhibitors like pargyline (Hess and Creese, 1987).

One of the most widely examined means of decreasing dopamine neurotransmission post-synaptically is by the administration of dopamine receptor antagonists, that prevent activation of the post-synaptic neuron by blocking the receptor from dopamine. Dopamine receptor antagonists were found clinically to alleviate the symptoms of schizophrenics, which led to the hypothesis that schizophrenia was caused by an overactivation of the dopamine system, presumably in the mesolimbocortical projection (Balderssarini, 1980)

Compounds that acted as antagonists at dopamine receptors were used not only as the most prescribed drugs to treat schizophrenia, but also subsequently served as tools to better understand the pharmacology, biochemistry and localization of dopamine receptors within the CNS. Kebabian and Calne (1979 ) hypothesized that there were two distinct subtypes of dopamine receptors, designated D1 and D2, mainly based on differences in their second messenger systems. Activation of the D2 receptor by dopamine had either no effect on adenylate cylase (AC) activity or produced a decrease, whereas D1 receptor activation produced an increase in AC activity.

It was later determined that standard compounds had varying affinities for the D1 and D2 subtypes. Haloperidol and other classic neuroleptics, including thioridizine and chlorpromazine, have a greater affinity for D2

receptors and act as antagonists. The agonists Ly17755 and quinperole have greater affinity for the D2 receptor, and inhibit AC through a G-protein mediated event. Other agonists, such as SKF 38393, act at the D1 receptor to stimulate AC activity through a different G-protein (Hess and Creese, 1987). The first D1 selective antagonist developed was SCH 23390 ((R)-(+)-8-chloro-2,3,4,5 tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol ), that was found to have over 100-fold selectivity for the D1 receptor *in vitro* (Iorio et al., 1983). Subsequently, other D1 receptor antagonists were developed, one of the most potent and selective beingSCH 39166 [(-)-trans-6,7,7a,8,9,13b-hexahydro-3-chloro-2-hydroxy-N-methyl-5-H-benzo[d]naphtho-{2,1b}azepine] (Chipkin et al., 1988).

Most currently available neuroleptics used to treat schizophrenia have higher affinity for D2 receptors *in vitro*. The side effects of these antipsychotic compounds following both long- and short-term use have been well documented. Chief among the side effects produced by acute administration of antipsychotics are movement disorders known collectively as extrapyramidal syndrome (EPS). In a percentage of patients receiving neuroleptics, long-term administration of neuroleptics can result in movement disorders known as tardive dyskinesia. Tardive dyskinesias are distinguished from EPS not only in time of onset and movement characteristics, but also in the lack of response to treatment with cholinergic antagonists, whereas EPS does respond to anticholinergic treatment (Baldisserini, 1980 ). In addition, tardive dyskinesias produced by prolonged administration of neuroleptics persist after the cessation of drug treatment, whereas EPS does not. The nigro-striatal pathway has been linked to the production of extrapyramidal syndrome after acute antipsychotic treatment (Role and Kelly, 1991).

Behaviorally, D2 dopamine receptor antagonists produce a distinct pattern of responding in the conditioned avoidance response (CAR) test in both rats and monkeys. All compounds that are known to act clinically as antipsychotics produce a decrease in the number of avoidances in the CAR along with an increase in the number of escapes, without a change in the rate of failures, defined as not jumping onto a raised platform within the trial period (Cook and Davidson, 1978).

The discovery of the selective D1 antagonists SCH 23390 (Iorio et al., 1983) and SCH 39166 (Chipkin et al., 1988) gave researchers new tools to further study the pharmacology and biochemistry of the dopaminergic system. These compounds demonstrated selectivity for D1 receptors *in vitro*, with differences in affinity of 100 - 1000 fold between D1 and D2 receptors. Behaviorally, both compounds produced the same response profile in the CAR as D2 antagonists (Iorio et al., 1983; Chipkin et al., 1988). It was theorized that compounds that demonstrated D1 affinity would have potential as antipsychotics without the risk of EPS. Behavioral models in Cebus monkeys demonstrated that SCH 39166 alone did not produce abnormal movements (Coffin et al., 1992), whereas Cebus monkeys became sensitized to abnormal movements following administration of D2 antagonists (Coffin et al., 1989).

More recently, five dopamine receptor proteins have been cloned and expressed in cell lines. These receptors have been designated D1-D5 and share similar characteristics with the pharmacological D1 and D2 receptors previously described by Kebabian and Calne. In fact, the five receptor subtypes have been divided into two families, with D1 and D5 receptors being classified as "D1-like" and the D2, D3 and D4 receptors as "D2-like". Activation of both the D1 and D5 receptors produces an increase in adenylate

cyclase activity (Van Tol et al., 1991). In addition, both the D1 and D5 receptor have selective affinity for both SCH 23390 and SCH 39166 (Tice et al., 1994). In contrast, the D2, D3 and D4 receptors share the same properties in that all have a high affinity for haloperidol. Whereas D2 activation causes a decrease in adenylate cyclase activity, the effector systems of the D3 and D4 receptor are poorly understood (Strange, 1993). Other compounds that have been proposed as novel antipsychotics have varying affinity for these new subtypes. Clozapine, for example, has been proposed to have up to a nine-fold greater affinity for D4 receptors than for the other four subtypes (Van Tol et al., 1991), whereas raclopride has been shown to have much lower affinity for the D4 receptor than for the D2 or D3 receptor (Strange, 1993). The functional role of these additional receptors *in vivo* has yet to be understood, as igands that are selective for the additional dopamine subtypes are just becominig available.

The localization of these five new dopamine subtypes has been determined using both the polymerase chain reaction (PCR) and in situ hybridization. These studies found that the mRNA for the D1 receptor was located in the caudate-putamen, nucleus accumbens, olfactory tubercle and frontal cortex. These same localizations were found for the mRNA of the D5 receptor, in addition to low amounts in the hippocampus, hypothalamus and frontal cortex. The mRNA for the D2 receptor was also found in caudate putamen, nucleus accumbens and olfactory tubercle, whereas the mRNA coding for the D3 receptor was also found in nucleus accumbens and olfactory tubercle. The D4 mRNA was found in low amounts in the frontal cortex and amygdala, as well as the retina (Strange, 1993). In addition, it is thought that in certain brain regions, both the D1 and D2 receptor families are located presynaptically, and function as autoreceptors to affect the

subsequent release of dopamine (Kandel, 1991). Antagonists acting at presynaptic receptors would result in an increase in dopamine release, whereas agonist binding at presynaptic receptors would inhibit dopamine release.

This chapter will detail the studies performed to examine the *in vivo* receptor occupancy of both D1 and D2 antagonists using a variety of *in vivo* methodologies. These studies will demonstrate, using *in vivo* techniques designed to measure specific binding in tissue, as well as selective destruction of specific receptor subtypes with alkylating agents, that SCH 39166 has selective affinity *in vivo* for the D1 receptor subtype. In fact, this selectivity is greater than that predicted by *in vitro* binding. The selectivity of SCH 39166 for D1 receptors will also be demonstrated following chronic treatment using an *ex vivo* methodology. Finally, an *in vivo* autoradiographic procedure will be described which also confirms the selectivity of SCH 39166 for D1 receptors. The data to be presented will be correlated with both *in vitro* and behavioral data, as well as human PET data, to show the utility of these *in vivo* methods as not only providing a means to assess the affinity of compounds for receptors preclinically, but also as a means to determine possible dose ranges for subsequent clinical trials.

## II. IN VIVO BINDING TO D1 AND D2 RECEPTORS

The first studies to examine the *in vivo* affinity of dopamine antagonists for D1 and D2 receptors *in vivo* used $^{125}$I-SCH 38840, another D1 antagonist (McQuade et al., 1988a; 1988b). These studies used subcutaneously administered $^{125}$I-SCH 38840 in competition with D1 and D2 dopamine antagonists, which were also administered systemically.

These initial studies were designed to develop an *in vivo* binding methodology to examine the CNS penetrability of SCH 39166 and to determine the receptor subtype selectivity of SCH 39166 for D1 receptors in the CNS. In these studies, the affinity of SCH 39166 and related compounds, as well as compounds known to be D2 selective, were determined using this *in vivo* method and compared to both their *in vitro* affinity and behavioral profiles using the rat conditioned avoidance response (CAR) test. These studies are reported in published papers from this laboratory (McQuade et al., 1991a; 1991b and 1992) and are summarized here.

## METHODS

### SUBJECTS:

The subjects for all studies were male Sprague-Dawley rats (Charles River), weighing 225-300 grams at the time of the study. The animals were maintained on a normal light-dark cycle and had free access to food and water.

### MATERIALS:

For *in vitro* studies, $^{3}$H-SCH 23390 and $^{3}$H-spiperone were purchased from New England Nuclear (Boston, MA). For *in vivo* studies, $^{125}$I-SCH 38840 and $^{3}$H-SCH 39166 were synthesized by Amersham (Arlington Heights, Illinois). $^{3}$H-Raclopride was purchased from New England Nuclear (Boston, MA). SCH 38840, SCH 23390, SCH 39166, SCH 15198, SCH 12679, SCH 23389 and SCH 40853 were synthesized in the chemistry department at the Schering-Plough Research Institute. Raclopride was a gift from Astra Research Center (Sordertalje, Sweden), while A-66359 was donated by Abbott Laboratories (Chicago, Illinois). All other compounds were purchased from Research Biochemicals (Natick, MA).

PROCEDURES:

a.) In vitro binding:

Receptor binding studies for the D1 and D2 receptors were performed using $^{3}$H-SCH 23390 and $^{3}$H-spiperone, respectively, according to the method of Billard et al. (1984). First, striatal membranes were prepared by homogenizing rat striatal tissue in 50 volumes of cold 50 mM Tris-HCl, pH 7.4. The homogenates were then centrifuged at 20,000 x g for 20 min. The resulting pellet was resuspended in Buffer A and spun again at the same speed. The final pellet was suspended in Buffer A containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Buffer B). Then, in competition studies, these membranes were incubated with approximately 0.3 nM of each ligand and increasing concentrations of competing drugs in Buffer B. Non-specific binding was defined for D1 and D2 receptors by addition of 10 uM SCH 23390 and 10 uM butaclamol, respectively. After incubation at 37$^{0}$C for 20 min, the reaction was terminated by rapid filtration over GF/B glass fiber filters using a Bradel cell harvester and washed with cold Buffer A. The filters were incubated overnight in scintillation cocktail and the bound radioactivity determined in a liquid scintillation counter. The concentration of competing drug which inhibited specific binding of the radioligand by fifty percent ($IC_{50}$) was determined for each drug using the EBDA curve fit program (McPherson, 1985). These procedures were performed largely by the biochemistry section of the CNS pharmacology department, consisting of myself, Vilma Ruperto, Bill Billard and Gordon Crosby.

b.) In vivo binding:

D1 receptors: Two different procedures were used to examine the *in vivo* binding of compounds to D1 dopamine receptors. The original procedure used $^{125}$I-SCH 38840 (25 uCi or 14 pmoles per rat) injected s.c.

simultaneously with competing compounds in a volume of 500 uL. One hour later the animals were sacrificed and the striatum, frontal cortex and cerebellum were dissected, weighed and bound radioactivity determined with a gamma counter. Data were expressed as (fmoles bound/g tissue) /(uCi injected/kg body weight). Specific binding was determined by subtracting values obtained in the cerebellum, an area with low dopamine receptor density, from both cortical and striatal values. For competition studies, graphed data are expressed as percent bound in the absence of drug (% control). These procedures were performed largely by Robert McQuade, but I assisted in later studies to complete this project. These studies are reported in McQuade et al. (1988b and 1991a).

The procedure for determination of D1 receptor binding was modified upon the subsequent availability of $^{3}$H-SCH 39166. $^{3}$H-SCH 39166 (15 uCi) and competing drugs were administered subcutaneously in a volume of 500ul. One hour following 3H-SCH 39166 administration, the cortex, striatum, and cerebellum were dissected, weighed and each homogenized in 5 ml of 50 mM Tris-HCl, pH 7.4 using a Polytron. Aliquots of the homogenates were then added to glass vials with Scintisol or Ready Safe scintillation fluid (Beckman ) and bound radioactivity determined in a Beckmann scintillation counter. Data were expressed as dpm/mg tissue and cerebellar values were again subtracted from cortical and striatal values to account for non-specific tissue distribution. For time course studies, specific binding at each of seven time points (0.5, 1, 2, 3, 4, 6 and 8 hours following 3H-SCH 39166 administration) was determined by subtracting the binding found in the presence of 1 umole SCH 23390 from that obtained with vehicle treatment. For competition studies, data were expressed as percent control in the absence of drug. The $ED_{50}$ was defined as the dose of drug which inhibited 50% of control binding,

and was also determined by the EBDA computer program. These studies were performed by myself under the direction of Robert McQuade. They are reported in three separate publications (McQuade et al., 1991b; McQuade et al., 1992)

D2 receptors: The procedure for *in vivo* binding of compounds to D2 receptors was similar to that used for D1 receptors with $^{3}$H-SCH 39166. $^{3}$H-raclopride (15 uCi) was injected subcutaneously with either vehicle or competing drugs in a volume of 500ul. One hour later the cortex, striatum, and cerebellum were dissected, weighed, homogenized and analyzed as above. These studies were performed by me under the guidance of Robert McQuade and are reported in McQuade et al., 1992.

c.) Rat conditioned Avoidance Response (CAR) test:

Rats were trained to jump onto a platform 6.75 inches above a grid floor of an experimental chamber in response to a 5 sec tone in order to avoid a 10 sec foot shock (0.6 mA). Each session consisted of 20 trials, each separated by 15 sec. Two experimental sessions were conducted on consecutive days. An avoidance response was recorded if the animal jumped onto the platform before the end of the tone. An escape was scored if the rat jumped to the platform after the shock had begun, and a failure was recorded if the animal failed to jump onto the platform during the tone or the shock. The minimal effective dose (MED) was defined as the lowest dose which produced a statistically significant decrease in the number of avoidances. These studies were performed by the behavioral pharmacology group of the CNS research department at Schering-Research Institute under the direction of Vicki Coffin.

## RESULTS

The results of the time course study of $^{3}$H-SCH 39166 binding to striatum in rats is shown in Figure 1. The peak of specific binding occurred at one hour following s.c. administration and returned to background levels by eight hours post-administration. The binding to cortex remained near background levels for the length of the time course study (data not shown).

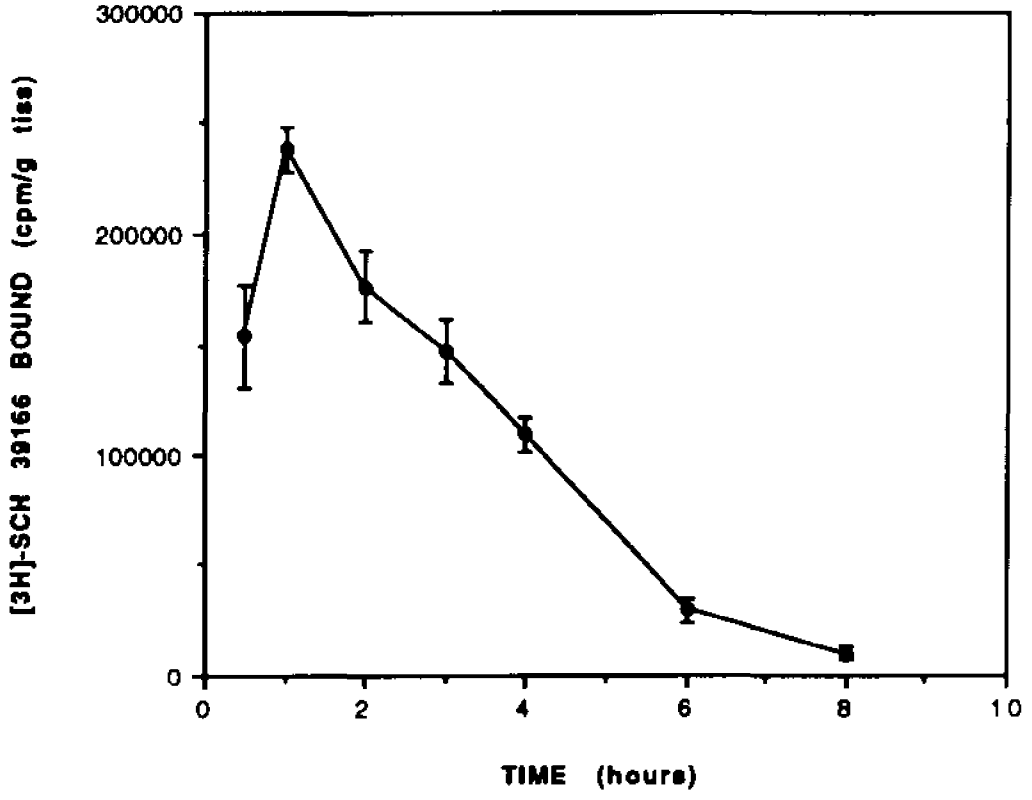



**FIGURE 1:** Time course of *in vivo* binding of $^{3}$H-SCH 39166 to rat striatum. Data represent specific binding of $^{3}$H-SCH 39166, determined by subtracting binding values obtained in the presence of 1 umole SCH 23390 from vehicle treated animals at each time point. Each point represents the mean (± S.E.) of at least three animals

Table 1 summarizes the in $ED_{50}$, $K_i$ and MED value obtained for *in vivo* receptor binding, *in vitro* binding and CAR tests, respectively, for a variety of

compounds. For each compound, the MED for CAR activity, the $K_i$ value for *in vitro* binding affinity for both D1 and D2 receptors in rat striatum, and the $ED_{50}$ for *in vivo* binding at D1 and D2 receptors in rat striatum were determined according to the methods described above. The drugs tested

**Table 1: COMPARISON OF THE IN VITRO AND IN VIVO BINDING ACTIVITIES OF SELECTED COMPOUNDS AT DOPAMINE D-1 AND D-2 RECEPTORS WITH THEIR BEHAVIORAL ACTIVITY IN THE RAT CAR TEST**

| COMPOUND | CAR ACTIVITY MED (sc) (mg/kg) | IN VITRO BINDING ACTIVITY $K_i$ (nM) | | IN VIVO BINDING ACTIVITY $ED_{50}$ (mg/kg, sc) | |
|---|---|---|---|---|---|
| | | D1 | D2 | D1 | D2 |
| SCH 23390 | 0.01 | 0.3 | 760 | 0.005 | > 100 |
| SCH 39166 | 0.03 | 1.3 | 570 | 0.016 | > 100 |
| SCH 23389 | 0.10 | 1.1 | 1062 | 0.022 | ND |
| SCH 15198 | 0.10 | 2.4 | 3770 | 0.021 | 13.65 |
| SCH 38840 | 1.00 | 1.5 | 2377 | 0.176 | ND |
| SCH 40853 | 1.00 | 3.2 | 4930 | 0.282 | ND |
| A-66359 | 3.00 | 24.7 | 2580 | 0.468 | ND |
| SCH 12679 | 5.60 | 491 | 5218 | 0.600 | 90.76 |
| Thioridazine | 30.0 | 59.0 | 9.1 | 3.35 | 8.74 |
| Chlorpromazine | 3.00 | 74.0 | 8.2 | 1.30 | 0.651 |
| Perphenazine | 0.10 | 29.9 | 1.3 | 4.79 | 0.041 |
| Haloperidol | 0.10 | 835 | 1.8 | > 100 | 0.013 |
| Raclopride | 0.03 | 22720 | 7.6 | > 100 | 0.002 |

included D1 antagonists of at least three molecular types (SCH 23390, SCH 39166, SCH 23389, SCH 15198, SCH 38840, SCH 40853 and A-66359), a pro-drug of a D1 antagonist (SCH 12679), two selective D2 antagonists (haloperidol and raclopride) and three mixed D1/D2 antagonists (thioridazine, chlorpromazine and perphenazine).

The $ED_{50}$ values for *in vivo* binding for each drug in Table 1 represent the inhibition of $^{3}H$-SCH 39166 produced by that compound in the striatum. Although the amount of specific binding of $^{3}H$-SCH 39166 in cortex was measurable, it represented approximately one-tenth of that seen in the striatum. Using $^{3}H$-SCH 39166 *in vitro*, the density of D1 receptors in rat cortex has been shown to be low (Wamsley et al., 1991). None of the compounds tested were shown to significantly inhibit this low specific binding at the range of doses tested for activity in striatum.

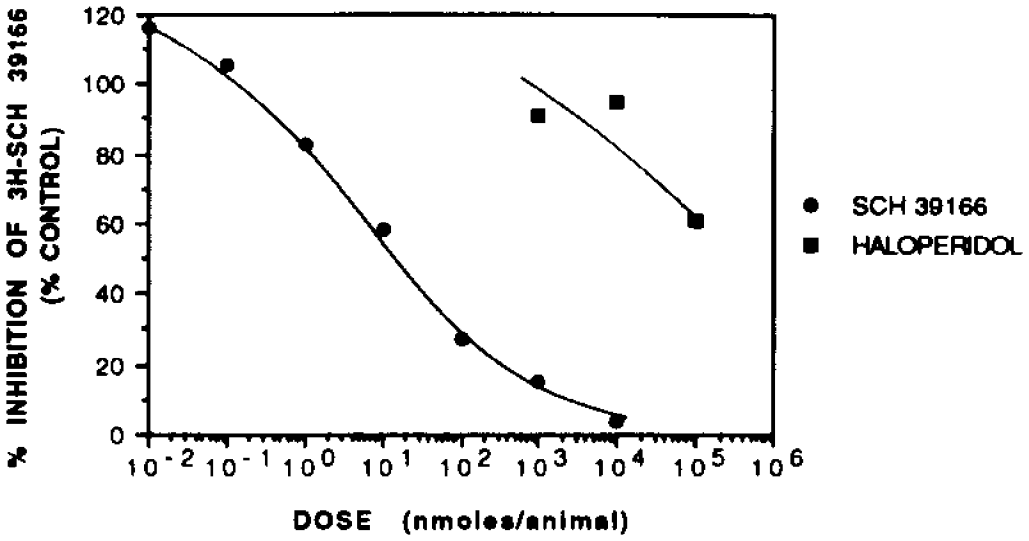
All of the seven D1 dopamine antagonists tested inhibited $^{3}H$-SCH 39166 binding in striatum. SCH 23390 was the most potent of these, with an $ED_{50}$ of 0.005 mg/kg, followed by SCH 39166 with an $ED_{50}$ of 0.016 mg/kg. Neither haloperidol nor raclopride showed inhibition of $^{3}H$-SCH 39166 binding at doses of more than 100 mg/kg.

In contrast, most of the D1 antagonists were unable to inhibit the binding of $^{3}H$-raclopride at D2 receptors at doses in excess of 100 mg/kg. As would be expected, both haloperidol and raclopride were active in this assay, with raclopride showing greater potency than haloperidol.

The mixed D1/D2 antagonists inhibited both $^{3}H$-SCH 39166 and $^{3}H$-raclopride to varying degrees. Both chlorpromazine and perphenazine exhibited greater affinity for $^{3}H$-raclopride than for $^{3}H$-SCH 39166 *in vivo*, while thioridazine had a slightly greater D1 affinity.

Dose-response curves for inhibition of SCH 39166 at D1 and D2 receptors using the *in vivo* binding procedure are shown in Figures 2a and 2b. SCH 39166 produced a dose-dependent inhibition of $^{3}$H-SCH 39166 binding to D1 receptors, but did not inhibit the binding of $^{3}$H-raclopride to D2 receptors, yielding a greater than 1000 fold selectivity of SCH 39166 for the D1 receptor *in vivo*. Haloperidol, a D2 receptor antagonist, did not inhibit $^{3}$H-SCH 39166 binding, but haloperidol did inhibit $^{3}$H-raclopride binding in a dose-dependent fashion, with a D2 selectivity ratio *in vivo* of greater than 1000-fold over the D1 receptor..

