DOPAMINERGIC MODULATION OF THE SEPTOHIPPOCAMPAL CHOLINERGIC SYSTEM

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The central cholinergic system is thought to be an integral component of the neural circuitry involved in mnemonic processes. The septohippocampal cholinergic pathway is considered crucial in this respect. In addition, however, other neurotransmitter systems are also likely involved in learning and memory either by interacting with the cholinergic system or on their own. Dopamine is postulated to be one such neurotransmitter. Accordingly, this thesis examines the possible interactions between dopamine and acetylcholine in the hippocampus, in particular in relation to cognitive processes.

Using a fimbriaectomy / receptor autoradiography approach, we examined the location of hippocampal dopamine receptors and found that a proportion of dopamine D1-like receptors are likely located on cholinergic terminals in the hippocampus. Moreover, the stimulation of D1-like, but not D2-like, receptors enhanced in vivo hippocampal acetylcholine release, in a phasic manner. The loci for this action is apparently at the level of the cholinergic terminals within the hippocampus.

There are at least two members of the D1-like family of dopamine receptors, namely D1 and D5 subtypes. Owing to the unavailability of selective ligands, we utilized a combined antisense-in vivo dialysis approach in order to ascertain which of these two receptors is involved in modulating hippocampal acetylcholine release. It appears that the D5 receptor subtype is responsible for this function. Interestingly, this is the first evidence of a possible function for the dopamine D5 receptor subtype in the mammalian brain.

This dopaminergic modulation of hippocampal acetylcholine release is preserved as the animal ages. Interestingly, stimulation of D1-like receptors attenuated the memory deficits observed in aged rats in the Morris water maze task. Thus the hippocampal dopamine-acetylcholine interactions likely have a cognitive significance.

Finally, dopamine D1-like receptors were also found in the hippocampal formation of monkey and human brains. It is conceivable, therefore, that dopamine acting via these receptors might serve similar functions in primates as those described above for the rat.

Taken together, the results presented in this thesis provide information about the heteroregulation of the cholinergic synapses in the hippocampus. Most importantly, this work suggests a novel approach to alleviate age-associated memory deficits by stimulating D5 receptors.

RESUME

L'innervation cholinergique est considérée comme un élément essentiel des voies nerveuses impliquées dans les processus mnésiques. En particulier, le faisceau cholinergique septo-hippocampique jouerait un rôle primordial. Par ailleurs, d'autres neurotransmetteurs seraient également impliqués soit directement ou en interaction avec le système cholinergique. La dopamine est l'un des candidats. Le but de cette thèse est donc d'examiner une éventuelle interaction entre la dopamine et l'acétylcholine au niveau de la formation hippocampique, en relation notamment avec les processus cognitifs.

Par fimbriaectomie/autoradiographie des récepteurs, nous avons observé que des récepteurs dopaminergiques de type D1 sont possiblement localisés sur les terminaisons cholinergiques de l'hippocampe. De plus, la stimulation des récepteurs de type D1, contrairement à celle des récepteurs de type D2, augmente la libération d'acétylcholine, et ce de manière phasique. Cette action serait localisée au niveau des terminaisons cholinergiques situées dans l'hippocampe.

La sous-famille de type D1 des récepteurs dopaminergiques comporte les récepteurs D1 et D5. L'absence de ligands sélectifs pour ces 2 sous-types de récepteurs nous a conduit à utiliser la combinaison "antisense-dialyse in vivo" afin de déterminer le sous-type impliqué dans la libération de l'acétylcholine. Les résultats suggèrent que les récepteurs D5 sont responsables de cette fonction. Cela constitue la première démonstration d'un rôle possible des récepteurs D5 dans le cerveau des mammifères.

La modulation par la dopamine de la libération d'acétylcholine hippocampique persiste chez lè rat âgé. La stimulation des récepteurs de type D1 atténue les déficits mnésiques observés chez le rat âgé dans le test de Morris. L'interaction entre la dopamine et l'acétylcholine dans l'hippocampe semble donc être fonctionelle en terme de comportement.

Enfin, nous avons observé que les récepteurs de type D1 sont présents dans la formation hippocampique du singe et chez l'homme. Il est donc probable que la dopamine, en interagissant avec ces récepteurs, exerce des fonctions similaires chez le primate à celles décrites chez le rat.

En conclusion, les résultats présentés dans cette thèse fournissent des informations sur l'hétérorégulation des synapses cholinergiques dans l'hippocampe. Ces travaux suggèrent une nouvelle approche dans le traitement des déficits mnésiques par la stimulation des récepteurs D5.

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This thesis is dedicated to my late father who gave me wings and graciously let me out of the nest early and to my mother who by her hard work and determination led by example.

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Candidates have the option of including as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts: in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

CONTRIBUTION OF THE AUTHORS ON CO-AUTHORED PAPERS

As co-supervisors of my doctoral training, Drs Rémi Quirion and Pierrette Gaudreau are co-authors on all the manuscripts presented in this thesis.

1) Local Modulation of Hippocampal Acetylcholine Release by Dopamine D1 receptors: A combined Receptor Autoradiography and in vivo dialysis study.
Ali I. Hersi, Jean W. Richard, Pierrette Gaudreau, and Remi Quirion (J Neurosci 15:7150-7157).

Mr. Jean W. Richard assisted in generating the fimbriaectomized animals as well as in the in vivo dialysis experiments.

2) Antisenses Suggest a Role for the Dopamine D5 Receptors in the Modulation of Hippocampal Acetylcholine Release.
Ali I. Hersi, Lalit Srivastava, Pierrette Gaudreau, and Remi Quirion (To be submitted).

Dr Lalit Srivastava participated in the design of the antisense oligodeoxynucleotides. He also advised on the development of an optimum protocol for the antisense-in vivo dialysis experiments.

3) Dopamine D1 Receptor Ligands Modulate Cognitive Performance and Hippocampal Acetylcholine Release in Memory-impaired Aged Rats.
A. I. Hersi, W. Rowe, P. Gaudreau, and R. Quirion (Neuroscience 69:1067-1074).

Mr Wayne Rowe performed the initial experiments classifying the animal colony in to aged-memory-impaired (AI) and aged-memory-unimpaired cohorts. He also assisted in conducting the experiments which assessed the effects of D1 ligands on the Morris Water Maze performance of the AI animals.

4) Distribution of D1-like Receptors in the Hippocampal Formation of Rat, Monkey and Human Brains.

Ali I. Hersi, Danielle Jacques, Pierrette Gaudreau, and Remi Quirion (To be submitted).

Dr Danielle Jacques sectioned the human tissue and assisted in the analysis of the autoradiographic data.

CHAPTER 1
General Introduction

PREFACE TO CHAPTER 1

This general introduction discusses the basic characteristics of the central dopamine and acetylcholine systems. First, the history, neurochemistry, anatomy and receptors of dopamine are addressed. Next, the neurochemistry and receptors of acetylcholine as well as the anatomy and the possible functions of the septohippocampal cholinergic innervation are discussed. In the final section, the rationale and objectives of this thesis are set forth.

1. CENTRAL DOPAMINERGIC SYSTEMS

1.1 Dopamine - A history[1]

3-hydroxytyramine (dopamine, DA) is a neurotransmitter of great importance in the central nervous system (CNS). The establishment of this fact, however, has been a rather recent event, relative to other classical neurotransmitters. In 1938 the enzyme dopa decarboxylase was discovered and shown to be involved in the formation of the catecholamines epinephrine (E) and norepinephrine (NE) with dopamine as an intermediary (Blaschko, 1939; Holtz et al., 1938). Due to an apparent weak sympathomimetic activity, however, dopamine was not considered an active entity in its own right. Rather, dopamine was designated as a precursor of the other two catecholamines, namely E and NE.

About twenty years later, this picture altered drastically. In 1957 Montagu isolated a compound that he termed "X" from brain extracts with chromatographic properties similar to dopamine (Montagu, 1957). Subsequently, as a result of a series of monoamine depletion experiments utilizing reserpine, Blaschko, Carlsson and colleages found dopamine to occur normally in the brain at levels comparable to NE and proposed dopamine as an agonist in its own right in the brain (Carlsson et al., 1958). Shortly afterwards, these and other investigators using techniques such as histofluorescence showed a unique distribution profile for dopamine that was markedly different from NE (Carlsson, 1959). In particular, the highest amounts of DA were found in the basal ganglia. Around this same time period and in relatively quick succession, a number of other discoveries were made which established DA as a bonafide CNS active compound once and for all.

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[1]Based on reviews by Anden, 1979; Carlsson, 1987 and 1993

For example, low levels of dopamine in the striatum of Parkinsonian patients and a therapeutic effect of L-dihydroxyphenylacetic acid (the immediate precursor of DA) in this disorder were demonstrated (Barbeau et al., 1962; Birkmayer and Hornykiewics, 1962; Ehringer and Hornykiewics, 1960). Most importantly, the major neuroleptics (antipsychotics) such as chloropromazine and haloperidol were found to accelerate the turnover of dopamine and this was interpreted as a negative feedback response to postsynaptic dopamine receptor blockade (Carlsson and Lindqvist, 1963). This, in part, ushered in the modern era of psychopharmacology.

Today, DA is implicated in a myriad of brain functions; both normal and pathological. In most animals DA is thought to modulate a variety of processes including motor, cognition, neuroendocrine regulation, arousal, feeding and addiction among others. In addition, a growing list of neurological and psychiatric disorders such as tardive dyskenisia, Gilles de la Tourette syndrome, Huntington's chorea, bipolar affective disorder, schizophrenia and hyperprolactinemia are thought to involve dysfunctions of the dopaminergic system. These observation have served to underscore the significance of dopamine transmission in the CNS.

1.2 Dopamine Neurochemistry[2]

1.2.1 General

To date the most extensive work has been carried out in the nigrostriatal DA sytem. Although biochemical heterogeneity exists among the various DA cell groups, enough basic similarities exist between all DA systems in terms of synthesis, release and metabolism. Therefore, general neurochemical characteristics of DA neurons, which in most part are derived from the nigrostriatal system, are considered below.

1.2.2 Synthesis

Dopamine is synthesized from the essential amino acid tyrosine which must be transported across the blood-brain-barrier and into DA neurons. A number of conditions including dietary restriction and the presence of other amino acids that compete with tyrosine for transport into the brain can affect tyrosine availability and thus dopamine synthesis. The first step in the formation of DA is the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-Dopa) catalyzed by the enzyme tyrosine hydroxylase (TH). This enzyme which appears to be a unique constituent of catecholamine-containing neurons and chromaffin cells is stereospecific and requires molecular O_2 and Fe^{2+}. TH utilizes tetra-hydropteridine (DMPH4) as a cofactor in the hydroxylation reaction of L-tyrosine. Next, L-dopa is converted to DA by L-aromatic acid decarboxylase. This enzyme requires pyridoxal phosphate (vitamin B_6) as a cofactor and has a very rapid turnover rate so that, under normal circumstances, L-DOPA levels in the brain are negligible.

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[2]*Adapted from Cooper et al., 1991; Roth and Elsworth, 1987 and 1995*

The rate limiting step in the biosynthesis of DA is the formation of L-dopa by TH. Consequently, mechanisms for regulating DA levels involve primarily the manipulation of the activity of this enzyme (Cooper et al., 1991). Four such mechanisms exist :

A) End-product inhibition : DA and other catecholamines inhibit TH activity by displacing the DMPH4 cofactor from its binding site on the enzyme.

B) Co-factor availability : TH activity is dependent on the availabilty of an adequate concentration of the DMPH4 cofactor. Endogenous levels of DMPH4 are such that TH is normally unsaturated with respect to this cofactor. Therefore, manipulation of the formation of this cofactor provides a possible way to regulate the activity of TH.

C) Phosphorylation by impulse flow : Dopamine neurons are similar to other monoaminergic neurons in that they respond to increased impulse flow by increasing neurotransmitter synthesis. During periods of increased impulse flow, the efficiency of TH is increased primarily via a kinetic activation that increases the affinity of the enzyme for its cofactor and decreases its affinity for the normal end product inhibitor, DA.

D) Feed-back inhibition by synthesis-modulating autoreceptors: Stimulation of nerve-terminal, and possibly somatodendritic, autoreceptors inhibits DA synthesis. Although, the mechanistic basis of this autoregulation is not well established at the present time, a reduction in TH phosphorylation has been suggested as a possibility (Wolf and Roth, 1990). Interestingly, these autoreceptors are differentially expressed in the various DA systems (see section 1.3).

1.2.3 Release

DA is released in an exocytotic manner following action potential invasion. The magnititude of DA release is a function of the rate and pattern of impulse flow (Bean and Roth, 1991; Gonon, 1988). Moreover, the rate of dopamine release is coupled to its rate of synthesis. Generally, this release is regulated by autoreceptors that appear distinct from the synthesis-regulating-autoreceptors discussed above. Stimulation of these receptors, which belong to the D2-like family (see section 1.4.2.3), inhibits DA release. In general, this is thought to be due to increased potassium conductance and thus hyperpolarization of target cells (Wolf and Roth, 1990).

In addition, however, there have been reports of a nonexocytotic mode of DA release (Arbuthnott et al., 1990; Fischer and Cho, 1979; Heikkila et al., 1975; Raiteri et al., 1979). This is a carrier-mediated process by which DA is either accumulated (see section 1.2.5) or released depending on the existing concentration gradient. This process is inhibited by drugs such as cocaine and nomifensine which inhibit DA uptake. At the present time it is not fully clear if the same protein is involved in both uptake and release.

1.2.4 Metabolism

Following release, the action of DA is terminated by reuptake into the presynaptic terminals via a high-affinity transporter (section 1.2.5). Once inside the neuron, dopamine is either packaged into synaptic vesicles for rerelease or catabolized into dihydrophenylacetic acid (DOPAC) by intraneuronal monoamine oxidase (MAO). This enzyme which converts catecholamines to their corresponding aldehydes is intraneuronally found in a particle-bound form, largely localized in the outer

membrane of mitochondria. In human and rat brains MAO exists in at least two forms designated type A and B based on substrate specificity and sensitivity to inhibitors. However, DA appears to be a good substrate for both forms of the enzyme.

Next, the MAO-generated DOPAC diffuses from the neuron and is converted into homovanillic acid (HVA) by extraneuronal catechol-o-methyltransferase (COMT). COMT is a relatively nonspecific enzyme that catalyzes the transfer of methyl groups from S-adenosyl methionine to the m-hydroxyl group of catecholamines and various other catechol-containing compounds. In addition to S-adenosyl methionine, this enzyme requires Mg^{2+} for activity. In some instances, released DA is converted extraneuronally into 3-methoxytyramine (3-MT) by COMT and in turn into DOPAC by extraneuronal MAO. The major DA metabolite is HVA in primates and DOPAC in rat. Changes in the concentration of these metabolites have been used extensively as indices of DA neuronal activity.

Manipulation of the enzymes involved at this stage of the life cycle of DA influences transmitter dynamics (Fig 1). For instance, inhibition of MAO by drugs such as pargyline or COMT by tropolone increases DA levels. However, although such drugs provide useful pharmacological tools for modifying DA activity, their actions are not confined to DA neurons but will also interact with other catecholamines.

1.2.5 DA Transporter[3]

The dopamine plasme membrane transporter (DAT) is a unique constituent of dopaminergic nerve terminals. DAT is a glycoprotein with an apparent molecular weight of approximately 80 kd. It is a member of a family of substrate-specific, high affinity membrane transporters. The functions of DAT are temperature and Na^+/Cl^+ dependent and can be inhibited by various pharmacological agents, including cocaine. DAT is capable of transporting DA in either direction depending on concentration gradient. Therefore, in addition to DA-action-termination, DAT can participate in the release of this neurotransmitter. Recently, the DAT gene has been cloned from rat, bovine and human brains (Kilty et al., 1991; Shimada et al., 1991; Usdin et al., 1991; Vanderbergh et al., 1992). Based on sequence information, DAT is postulated to contain twelve putative transmembrane domains of 20-24 amino acids. Moreover, DAT is differentially distributed, has differential glycosylation patterns and probably dissimilar kinetic properties in the various central dopaminergic systems (see section 1.3). Although, the mechanism of transport of this carrier is not established at the present time, a sequential binding of ions and substrate leading to a conformational change whereby the extracellular binding face of the protein is flipped intracellularly has been postulated (Hoffman, 1994)

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[3]*Recently reviewed by Boja et al., 1994; Hoffman, 1994*

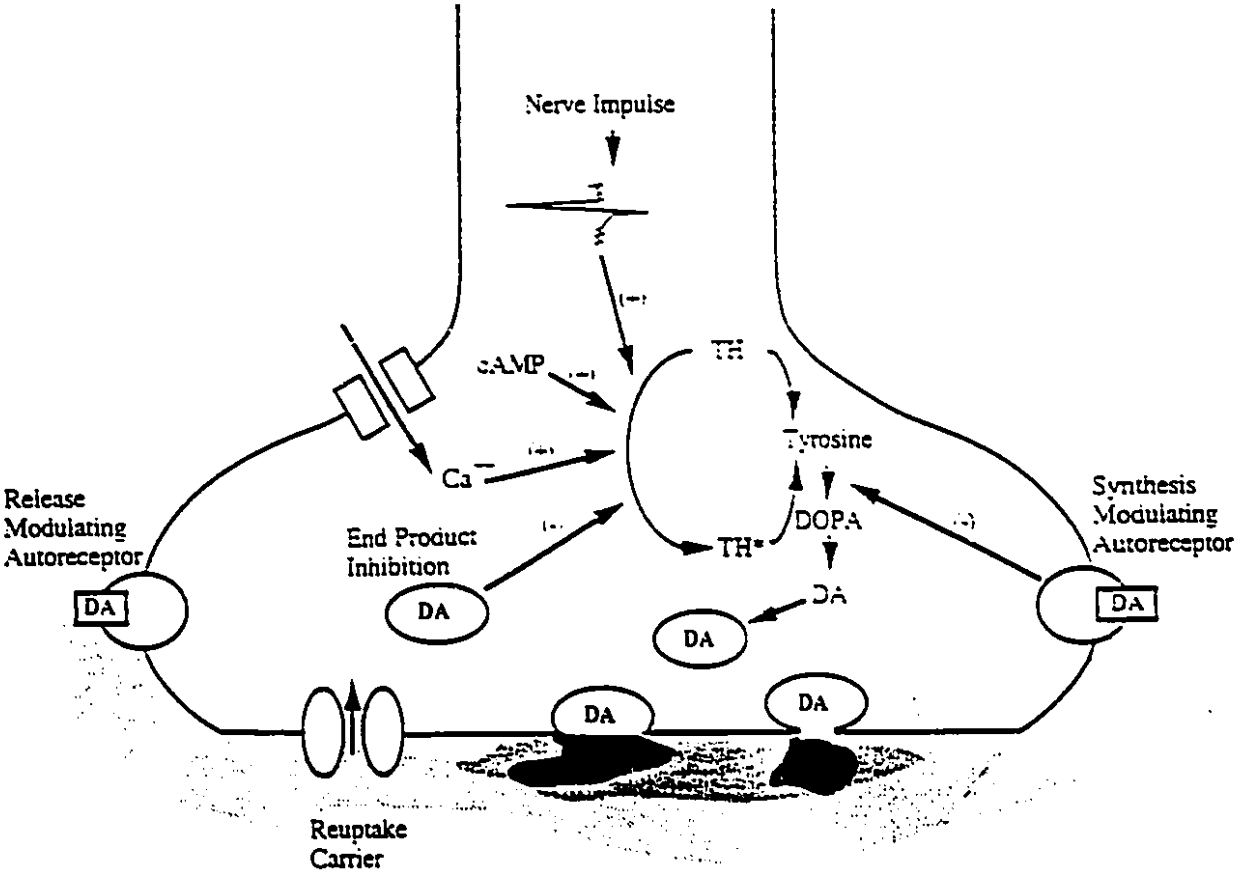

Fig 1. *Schematic model of a prototypic dopaminergic nerve terminal illustrating the life cycle of dopamine and mechanisms which modulate dopamine synthesis and release. Adapted from Roth and Elsworth, 1995.*

1.3 Distribution of Central Dopaminergic Innervation

1.3.1 General

Since the original histochemical detection of monoamine-containing neurons in the brain during the 1960's, considerable advances have been made in delineating the anatomy of CNS DA neurons. The advent of modern neuroanatomical techniques from histofluorescence to immunocytochemistry to retrograde tracing has led to the appreciation of the striking heterogeneity of these neurons. DA subsystems can be distinguished on the basis of various criteria including the location of the projecting neurons, intrinsic characteristics of these neurons (such as shape, dendritic arborization, colocalization of other neurotransmitters and the presence of autoreceptors), extrinsic afferents, area of projection and their reactivity to pharmacological and enviromental manipulations (Roth and Elseworth, 1995). On these bases, the central dopamine systems have been variably divided in to as many as twenty or more subsystems. For the purposes of this thesis, however, only two general divisions are made and briefly discussed. These are : the dopamine neurons of midbrain origin collectively called the mesotelencephalic dopamine system and those with their cell bodies in the hypothalamus.

1.3.2 Mesotelencephalic DA system[4]

The dopamine neurons of the ventral mesencephalon are popularly designated A8, A9 and A10 following the original classification of catecholamine-containing neurons by Dahlstrom and Fuxe (1964). The A9 (substantia nigra par compacta, SN) cells project principally to the striatum whereas those of A10 (ventral tegmentum, VTA) provide the main dopaminergic projection to mesocortical and mesolimbic areas

[4]*Based on review by Roth and Elsworth, 1987*

of the brain. The A8 (retrorubral field, RRF) DA neurons contribute to the nigrostriatal and mesolimbic but not mesocortical projections. It is important to point out, however, that there are no clear anatomical boundaries between these subsystems and in fact there are considerable overlaps between their areas of projection. At best, these systems might be thought of as interdigitating.

Recently, a number of brain areas that were previously considered devoid of DA input were shown to contain DA terminals. One such structure is the hippocampus (Verney et al., 1985). In the rat, the hippocampus receives relatively sparse DA innervation arising mainly from the VTA but also in part from the SN. The temporal (ventral and caudal) part of the hippocampal formation receives the bulk of the dopaminergic innervation whereas no fibers were observed in the septal pole. Very few positive axons are visualized in the hilus of the gyrus dentatus and the CA3 field. The most innervated area is the ventral part of the subiculum, in particular the prosubiculum. The ventral CA1 field (stratum oriens) also receives dopaminergic innervation. The presence of dopaminergic innervation in the hippocampal formation provides the basis of this thesis (see section 3).

There are considerable pharmacological differences between members of the mesotelencephalic DA systems. Many of these differences appear to be as a consequence of autoreceptor function. For example, although all nigrostriatal, mesocortical and mesolimbic neurons examined to date possess neurotransmitter-release-regulating autoreceptors, this is not the case when DA-synthesis-regulating autoreceptors are considered. The dopamine neurons which project to the cingulate and prefrontal cortices as well as the amygdala appear to be devoid of this type of autoreceptor. Generally, DA autoreceptors are pharmacologically classified as D2-like type (see section 1.4.2.3). It is possible that synthesis-regulating and release-regulating

autoreceptors belong to two distinct protein receptors of the D2-like family. Alternatively, the same receptor species which is nevertheless coupled to different second messenger systems in the various tissues could be involved in these two different functions. Finally, midbrain DA neurons also differ in the nature of their interaction with other neurotransmitters.

In general, the nigrostriatal pathway is thought to be responsible for motor control and the degeneration of the DA cell bodies in the pars compacta of the substantia nigra leads to Parkinson's disease. The mesocortical and mesolimbic DA systems are thought to be involved in cognition, learning and memory as well as motivated behaviors and reward. Dysfunction of these latter two systems is implicated in a number of psychiatric disorders including schizophrenia.

1.3.3 Hypothalamic DA System

The anatomy and functional properties of DA neurons in the hypothalamus have been extensively reviewed recently (Moore and Lookingland, 1995). In brief, four subdivisions of this system are currently recognized. The best studied of these comprise the tuberoinfundibular dopaminergic system (TIDA) whose cell bodies are located in the mediobasal hypothalamus with projections terminating in the external layer of the median eminence. The second set of neurons are located in rostral periventricular nucleus. These neurons are designated as incertohypothalamic (IHDA) and project out of the hypothalamus to such areas as the central nucleus of the amygdala. Next, are the periventricular-hypophysial dopaminergic neurons (PHDA) with cell bodies in the periventricular nuclei projecting to posterior as well as intermediate lobe of the pituitary. The remaining periventricular DA neurons project laterally into adjacent regions such as the medial preoptic and anterior hypothalamus.

There are number of unique neurochemical characteristics of hypothalamic DA systems when compared to their mesotelencephalic counterparts. For example, DA neurons such as those from the TIDA do not form synapses but rather release DA directly into perivascular spaces in the median eminence and thus into the blood of the hypophysial portal system. Furthermore, the dopamine uptake system in TIDA and PHDA has a lower affinity for DA than the nigrostriatal pathway. This could in part be because since DA from these hypothalamic neurons is carried to a distant site of action by the blood stream, a different mode of action-termination is required other than reuptake in to the releasing terminals, as is the case for the nigrostriatal pathway. Here, an alternate mode of signal transmission than the classical synaptic one is called for. This is termed volume transmission and has been amply demonstrated in the TIDA system. Interestingly, the hippocampal dopaminergic innervation has been postulated to utilize a similar mode of transmission.

There are also differences between the various hypothalamic dopamine subsystems. The activity of TIDA neurons is not controlled by autoreceptor-mediated-mechanisms whereas that of IHDA neurons is. In this respect, IHDA neurons resemble the nigrostriatal neurons rather than the anatomically related TIDA neurons. TIDA neurons regulate the release of prolactin from the anterior pituatary and are, in part, regulated by this hormone. PHDA neurons modulate the release of various hormones including α-melanocyte stimulating hormone and their activity is sensitive to changes in blood osmolarity. The functions of the two other hypothalamic DA subsystems are currently unkown.

1.4 Dopamine Receptors

1.4.1 A Historical Prespective

The concept that most drugs, hormones and neurotransmitters produce their biological effects by interacting with receptor "substances" in cells was introduced by Langley as early as 1905. This was based on the observation that some drugs mimicked while others prevented biological response. Today, this concept forms a cornerstone in biology and macromolecules that fit receptor criteria have been identified, isolated and cloned for a long list of molecules, including neurotransmitters.

Two years following the identification of DA as a bonafide transmitter in the central nervous system, the existence of specific receptors for this amine was suggested (Ernst, 1965; van Rossum, 1966). Soon, it was discovered that DA stimulates the activity of the enzyme adenylate cyclase (AC) and this property was used as an indirect method to assay for DA receptor function (Brown and Makman, 1972; Kebabian et al., 1972; Kebabian and Greengard, 1971; McAfee et al., 1971). However, not all the actions of DA could be linked to this enzyme and thus the idea of multiple DA receptors was introduced.

The advent of specific radioreceptor assay in the mid 1970's revolutionized the study of receptor functions, including those of DA (Burt et al., 1975 and 1976; Creese et al., 1975; Seeman et al., 1975). Based on the differential potencies of a series of agonists and antagonists, DA receptors were classified (and practically, this classification is still valid today) into two general subtypes, namely D1-like and D2-like (Kebabian and Calne, 1979). In this schema, D1-like receptors were responsible for the stimulation of AC and had a low affinity for butyrophenone neuroleptics, whereas D2-like receptors had no effect on or inhibited AC and possesses high affinity

for butyrophenones and substituted benzamides (Kebabian and Calne, 1979; Stoof and Kebabian, 1984).

The next major advance in DA receptors came from molecular biology with the development of cloning techniques in the 1980's. To date, at least five functional DA receptor subtypes have been cloned from a variety of species and tissues. There are many recent excellent reviews dealing with different aspects of DA receptor properties (Jackson and Westlind-Danielsson, 1994; Gingrich and Caron, 1993; Niznik and Van Tol, 1992; Strange, 1991;). Based on these reviews and for the purposes of this thesis some of these characteristics are summarily discussed below.

1.4.2 Dopamine Receptor Subtypes

1.4.2.1 General Features

DA receptors belong to the superfamily of G-protein-coupled receptors which includes among many others ß-adrenergic, muscarinic and rhodopsin receptors. From the amino acid sequence, a general topology is predicted for the members of this family (Fig 2). This topology is characterized by extracellular N-terminus, an intracellular carboxyl tail and the putative presence of seven trans-membrane (TM) helices which would form a narrow dihedral hydrophobic cleft surrounded by three extracellular and three intracellular loops. The TM helices are believed to form the major binding determinants for this class of receptors. Based on chimeric and deletion studies, the third cytoplasmic loop is proposed to confer interaction with G-proteins (reviewed by Dohlman et al., 1991). Several structural features which allow for post-transational modifications or potential regulatory control are common between the G-protein coupled receptors. These include, the presence of asparagine-linked (N-linked)

glycosylation sites, consensus sites for phosphorylation by regulatory kinases and a conserved cysteine in the carboxyl tail where anchoring via palmitolylation is thought to occur, thus giving rise to a possible fourth cytoplasmic loop (O'Dowd et al., 1989). In addition, all of the DA receptor subtypes share several conserved residues within their TM region which are thought to be the minimal requirement for catecholamine binding: the two serine residues in the putative fifth TM segment thought to be involved in recognition of the two hydroxyl groups of catecholamines and the aspartic acid residue in the third TM segment, which is thought to act as a counterion for the amine moiety in biogenic amines (Strader et al., 1987 and 1989).

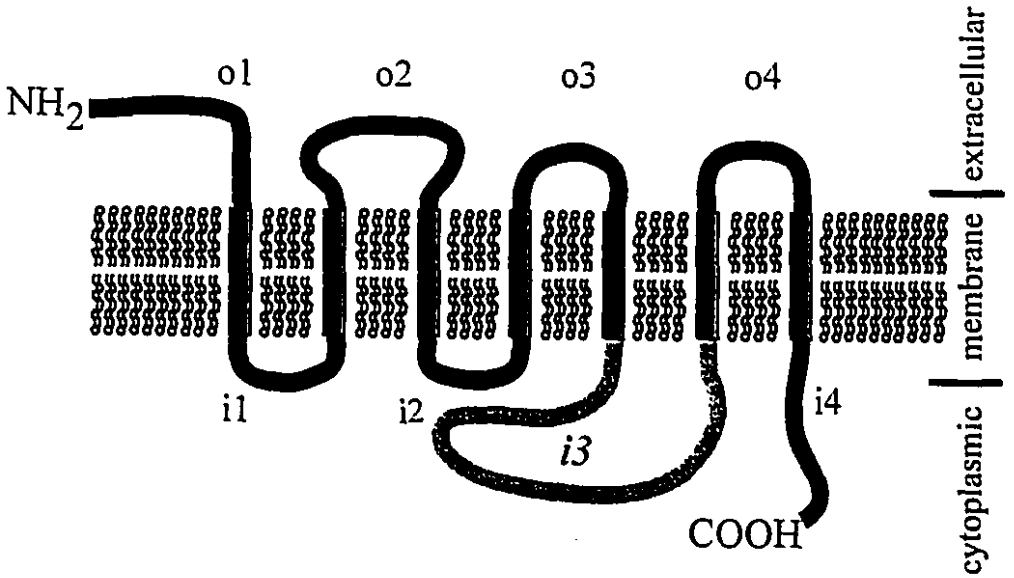

Fig 2. *A representative model of G-binding protein topography.*

1.4.2.2 D1-like DA Receptors

On the basis of structural, biochemical and pharmacological similarities, two DA receptors belong to this family, namely D1 (D1a) and D5 (D1b). The D1 gene is found on chromosome 5 (Sunahara et al., 1990) and the D5 gene locus is on the short arm of chromosome 4 (Tiberi et al., 1991). These two D1-like receptors share an overall sequence homology of 60%. When this comparison is restricted to the hydrophobic TM regions the homology increases to about 80%. In common with most other G-protein coupled receptors, D1/D5 receptors lack introns in their coding region, although the D1 gene has been reported to contain an intron in the 5' untranslated region (Minowa et al., 1992). When compared to other members of DA receptor family, D1/D5 receptors have a relatively short intracellular loops and long carboxy terminals. This structural arrangement is common among receptors that function by signalling via the G-protein Gs (Strange, 1993).

A unique property of the D5 receptor is the presence of two pseudogenes in the human genome (Grandy et al., 1991; Nguyen et al., 1991; Weinshank et al., 1991). These two pseudogenes contain an 8-base-pair insertion that leads to a frame shift and a premature stop-codon, and thus a truncated and presumably nonfunctional receptor. So far, these pseudogenes have been demonstrated only in humans, suggesting that the evolution of these genes might be a recent event restricted to primates.

The similar structure of these two receptor subtypes leads to similar pharmacological profiles. Both D1 and D5 receptors bind benzazepine ligands such as SCH 23390 and SKF 38393 and their analogues with high affinity and have a low affinity for butyrophenones (spiperone, haloperidol) and substituted benzamides such as sulpiride (Niznik and Van Tol, 1992). Interestingly, however, these two receptors have different binding affinities for the endogenous ligand, DA. In transfected cells,

the D5 receptor has a 3 to 10 fold higher affinity for DA than the D1 receptor (Sunahara et al., 1991). At present, there are no known compounds (with the exception of DA, of course) that can sufficiently distinguish between the two members of the D1-like family. This situation, in part, frustrates attempts to assign specific functions to either subtype.

The free energy of binding of DA to its receptors initiates a cascade of events that result in the external information carried by the DA molecule being transduced over the plasma membrane and then translated into an intracellular response. The first step in this cascade is mediated by molecules collectively termed second messengers. The adenylate cyclase (AC) system is the earliest discovered second messenger system (Rall and Sutherland, 1962). The pharmacologically-defined D1-like receptors were originally shown to stimulate AC activity. Subsequently, both cloned members of this family were shown to do the same via interaction with Gs G-proteins (Dearry et al., 1990; Grandy et al 1991; Monsma et al., 1990; Sunahara et al., 1990 and 1991; Tiberi et al., 1991; Zhou et al., 1991). Currently there are controversial suggestions of other second messenger systems being coupled to the D1/D5 receptors, in particular the activation of phospholipase C (Jarvie et al., 1994). Alternatively, it is possible that there are additional as-yet-uncloned subtypes of this family of DA receptors that signal via systems other than the AC pathway.

The D1 subtype is the most abundant DA receptor in the brain. The mRNA of this receptor (4.2 Kb) is widely distributed in the mammalian brain (Fremeau et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991; Weiner et al., 1991). High levels of D1 mRNA are found in the caudate-putamen, nucleus accumbens, the olfactory tubercule, amygdala and island of Caleja whereas lower levels are detected in the septum, hypothalamus, cortex and hippocampus among other regions. In general, the

distribution of the D1 receptor protein ascertained by autoradiography and immunocytochemistry agrees well with that of the mRNA. A clear exception to this rule is the substantia nigra where high levels of D1 receptors but sparse or nonexistent D1 mRNA are found (Fremeau et al., 1991). It is thought that these D1 receptors function as presynaptic heteroreceptors on striatal projections whose cell bodies contain abundant D1 mRNA.

The D5 receptor mRNA (3.0-3.7 Kb) is expressed at a level that is many folds lower than the D1 subtype. Moreover, unlike the D1 receptor, the distribution of the D5 receptor is reportedly highly restricted. The D5 mRNA is detected only in the hippocampus, the hypothalamus and the parafascicular nucleus of the thalamus (Tiberi et al., 1991). Nevertheles, a recent higher resolution immunoctyochemical evidence in primate brain has shown the presence of D5 receptor protein in additional structures such as the cortex and caudate (Bergson et al., 1995a). However, there is a possibility that the antibodies utilized in these studies might be labelling other proteins in the mammalian brain that have similar epitopes as the D5 receptor. At any rate, at the present time it appears that D5 receptor brain distribution might not be as restricted as had been proposed originally.

Both D1 and D5 receptors have been found at extra-synaptic sites such as cell bodies and have been proposed to partake in nonsynaptic modes of communication, namely volume transmission (Bergson et al., 1995b, Smiley et al., 1994). Interestingly, the D1 and D5 receptor proteins are frequently co-expressed in the same pyramidal neurons both in the neocortex and hippocampus. However, although the distribution of the two members of the D1-like family overlaps somewhat, at higher resolution, a differential and distinct cellular and subcellular localization is observed

(Bergson et al., 1995a). Therefore, although D1 and D5 receptors have similar pharmacological properties, they are most likely not functionally redundant.

1.4.2.3 D2-like DA Receptors

This family of DA receptors contains at least three subtypes, namely D2, D3 and D4 (Dal Toso et al., 1989; Sokoloff et al., 1990; Van Tol et al., 1991). Each of these receptors is similar in size, containing about 420 amino acids with 120 of these amino acids identical, giving an overall homology of 30%. This sequence homology rises to between 51% to 75% when the TM segments are considered. The common structural elements of these receptors include a similarly sized amino terminus, a large third cytoplasmic loop and a very short carboxy tail terminating at the same cysteine residue. This type of structure appears to be characteristic of receptors that couple to G_i (see below).

The inclusion of introns in the protein coding region distinguishes the D2-like receptors from most members of the G-protein coupled superfamily. The D2 receptor coding region is interrupted by five to six introns (Dal Toso et al., 1989; Selbie et al., 1989), the D3 by five (Sokoloff et al., 1990) and the D4 receptor by four introns (Van Tol et al., 1991). Furthermore, most of the introns in the D2-like receptor genes are located in similar positions, indicating a common phylogenic origin. Introns are thought to allow the generation of receptor variants by alternative splicing. For example, the D2 receptor exists in two alternatively spliced forms (Dal Toso et al., 1989). These two receptor isoforms differ by the inclusion in one of additional 29 amino acids in the third cytoplasmic loop. Splice variants of D3 receptor have also been detected (Giros et al., 1991). Thus far, no alternative spliced products of the D4 receptor have been reported.

All members of the D2-like family bind butyrophenones (e.g. spiperone) and substituted benzamides (e.g. sulpiride) with high affinity and show exceedingly low affinity for D1 prototypic compounds such SCH 23390 and SKF 38393 (Niznik and Van Tol, 1992). Interestingly, D2 and D4 but not D3 receptors exhibit agonist-binding profile sensitivity to guanine nucleotides. Unlike the D1 family, there are several antagonists that can distinguish between the different members of the D2 family. For example, the compound 7-OH-DPAT is under certain conditions selective for the D3 receptor whereas the atypical neuroleptic, clozapine, has the highest affinity for the D4 subtype (Sokoloff et al., 1992; Van Tol et al., 1991) .

The D2 receptor inhibits adenylate cyclase via interaction with Gi/Go proteins (Duman and Nestler, 1995). During the last fifteen years, however, many other second messenger systems have been shown to couple with the D2 receptor (Memo, 1992; Vallar and Meldolesi, 1989). These include, the phospholipase C pathway, arachidonic acid and metabolites, Na+/K+-ATPase, the Na+/K+ antiporter and several distinct K+ and Ca2+ currents. In contrast, the second messenger systems coupled to D3 and D4 receptors are not as clear. This may arise, in part, from the nature of the G-protein complement of the cell systems used for transfection of these receptors as well as the unavailabilty of tissues that express one subtype exclusively. At any rate, the D4 receptor subtype has been reported to couple to inhibition of AC activity (Tang et al., 1994) whereas the second messenger system involved in transducing the DA signal via the D3 receptor is essentially unknown, at the present time.

The members of the D2-like family exhibit differential distribution patterns. The D2 subtype is the most abundant of this class of receptors in the brain (Bunzow et al., 1988; Giros et al., 1989; Meador-Woodruff et al., 1989; Mengod et al., 1989; Monsma et al., 1989 and 1990; Mansour et al., 1990; Najleranim et al., 1989; Weiner

and Brann 1989; Weiner et al., 1991). High D2 mRNA (2.9 Kb) levels are found in the basal ganglia structures such as the caudate-putamen, nucleus accumbens and olfactory tubercle but also in septal regions, amygdala, mammilary nucleus and a number of regions in the mesencephalon. Lower levels have been reported in cortical and hippocampal areas. In general, there appears to be no significant differential distribution between the two D2 receptor isoforms.

The D3 and D4 receptors are expressed at a level which is one to two orders of magnitude less than the D2 receptor (for a recent review see Jackson and Westlind-Danielsson, 1994). D3 mRNA (8.3 kb) levels are reportedly abundant in the island of Calleja, vertical limb of the diagonal band, the bed nucleus of the stria terminalus, some thalamic nuclei and the medial mammilary nucleus. The mRNA for the D4 subtype is enriched in the frontal cortex, amygdala, olfactory bulb, mesencephalon and medulla oblongata. Receptor protein localization studies, generally, agree with this overall distribution profile of the mRNA for the members of the D2-like family.

1.4.3 D1 and D2 Receptor Interactions

There is an extensive body of behavioral, electrophysiological and biochemical evidence that documents the existence of a functional interaction between D1-like and D2-like families of DA receptors (for a recent review see Waddington et al., 1994). The nature of this interaction varies from synergistic to co-operative to antagonistic and is apparently species specific (Waddington, 1993). Conceptually, two possibilities exist as to the basis of this phenomenon. Either the interacting D1-like and D2-like receptors are anatomically linked or communicate downstream via more integrative mechanisms.

In support of the first possibility, in situ hybridization studies of D1 and D2 mRNA have suggested that both receptors may be at times co-expressed in the same neurons in areas like the striatum (Lester et al., 1993; Mansour et al., 1991; Weiner et al., 1991). There is also evidence that these two families of receptors interact at the level of the transduction mechanisms such as the regulation of AC activity (Waddington et al., 1994). Although the mechanistic basis for this interaction is not clear at the present time, it has been suggested that colocalized D1/D2 receptors may share the $\beta\gamma$ subunit of the heterotrimeric G-protein used in their respective signalling mechanisms (Seeman et al., 1989). The shuttling back and forth of the $\beta\gamma$ subunit between the two receptor subtypes is thought to represent a possible mechanism via which occupancy of this subunit by one receptor subtype keeps the other receptor subtype in a state that has low affinity for DA, in effect desensitizing the receptor.

As it relates to the integrative concept, there is a very pertinent neurochemical example, considering the nature of this thesis. This is the regulation of striatal hippocampal acetylcholine (ACh) release by DA (for review see Stoof et al., 1992; Lehmann and Langer, 1983; This thesis, General Discussion). The activation of D2 receptors inhibits striatal ACh release locally within the striatum. In contrast, D1 receptor activation reportedly stimulates striatal ACh release by an indirect mechanism via action on cells other than cholinergic interneurons (but see General Discussion). Finally, the individual member(s) (i.e D1-D5) of the D1-like and D2-like receptor families involved in these functional interactions is(are) not clear at the present time.

2. CENTRAL CHOLINERGIC SYSTEMS

2.1 General

The neurophysiological activity of ACh has been known since the turn of the century and its neurotransmitter role since the mid-1920's (for review see Holmstedt, 1975). It is ironic, therefore, that the delineation of the property of the systems subserved by this substance lagged behind those of other more recently identified neurotransmitters, such as the biogenic amines. In the last two decades, as a result of the introduction of sophisticated methods to identify ACh, there has been an explosion in knowledge concerning the anatomy, chemistry, pharmacology and functions of central cholinergic neurons.

Currently, cholinergic action is intimately implicated in a wide array of central processes including motor functions, nociception, temperature regulation, water intake as well as more species-specific behaviors such as grooming, aggression, social play, response to novelty and sexual behaviors (for review see Bartus et al.,1987) In addition, more complex behaviors such as depression, delerium, response to stress, arousal, sleep and dreaming as well learning and memory have been shown to have a cholinergic component. Most aspects of the biology of the central cholinergic systems have been recently reviewed extensively (Arneric et al., 1995; Ehlert et al., 1995; Mesulam, 1995; Reiner and Fibiger, 1995; Richelson, 1995). For the purposes of this thesis, only key characteristics of the biochemistry of the central cholinergic systems and the anatomy as well as possible functions of the hippocampal cholinergic innervation are considered below.

2.2 Acetylcholine Neurochemistry[5]

2.2.1 ACh Synthesis

ACh is synthesized from acetyl coenzyme A (AcCoA) and choline. The AcCoA used for this reaction is thought to be derived from glucose or citrate metabolism. Regardless of its source, AcCoA is primarily synthesized in mitochondria and is transported out into the cytoplasm where ACh synthesis occurs. On the other hand, choline is transported to the brain primarily by the blood, although ACh hydrolysis and membrane phospholipids can also serve as sources. Choline crosses cell membranes primarily by a high-affinity carrier mediated transport thought to be specific to cholinergic neurons but also via a low-affinity non-specific passive diffusion process.

The synthetic reaction of ACh wherein an acetyl group is transferred from AcCoA to choline is catalyzed by the enzyme choline acetyl-transferase (ChAT) (Nachmansohn and Machado, 1943). In vivo, ChAT, a protein of 66-70 kD molecular weight, is likely present in the cytoplasm in a soluble form (Benishin and Carrol, 1983). In addition, however, a particulate form of ChAT with kinetic characteristics similar to those of the soluble form has been identified (Benishin and Carrol, 1983).

There are three mechanisms for regulating ACh synthesis; feedback inhibition by ACh of ChAT, mass action and the availabilty of AcCoA and/or choline. Of these, the major regulatory factor is thought to be choline levels and thus high-affinity choline transport.

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[5]*Based on reviews by Cooper et al., 1991; Israel and Dunant, 1993; Massoullie et al 1993*

2.2.2 ACh Release

Most of the newly synthesized ACh is packaged into vesicles. This is an active process driven by an outward proton gradient that is generated by a bicarbonate-activated Ca2+ or Mg2+ ATPase. These vesicles serve as storage depots but also participate in evoked release via exocytosis. Depolarization results in Ca2+ influx into the neuron which eventually leads to the ACh-containing-vesicles in opposition to the terminal to fuse with the presynaptic membrane thus releasing ACh into the synaptic cleft. Subsequently, the presynaptic membrane is pinocytotically recaptured, vesicles resynthesized and repleted with ACh for the next quantal release.

There is another pool of ACh, however, that is found in the cytoplasm (Israel and Dunant, 1979). It has been proposed that evoked release occurs from this free cytoplasmic pool via a gating mechanism in the plasma membrane (for review see Israel and Dunant, 1993). Although all the steps in this gating mechanism are not clearly understood at the present time, a protein named mediatophore which was originally isolated from the Torpedo electric organ but also found in mammalian nervous tissue has been implicated in this process (Israel et al., 1986). When this protein is incorporated into ACh-loaded proteoliposomes, the addition of calcium and the calcium ionophore A23187 to the medium triggers the release of the transmitter.

Similar to DA, there are presynaptic release-regulating autoreceptors on the cholinergic synapse (for review see de Lorez Arnais, 1993). These receptors belong to the muscarinic family of cholinergic receptors and are pharmacologically classified as M2-like (see section 2.3). At the present time, it is not clear which of the two subtypes (m2 or m4) of these M2-like muscarinic receptors is responsible for this action. In general, the stimulation of these receptors inhibits whereas their antagonism increases ACh release.

Through the years, there have been a number of methods employed in studying ACh release. In 1938, Mann, Tennenbaum and Quastel demonstrated the synthesis and release of ACh in cerebral cortical slices. Since then this method has been extensively used. Another in vitro approach upon which much of the current understanding on the release of ACh from the brain is based involves the use of nerve ending particles, synaptosomes. There have also been a number of attempts to measure ACh release in vivo which culminated in the technique of microdialysis (Westernik et al., 1987).

Microdialysis is a relatively new technique that is now being used to determine cholinergic activity in awake and freely moving animals. In brief, this procedure involves the stereotaxic implantation of a small dialysis membrane in the brain of an experimental animal. Perfusion of the dialysis tube with a physiological solution forms a closed flow system, which is separated from the nervous tissue. Small molecules which have dialysed through the membrane can then be collected in the effluent dialysate. The dialysate is assayed for ACh in a variety of ways. In addition, this technique is suitable for the application of minute quantities (through the dialysis probe) of pharmacologically active compounds to restricted brain regions. Brain dialysis, which is extensively employed in the current thesis, offers considerable promise for measuring basal and evoked ACh release under various experimental conditions.

2.2.3 ACh Metabolism

The action of ACh is terminated by its hydrolysis to choline and acetic acid. In the brain, this reaction is catalysed primarily by the enzyme acetylcholinesterase (AChE). AChE occurs in both water-soluble and membrane bound state and is one of the most efficient enzymes extant. It has a turnover of 150 microseconds, equivalent to hydrolyzing 5000 ACh molecules per second per enzyme molecule. In other words, most of the released ACh is catabolized very rapidly. Therefore, in most studies examining the release of ACh, at least a partial inhibition of this enzyme is required to obtain measurable ACh levels.

2.3 Cholinergic Receptors[6]

Actions of acetylcholine are mediated via two quite distinct classes of receptors, namely muscarinic (mAChRs) and nicotinic (nAChRs). These two classes of cholinergic receptors were originally defined on the basis of tissue response to certain agonists and antagonists (Dale, 1914). Either a mimic or antagonism of acetylcholine response caused by the alkaloids muscarine and atropine, respectively, characterized mAChRs. On the other hand, nAChRs were defined by an acetylcholine-like response induced by nicotine and the blockade of cholinergic response by d-tubocurarine. Today, much more is known about both classes of cholinergic receptors.

In the CNS, the majority of cholinergic receptors belong to the mAChRs type. To date, five (m1-m5) mAChRs subtypes have been cloned and shown to exist in the CNS (for recent review see Ehlert et al., 1995). Like the DA receptors, mAChRs belong to the family of G-protein binding seven-transmembrane-domain receptors. Moreover, as is the case with the dopaminergic receptor family, there are no ligands

[6]*Recently reviewed by Arneric et al., 1995; Ehlert et al., 1995*

that can identify each mAChR exclusively. In general, mAChRs are classified in to two pharmacological groups; M1-like (m1, m3 and m5) and M2-like (m2 and m4).

In contrast, nAChRs are ligand-gated ion channel receptors composed of multiple subunits (Arneric et al., 1995). To date, ten genes encoding putative subunits of neuronal nAChRs have been identified in the mammalian brain. This provides for a multitude of potential combinations suggesting that many functional subtypes of neuronal nAChRs are possible. However, the negative connotations associated with the use of nicotine in tobacco products and the ensuing health problems diminished interest in nAChRs. Recent reports implicating nAChRs in cognitive processes (Arneric and Williams, 1993) have rekindled interest in these receptors and their pharmacology is beginning to emerge.

2.4 The Septo-hippocampal Cholinergic System

2.4.1 Anatomy

Neurons in the basal forebrain provide cholinergic innervation to the hippocampal formation (Semba and Fibiger, 1993). Specifically, neurons in the medial septum and the vertical limb of the nucleus of the diagonal band of Broca provide this innervation via the fimbria-fornix. The principal hippocampal cells, pyramidal neurons and granule cells, receive dense cholinergic innervation. The septo-hippocampal cholinergic fibers form a pericellular plexus around the bodies of these cells and make symmetric synaptic contacts on the cell bodies of both types of neurons. Cholinergic fibers are also found in other layers such as the stratum oriens, and stratum radiatum of the hippocampus as well as the molecular layer of the dentate gyrus where they make

contacts on dendritic shafts and spines. The target cells also include GABAergic and peptidergic nonpyramidal neurons.

2.4.2 Functions

Because most regions of the hippocampal formation receive intense cholinergic innervation, many aspects of hippocampal function are influenced by cholinergic neurotransmission. Paramount among these are the neural mechanisms underlying mnemonic processes. The hippocampal formation has been linked to memory function especially since patient H. M. was first described (Scoville and Milner, 1957). This patient exhibited a severe anterograde amnesia that was dissociated from other perceptual and cognitive abilities following a bilateral surgical removal of the medial temporal lobe. Since then, similar observation have been described in cases involving damage restricted to the hippocampus proper (Zola-Morgan et al., 1986; Victor and Agamanolis, 1990). In addition, a hallmark of Alzheimer's disease (AD) is the profound loss of cortical and hippocampal cholinergic innervation and the concommitent memory deficits (Davies and Maloney, 1976; Coyle et al., 1983). These clinical observations are backed up by a wealth of evidence from experimental animals including non-human primates and rodents (for review see Zola-Morgan and Squire, 1993). Taken together, these findings support the view that the hippocampal cholinergic system is involved in mnemonic processes (but see Fibiger, 1991).

The role of acetylcholine in hippocampal long term potentiation (LTP) may provide a mechanistic link between cholinergic pathways and memory. LTP is a phenomenon which was first described in the perforant path-granule cell synapses of the rabbit hippocampus and refers to an enduring increase in synaptic efficacy following a brief train of high frequency stimulation (Bliss and Lomo, 1973). This

phenomenon has been shown to persist for days or even weeks (Bliss and Gardner-Medwin, 1973) and it is this persistence, resulting from a relatively modest train of stimuli, which suggests that LTP may provide a cellular basis for learning and memory. Cholinergic systems in the hippocampus lower the threshold requirement for LTP induction by evoking theta rhythms (Bland, 1986; Huerta and Lisman, 1993). In this way hippocampal cholinergic stimulation may facilitate learning and memory.