The *in vitro* activity of the thirteen representative D1, D2 and mixed D1/D2 antagonists is also shown in Table 1. The seven D1 antagonists exhibit significantly greater affinity for the D1 receptor, as compared with their D2 affinities versus $^{3}$H-spiperone. The D2 antagonists haloperidol and raclopride, on the other hand, exhibit greater affinity for the D2 receptor than the D1 receptor. The mixed antagonists all appear to have greater affinity for the D2 receptor subtype than D1, as the $IC_{50}$ values obtained for the ability of these compounds to displace $^{3}$H-SCH 39166 were higher than those obtained for $^{3}$H-spiperone. The ratio of the D2 to D1 affinity of the mixed antagonists is no greater than 25 fold, as compared with the minimum 100 fold differences in affinities for the selective D1 and D2 antagonists.



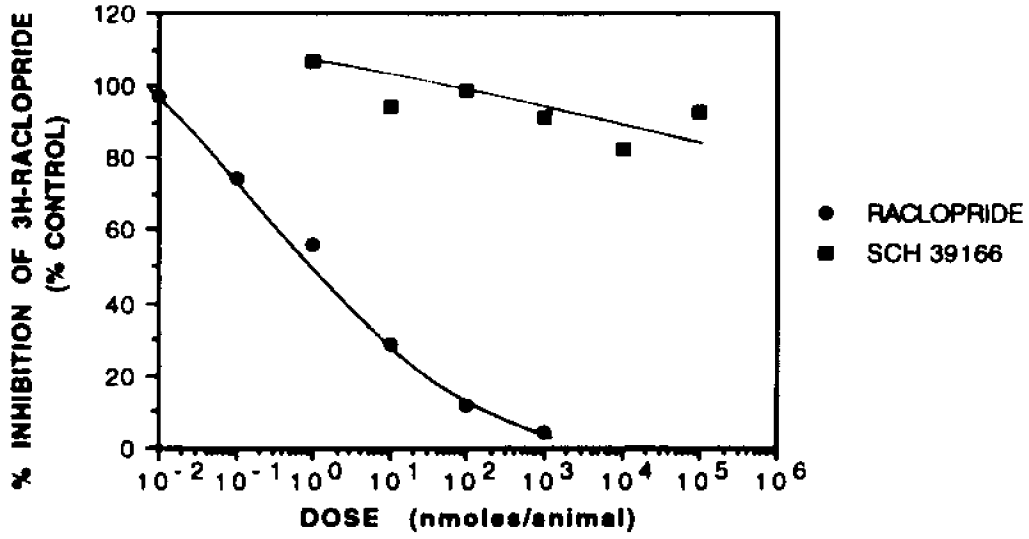




**FIGURE 2 :** a.) Representative dose response curves for SCH 39166 and haloperidol inhibition of $^{3}$H-SCH 39166 *in vivo*. b.) Representative dose response curves for SCH 39166 and raclopride versus $^{3}$H-raclopride *in vivo*.

Finally, the behavioral activity of the D1, D2 and mixed D1/D2 antagonists was assessed in the CAR test and summarized in Table 1. The most potent compound tested was SCH 23390, with an MED of 0.01 mg/kg. This was followed closely in order of decreasing potency by SCH 23390 = raclopride > haloperidol = perphenazine = SCH 23389 = SCH 15198 > SCH 38840 = SCH 40853 > chlorpromazine = A-66359 > SCH 12679 > thioridazine.

To determine how well *in vivo* receptor occupancy at the D1 and D2 receptors is correlated with behavioral activity, separate correlation coefficients were determined for the MED values in the CAR test versus the $ED_{50}$ values *in vivo* binding to each receptor subtype.

In the first set of correlations, *in vivo* binding $ED_{50}$'s versus $^{3}H$-SCH 39166 for the D1 and non-selective dopamine antagonists were compared with their MED values in the CAR. Figure 3 shows the significant positive correlation between doses of these antagonists which occupy 50% of D1 receptors and doses of these compounds which produce behavioral deficits in the CAR ($r = 0.973$, $p < 0.001$).

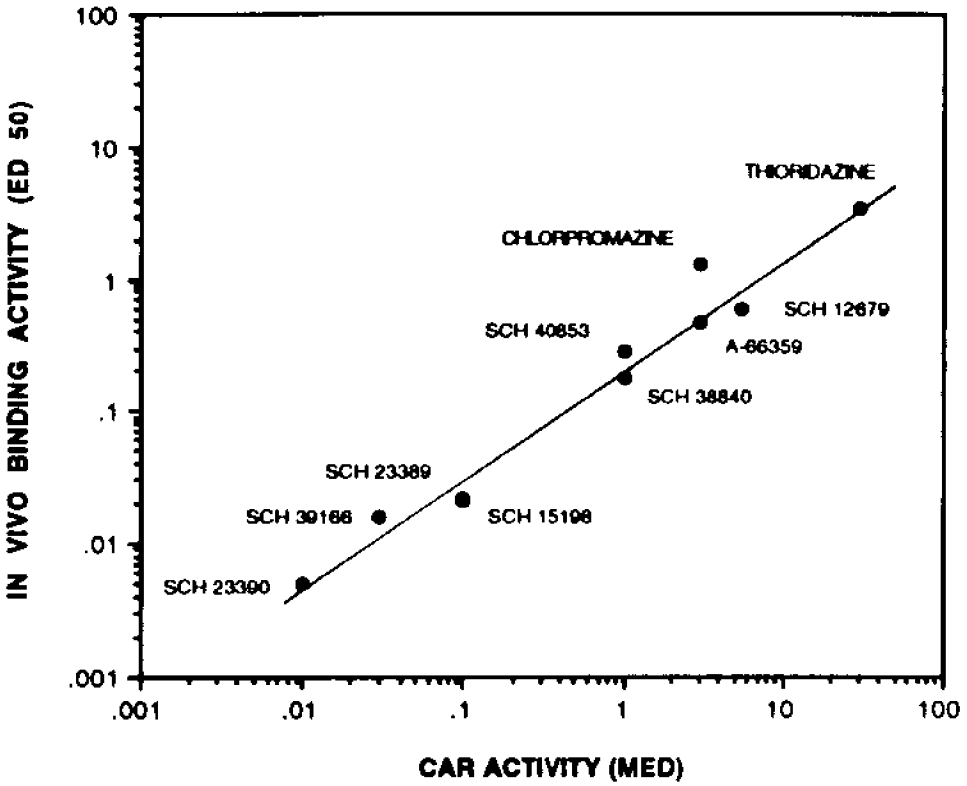



**FIGURE 3:** Correlation of D1 *in vivo* binding affinity and behavioral activity in the rat CAR test. The compounds included in this correlation were either selective for D1 receptors *in vivo*, or had < 10 fold selectivity for the D2 receptor, as determined by comparing their affinity for $^{3}$H-SCH 39166 to $^{3}$H-raclopride.

Similarly, when the $ED_{50}$ values for 50% inhibition of *in vivo* $^{3}$H-raclopride binding of D2 selective antagonists are plotted against their corresponding MED values in the CAR, a significant positive correlation emerges ($r = 0.975$, $p < 0.005$). The graphs of this significant correlation can be seen in Figure 4. These results suggest that both D1 and D2 receptor occupancy may mediate the behavioral effects in the CAR produced by dopamine antagonists.

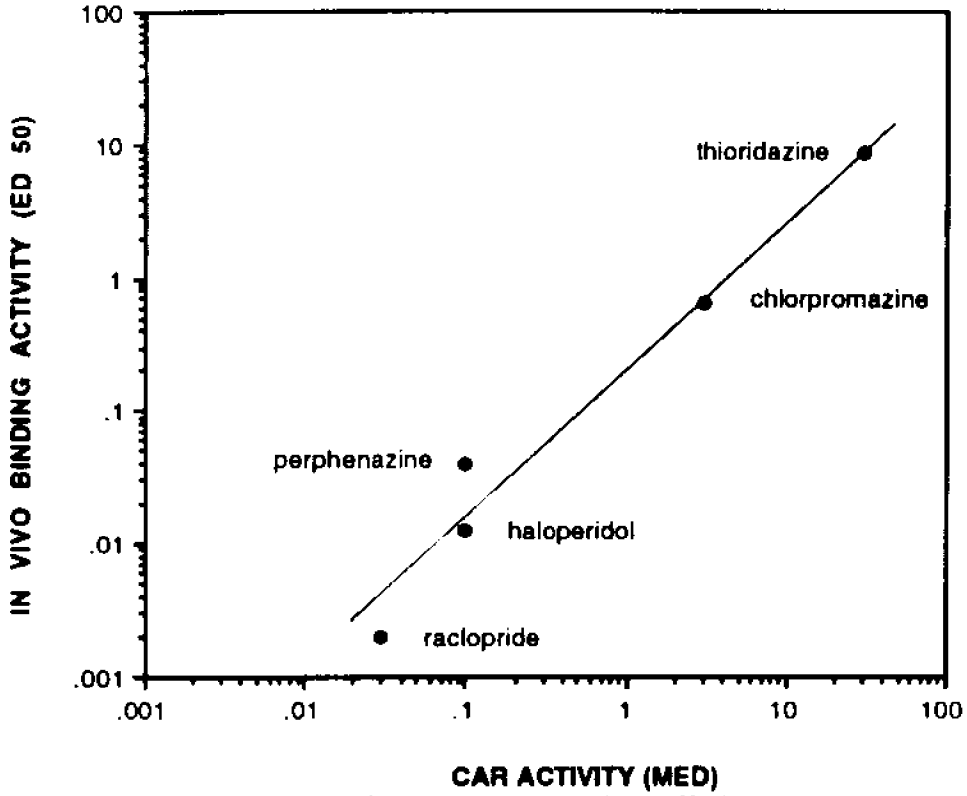



**FIGURE 4:** Correlation of D2 *in vivo* binding affinity with behavioral activity in the rat CAR test. Compounds included in this correlation had greater affinity for $^{3}$H-raclopride *in vivo*, as compared to their $ED_{50}$ values versus $^{3}$H-SCH 39166.

## DISCUSSION

These studies report the *in vivo*, *in vitro* and behavioral activity of selective D1 antagonists, including SCH 39166 and SCH 23390, selective D2 antagonists such as raclopride and haloperidol, and antagonists with affinity for both receptors. *In vitro*, SCH 39166 was shown to bind selectively to D1 receptors, with an affinity constant similar to the prototypic D1 antagonist SCH 23390. SCH 39166 not only has a similar *in vivo* selectivity to SCH 23390, but SCH 39166 also has much lower affinity for the $5HT_2$ receptor (Chipkin et al., 1988). The lower affinity of SCH 39166 for serotonergic receptors makes

it more useful as a ligand for the purposes of binding studies, as $^{3}$H-SCH 23390 had demonstrated significant binding in the cortex both *in vitro* (Alburges et al., 1992) and *in vivo* (McQuade et al., 1988b). In addition, SCH 23390 was found *in vivo* to inhibit $^{125}$I-SCH 38840, a D1 antagonist, with a Hill coefficient less than unity, indicating that SCH 23390 was binding to more than one receptor. When SCH 23390 was co-administered with ketanserin, the inhibition of $^{125}$I-SCH 38840 seen was greater then either compound administered alone (McQuade et al., 1988b).

The results of the *in vivo* binding studies indicate that the D1 antagonists SCH 23390 and SCH 39166 cross the blood-brain barrier and bind to dopaminergic receptors within the CNS. The binding of $^{3}$H-SCH 39166 within the CNS is specific to D1 receptors, in that known D1 dopamine antagonists can displace the ligand, while D2 antagonists cannot at reasonably active doses. Similarly, SCH 39166 did not displace $^{3}$H-raclopride, a D2 antagonist, at doses of over 100 mg/kg, while the D2 antagonists haloperidol and raclopride did inhibit this ligand, indicating that these D2 selective compounds cross the blood-brain barrier and bind to D2 receptors in the CNS.

When the ratio of D1 over D2 receptor affinity obtained *in vitro* is compared with that found using *in vivo* receptor binding methods, it is found that compounds which are D1 selective have much greater affinity for D1 receptors *in vivo* compared to their *in vitro* affinities. SCH 23390 and SCH 39166, for example, have up to 10,000-fold affinity for D1 receptors *in vivo* compared with 2500 and 440-fold, respectively. Similarly, the D2 selective antagonists haloperidol and raclopride have greater than 10,000-fold affinity for D2 receptors *in vivo* compared with 460 and 3000-fold *in vitro*. These results reflect pharmacokinetic effects, such as metabolism, that influence the amount of drug which gets to the receptor. These data suggest that *in vitro*

affinity determinations are not necessarily predictive of the degree of *in vivo* selectivity. In addition, the results of this study suggest that a compound must be at least 20-fold selective *in vivo* to displace either a D1 or D2 selective ligand preferentially. Compounds less than twenty-fold selective tend to displace both $^{3}$H-SCH 39166 and $^{3}$H-raclopride, while compounds with greater than 20-fold selectivity *in vivo* will displace only one or the other.

The significant correlation between the $ED_{50}$'s for compounds active at D1 receptors *in vivo* and their activity in the CAR, as well as the comparable correlation between D2 active compounds and their CAR actitivies, indicates that there may be a relationship between the binding of compounds to D1 and D2 receptors in the striatum, and most likely other areas, and behavioral activity in the rat CAR test. Dopamine receptor occupancy in the striatum appears to play a role in the behavioral effects demonstrated by dopamine antagonists in the CAR.

Furthermore, a comparison of the $ED_{50}$ values *in vivo* , which indicate that 50% of the receptors are occupied, and MED values in the CAR suggests that greater than 50% of the dopamine receptors must be occupied *in vivo* to produce a minimal behavioral effect. The need for greater than 50% receptor occupancy to produce a behavioral effect applies to all compounds tested, whether D1, D2 or mixed D1/D2 antagonists. It has also been demonstrated in clinical *in vivo* studies using positron emission tomography (PET), in patients treated with antipsychotics, that greater than 50% of dopamine receptors are occupied at therapeutic doses (Sedvall, 1988). This suggests that as *in vivo* binding methodologies are useful in correlating receptor occupancy levels with behavioral effects, they may therefore be useful in determining dose ranges for clinical trials to determine therapeutic effects.

The first series of experiments reported here demonstrated the selectivity of a novel D1 dopamine antagonist using both *in vitro* and *in vivo* methodologies. The second type of experiment, reported below, examines the selectivity of SCH 39166 for the D1 receptor using an *ex vivo* methodology, the ability of SCH 39166 to protect D1 receptors from the alkylating agent EEDQ.

## III. SELECTIVE PROTECTION OF D1 RECEPTORS BY SCH 39166

This study was designed to examine the *in vivo* selectivity of SCH 39166 for D1, D2 and $5HT_2$ receptors, by measuring the ability of SCH 39166 to protect these three receptors from inactivation by the alkylating agent N-ethoxycarbonyl-2-ethoxy-1,2 dihydroquiniline (EEDQ). EEDQ binds irreversibly to certain receptors and inactivates them by changing charged groups of amino acid side chains. This agent has been shown to inactivate serotonergic, adrenergic, cholinergic and dopaminergic receptors to varying degrees, as measured by receptor density values obtained following saturation analyses (Meller et al., 1985; Hess et al., 1986; Norman & Creese, 1986). The 5HT2 receptor was added, in addition to the D1 and D2 receptors, because previous studies had shown the SCH 23390 has some degree of affinity for this receptor (Chipkin et al., 1988, McQuade et al., 1988b). The rationale of this approach is that if SCH 39166 is D1 selective, then pretreatment of rats with behaviorally active doses should protect D1 receptors from inactivation by EEDQ, as indicated by no change in $B_{max}$ values in subseqent saturation analyses. In contrast,D2 or 5HT2 receptors, which bind SCH 39166 with much lower affinity, will be inactivated, resulting

in a decrease in $B_{max}$. Compounds selective for D2 and 5HT2 receptors were tested as well to ensure the accuracy of the model.

## METHODS

SUBJECTS: Same as Experiment 1.

MATERIALS:

SCH 39166 was synthesized by the CNS chemistry group at Schering-Plough Research Institute. Haloperidol, EEDQ, ketanserin, and methysergide were purchased from Research Biochemicals (Natick, Ma). For subsequent *in vitro* binding studies, the materials for the D1 and D2 binding assays were the same as those for experiment 1. In addition, $^{3}$H-ketanserin was used for 5HT2 saturation studies and was purchased from New England Nuclear (Boston, Ma).

PROCEDURES:

a.) EEDQ treatment studies:

These studies were performed according to previously described methods (Meller et al., 1985; Hess et al., 1986). Rats were pre-treated with 0.4 % aqueous methylcellulose vehicle, one of 4 doses of SCH 31966 (0.01, 0.1, 1.0 and 10.0 mg/kg, s.c.), ketanserin (50 mg/kg, s.c.) or haloperidol (1.0 mg/kg, s.c.). Either 30 minutes or one hour later (for haloperidol-treated animals), a vehicle containing 50% saline and 50% ethanol, or EEDQ (12 mg/kg, i.p.) in the same vehicle was administered. Following four hours or 24 hours (for the haloperidol-treated animals), the rats were sacrificed and the frontal cortex and striatum were dissected and weighed. Twenty-four hours was needed for the haloperidol to dissociate from protected receptors in animals receiving this treatment, as had been previously reported (Meller et al., 1985).

b.) In vitro binding studies:

For D1 and D2 binding studies, membranes from the striata of animals in each of the treatment groups above (vehicle plus or minus EEDQ, SCH 39166 at four doses, ketanserin or haloperidol, all plus EEDQ) were prepared as in experiment 1. Binding studies for the D1 and D2 receptors were performed by incubating striatal membranes from each of the treatment groups, prepared as in experiment 1, with increasing concentrations of $^3$H-SCH 23390 and $^3$H-spiperone, respectively. All other conditions were the same as in experiment 1. The data were analyzed according to the method of Scatchard (1949) and $K_d$ and $B_{max}$ values determined.

Saturation studies for the 5HT2 receptor were performed using the frontal cortex tissue collected from each of the treatment groups described above. The membranes were prepared in the same way as striatal membranes, by homogenizing with 50 volumes of Tris-HCl, pH 7.4 (Buffer A) and centrifuging at 20,000 x g for 20 min, after which the resulting pellet was resuspended in buffer A and spun again. The final pellet was resuspended in buffer A containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Buffer B). The *in vitro* binding assay was performed as above, with the exception that increasing concentrations of $^3$H-ketanserin were incubated with frontal cortex membranes for 20 min at 37$^0$C. Non-specific binding in these studies was determined in the presence of 10 uM methysergide. The assay was terminated as above. The data were again analyzed according the the method of Scatchard and the $K_d$ and $B_{max}$ values determined. All procedures were performed by myself with some assistance from Cheryl Anderson under the supervision of Robert McQuade and appear in press (McQuade et al., 1991a).

## RESULTS

Rats were pretreated with SCH 39166, ketanserin, haloperidol, or vehicle, and then subsequently treated with either vehicle or EEDQ. Frontal cortex and striatal tissue were collected and saturation analyses performed to determine D1, D2 and 5HT2 affinity and receptor density. The results are summarized in Table 2. No significant effects of drug treatments were found on $K_d$ values, with the exception that the 10 mg/kg dose of SCH 39166 produced a significant increase in affinity of $^3$H-SCH 23390 for the D1 receptor when measured by Scatchard analysis. This finding most likely represents the presence of residual drug bound to the receptor at sacrifice.

In Figure 5a, a significant decrease in the $B_{max}$ of D1 receptors was seen in vehicle-treated animals administered EEDQ, with over 80 % of the receptors being inactivated by the alkylating agent. SCH 39166 dose-dependently protected D1 receptors from inactivation, producing a significant difference from EEDQ-treated vehicle animals at all doses tested, from 0.01 to 10 mg/kg ($p. < 0.05$, Duncan's multiple range test).

The data for D2 receptor density indicate that SCH 39166 did not protect D2 receptors from EEDQ inactivation at any dose tested. These data are shown in Figure 5b. As shown in Figure 6a, rats pretreated with haloperidol prior to EEDQ administration showed a significant decrease in receptor density compared with EEDQ-treated rats that had received vehicle, indicating that haloperidol had protected the D2 receptors from inactivation.

SCH 39166 pretreatment at 10 mg/kg decreased 5HT2 receptor density. The lower doses of SCH 39166 produced no significant protection of $5HT_2$ receptors from EEDQ inactivation (Figure 5c). Ketanserin, when administered prior to EEDQ, did afford significant protection from EEDQ inactivation at 50 mg/kg (see Figure 6b).

**TABLE 2: SCH 39166 protection of D-1, D-2 AND 5HT-2 receptors from EEDQ inactivation**

| Treatment | Kd (nM) | Receptor Density (% Control) |
|---|---|---|
| **D-1 Receptors** | | |
| Vehicle | 0.64 + 0.11 | 100 |
| 12 mg/kg EEDQ | 1.15 + 0.42 | 14.4 + 3.7 a |
| SCH 39166 + EEDQ | | |
| 0.01 mg/kg | 0.60 + 0.12 | 35.4 + 2.7 a,b |
| 0.10 mg/kg | 0.67 + 0.25 | 64.2 + 6.4 a,b |
| 1.00 mg/kg | 0.65 + 0.13 | 89.5 + 5.8 b |
| 10.0 mg/kg | 2.66 + 0.10 a,b | 90.4 + 9.9 b |
| **D-2 Receptors** | | |
| Vehicle | 0.21 + 0.06 | 100 |
| 12 mg/kg EEDQ | 0.32 + 0.07 | 26.3 + 4.7 a |
| SCH 39166 + EEDQ | | |
| 0.01 mg/kg | 0.33 + 0.17 | 33.8 + 3.1 a |
| 0.10 mg/kg | 0.29 + 0.06 | 32.2 + 4.4 a |
| 1.00 mg/kg | 0.53 + 0.04 | 23.3 + 4.2 a |
| 10.0 mg/kg | 0.23 + 0.05 | 19.1 + 2.0 a |
| **5HT-2 Receptors** | | |
| Vehicle | 1.78 + 0.49 | 100 |
| 12 mg/kg EEDQ | 2.60 + 0.70 | 15.9 + 2.1 a |
| SCH 39166 + EEDQ | | |
| 0.01 mg/kg | 1.53 + 0.40 | 23.7 + 11.5 a |
| 0.10 mg/kg | 1.15 + 0.40 | 9.9 + 1.2 a |
| 1.00 mg/kg | 2.24 + 0.60 | 19.8 + 8.0 a |
| 10.0 mg/kg | 1.41 + 0.15 | 37.9 + 4.4 a,b |

[a] Significantly different from control ($P < 0.05$, Duncan's multiple range test)

[b] Significantly different from EEDQ ($P < 0.05$, Duncan's multiple range test)

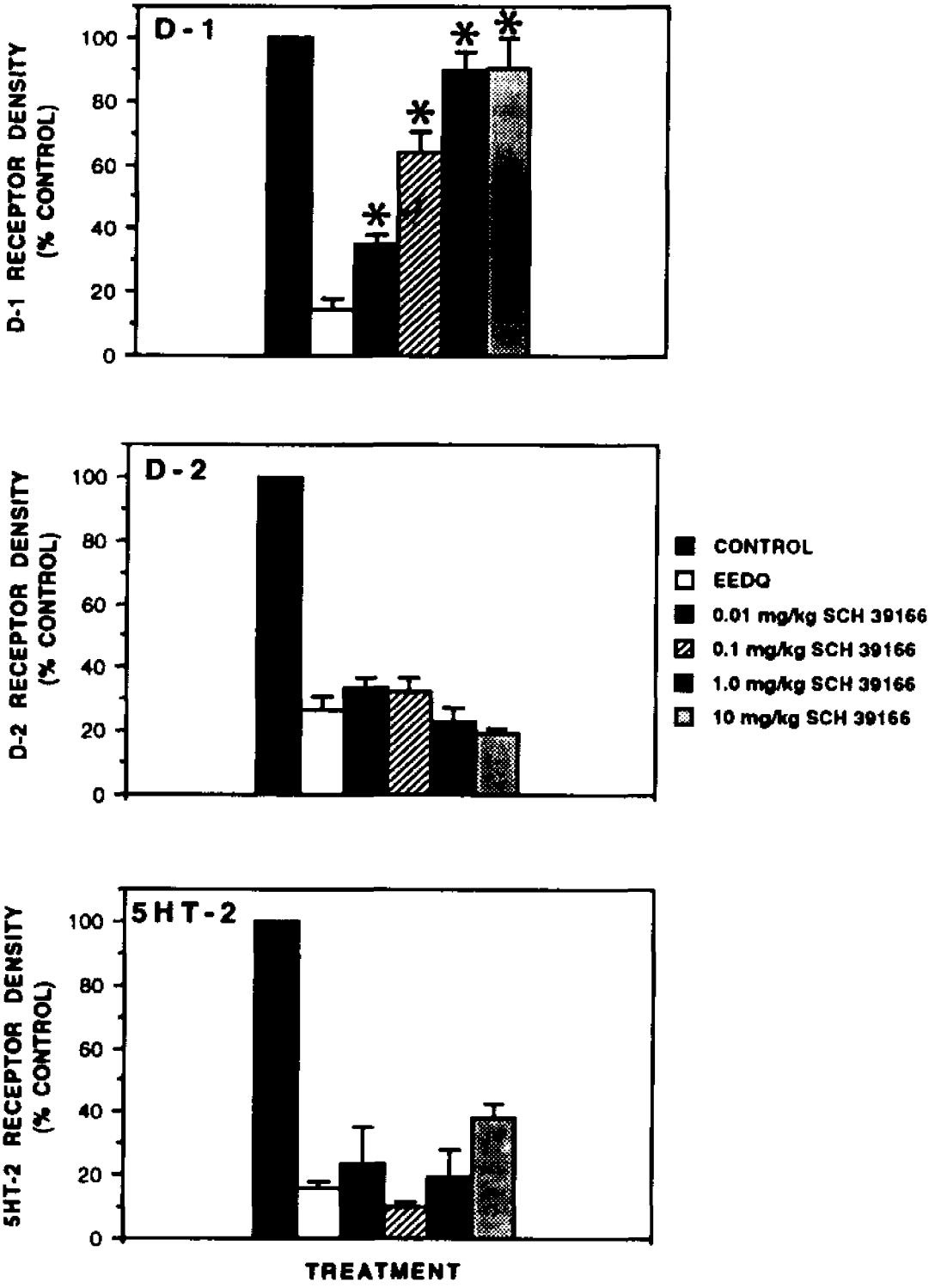



**FIGURE 5:** Ability of SCH 39166 to protect: a.) D1; b.) D2 and; c.) 5HT2 receptors from inactivation by EEDQ. See text for procedural details.