3. RATIONALE AND OBJECTIVES

As stated earlier, preponderance of evidence implicates the central cholinergic systems in mnemonic processes. For example, one of the hallmarks of AD is the loss of cholinergic integrity in cortical areas and concomitant memory deficits. Recently, however, a cholinergic deficit comparable to that seen in AD has been observed in cortical areas of non-demented Parkinsonian patients (Aubert et al, 1992). The Parkinsonian disorder is primarily due to a loss of dopaminergic cells in the substantia nigra and a subsequent diminution of dopaminergic tone in the basal ganglia (for recent review see Cornford et al., 1995). Nowadays, as a matter of course, Parkinsonian patients are administered some form of dopaminergic therapy to counter-balance the dopaminergic deficit. Could it be possible, then, that this dopaminergic therapy either directly, or indirectly via an interaction with the cholinergic system, masked the cognitive deficits that would have been expected in the non-demented parkinsonian patients given the cholinergic losses in cortical and hippocampal areas? The present thesis adresses this question by examining the possible dopaminergic modulation of the septohippocampal cholinergic system. This system, in addition to being implicated in mnemonic processes, is one of the cortical structures most affected in degenerative diseases such as AD.

In chapter 2, the existence and nature of a possible interaction between hippocampal DA and ACh were investigated using methods including fimbriaectomy, receptor autoradiography and in vivo dialysis. In chapter 3, in an attempt to ascertain which receptor subtype is responsible for the dopaminergic modulation of hippocampal cholinergic activity, we utilized a combined antisense-in vivo dialysis approach focusing on the D1/D5 receptors.

Next (Chapter 4), we investigated the behavioral sequelea of hippocampal DA-ACh interaction. We utilized aged-memory-impaired (AI) and aged-memory-unimpaired (AU) animals and the Morris Water Maze (MWM) task. These animals are thought to model some aspects of the memory deficits observed in aged population. The MWM task was chosen to examine spatial memory, a hippocampal function that is compromised in certain age-related cognitive deficits.

The series of studies described above were all conducted in the rat. Therefore, we also investigated the potential existence of such DA/ACh interactions in other species (chapter 5). This was done by examining the distribution profile of the implicated receptors in the septohippocampal area of monkey and human brains as an index of the likelihood that these receptors might serve functions similar to those described for the rat.

This thesis is presented as a series of manuscripts. Thus more details on the objectives of each study are provided in the preface, abstract and introduction sections of each chapter. A general discussion is presented as the last chapter.

CHAPTER 2
Dopaminergic Modulation of Hippocampal ACh Release

PREFACE TO CHAPTER 2

Dopamine has been demonstrated to regulate acetylcholine release from striatal interneurons. In this chapter, we investigated the existence as well as the nature of such a regulation in the septohippocampal cholinergic system, a system that is intimately involved in mechanisms thought to underlie mnemonic processes.

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LOCAL MODULATION OF HIPPOCAMPAL ACETYLCHOLINE RELEASE BY DOPAMINE D1 RECEPTORS : A COMBINED RECEPTOR AUTORADIOGRAPHY AND *IN VIVO* DIALYSIS STUDY

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Key words:

Acetylcholine; Dopamine; Hippocampus; *in vivo* Dialysis; Receptor autoradiography, D1 receptor

ABSTRACT

The modulation of in vivo hippocampal acetylcholine (ACh) release by dopaminergic D1 and D2 receptors was examined in this study. Additionally, in an attempt to ascertain the location of these receptors in relation to hippocampal cholinergic terminals, fimbriaectomy and quantitative autoradiography were used. Following unilateral fimbriaectomy, whereby at least 50% of hippocampal cholineacetyltransferase (ChAT) activity was lost, a significant ipsilateral decrease in D1/[^{3}H] SCH23390 binding was observed in the molecular layer of the dentate gyrus while hippocampal D2/[^{3}H] raclopride binding was unaffected. The effects of prototypical D1 and D2 receptor agonists and antagonists on hippocampal ACh release were examined next using *in vivo* dialysis in freely moving rats. The D1 agonist SKF 38393 (10μM-100μM) administered directly into the hippocampus via the dialysis probe stimulated ACh release in a concentration dependent manner. The effect of the agonist was blocked by the coadministration of the D1 receptor antagonist SCH 23390 (1μM), which by itself failed to modulate ACh release. In contrast, neither the D2 agonist quinpirole (1-10μM) nor the D2 antagonist sulpiride (1-10μM) had any direct effect on hippocampal ACh release. Additionally, the infusion of these D1 and D2 drugs in the septal area failed to affect hippocampal ACh release. Taken together, these results suggest that a proportion of hippocampal D1 receptors are located on cholinergic nerve terminals and that dopamine, acting via D1 receptors, can locally stimulate hippocampal ACh release.

INTRODUCTION

Dopamine (DA) is thought to modulate the activity of the septo-hippocampal cholinergic pathway (Costa et al., 1983; Robinson et al,. 1979). Infusions of dopamine antagonists into the septum increase the firing rate of this pathway (Robinson et al., 1979) and elevate acetylcholine (ACh) turnover (Robinson et al., 1979) and high affinity choline uptake (Durkin et al., 1986) in the hippocampus. Similar effects are also observed following either intraseptal 6 - hydroxydopamine injection (Robinson et. al., 1979) or the destruction of the ventral tegmental A10 dopaminergic neurons (Galey et al., 1985; Robinson et.al., 1979). It has been suggested that A10 dopaminergic neurons projecting to the lateral septum interact with cholinergic fibers originating from the medial septal nucleus, possibly via septal GABAergic interneurons, to bring about this inhibitory influence (Wood, 1985). On the other hand, a variety of wide ranging studies including morphological (Scatton et al., 1980; Simon et al., 1989; Verney et al., 1985), electrophysiological (Gribkoff et al., 1984; Smialowski et al., 1987; Stanzione et al., 1984) and biochemical (Bischoff, 1979; Ishikawa, 1982) approaches provide evidence suggesting that the hippocampus receives direct dopaminergic innervation. This innervation apparently originates mainly from the ventral tegmental area (VTA; and to a lesser extent from the substantia nigra) and ascends in the medial forebrain bundle to innervate various limbic structures, including the hippocampus (Verney et al., 1985).

Dopamine receptors have been classified in two broad families (D1-like and D2-like) on the basis of the activity of various agonists and antagonists (Kebabian and Calne, 1979; Seeman 1980). More recently, molecular cloning techniques have shown that the D1 family comprises two receptors (d1 and d5) while the D2 family consists of at least three different receptor proteins (d2, d3 and d4) (for a recent review see Niznik and Van Tol, 1992). D1 receptors activate adenylate cyclase whereas members of the D2 receptor class have been shown to couple to numerous effector systems, including the inhibition of

adenylate cyclase and the activation of potassium channels, among others (Monsma et al., 1990; Sibley and Monsma, 1992). Both the D1 and the D2 receptor subtypes have been localized in the septum as well as the hippocampus of various mammalian species including the rat (Bischoff et al., 1980; Bruink et al., 1986; Dawson et al. 1986; Grilli et al., 1988; Mengod et al., 1992; Tiberi et al., 1991).

Given this background, it is thus likely that dopamine may act as a modulator of the septo-hippocampal cholinergic pathway both at the level of the cell bodies in the septal area and at the nerve terminals within the hippocampus. In the present study, these two possibilities were investigated directly by examining the effects of local administration of selective dopamine D1 and D2 receptor agonists and antagonists on hippocampal ACh release using *in vivo* dialysis in freely moving rats as well as by evaluating the effects of unilateral fimbriaectomy on these receptors in the hippocampus as monitored by quantitative autoradiography. Our results suggest that a certain proportion of pharmacologically - defined D1 receptors are located on cholinergic nerve terminals and that dopamine may act, via D1 receptors, as a stimulant of ACh release at level of the cholinergic nerve terminals within the hippocampus.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (250-350g) obtained from Charles River Canada (St. Constant, Quebec, Canada) were maintained on a 12 hr light-dark cycle (light on at 7:00 a.m.) in temperature and humidity controlled rooms for at least 3-4 days prior to surgery. Animals were fed standard laboratory chow and had access to tap water ad libidum. Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care (CCAC).

The dialysis probes were made from AN69 Hospal fibers (molecular weight cut off < 60,000 i.d. = 220 um, o.d. = 310 um). SCH23390 HCl, SKF38393 HCl, quinpirole HCl and sulpiride were obtained from RBI (Watick, MA, U.S.A.). Neostigmine Bromide was purchased from BAS (West Lafayette, IN, U.S.A). [^{3}H] SCH23390 (80.7Ci/mmol), [^{3}H] raclopride (70.0Ci/mmol), ^{3}H-Hyperfilms and microscale standards were purchased from Amersham Canada (Oakville, Ontario, Canada). The deuterated variant of ACh, [$^2H_4$] ACh bromide [$(CH_3)_3NBrCD_2CD_2OC$-(0)CH_3], used as internal standard for ACh determination was obtained from Merck, Sharp and Dohme Isotopes (Montreal, Quebec, Canada). Developer (D-19) and fixer (Rapid Fix) were obtained from Kodak Chemical Inc. (Montreal Quebec, Canada). All other reagents and chemicals were of HPLC or GC-MS grade and purchased from either Fisher Scientific Co. (Montreal, Quebec, Canada) or Aldrich Chemicals (Chicago, IL, U.S.A.).

Fimbriaectomy

The fimbria fornix of male rats was unilaterally interrupted by a knife cut lesion under sodium nembutal anesthesia (50mg/kg). Briefly, at a 90 degree angle and coordinates of 1mm behind Bregma and 3.0mm lateral to the midline suture (Paxinos and Watson, 1982), a leukotome knife (Kopf Instruments) was lowered via an opening in the

skull to a depth of 4.0mm below dura. The wire in the knife was then extended under the fimbria and the leukotome slowly brought back to the dura. For sham operated animals, the leukotome was lowered and brought back to the surface as above but without extending the wire. The animals were allowed to recover from anesthesia under a warm light and individually housed according to CCAC guidelines. Two weeks post surgery, the animals were sacrificed and the efficacy of the lesions was assessed by determining hippocampal ChAT activity.

Hippocampal ChAT activity

Hippocampal punches from 300μm brain slices of the lesioned and sham operated animals were assayed for ChAT activity. Homogenates from these punches were incubated for 15 min in a buffer containing [^{14}C] acetyl CoA as previously described in detail (Araujo et al., 1988) using the method of Fonnum (1969) as modified by Tucek (1978). The animals that were used in subsequent receptor autoradiographic studies showed hippocampal ChAT activity losses of 51±4% on the lesioned side as compared to the contralateral hippocampi.

Dopamine Receptor Autoradiography

The status of hippocampal dopaminergic receptors following fimbriaectomy was assessed as described in detail elsewhere (Debonnel et al., 1990). In brief, following sectioning at -17°C, 20μm hippocampal slices were incubated for 60 min at room temperature in 50mM Tris HCL buffer (pH 7.4) containing 120mM NCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$ and 1.0nM [^{3}H] SCH 23390 for D1 receptors or 5.7nM [^{3}H] raclopride for D2 receptors. Serial sections were incubated in this buffer but with the addition of 1μM SCH 23390 or 1μM (+) butaclamol to ascertain the specificity of the D1 or D2 radioligand binding, respectively. The sections were then rinsed 5times (2 min each) in fresh ice cold buffer. Buffer salts were removed by a rapid dip in ice cold

distilled water and the sections rapidly air dried. Autoradiograms were generated by apposing the sections alongside with tritium standards to tritium sensitive films for four weeks. The films were then developed as described before (Quirion et al., 1981) and [^{3}H] SCH 23390 and [^{3}H] raclopride binding quantified (fmol/mg tissue wet weight) using computer assisted microdensitometric image analysis system (MCID System, Imaging Research Inc., St-Catherines, Ontario, Canada). Anatomical areas were identified according to the Paxinos and Watson's atlas (1982). Significant differences between experimental groups (n=4 in each group) were determined by a one way analysis of variance (ANOVA).

Probe implantation and hippocampal *in vivo* dialysis

Male Sprague Dawley rats (250-350g) were anesthetized with sodium nembutal (50mg/kg,). Transverse probes (Damsma et al., 1987) were stereotaxically implanted in the dorsal hippocampus and for septally-manipulated animals also in the lateral septum (Giovannini et al., 1994) at coordinates of 3.8mm posterior to bregma and 3.5mm below the skull for the hippocampus and 0.7mm anterior to bregma and 4.5mm below the skull for the septum (Paxinos and Watson, 1982). The animals were individually housed and allowed to recover from surgery for 2 days prior to their use in the *in vivo* dialysis experiments. Each animal was dialysed only once.

At the beginning of each dialysis experiment, animals were placed in lidless cages and connected to a BAS microliter syringe pump in a manner as to allow them to freely move in the cages. The probes were perfused for a one hour wash out period at a flow rate of 2.34 µl/min with an Ungerstedt-Ringer solution (127mM NaCl, 2.5mM KCl, 1.0mM $MgCl_2$, 1.3mM $CaCl_2$, pH 7.4) containing 5µM neostigmine bromide, a cholinesterase inhibitor. Twenty five minute dialysate fractions were collected into a 1ml glass vials containing 46µl of 0.1N HCl and 50 pmoles of deuterated ACh as internal standard.

Following about three hours of baseline hippocampal ACh release, drugs of interest were tested by inclusion in the perfusion Ringer solution for the remainder of the dialysis experiment. For septally-manipulated animals, the drugs of interest were perfused into the septum in a Ringer solution lacking neostigmine. The samples were frozen immediately and stored at -80^{o}C until assayed by gas chromatography/mass spectroscopy (GC/MS). Following most experiments, probe location was verified by standard histological examination of the brain.

In the experiment designed to evaluate the potential diffusion of the locally applied drugs, the *in vivo* dialysis setup was as described above for hippocampally-manipulated animals except that [^{3}H] SCH 23390 (1μM) was used and no samples were collected. The animals were sacrificed immediately following the termination of the experiment. Twentyμm slices of the brains of these animals were exposed to tritium sensitive Hyperfilms for ten days.

GC/MS analysis of ACh

ACh content of the dialysate fractions was determined by GC-MS as described in detail by Marien and Richard (1990). Briefly, frozen samples were lyophilized overnight, reconstituted in 250μl acetonitrile, capped, heated at 80^{o}C for 30 minutes and dried under a gentle stream of nitrogen gas. Quaternary amines present in the samples were demethylated by adding 250μl sodium benzene thiolate solution (160mg in 18ml of redistilled methyl ethyl ketone and 35μl glacial acetic acid) under a flow of nitrogen and reacting at 80^{o}C for 45min. Samples were then extracted into 35μl citric acid and washed twice with 250μl pentane. Finally the samples were extracted into 80μl of ethyl acetate and concentrated down to 3-5μl volume before being injected in to the GC-MS (Hewlett-Packard 5987b). The demethylated derivatives of ACh were analysed by selective ion

monitoring of 132 atomic mass units (amu) for endogenous and 136 amu for the internal standard, [$^2H_4$]ACh.

The amount of endogenous ACh in each dialysate was calculated (Jenden and Hanin, 1974) from the peak area ratio of endogenous vs deuterated internal standard. Calculations were not corrected for the recovery of ACh by each dialysis probe. Sample ACh content was expressed as a percentage of average baseline (eight sample collections preceding drug infusion). Significant differences between experimental groups were determined by a one way analysis of variance (ANOVA).

RESULTS

Effect of fimbriaectomy on hippocampal D1 and D2 receptor binding sites.

A significant loss (18.2%, p=0.0038) of D1/[^{3}H] SCH23390 binding was observed in the molecular layer of the dentate gyrus of the ipsilateral hippocampus in lesioned animals (29.9 $\pm$ 0.8 vs 36.6 $\pm$ 1.1 fmol/mg tissue wet weight) (Fig 1A, 1B). No detectable differences were observed in [^{3}H] SCH 23390 binding in CA1, CA2 and CA3 subfields between the ipsi- and contra-lateral hippocampi of fimbriaectomized rats (Fig 1A, 1B). [^{3}H] SCH 23390 binding levels were similar in the hippocampus of sham operated animals as compared to the contralateral hippocampi of the fimbriaectomized animals (data not shown).

Hippocampal D2/[^{3}H] raclopride binding was considerably lower than that of D1/[3H] SCH 23390. Using [^{3}H] raclopride, the only detectable amount of specific binding in the dorsal hippocampus was observed in the CA1 and subicular regions (Fig 1C). No differences were seen between ipsi- and contra-lateral hippocampi of unilaterally fimbriaectomized animals (Fig1D). Additionally, no detectable changes of either [^{3}H] SCH 23390 or [^{3}H] raclopride binding levels were observed in the septum or cortical regions as a result of unilateral fimbriaectomy. In all the animals used here, ChAT activity in the ipsilateral hippocampi decreased by 51$\pm$4% as compared to the contralateral hippocampi or the hippocampus of sham operated animals.

Local dopaminergic receptor modulation of hippocampal ACh release

The average basal efflux of ACh from the dorsal hippocampus was 4.8 pmol/25min (n=52). Moreover, there were no appreciable differences in basal ACh release between the various experimental groups studied here. Hippocampal intraprobe administration of the active enantiomer of the selective D1 agonist (+) SKF 38393 (10-100μM), but not the inactive enantiomer (10μM), increased ACh release in a

concentration dependent manner (Fig 2A). The D1 antagonist SCH 23390 (1-10μM), by itself, had no appreciable effect on hippocampal ACh release in freely behaving animals (Fig2B). However, the coinfusion of the D1 antagonist (1μM) with the D1 agonist (10μM) blocked the stimulatory effect of the latter (Fig2C).

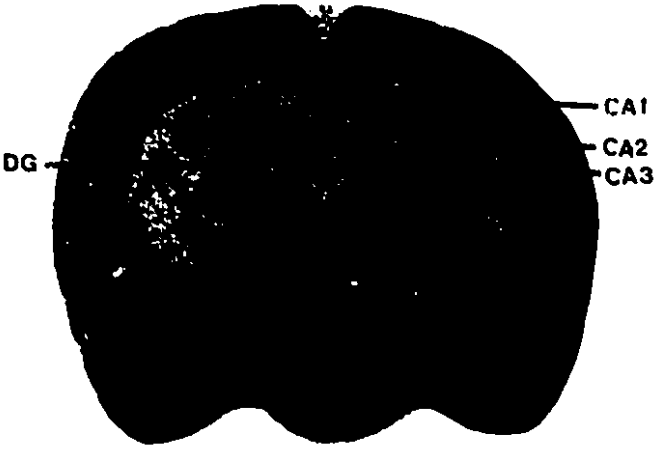
Under the conditions used here, neither the D2 agonist quinpirole HCl (1-10μM) (Fig3A) nor the D2 antagonist sulpiride (1-10μM) (Fig3B) significantly modified hippocampal ACh release. Interestingly, when infused in to the lateral septum neither the D1 nor D2 drugs had an effect on hippocampal ACh release (Fig 4). No appreciable obvious behavioral sequelae (locomotion, sniffing, grooming, etc.) were induced by these dopaminergic drugs administered via the probe.

Diffusion of hippocampally-infused [^{3}H] SCH23390

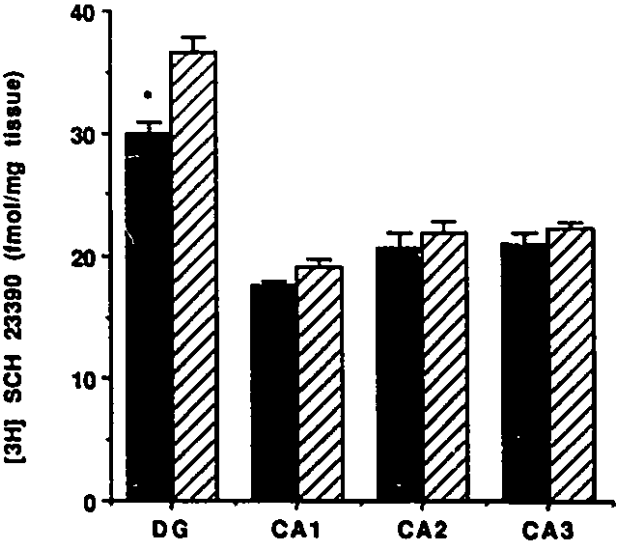
[^{3}H] SCH 23390 (1μM; 1uCi) infused via the probe for 4hrs showed virtually no diffusion beyond the hippocampal area immediately in contact with the dialysis probe (Fig5). Considering that this concentration of the unlabelled D1 antagonist blocked the stimulation of hippocampal ACh release induced by the D1 agonist SKF 38393, it suggests that this effect is occuring locally.

Figure 1. Effect of fimbriaectomy on hippocampal D1/[^{3}H] SCH 23390 and D2/[^{3}H] raclopride binding. Data represent mean ± S.E.M. from four different animals expresses in fmol/mg tissue, wet weight. Sections from unilaterally fimbriaectomized rats were incubated with 1.0nM [^{3}H] SCH 23390/D1 (A,B) or [^{3}H] raclopride/D2 (C,D) as described in Methods. Non specific binding was defined in the presence of 1μM SCH 23390 or 1μM (+) butaclamol for D1 and D2, respectively. Autoradiograms (A,C) generated by apposing the sections against tritium sensitive films were subsequently quantified (B,D) using computer assisted image analysis system. The histograms represent specific labelling obtained by subtracting non specific from total binding. Filled and shaded bars represent the ipsi- and contralateral, respectively. Statistical analysis was evaluated by one way analysis of variance (ANOVA). * $p<0.05$; significantly different from non lesioned contralateral side The bar equals 2cm. Abbreviations: CA, Ammons horn; CPu, Caudate-Putamen; DG, Dentate gyrus; Sub, subiculum.

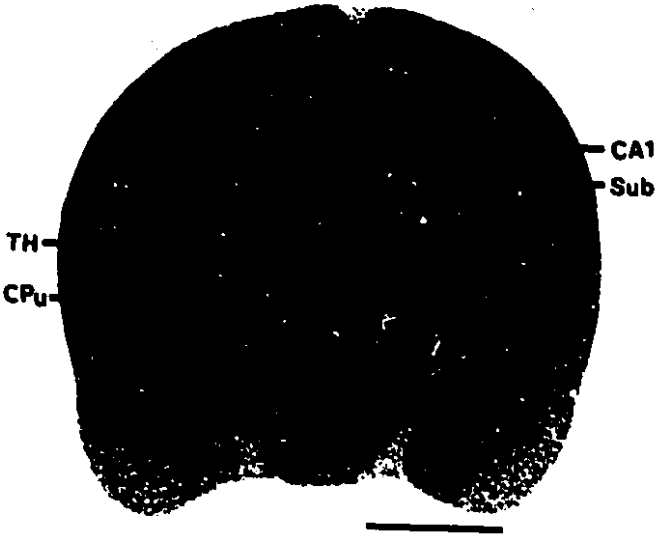
A


DG
CA1
CA2
CA3

B


[3H] SCH 23390 (fmol/mg tissue)
40
30
20
10
0
DG
CA1
CA2
CA3

C


CA1
Sub
TH
CPu

D

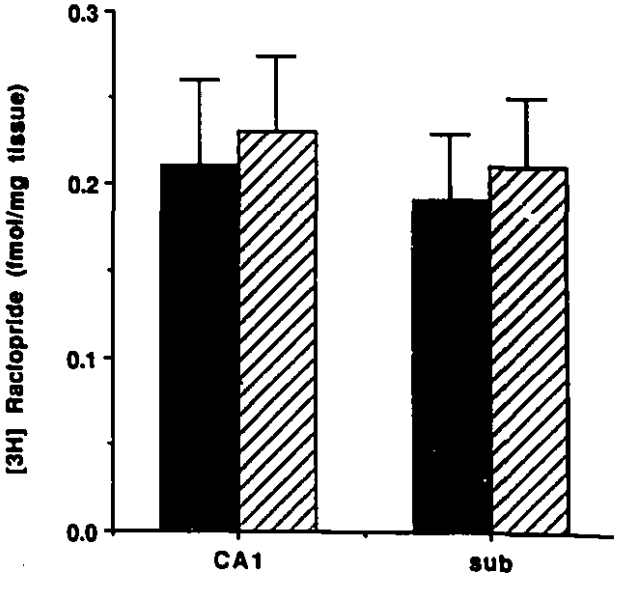
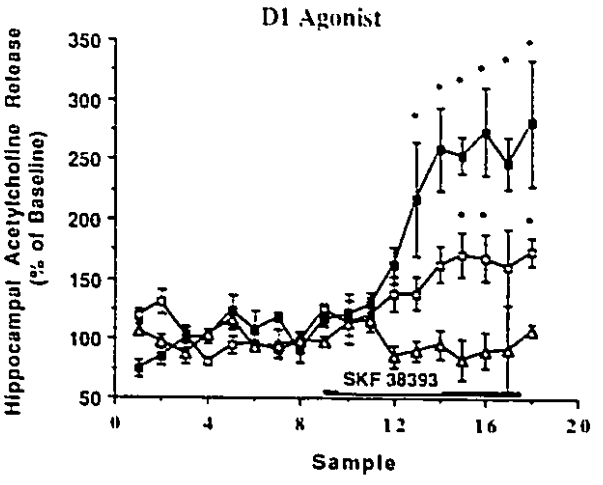
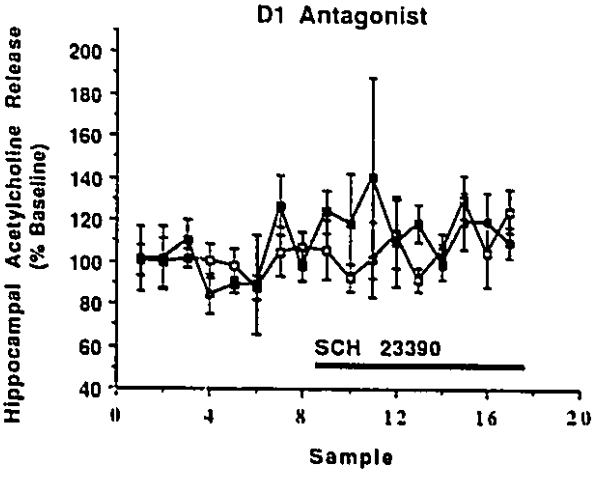

[3H] Raclopride (fmol/mg tissue)
0.3
0.2
0.1
0.0
CA1
sub

Figure 2. Effects of the infusion of the D1 agonist (+)SKF 38393 (A: 10μM, □ ; 100μM, ■ or its inactive enantiomer (-)SKF 38393 10μM◆), the D1 antagonist SCH 23390 (B: 1μM □ ; 10μM ■) or their combination (C: 10μM (+)SKF 38393 and 1μM SCH 23390) on hippocampal ACh efflux. Data represent mean ± S.E.M. (n=4 for each concentration). Following baseline determination, ringer solution containing the drug of interest was perfused through the probe implanted in the hippocampus for the period indicated by the black bars (upto 4hrs). Dialysate ACh content, measured by GC-MS, is expressed as a percent of baseline. Baseline was calculated from the average of eight samples preceding drug infusion. Statistical evaluation was evaluated by one way analysis of variance (ANOVA). * $p<0.001$; significantly different from baseline. Only the D1 agonist induced significant effects (A) that were blocked by the a co-infusion of the antagonist(C).

A


D1 Agonist
Hippocampal Acetylcholine Release
(% of Baseline)
350
300
250
200
150
100
50
SKF 38393
0
4
8
12
16
20
Sample

B


D1 Antagonist
Hippocampal Acetylcholine Release
(% Baseline)
200
180
160
140
120
100
80
60
40
SCH 23390
0
4
8
12
16
20
Sample

C

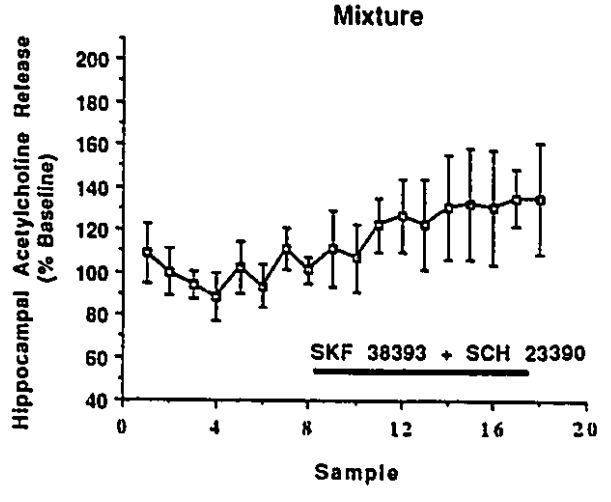
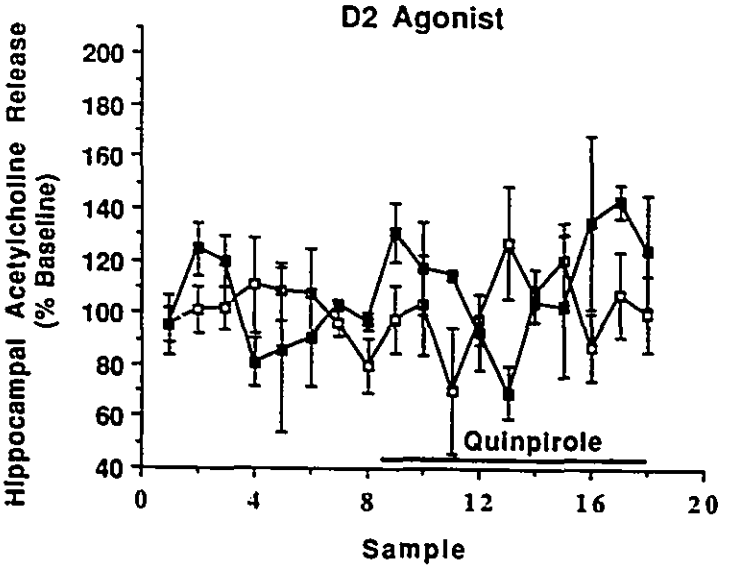

Mixture
Hippocampal Acetylcholine Release
(% Baseline)
200
180
160
140
120
100
80
60
40
SKF 38393 + SCH 23390
0
4
8
12
16
20
Sample

Figure 3. Effects of the infusion of the D2 agonist quinpirole (A: 1μM, ▫ ; 10μM ■) and the D2 antagonist sulpiride (B: 1μM, ▫ ; 10μM, ■) on hippocampal ACh efflux. Data represent mean ± S.E.M. (n=4 for each concentration). Following baseline determinations, ringer solution containing the drug of interest was perfused through the probe implanted in the hippocampus for the period indicated by the black bars (upto 4hrs). Dialysate ACh content, measured by GC-MS, is expressed as a percent of baseline. Baseline was calculated from the average of eight samples preceding drug infusion.

A


D2 Agonist
Hippocampal Acetylcholine Release (% Baseline)
200
180
160
140
120
100
80
60
40
Quinpirole
0
4
8
12
16
20
Sample

B

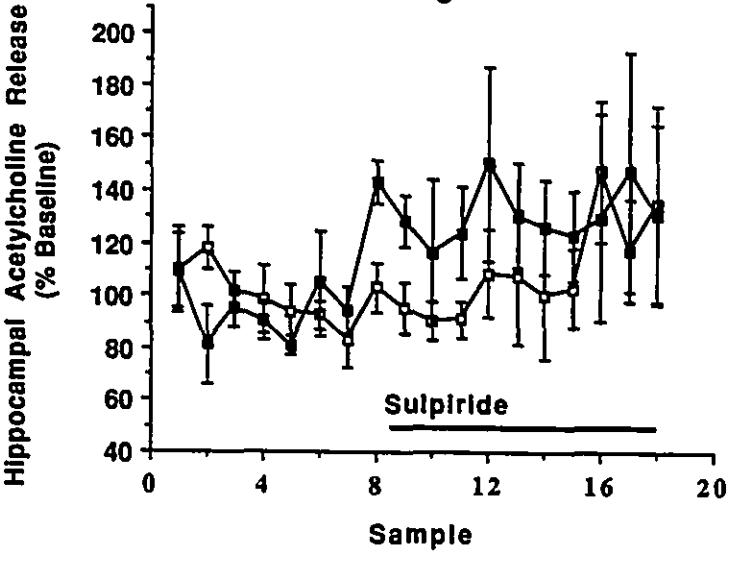
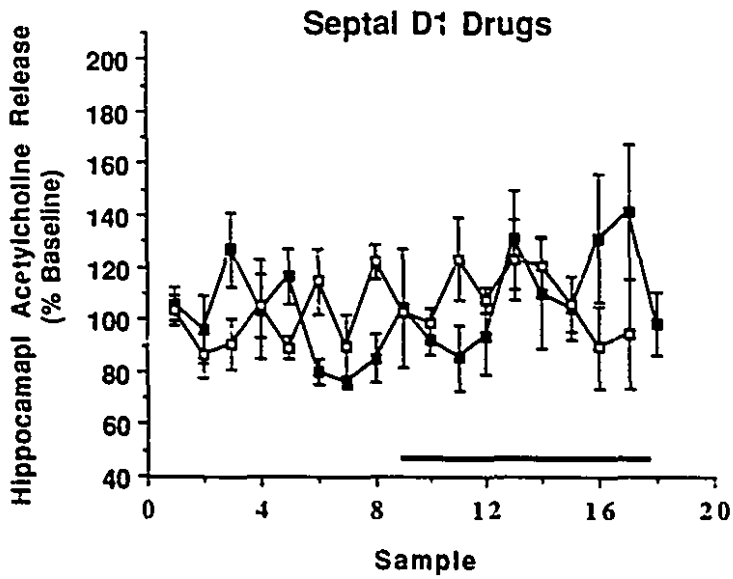

D2 Antagonist
Hippocampal Acetylcholine Release (% Baseline)
200
180
160
140
120
100
80
60
40
Sulpiride
0
4
8
12
16
20
Sample

Figure 4. Effects of the septal infusion of the D1 ligands (A: SKF 38393 10μM, ■ ; SCH 23390 10μM □) and the D2 ligands (B: Quinpirole 10μM, ■; Sulpride 10μM, □) on hippocampal ACh efflux. Data represent mean ± S.E.M. (n=4 for each ligand). Following baseline determinations, ringer (minus neostigmine) solution containing the drug of interest was perfused through the probe implanted in the septum for the period indicated by the black bars (upto 4hrs). Samples were collected from the probe implanted in the dorsal hippocampus. Dialysate ACh content, measured by GC-MS, is expressed as a percent of baseline. Baseline was calculated from the average of eight samples preceding drug infusion.

A


Septal D1 Drugs
Hippocampal Acetylcholine Release
(% Baseline)
200
180
160
140
120
100
80
60
40
0
4
8
12
16
20
Sample

B

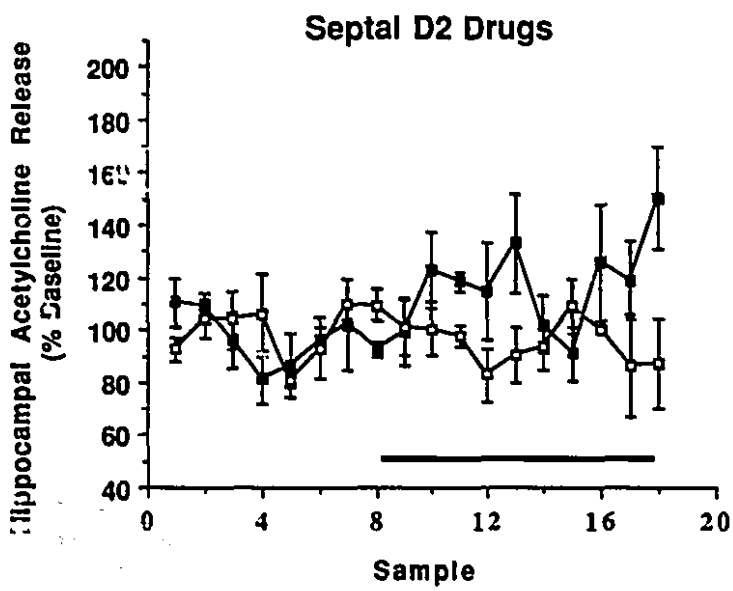
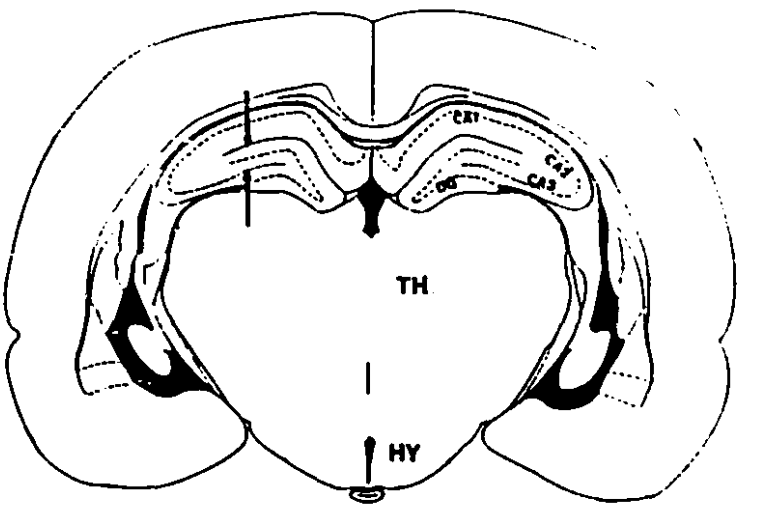

Septal D2 Drugs
Hippocampal Acetylcholine Release
(% Baseline)
200
180
140
120
100
80
60
40
0
4
8
12
16
20
Sample

Figure 5. Diffusion of trans-hippocampally infused [^{3}H] SCH 23390 in brain parenchyma. [^{3}H] SCH23390 (1μM; 1mCi/mmol) was perfused for 4hrs in the dorsal hippocampus as described in the Methods. 20μm brain sections from these animals were exposed to tritium sensitive films and photomicrographs taken. (A) shows the extent of diffusion of the radioactive material and (B) a schematic representation of the section in A. The arrows mark the location of the dialysis probe. It is rather evident that diffusion was minimal and the infused radioactivity remained in close proximity to the dialysis probe. Abbreviations: CA, Ammons horn; DG, Dentate gyrus; HY, hypothalamus and TH, Thalamus.

A

B



DISCUSSION

A variety of studies have demonstrated the presence of direct dopaminergic innervation into the hippocampus. This innervation is concentrated in the ventral subiculum, in particular in the presubiculum (Verney et. al., 1985). Hippocampal D1 receptors, on the other hand, are mainly located in the molecular layer of the dentate gyrus and the dorsal hippocampus (Dawson et al., 1986; Grilli et al., 1988; Tiberi et al., 1991). In the present study, we observed that a significant portion of D1 receptors in the molecular layer of the dentate gyrus was lost following fimbriaectomy. This suggests that a proportion of hippocampal D1 receptors are located presynaptically on afferent terminals. Moreover, a concomitant reduction in hippocampal ChAT activity was observed as a result of the lesion. Considering that a major cholinergic input carried via the fimbrial system terminates in the molecular layer of the dentate gyrus (Fibiger, 1982; Butcher and Woolf, 1986), and that there are very few, if any, dopaminergic terminals in this area of the hippocampus (Verney et al., 1985), it is logical to assume that at least some of the lost D1 receptors are located on cholinergic nerve terminals. Interestingly, in senile dementia of the Alzheimer's Type (SDAT), a marked reduction in hippocampal D1 receptors has been reported (Cortes et al., 1988), with the highest loss (89%) seen in the molecular layer of the dentate gyrus. It is well known that one of the hallmarks of SDAT is the destruction of basal forebrain cholinergic neurons which, in part, project to the hippocampus (Davies and Maloney, 1976; Coyle et al., 1983; Whitehouse et al., 1982). Therefore, in agreement with these studies, the reduction in hippocampal D1 receptors observed here could be due to the loss of presynaptically located receptors resulting from the cholinergic denervation. Nevertheless, the possibility that some or all of these D1 receptors are located on afferents other than cholinergic that are contained within the fimbria-fornix cannot be excluded presently.

D2 receptors, on the other hand, are found in the hippocampus in very low amounts compared to D1 receptor levels (Fig 1D). In the dorsal hippocampus, D2/[3H] raclopride binding sites are mainly located in the CA1 and subiculum regions (Fig 1C). In the present study, no alteration in hippocampal D2 receptor densities were observed following fimbriaectomy. It would thus appear that this dopamine receptor subtype is not directly associated with the septo-hippocampal cholinergic nerve projection.

Recently, the technique of *in vivo* dialysis has been used to study the possible regulation by dopaminergic drugs of the septo-hippocampal cholinergic pathway. Systemic administration of dopaminergic drugs has shown that dopamine potentially stimulates hippocampal ACh release via both D1 and D2 receptor subtypes (Imperato et al., 1993) or the D1 subtype alone (Day and Fibiger, 1994) in young animals, and at least via D1 receptors in aged-memory impaired rats (Hersi et al., 1994). A number of possible loci exist for this apparent interaction between DA and ACh. Given the direct dopaminergic innervation of the hippocampus and the putative localization of D1 receptors on hippocampal terminals (see above), a direct modulation of hippocampal ACh release by DA drugs is a likely possibility.

The D1 receptor agonist (+) SKF 38393 applied directly into the hippocampus via the dialysis probe stimulated, in a concentration dependent manner, ACh release. This effect was blocked by the D1 antagonist SCH 23390, attesting to the specificity of the effect for the D1 receptor family. However, SCH 23390 infused alone in the dialysis probe did not alter hippocampal ACh levels. These findings suggest that the D1 receptors located in the dorsal hippocampus can enhance ACh release and that this action is likely phasic in nature, in view of the lack of effect of the D1 antagonist alone.

A question arises, however, as to the extent of diffusion of locally applied drugs in the *in vivo* dialysis paradigm used here. This is especially pertinent considering the relatively long period of drug infusion. Administration of [^{3}H] SCH 23390 under conditions identical to those used for the non-radioactive drugs, showed that diffusion is rather minimal and that the ligand is mostly, if not exclusively, found in the area surrounding the dialysis probe (Fig 5). Thus, it would appear that local interaction can account for the effects of D1 drugs on hippocampal ACh release as observed in the present study or following systemic administration (Day and Fibiger, 1994; Hersi et al., 1994; Imperato et al., 1993).

In contrast to D1 drugs, neither the stimulation nor the blockade of D2 receptors by local, intraprobe infusion of prototypical drugs into the dorsal hippocampus had any effect on ACh release.

Another possible locus for ACh / DA interaction is the lateral septal area. Dopaminergic projections arising from the VTA and terminating in the lateral septum were postulated to interact with the cholinergic cell bodies of the medial septum that give rise to the cholinergic septo-hippocampal pathhway (Wood, 1985). In the present study, the manipulation of neither D1 nor D2 receptors in the septal area had any effect on hippocampal ACh release. Thus, it would appear that dopamine stimulates hippocampal ACh release by acting on D1 receptors located in the hippocampus. Moreover, although the lesion data presented here suggests that these D1 receptors are located on cholinergic terminals the possibility of transsynaptic action involving interneurons or other non-cholinergic afferents cannot be ruled out at the present time.

In the classical view of the synapse, a close juxtaposition must exist between the nerve fiber terminals enriched with a given neurotransmitter and its post- synaptic

receptors. However, this is clearly not always the case and may even be the exception (Beaudet and Descarries, 1978). Discrepancies between the localization of receptors and the distribution of the relevant neurotransmitter has been referred to as the mismatch issue (Kuhar, 1985; Herkenham, 1987). In the present situation, the hippocampal dopaminergic innervation, arising mainly from the VTA and the substantia nigra, is mostly restricted to the ventral hippocampus (Verney et al., 1985). In contrast, hippocampal dopaminergic receptors are predominantly found in the dorsal hippocampus (Dawson et al., 1986; Grilli et al., 1988; Tiberi et al., 1991; this study). Interestingly, however, more than 40% of the dopamine present in the hippocampus has been proposed to be located within dorsal noradrenergic terminals (Bischoff et al., 1979). It may be conceivable that, under certain conditions, dopamine and noradrenaline may be co-released from the terminals of the latter present in the dorsal hippocampus. It is also tempting to suggest that crossover between neurotransmitters may exist to partly account for the apparent mismatches. For instance, under excessive cell firing conditions, the release of noradrenaline in quantities large enough to saturate its own receptors may lead to the binding of this neurotransmitter to other related receptor families such as those for dopamine. In this way, a secondary level of complexity would be added to code for signal strength and more effective transmission.

Another intriguing possibility to account for apparent ligand-receptor mismatches is known as volume transmission (VT). In contrast to the classical synaptic mode of signal transmission, VT refers to the diffusion of chemical signals in the extracellular fluid. A neurotransmitter could thus act at a considerable distance from its site of release (for a recent review see Benfenati and Agnati, 1991). For example, it has been reported that when transient parkinsonism is induced in cats following MPTP administration, dopamine released from spared ventral striatum terminals can diffuse over a distance of 5.5 to 7.0mm to the more extensively denervated dorsolateral striatum

(Schneider et al., 1994). Similarly, dopamine released from fiber terminals located in the ventral hippocampus could diffuse to the dorsal part where the dopaminergic D1 receptors are mostly found. However, direct evidence in support of this possibility have yet to be provided. Interestingly, D1 receptors have, in the past, been linked with volume transmission in areas such as the median eminence and globus pallidus (Fuxe et al., 1988).In any case, it is evident from the data reported here that the activation of D1 receptors present in the dorsal hippocampus can modulate ACh release and are hence fully functional.

Finally, it should be added that the physiological significance of the dopaminergic system in the hippocampus is not yet clear. Intrahippocampal injections of dopamine receptor agonists evoke theta rhythms in hippocampal electroencephalograms (Smialowski, 1985). This type of rhythmic oscillation is known to occur during periods of learning (Winson, 1978). Moreover, during theta oscillation, hippocampal synapses are in a state of heightened plasticity and the stimulatory requirements for the induction of long term potentiation (LTP) are markedly reduced (Huerta and Lisman, 1993). Interestingly, a well established role of the hippocampal cholinergic nerve terminals is to elicit theta rhythms (Bland, 1986). Dopamine modifies LTP in the Schaffer collateral pathway of the rat hippocampus via D1 receptors (Huang and Kandel, 1995) and as shown in the present report stimulates cholinergic neurons via the activation of these same D1 receptors. In addition, mnemonic deficits in aged rats can be attenuated by D1 receptor agonists and this effect was proposed to be mediated by the release of ACh in the hippocampus (Hersi et al., 1994). Therefore, it appears that a role for DA in the hippocampus could, at least in part, be associated with learning and memory, likely via the modulation of hippocampal cholinergic functions.

In summary, a certain proportion of hippocampal D1 receptors appears to be located directly on septo-hippocampal cholinergic nerve terminals. Moreover, *in vivo* hippocampal ACh release is facilitated by the local stimulation of D1 but not D2 receptors. Recent data have clearly shown that the D1 receptor family comprises both the d1 and d5 subtypes (see Introduction). At present, it is unclear which of these two subtypes is involved in the modulation of hippocampal ACh release as selective probes have yet to be developed to discriminate between these members of the D1 receptor family. Other strategies such as the use of functional receptor antibodies or oligonucleotide antisenses would have to be used.

ACKNOWLEDGMENT

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CHAPTER 3
D5 Receptors and Hippocampal ACh Release

PREFACE TO CHAPTER 3

Dopamine acts via two classes of receptors, namely D1-like and D2-like. The D1-like family, which in the preceding chapter was shown to mediate the dopaminergic modulation of hippocampal acetylcholine release, is comprised of two members, D1 and D5, with similar pharmacological profiles. Recently, a novel technique (antisense knockdown) which is potentially capable of discriminating between closely related proteins has been developed. In this chapter, we utilized a combined antisense-in vivo dialysis approach to determine which of the two dopamine D1-like receptors is involved in modulating hippocampal acetylcholine release.

ANTISENSES SUGGEST A ROLE FOR DOPAMINE D5 RECEPTORS IN THE MODULATION OF HIPPOCAMPAL ACETYCHOLINE RELEASE

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Key Words : Acetylcholine, dopamine, hippocampus, in vivo dialysis, antisense, D5 receptor.

ABSTRACT

The receptor subtype responsible for the D1 receptor agonist-induced stimulation of hippocampal acetylcholine (ACh) release was investigated by a combined antisense-in vivo dialysis approach. Phosphorothioate-modified antisense oligodeoxynucleotides targeted against the D1 or D5 subtypes of the D1-like family of dopamine receptors were continuously infused into the third ventricle for three days at a dose of 1μg/hr. Both antisenses reduced hippocampal [^{3}H] SCH 23390/D1-like receptor binding while not altering [^{3}H] raclopride/D2-like binding. However, only the antisense directed against the D5 receptor was able to block the increase in hippocampal ACh release resulting from the local administration of the D1-like receptor agonist, SKF 38393 (100μM). Administration of an oligonucleotide having the same proportion of bases as the D5 antisense but in a random sequence failed to either decrease [^{3}H] SCH 23390 binding or block SKF 38393-induced increase in ACh release. Taken together, these findings suggest that the receptor subtype involved in the dopaminergic regulation of hippocampal cholinergic activity is of the D5 subtype. To our knowledge, this is the first time that a functional role has been demonstrated for the D5 receptor in the mammalian brain.

INTRODUCTION

Dopamine neurotransmission has been implicated in a myriad of brain functions. These actions of dopamine are mediated via a family of G-protein coupled receptors that are generally divided into two groups, namely D1-like and D2 like receptors (Kebabian and Calne, 1979; Seeman, 1980, Dohlman et al., 1991). Lately, the D1-like receptors and their possible involvement in cognition have generated much excitement (Desimone, 1995). For instance, an exquisite modulation of working memory in monkeys by antagonizing D1-like receptors in the prefrontal cortex has been reported (Williams and Goldman-Rakic, 1995). D1-like receptor activation has also been implicated in long term potentiation in the Schaffer collateral pathway of the rat hippocampus (Huang and Kandel, 1995). Recently, we have shown that stimulation of these receptors can attenuate memory deficits in the aged rat possibly through the stimulation of hippocampal cholinergic activity (Hersi et al., 1995a; 1995b).

There are at least two cloned members of the D1-like family of dopamine receptors, namely D1 and D5 (for recent reviews see Civelli et al., 1993; Gingrich and Caron 1993). At the present time, no pharmacological ligands are available that can distinguish between these two receptor subtypes. Therefore, it has not been possible to ascertain which of these two D1-like receptors is involved in the functions described above. In this study, we utilized a combined antisense-in vivo dialysis approach to determine the D1-like receptor subtype involved in stimulating hippocampal acetylcholine release. Interestingly, it appears that it is the D5 receptor that is involved.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (250-350g) obtained from Charles River Canada (St. Constant, Quebec, Canada) were maintained on a 12 hr light-dark cycle (light on at 7:00 a.m.) in temperature and humidity controlled rooms for at least 3-4 days prior to surgery. Animals were fed standard laboratory chow and had access to tap water ad libidum. Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care (CCAC).

From the cDNA sequences of the D1 and D5 dopamine receptors (Zhou et al., 1990; Tiberi et al., 1991), 24-mer and 23-mer phosphorothioate-modified oligodeoxynucleotides were designed, respectively. The antisense was targeted to the area of these receptors spanning the initiation codon: D1 : 5' GTTAGGAGCCATCTTCCAGAAGGA 3' , D5 : 5' AGCATGTCGCGCTGAGTAGCTCG 3'. These selected sequences have very low homology with any other known cDNA sequences found in the Gene Bank database and are predicted to form no stable homodimers under physiological conditions. A phosphorothioate-modified oligodeoxynucleotide having the same proportion of DNA bases as the D5 sequence but in a scrambled order (5' CTAGGAAACGGGTTCGCGCCTTG 3') was similarly prepared. The antisense oligodeoxynucleotides were synthesized and purified by Oligo-ETC (Willsonville, Or, U.S.A).

The dialysis probes were made from AN69 Hospal fibers (molecular weight cut off < 60,000 i.d. = 220 μm, o.d. = 310 μm). Alzet minipumps (model 1003D) were purchased from Alza corporation (Palo Alto, CA, U.S.A). (+) SKF38393 HCl was

obtained from RBI (Watick, MA, U.S.A.). Neostigmine bromide was purchased from BAS (West Lafayette, IN, U.S.A). [^{3}H]SCH23390 (80.7Ci/mmol), [^{3}H]raclopride (70.0Ci/mmol), ^{3}H-Hyperfilms and microscale standards were purchased from Amersham Canada (Oakville, Ontario, Canada). Developer (D-19) and fixer (Rapid Fix) were obtained from Kodak Chemical Inc. (Montreal, Quebec, Canada). All other reagents and chemicals were of HPLC or GC-MS grade and purchased from either Fisher Scientific Co. (Montreal, Quebec, Canada) or Aldrich Chemicals (Chicago, IL, U.S.A.).

Probe and perfusion pump implantation and hippocampal *in vivo* dialysis

Rats were anesthetized with sodium nembutal (50mg/kg,). Transverse probes (Damsma et al., 1987) were stereotaxically implanted in the dorsal hippocampus at coordinates of 3.8mm posterior to bregma and 3.5mm below the skull. At the same time, the injection cannula of an Alzet minipump was placed in the third ventricle at co-ordinates of 1.8mm posterior to bregma and 3.8mm below the skull (Paxinos and Watson, 1982). The antisense oligonucleotides were dissolved in sterile Ringer solution (1μg/μl) and the Alzet mini-pumps loaded according to the manufacturer's guidelines. These pumps were housed in a skin pouch created between the shoulder blades of the animal. The pumps continuously delivered at a rate of 1μl/hr (1μg/μl) for an average of 65 hrs prior to the use of the animals in the *in vivo* dialysis experiments. Each animal was dialysed only once.