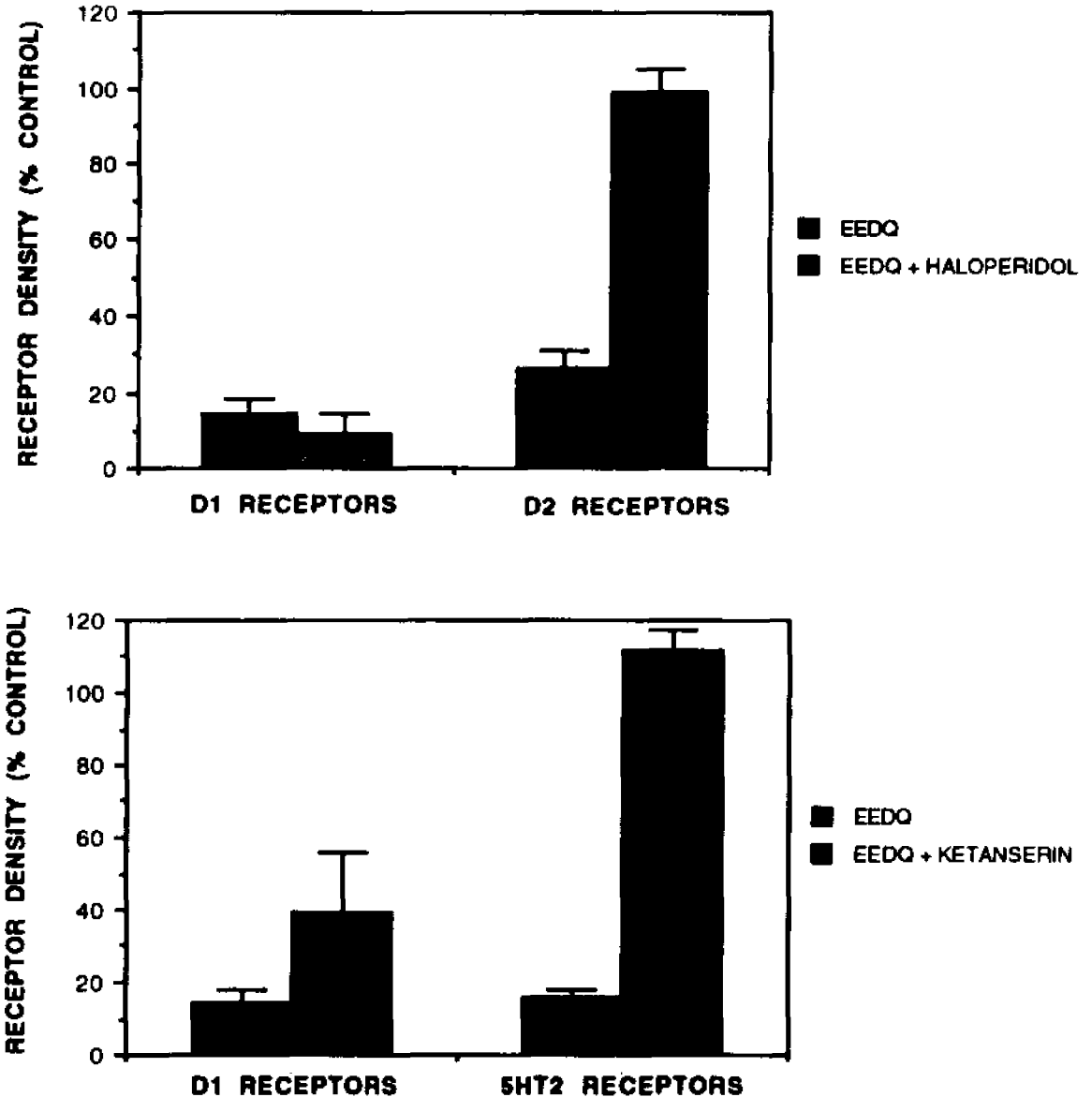



**FIGURE 6:** Ability of a.) haloperidol (1.0 mg/kg s.c.) and b.) ketanserin (50 mg/kg s.c.) to protect D1 and D2 or D1 and 5HT2 receptors from EEDQ inactivation. Data are expressed as the percentage of density in the absence of EEDQ treatment (% control) and represent the mean ± S.E. of at least three animals. * Significantly different from EEDQ ($p < .05$, Duncan's multiple range test).

## DISCUSSION

The data from this experiment indicate that SCH 39166 produced significant protection of D1 receptors from EEDQ inactivation at doses which are behaviorally active. The results suggest that SCH 39166 was binding selectively to the D1 receptor at the time of EEDQ administration, thereby preventing the alkylating agent from binding to, and subsequently inactivating, the D1 receptor.

No significant protection of the D2 receptor from EEDQ inactivation was afforded by SCH 39166 at the doses tested. Pretreatment with the D2 antagonist haloperidol did protect D2 receptors from alkylation by EEDQ, however, and served as a positive control validating the method.

Similarly, pretreatment with the 5HT2 antagonist ketanserin protected 5HT2 receptors from inactivation by EEDQ. SCH 39166, at 10 mg/kg, also produced partial protection of the 5HT2 receptor from EEDQ inactivation. The difference in the minimum dose of SCH 39166 which produced D1 receptor protection (0.01 mg/kg) and that which produced 5HT2 receptor protection (10.0 mg/kg) suggests that, *in vivo*, SCH 39166 is 1000 times more selective for D1 receptors as compared with 5HT2 receptors.

The results from experiments 1 and 2 indicate that SCH 39166 is D1 selective using two different types of *in vivo* methodologies. In experiment 1, an *in vivo* binding procedure was described which provided a direct measure of receptor occupancy. This *in vivo* binding procedure compared D1, D2 and mixed D1/D2 antagonists for activity at both D1 and D2 receptors. The procedure was able to discern known D1 and D2 antagonists by their affinity for $^{3}$H-SCH 39166 and $^{3}$H-raclopride *in vivo*. This procedure indicated that SCH 39166 is a D1 selective antagonist, with an *in vivo* affinity for the D1 receptor greater that 1000 times its D2 affinity. In addition, the strong

correlation between behavior and D1 receptor occupancy demonstrated in experiment 1 suggests that 39166 is producing its behavioral effects in the CAR via D1 receptors.

The second experiment used a different *in vivo* procedure to demonstrate the D1 selectivity of SCH 39166. This experiment used an indirect measure of receptor selectivity, the ability of a compound to protect receptors from destruction by a chemical alkylating agent. Using this technique, it was demonstrated that SCH 39166 protects D1 receptors selectively, but not D2 or 5HT2 receptors.

This study combined *in vivo* with *in vitro* techniques to answer the question of receptor selectivity. The next experiment examines the selectivity of SCH 39166 using a similar strategy, by determining its ability to selectively up-regulate D-1 receptors following chronic treatment.

## IV. SELECTIVE RECEPTOR UP-REGULATION WITH CHRONIC TREATMENT OF SCH 39166

Chronic administration of both agonists and antagonists of neurotransmitters has been shown to produce changes in receptor densities. Agonists generally produce decreases, or down-regulation, of the receptor number following chronic administration. Conversely, antagonists produce up-regulation, or an increase in receptor density, with repeated dosing. The changes seen in receptor number are specific only to the receptor(s) to which the drug binds, and usually are not accompanied by changes in the affinity of the receptor(s). The phenomena of up- and down-regulation of receptors have been demonstrated using compounds selective for a number of different

neurotransmitters, including those agents which bind to dopaminergic receptors (Creese and Sibley, 1980).

Five distinct dopamine receptors have been identified by their binding and second messenger properties (Kebabian, 1979; Billard et al., 1984) or by cloning techniques (Sokoloff et al., 1990; Sunahara et al., 1991; Van Tol et al., 1991). Compounds which are selective for the D2 receptor subtype, such as haloperidol, have been shown to produce up-regulation of D2 receptors, and not D1 receptors, in rats. Selective up-regulation of D1 receptors was likewise demonstrated in rats following chronic administration of the D1 selective antagonist, SCH 23390 (Chipkin et al., 1987). At the current time, there are no data on drug-induced changes in the density of D3, D4 or D5 receptors.

More recently, a D1 selective antagonist, SCH 39166 ((-)-trans-6,7,7a,8,9,13b-hexahydro-3-chloro-2-hydroxy-N-methyl-5-H-benzo[d]naphtho-(2,1b)azepine), has been characterized (Chipkin et al., 1988) *In vitro*, SCH 39166 was demonstrated to be 270 and 88 times more potent at D1 receptors than at D2 or $5HT_2$ sites, respectively (Chipkin et al., 1988). *In vivo*, SCH 39166 was shown to bind selectively to D1 receptors, in rat striatum, and selectively protected D1 receptors from inactivation by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (McQuade et al., 1991a). Behaviorally, SCH 39166 exhibited a preclinical profile similar to other antipsychotics and has been shown to possess a longer duration of action in primates than its predecessor, SCH 23390 (Chipkin et al., 1988).

Despite the wealth of studies describing the biochemical and behavioral effects of SCH 39166, there are no data in primates which biochemically confirm the D1 mechanism of the drug. Therefore, the present studies were designed to examine the ability of SCH 39166 to produce a selective up-

regulation of D1 dopamine receptors in Rhesus monkeys. The Rhesus monkey brain tissue used in these studies became available as the result of preclinical toxicology studies required by the FDA. Selective up-regulation of D1 receptors by SCH 39166 would provide the first biochemical confirmation of its *in vivo* D1 selectivity and CNS penetrability in non-human primates.

## METHODS

### SUBJECTS:

Twenty-four male and female Rhesus monkeys were separated into four groups (3 male and 3 female per group) and treated orally with either vehicle or SCH 39166 (3, 12 or 48 mg/kg) daily for three months. Twenty-four hours following the last treatment, all animals were sacrificed and caudate, putamen and frontal cortex tissues were dissected and frozen at -80°C until processed into membranes. The monkeys used for this study were part of a toxicology study required by the FDA and were treated according to all AALAC standards.

### PROCEDURE:

Membranes from the three brain regions for each of the three Rhesus monkeys were prepared by homogenizing the tissue in 40 volumes (w/v) of 50 mM Tris-HCl, pH 7.4 (Buffer A), using a Brinkman Polytron. Following centrifugation at 20,000 x g for 10 min, the supernatant was discarded and the pellet was resuspended in Buffer A and centrifuged again at the same speed. The supernatant was again discarded and the resulting pellet was resuspended in Buffer A containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. The final protein concentrations of the membranes from each group were determined using a bicinchoininic (BCA) assay kit (Pierce).

Membrane aliquots were frozen at -80°C until used in subsequent binding studies.

Saturation analyses of binding to D1, D2 and $5HT_2$ receptors were performed using $^3H$-SCH 23390, $^3H$-spiperone , and $^3H$-ketanserin, respectively. Aliquots of both caudate and putamen membranes were incubated with increasing concentrations of $^3H$-SCH 23390 and $^3H$-spiperone, while frontal cortex membranes were incubated with $^3H$-ketanserin, for 20 min at 37°C. Nonspecific binding at each concentration of ligand was determined in the presence of either 1 uM SCH 23390, 10 uM butaclamol or 10 uM methysergide for the D1, D2, and $5HT_2$ receptors, respectively. Following incubation, the reactions were terminated by filtration over GF/B glass fiber filters. The filters were subsequently washed with cold Buffer A using a Skatron filtration apparatus. Ready-Safe scintillant was added to the filters and the vials were incubated overnight. A Beckman liquid scintillation counter (50% efficiency) was used to determine the amount of bound radioligand. The data from each group were then analyzed according to the method of Scatchard (1949), and the values for receptor affinity ($K_D$) and receptor density ($B_{max}$) for each treatment were determined using linear regression analysis. These studies were performed and analyzed by the author, and the manuscript prepared and published with myself as first author (Duffy, 1988).

## RESULTS

Membranes prepared from the caudate and putamen of Rhesus monkeys which had been chronically treated with SCH 39166 (3, 12 and 48 mg/kg, po) exhibited a significant, dose-dependent increase in D1 receptor density in both the caudate and putamen (Figure 7). In the putamen, the increase in

$B_{max}$ was statistically significant ($p < 0.05$, Duncan's multiple range test) at all three doses of SCH 39166 tested, while in the caudate, only the 12 and 48 mg/kg doses produced a significant up-regulation. Analysis of the affinity constants of D1 receptors in the caudate and putamen of Rhesus monkeys (Figure 7) indicated a significant increase in $K_d$ in both regions at only the 48 mg/kg dose of SCH 39166 ($p < 0.05$, Duncan's multiple range test) . When the plasma levels of both SCH 39166 and its major metabolite were determined, measurable amounts of the drug derived material were present 24 hours after the last administration only in those animals which had received the 48 mg/kg dose (unpublished data). The increase in $K_d$ at the 48 mg/kg dose, therefore, is most likely the result of residual drug still bound to the D1 receptors.

An examination of the caudate and putamen from Rhesus monkeys for changes in D2 receptor number and affinity (Figure 8) revealed no significant differences between treatment groups. Likewise, there was no significant change in either the density or affinity of 5HT2 receptors in the frontal cortex of Rhesus monkeys at any of the doses of SCH 39166 tested (Figure 9). These data indicate that chronic treatment with SCH 39166 did not result in changes in the binding characteristics of these receptor subtypes.

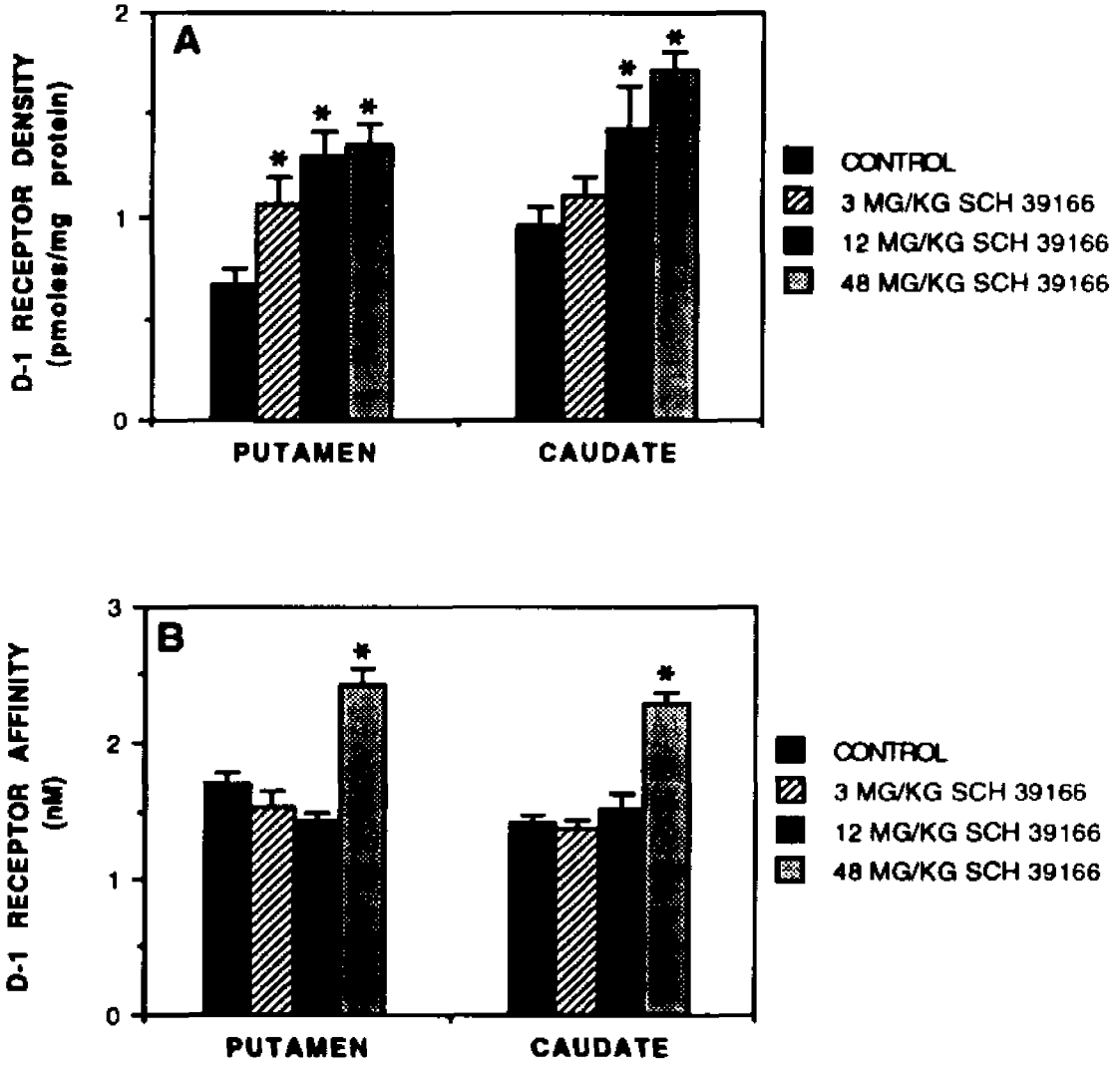



**FIGURE 7:** D1 receptor density (A) and affinity (B) determined from saturation analyses using $^{3}$H-SCH 23390 in caudate and putamen of rhesus monkeys chronically treated with SCH 39166. Each group was composed of samples from 6 individual animals, 3 male and 3 female. Significant increases in receptor $B_{max}$ or $K_d$, when compared to vehicle treatment, are indicated by an *.

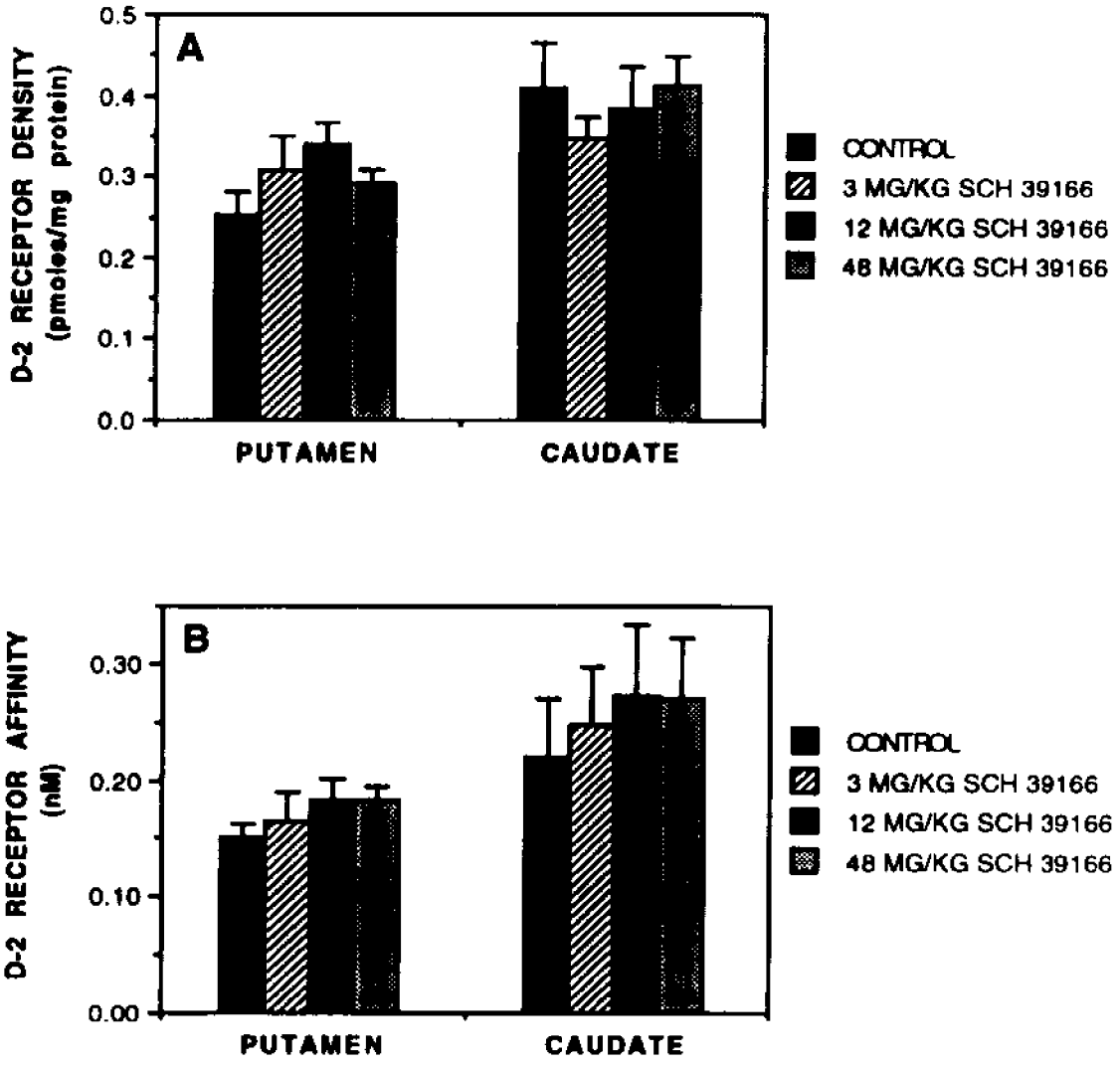



**FIGURE 8:** D2 receptor density (A) and affinity (B) measured in caudate and putamen of the the same animals as in Figure 1 using $^{3}$H-spiperone. No significant changes in either density or affinity were determined.