At the beginning of each dialysis experiment, animals were placed in lidless cages and connected to a BAS microliter syringe pump in a manner as to allow them to freely move in the cages. The probes were perfused for a one hour wash out period at a flow rate of 2.34 μl/min with an Ungerstedt-Ringer solution (127mM NaCl, 2.5mM

KCl, 1.0mM $MgCl_2$, 1.3mM $CaCl_2$, pH 7.4) containing 5μM neostigmine bromide, a cholinesterase inhibitor. Twenty five minute dialysate fractions were collected into a 1ml glass vials containing 46μl of 0.1N HCl and 50 pmoles of deuterated ACh as internal standard. Following about three hours of baseline hippocampal ACh release, 100μM (+)SKF 38393 was administred via the probe by inclusion in the perfusion Ringer solution for the remainder of the dialysis experiment. The samples were frozen immediately and stored at -80^{o}C until assayed by gas chromatography/mass spectroscopy (GC/MS) as described previously (Hersi et al., 1995a). Following most experiments, probe location was verified by standard histological examination of the brain.

Dopamine Receptor Autoradiography

The status of hippocampal dopaminergic receptors following antisense infusion was assessed as described in detail elsewhere (Debonnel et al., 1990). In brief, brains were removed immediately after the conclusion of the dialysis experiment and stored at -80^{o}C. Following sectioning at -17^{o}C, 20μm slices were washed twice (15min each time) at room temperature in 50mM Tris HCl buffer (pH 7.4) containing 120mM NCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$. These sections were then incubated for 60min at room temperature in this buffer containing 1.0nM [^{3}H] SCH 23390 for D1-like receptors or 5.7nM [^{3}H] raclopride for D2 (D2/D3) receptors. Serial sections were incubated in this latter buffer but with the addition of 1μM SCH 23390 or 1μM (+) butaclamol to ascertain the specificity of the D1 or D2 radioligand binding, respectively. The sections were then rinsed 5 times (2 min each) in fresh ice cold buffer. Buffer salts were removed by a rapid dip in ice cold distilled water and the sections rapidly air dried. Autoradiograms were generated by apposing the sections alongside with tritium standards to tritium sensitive films for four weeks. The films

were then developed as described before (Quirion et al., 1981) and [^{3}H] SCH 23390 and [^{3}H] raclopride binding quantified (fmol/mg tissue wet weight) using computer assisted microdensitometric image analysis system (MCID System, Imaging Research Inc., St-Catherines, Ontario, Canada). Anatomical areas were identified according to Paxinos and Watson's atlas (1982). Significant differences between experimental groups (n=4 in each group) were determined by a one way analysis of variance (ANOVA).

RESULTS

Antisense effects on animal behaviour

At the concentration primarily used here (1μg/hr for three days) no gross abnormal behaviors were observed in the animals which appeared generally healthy, gained weight and were responsive following the recovery period from surgery. At a higher antisense concentration (5μg/hr), a general hypoactivity as well as significant loss of weight were observed in the treated animals. This was especially true of the rats receiving the anti-D1 oligonucleotide. Accordingly, the 1μg/hr antisense dose was routinely administered.

Effect of antisense oligonucleotides on dopaminergic receptor levels

DA receptor levels were determined by autoradiography in sections obtained from the animals used in the dialysis experiments. Both the anti-D1 and anti-D5 receptor oligonucleotides significantly reduced [^{3}H] SCH 23390/D1-like binding sites in the hippocampal formation (Fig 1). The D5 receptor antisense decreased labelling by 31.8$\pm$3.6% in the dentate gyrus and 27.1$\pm$3.3% in the Ammon's horn area of the hippocampus. Similarly, the D1 receptor antisense reduced D1-like binding by 30.1$\pm$12.4% in the dentate gyrus and 15.1$\pm$5.3% in the Ammon's horn. In contrast, the scrambled D5 receptor antisense oligonucleotide had no significant effect on the levels of these receptors in any of the brain regions examined. A trend towards a reduction in [^{3}H] SCH 23390 binding sites was seen in the medial septal area; however, it failed to reach statistical significance. No alterations in the levels of D1-like receptors were seen in the dorsal striatum as a result of the administration of any of these antisense oligodeoxynucleotides.

Moreover, neither the anti-D1, anti-D5 nor the scrambled anti-D5 oligonucleotides reduced total [^{3}H] raclopride/D2-like binding in the hippocampus, medial septum or dorsal striatum (data not shown).

Effect of D1/D5 antisense on the D1-like agonist-induced-stimulation of hippocampal ACh release

The average basal level of hippocampal ACh release under the conditions used here was 2.6±0.4 pmoles/25min. None of the antisense treatments significantly altered this basal release (Fig 2A). In control animals, hippocampal ACh release was elevated by about two fold following the local administration of the D1 receptor agonist SKF 38393 (100μM) in a fashion similar to that described previously (Hersi et al., 1995a). Infusion of the D1 receptor antisense (1μg/hr) did not alter the D1 agonist effect (Fig 2B). In contrast, the anti-D5 receptor oligonucleotide (1μg/hr) significantly blocked the SKF 38393-induced-stimulation of hippocampal ACh release (Fig 2C). An oligonucleotide composed of the same bases as the anti-D5 receptor antisense, but in scrambled manner failed to block the increase in hippocampal ACh release (Fig 2C).

Figure 1. Effect of antisense treatments on hippocampal D1-like/[^{3}H] SCH 23390 binding sites. Brain sections obtained from rats immediately upon the completion of the dialysis experiments were incubated with 1.0nM [^{3}H] SCH 23390 as described in Materials and Methods. Non specific binding was determined in the presence of 1μM SCH 23390. Autoradiograms generated by apposing the sections against tritium sensitive films were subsequently quantified using computer assisted image analysis. The histograms represent specific labelling obtained by subtracting non specific from total binding. Data are mean $\pm$ S.E.M. from four different animals for each treatment expressed as percent of control Ringer infusion. Statistical analysis was evaluated by one way analysis of variance (ANOVA). * $p<0.05$. Abbreviations: CA, Ammons horn; DG, Dentate gyrus; Dstr, dorsal striatum; Msep, medial septum. Ringer, ■ ; AntiD5 Scrambled, ; Anti-D1, ; Anti-D5, .

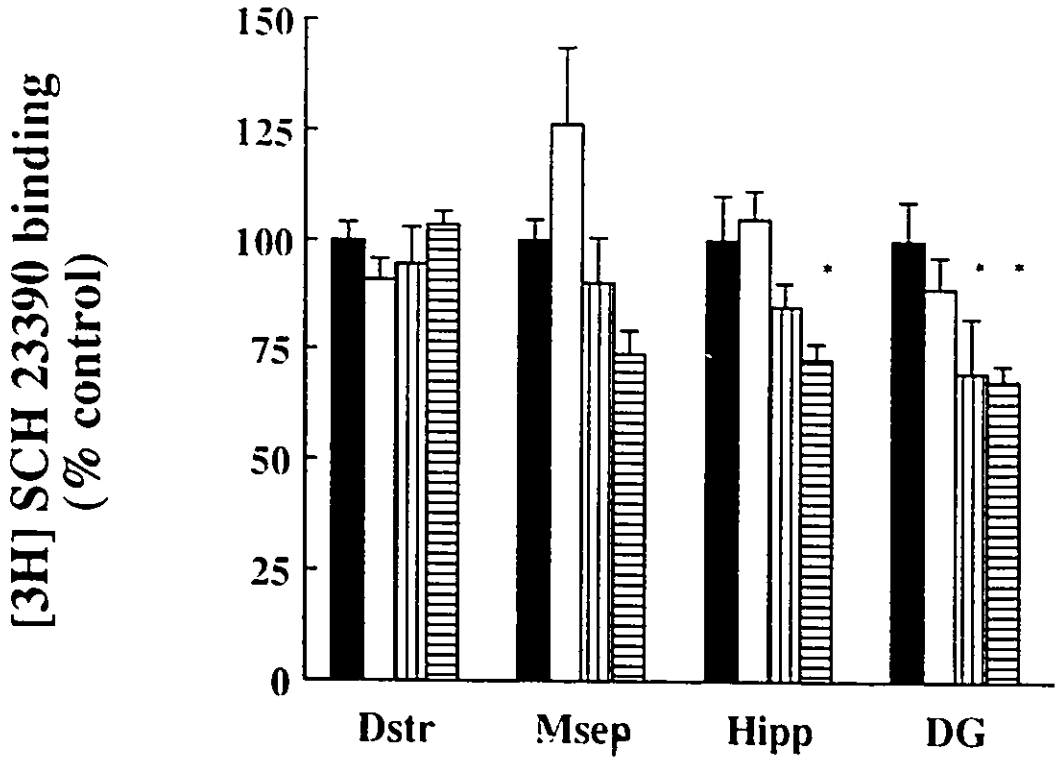
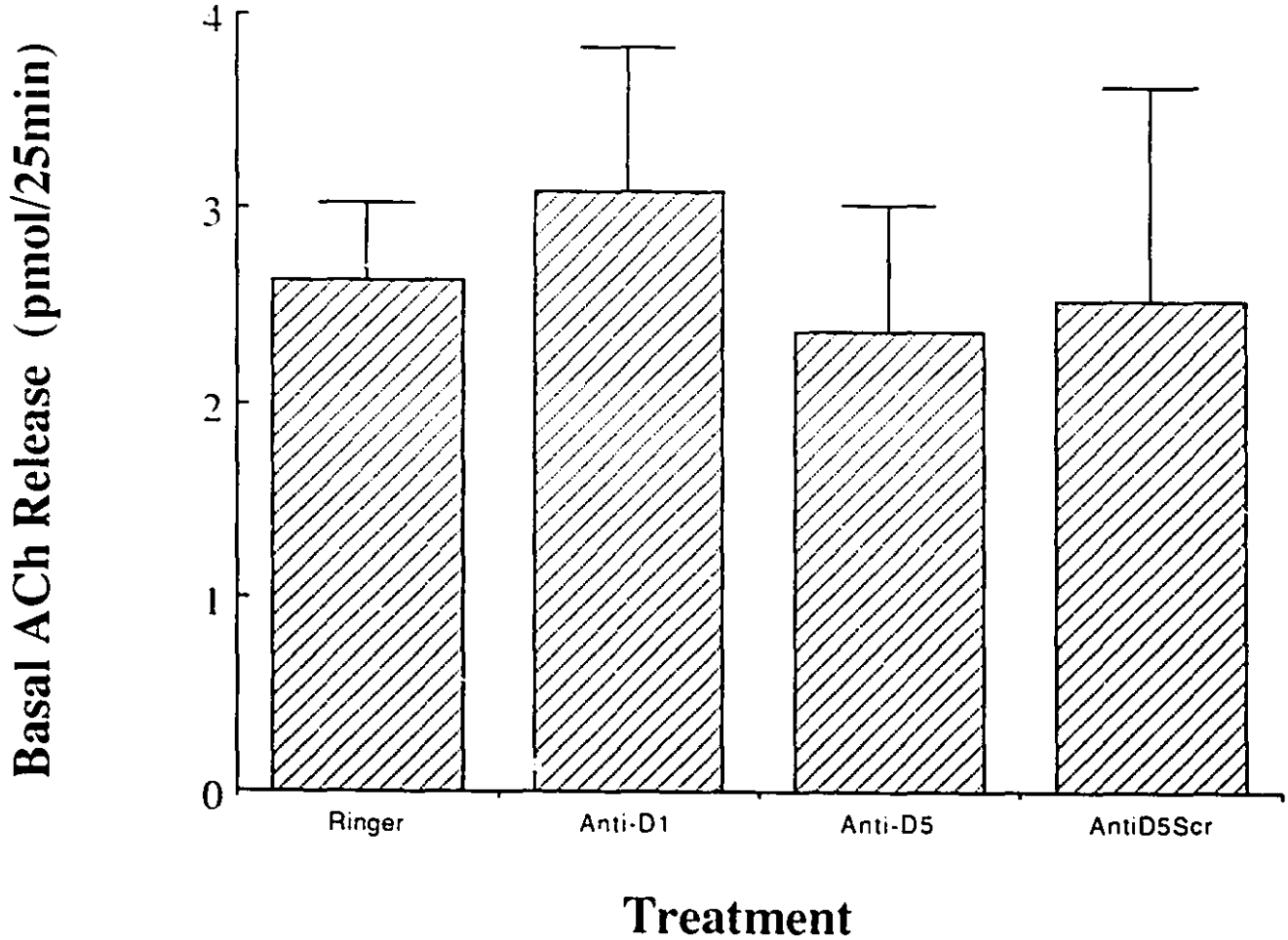
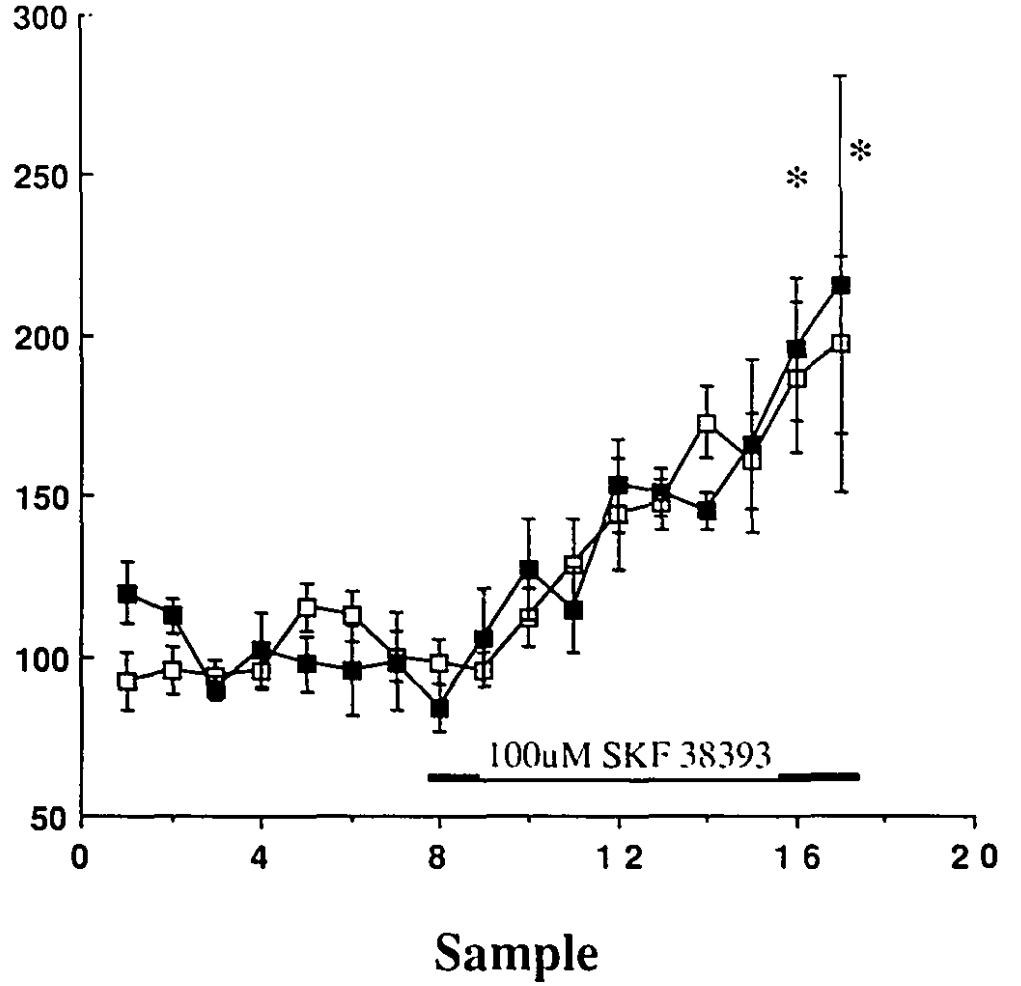

[3H] SCH 23390 binding
(% control)
150
125
100
75
50
25
0
Dstr
Msep
Hipp
DG
*
* *

Figure 2. Effects of antisense treatment on basal (A) and SKF 38393 stimulated (B,C) hippocampal ACh release. Following baseline determination, Ringer solution containing 100μM SKF 38393 was perfused through the probe implanted in the hippocampus for the period indicated by the black bars (up to 4hrs). Dialysate ACh content, measured by GC-MS, is expressed as a percent of baseline. Basal release was calculated from the average of eight samples preceding drug infusion. Data represent mean $\pm$ S.E.M. (n=4 for each group). Statistical significance was evaluated by one way analysis of variance (ANOVA). * $p<0.05$ from basal release. Only the D5 antisense treatment blocked the effect of SKF 38393 on ACh release, the D1 and D5 scrambled having no effect.

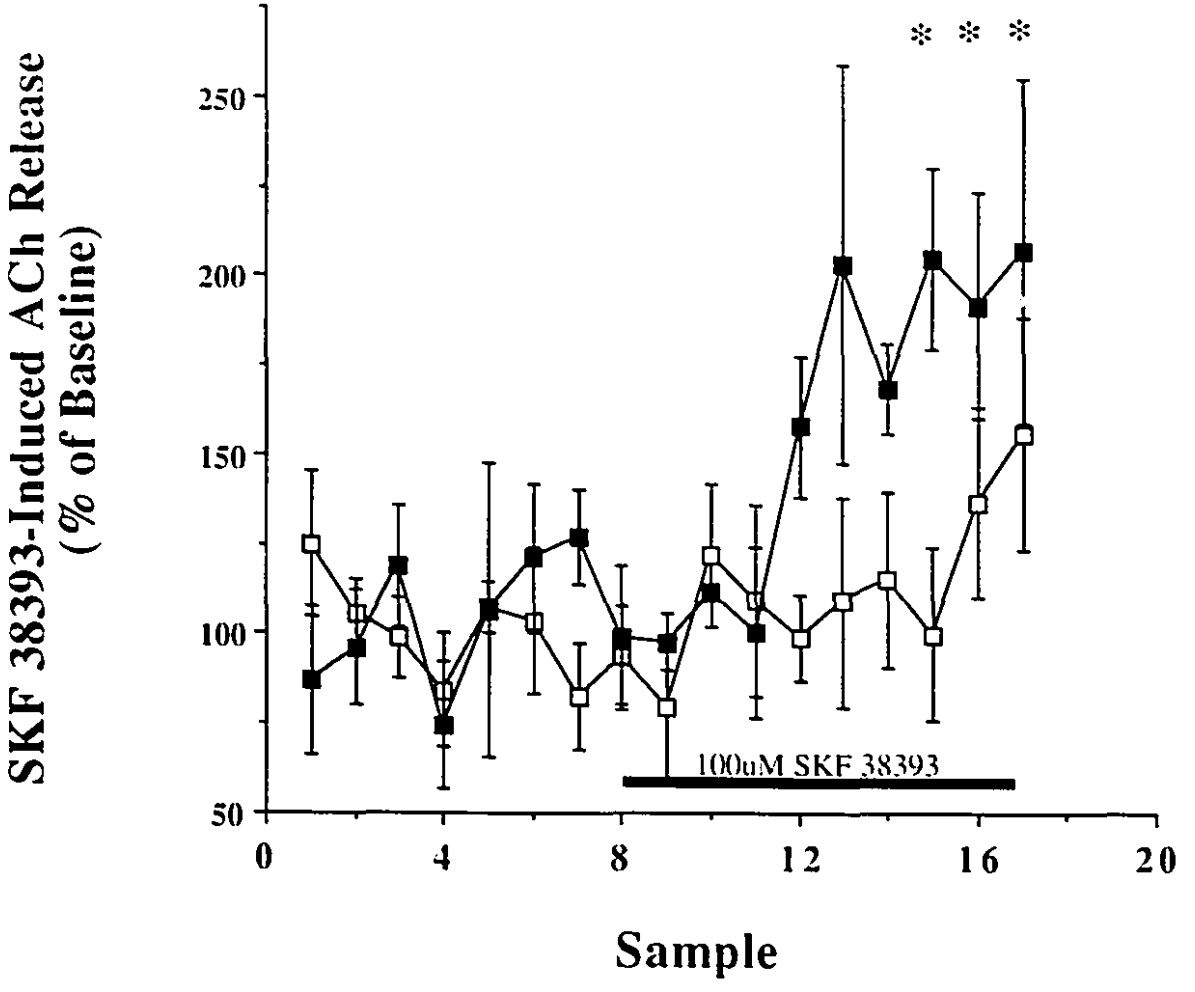
A


Basal ACh Release (pmol/25min)
4
3
2
1
0
Ringer
Anti-D1
Anti-D5
AntiD5Scr
Treatment

B


SKF 38393-Induced ACh Release
(% Baseline)
300
250
200
150
100
50
0
4
8
12
16
20
100uM SKF 38393
*
*
Sample

C


SKF 38393-Induced ACh Release
(% of Baseline)
250
200
150
100
50
0
4
8
12
16
20
Sample
100uM SKF 38393
* * *

DISCUSSION

Dopamine modulates hippocampal cholinergic activity (Day and Fibiger, 1994; Imperato et al., 1993). Specifically, local stimulation of D1-like receptors enhances hippocampal ACh release (Hersi et al., 1995a). On the basis of structural, biochemical and pharmacological similarities, two receptors members belong to this family of DA receptors, namely D1 (D1a) and D5 (D1b) (Civelli et al., 1993; Gingrich and Caron 1993). Unfortunately, no drug is currently available which can clearly distinguish between these two receptors. Therefore it is difficult to ascribe a particular biological function to either of the two subtypes. This is specially true for brain structures enriched with both the D1 and D5 receptors. The hippocampal formation is such a structure where the mRNA as well as the proteins for D1 and D5 receptors have been localized. (Bergson et al., 1995; Fremeau et al., 1991; Sunahara et al., 1991; Tiberi et al., 1991; Weiner et al., 1991). A novel approach to discriminate among closely related receptor subtypes is the use of antisense oligonucleotides targeted to specific mRNAs (Whitesell et al., 1993). In the present study, we utilized a combined antisense-in vivo dialysis approach to ascertain which of the two D1-like receptors is involved in stimulating hippocampal ACh release. Infusion of phosphorothioate-modified oligodeoxynucleotides specific to either the D1 or D5 receptor subtype reduced hippocampal [^{3}H] SCH 23390/D1-like receptor binding. However, only the D5 receptor antisense was able to block SKF 38393-induced increase in hippocampal ACh release.

[^{3}H] SCH 23390/D1-like binding in the rat hippocampal formation is primarily found in the dentate gyrus (Dawson et al., 1986; Grilli et al., 1988; Hersi et al., 1995a; Tiberi et al., 1991). Both D1 and D5 receptor antisense oligonucleotides reduced [^{3}H] SCH 23390 binding by about 30% (Fig 1). Given the fact that the radioligand used here recognizes both receptor subtypes equally well (Tiberi et al.,

1991), this decrease is likely higher. Moreover, it appears that these two antisenses affect different population of D1-like receptors, given their differential effect on hippocampal cholinergic function (see below). However, additional studies examining the effect of these oligonucleotides on the respective mRNAs of these two receptors as well as concomitant administration of the two antisenses will be necessary to substantiate this assertion.

Although the ventricular compartments link most parts of the brain, maximal alteration of various receptors following antisense infusion has consistently been observed in areas adjacent to the ventricle of administration (Karle and Nielsen, 1995; Sakai et al., 1994; Weiss et al., 1993). This is likely due to the generation of a concentration gradient at the site of infusion, the surrounding tissues being exposed to higher levels of antisenses. Given our purpose we chose the third ventricle as infusion site. As expected, neither of the two antisense oligonucleotides had an effect on D1-like receptor binding in areas relatively distant to the ventricle such as the dorsal striatum (Fig 1). However, a trend toward a decrease in [3H] SCH 23390 binding was noted in the medial septum (Fig 1). The perikarya of the septohippocampal cholinergic innervation are found in this area. Therefore, it is possible that antisenses taken up by terminals adjacent to the site of infusion were retrogadely transported into the septal area.

Administration of the scrambled-sequence D5 antisense did not affect D1 receptor binding (Fig 1). Moreover, all the antisense treatments used here failed to decrease the levels of [^{3}H] raclopride/D2-like binding in the hippocampal formation. Taken together, these observations argue against the possibility that the infusion of antisenses have non-specific effects such as neuronal toxicity and overall inhibition of protein synthesis.

None of the antisense treatments utilized here had an effect on basal hippocampal ACh release (Fig 2a). In addition to excluding nonspecific toxicity induced by the antisense administration, this observation corroborates our previous finding that the dopaminergic regulation of hippocampal cholinergic activity is not tonic in nature (Hersi et al., 1995a). Only the D5 receptor antisense but not the D1 nor the scrambled D5 antisense treatments blocked SKF 38393-induced stimulation of ACh release in the hippocampus (Fig 2b and 2c). Thus, it appears that DA modulates hippocampal cholinergic terminal activity via the D5 receptor subtype. Additional experiments utilizing antisense sequences targeted against other portions of the D1 and D5 receptors are needed to categorically exclude the D1 receptor from this function.

The D5 receptor mRNA has been shown to possess a unique and restricted distribution compared to its D1 counterpart (Tiberi et al., 1991). Detectable levels of D5 receptor mRNA are found only in the lateral mammilary nuclei, the anterior pretectal nuclei and the hippocampal formation. On this basis, it has been postulated that this receptor might be involved in limbic function including learning and memory (Tiberi et al., 1991). Recently, it was reported that the D5 receptor protein might also be localized on cholinergic interneurons in the caudate nucleus and in the basal forebrain where cholinergic perikerya that innervate cortical areas are found (Bergson et al., 1995). It is well known that DA modulates cortical and hippocampal cholinergic activity by acting on D1-like receptors (Day and Fibiger, 1992, 1993 and 1994; Hersi et al., 1995a). Moreover, we have shown here that the receptor involved in this function in the hippocampus is likely of the D5 subtype. Given that central cholinergic innervation is considered as an integral component of the circuitry underlying mnemonic processes, it seems that a putative function of the D5 receptor subtype in the mammalian brain could be the regulation of memory. Moreover, it should now be feasible, using antisenses, to ascertain the receptor subtype involved in other D1-like

modulation of mnemonic processes such working memory in the prefrontal cortex (William and Goldman-Rakic, 1995) and LTP in the Schaffer collateral pathway of the hippocampus (Huang and Kandel, 1995) using similar approaches.

In summary, administration of specific antisense oligonucleotides directed against regions spanning the initiation codon of the D1 and D5 receptors reduced the levels of D1-like binding sites in the hippocampus. However, only the D5 antisense blocked the SKF 38393 induced stimulation of hippocampal ACh release. The behavioral consequences, in terms of learning and memory, of this novel D5 function are being investigated at the present time.

ACKNOWLEDGMENT

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CHAPTER 4
Hippocampal DA-ACh Interactions and Spatial Memory

PREFACE TO CHAPTER 4

As described in the preceeding two chapters, stimulation of D1-like receptors enhances hippocampal acetylcholine release. In this chapter, we studied the behavioral significance of this effect, utilizing rats that become memory impaired as a function of age.

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Dopamine D1 Receptor Ligands Modulate Cognitive Performance and Hippocampal Acetylcholine Release in Memory Impaired Aged Rats

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Key Words: D1 receptor, Acetylcholine, Spatial Memory, Aging, Hippocampus, In vivo Dialysis,.

ABSTRACT

The possible modulation by D1 drugs of learning abilities of a population of aged memory impaired animals was investigated in the present study. The level of D1/[^{3}H]SCH 23390 receptors was first examined by quantitative autoradiography to ascertain if cognitive deficits seen in these animals could be related to alterations in the levels of these receptors. No significant differences in [^{3}H]SCH 23390 binding were observed in any of the brain areas examined between young, and aged memory-unimpaired (AU) and aged memory-impaired (AI) animals. However, the cognitive deficits of the AI rats were modulated by D1 drugs. The D1 agonists SKF 38393 and SKF 81297 (3.0mg/kg i.p.) significantly reduced the latency period to find a hidden platform in the Morris Water Maze, reflecting improved cognitive functions, while the D1 antagonist SCH 23390 (0.05mg/kg i.p.) had no overall significant effect. Moreover, the D1 agonist SKF 38393 increased whereas the antagonist inhibited *in vivo* hippocampal ACh release.

Taken together, these results suggest that functional hippocampal ACh-DA interactions exist in aged memory impaired rats. More importantly, the cognitive deficits seen in the AI rats can be attenuated by stimulation of D1 receptors, hence suggesting an alternate approach to alleviate the cognitive deficits seen in the aged brain.

INTRODUCTION

A substantial body of evidence has implicated the central cholinergic system as an integral component of the neural circuitry associated with learning and memory (for recent reviews, see 14,42 and 49). For example, the systemic administration of muscarinic cholinergic receptor antagonists disrupts the performance of animals in a variety of tasks such as operant matching / nonmatching to sample[5,46] and spatial memory tasks including the radial arm maze [6,41,54] and Morris water Maze [9,55,57]. In addition, lesion studies have, in general, highlighted the importance of the cholinergic system in learning and memory. For instance, lesions of the cholinergic neurons of the nucleus basalis magnocellularis or the medial septum / diagonal band of Broca disrupt the performance in memory tasks sensitive to cholinergic blockade [4,18,38,56]. Besides acetylcholine (ACh), however, several other neurotransmitter systems likely play a role in learning and memory (for review see 14). One such neurotransmitter is dopamine (DA).

There is a well characterized reciprocal interaction between acetylcholine and dopamine in the control of normal motor activity at the level of the basal ganglia (for recent review see 50). More recently, it has become evident that complex interactions also exist between ACh and DA in the normal functioning of memory and cognition. Mnemonic deficits brought on by denervation or pharmacological blockade of central cholinergic systems can be modulated by dopaminergic drugs. For example, memory deficits induced by muscarinic receptor antagonists are attenuated by DA antagonists[32] acting on D1 receptors[28] whereas cognitive impairments resulting from nicotinic receptor blockade are exacerbated by D2 receptor antagonists[33,34]. Conversely, impairments in avoidance learning[1], operant responding[29] and conditioned avoidance[2] caused by the systemic administration of the nonspecific DA receptor antagonist

haloperidol are attenuated by the muscarinic antagonist scopolamine, the latter at doses that do not have effects by themselves.

Lately, however, questions have been raised about the validity of equating mnemonic deficits induced by acute pharmacological manipulations or discrete mechanical or chemical lesions with those occurring during the normal aging process[16,22]. For instance, the cholinergic deficits observed in aging occur within a context of age and/ or disease-related pathologies in other neurotransmitter systems. Even more relevant here is the observation that the well characterized reciprocal antagonistic interaction between ACh and DA in the basal ganglia is altered in senescent animals[27]. For example, the dopaminergic agonist apomorphine inhibited K+-evoked ACh release from striatal slices of young and middle-aged, but not old animals. Similarly, the muscarinic agonists, oxotremorine and carbachol, inhibited DA-stimulated adenylate cyclase cell preparations obtained from young and middle aged, but not from old rats[27].

Therefore, the purported ACh/DA interactions in cognitive processes seen in young animals must be reinvestigated in a model that better approximates the conditions of the aged brain. Memory loss in the aged human population varies widely from the so-called successful aging whereby an individual performs most memory tasks as well as young cohorts to the severe deficits seen, for example, in Alzheimer's Disease (AD). Analogously, about 30% of aged (24-25 mo.) Long Evans rats exhibit impairments in spatial memory as evidenced by a poor performance in tasks such as the Morris Water Maze[19,25,45]. It is believed that these aged memory-impaired rats better represent the global dynamics of cognitive alterations occurring in aging in certain sub-groups. The neuroanatomical structure most implicated in spatial memory is thought to be the hippocampal formation[8,36]. Interestingly, the majority of DA receptors in the hippocampus are of the D1 (d1/d5) subtype[10,40]. Accordingly, in this study, we

examined (i) the level of D1 receptor as a function of age and cognitive status, (ii) whether the memory deficits that characterize the AI animals can be modulated with D1 drugs and (iii) the effect of D1 receptor agonist and antagonist on *in vivo* hippocampal ACh release.

EXPERIMENTAL PROCEDURES

Animals and Materials

Male Long Evans rats (3-6 mo. old) were obtained from Charles River Canada (St. Constant, Quebec, Canada) and aged in our animal facilities. The animals were maintained on a 12 hr light-dark cycle (light on at 7:00 a.m.) in temperature and humidity controlled rooms. Animals were fed standard laboratory chow and had access to tap water ad libitum. Any animals showing signs of respiratory ailment were removed from the study. Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care (CCAC).

The concentric dialysis probes (cut off 20,000 daltons and a surface of 2mm in length and 0.5mm in diameter) were from BAS (West Lafayette, IN, U.S.A.) and the transverse probes were made from AN69 Hospal fibers (molecular weight cut off < 60,000, i.d. = 220μm, o.d. = 310μm). Physostigmine sulfate was from Sigma Chemical Co.(St. Louis, Mo, U.S.A) while SCH23390 HCl, SKF 81297 HBr and SKF38393 HCl were obtained from RBI (Natick, MA, U.S.A.). [^{3}H] SCH23390 (80.7Ci/mmol), 3H-Hyper-films and microscale standards were purchased from Amersham Canada (Oakville, Ontario, Canada). The deuterated variant of ACh, [$^2H_4$] ACh bromide [$(CH_3)_3NBrCD_2CD_2OC$-(0)CH_3], which was used as internal standard for ACh determination was obtained from Merck, Sharp and Dohme Isotopes (Montreal, Quebec, Canada). Developer(D-19) and fixer (Rapid Fix) were obtained from Kodak Chemical Inc. (Montreal Quebec, Canada). All other reagents were of HPLC or GC-MS grade and purchased from either Fisher Scientific Co. (Montreal, Quebec, Canada) or Aldrich Chemicals (Chicago, IL, U.S.A.).

Behavioral Screening

Young and 24-25 mo old aged rats (n=132) were evaluated for their learning capacities using the well established Morris maze task [19,37,45] and as previously described by our group[25,48]. Briefly, animals are required to find a submerged platform (2cm) in a pool (1.6-m dia.) of water that has been made opaque by the addition of powdered skim milk. The animals solve the task using only distal spatial cues provided in the testing room. Rats were given 15 trials over 5 successive days (3 trials per day; maximum trial duration of 2 minutes; inter-trial interval about 20 minutes) with the platform submerged. At the end of this five day period, the aged animals are categorized as age-impaired (AI) or aged-unimpaired (AU) based on their performance with respect to the young cohorts. As reported earlier[25]aged animals demonstrate great individual differences in their learning capabilities (Fig1). Except for those used in D1 drug behavioral testing, all animals were immediately run on probe trials on the 5th day with the platform elevated 2cm above the surface of the water. These trials were conducted to ensure that the impairments were not related to any visual defects or to the animal's inability to perform the motor demands of the task[19,37,45]. For D1 drug testing, the animals catagorized as AI were subdivided into groups of overall equal performance and given three trials a day for three days starting at day 6 preceded by i.p. injection of the drug of interest (see legend to Table 1 for further details). These animals were given probe trials immediately following termination of testing on the last day. In these probe trials, first the platform was altogether removed to determine the length of time that the animal spends in the training quadrant for a trial length of 30 seconds. Then the platform was reinstated and also made visible to the animal to check for any visual or motor deficits. The drug employed here were chosen based on earlier studies that showed the effectiveness of such doses in similar behavioral paradigms[35].

D1 Receptor Autoradiography

The status of D1 ([^{3}H] SCH 23390) receptors in AI, AU and young animals was assessed using a method described in detail elsewhere[12]. In brief, 20µm brain sections were incubated for 60 min at room temperature in 50mM Tris HCl buffer (pH 7.4) containing 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$ and 1.0nM [^{3}H] SCH 23390. Consecutive sections were also incubated in the presence of 1µM SCH 23390 to ascertain the specificity of the labelling. The sections were then rinsed five times (2 min each) in fresh ice cold buffer. The buffer salts were removed by a rapid dip in ice cold distilled water and the sections air dried. Autoradiograms were generated by apposing the sections alongside with tritium standards to tritium sensitive films for four weeks. The films were then developed as described before[47] and [^{3}H]SCH 23390 specific labelling quantified (fmol/mg tissue wet weight) using computer assisted microdensitometric image analysis system (MCID System, Imaging Research Inc., St-Catherines, Ontario, Canada). Anatomical areas were identified and named according to Paxinos and Watson[44]. Significant differences between experimental groups were determined by a one way analysis of variance (ANOVA); $p<0.05$ being considered significant.

Probe implantation and hippocampal *in vivo* dialysis

AI rats were anesthetized with nembutal (50mg/kg, i.p.). Guide cannulas (BAS, West Lafayette, IN, U. S. A.) were stereotaxically implanted in the dorsal hippocampus as described elsewhere[48] . The animals were individually housed and allowed to recover from surgery for 2-3 days prior to their use in the *in vivo* dialysis experiments. 16-20 hours before dialysis, a vertical probe was implanted via the guide cannula so as to give a final depth of 3mm below the dura. Young (5 months old) and some AI rats were implanted with transverse probes[9b] in the dorsal hippocampus as described elsewhere[23]. Each animal was dialysed only once. At the beginning of each dialysis experiment, animals were placed in lidless cages and connected to a BAS microliter syringe pump in a

manner as to allow them to freely move in the cages. The probes were perfused for a one hour wash out period at a flow rate of 2.34 ul/min with an Ungerstedt-Ringer solution (125mM NaCl, 3.0mM KCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 1.5mM NaH_2PO_4 pH 7.4) containing 75μM physostigmine[48]. Twenty minute dialysate fractions were collected into a 1ml glass vials containing 46 ul of 0.1N HCl and 50 pmoles of deuterated ACh as internal standard. The samples were frozen immediately and stored at -80°C until assayed by gas chromatography/mass spectroscopy (GC-MS). SKF 38393(3.0mg/ml) and SCH23390 (0.05mg/ml) were dissolved in 0.9% saline solution and injected i.p. in a volume of 1ml/kg. Following most experiments, probe location was verified by standard histological examination of the brain.

GC-MS analysis of ACh

ACh content of the dialysate fractions was determined by GC-MS as described by Marien and Richard[31]. Briefly, frozen samples were lyophilized overnight, reconstituted in 250ul acetonitrile, capped, heated at 80°C for 30 minutes and dried under a gentle stream of nitrogen gas. Quaternary amines present in the samples were demethylated by adding 250ul sodium benzene thiolate solution (160mg in 18ml of redistilled methyl ethyl ketone and 35ul of glacial acetic acid) under a flow of nitrogen and reacting at 80°C for 45min. Samples were then extracted into 35ul of citric acid and washed twice with 250ul of pentane. Finally the samples were extracted into 80ul of ethyl acetate and concentrated down to 3-5ul volume before being injected in to the GC-MS (Hewlett-Packard 5987b). The amount of endogenous ACh in each dialysate was calculated from the peak area ratio of endogenous vs deuterated internal standard[26]. Calculations were not corrected for the recovery of ACh by each dialysis probe. Sample ACh content was expressed as a percentage of average baseline (six sample collections preceding drug administration). Significant differences between experimental groups were determined by a one way analysis of variance (ANOVA). $p<0.05$ being considered significant.

RESULTS

Behavioral Screening of Aged Animals

Cognitive performances of young (6mo ; n=10) and aged (24-25 mo ; n=132) male Long Evans rats in the Morris Water Maze were examined. An animal was designated as aged-impaired (AI) if the latency to find the platform on each of the test days 2-5 was >2 SD higher than the mean of the young animals. Aged-unimpaired (AU) animals showed latencies over this period that were <0.5 SD higher than the mean for the young animals. A significant portion (33%) of aged animals from our colony demonstrates significantly impaired performance whereas 27% of the aged animals were clearly unimpaired. An analysis of variance (with Scheffe post hoc test) shows that, as a group, the AI differ significantly from both the young and AU animals on test days 2-5 (Fig1). No differences were observed between young and AU animals. The performance of these animals has also been examined in terms of the distance travelled before locating and climbing onto the platform. An analysis of these data shows the same pattern of differences as the latency figures (data not shown). When these same animals are provided an opportunity to swim towards a platform that has been raised above the level of the water (visually-cued condition), there are no differences in latency or distance measures (data not shown) indicating that the differences between groups are not related to swimming abilities (Issa et al., 1990).

Dopaminergic D1 / [^{3}H] SCH23390 receptors and aging

No clear differences in D1/[^{3}H] SCH23390 binding densities were observed between the AI and AU groups in any of the brain regions examined (Fig 2). Similarly, aging per se does not seem to alter D1/[3H]SCH23390 binding site levels as evidenced by comparable binding densities between the aged and young cohorts (Fig 2).

Behavioral effects of SKF 38393, SKF 81297 and SCH 23390 in the aged impaired rats

Administration of the D1 agonists SKF 38393 and SKF 81297 (3.0mg/kg i.p) to AI animals fifteen minutes before trial resulted in a significant reduction (44% and 51%, respectively; $p<0.05$) in the latency to find the submerged platform compared to saline treated AI controls (Table 1). The D1 antagonist SCH 23390 (0.05mg/kg i.p.) induced an average 33% increase in latency over the three days of testing (Table 1). This overall increase failed to reach statistical significance. However, when performance trend across the three days of testing was examined for the antagonist, a worsening of the deficit of the AI animals which reaches statistical significance on the last day of testing is observed (Table 1). In probe trials wherein the platform was removed and the percentage of time spent in the training quadrant was examined, no significant differences were observed between groups (data not shown). There were no apparent differences in swim speed between experimental groups following the administration of either the D1 agonists or the antagonist (data not shown). Moreover, during probe trials wherein the submerged platform was made visible to the animals, there were no differences in latency to find the platform following drug administration (data not shown).

Effect of SKF 38393 and SCH 23390 on hippocampal ACh release

Administration of the selective D1 agonist SKF 38393 (3.0mg/kg i.p.) increased hippocampal ACh release by about two fold over baseline in both young and AI rats (Fig 3A, 3B). The maximal increase in ACh release was observed approximately 40min after the administration of the D1 agonist. On the other hand, the D1 antagonist SCH 23390 (0.05mg/kg i.p.) transiently inhibited ACh release in young animals whereas in AI rats this inhibition persisted and reached statistical significance by about two hours following drug administration (Fig3A, 3B).

Fig 1. Behavioral Screening of Aged Animals: Cognitive performances of male young (6mo. n=10) and aged (24-25 mo. n=132) Long Evans rats in the Morris Water Maze were examined as described in Methods and Results sections. A significant portion (33.2%) of aged animals from our colony demonstrates significantly impaired performance whereas 27.5% of the aged animals show clearly unimpaired performance. $^{*}p<0.01$ compared to Y animals. AI, □ ; AU, ■ ; Young, ▼ .

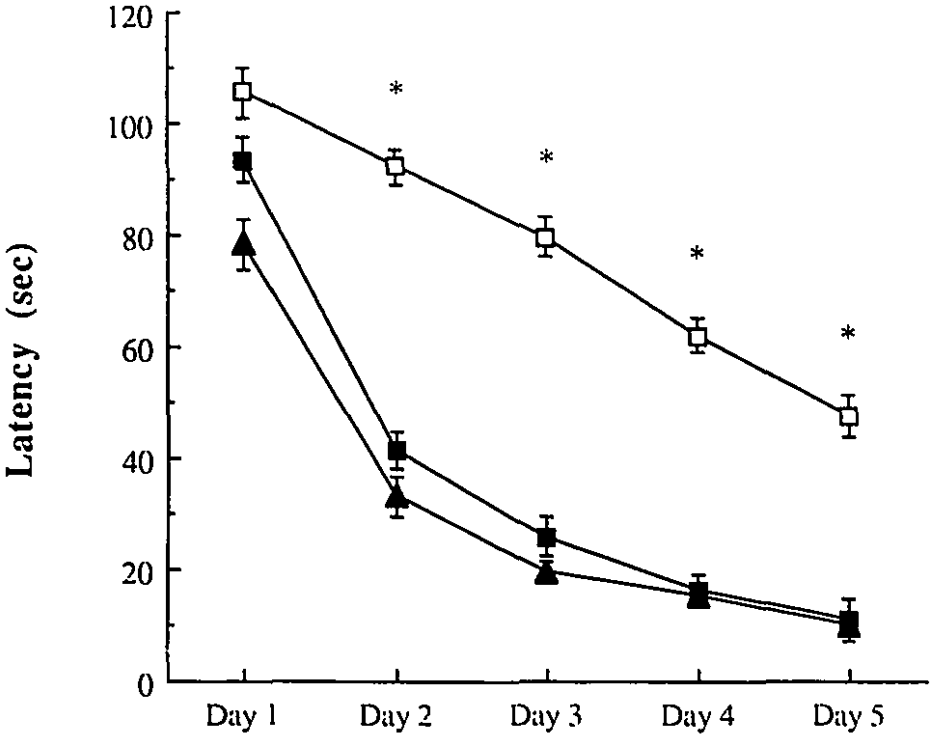

Latency (sec)
120
100
80
60
40
20
0
Day 1
Day 2
Day 3
Day 4
Day 5
*
*
*
*

Fig 2. Effect of aging and spatial memory impairment on D1 receptor binding levels: Sections from AI, AU and young animals were incubated with 1.0nM ^{3}H SCH 23390 as described in the Materials and Methods section. The histograms represent specific labelling obtained by subtracting nonspecific from total binding. Data represent mean ± S.E.M. from six animals per group. No significant difference was observed in [^{3}H]SCH23390 binding in any of the regions studied. DG, dentate gyrus of dorsal hippocampus; FCTX, frontal cortex; HIPP, CA1/CA2/CA3 subfields of dorsal hippocampus; NACC, nucleus accumbens; OT, olfactory tract; PCTX, Parietal Cortex; STR, Striatum and TCTX, temporal cortex. AI, □ ; AU, ▨ ; Young, ■ .

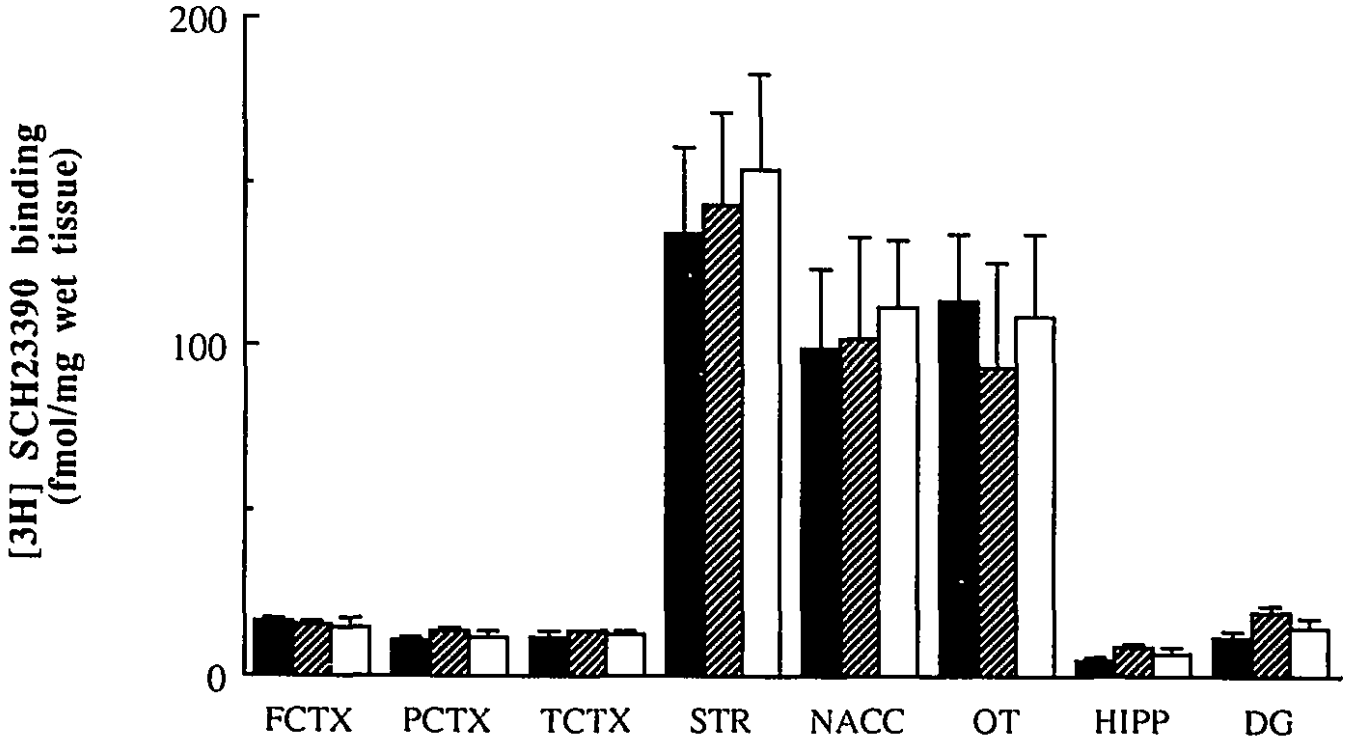
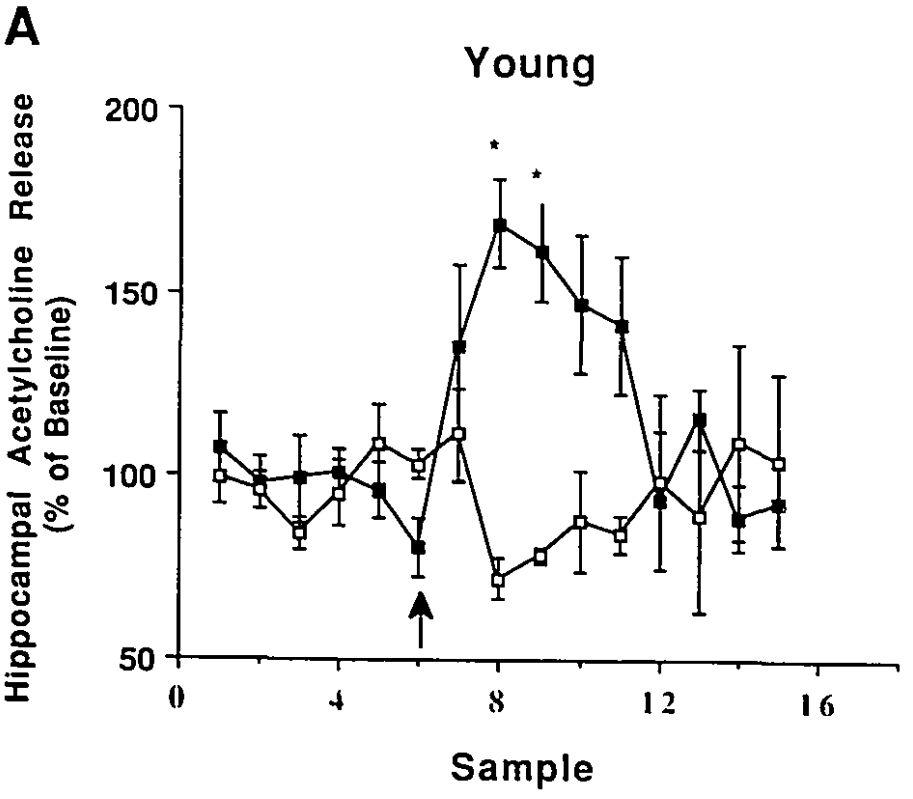

[3H] SCH23390 binding
(fmol/mg wet tissue)
200
100
0
FCTX
PCTX
TCTX
STR
NACC
OT
HIPP
DG

Fig 3. Effect of D1 receptor agonist, SKF 38393, and antagonist, SCH 23390, on in vivo hippocampal ACh release of Young and AI rats: Data represent group mean + S.E.M. SKF 38393 (n=4 for AI and n=5 for young); SCH 23390 (n=5 for AI and n=4 for young). 0.9% Saline solution containing either SKF 38393 (3.0mg/kg ■) or SCH 23390 (0.05mg/kg □) were injected i.p. following baseline collection (as indicated by the arrow). Dialysate ACh content, measured by GC/MS, is expressed as a percent of baseline. Baseline is the average of six samples preceding drug application. *$p<0.05$: significantly different from baseline.