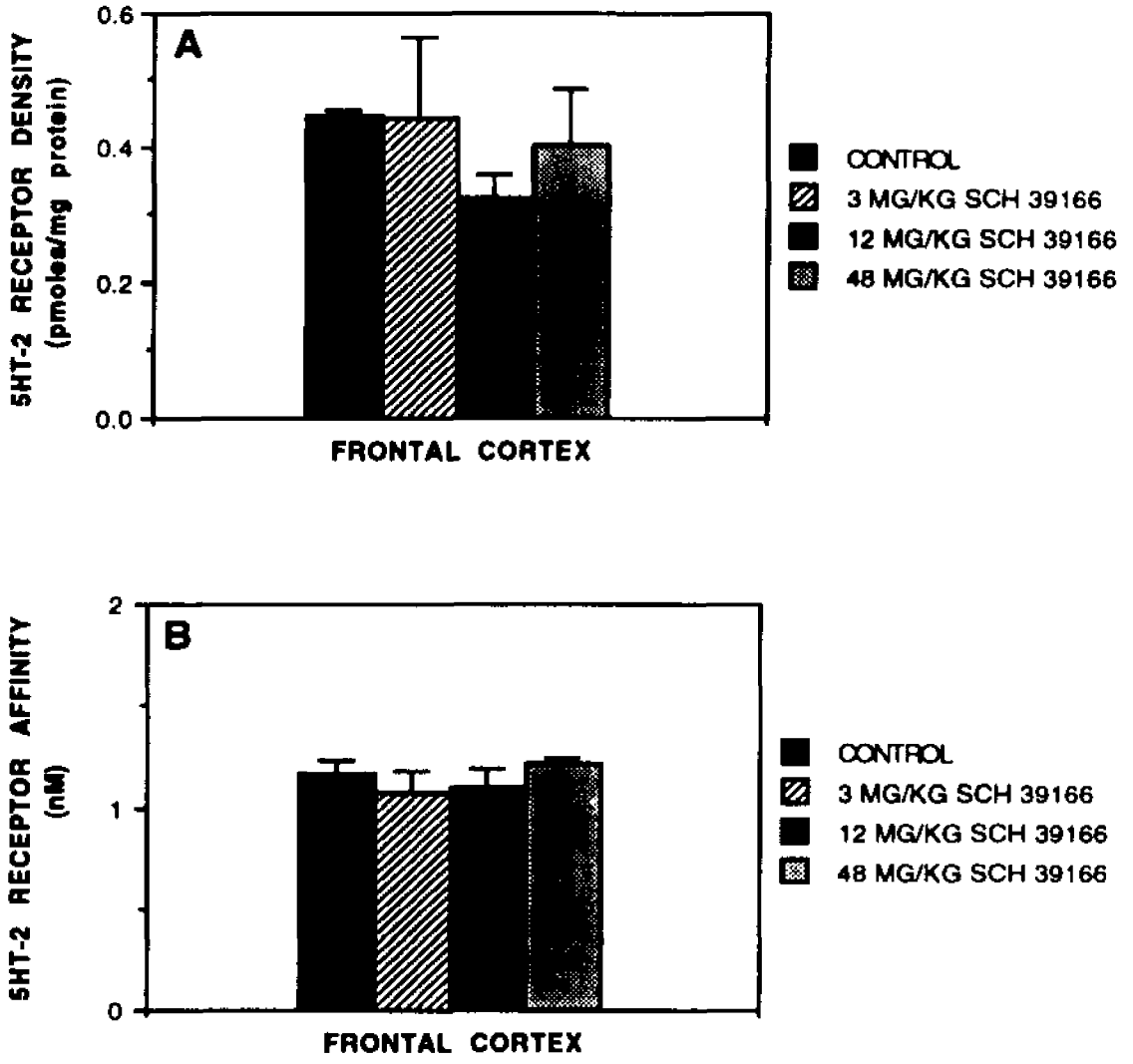



**FIGURE 9:** $B_{max}$ and $K_D$ values obtained from saturation studies using $^3H$-ketanserin in frontal cortex of rhesus monkeys. Each group was composed of 3 animals. No significant differences between treatment groups were noted.

## DISCUSSION

Three specific conclusions can be drawn from this work: first, SCH 39166 is able to cross the blood-brain barrier in primates. Although this observation is obvious, it is the first biochemical data to indicate that the drug does enter the CNS of monkeys and is not metabolized to a non-penetrable analogue.

This conclusion was confirmed by Sedvall and coworkers (1991) who used positron emission tomography to localize radioactive SCH 39166 to the striatum and neocortex of Cynomolgus monkeys.

The second conclusion is that SCH 39166 binds selectively to D1 receptors in the striatum of Rhesus monkeys. This conclusion is based on the selectivity of the up-regulation of D1 receptors. While the lack of an effect at D2 receptors was not unexpected, the inability of SCH 39166 to affect $5HT_2$ receptor density suggests that SCH 39166, unlike SCH 23390, does not bind to $5HT_2$ receptors, *in vivo*. These data are similar to findings in the rat, where SCH 39166 was demonstrated to be more selective for D1 receptors, as opposed to $5HT_2$ receptors, *in vivo*, than was its analogue, SCH 23390 (McQuade et al., 1988b). Likewise, autoradiographic studies using rat brains demonstrated that $^3$H-SCH 39166 labeled lamina IV of the cortex and the choroid plexus less densely than did $^3$H-SCH 23390, again indicating the poor affinity of SCH 39166 for 5HT receptors (Wamsley et al., 1991).

Finally, these studies demonstrate that SCH 39166 is functioning as an antagonist at the D1 site in monkeys. As mentioned above, neurotransmitter agonists produce down-regulation of receptors following repeated administrations, while antagonists increase the number of binding sites (Creese and Sibley, 1980).

The finding that SCH 39166 binds selectively *in vivo* to D1 dopamine sites has important implications for its potential clinical use as an antipsychotic. The currently available antipsychotics are either non-selective dopamine antagonists or are D2 selective. One of the side effects of acute treatment associated with these antipsychotics is the development of a movement disorder, referred to as extrapyramidal syndrome. Studies in Cebus monkeys have demonstrated that these movement disorders are produced by repeated

administration of the D2 antagonist, haloperidol, but are not seen following repeated treatment with SCH 39166 (Coffin et al., 1991). The studies presented herein confirm that SCH 39166 is D1 selective in non-human primates and suggest that this D1 selectivity is the mechanism responsible for its improved side-effect profile

The first three experiments have demonstrated the D1 selectivity of SCH 39166 *in vivo* by examining its receptor occupancy and kinetics following both acute and chronic administration. The final study in this series will describe an *in vivo* autoradiographic procedure which allows for the visualization of the selectivity of SCH 39166 for D1 receptors in smaller brain regions simultaneously in a single animal.

## V. IN VIVO AUTORADIOGRAPHY USING $^{3}$H-SCH 39166

The benzonaphthazephine SCH 39166 ([(-)-trans-6,7,7a,8,9,13b-hexahydro-3-chloro-2-hydroxy-N-methyl-5H-benzo[d]naphtho-[2,1b]azepine]) has been characterized as a selective D1 antagonist. *In vitro*, SCH 39166 has over one-hundred fold selectivity for D1 receptors, as compared to D2 receptors (Chipkin et al., 1988). In addition, SCH 39166 has a much lower affinity for serotonergic receptors than the prototype D1 antagonist, SCH 23390 (McQuade et al., 1988; Taylor et al., 1991). *In vivo* binding studies have demonstrated that SCH 39166 penetrates the blood-brain barrier and binds selectively to D1 receptors in the striatum, an area of the brain rich in dopamine receptors (McQuade et al, 1991a). In addition, $^{3}$H-SCH 39166 was synthesized and found to selectively bind to D1 receptors both *in vitro* and *in vivo* (McQuade et al, 1991b).

*In vitro* autoradiographic studies have determined the localization of $^{3}$H-SCH 39166 binding in the CNS to the caudate and putamen in rats (Alburges et al., 1992) and humans (Hall et al., 1993), in addition to the substantia nigra and entopeduncular nucleus, structures associated with the nigro-striatal pathway (Wamsley et al., 1991). In these studies, sections of rat brain were treated with $^{3}$H-SCH 39166 *in vitro* in the presence or absence of competing drugs, washed and then the bound radioactivity was quantified. The localization of D1 and D2 receptors using subtype-specific antibodies in rat, monkey, and human brain was also found to be primarily in the striatum, substantia nigra and olfactory bulb (Levey et al., 1993).

The current studies report the use of an *in vivo* autoradiographic methodology in which $^{3}$H-SCH 39166 is administered directly to rats in the presence or absence of competing drugs. After incubation, sacrifice and brain removal, sections of brain are then mounted to slides and the bound radioactivity quantified directly. Using this methodology, it is possible to determine if $^{3}$H-SCH 39166 crosses the blood-brain barrier, binds within the CNS selectively to D-1 receptors, and can be displaced by D-1 antagonists. The advantage to this methodology over standard *in vivo* binding studies is that the binding of $^{3}$H-SCH 39166 can be quantified in a large number of regions simultaneously in the same slide, as well as in smaller regions that would be difficult to dissect.

## METHODS

SUBJECTS: Male Sprague Dawley rats (Charles River) weighing 200-250 grams were used for all studies.

PROCEDURE:

Rats were injected subcutaneously with one of the following treatments: vehicle (0.4% aqueous methylcellulose); SCH 23390 at doses of 0.0016, 0.016 and 0.16 mg/kg; 0.0014, 0.014, 0.14, 0.42 and 1.4 mg/kg SCH 39166; 150 mg/kg of haloperidol; or 200 mg/kg ketanserin. The drugs were dissolved in a volume of 300 ml, which was added to 200 µCi (µl) of $^{3}$H-SCH 39166 (76 Ci/mmole - specially synthesized by Amersham) for a total injection volume of 500 µl. The doses of SCH 39166 and SCH 23390 chosen were shown to be both behaviorally active in the CAR and to inhibit the binding of $^{3}$H-SCH 39166 *in vivo* (Chipkin et al., 1988; McQuade et al., 1991b).

One hour following injection, rats were sacrificed by decapitation, the brains were removed and placed in ice-cold saline. The most lateral portions of the cortex of each brain were removed and the brain was then mounted onto a chuck using OCT embedding compound (Miles) so that the sagittal plane was exposed. Twelve micron thick sections were cut using a cryostat mounted inside a refrigerated cabinet (Zeiss) and thaw-mounted onto gelatin coated slides. Ten slides were obtained from each brain, two from each of five regions which were from 80-100 microns apart.

One slide from each region (five total for each treated rat) was then placed in an X-ray cassette with Hyperfilm (Amersham) and incubated at -80$^{o}$C for 10-12 weeks. In addition, each cassette contained commercially available tritium standards (New England Nuclear, Boston, Ma.) so that the amount of bound radioactivity which interacted with the emulsion of the film could then be quantified directly for each sample.

Following the incubation period, the film was developed using a Kodak D19 developer, and fixed using 30% sodium thiosulfate. The film was air dried and the bound radioactivity of the image produced by each slide was

quantified using a microcomputer imaging densitometry system (Imaging Research Inc., Ontario, Canada) which was interfaced with a Compaq 386 computer. For each region, several readings from different slides were taken and averaged. The data were then converted to fmol/mg tissue by standardization with the tritium standards. Mean values for each treated rat from each region were determined for statistical analysis. These studies were designed and performed by myself. I was helped with the density determinations by Mary Hunt from James Wamsley's laboratory. The data were analyzed by myself and the manuscript prepared for publication (Duffy, et al., 1995).

## RESULTS

For each treated rat, fmol/mg tissue values for five different brain regions were obtained by averaging multiple readings from several slides. The regions of interest included the cortex (C), the caudate putamen (CP), the substantia nigra (SN), the nucleus accumbens (NA) and the olfactory tubercle (OT).

Three separate rats were used for each of the 11 treatments: control rats, which received methylcellulose vehicle plus 200 mCi $^{3}$H-SCH 39166; haloperidol at 150 mg/kg; 200 mg/kg of ketanserin; 0.0016, 0.016, and 0.16 mg/kg SCH 23390 and; 0.0014, 0.014, 0.14, 0.42 and 1.4 mg/kg SCH 39166. Data analysis was performed on these average readings.

A summary of the average fmol/mg tissue values for selected regions can be found in Table 1. No significant inhibition of $^{3}$H-SCH 39166 was found in the cortex of any of the rats tested, regardless of treatment or dose. The average fmol/mg value for cortical regions across all treatments was 9.44 $\pm$ 0.50, and therefore the individual values are not included in the summary

**TABLE 3: Average fmole/mg tissue values of $^{3}$H-SCH 39166 binding in selected regions in rat brain using in vivo autoradiography.**

| COMPOUND | DOSE (mg/kg) | CP | SN | ACC | OT |
|---|---|---|---|---|---|
| **Control** | 0.0 | 33.7 ± 5.7 | 23.7 ± 4.0 | 26.7 ± 4.8 | 30.0 ± 5.9 |
| **SCH 23390** | 0.0016 | 35.9 ± 6.4 | 27.1 ± 3.5 | 29.6 ± 5.5 | 35.1 ± 10.2 |
| | 0.016 | 26.5 ± 2.5 | 22.9 ± 2.2 | 21.8 ± 3.2 | 25.5 ± 2.7 |
| | 0.16 | 10.3 ± 0.8* | 10.4 ± 1.5* | 10.4 ± 1.5* | 9.5 ± 0.6* |
| **SCH 39166** | 0.0014 | 35.9 ± 7.7 | 27.0 ± 6.3 | 29.0 ± 6.9 | 33.0 ± 5.7 |
| | 0.014 | 31.5 ± 7.3 | 22.2 ± 5.3 | 26.5 ± 6.9 | 25.5 ± 6.7 |
| | 0.14 | 15.6 ± 2.7* | 13.6 ± 1.5* | 15.5 ± 3.2* | 14.3 ± 2.3* |
| | 0.42 | 7.9 ± 0.9* | 6.9 ± 1.0 * | 6.9 ± 1.2* | 9.7 ± 0.9* |
| | 1.4 | 9.8 ± 4.0* | 8.6 ± 2.9* | 6.0 ± 4.2* | 5.7 ± 4.4* |
| **Haloperidol** | 150 | 28.5 ± 5.7 | 23.0 ± 5.6 | 22.2 ± 4.1 | 25.7 ± 4.6 |
| **Ketanserin** | 200 | 37.2 ± 15.5 | 26.0 ± 9.6 | 31.8 ± 10.7 | 34.5 ± 12.4 |

* Significantly different from control based on analysis of variance with Dunnett's post hoc test, $p < 0.05$

table. This value was approximately equal to the non-specific binding of $^{3}$H-SCH 39166 in other brain regions and suggests that the density of D1 receptors in the cortex of rats is extremely low.

In CP, SN, NA and OT, however, SCH 39166 produced a dose-dependent inhibition of $^{3}$H-SCH 39166 binding. The fmol/mg tissue values for these regions were converted to percent inhibition produced in the absence of drug (% control) and analyzed using the Ligand computer program (McPherson, 1985) to determine $IC_{50}$ values, defined as the dose at which 50% inhibition of control, or specific, binding occurs. The $IC_{50}$ values for inhibition of $^{3}$H-SCH 39166 by SCH 39166 for CP, SN, NA, and OT were approximately equivalent, ranging from 0.11 to 0.15 mg/kg. Dose-response curves for the displacement of $^{3}$H-SCH 39166 by SCH 23390 and SCH 39166 can be seen in Figure 10.

SCH 23390 also produced significant inhibition of $^{3}$H-SCH 39166 in all the structures associated with dopamine receptors which were examined. For SCH 23390, the $IC_{50}$ values for CP, SN, NA and OT were again approximately equivalent, ranging from 0.05-0.07 mg/kg. Representative autoradiograms are shown in Figure 11.

Neither haloperidol nor ketanserin produced significant inhibition of $^{3}$H-SCH 31966 in any region at the doses tested. These compounds were used as controls to ensure that the binding of $^{3}$H-SCH 39166 and its displacement were selective for the D-1 receptors subtype.

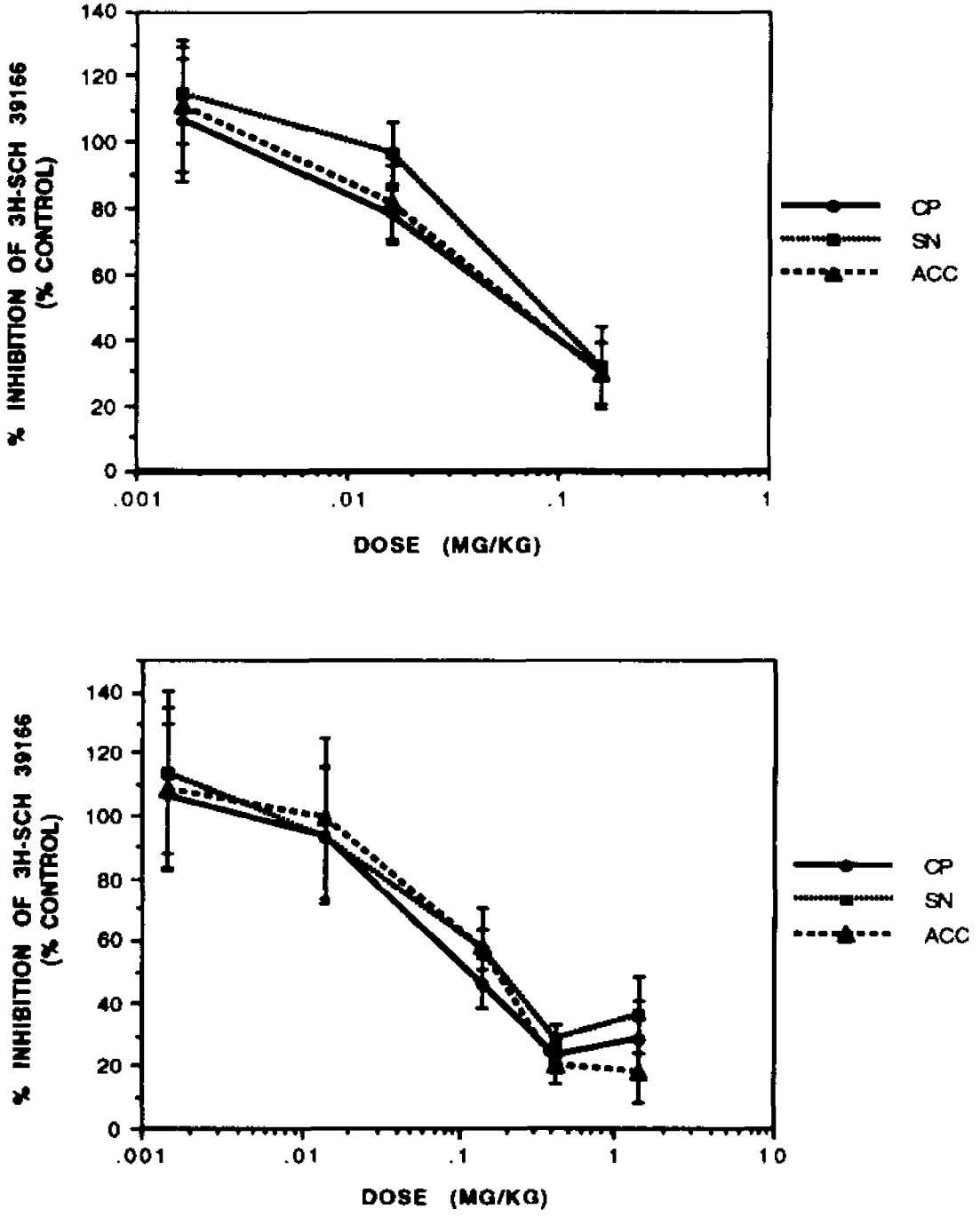
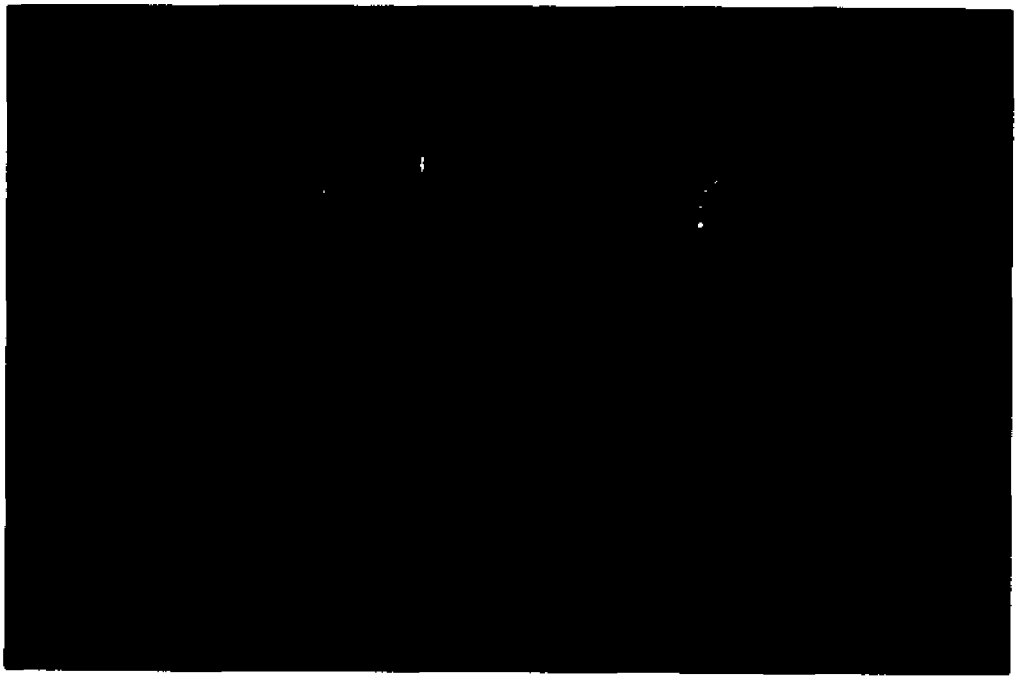



**FIGURE 10:** Dose response curves for SCH 23390 and SCH 39166 inhbition of $^{3}$H-SCH 39166 in three regions of rat brain using *in vivo* autoradiography technique detailed in text. Each point represents mean % control ± S.E. of three animals.

a.)



b.)

**Figure 11:** Selected color-enhanced autoradiograms obtained using the *in vivo* autoradiographic prodedure described in methods. a.) Dose-dependent displacement of $^{3}$H-SCH 31966 by SCH 23390. Bar at right gives concentrations of ligand. b.) Competition of 150 mg/kg ketanserin and 200 mg/kg haloperidol v. $^{3}$H-SCH 39166. No significant displacement was found.

## DISCUSSION

The findings of this study indicate that $^{3}$H-SCH 39166, when administered systemically, penetrates the blood-brain barrier and binds within the central nervous system. The blood-brain barrier penetration of $^{3}$H-SCH 39166 agrees with previous studies using *in vivo* binding methodologies (McQuade et al., 1991b).

The results also indicate that systemic administration of $^{3}$H-SCH 39166 results in binding of the compound to structures in the nigro-striatal system, including the caudate-putamen, substantia nigra and associated regions. These structures have previously been described to have high densities of dopamine receptors (Wamsley et al., 1991). The regions to which $^{3}$H-SCH 39166 binds with high affinity *in vivo* are similar to those found for *in vitro* studies (Alburges et al., 1992), which would provide further evidence for the presence of large populations of D1 dopamine receptors in the nigro-striatal system.

No significant binding of $^{3}$H-SCH 39166 was found in the cortex, and the low levels which were present were not displaced by either dopamine antagonists or serotonergic antagonists, suggesting that the cortical levels represent non-specific binding. This finding is supported by *in vitro* autoradiographic studies (Alburges et al., 1992), which compared $^{3}$H-SCH 23390 to $^{3}$H-SCH 39166 and found significant binding of $^{3}$H-SCH 23390 in cortex, the result of its high affinity for the 5HT2 receptors in this region. The high affinity of $^{3}$H-SCH 23390 for serotonergic receptors has made $^{3}$H-SCH 39166 a better ligand for *in vivo* studies of D1 receptor binding (McQuade et al., 1988). Similarly, studies examining the binding of $^{11}$C-labeled SCH 39166 (Halldin et al., 1991) using positron emission tomography (PET) in monkey brain found increased uptake in striatum but not in cerebellum. The

uptake of $^{11}$C- SCH 39166 in striatum could be displaced by SCH 23390, but not by ketanserin (Sedvall et al., 1991).

$^{3}$H-SCH 39166 can be displaced *in vivo* by the D1 dopamine antagonists SCH 23390 and SCH 39166 at doses which are behaviorally active. The minimum effective dose (MED) for activity in the conditioned avoidance response test (CAR) for subcutaneous administration of SCH 23390 is 0.01 mg/kg, while the MED for subcutaneous SCH 39166 administration is 0.03 mg/kg (Chipkin et al., 1988). These findings correlate with *in vivo* binding data, where the ratio of the MED in the CAR test for SCH 23390 and SCH 39166 (0.01:0.03 mg/kg) is the same as the ratio found for *in vivo* displacement of $^{3}$H-SCH 39166 by these compounds (0.005:0.016 mg/kg) (McQuade, et al, 1992). In the studies reported here using *in vivo* autoradiographic techniques, the doses of SCH 23390 and SCH 39166 which produced fifty percent inhibition of $^{3}$H-SCH 39166 (approximately 0.05 and 0.1 mg/kg, respectively) were higher than those using *in vivo* receptor binding procedures. The ten-fold difference in $IC_{50}$ values between the two *in vivo* methodologies may simply reflect the ten-fold greater concentration of ligand administered in the *in vivo* autoradiographic procedure (200 mCi per animal) versus that administered for *in vivo* binding studies (15 mCi per animal). For these studies, a higher ligand concentration was necessary to see an image on the film, and therefore greater concentrations of competing drug would be necessary to displace the ligand. The $IC_{50}$ values for SCH 23390 and SCH 39166 reported here are only slightly greater than those reported to produce a significant behavioral effect in the CAR. In addition, the ratio of the $IC_{50}$'s of SCH 23390 to SCH 39166 remains nearly the same, which lends further support to the contention that the behavioral effect produced by SCH 39166 is mediated through dopamine D1 receptors.

No significant displacement of $^{3}$H-SCH 39166 was found for either the D2 antagonist haloperidol or the $5HT_2$ antagonist ketanserin, which agrees with previously published data using both *in vivo* binding methodologies (McQuade et al., 1991b), as well as up-regulation studies showing that chronic treatment of monkeys with SCH 39166 produced significant up-regulation of D1 receptors in striatum, but no up-regulation of either D2 or $5HT_2$ receptors (Duffy et al., 1992). *In vitro* autoradiographic studies also demonstrated no up-regulation of either D2 or 5HT2 receptors following chronic SCH 39166 treatment (Lappalainin et al., 1992).

More recently, cell lines expressing the five dopamine receptor subtypes D1-D5 have become available (Strange, 1993). The affinity of SCH 39166 and SCH 23390 for these five receptor subtypes was examined. SCH 39166 and SCH 23390 had equal affinity for both the D1 and D5 receptor subtypes, with much lower affinity for the D2, D3 and D4 receptors, which bound haloperidol with high affinity (Tice et al., 1994). Dopamine D5 receptors are found within the CNS in many of the same regions as D1 receptors, but their functional significance has yet to be understood (Strange, 1993). While it is presumable that $^{3}$H-SCH 39166 would bind to D5 receptors *in vivo* as well as D1, there is yet no method to separate D1 and D5 binding because no selective ligand for the D5 receptor has been found.

Overall, this study provides further evidence that SCH 39166 crosses the blood-brain barrier and binds selectively to D1 dopamine receptors. The doses at which $^{3}$H-SCH 39166 is displaced using this *in vivo* autoradiographic method are similar to those which produce physiologic activity of the drug in the CAR test. These finding further suggest that the regions to which the compound binds, namely those in the nigro-striatal dopaminergic system, are those same regions which are responsible for its behavioral activity. Finally,

the *in vivo* autoradiographic procedure described here allows for the characterization of binding properties of compounds in multiple regions simultaneously in the same animal, and may be applied to examining other receptor systems as well.

## VI. SUMMARY OF FINDINGS FROM STUDIES IN CHAPTER 2

The first four experiments detailed the *in vivo* methodologies used to examine the CNS penetrability, localization and selectivity of SCH 39166 for D1 dopamine receptors in rat striatum and associated structures. In addition, the studies reported here discussed the correlation between receptor occupancy and behavioral effects produced by SCH 39166, and suggested that the behavioral effects produced by SCH 39166 in the rat CAR were mediated through its interaction with D1 dopamine receptors in the striatum and associated structures.

The relative potencies of the compounds *in vivo* match their *in vitro* affinities fairly accurately. When the $ED_{50}$ for *in vivo* binding is compared to the $IC_{50}$ for inhibition of $^{3}$H-SCH 23390 *in vitro*, the order or potency for the compounds is quite similar, with SCH 23390 being the most active and raclopride the least active at D1 receptors. These data indicate that the inhibition of $^{3}$H-SCH 39166 produced by these compounds *in vivo* is at the D1 receptor.

The ability of a compound to cross the blood brain barrier and bind to receptors within the CNS is crucial to demonstrating activity of the compound *in vivo*. All of the studies reported here demonstrate that SCH 39166 crosses the blood-brain barrier and binds to receptors within the CNS. In experiment 1, D1 antagonists were shown to displace $^{3}$H-SCH 39166 from D1 receptors

in the striatum dose-dependently following subcutaneous administration. Similarly, compounds which are D2 antagonists displaced $^{3}$H-raclopride, a D2 selective ligand, from D2 receptors. Compounds of mixed D1/D2 affinity had variable degrees of affinity for the two receptors *in vivo*. In experiment 2, SCH 39166 was shown to cross the blood-brain barrier and bind to receptors in the striatum, which resulting in the selective protection of D1 receptors from inactivation by EEDQ. Experiment 3 demonstrated that SCH 39166 produced an up-regulation of only D1 receptors in rhesus monkey striatum, again indicating that the compound must have crossed the blood-brain barrier and bound to D1 receptors in the striatum to produce the subsequent up-regulation upon chronic administration. Finally, the binding of $^{3}$H-SCH 39166 to receptors in the CNS was visualized not only in the striatum but in other structures associated with dopamine pathways, including the substantia nigra and entopeduncular nucleus, using an *in vivo* autoradiographic method.

The selectivity of SCH 39166 for D1 receptors in the rat striatum and associated structures was also demonstrated in these experiments via a number of *in vivo* methods. First, SCH 39166 was found to selectively occupy D1 receptors in the striatum, but not D2 receptors, as measured by comparing its ability to displace $^{3}$H-SCH 39166 to its affinity for $^{3}$H-raclopride. In the first experiment, the $ED_{50}$ for SCH 39166 at D1 receptors *in vivo* was 0.016 mg/kg, compared with an $ED_{50}$ of greater than 100 mg/kg for D2 receptors, yielding up to a one thousand-fold greater affinity of SCH 39166 for the D1 receptor than the D2 receptor. The same holds true for other compounds which were demonstrated *in vitro* to be D1 selective, which have selectivity of about 100-fold for the D1 receptor compared to their D2 affinity. In contrast, compounds which are known to be D2 selective, including haloperidol and raclopride, had no affinity for the D1 receptor at doses up to 100 mg/kg, but

were found to be highly selective for the D2 receptor, with $ED_{50}$ values of 0.013 and 0.002, respectively, for displacing $^{3}H$-raclopride.

The selectivity of SCH 39166 for D1 receptors *in vivo* was also demonstrated using *in vivo* autoradiography. SCH 39166 was found to dose-dependently displace $^{3}H$-SCH 39166 from nigro-striatal structures. The D1 antagonist SCH 23390 was also found to dose-dependently displace $^{3}H$-SCH 39166 *in vivo* using this procedure, but the D2 antagonist haloperidol and the $5HT_2$ antagonist ketanserin could not, which further demonstrates the specificity of SCH 39166 for the D1 receptor.

In addition, the *in vivo* selectivity of SCH 39166 for the D1 receptor in striatum was demonstrated using two other indirect measures, the ability of SCH 39166 to selectively protect D1 receptors from inactivation by the aklyating agent EEDQ and the ability of SCH 39166 to selectively up-regulate D1 receptors. The first of these studies demonstrated that the D1 antagonist SCH 39166 will protect D1 receptors from EEDQ inactivation, but not D2 or $5HT_2$ receptors. In addition, the D2 antagonist halperidol was shown to protect D2, but not D1 or $5HT_2$ receptors, while the $5HT_2$ antagonist ketanserin protected $5HT_2$ receptors from EEDQ inactivation, but not D1 or D2 receptors. The second study used a similar rationale, in that SCH 39166 was found to up-regulate D1 receptors, but not D2 or $5HT_2$ receptors, in rhesus monkey striatum.

Perhaps the most potentially far-reaching finding of these studies is the determination of a significant correlation between receptor occupancy and the production of behavioral effects for both D1 and D2 antagonists. As detailed in experiment 1, when separate correlation coefficients were calculated between the $ED_{50}$ *in vivo* and the MED in the CAR for both D1 and D2 antagonists, both correlations were found to be significant. This finding can

be interpreted several ways. First, it suggests that the occupation of both D1 and D2 dopamine receptors in the striatum is mediating the behavioral effects produced by these compounds in the CAR. Second, the significant correlation further suggests that at least 50% of the dopamine receptors in the striatum must be occupied in order to produce this behavioral effect. In addition, if the CAR is predictive of antipsychotic activity of a compound in clinical populations (Cooke and Davidson, 1979), then the activity of D1 antagonists in the CAR suggests that they may be efficacious antipsychotics. And, finally, if at least 50% of the receptors need to be occupied at a given dose to produce an effect in a behavioral test predictive of antipsychotic activity, then perhaps the $ED_{50}$ found for a given compound using *in vivo* binding assays may provide a dose range of the compound which could be tested in clinical trials in humans.

# CHAPTER 3

# OVERVIEW OF CHOLINERGIC PHARMACOLOGY IN THE CNS AND THE USE OF IN VIVO METHODOLOGIES TO ASSESS THE AFFINITY OF COMPOUNDS FOR MUSCARINIC RECEPTORS

## I. INTRODUCTION

Initial research on muscarinic cholinergic receptors concentrated on their pharmacological characterization. Through the development of receptor binding techniques in the 1970's, coupled with the availability of radiolabeled ligands that bound specifically to a given class of membrane-bound receptors, it was possible to characterize the binding of compounds at muscarinic receptors. These initial studies led to the discovery that certain muscarinic receptor populations demonstrated differences in their affinity for certain compounds (Birdsall and Hulme, 1983), leading to the classification of muscarinic receptor subtypes.

The muscarinic M1 cholinergic receptor has been defined as possessing a high affinity for the antagonist pirenzepine, whereas the M2 receptor has a low affinity for this compound and a high affinity for the antagonist AFDX-116 (Wang et al., 1987; Micheletti et al., 1987; Birdsall et al., 1988). A muscarinic receptor designated M3 has now been proposed in both rat and human brain that has a higher affinity for antagonist 4-DAMP than the M1 receptor (Bonner, 1990). An M4 receptor has also been proposed (Waelbroeck et al., 1990). Currently no agonists have been found to be consistently selective for these three subtypes.

When the second messenger properties of the three muscarinic receptor subtypes were examined, it was found that M1 receptors are coupled to a G-protein that mediates an increase in phosphatidyl inositol (PI) hydrolysis in response to agonist binding. This increase in PI metabolism can be reversed by cholinergic antagonists. At M2 receptors, agonist binding has been linked with an inhibition of adenylyl cyclase, an enzyme that mediates a different second messenger system (Gil & Wolfe, 1985), presumably through a different G-protein (Wheatley et al., 1988). Studies describing the second messenger properties of the M3 receptor have found that it weakly stimulates PI hydrolysis, as well as arachidonic acid metabolism (Bonner, 1990).

The localization of the different pharmacological muscarinic receptor subtypes in the CNS has been achieved through the use of techniques such as autoradiography. Early studies used radiolabeled muscarinic compounds for *in vitro* autoradiographic studies to determine the distribution of the pharmacological muscarinic receptor subtypes M1, M2 and M3 within the central nervous system. Studies were done examining the ability of pirenzepine to displace $^{3}$H-quinuclidinyl benzilate (QNB) from rat brain slices apposed to tritium-sensitive film. It was found that pirenzepine displaced $^{3}$H-QNB with relative potencies being greatest in hippocampus > cortex > thalamus (Messer & Hoss, 1987). Other studies have confirmed the distribution of M1 receptors in the cortex, hippocampus and thalamus, in addition to low amounts in the midbrain (Ehlert & Tran, 1990). Using the M2 selective ligand $^{3}$H-AFDX-116, M2 receptors have been found in high densities in rat brain in many medullary and midbrain regions, including the facial, trigeminal, and hypoglossal nuclei, as well as septal nuclei, the olfactory bulb and some thalamic nuclei, in addition to lower levels in the cerebellum, striatum and some cortical regions (Regenold et al., 1987). Other

studies using the same ligand, however, have localized high densities of M2 receptors in the parietal cortex and dentate gyrus, as well as frontal cortex and hippocampus, and striatum (Wang et al., 1989). Most studies agree, however, that the M1 and M2 receptor subtypes have a discreet localization, with M1 receptors concentrated in the telencephalic regions, including the frontal cortex and hippocampus, whereas M2 receptors are concentrated in the cerebellum and brainstem (Miyoshi et al., 1989). Whereas the m1 receptor is localized almost exclusively in the CNS, the M2 receptor is found in many peripheral tissues, including the heart and vas deferens (Hulme et al., 1990). The localization of the M3 receptor has been found in the periphery, particularly the exocrine glands (Mei et al., 1989).

The reason some of these localization studies have differing results may be due to the poor selectivity of the ligands used. For example, pirenzepine has less than one hundred-fold greater affinity for M1 receptors than M2 receptors, and subsequent discovery of additional receptor subtypes has found that pirenzepine also binds to the m3 and m4 receptor in cultured cells. Similarly, AFDX-116 has greater affinity for the M2 receptor, but has also been shown to bind the m4 muscarinic receptor subtype recently discovered using molecular cloning techniques.

Advances in molecular biology have led to the discovery of five genes coding for different muscarinic receptors, that have been designated m1 through m5 (Bonner et al., 1987; Peralta et al., 1987; Bonner et al., 1988; Liao et al., 1989) All of these genes code for a receptor protein composed of seven transmembrane domains. Whereas there appears to be a large amount of conservation within the transmembrane segments of the receptors, the difference between receptor subtypes appears to reside in other areas, particularly the third cytosplasmic loop (Bonner et al., 1987). This loop has

been proposed to be important in explaining the differences in the second messenger properties of the receptor subtypes, as well as their high affinity agonist states (Hulme et al., 1990). The m1, m3 and m5 subtypes have been found, in a variety of cell lines, to stimulate PI metabolism, in a manner that is pertussis toxin insensitive, in response to agonist binding (Lai et al., 1988; Peralta et al., 1987). The m1, m3 and m5 clones have also been linked to a stimulation of arachidonic acid release, that is again pertussis toxin insensitive, and to the release of intracellular calcium, again upon agonist binding. These clones have also been found to slightly increase cyclic AMP levels. The m2 and m4 receptor clones both weakly stimulate PI and inhibit cyclase both in a manner sensitive to pertussis toxin (Jones et al., 1991).

Compounds that are known to possess muscarinic activity have been used as tools to describe the binding properties of these new muscarinic cholinergic receptor subtypes. Pirenzepine, an antagonist, has been shown to possess a high affinity for m1 receptors and a low affinity for m2 receptors, with intermediate affinity for the m3-m5 receptors. AFDX-116 and its related analogs, such as AFDX-384, have been shown to possess a higher affinity for m2 receptors than m1, m3 and m5 in competitive binding studies (Watson et al., 1986; Dorje et al., 1991). More recently, another m2 selective antagonist, methoctramine, has been reported (Michel & Whiting, 1988; Watson et al., 1992). Another antagonist, 4-DAMP, has been shown to that higher affinity for m3 receptors in binding studies using radiolabeled ligands (Michel et al., 1989). More recently, a 4-DAMP mustard has been synthesized and been reported to be an alkylating agent that forms an aziridinium ion and irreversibly inactivates m3 receptors (Thomas and Ehlert, 1992; Giffin et al., 1993). No selective antagonists have been found for the m4 and m5 receptor subtypes.

The localization of the mRNA coding for the five muscarinic receptor subtypes has been achieved through in situ hybridization. In situ hybridization studies involve the construction of a probe consisting of a portion of the mRNA for a given receptor subtype. This probe is then labeled and exposed to tissue, and localized using autoradiographic techniques (Weiner and Brann, 1990). The limitations of the results of these studies are that only the message coding for the receptor protein, and not the protein itself, can be detected. Using this technique, it has been demonstrated that the mRNA of all five genes can be found in rat brain in the following locations: m1 mRNA is located in the cerebral cortex and the dentate gyrus and hippocampus; m2 mRNA in basal forebrain, septum and hindbrain; m3 mRNA in cerebral cortex and hippocampus; m4 mRNA in caudate putamen and nucleus accumbens; and m5 mRNA in hippocampus, substantia nigra and globus pallidus (Weiner and Brann, 1990). Similar studies have confirmed the presence of m1, m3 and m4 mRNA in cortex, hippocampus and striatum, in addition to the localization of m3 to salivary glands. The m2 mRNA was found mostly in peripheral tissues, including the heart and intestine (Mei et al., 1989).

More recently, studies have examined the localization of muscarinic receptors m1-m5 through the production of subtype-specific antibodies. In these studies, a fusion protein is constructed by expressing in bacteria the DNA encoding a portion of the third cytoplasmic loop, that has been found to differ for each muscarinic receptor subtype (Bonner et al., 1987). This fusion protein is then purified and used as an antigen to raise antisera in rabbits. The antisera against each receptor subtype were then screened in chinese hamster ovary cells expressing each of the receptor subtypes m1-m5 to test for selectivity of the antisera. The antisera specific for each receptor subtype were then incubated with labeled, solubilized receptors prepared from

different regions of the brain and periphery and the labeled receptors were immunoprecipitated (Levey et al., 1990). This technique allows for the determination of the percentage of each muscarinic receptor subtype within a given region. Using this technique, antisera raised to a fusion protein containing the third cytoplasmic loop of the m2 receptor labeled receptors in the heart, brain stem, hindbrain and midbrain regions, including the cerebellum, pons, medulla and thalamus. Much lower precentages were found in cortex, hippocampus and striatum (Li et al., 1991). Similar studies using antisera for the rat m1 receptor found it localized primarily in cortex, hippocampus, striatum and olfactory tubercle, whereas areas found to be high in m2 receptors had low m1 receptor density (Wall et al., 1991a). Antisera developed against a portion of m3 receptor labeled cortex, hippocampus, striatum and olfactory tubercle as well, with low densities in hindbrain regions (Wall et al, 1991b). Finally, m4 antisera were found to label receptors predominantly in striatum and olfactory tubercle, whereas low levels of m5 receptors were detected in several brain regions (Yasuda et al., 1993). A similar study examining the distribution of all five muscarinic receptors in peripheral tissues found high levels of m1 in sympathetic ganglia and submaxillary glands, whereas m3 was also high in the submaxillary gland but not other tissues. The antisera prepared against the m4 receptor labeled receptors in the lung and ileum, whereas the m2 receptor receptor was found in many peripheral tissues, including the sympathetic ganglia and ileum (Dorje et al., 1991). The findings of these studies generally agree with those found for in situ hybridization studies, in that m1 and m3 predominate in cortex, striatum and hippocampus, whereas m2 is found primarily in hindbrain regions.

However, whereas the distribution of muscarinic receptor subtypes is discreet, it is important to note that the receptors are not totally segregated from one another. In fact, it has been proposed that m1 receptors may be post-synaptic, whereas m2 receptors in some regions have been proposed to be autoreceptors that may lie on the pre-synaptic cholinergic terminal (Lapchak et al., 1989). Peripherally, m2 receptors were found to be presynaptic in guinea pig airways (Watson et al., 1992). In addition, lesions of the fimbria-fornix that produce deafferentation of the dorsal hippocampus resulted in an increase in m1 and m3 receptors in hippocampus, that was interpreted as a compensatory up-regulation due to the loss of cholinergic input. This finding then implies that the m1 and m3 receptor are post-synaptic. The study also found that m2 receptors decreased, suggesting that the lesion that destroyed the cholinergic input to the hippocampus produced a loss of presynaptic m2 receptors (Wall et al., 1994). The localization of m2 as a presynaptic autoreceptor in rat striatum has recently been demonstrated (Billard et al., 1995) by correlating potencies for compounds to enhance acetylcholine release *in vivo* by microdialysis with *in vitro* receptor binding affinity data. In these studies, AFDX-116 was found to be more potent than pirenzepine in producing an increase in acetylcholine release, as measured by *in vivo* microdialysis, and has a greater affinity for the m2 receptor *in vitro.*

Research suggests that the cloned m1 clone possesses many of the same properties that characterize the M1 receptor, including both its greater affinity for pirenzepine and its link to the PI second messenger system (Lai et al., 1988). Similarly, the m2 clone is thought to be comparable to the M2 receptor. Less work has been done to further characterize the properties of the m3 through m5 clones, although it has been suggested that the m3 gene and M3 receptor possess comparable qualities (Bonner, 1990). It is now

generally accepted that the genes coding for the m1 through m5 clones represent the receptors either already characterized *in vivo* or, in the case of the m4 and m5, have yet to be pharmacologically characterized *in vivo*. Therefore, a standard classification of m1-m5 is now generally used to refer to muscarinic receptors either pharmacologically or from a molecular perspective.

The study to be presented will describe the development of a methodology to examine the affinity of known cholinergic antagonists for muscarinic receptors *in vivo*. Many of the same principles that were applied to the study of binding to dopaminergic receptors *in vivo*, described in Chapter 2, were utilized for these studies. Both the ligand and competing compounds were administered subcutaneously in order to assess blood-brain barrier penetrability. A variety of tissues were collected and compared for their level of specific binding and the time course of that binding. And, finally, antagonists were administered and compared for their ability to displace the ligand from various brain tissues.

Unlike the studies in Chapter 2, however, the ligand used in these studies to examine muscarinic receptors *in vivo*, $^{3}$H-scopolamine, is not selective for any of the five muscarinic receptor subtypes. In fact, there is no currently available radiolabeled ligand that crosses the blood-brain barrier in sufficient quantities to bind selectively to muscarinic receptor subtypes *in vivo*. Nonetheless, the studies were undertaken to establish the ability of the ligand to cross the blood-brain barrier and bind to muscarinic receptor within the CNS specifically and to be displaced by muscarinic antagonists. In addition, a comparison of the relative potency of the antagonists can be made and compared with their relative potency *in vitro*, to determine if any potential pharmacokinetic factors exist that could impair the ability of these antagonists

to bind to receptors in the CNS. Also, the kinetics of the binding of the $^{3}$H-scopolamine to muscarinic receptors *in vivo* can be examined and compared to that found *in vitro*. Finally, as muscarinic receptors have been strongly associated with learning and memory (Haroutunian et al., 1985), a correlation between *in vivo* muscarinic receptor occupancy of standard cholinergic antagonists and effective doses of these same compounds in a behavioral test of learning and memory, such as the passive avoidance response (PAR) test, can be assessed.

## II IN VIVO BINDING TO MUSCARINIC RECEPTORS

In this study a methodology to examine the binding of compounds to muscarinic receptors *in vivo* will be described. Early studies using *in vivo* methodologies to examine muscarinic cholinergic receptors involved injecting large concentrations of $^{3}$H-QNB intravenously (i.v.) in rats, and then determining the bound radioactivity (Yamamura et al., 1974). Using this methodology, the binding of $^{3}$H-QNB was found to be saturable, and could be displaced by atropine (60 mg/ kg, i.m). In addition, the binding of $^{3}$H-QNB was found to be highest in striatum, cortex and hippocampus and lowest in cerebellum. Finally, the time course of binding was similar in the striatum and hippocampus, with peak specfic binding at 2.5 minutes which remained unchanged out to the 24 hour time point, while the time course in the cerebellum fell off rapidly.