A
Young
Hippocampal Acetylcholine Release
(% of Baseline)
200
150
100
50
0
4
8
12
16
Sample

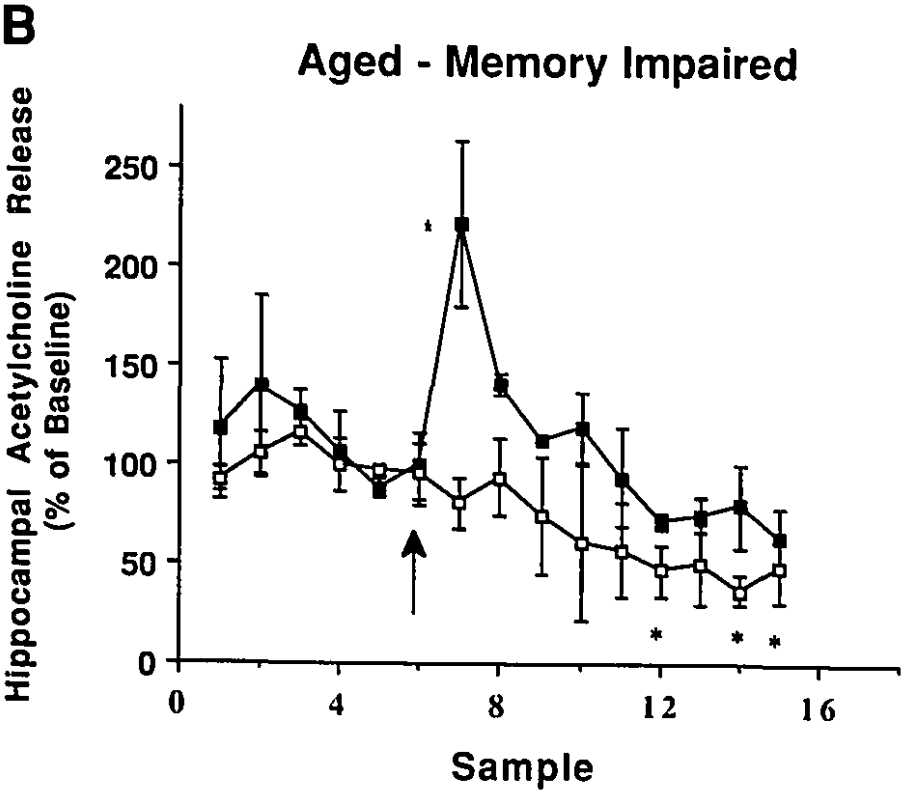

B
Aged - Memory Impaired
Hippocampal Acetylcholine Release
(% of Baseline)
250
200
150
100
50
0
0
4
8
12
16
Sample

Table 1 . Effect of SKF 38393, SKF 81297 and SCH 23390 on latency period/learning abilities of AI animals in the Morris Water Maze

	Latency (sec)			
Group	**Day1**	**Day2**	**Day3**	**Overall**
Saline	43.3 ± 11.06	26.58 ± 4.16	24.8 ± 11.06	33.20 ± 5.7
SKF 38393	23.07 ± 4.01	20.64 ± 5.26	11.49 ± 3.17	18.80 ± 2.50*
SKF81297	24.42 ± 11.20	11.28 ± 3.76	10.93 ± 2.10	16.09 ± 4.21*
SCH 23390	44.7 ± 10.73	36.78 ± 8.46	48.87 ± 10.85*	44.40 ± 5.80

Animals that were designated as AI as described in Fig 1 were utilized. These AI rats were injected (i.p.) either SKF 38393 (3.0mg/kg), SKF 81297(3.0mg/kg), SCH 23390 (0.05mg/kg) or vehicle (0.9% saline) 15min prior to the first testing of the day. Three trials separated by ten minutes rest period were administered per animal. Each animal underwent a total of nine trials over a three day period. Data represent group mean ± S.E.M. of five different animals except for SKF 81297 group where n=3. *$p<0.05$.

DISCUSSION

Various studies using pharmacological manipulations have suggested the existence of complex interactions between ACh and DA in learning and memory processes. However, questions have been raised against equating memory deficits induced by pharmacological manipulations with those occurring during normal aging and in disease states. In our studies, we sought to address this point by examining ACh/DA interactions in the AI rat model. The majority of DA receptors in the hippocampus, a structure most implicated in learning and memory, are of the D1 (d1/d5) subtype[10,40]. Therefore, we examined the status of brain D1 receptor levels in AI *vs* AU, and in young animals. No apparent differences in the densities of D1 binding sites were observed in any of the brain areas examined between AI and AU animals. Furthermore, no significant differences in hippocampal D1 binding levels were observed between the young and aged cohorts. However, the D1 agonists SKF 38393 and SKF 81297 improved the cognitive abilities of the AI rats in the Morris Maze task. D1 drugs also modulated hippocampal ACh release, thus suggesting a possible mechanism for the observed behavioral effects.

Numerous studies have examined the status of various neurotransmitters in AI animals by monitoring changes in receptor levels. The most consistent finding appears to be that of rather normal densities. For example, the present consensus is that the total number of muscarinic receptors does not change in AI compared to AU and young animals[13,30,51] but also see [7,20], although a specific subtype may be altered[3]. In the present study, we report that levels of dorsal hippocampal D1 receptors are apparently unchanged during aging and in the AI rats. This finding also suggests that endogenous DA levels are not altered in these animals. In fact, it has been shown that neither the levels of DA nor those of its metabolites are altered in AI animals[20]. Nevertheless, the maintenance of normal receptor densities does not necessarily mean that these receptors

are functionally intact. For instance, the efficiency of receptor transduction mechanisms could be altered with age, and in various disease processes[17,39,43,52]. In fact, although cortical D1 receptor densities are unchanged in AD, a reduction in the number of the high affinity agonist state of these receptors has been reported[15]. The functional significance of this finding remains to be established.

The D1 agonists SKF 38393 and SKF 81297 significantly improved the cognitive performance of AI animals in the Morris Water Maze task. On the other hand, the antagonist SCH 23390 had no overall significant effect on the memory deficits of these animals, although a worsening of the performance trend of the AI animals across days was observed. In order to demonstrate a statistically significant overall effect of the antagonist at the dose used, an increase in the latency between drug administration and behavioral testing would likely be required to coincide with the time of likely inhibition (2hrs) of hippocampal ACh release (Fig 3B). Alternatively, there could be a ceiling phenomenon in the magnitude of the deficit that can be demonstrated in this task. In addition to measuring spatial learning ability, the Morris Maze task also involves both visual and motor components[19,37,45]. However, at the doses used in the present study, neither D1 drugs had an effect on visually cued performance as evidenced by probe trials. Similarly motor side effects were absent as the swim speeds between drug-treated and saline-treated AI animals were comparable. Thus, it seems likely that the behavioral effects of the D1 drugs in the AI rats resulted from the modulation of cognitive abilities, although effects on attentional and/or motivational mechanisms cannot be excluded at this time.

There is a series of studies, most notably by Levin and collaborators (see ref 28 for a review), that strongly suggest the existence of interactions between ACh and DA in learning and memory processes in young animals. With regards to D1 receptors, these

investigators found that scopolamine-induced radial-arm maze choice accuracy deficits are reversible by the co-administration of the D1 antagonist SCH23390[28,32] . In contrast, we report here that spatial memory deficits of the AI rat are reversible by the D1 agonists SKF 38393 and SKF 81297. One possible explanation for this apparent discrepancy relates to the type of memory being examined by the tasks used in these respective studies. Although both radial arm maze and the Morris Water Maze evaluate spatial working memory, the former is of the ``effortful`` type as compared to the later which examines memory of rather ``automatic`` nature[8,21,36]. Therefore, it is quite conceivable that these two types of mnemonic processes are subserved by distinct neuroanatomical structures or pathways that might be differentially sensitive to dopaminergic drugs. Another more likely possibility relates to the nature of the memory deficit being investigated. For instance, concerns have been raised against equating memory deficits induced by pharmacological manipulations with those occurring as the result of normal aging[16]. For example, whereas muscarinic antagonists like scopolamine produce an acute and short lasting blockade of cholinergic receptors, aging is a slowly progressing, irreversible process that involves, in addition to changes in pre and postsynaptic cholinergic functions[20,48], substantial alterations in various other neurotransmitter systems[16]. Accordingly, caution must be exercised when generalizing from data based on acute pharmacological manipulation in young and healthy animals to cognitive deficits seen in aging.

A number of possible mechanisms could explain the behavioral effects of the D1 drugs in the AI animals. Most prominent among these, however, is the septo-hippocampal cholinergic projection. This pathway is thought to be a crucial component of the circuitry involved in the type of memory examined by the Morris Water Maze[8,36]. Interestingly, the modulation of hippocampal ACh release by the D1 drugs in the AI animals (Fig4B) mirrored the effects of these same drugs on spatial learning abilities (Table1). This

suggests that hippocampal ACh activity may underlie, at least in part, the changes in the cognitive performance of these animals. A possible site of action of the D1 drugs in modulating hippocampal ACh release is the septal nuclei. It is well established that ventral tegmental area (VTA) dopaminergic perikarya send projections to the lateral septum[53]. These dopaminergic terminals are thought to inhibit, via GABAergic interneurons, the activity of medial septal neurons which give rise to the septo-hippocampal cholinergic pathway[58]. However, if indeed this was the case effects opposite to those observed here would have been expected; the D1 agonist, via its stimulation of the release of GABA, indirectly inhibiting hippocampal ACh release. A more likely locus for the interaction between D1 receptors and hippocampai ACh release is within the hippocampus. In fact, the existence of direct, albeit sparse, dopaminergic innervation in the hippocampus arising from the VTA and the substantia nigra has been established[53]. The hippocampal formation is also relatively enriched with D1 receptors and a proportion of these receptors is postulated to reside directly on dorsal hippocampal cholinergic terminals, where D1 receptors were shown to locally modulate ACh release[23]. Apart from the hippocampus, however, the frontal cortex is also likely involved in the performance of the Morris Water Maze task and interestingly D1 drugs have previously been shown to modulate cortical ACh release in a manner analogous to the hippocampus[10b,10c]. Therefore, in addition to the hippocampus, the frontral cortex could be a possible site for the behavioral effects of the D1 drugs utilized in our study. Moreover, the effect of these D1 drugs on other neurotransmitters remains to be investigated.

Finally, our findings on hippocampal ACh release in AI animals are in general very similar to those seen in young animals[11,24,this study]. Thus, unlike the reciprocal interactions between striatal ACh and DA innervations which are reportedly affected in

aging[27], hippocampal ACh/DA D1 receptor interactions appear to be generally maintained as the animal ages and becomes cognitively impaired.

CONCLUSIONS

The spatial learning deficit seen in the AI rats was significantly attenuated by the D1 agonists SKF 38393 and SKF 81297. Our data also suggest that an underlying mechanism could relate to the modulation of hippocampal ACh release. In light of the relatively limited success of cholinesterase inhibitor-based therapies in age associated memory deficits, an interesting alternate possibility could be the use of D1 agonists.

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CHAPTER 5
Hippocampal Distribution of D1-like Receptors

PREFACE TO CHAPTER 5

The rationale of the present thesis stemmed from data obtained from human post mortem tissues. On the other hand, the work delineated in the preceding three chapters was conducted in rats. Therefore, in an attempt to determine the potential applicability of the rodent data to other species including humans, the existence and distribution of the dopamine receptors implicated in hippocampal acetylcholine release were examined in monkey and human brains as an index that these receptors might be serving similar functions in primates as in the rat.

Comparative distribution of D1-like receptors in the hippocampal formation of rat, monkey and human brains

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Key Words Receptor Autoradiography; Hippocampus; D1 receptors; Monkey; Human; Rat.

ABSTRACT

The distributional profile of [^{3}H] SCH 23390/dopamine D1-like receptors in the hippocampal formation of the rat, monkey and human brains was examined by quantitative receptor autoradiography. D1-like receptors were found in the hippocampus of all three species. In the rat, the majority of these receptors were located in the molecular layer of the dentate gyrus whereas the Ammon's horn subfields exhibited lower levels of D1-like binding sites. Interestingly, this distribution profile was reversed in the primate hippocampus. In both the monkey and human hippocampus, the highest D1-like receptor density was seen in the CA1 followed by the CA3 subfields, with the dentate gyrus containing lower levels. These results demonstrate that, in contrast to the situation seen in the basal ganglia, D1-like receptor distribution in the hippocampal formation may not be evolutionary conserved.

INTRODUCTION

Dopamine (DA) neurons in the ventral tegmental area project to a number of cortical and subcortical structures (Bjorklund and Lindvall, 1984; Lindvall and Bjorklund, 1983). These dopaminergic projections which are referred to as the mesocorticolimbic system have been implicated in a number of brain functions such as cognition, motivation and reward (for reviews see Bozarth, 1989; Decker and McGaugh, 1991; Phillips et al., 1989; Wise and Hoffman, 1992). Mesocorticolimbic dysfunction is also thought to contribute to a number of psychopathological conditions including schizophrenia and affective disorders (Willner et al., 1989).

As a result, the characteristics of this dopaminergic innervation have been studied in a variety of species including rat, monkey and human (Fallon and Loughlin, 1987; Goldman-Rakic et al., 1992). For example, DA modulates long term potentiation in the Schaffer collateral pathway of the rat hippocampus via D1-like receptors (Huang and Kandel, 1995). Moreover, we recently reported that D1-like receptors modulate hippocampal acetylcholine (ACh) release in the rat and that this action may impact on cognitive abilities of the aged rat (Hersi et al., 1995a and 1995b). An antisense approach revealed the likely involvement of the D5 subtype of the D1-like receptor family in these effects (Hersi et al., 1996). However, while it is true that basic commonalties of DA mesocorticolimbic innervation exist across species, there are also important differences. For example, the neocortical dopamine system appears to be more extensive in the primate than in the rat brain (Roth and Elsworth, 1995; Roth et al., 1987). A recent report also suggests the presence of differential distribution of dopamine receptor subtypes in the primate cortex compared to that of the rat (Huntley et al., 1992).

In order to establish if the data on the effects of D1-like receptors on hippocampal ACh release in the rat may be applicable to other species including man, we investigated the comparative distribution of [^{3}H] SCH 23390/D1-like receptor in the hippocampal formation of the rat, monkey and man using quantitative receptor autoradiography.

MATERIALS AND METHODS

Materials

Male Long Evans rats (250-350g) obtained from Charles River Canada (St. Constant, Quebec, Canada) were maintained on a 12 hr light-dark cycle (light on at 7:00 a.m.) in temperature and humidity controlled rooms for at least 3-4 days prior to sacrifice. Animals were fed standard laboratory chow and had access to tap water ad libitum. Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care (CCAC). Brains of monkeys (*Callithrix Jacchus*) were kindly provided by Sanofi (France). Blocks of normal control human brains from four individuals (3 males, 1 female; 74±3 years; neuropathological examination revealed no evidence of neurological disorders, e.g. cell loss, plaques, tangles, excessive gliosis) of approximately $3cm^3$ containing various regions were obtained from the Douglas Hospital Research Centre Brain Bank. Post mortem intervals ranged from 6 to 26 hrs. These brain blocks were frozen in isopentane, cooled on dry-ice and stored at -80°C as previously described (Quirion et al, 1987).

[^{3}H] SCH23390 (80.7Ci/mmol), ^{3}H-Hyperfilms and microscale standards were purchased from Amersham Canada (Oakville, Ontario, Canada). SCH 23390 HCl was obtained from RBI (Watick, MA, U.S.A.). Films, developer (D-19) and fixer (Rapid Fix) were obtained from Kodak Chemical Inc. (Montreal Quebec, Canada). All other reagents and chemicals were of HPLC or GC-MS grade and purchased from either Fisher Scientific Co. (Montreal, Quebec, Canada) or Aldrich Chemicals (Chicago, IL, U.S.A.).

Dopamine D1 Receptor Autoradiography

The distribution of dopaminergic D1-like receptors in the hippocampal formation and striatum of rat, monkey and human brains was assessed as described in detail elsewhere (Debonnel et al., 1990). In brief, following slicing at -17°C, 20μm sections mounted on gelatin coated slides were washed twice (15min each time) at room temperature in 50mM Tris HCl buffer (pH 7.4) containing 120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$. These sections were then incubated for 60min at room temperature in the buffer in the presence of 1.0nM [^{3}H] SCH 23390. Adjacent sections were incubated in the presence of the radioligand and 1μM SCH 23390 to determine the level of specific binding. The sections were then rinsed five times (2 min each) in fresh ice cold buffer. Buffer salts were removed by a rapid dip in ice cold distilled water and the sections rapidly air dried. Autoradiograms were generated by apposing the sections alongside with tritium standards to tritium sensitive films for 4-8 weeks. The films were then developed as described before (Quirion et al., 1981) and [^{3}H] SCH 23390 binding quantified (fmol/mg tissue, wet weight) using computer assisted microdensitometric image analysis system (MCID System, Imaging Research Inc., St-Catherines, Ontario, Canada). Anatomical areas were identified according to the Paxinos and Watson's (1982), DeArmond et al. (1989) and Gergen and Maclean (1962) atlases.

RESULTS AND DISCUSSION

The distribution of [^{3}H] SCH 23390/D1-like receptors in the hippocampal formation of the rat, monkey and human brains was examined by quantitative receptor autoradiography. Our results demonstrate that D1-like receptors are expressed in the hippocampal formation of all three species. However, differences in the distribution profile of these receptors are seen between the species.

The most extensive investigations examining the discrete localization of DA receptors have been carried out in the basal ganglia (for example, Richfield et al., 1987; Camps et al., 1990). Hence, in the present study, we examined [^{3}H] SCH 23390/D1-like binding sites in the caudate/dorsal striatum and used it as control. As reported in these earlier studies, the highest density of D1-like receptors in mammalian brain is found in basal ganglia structures such as the caudate and putamen (Table 1). Moreover, the distributional profile of D1-like receptors in these structures appears similar across species (Fig 1).

The hippocampal formation of the three species studied here also expressed D1-like receptors (Fig 1). Unlike in the basal ganglia structures, however, their pattern of distribution was species-dependent (Table 1). In the rat, the highest densities of D1-like receptors were seen in the molecular layer of the dentate gyrus, followed by the pyramidal cell layer of the CA3 and CA1 subfields. In contrast, in the monkey and human hippocampi, [^{3}H] SCH 23390/D1-like receptor levels were highest in the CA1 area (stratum oriens, pyramidale and radiatum) followed by the CA3 subfield (Fig 1). Lower amounts were seen in the molecular layer of the dentate gyrus. Therefore, it appears that the distributional profile of D1-like receptors is evolutionarily conserved in the basal ganglia but not in the hippocampal formation (Camps et al., 1990).

The D1-like family of DA receptors consists of two members, namely D1 and D5 (Jackson and Westlind-Danielsson, 1994). The mRNA of both these receptors were shown to be expressed in the hippocampal formation (for recent reviews see Mansour and Watson, 1995; Meodor-Woodruff et al., 1994a and 1994b). In the rat, the D1 receptor mRNA is found primarily in the granular cell layer of the dentate gyrus with no detectable levels in other areas of the hippocampal formation. In contrast, the D5 receptor mRNA is seen throughout the rat hippocampus, both in the granular cell layer of the dentate gyrus and the pyramidal cell layer of the Ammon's horn. In the human hippocampus, D1 receptor mRNA is expressed in the subiculum and the pyramidal cell layer of the CA1 subfield (Meador-Woodruff et al., 1994a). Similar to the rat, D5 receptor messages in the human hippocampus are reportedly seen both in the dentate gyrus and in the pyramidal cell layers (CA1-CA4).

However, due to translational efficiencies, turnover rates and possible receptor protein transport, mRNA levels and distribution do not necessarily reflect that of their respective receptor proteins. Selective ligands/antibodies targeted against the individual receptor proteins are necessary to map the precise location of these proteins. Presently, there are no selective pharmacological ligands available that can distinguish between the two members of the DA D1-like receptor family. Instead, the use of receptor subtype-specific antibodies to determine receptor distribution has started recently (Bergson et al., 1995; Ciliax et al., 1994). Preliminary results from this immunocytochemical approach suggest that in the rat D1 receptors are found in the CA1 subfield and not in the dentate gyrus (Ciliax et al., 1994). This may be taken as an indication that [^{3}H] SCH 23390/D1-like binding found in the dentate gyrus of the rat brain (Dawson et al., 1986; This study) belongs to the D5 receptor subtype. Currently, no immunocytochemical information is available regarding either the distribution of the D5 receptor in the rat or the D1 and D5 receptor proteins in the

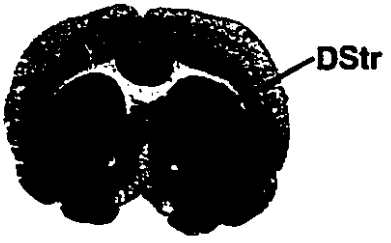
human hippocampus. In the monkey hippocampus, both D1 and D5 receptor proteins are reportedly present in the pyramidal layers (CA1-CA4) as well as in the dentate gyrus (Bergson et al., 1995). It is not possible, at this time, to ascertain the respective contribution(s) of the two D1-like receptor subtypes in the species-dependent distributional profile of [^{3}H] SCH 23390/D1-like binding sites reported in the present study.

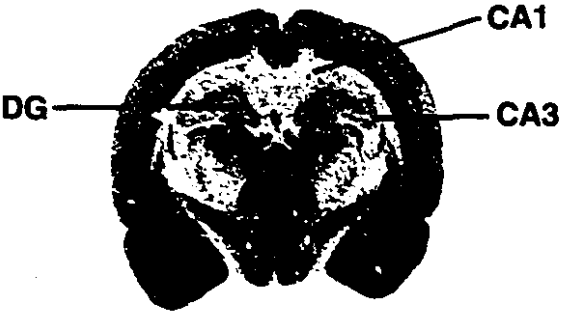
The functional significance of the species-dependent differential distribution of [^{3}H] SCH 23390/D1-like receptors in the hippocampal formation is unclear. We have recently shown that D1-like hippocampal receptors are involved in modulating ACh release in the rat as monitored by in vivo dialysis employing transverse probes (Hersi et al., 1995a). These probes are implanted in such a way that they traverse both the dentate gyrus and the various Ammon's horn subfields of the hippocampus (Damsma et al., 1987). As a result, the effect on ACh release elicited by the stimulation of D1-like receptors could be due to action in any subregion/laminae of the hippocampal formation. Therefore, the presence of [^{3}H] SCH 23390/D1-like receptors in the hippocampus of monkey and human brains suggests that DA acting via these receptors might also stimulate hippocampal ACh release in primates. Given the differential distribution pattern of D1-like receptors across species, however, the validation of this hypothesis must await the conductance of functional investigations in primates. Fortunately, this is now technically feasible with the development of in vivo dialysis techniques in the monkey brain (Kolachana et al., 1994).

In summary, [^{3}H] SCH 23390/D1-like receptors are expressed in the rat, monkey and human hippocampi. However, a differential distribution profile of these receptors was observed between the primate and rodent species studied.

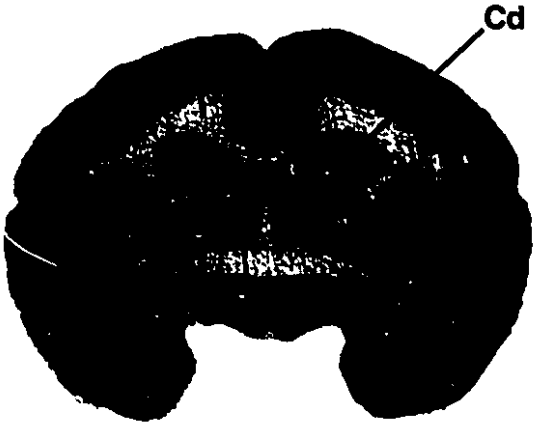
Figure 1. Distribution profile of D1-like/[^{3}H] SCH 23390 binding sites. Rat, monkey and human brain sections were incubated with 1.0nM [^{3}H] SCH 23390 as described in the Materials and Methods. Autoradiograms were generated by apposing the sections against tritium sensitive films for 6-8 weeks (A-C). The bar equals 1cm. Abbreviations: CA, Ammon's horn; Cd, Caudate; DG, Dentate gyrus; Str, Dorsal striatum.

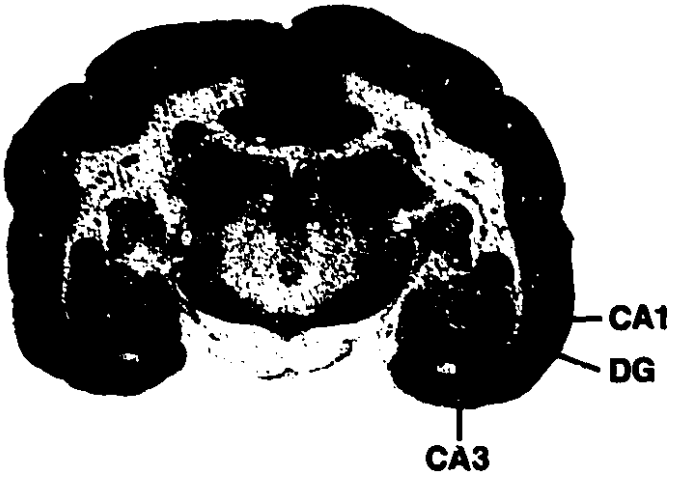
Rat



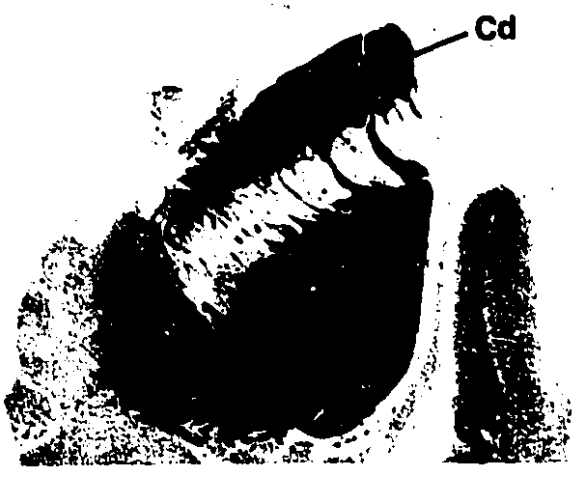


Monkey





Human



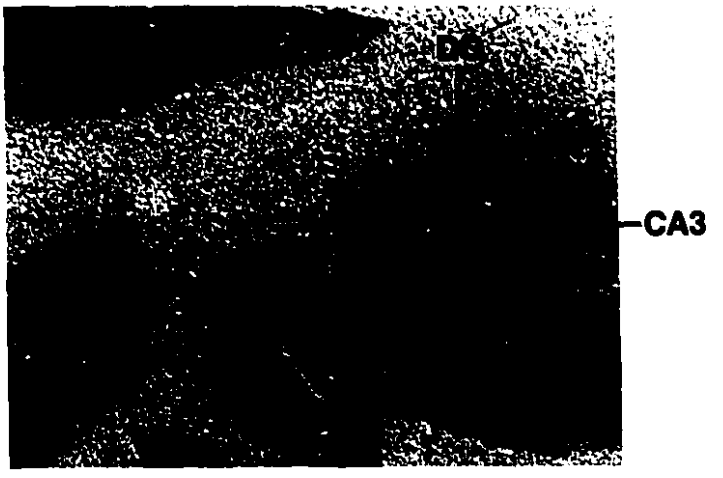


TABLE 1: Quantitative distribution of SCH 23390/D1-like receptor binding sites

Area	Rat	Monkey	Human
Dorsal striatum / Caudate	143±28	126.2±10.4	71.0±11.8
Hippocampal formation			
Dentate gyrus	19±1.7	14.4±0.6	13.2±0.5
CA1 subfield	9.0±0.5	39.7±5.8	22.1±2.5
CA3 subfield	7.0±0.4	16.2±2.2	14.7±1.5

The autoradiograms generated as described in the legend to figure 1 were subsequently quantified using computer assisted image analysis system. The data are expressed as fmol/mg tissue wet weight and represent mean specific labelling ± S.E.M. Rat, n=5; Human, n=4; Monkey, n=2.