Subsequent studies used autoradiographic procedures, in which $^{3}$H-QNB was administered i.v. to rats, after which coronal sections of the brain were mounted onto slides and apposed to tritium sensitive film for varying amounts of time. These studies confirmed the previous findings of high

concentrations of $^{3}$H-QNB accumulation in the hippocampus, particularly those regions receiving dendritic input, as well as the striatum and cortex. Again, this accumulation of $^{3}$H-QNB was displaced by atropine treatment (Kuhar and Yamamura, 1975; Kuhar and Yamamura, 1976).

Another early study looked at the accumulation of $^{3}$H-dexetimide, another muscarinic antagonist with lower affinity than QNB, in various brain regions following i.v. administration. Again, the highest concentrations were found in the cortex, striatum and hippocampus, as well as in the nucleus accumbens and olfactory tubercle. The cerebellum was again found to have the lowest concentrations of $^{3}$H-dexetimide. Unlabeled dexetimide was able to displace $^{3}$H-dexetimide from all brain regions except cerebellum while levetimide, its inactive stereoisomer, did not (Laduron and Janssen, 1979).

The *in vivo* binding methodology to be described uses small quantities of newly available radioligand, $^{3}$H-scopolamine, a non-selective muscarinic antagonist. Using this methodology, the binding of $^{3}$H-scopolamine to muscarinic receptors *in vivo* was characterized in conscious, free-moving rats and standard muscarinic antagonists were compared for their ability to displace the ligand from receptors in both the CNS and periphery. In addition, the kinetics of $^{3}$H-scopolamine for muscarinic receptors *in vivo* will be compared with its *in vitro* kinetics. Finally, the ability of muscarinic antagonists to displace $^{3}$H-scopolamine *in vivo* will be compared with both their *in vitro* affinity, as well as their behavioral effects in a standard model of memory in rodents, the passive avoidance response (PAR).

**METHOD**

SUBJECTS:

Male Sprague-Dawley rats (Charles River) weighing 200-250 grams at testing were used for all studies.

MATERIALS:

$^{3}$H-scopolamine was purchased from Amersham International (Buckinghamshire, England), while all other compounds were purchased from either Research Biochemicals (Natick, Massachusetts) or Sigma Chemicals (St. Louis, Missouri).

PROCEDURES

In vivo binding studies:

For time-course studies, pairs of rats received either vehicle or 100 mg/kg scopolamine subcutaneously followed by 10 uCi (10ul) $^{3}$H-scopolamine in a volume of 500 ul (dose equal to approximately 0.15 ug/kg) At predetermined time points following the injections ( 5, 15, 30 min., 1, 2, 4, 6 and 24 hours), one control and one scopolamine-treated rat were sacrificed by decapitation. The brain and heart were removed and the cortex, striatum, hippocampus and cerebellum were dissected. The regions were then weighed and homogenized in 10 volumes 10 mM Na/K PO4, pH 7.4 using a Polytron at setting 6. Alliquots (250ul) were then filtered over glass fiber filters and washed with 2 X 5 mls of cold buffer. In addition, plasma samples were collected to determine blood levels of $^{3}$H-scopolamine. The data from each brain region and heart at each time point were expressed as dpm/mg tissue weight. Specific binding for each time point was determined by subtracting the binding in the presence of scopolamine from that obtained for vehicle-treated animals. Each time point represents the mean of 3 vehicle or 3 scopolamine-treated rats.

For competition studies, rats again received both 10 uCi $^3$H-scopolamine in a volume of 500 ul and either vehicle or various doses of test compounds s.c., in two separate injections. One hour following administration of $^3$H-scopolamine and test compounds, the rats were sacrificed and both the brain and heart removed, dissected, weighed, homogenized and filtered as above. For competition studies, data from each brain region and heart were expressed as dpm/mg tissue weight for each treated animal. These were then compared to vehicle controls and expressed as percent inhibition in the absence of drug. The $IC_{50}$, the concentration at which 50 percent inhibition occurred, was then determined for each drug. Each dose is represented by at least 3 rats. All studies were performed by myself and are being prepared for publication (Duffy and McQuade, 1996).

In vitro binding studies:

Binding studies with $^3$H-scopolamine were conducted using membranes prepared from rat cortex, heart and cerebellum. All membranes were prepared by homogenizing each tissue in 40 volumes 10 mM Na/K phosphate, pH 7.4 (Buffer A) using a Polytron at setting 6. Membranes were then centrifuged at 40,000 x g for one hour, except heart membranes, which were first spun at 1200 x g to remove debris, after which the supernatent was spun as above. The resulting pellet was then resuspended in Buffer A at 25 mg/ml for cortex and 100 mg/ml for cerebellum and heart membranes (wet weight) and frozen at -80°C until used for subsequent studies.

To determine association rates of $^3$H-scopolamine, a 100 pM concentration of $^3$H-scopolamine was incubated with membranes from cortex (0.25 mg/assay), cerebellum and heart (0.5 mg/assay) for up to 90 minutes at 25°C. The dissociation rates of $^3$H-scopolamine from each tissue were determined by adding 100 uM atropine once equilibrium had been reached,

and then determining specific binding at various timepoints thereafter. Data were expressed as dpm specifically bound over time.

Saturation studies were performed by incubating increasing concentrations of $^3$H-scopolamine (10 - 1000 pM) with cerebellar and heart membranes and then incubating at 25°C for one hour. Reactions for all studies were terminated by filtration over GF/C filters using a Brandel cell harvester. Filters were then incubated overnight in Ready-Safe scintillation fluid (Beckman) and bound radioligand determined using a Beckman scintillation counter. Dissociation constants ($K_d$) and receptor number ($B_{max}$) were determined according to the method of Scatchard (1949).

In addition, saturation studies of $^3$H-scopolamine were performed on cloned human muscarinic receptors m1-m5 which had been transfected into Chinese Hamster Ovary (CHO) cells. For these studies, increasing concentrations of $^3$H-scopolamine were incubated with varying concentrations of each of the five cloned muscarinic receptor cell membranes, which had been previously prepared using the same procedure as for cortical membranes above. The concentration of membranes used for each receptor subtype varied with the expression level of the cell line. The reactions were terminated as above by rapid filtration over GF/C filters using a Brandel cell harvester and the bound radioactivity determined as above. The $K_d$ and $B_{max}$ values for each of the five cloned receptors were determined as above. As with the *in vivo* studies, the *in vitro* work was performed by myself.

Passive avoidance response (PAR):

The PAR test was conducted in a chamber consisting of an illuminated compartment separated from a dark compartment by a guillotine door. On training day, rats were placed in the lit chamber and, after entering the dark compartment, the door was closed and a 2 mA footshock was administered

for 3 sec. Twenty four hours later, retention testing was conducted. Rats were administered test compounds s.c. and, thirty minutes later, were again placed into the lit chamber and the latency for entry into the dark chamber was recorded. The minimal effective dose (MED) for each compound was defined as the lowest dose which produced a significant decrease in latency to enter the dark chamber when compared with control rats, who typically do not enter the dark chamber for the entire 3 minute test interval. These studies were performed by the behavioral pharmacology section of the CNS pharmacology department under the direction of Vicki Coffin and appear in a recent publication (Smith et al., 1994).

## RESULTS

The results of the time course studies using the *in vivo* binding procedure are shown in Figure 12. The results for cortex, striatum and hippocampus all demonstrate peak specific binding at between one and two hours following subcutaneous administration. While the binding of $^{3}$H-scopolamine in these regions decreases with time, it remains at a fairly significant level at all time points tested, out to twenty four hours following $^{3}$H-scopolamine administration (Figure 12a). In both the cerebellum and heart, however, the time course peaks within one hour, then drops off rapidly and reaches background levels by two hours following $^{3}$H-scopolamine adminstration (Figure 12b)

In order to determine if the binding of $^{3}$H-scopolamine in brain at 24 hours was specific, a study was performed in which $^{3}$H-scopolamine was administered to pairs of rats. Either 2, 4, 6 or 24 hours following $^{3}$H-scopolamine administration, one of the pair of rats received 100 mg/kg scopolamine, while the other received vehicle. The animals were sacrificed

one half hour later and the bound radioactivity determined in brain and heart using the methods described above. The results are shown in Figure 13. The study indicates that cold scopolamine was able to displace $^{3}$H-scopolamine, which had been administered up to 24 hours earlier, to almost background levels. This suggests that the $^{3}$H-scopolamine bound to receptors in brain at 24 hours has not been metabolized, but is in its intact state. As expected, no evidence of binding was seen in the heart at any time point tested, which correlates with the initial time course study indicating that the specific binding in heart returned to background levels by 2 hours, the earliest time point tested (data not shown).

To further examine if the time course of binding of $^{3}$H-scopolamine in cortex, cerebellum and heart was correlated with that found *in vitro*, both association and dissociation studies were conducted using membranes prepared from these three tissues. Results from representative studies are shown in Figure 14. While association of $^{3}$H-scopolamine to muscarinic receptors in cortex, heart and cerebellum were similar (Figure 14a), the dissociation rates for $^{3}$H-scopolamine differed in the three tissues. Fifty percent of the dpm bound to cortical membranes dissociated by one hour in cortex, while the amount of time for fifty percent of the $^{3}$H-scopolamine bound to both cerebellum and heart was only about five minutes (Figure 14b).

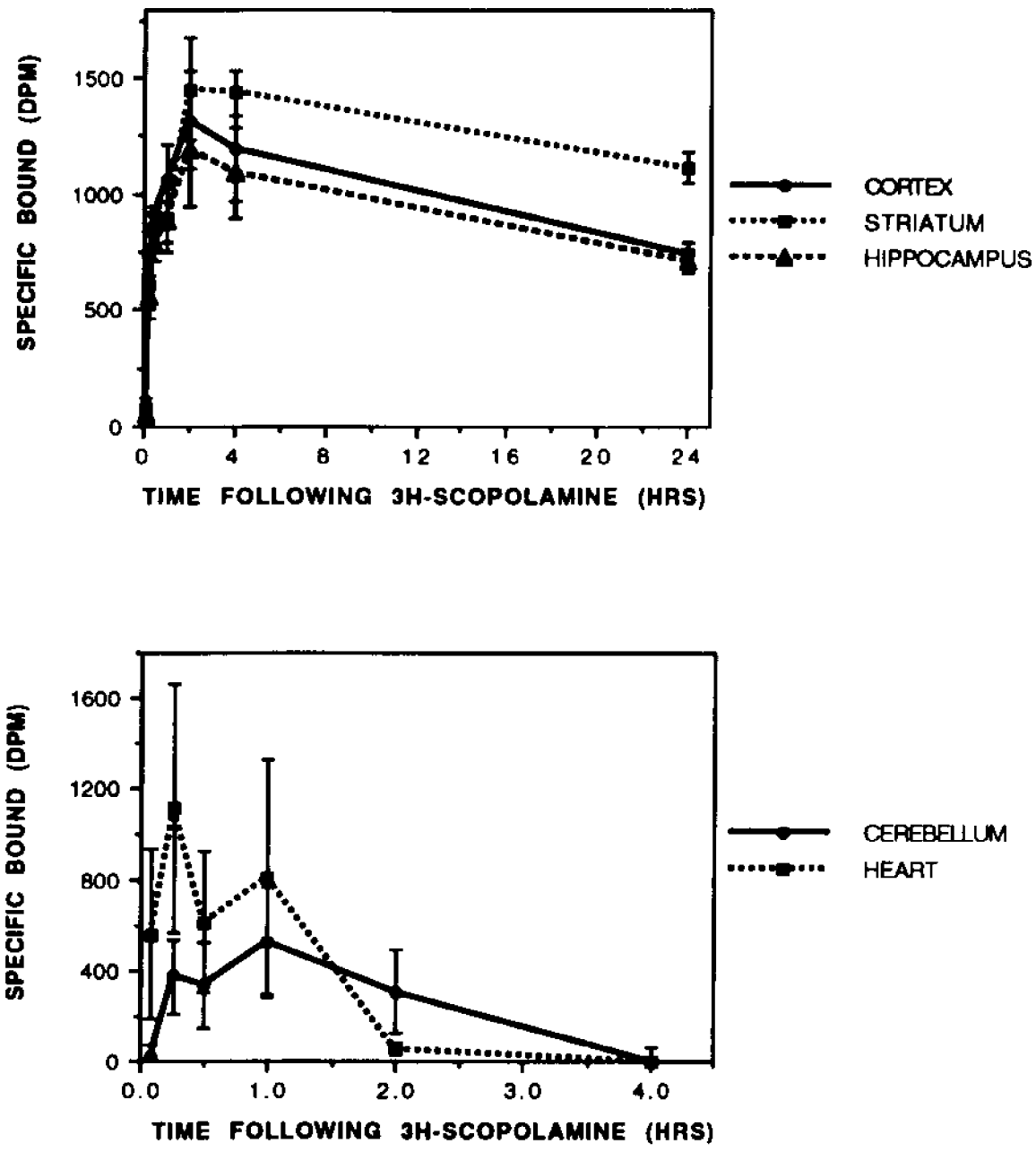



**FIGURE 12:** Time course of *in vivo* binding of $^{3}$H-scopolamine in rat brain. Each point represents specific bound in three pairs of rats. a.) Specific bound across time in cortex, hippocampus and striatum. b.) Specific bound in cerebellum and heart.

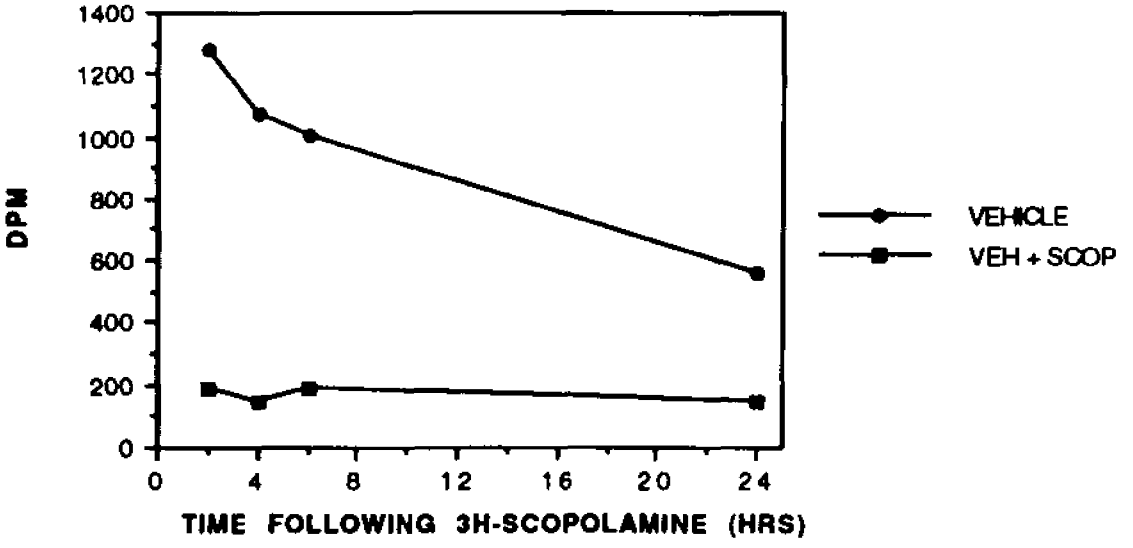



**FIGURE 13:** Ability of scopolamine to displace $^{3}$H-scopolamine when administered up to 24 hours following $^{3}$H-scopolamine. Rats were given $^{3}$H-scopolamine and then, at time points above, were given either vehicle or 100 mg/kg scopolamine. Amount of $^{3}$H-scopolamine bound in whole brain was then assessed according to methods above.

Previous studies (Miyoshi et al., 1989, Hulme et al., 1990b) have demonstrated that both cerebellum and heart contain predominantly m2 receptors. To determine if the affinity for $^{3}$H-scopolamine was the same in heart and cerebellum, saturation studies were performed using heart and cerebellar membranes. The affinity of $^{3}$H-scopolamine for the two tissues was quite similar, with average $K_d$ values of 448 pM and 534 pM for cerebellum and heart, respectively, while the affinity of $^{3}$H-scopolamine in cortex was found to be 10 fold lower, with a $K_d$ of 40 pM.

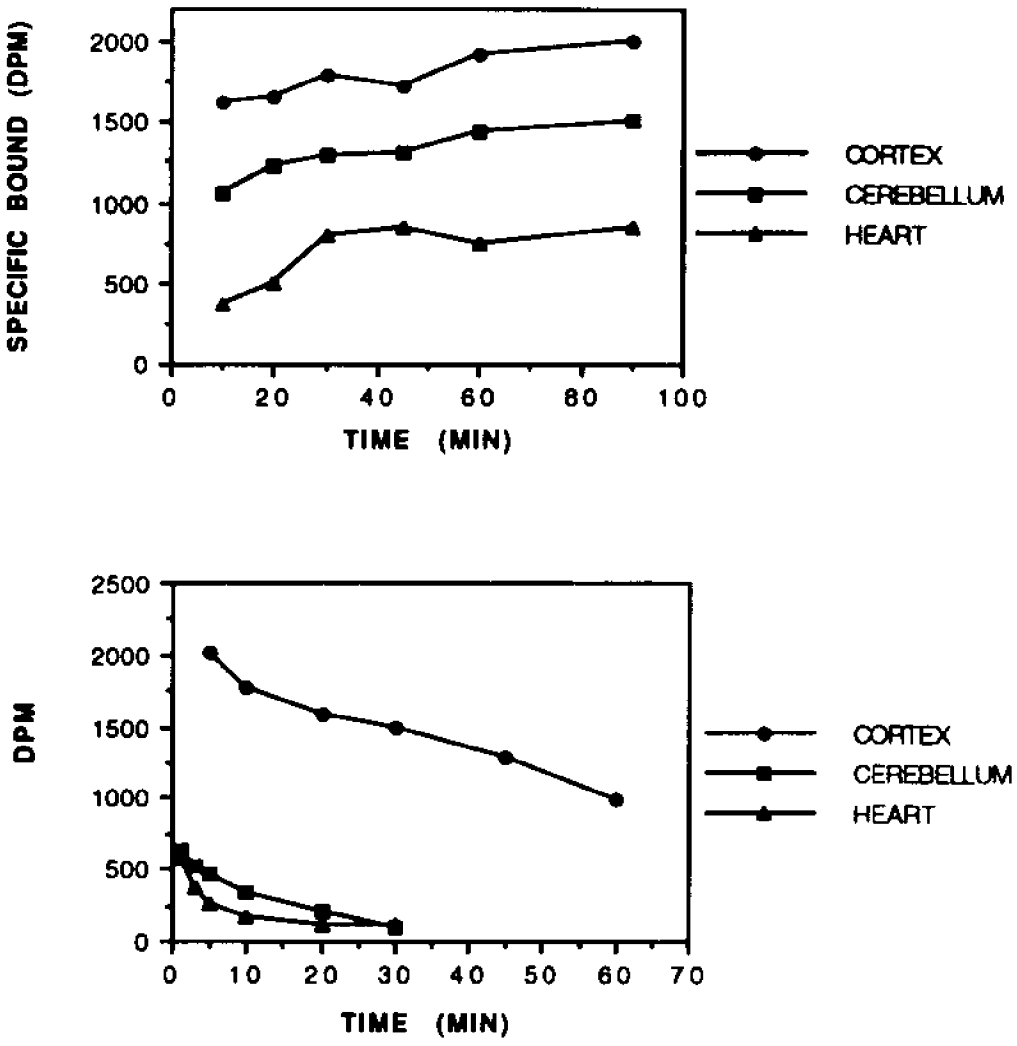



**FIGURE 14:** Representative studies of a.) association and b.) dissociation of $^{3}$H-scopolamine from cortex, cerebellum and heart membranes. $^{3}$H-scopolamine was incubated with either cortex, cerebellum, or heart membranes to determine association rates. Dissociation rates were determined by addition 100 uM atropine one hour after incubation and then terminating the reaction at various time points thereafter. Assays were performed at 25 ° C.

Competition studies were performed to determine the ability of the standard muscarinic antagonists scopolamine and atropine, as well as their

quaternary analogs methylscopolamine and methylatropine, to displace $^{3}H$-scopolamine from muscarinic receptors in a number of brain regions and heart using the *in vivo* binding method described above. In addition, the antagonist dexetimide and its inactive enantiomer levetimide were also tested for their ability to displace $^{3}H$-scopolamine *in vivo*. The results are summarized in Table 3.

**TABLE 3: $IC_{50}$ values (mg/kg) for in vivo binding of muscarinic antagonists to different rat brain regions.**

| | REGION | | | | |
|---|---|---|---|---|---|
| **COMPOUND** | CORTEX | STRIATUM | HIPPOCAMPUS | CEREBELLUM | HEART |
| **SCOPOLAMINE** | 0.08 | 0.27 | 0.11 | 0.004 | 0.001 |
| **METHYLSCOPOLAMINE** | 100 | 74.4 | 69.4 | 30.46 | 0.001 |
| **ATROPINE** | 12.09 | 31.87 | 12.45 | 0.23 | 0.001 |
| **METHYLATROPINE** | >100 | >100 | >100 | >100 | 0.001 |
| **DEXETIMIDE** | 0.51 | 0.83 | 0.61 | 0.02 | 0.003 |
| **LEVETIMIDE** | >100 | >100 | >100 | >100 | 0.7 |

The $IC_{50}$ values in cerebellum for the muscarinic antagonists shown in Table 3 are lower than for other regions. To determine if this effect was the result of a greater affinity of these compounds for m2 receptors in the cerebellum or merely reflects a difference in the affinity of $^{3}H$-scopolamine for the different muscarinic receptor subtypes, saturation studies were performed

to assess the affinity of $^{3}$H-scopolamine for the five muscarinic receptors which had been transfected into chinese hamster ovary cells. The results of these studies are summarized in Table 4.

**TABLE 4: Summary of saturation studies of $^{3}$H-scopolamine in muscarinc receptor clones in CHO cell membranes.**

| | Kd (pM) | | | | |
|---|---|---|---|---|---|
| | **m1** | **m2** | **m3** | **m4** | **m5** |
| **AVG.** | 93.9 | 433.2 | 80.0 | 110.5 | 189.7 |
| **S.E.** | 7.2 | 72.3 | 6.1 | 34.7 | 12.1 |
| | **Bmax (fmol/mg pro)** | | | | |
| | **m1** | **m2** | **m3** | **m4** | **m5** |
| **AVG.** | 2530.7 | 618.6 | 1456.6 | 1561.2 | 613.2 |
| **S.E.** | 66.3 | 79.6 | 85.7 | 159.1 | 40.6 |

In order to correlate the $IC_{50}$ values for *in vivo* binding with the MED values found in the PAR, the dpm/mg values for whole brain were calculated by combining the dpm values for each brain region, as well as the remainder of the brain not used in regional analysis, and then combining tissue weights for the individual regions to determine a whole brain dpm/mg tissue value. The $IC_{50}$ values for whole brain for these compounds and their corresponding MED values in the PAR are shown in Table 5. A Spearman rank-order correlation was significant ($r_s = 1.0$, $p < 0.05$). A summary graph showing the inhibition of $^{3}$H-scopolamine binding in whole brain by the two classic muscarinic antagonists and their quaternary amines is shown in Figure 15.

**TABLE 5: Whole brain $IC_{50}$ values for in vivo binding vs. $^3H$-scopolamine and MED values for PAR of muscarinic antagonists administered subcutaneously.**

| COMPOUND | IC50 (MG/KG) WHOLE BRAIN | MED (MG/KG) IN PAR |
|---|---|---|
| SCOPOLAMINE | 0.08 | 0.1 |
| METHYLSCOPOLAMINE | 55.83 | 10 |
| ATROPINE | 9.36 | 1 |
| METHYLATROPINE | >100 | >100 |
| DEXETIMIDE | 0.36 | ND |
| LEVETIMIDE | >100 | ND |

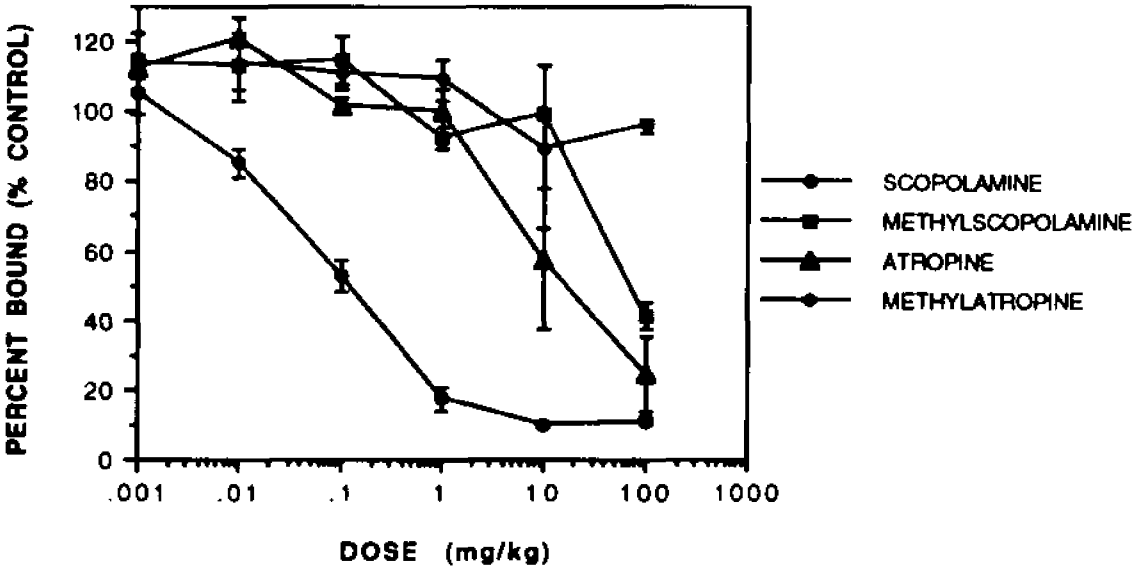



**FIGURE 15:** Summary of *in vivo* binding of muscarinic antagonists v. $^3H$-scopolamine in rat whole brain. Each point represents mean ± S.E. of at least three rats at that dose.

## DISCUSSION

The data presented in this study is a compilation of *in vitro*, *in vivo* and behavioral work which attempts to demonstrate the viability of an *in vivo* receptor binding methodology using $^{3}$H-scopolamine as a measure of muscarinic receptor occupancy. These data are not comprehensive, but are presented as an initial report of the use of $^{3}$H-scopolamine in an *in vivo* procedure.

The results of these studies indicate that $^{3}$H-scopolamine penetrates the blood brain barrier and binds to muscarinic receptors within the CNS. The binding of $^{3}$H-scopolamine is found throughout the CNS and periphery in regions associated with muscarinic receptor localization, including the cortex, striatum, hippocampus, cerebellum and heart. In addition, the binding of $^{3}$H-scopolamine in the CNS can be selectively displaced by muscarinic antagonists which cross the blood-brain barrier, such as atropine, but not by quaternary amines which do not cross, such as methylatropine. The results presented here then illustrate that the binding of $^{3}$H-scopolamine to muscarinic receptors using this procedure is specific and appropriately localized.

The results of the time course study demonstrate that in cortex, striatum and hippocampus, the binding of $^{3}$H-scopolamine to muscarinic receptors is found to be at high levels up to 24 hours post-administration. These results are similar to those described initially for $^{3}$H-QNB binding using an *in vivo* procedure (Yamamura et al., 1974), and more recent dissociation experiments examining wash out kinetics in various tissues following i.v. $^{3}$H-QNB administration (Gibson et al., 1991). One interpretation of this finding is

that $^{3}$H-scopolamine is somehow metabolized to an inactive form at some point following s.c. administration. However, when an active dose of scopolamine (100 mg/kg) was found to displace $^{3}$H-scopolamine to background levels when administered 24 hours later, it suggests that the $^{3}$H-scopolamine which is bound to muscarinic receptors in cortex, striatum and hippocampus at 24 hours has not been metabolized to an inactive form. Therefore, this study indicates that $^{3}$H-scopolamine is still actively bound to muscarinic receptors at significant levels at least 24 hours following administration. The amount of $^{3}$H-scopolamine injected initially (0.15 ug/kg) is so small as to not produce any behavioral effects by itself, so it is possible that it remains available to bind to muscarinic receptors in a equilibrium state. The off-rate of $^{3}$H-scopolamine from cortical tissue was confirmed to be quite slow using *in vitro* methods, so the slow dissociation of $^{3}$H-scopolamine from muscarinic receptors in this region is not an artifact of using an *in vivo* methodology. A recent study demonstrated that a single dose of atropine (500 mg/kg) administered up to 7 days prior was still available to displace $^{125}$I-dexetimide from cortex (Uno et al., 1991), which confirms that muscarinic receptor blockade by antagonists can be of long duration.

The *in vivo* time course studies also demonstrated that the dissociation of $^{3}$H-scopolamine from muscarinic receptors in cerebellum and heart occurred more quickly than in the other tissues. This finding concurs with the time course in cerebellum reported by Yamamura (1974) and later by Gibson et al. (1991) using $^{3}$H-QNB *in vivo*. In addition, the fast dissociation rates of $^{3}$H-scopolamine from cerebellum and heart were confirmed using *in vitro* assays. As cerebellum and heart are areas known to contain predominantly m2 receptors (Hulme et al., 1990b), studies were undertaken to determine if the muscarinic receptors in heart and cerebellum are the same subtype and

whether the kinetics of $^{3}$H-scopolamine are different at the m2 receptor compared to the other muscarinic receptor subtypes.

The first issue of whether the muscarinic receptors in cerebellum and heart have the same kinetics was addressed using *in vitro* studies in these tissues. Both the assocation and dissociation rates of $^{3}$H-scopolamine in the two tissues were the same, as were the $K_d$ and $B_{max}$ values determined by saturation analysis of membranes prepared from the two tissues. It seems likely that the same muscarinic receptor found in heart is that found in cerebellum, and it is of the m2 subtype.

The difference in dissociation rates for cerebellum and heart, areas known to be predominantly m2, compared with that seen in cortex suggests that there is a difference in the kinetics for the m2 receptor compared with the other muscarinic receptor subtypes. Saturation studies using cloned muscarinic receptors suggested a difference in affinity for $^{3}$H-scopolamine of over four-fold between the m2 receptor and the m1 and m4 receptor, the other receptors which predominate in the CNS. Other studies using muscarinic receptor clones report up to a five-fold lower affinity of both methylscopolamine (Buckley et al., 1989) and atropine (Peralta et al., 1987) for m2 receptors compared with other muscarinic receptor subtypes. However, saturation studies performed comparing the affinity of $^{3}$H-scopolamine for cerebellum and heart, approximately 500 pM, with that of cortex, approximately 40 pM (data not shown), suggest up to a ten-fold lower affinity of $^{3}$H-scopolamine for the m2 receptor compared with the subytpes located in the cortex, including m1 and m4. Other studies have confirmed a ten-fold lower affinity of scopolamine for m2 muscarinic receptors *in vitro* (Billard et al., 1995).

The results of the competition studies indicate that in the CNS, regardless of region, the order of potency is scopolamine > dexetimide > atropine > methylscopolamine > methylatropine = levetimide. In the heart, the drugs appear to be largly equipotent, with the exception of levetimide, which is over 100-fold less potent that its active enantiomer dexetimide. The fact that all the drugs tested but levetimide are equipotent peripherally suggests that the difference in their affinity for central muscarinic receptors is a function of their availability within the CNS, i.e. ability to cross the blood-brain barrier. A difference in the bioavailability of the compounds for muscarinic receptors in the CNS *in vivo* is further suggested by *in vitro* binding studies performed in our lab in cortical membranes using $^{3}$H-QNB as the ligand, which found that scopolamine, atropine, and their quaternary amines are equipotent for muscarinic receptors *in vitro* (data not shown). In addition, scopolamine, atropine methylscopolamine and methylatropine are all active behaviorally when administered icv (Smith et al., 1994).

Regional potency of the compounds tested *in vivo* shows that all compounds appear to be more potent in cerebellum than in other areas. The lower $IC_{50}$ values in the cerebellum could be simply that the affinity of $^{3}$H-scopolamine for m2 receptors is lower than for other muscarinic receptors. If the affinity of the ligand is lower for one receptor subtypes than for the others, it would therefore take less competing compound to displace the ligand from that receptor because it does not bind as well, with the result of an apparently lower $IC_{50}$. The results of the saturation studies in the cloned muscarinic receptor membranes suggest that the latter effect had taken place. In these saturation studies in cloned muscarinic receptors, $^{3}$H-scopolamine had a 4-5 fold lower affinity for m2 receptors compared with m1 and m4, the other receptors which predominate in the CNS. When saturation studies were

performed in cerebellum and heart and the values compared with those previously obtained in cortex, a difference of at least 10 fold between the affinity of $^{3}$H-scopolamine for the m2 receptors which predominate in cerebellum and heart compared with other muscarinic receptor subtypes was found. Taken together, these results suggest that the $IC_{50}$ values obtained in cerebellum using the *in vivo* procedure are overestimates based on the lower affinity of $^{3}$H-scopolamine for m2 receptors. If the affinity of $^{3}$H-scopolamine at m2 receptors *in vivo* is 10 fold lower than at other receptor subtypes, then many of the $IC_{50}$ values obtained in cerebellum would be consistent with those obtained in other regions.

When the whole brain $IC_{50}$ values for scopolamine, atropine, methylscopolamine and methylatropine are compared with the the MED values obtained in the PAR test, a significant correlation emerges. Although the sample size is small, the result is encouraging because it suggests a relationship between *in vivo* muscarinic receptor occupancy and a behavioral effect associated with muscarinic receptors. More compounds will need to be tested both *in vitro* and *in vivo* to see if the correlation between the two is sustained.

Overall, the findings of this study suggest a viable method for the study of receptor occupation *in vivo*. The data presented demonstrate that standard muscarinic antagonists displace $^{3}$H-scopolamine in a dose dependent fashion, and that this displacement is selective to muscarinic antagonists which cross the blood-brain barrier. In addition, the results obtained using this method correlate well both with *in vitro* kinetic studies and with data obtained in a behavioral test known to be sensitive to muscarinic antagonists. This method may be used to determine not only the selectivity of unknown compounds for muscarinic receptors *in vivo*, but also may allow for a

determination of pharmacokinetic effects which may influence the affinity of a compound for muscarinic receptors *in vivo*.

Finally, the *in vivo* methodology described here has applications for other receptor systems in the CNS and periphery. Just the ability to determine the CNS penetrability of a compound alone makes this a useful technique. In combination with lesion studies and selective inactivating agents, as well as selective radioligands, this model has a potential to provide a great deal of information about receptor occupancy *in vivo* using whole animal preparations. The implications of the data presented here and future directions for this type of *in vivo* model, as well as the utility of *in vivo* models as a whole, will be discussed in the next chapter.

# CHAPTER 4

# DISCUSSION: UTILITY OF IN VIVO METHODOLGIES

The major conclusions to be drawn from these studies are that *in vivo* binding methods can be used to determine the penetration of a ligand across the blood-brain barrier, to measure its selective binding to appropriate receptors, and to quantify its dose-dependent displacement by selective antagonists. All of these results can be obtained using *in vivo* procedures that maintain the integrity of both the blood-brain barrier and the interconnections within the CNS.

In Chapter 2, the studies examining the *in vivo* binding of $^{3}$H-SCH 39166 and $^{3}$H-raclopride to D1 and D2 dopamine receptors in rat striatum demonstrated that known D1 selective antagonists displaced $^{3}$H-SCH 39166 with high affinity, but not $^{3}$H-raclopride, whereas known D2 antagonists displaced $^{3}$H-raclopride, but not $^{3}$H-SCH 39166. This finding demonstrates that $^{3}$H-SCH 39166 and $^{3}$H-raclopride bind selectivity to D1 and D2 receptors, respectively, and that they can be displaced in a dose-dependent fashion with high affinity only by the appropriately selective D1 and D2 antagonists. Further, these studies demonstrate that these D1 and D2 receptors are located in high concentration in rat striatum, an area previously associated with the presence of dopamine receptors, and in much lower concentrations in the cortex or cerebellum.

The results of the studies using an *in vivo* binding methology to examine the selectivity of SCH 39166 for D1 receptors were corroborated using both *ex vivo* and *in vitro* methodologies. In the EEDQ study, SCH 39166

selectively protected D1 receptors in rat striatum from inactivation by the alkylating agent, but not D2 and $5HT_2$ receptors The EEDQ study used an *ex vivo* procedure, in which the rats were treated with SCH 39166 +/- EEDQ, the tissues removed and examined for changes in $B_{max}$ using *in vitro* techniques. SCH 39166 was found to be D1 selective at dopamine receptors in rat striatum using this procedure as well. Neither D2 nor $5HT_2$ receptors were protected from EEDQ inactivation following treatment with SCH 39166.

Similarly, Experiment 3 used an *ex vivo* procedure to examine the D1 selectivity of SCH 39166 in rhesus monkeys. SCH 39166 was administered chronically to rhesus monkeys and the striatum was later dissected and analyzed for changes in Bmax *in vitro.* Selective up-regulation of D1 receptors was found following chronic SCH 39166 treatment, as seen by increases in $B_{max}$ in striatum. No up-regulation of either D2 or $5HT_2$ receptors was found, again confirming the D1 selectivity of SCH 39166. In addition, this study confirms the D1 selectivity of SCH 39166 in a non-human primate.

Finally, another *in vivo* methodolgy confirmed the D1 selectivity of SCH 39166 in rat striatum. Using an *in vivo* autoradiographic procedure, $^3$H-SCH 39166 crossed the blood-brain barrier and bound to structures associated with dopamine receptors, including the substantia nigra and nucleus accumbens, and was displaced selectively by SCH 23390 and SCH 39166, but not by D2 or $5HT_2$ antagonists. An additional advantage to this technique is that it allows for the visualization of dopamine pathways in intact slices. Regardless of the procedure used, the same results were obtained, that provides further validity for *in vivo* methodologies and demonstrates their utility in examining the pharmacology of receptor systems.

The results reported in Chapter 3 using *in vivo* methodologies to examine muscarinic receptor pharmacology are more preliminary, mainly because there are no selective ligands available that cross the blood-brain barrier. However, the results of these studies do indicate that $^{3}$H-scopolamine, a muscarinic antagonist, crosses the blood-brain barrier when administered systemically, and binds to muscarinic receptors in the CNS. The regions to which $^{3}$H-scopolamine binds are associated with the presence of muscarinic receptors both in the CNS and peripherally in the heart. In addition, $^{3}$H-scopolamine is displaced selectively by muscarinic antagonists that cross the blood-brain barrier and it is not displaced by those that do not cross. The regional differences in the $EC_{50}$'s obtained using *in vivo* procedures can be partly explained by differences in the affinity of $^{3}$H-scopolamine in heart and cerebellum compared with cortex. The off-rate of $^{3}$H-scopolamine is faster in cerebellum and heart, areas with predominantly m2 receptors, when compared with m1 and m4 receptors in the cortex. In addition, the faster off-rate of $^{3}$H-scopolamine in cerebellar tissue was confirmed when $^{3}$H-scopolamine was shown to have a lower affinity for m2 receptors than for the other four cloned muscarinic receptors. The lower affinity for $^{3}$H-scopolamine at m2 receptors may explain why the $EC_{50}$'s for compounds in cerebellum *in vivo* were lower. If the ligand binds with lower affinity and comes off the m2 receptor at a faster rate than other subtypes, than less competing drug would be needed to displace the ligand, that would result in a lower $EC_{50}$ and apparently greater affinity of the drug for m2 receptors. The results of these *in vitro* procedures help explain the data obtained *in vivo*.

If similar results are obtained using *in vivo* and *in vitro* procedures, what is the utility of *in vivo* procedures? One of the major uses of *in vivo*

procedures is the determination of CNS penetrability of compounds. Both the compound of interest and the radiolabeled ligand for a given receptor are administered sytemically. If the compound is able to displace the label from brain homogenates, then the compound can be said to penetrate the blood-brain barrier. Thus, *In vivo* binding can be used as a rapid screen to determine the CNS penetrability of many compounds. Determinations of CNS penetrability are not only important for basic research of the pharmacology of receptors within the brain, but are crucial for applied research, particularly drug development.

The most promising use of *in vivo* procedures comes from correlating receptor occupancy with behavioral effects seen by the drug of interest. In the studies of D1 dopamine selectivity in Chapter 3, doses of either D1 or D2 antagonists that produced fifty percent occupancy of dopamine receptors were highly correlated with doses . were minimally effective in producing behavioral effects in the conditioned avoidance response (CAR) test. Similarly, a promising correlation was found between $EC_{50}$ values for *in vivo* binding of muscarinic antagonists and their corresponding MED values in the passive avoidance response (PAR) test. While the significance of these correlations are open to interpretation, they do suggest that the behavioral effects produced by these drugs are at least in part related to their binding to the appropriate receptors, dopamine D1 or D2 receptors for the CAR and muscarinic receptors for the PAR. It is the ability to correlate biochemistry with behavior, thereby suggesting a functional role for receptors, that makes the use of *in vivo* methods so potentially powerful.

Yet for all the potential of *in vivo* methods, they are only as powerful as the tools available. The dopamine studies represent a more ideal circumstance, with more discreetly localized receptor subtypes and highly

selective ligands making the use of *in vivo* procedures to study this system less complicated. The *in vivo* binding studies of the muscarinic receptors in the CNS, on the other hand, were complicated by the lack of selective ligands for the five muscarinic subtypes and their more widely distributed and overlapping distribution. This is not to say that the *in vivo* procedures used did not yield any information of muscarinic receptor occupancy in the CNS, but selectivity of compounds for different receptor subtypes is not possible given the current tools.

Overall, the results of these studies illustrate the usefulness of *in vivo* methodologies to study the pharmacology and biochemistry of receptors in the CNS. In these studies, different types of *in vivo* procedures yielded similar results, providing face validity for the results obtained using *in vivo* methods. Finally, the utility of *in vivo* methods for determining the correlation of behavioral effects and biochemical receptor occupancy allow for the determination of the functional role of receptor systems in behavior. These methods may be useful for examining other receptor systems in both the CNS and periphery, where appropriately selective ligands are available.

## REFERENCES


Alburges, M.E., Hunt, M.E., McQuade, R.D. and Wamsley, J.K. (1992) D1-receptor antagonists: Comparison of $^{3}$H-SCH 39166 to $^{3}$H-SCH 23390. J. Chem. Neuroanatomy, 5: 357-366.

Atweh, S and Kuhar, M.J. (1977) Autoradiographic localization of opiate receptors in rat brain: The telencephalon. Brain Res., 134: 393-405.

Balderssarini, R.J. (1980) Drugs and the treatment of psychiatric disorders. In: Goodman and Gilman's the Pharmacological Basis of Therapeutics (Alfred Goodman Gilman, Louis Goodmanand Alfred Gilman, eds.), Macmillan: New York, 3391-445.

Billard, W., Ruperto, V., Crosby, G., Iorio, L.C. and Barnett, A. (1984) Characterization of the binding of $^{3}$H-SCH 23390, a selective D-1 receptor antagonist ligand, in rat striatum. Life Sci. 35: 1885-1893.

Billard, W., Binch, H., Crosby, G. and McQuade, R.D. (1995) Identification of the primary muscarinic autoreceptor subtype in rat striatum as m2 through a correlation of in vivo microdialysis and in vitro binding data. J. Pharmacol. Exp. Ther., 273: 273-279.

Birdsall, N.J.M. and Hulme, E.C. (1983) Muscarinic receptor subclasses. Trends in Pharmacol. Sci., 4: 459-463.

Birdsall, N.J.M., Curtis, C.A.M., Eveleigh, P., Hulme, E.C., Pedder, E.K., Poyner, D. and Wheatley, M. (1988) Muscarinic receptor subtypes and the selectivity of agonists and antagonists. Pharmacology, 37 (suppl): 22-31.

Bonner, T.I. (1990) New subtypes of muscarinic acetylcholine receptors. Trends in Pharmacol. Sci., Suppl: Subtypes Muscarinic Receptors IV, pp. 11-15.

Bonner, T.I., Buckley, N.J., Young, A.C. and Brann, M. (1987) Identification of a family of muscarinic acetylcholine receptor genes. Science, 237: 527-532.

Bonner, T.I., Young, A.C., Brann, M.R. and Buckley, N.J. (1988) Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. Neuron, 1: 403-410.

Buckley, N.J., Bonner, T.I., Buckley, C.M. and Brann, M.R. (1989) Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. Mol. Pharmacol., 35: 403-410

Chang, R.S and Snyder, S.H. (1978) Benzodiazepine receptors: Labeling in intact animals with $^{3}$H-Flunitrazepam. Eur. J. Pharmacol., 48: 213-218.

Chipkin, R.E., McQuade, R.D. and Iorio, L.C. (1987) D1 and D2 dopamine binding site up-regulation and apomorphine-induced sterotypy. Pharmacol. Biochem. Behav. 28: 477-482.

Chipkin, R.E., Iorio, L.C., Coffin, V.C., McQuade, R.D., Berger, J.G. and Barnett, A. (1988) Pharmacological profile of SCH 39166: A dopamine D1 selective benzonaphthazepine with potential antipsychotic activity. J. Pharmacol. Exp. Ther., 247: 1093-1102.

Coffin, V.L., M.B. Latranyi and R.E. Chipkin (1989) Acute extrapyramidal syndrome in Cebus monkeys: Development mediated by dopamine $D_2$ but not $D_1$ receptors. J. Pharmacol. Exp. Ther., 249: 769.

Coffin, V.L., McHugh, D., Chipkin, R.E. and Barnett, A. (1991) SCH 39166, a potential antipsychotic drug, does not evoke movement disorders in Cebus monkeys. Neurochem. International, 20: 141S-145S

Cohen, V.I., Jin, B., Gitler, M.S., de la Crus, R.A., Boulay, S.F., Sood, V.K., Zeeberg, B.R. and Reba, R.C. (1995) Novel potent and m2-selective antimuscarinic compounds which penetrate the blood-brain barrier. Eur. J. Med Chem., 30: 61-69.

Cook, L. and A.B. Davidson (1978) Behavioral pharmacology: Animal models involving aversive control of behavior, in: Psychopharmacology: A Generation of Progress, eds. M.A. Lipton, A. Dimascio and K. Killam (Raven Press, New York) p. 563.

Coppens, H.J., Sebens, J.B. and Korf, J. (1995) Catalepsy, Fos protein and dopamine receptor occupancy after long-term haloperidol treatment. Pharm. Biochem and Behav., 51: 175-182.

Dorje, F., Levey, A.I. and Brann, M.R. (1991) Immunological detection of muscarinic receptor subtype proteins (m1-m5) in rabbit peripheral tissues. Mol. Pharm., 40: 459-462.

Dorje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschlet, E. and Brann, M.R. (1991) Antagonist binding profiles of five cloned human muscarinic receptor subtypes. J. Pharmacol. Exp. Ther., 256: 727-733.

Duffy, R.A., Kaminska, G., Chipkin, R.E. and McQuade, R.D. (1988) Selective up-regulation of D1 dopamine receptors following chronic administration of SCH 39166 in primates. Pharmacol. Biochem. Behav., 41: 235-238.

Duffy, R.A., Hunt., M.A., Wamsley. J. and McQuade, R.D. In vivo autoradiography of $^{3H}$-SCH 39166 in rat brain: Selective displacement by D1 antagonists. Brain Res., submitted, 1995.

Duffy, R.A. and McQuade, R.D. In vivo binding of $^{3H}$-scopolamine in rat brain. Manuscript in preparation, 1996.

Ehlert, F.J. and Tran, L.P. (1990) Regional distribution of M1, M2 and non-M1, non -M2 subtypes of muscarinic binding sites in rat brain. J. Pharmacol. Exp. Ther., 255: 1148-1157.

Gerlach, J.L. and McEwen, B.S. (1972) Rat brain binds adrenal steroid hormone: Radioautography of hippocampus with corticosterone. Science, 175: 1133-1136.

Gibson, R.E., Zeeberg, B.R., Melograna, J.M., Wang, T.F., Ruch, J., Braun, A. and Reba, R.C. (1991) In vivo dissociation kinetics of $^{3}$H-quinuclidinyl benzilate: Relationship to muscarinic receptor concentration and in vitro kinetics. Brain Res., 553: 110-116.

Gil, D.W. and Wolfe, B.B. (1985) Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinostide breakdown and inhibition of adenylate cyclase. J. Pharmacol. Exp. Ther., 232: 608-616.

Gitler, M.S., de la Cruz, R., Zeeberg, B.R. and Reba, R.C. (1994) $^{3}$H-QNB displays in vivo selectivity for the m2 subtype. Life Sci., 55: 1493-1508.

Griffin, M.T., Thomas, E.A. and Ehlert, F.J. (1993) Kinetics of activation and in vivo muscarinc receptor binding of N-(2-bromoethyl)-4-peperidinyl diphenylacetate: An analog of 4-DAMP mustard. J. Pharmacol. Exp. Ther., 266: 301-305.

Hall, H., Halldin, C. and Sedvall, G. (1993) Binding of $^{3H}$-SCH 39166 to post mortem brain tissue. Pharmacol. Toxicol., 72: 152-158.

Halldin, C., Farde, L., Barnett, A. and Sedvall, G. (1991) Synthesis of carbon-11 labelled SCH 39166, a new selective dopamine D1 receptor ligand, and preliminary PET investigations. Inter. J. of Radiation Appl. Instrument - Part A, Appl. Radiation and Isotopes, 42: 451-455.

Hamblin, M.W. and Creese, I. (1983) Behavioral and radioligand binding evidence for irreversible dopamine receptor blockade by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. Life Sci. 32: 2247-2255.

Haroutunian, V., Barnes, E. and Davis, K. (1985) Cholinergic modulation of memory in rats. Psychopharmacology, 87: 266-271.

Hess, E.J., Albers, L.J., Le, H. and Creese, I. (1986) Effects of chronic SCH 23390 treatment on the biochemical and behavioral properties of D1 and D2 dopamine receptors: Potentiated behavioral responses to a D2 dopamine agonist after selective D1 dopamine receptor upregulation. J. Pharmacol. Exp. Ther. 238: 846-854.

Hess, E.J. and Creese, I. (1987) Biochemical characterization of dopamine receptors. In Receptor biochemistry and Methodology Vol 8: Dopamine receptors (editors I. Creese and C. Fraser), Alan J. Liss: New York, 1-27.

Hiramuatsu, Y., Kawai, R., Reba, R.C., Simon, T.R., Baum, B.J. and Blasberg, R.G. (1993) Kinetic analysis of rat parotid gland muscarinic receptors in vivo: Comparison with brain and heart. Amer. J. Physiol., 264: G541-G552.

Hiramatusu, Y., Kawai, R., Reba, R.C., Blasberg, R.G. and Baum, B.J. (1994) Kinetic analysis of rat exocrine gland muscarinic receptors in vivo. J. Pharmacol. Exp. Ther., 269: 1205-1212.

Hollt, V., Dum, J., Blasig, J., Schubert, P. and Herz, A. (1975) Comparison of in vivo and in vitro parameters of opiate receptor binding in naive and tolerant/dependent rats. Life Sci., 16: 1823-1828.

Hollt., V., Czlonkowski, A., and Herz., A. (1977) The demonstration in vivo of specific binding siters for neuroleptic drugs in mouse brain. Brain Res., 130: 176-183.

Hollt, V. and Schubert, R. (1978) Demonstration of neuroleptic receptor sites in mouse brain by autoradiography. Brain Res., 151: 149-153.

Hulme, E.C., Curtis, C.A.M., Wheatley, M., Aitken, A. and Harris, A.C. (1990a) Localization and structure of the muscarinic receptor ligand binding site. Trends Pharmacological Sciences Supplement Subtypes Muscarinic Receptors IV, pp. 22-25.

Hulme, E.C., Birdsall, N.J.M. and Buckley, N.J. (1990)b Muscarinic receptor subtypes. Ann. Rev. Pharmacol. Toxicol., 30: 633- 673.

Hunt, S.P. and Schmidt, J. (1978a) The electron microscopic autoradiographic localization of a-bungarotoxin binding sites within the central nervous system of the rat. Brain Res., 152: 152-159.

Hunt, S.P. and Schmidt, J. (1978b) Some observation on the binding patterns of a-bungarotoxin in the central nervous system of the rat. Brain Res., 157: 213-232.

Iorio, L.C., A. Barnett, F.H. Leitz, V.P. Houser and C.A. Korduba (1983) SCH 23390, a potential benzazepine antipsychotic with unique interactions on dopaminergic systems, J. Pharmacol. Exp. Ther. 226, 462-468.

Jagust, W.J. (1994) Functional imaging in dementia: An overview. J. Clin Psychiatry, 55: 11(suppl), 5-11.

Jobst, K.A., Hindley, N.J., King, E. and Smith, A.D. (1994) The diagnosis of Alzheimers's disease: A question of image? J. Clin. Psychiatry, 55: 11 (suppl), 22-31.

Jones, S.V., Levey, A.I., Weiner, D.M., Ellis, J., Novotny, E., Yu, S., Dorje, F., Wess, J. and Brann, M. (1991) Muscarinic acetylcholine receptors. In Molecular biology of receptors which couple to G-proteins (Ed. Brann, M.R.). Birkhauser: Boston.

Kandel, E.R. (1991) Transmitter release. In: Principles of Neural Science, Vol 3 (Eric R. Kandel, James H. Schwartz and Thomas M. Jessel, eds.), Appleton and Lange: Norwalk Conn., 194-212.

Kebabian, J. and Calne, D.B. (1979) Multiple receptors for dopamine. Nature. 277: 93-98.

Klemm, N., Murrin, L.C. and Kuhar, M.J. (1979) Neuroleptic and dopamine receptors: Autoradiographic localization of $^{3H}$-spiperone in rat brain. Brain Res., 169: 1-9.

Kuhar, M.J. and Yamamura, H.I. (1975) Light autoradiographic localisation of cholinergic muscarinic receptors in rat brain by specific binding of a potent antagonist. Nature, 253: 560-561.

Kuhar, M.J. and Yamamura, H.I. (1976) Localization of cholinergic muscarinic receptors in rat brain by light miscroscopic radioautography. Brain Res., 110: 229-243.

Kuhar, M.J., Murrin, L.C., Malouf, A.T. and Klemm, N. (1978) Dopamine receptor binding in vivo: The feasibility of autoradiographic studies. Life Sci., 22: 203-210.

Kuhar, M.J., DeSouza, E.B. and Unnerstall, J.R. (1986) Neurotransmitter receptor mapping by autoradiography and other methods. Ann Rev. Neurosci., 9: 27-59.

Laduron, P.M. and Leysen, J. (1977) Specific in vivo binding of neuroleptic drugs in rat brain. Biochem. Pharmacol., 26: 1003-1007.

Laduron, P.M. and Janssen, P.F.M. (1979) Characterization and subcellular localization of brain muscarinic receptors labelled in vivo by $^{3H}$-dexetimide. J. Neurochem., 33: 1223-1231.

Lai, J., Mei, L, Roeske, W.R., Chung, F., Yamamura, H.I. and Venter, J.C. (1988) The cloned murine m1 muscarinic receptor is associated with the hydrolysis of phosphatidylinositols in transfected murine B82 cells. Life Sci., 42: 2489-2502.

Lapchak, P.A., Araujo, D.M., Quirion, R. and Collier, B. (1989) Binding sites for $^{3H}$-AFDX-116 and effect of AFDX-116 on endogenous acetylcholine release from rat brain slices. Brain Res., 496: 285-294.

Lappalainen, J., Hietala, J., Pohjalainen, T. and Syvalahti, E. (1992) Regulation of dopamine D1 receptors by chronic administration of structurally different D1 receptor antagonists: A quantitative autoradiographic study. Eur. J. Pharmacol., 210: 195-200.

Levey, A.I., Stormann, T.M. and Brann, M.R. (1990) Bacterial expression of human muscarinic receptor fusion proteins and generation of subtype-specific antisera. FEBS, 275: 65-69.

Levey, A.I., Kitt, C.A., Simonds, W.F., Price, D.L. and Brann, M.R. (1991) Indentification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J. Neurosci., 11: 3218-3226.

Levey, A.I., Hersch, S.M., Rye, D.B., Sunhara, R.K., Niznik, H.B., Kitt, C.A., Price, D.L., Maggio, R., Brann, M.R. and Ciliax, B.J. (1993) Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. Proceed. Nat. Acad. Sci. U.S. Amer., 90: 8861-8865.

Li, M., Yasuda, R.P., Wall, S.J., Wellstein, A. and Wolfe, B.B. (1991) Distribution of m2 muscarinic receptors in rat brain using antisera selective for m2 receptors. Mol. Pharmacol., 40: 28-35.

Liao, C., Themmen, A.P.N., Joho, R., Barberis, C., Birnbaumer, M. and Birnbaumer, L. (1989) Molecular cloning and expression of a fifth muscarinic acetylcholine receptor. J. Biol. Chem., 264: 7328-7337.

London, E.D., Scheffel, U., Kimes, A.S. and Kellar, K.J. (1995) In vivo labeling of nicotinic acetylcholine receptors in brain with $^{3}$H-epibatidine. Eur. J. Pharmacol., 278: R1-R2.

Martin, J.H., Brust, J.C. and Hilal, S. (1991) Imaging the Living Brain. In: Principles of Neural Science, Vol 3 (Eric R. Kandel, James H. Schwartz and Thomas M. Jessel, eds.), Appleton and Lange: Norwalk Conn., 309-324.

Matsumura, K., Uno, Y., Scheffel, U., Wilson, A.A., Dannals, R.F. and Wagner, H.N. Jr. (1991) In vitro and in vivo characterization of 4-[125I]Iododexetimide binding to muscarinic cholinergic receptors in the rat heart. J. Nucl. Med., 32: 76-80.

Mayberg, H.S. (1994) Clinical correlates of PET and SPECT-identified defects in dementia. J. Clin. Psychiatry, 55: 11(suppl), 12-21.

Mazziotta, J.C. (1994) Mapping human brain activity in vivo. West. J. Med., 161: 273-278.

McPherson, G.A. (1985) Kinetic, EBDA, ligand, lowry: A collection of radioligand binding analysis programs. Elsevier-Biosoft.

McQuade, R.D., N. Amlaiky, M. Caron, R. Chipkin, L. Iorio and A. Barnett (1988a) Characterization of the radioiodinated analogue of SCH 23390: In vitro and in vivo D-1 dopamine receptor binding studies, Life Sci. 43, 1151.

McQuade, R.D., Ford, D., Duffy, R.A., Chipkin, R.E., Iorio, L.C. and Barnett, A. (1988b) Serotonergic component of SCH 23390: In vitro and in vivo binding analyses. Life Sci., 43: 1861-1869.

McQuade, R.D., Duffy, R.A., Coffin, V.C., Chipkin, R.E. and Barnett, A. (1991a) In vivo binding of SCH 39166: A D1 selective antagonist. J. Pharmacol. Exper. Ther., 257: 42-49.

McQuade, R.D., Duffy, R.A., Anderson, C.C., Crosby, G.C., Coffin, V.C., Chipkin, R.E. and Barnett, A. (1991b) $^{3}$H-SCH 39166, a new D1 selective radioligand: In vitro and in vivo binding analyses. J. Neurochem., 57: 2001-2009.

McQuade, R.D., Duffy, R.A., Coffin, V.C. and Barnett, A. (1992) In vivo binding to dopamine receptors: A correlate to potential antipsychotic activity. Eur. J. Pharmacol., 215: 29-34.

Mei, L., Roeske, W.R. and Yamamura, H.I. (1989) Molecular pharmacology of muscarinic receptor heterogeneity. Life Sci., 45: 1831-1851.

Meller, E., Bohmaker, K., Goldstein, M. and Friedhoff, A.J. (1985) Inactivtion of D1 and D2 dopamine receptors by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in vivo: Selective protection by neuroleptics. J. Pharmacol. Exp. Ther. 233: 656-662.

Messer, W.S. Jr. and Hoss, W. (1987) Selectivity of pirenzepine in the central nervous system. I. Direct autoradiographic comparison of the regional distribution of pirenzepine and carbamylcholine binding sites. Brain Res., 407: 27-36.

Michel, A.D. and Whiting, R.L. (1988) Methoctramine, a polymethylene tetraamine, differentiates three subtypes of muscarinic receptor in direct binding studies. Eur. J. Pharmacol., 145: 61- 66.

Michel, A.D., Stafanich, E. and Whiting, R.L. (1989) Direct labeling of rat M3-muscarinic receptors by $^{3H}$-4-DAMP. Eur. J. Pharmacol., 166: 459-466.

Micheletti, R., Montagna, E. and Giachetti, A. (1987) AFDX-116, a cardioselective muscarinic antagonist. J. Pharmacol. Exper. Ther., 241: 628-634.

Mitchelson, F. (1988) Muscarinic receptor differentiation. Pharmacol. Ther., 37: 357-423.

Miyoshi, R., Kito, S and Shimoyama, M. (1989) Quantitative autoradiographic localization of the M1 and M2 subtypes of muscarinic acetylcholine receptors in the monkey brain. Japanese J. Pharmacol., 51: 247-255.

Murrin, L.C. and Kuhar, M.J. (1979) Dopamine receptors in rat frontal cortex: An autoradiographic study. Brain Res., 177: 279-285.

Murrin, L.C., Coyle, J.T. and Kuhar, M.J. (1980) Striatal opiate receptors: Pre- and postsynaptic localization. Life Sci., 27: 1175-1183.

Murrin, L.C. (1981) Neurotransmitter receptors: Neuroanatomical localization through autoradiography. Internat. J. Neurobiol., 22: 111-171.

Murrin, L.C., Gale, K. and Kuhar, M.J. (1979) Autoradiographic localization of neuroleptic and dopamine receptors in the caudate-putamen and substantia nigra: Effects of lesions. Eur. J. Pharmacol. 60: 229-235.

Norman, A.B., and Creese, I. (1986) Effects of in vivo and in vitro treatments with N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline on putative muscarinic receptor subtypes in rat brain. Mol. Pharmacol., 30: 96-103.

Pedigo, N.W., Schallert, T., Overstreet, D.H., Ling, N.C., Ragan, P., Reisine, T.D. and Yamamura, H.I. (1979) Inhibition of in vivo $^{3H}$-spiperone binding by the proposed antipsychotic des-tyr-g-endorphin. Eur. J. Pharmacol., 60: 359-364

Peralta, E.G., Ashkenazi, A., Winslow, J.W., Smith, D.H., Ramachandran, J. and Capon, D.J. (1987) Distinct primary structures, ligand binding properties and tissue specific expression of four human muscarinic acetylcholine receptors. EMBO J, 6: 3923-3929.

Pert, C.B., Kuhar, M.J. and Snyder, S.H. (1975) Autoradiographic localization of the opiate receptor in rat brain. Life Sci., 16: 1849-1854.

Pert, C.B.,, Kuhar, M.J. and Synder, S.H. (1976) Opiate receptor: Autoradiographic localization in rat brain. Proceed. Natl. Acad. Sci. USA, 73:3729-3733.

Regenhold, W., Araujo, D and Quirion, R. (1987) Direct visualization of brain M2 muscarinic receptors using the selective antagonist $^{3H}$-AFDX-116. Eur. J. Pharmacol., 144: 417-419.

Role, L.W. and Kelly, J.P. (1991) The brain stem: Cranial nerve nuclei and the monoaminergic system. In: Principles of Neural Science, Vol 3, (Eric R Kandel, James H. Schwartz and Thomas M. Jessel, eds.) Appleton and Lange: Norwalk, Conn., 683-699.

Scatchard, G. (1949) The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. 51: 660-674.

Sedvall, G., L. Farde and H. Hall (1988) D1 and D2 dopamine receptors in the human brain as studied by in vitro analysis and PET, in: Central and Peripheral Dopamine Receptors: Biochemistry and Pharmacology, eds. P.F. Spano, G. Biggio, G. Toffano and G.L. Gessa (Liviana Press, Padova, Italy) p. 81.

Sedvall, G., Farde, L., Barnett, A., Hall, H. and Halldin, C. (1991) $^{11}$C-SCH 39166, a selective ligand for visualization of dopamine D1 receptor binding in the monkey brain using PET. Psychopharmacology, 103: 150-153.

Seeger, T.F., Sforzo, G.A., Pert, C.B. and Pert, A. (1984) In vivo autoradiography: Visualization of stress-induced changes in opiate receptor occupancy in the rat brain. Brain Res.: 305: 303-311.

Seeman, P. (1987) Dopamine receptors and the dopamine hypothesis of schizophrenia, Synapse, 1: 133.

Sergent, J. (1994) Brain-imaging studies of cognitive functions. Trends in Neurosci. 6: 221-226.

Shulman, R.G., Rothman, D.L. and Blamire, A.M. (1994) NMR Studies of human brain function. Trends in Biolog. Sci., 19: 522-526.

Smith, R.D., Grzelak, M.E., and Coffin, V.L. (1994) Methylatropine blocks the central effects of cholinergic antagonists. Behavioural Pharmacology, 5: 167-175.

Sokoloff, P., Giros, B., Marpres, M.P., Bouthenet, M.L. and Schwartz, J.C. (1990) Molecular cloning and characterization of a novel dopamine receptor ($D_3$) as a target for neuroleptics. Nature, 347: 146-151.

Strange, P.G. (1992) Studies on the structure and function of D2-dopamine receptors. Biochemical Society Transactions, 20: 126-130.

Stumpf, W.E. (1968) Estradiol-concentrating neurons: Topography in the hypothalamus by dry-mount autoradiography. Science, 162: 1001-1003.

Sunahara, R.K., Guan, H.-C., O'Dowd, B.F., Seeman, P., Laurier, L.G., Ng, G., George, S.R., Torchia, J., Van Tol, H.M.H. and Niznik, H.B. (1991) Cloning of the gene for a human dopamine $D_5$ receptor with higher affinity for dopamine than $D_1$. Nature, 350: 614-619.

Taylor, L.A., Tedford, C.E., and McQuade, R.D. (1991) The binding of SCH 39166 and SCH 23390 to 5-HT1C receptors in porcine choroid plexus. Life Sci., 49: 1505-1511.

Thomas, E.A. and Ehlert, F.J. (1992) Irreversible effects of 4-DAMP mustard on muscarinic receptors in vivo. Proceed. West. Pharmacol. Soc., 35: 233-237.

Tice, M.A.B., Hashemi, T., Taylor, L.A., Duffy, R.A. and McQuade, R.D. (1994) Characterization of the binding of SCH 39166 to the five cloned dopamine receptor subtypes. Pharmacol., Biochem. and Behav., 49: 567- 571.

Tonnaer, J.A., van Vugt, M.A., de Boer, T. and de Graaf, J.S. (1987) Differential interactions of muscarinic drugs with binding sites of $^{3H}$-pirenzepine and $^{3H}$-quinuclidinyl benzilate in rat brain tissue. Life Sci., 40: 1981-1987.

Uno, Y., Matsumura, K., Scheffel, U., Wilson, A.A. Dannals, R.F. and Wagner, H.N. Jr. (1991) Effects of atropine treatment on in vitro and in vivo binding of 4-[125I]-dexetimide to central and myocardial muscarinic receptors. Eur. J. Nuclear Med., 18: 447-452.

Vanderschuren, L., Stein, E.A., Weigand, V.M. and Van Ree, J.M. (1995) Social play alters regional brain opiod receptor binding in juvenile rats. Brain Res., 680: 148-156.

Van Tol, H.M.H., Bunzow, J.R., Guan, H.-C., Sunhara, R.K., Seeman, P., Niznik, H.B. and Civelli, O. (1991) Cloning of the gene for a human dopamine $D_4$ receptor with high affinity for the antipsychotic clozapine. Nature, 350: 610-614; 1991.

Waelbroeck, M., Tastenoy, M., Camus, J. and Christophe, J. (1990) Binding of selective antagonists to four muscarinic receptors (M1 to M4) in rat forebrain. Mol. Pharmacol., 38: 267-273.

Wall, S.J., Yasuda, R.P., Hory, F., Flagg, S., Martin, B.M., Ginns, E.I. and Wolfe, B.B. (1991a) Production of antisera selective for m1 muscarinic receptors using fusion proteins: Distribution of m1 receptors in rat brain. Mol. Pharmacol., 39: 643-649.

Wall, S.J., Yasuda, R.P., Li, M. and Wolfe, B.B. (1991b) Development of antiserum against m3 muscarinic receptors: Distribution of m3 receptors in rat tissues and clonal cell lines. Mol. Pharmacol., 40: 783-789.

Wall., S.J., Wolfe, B.B. and Kromer, L.F. (1994) Cholinergic deafferentation of doral hippocampus by fimbria-fornix lesioning differentially regulates subtypes (m1-m5) of muscarinic receptors. J. Neurochem., 62: 1345-1351.

Wamsley, J.K., Hunt, M.E., McQuade, R.D. and Alburges, M.E. (1991) [$^{3}$H] SCH 39166, a D1 dopamine receptor antagonist: Binding characteristics and localization. Exper. Neurol., 111: 145-151.

Wang, J., Roeske, W.R., Hawkins, K.N., Gehlert, D.R. and Yamamura, H.I. (1989) Quantitative autoradiography of M2 muscarinic receptors in the rat brain identified by using a selective radioligand $^{3H}$-AFDX-116. Brain Res., 477: 322-326.

Wang, J.X., Roeske, W.R., Gulya, K., Wang, W. and Yamamura, H.I. (1987) $^{3H}$-AFDX-116 labels subsets of muscarinic cholinergic receptors in rat brain and heart. Life Sci., 41: 1751-1760.

Watson, M., Roeske, W.R. and Yamamura, H.I. (1986) $^{3H}$-pirenzepine and (-)-$^{3H}$-quinuclydinyl benzilate binding to rat ceebral cortical and cardiac muscarinic cholinergic sites II. Characterization and regulation of antagonist binding to putative muscarinic subtypes. J.Pharmacol. Exper. Ther., 237: 419-427.

Watson, N., Branes, P.J. and Maclagan, J. (1992) Actions of methoctramine, a muscarinic m2 antagonist, on muscarinic and nicotinic cholinoceptors in guinea-pig airways in vivo and in vitro. Br. J. Pharm., 105: 107-112.

Weiner, D.M. and Brann, M.R. (1990) Distribution of m1-m5 muscarinic receptor mRNAs in rat brain. Trends Pharmacol. Sci., Suppl: Subtypes Muscarinic Receptors IV, p. 115.

Wheatley, M., Hulme, E.C., Birdsall, N.J.M., Curtis, C.A.M., Eveleigh, P., Pedder, E.K. and Poyner, D. (1988) Peptide mapping studies on muscarinic receptor structure and location of the ligand binding site. Trends Pharmacol. Sci., Suppl: Subtypes Muscarinic Receptors III, pp. 19-23, 1988.

Williamson, M.J. and Paul, S.M. (1978) Labelling of benzodiazepine receptors in vivo. Nature, 275: 551-553.

Yamamura, H.I., Kuhar, M.J. and Snyder, S.H. (1974) In vivo identification of muscarinic cholinergic receptor binding in rat brain. Brain Res., 80: 170-176

Yasuda, R.P., Ciesla, W., Flores, L.R., Wall, S.J., Li, M., Satkus, S.A., Weisstein, J.S., Spagnola, B.V. and Wolfe, B.B. (1993) Development of antisera selective for m4 and m5 muscarinic cholinergic receptors: Distribution of m4 and m5 receptors in rat brain. Mol. Pharmacol., 42: 149-157.