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CHAPTER 6
General Discussion

GENERAL DISCUSSION

6.1 General

The Webster dictionary defines interaction as mutual or reciprocal action or influence. To this, one can add, the operating principle of that prince of all organs, the brain (CNS). Nevertheless, it has been fashionable until the recent past to study the signaling molecules (neurotransmitters) of the CNS individually, as if they existed in isolation. Admittedly, this is (was) a necessary oversimplification, given the complexity of the nervous system. Fortunately, nowadays the state of the art of science is such that one can tackle the issue of the interaction of the various neurotransmitter and neuromodulator molecules in the brain. The present thesis is in that vein.

For instance, there is a considerable biochemical, electrophysiological and behavioral evidence suggesting the existence of significant cross-talk between DA and other neurotransmitters including noradrenaline, serotonin, GABA, excitatory amino acids, opiates, adenosine, cholecystokinin and others (for recent review see Jackson and Westlind-Danielsson, 1994). There is also an important interaction between central dopaminergic and cholinergic systems. The original indications of an interaction between these two systems were derived from clinical investigations in Parkinson's disease in the early sixties (Barbeau, 1962; McGeer et al., 1961). Muscarinic receptor antagonists, which decreased cholinergic tone, ameliorated Parkinsonian symptoms in the same manner as did dopamine receptor agonists, which increased dopaminergic tone. These findings led to the proposal that, under physiological conditions, DA and ACh tones are in balance, at least as far as the basal ganglia structures and the performance of motor functions are concerned. Today, much more is known about the nature as well as the extent of this DA-ACh cross-talk. Not surprisingly, the emerging picture is much more complex than the balancing-opposing concept envisaged. The

salient features of the components of this picture are discussed below in the context of this thesis.

6.2 Cholinergic modulation of central dopaminergic activity

ChAT-positive terminals are found in the substantia nigra and ventral tegmental brain areas (Gould et al., 1989) The source of this apparent cholinergic innervation has been shown to be the neurons of the pedunculopontine nucleus and laterodorsal tegmental nucleus (Bolam et al., 1991). These terminals make multiple asymmetric (presumably excitatory) contacts onto the dendrites of dopamine cells. In addition, cholinergic innervation is found in areas such as striatum, cortex and hippocampus which contain the terminal fields of these midbrain DA neurons (Fibiger and Vincent, 1987). Thus, a possibility for heteroreceptor modulation of DA by ACh exists. Interestingly, both types of cholinergic receptors (muscarinic and nicotinic) participate in this action (Calabresi et al., 1989; Lacey et al., 1990).

Activation of muscarinic receptors depolarizes midbrain dopamine neurons (Lacey et al., 1990). This excitation of dopaminergic neurons is mediated by M1-like receptors. Activation of M1-like receptors also enhances dopamine release from striatal slices and synaptosomes (de Belleroche and Gardner, 1983; Giorguieff et al., 1977; James and Cubeddu, 1984; Lehmann and Langer, 1982; Raiteri et al., 1984). Presumably, these receptors are located on striatal dopaminergic terminals as selective lesioning of rat midbrain DA neurons with 6-OHDA results in the loss of these receptors (Vilaro et al., 1990).

Nicotinic receptors are localized on nigrostriatal and mesolimbic DA neurons. These neurons contain [^{3}H]-nicotine binding sites and express various subunits of the nicotinic receptor (Clarke and Pert, 1985; Wada et al., 1989 and 1990). Selective lesioning of midbrain DA neurons results in the loss of these receptors both in the area of cell bodies as well as terminals. Thus it is likely that cholinergic nicotinic receptors occur at the level of cell bodies and / or dendrites as well as within the terminal fields of these midbrain DA neurons (Clarke and Pert, 1985).

Nicotinic receptor agonists also excite midbrain dopamine neurons (Calabresi et al., 1989). Nicotine administered systemically, intracerebroventricularly or iontophoretically increases both nigro-striatal and mesolimbic DA neuron firing (Clarke et al., 1985; Lichtensteiger et al., 1982). Furthermore, the nicotinic receptor antagonist mecamylamine decreases mesolimbic DA neuron firing rate, indicating that endogenous ACh may exert an excitatory effect on this DA system (Grenhoff et al., 1986). Nicotine also increases DA turnover in the striatum (Andersson et al, 1981) and stimulates DA and DOPAC release in the rat nucleus accumbens, as measured by in vivo dialysis (Brazell et al., 1990; Damsma et al., 1989) via a mecamylamine sensitive mechanism (Imperato et al., 1986).

To date most of the studies examining ACh-DA interactions in the CNS have been limited to the basal ganglia. This fact has both a historical as well as technical foundation. Historically, interest in DA-ACh interactions emerged as a result of their involvement in the extrapyramidal symptoms of Parkinson's disease (Barbeau, 1962; McGeer et al., 1961) and thus basal ganglia structures were targeted. It was also technically more feasible to examine DA-ACh interaction in these structures given the presence of relatively high contents of both of these neurotransmitters. Recently, sensitive methods to assay for ACh, DA and their products have become widely

available. As a result, information about DA-ACh interactions in other brain areas has started to be reported. For instance, ACh has been shown to modulate DA activity in the cortex and hippocampus. Systemic as well as local administration of nicotine stimulates cortical DA release (Summers and Giocobini, 1995; Toth et al., 1991). When applied locally through the dialysis probe, nicotine increases hippocampal DA efflux (Toth et al., 1991). Muscarinic receptors are also found on cortical DA terminals in the rat brain (Marchi and Raiteri, 1985). The stimulation of these receptors enhances cortical DA release (Marchi et al., 1987).

In addition to the electrophysiological and biochemical studies discussed above, there is also a wealth of behavioral evidence which suggest cholinergic modulation of central dopaminergic systems (for reviews see Levin et al., 1990; Yoemans, 1995). The most poignant of these relate to nicotine and the mesolimbic DA system. Nicotine is one of the most widely consumed psychotropic drugs, with an estimated 1 billion cigarette smokers worldwide (Clarke, 1993). Nicotine dependence, in the form of cigarette smoking, is responsible for approximately 10 million deaths every year worldwide (Centers for Disease Control, 1991). Although multiple mechanisms may underlie the nicotine addiction process, by far the most consistent finding seems to be that of mesolimbic dopamine involvement (Stolerman, 1991). There is an extensive literature that links an increased tone of this DA system to the rewarding effects of a variety of addictive substances (for review see Koob, 1992). Concerning nicotine, 6-OHDA destruction of mesolimbic DA neurons attenuates nicotine-induced locomotor activity (Clarke et al., 1988) and weakens nicotine self-administration in rats (Clarke et al., 1993; Singer et al, 1982). In addition, as described above, stimulation of nicotinic receptors can directly excite the mesolimbic dopamine system.

Finally, although much has been learned about the cholinergic modulation of central DA functions in the last decade, much more remains to be investigated (see conclusion). In spite of this, however, based on available data one can discern the outlines of a general principle : ACh receptors globally stimulate central DA systems.

6.3 Dopaminergic Modulation of Central Cholinergic Activity

6.3.1 The Striatum

On the basis of immunocytochemical, lesion as well as retrograde tracing studies, it has been established that the striatal cholinergic innervation is derived from intrinsic interneurons (Lehmann et al., 1979; McGeer et al., 1971; Satoh et al., 1983; Woolf and Butcher, 1981). Morphologically, these cholinergic interneurons belong to the large, aspiny class of striatal neurons and constitute 1-2% of the total cell population of the dorsal striatum (Phelps et al., 1985). In contrast, dopaminergic innervation of this structure is extrinsically derived (see Introduction).

Unlike the cholinergic innervation of midbrain DA neurons (see above), the anatomical evidence for a direct synaptic contact between dopaminergic nigrostriatal fibers and intrinsic cholinergic neurons is not conclusive, at the present time (Chang, 1988; Kubota et al., 1987; Pickel and Chan, 1990). Interestingly, however, ChAT and TH immunoreactive striatal terminals are found in close apposition and this has been proposed as an anatomical substrate for DA-ACh interaction (Pickel and Chan, 1990). Given the separating distance of more than 50nm (synaptic cleft size is 30-50nm) as well as the lack of synaptic membrane specialization on these terminals, a nonsynaptic mode of transmission is envisaged (see section 6.4). This situation is rather reminiscent

of the one in the hippocampal formation whereby a contact other than synaptic between DA and ACh systems is postulated (see Chapter 2).

A plethora of functional studies demonstrate that DA modulates the activity of the cholinergic cells in the striatum (for reviews see Lehmann and Langer, 1983; Stoof et al., 1992). For example, striatal ACh content is increased by DA receptor agonists and decreased by DA receptor antagonists (Guyenet et al., 1975). Conversely, DA agonists such as apomorphine inhibit both in vitro and in vivo ACh release in the striatum, an effect blocked by DA receptor antagonists (Sethy and Van Woert, 1974; Stadler et al., 1973). This dopaminergic inhibition of striatal cholinergic activity is apparently mediated via D2-like receptors located on cholinergic interneurons (Dawson et al., 1988; Enz et al., 1990; Le Moine et al., 1990; Scatton, 1982). This rather straight forward scenario has undergone major modifications in the past few years. These changes have been brought about by technological advance; in this case the development of in vivo dialysis techniques (see Introduction).

The in vivo dialysis method allows for the relative preservation of efferent and afferent connections. In this way, determination of global modulatory influences on the release of a neurotransmitter is possible. Utilizing this method, various investigators have demonstrated that DA, as was shown earlier by in vitro methods, does inhibit striatal ACh release in the awake animal via D2-like receptors (Ajima et al., 1990; Bertorelli and Consolo, 1990; Damsma et al., 1990a; De Boer et al., 1992; Marien and Richard, 1990; Westerink et al., 1990). In addition, however, a role for D1-like receptors in the regulation of striatal cholinergic activity has been observed. For instance, infusion of the D1-like receptor agonist, SKF 38393 increases hippocampal ACh release in a dose-dependent manner (Ajima et al., 1990; Bertorelli and Consolo,

1990; Damsma et al., 1990b). The location(s) of the involved receptors is(are) not clear at present time. Two schools of thought exist regarding this issue.

Some investigators, most notably Fibiger and colleagues, propose an extrastriatal site as the location of the enhanced striatal ACh release induced by D1-like receptor stimulation. This is, in part, based on the failure of locally administered D1-like receptor agonists and antagonists to influence ACh release in the striatum and the observation that 6-OHDA striatal DA denervation did not affect systemic-amphetamine-induced ACh release (Damsma et al., 1991; De Boer et al., 1992; Mandel et al., 1994). It is known that a corticostriatal projection utilizing glutamate/aspartate as a neurotransmitter plays an important role in the regulation of striatal cholinergic activity (Consolo et al., 1990; Wood et al., 1979). Moreover, local striatal application of the NMDA receptor antagonist MK-801 attenuates systemically-administered D1-like receptor agonist-induced increases in striatal ACh release (Damsma et al., 1991). Therefore, it has been hypothesized that the D1-like effect is mediated via the stimulation of D1 receptors located on corticostriatal neurons in the medial prefrontal cortex which leads to an increase in glutamate release from corticostriatal terminals that synapse with cholinergic interneurons in the striatum (Wilson et al., 1990).

Other studies, however, point to intrinsic striatal D1-like receptors as those involved in modulating ACh release in this structure. In these studies, the striatal, but not frontocortical, application of D1-like receptor agonists and antagonists does indeed modulate striatal ACh release (Ajima et al., 1990; Consolo et al., 1992; Zocchi and Pert, 1993). Recently, it has been reported that D1 receptor mRNA exists on large-sized, presumably cholinergic, striatal interneurons (Guennon and Bloch, 1992; Le Moine et al., 1991). Thus, it is conceivable that D1 drugs could directly induce their

effect on ACh release. Furthermore, partial hemisection caudal to the frontal cortex did not alter the ability of systemically administered D1-like receptor agonist, SKF 38393, to increase striatal ACh release, thus arguing against frontal cortical involvement (Zocchi and Pert, 1993).

It is important to point out that there are significant methodological differences between the two groups of studies mentioned above including drug dose and the composition of the dialysis solution which could possibly account for the discrepant results (De Boer et al., 1990; Westerink et al., 1990). It is possible, for example, that DA regulation of striatal ACh release is different depending on the basal activity levels of the cholinergic interneurons. Therefore, including a cholinesterase inhibitor (as is done in some of the studies discussed above) which results in the elevation of basal cholinergic tone might have an effect on the DA modulation of striatal ACh release. The interval between probe insertion and the dialysis experiment has also been reported to be crucial in demonstrating DA-ligand effects on striatal ACh release (Westerink et al., 1990). At present the optimum recovery period appears to be 48hrs. Fortunately, probes and assay methods which can detect ACh without the need to use cholinesterase inhibitors have become available recently. It may also be necessary to conduct a systematic investigation using one set of conditions, as has been done in the present thesis (chapter 2), to ascertain the locale of the receptors involved. Hopefully, the controversy surrounding the loci of DA D1-like effect on striatal ACh release could be resolved in the near future. In any case and regardless of the loci of action, however, it is clear that DA acting via D1-like receptors can stimulate striatal ACh activity.

Since it appears that D1-like and D2-like receptors have opposing effects, an important question arises as to the physiological nature of DA influence on striatal cholinergic activity. To address this issue, investigators have used approaches that

manipulate DA tone in the brain. The systemic administration of the indirect DA agonists amphetamine and cocaine has been shown to increase striatal ACh release (Damsma et al., 1991; Florin et al., 1992). Pretreatment with the D1 receptor antagonist, SCH 23390, completely inhibited the amphetamine effect. Blockade of D2-like receptors with sulpiride or haloperidol does also increase striatal ACh release but this effect is prevented by antagonism of D1-like receptors with SCH 23390 or SCH 39166 or by the depletion of DA stores with reserpine and α-methyltyrosine (Imperato et al., 1994) Furthermore, 6-OHDA lesioning of midbrain DA neurons induces a significant decrease in basal striatal release of ACh (Robertson et al., 1992). Taken together, these results suggest that in vivo striatal ACh release is under a tonic facilitatory control by dopamine acting on D1-like receptors.

What does this mean for the dogmatic concept of tonic inhibitory control of striatal ACh activity by DA acting via D2-like receptors ? D2-like receptor blockade results in a compensatory activation of dopaminergic neuronal firing and enhanced dopamine release (Bunney et al., 1973; Imperato and Di Chiara, 1985; Mereu et al., 1983; Zetterstrom et al., 1984). It is possible, therefore, that the enhancement in ACh release following D2-like receptor blockade is not due to the loss of inhibitory dopaminergic control on cholinergic neurons but consequent to the stimulant action of endogenously released DA on D1-like receptors. The converse is likely true, of course, for D2-like receptor stimulation (Imperato et. al., 1988; White, 1987). Nevertheless, there are some hints that under abnormal circumstances such as functional DA denervation or prolonged treatment with neuroleptics the inhibitory D2-like effect might become more prominent (Imperato et al., 1994; Morelli et al., 1986; Robertson et al., 1992).

Interestingly, DA modulation of cholinergic activity might not be uniform throughout the striatum. In mammals the striatum is composed of the caudate nucleus, the putamen, the nucleus accumbens , globus pallidus and some parts of the olfactory tubercle. Traditionally, the striatum has been subdivided into two functional parts, namely dorsal and ventral. The dorsal striatum is considered to be involved in sensorimotor functions because of its afferent connections from the somatosensory and motor cortices, whereas the ventral portion is thought to play a role in emotional and motivational processes in view of its anatomical association with limbic structures such as the limbic cortex, amygdala and hippocampus (Bjorklund and Lindvall, 1984; Groenewegen et al., 1991). Both the DA and ACh systems are nonhomogeneously distributed in the striatum (Stoof et al., 1992). Fortunately, with the help of techniques such as microdialysis it has become feasible to functionally microdissect the different striatal compartments. As a result, investigators have been able to show that the DA D2-like inhibitory effect on ACh release is either absent or greatly diminished in the ventral striatum compared to the dorsal portion of this structure (De Belleroche and Gardner, 1983; Stoof et al., 1987; Wedzony et al., 1988). Apparently, the same is true for the olfactory tubercle (Suarez-Roca et al., 1987). Therefore, it appears that there are differences in the regulatory effect of DA on cholinergic activity in the various regions of the striatal complex. In the context of the present thesis, it would be important to determine if the same is true for the hippocampal formation (i.e. dorsal vs ventral).

Finally, unlike the cholinergic modulation of striatal dopaminergic activity, a general principal is not very clear when DA modulation of ACh in this structure is considered. Nevertheless, a dualistic effect might be envisaged : normally stimulation via D1-like receptors and under certain circumstances inhibition via D2-like receptors.

6.3.2 Cortical Structures

Recently, DA has been shown to have a robust influence on neocortical cholinergic activity as reflected by ACh release. Non selective elevation of DA tone by the systemic administration of either amphetamine or apomorphine stimulates frontal cortical ACh release (Day and Fibiger, 1992). This effect was blocked by the D1-like receptor antagonist SCH 23390 but not by the D2-like receptor antagonist raclopride (Acques et al, 1994; Day and Fibiger, 1993). In addition, D1 agonists but not D2 agonists enhanced ACh release in this structure. Taken together, these results suggest that the receptor subtype involved in the effect of DA on ACh release is of the D1-like family. Interestingly, the local infusion of amphetamine in to the frontal cortex had no effect on ACh release, suggesting that the involved D1 receptors might have an extracortical localization (Day and Fibiger, 1992). To ascertain the locale of the involved receptors, more detailed studies using selective D1 ligands infused into discrete regions are warranted. Also, it will be important to determine the D1-like receptor subtype(s) involved using similar approaches as those utilized for the hippocampal formation (chapter 3).

Another structure that has received considerable attention lately is the hippocampal formation. In fact, the whole of the present thesis is concerned with this issue. Briefly, septohippocampal cholinergic activity is enhanced by stimulation of D1-like receptors located in the hippocampus (chapter 2). The D1-like receptor involved in this action appears to be of the D5 subtype (chapter 3). Likely, this DA-ACh interaction has cognitive significance (chapter 4). These issues were extensively discussed in the preceding chapters and are briefly expanded upon below.

The DA D1-like receptors involved in modulating hippocampal ACh release were postulated to reside on cholinergic terminals in the hippocampus (chapter 2).

Thus a possibility for a direct presynaptic modulation exists. To substantiate this, however, more direct evidence is deemed necessary. For example, a double labelling immunocytochemical study of ChAT and D1-like (D5) receptors in the hippocampus is called for. In addition, the tetrodotoxin (TTX) sensitivity of the D1-like effect on hippocampal ACh release should be assessed. This test has traditionally been used to differentiate between direct and indirect effect of molecules in the brain. However, it might not be feasible to undertake this in the in vivo dialysis setup as the administration of TTX in the living animal reduces ACh levels below detection limits. Alternatively, the TTX sensitivity test could be conducted in vitro using a synaptosomal preparation technique.

If the D1-like stimulation of hippocampal ACh release has a cognitive significance (chapter 4) and the receptor involved in stimulating ACh release is postulated to be of the D5 subtype (chapter 3), then it follows that the D5 receptor is involved in cognition. Once again, however, a more direct evidence is called for. As suggested at the end of chapter 3, this could be the examination of the effect of the D5 antisense oligonucleotides on spatial memory in the Morris Water Maze task both in young and aged animals. This notwithstanding, stimulation of D5 receptors may present a unique opportunity in alleviating age-associated memory deficits. The DA D1-like stimulation of hippocampal ACh release is preserved as the animal ages and becomes memory-impaired (chapter 4). Moreover, the relatively limited distribution of the D5 receptor (see Introduction) raises the possibility of minimum undesirable side effects from drugs targeted to this receptor subtype. Unfortunately, at the present time there are no agonists available which are selective enough for the D5 receptor as compared to the other members of the DA receptor family. Hopefully, given the level of interest in the field of cognition and DA, this situation should improve in the near future.

6.4 THE TRANSMISSION MODE OF DA

DA receptors in various brain areas have frequently, if not consistently, been shown to reside on non-synaptic membranes (Bergson et al., 1995a; Vizi and labos, 1991; Yung et al., 1995). Furthermore, there is often a mismatch between the location of these receptors and presumptive DA terminals (Dawson et al., 1986; Pickel and Chan, 1990; Smiley and Goldman-Rakic, 1994; Verney et al., 1985). This anatomical reality implies a mode of communication different from classical synaptic transmission. This alternate mode of communication has variably been called non-synaptic, parasynaptic or as of late volume transmission (VT) (Descarries et al., 1991; Fuxe et al., 1991; Schmitt, 1984). VT is characterized by transmitter (signal) diffusion in the extracellular fluid in a three dimensional fashion to act at distant sites. Apparently, in addition to DA, many other messenger molecules probably utilize VT (for a recent review see Agnati et al., 1995).

Interestingly, this concept has an illustrious history dating back to the beginning of modern neuroscience (for recent reviews see Jacobson, 1993; Shepperd, 1991). This is exemplified by the opposing views held by Golgi and Cajal concerning the modus operandi of the brain. Golgi supported the view of the brain as a global system whereas Cajal favored the theory of the brain as a collection of relatively independent elements (Cajal, 1906; Golgi, 1903). Today, it is clear that both modalities occur in the brain.

Implicit in these two views is the nature of the brain's signalling molecules as either neurotransmitters (NTs) or neuromodulators (NMs). One salient difference between NTs and NMs is the speed with which they act, whereby the former are faster-acting than the latter. However, the classification of most signalling molecules, including DA, as a NT or a NM is not clear-cut (Kupfermann, 1979). Nevertheless,

given its putative non-synaptic mode of communication, apparently occurring over longer distances and times than synaptic transmission, the designation of DA as a neuromodulator has been proposed over a decade ago (Lehmann and Langer, 1983). Neuromodulation is defined as the ability of neurons to alter their electrical properties in response to intracellular biochemical changes resulting from synaptic or hormonal stimulation and a neuromodulator as the substance that brings about such actions (Kazmarek and Levitan, 1987). There is an extensive electrophysiological and behavioral literature evidencing DA as a neuromodulator (for recent reviews see Le Moal and Simon, 1991; Mogenson and Yim, 1989). As a result DA neurons are thought to play a general regulatory (variably called enabling, focusing or gating) role, as opposed to other sets of neurons that are supposed to integrate certain functions (Mogenson and Yim, 1981; Simon and Le Moal, 1988). In this view, the DA code acquires its specific significance largely from the characteristics of the receiving cell. Consequently functions attributed to DA activity are as varied as the number of areas receiving DA projections. One of these areas, the hippocampal formation, is briefly considered next.

6.5 FUNCTION(S) OF DA IN THE HIPPOCAMPUS

Little is known about the functional role(s) of the mesolimbocortical input into the hippocampus. The reasons for this are manifold. Until recently, the DA content of the hippocampus was considered only as a precursor to noradrenaline synthesis (see General Introduction). Also, relative to other limbic structures, the hippocampus receives a rather sparse DA innervation (Verney et al., 1985). These facts delayed the focussing of attention on this structure. Traditionally, lesion studies have been used to ascertain the putative roles of a certain transmitter innervation. Applying this approach

to the hippocampal DA innervation, however, gives rise to results whose interpretation is problematic. This is due to the nature of the mesocorticolimbic DA innervation as well as the toxin(s) used. The most specific and widely used toxin for this purpose is 6-OHDA. Unfortunately, when this toxin is administered directly into the hippocampus, in addition to DA terminals it also injures the robust noradrenergic innervation found in this structure (Aston-Jones et al., 1984). Also, Injecting 6-OHDA into the area of the hippocampally-projecting DA cell bodies in the VTA/SN leads to the loss of DA input into multiple structures besides the hippocampus (see General Introduction). In spite of this and with the utilization of various direct and indirect approaches, the assignment of putative function(s) to the hippocampal DA innervation has began. One of these is the possible involvement of DA in epilepsy.

Epilepsy is a term used for a collection of clinically and pathologically diverse group of neurological disorders. This condition is characterized by the synchronization of electrical activity in a pocket of active neurons and excitatory neurotransmitters such as glutamate or inhibitory ones such as GABA are thought to play a central role in this process. In addition, monoamines such as DA apparently have a role in regulating the initiation and spread of seizure activity (for a recent review see Starr, 1996).

Microinjection studies investigating the site of action of DA drugs in the brain have suggested the hippocampal formation as a possible target. For instance, the dorsal hippocampal injection of the D2-like agonist quinpirole attenuated the severity and increased the latency of intrahippocampal pilocarpine or carbachol-induced seizures. Conversely, the D2-like antagonist raclopride augmented pilocarpine-induced seizures (Alam and Starr, 1993). On the other hand, local stimulation of hippocampal D1-like receptors does not alter seizure threshold to pilocarpine whereas the blockade of these receptors affords a modest protection against pilocarpine-induced convulsions (Alam

and Star, 1992). Thus, it appears that the hippocampal DA terminals may have a role in epileptogenesis and this is likely inhibitory in nature, mediated via D2-like receptors. (Starr, 1996).

It is tempting to invoke modulation of hippocampal ACh release as a possible mechanism for the involvement of DA in epilepsy. DA D2-like receptors were not shown to be involved in regulating ACh release in the hippocampus of young healthy animals (Chapter 2). Nevertheless, could the situation be similar to that of the striatum where under abnormal conditions a D2-like effect on ACh release might become more prominent (see section 6.3.1)? This possibility is specially intriguing given that all the epileptic activities discussed above were induced by the over-stimulation of hippocampal cholinergic activity. The validity of this supposition should be investigated by conducting ACh release experiments, similar to those described in chapter 2, in seizure-prone animals.

Another possible function for the DA innervation in the hippocampus is involvement in cognition. A relatively strong collection of electrophysiological, behavioral and biochemical evidence implicate hippocampal DA in the processes thought to underlie cognition. This potential DA-cognition connection forms one of the pillars of the present thesis and has been extensively discussed in the preceding chapters (chapters 1 and 4).

Finally, preliminary results suggest a role for hippocampal DA in reward and reinforcement. At the cellular level, spontaneous bursting of CA1 pyramidal neurons in hippocampal slices can be reinforced with contingent injections of DA and cocaine. In the intact animal, CA1 field administration of DA reinforces self-administration behavior (Stein and Belluzzi, 1989). There is also a possibility of an interaction

between the opioid and DA systems in the hippocampus in reward processes. There is a great deal of evidence that opioid reward effects are, at least in part, mediated by the activation of midbrain DA systems, in particular VTA (for reviews see Bozarth, 1989; Cooper, 1989; Wise, 1989). Although the involvement of the DA VTA tern. the hippocampus has not yet been addressed, an anatomical substrate exist given the presence of opioid reward-related circuitry in the hippocampus.

6.6 CONCLUSION

The present thesis examined the existence, nature and functional significance of modulation of septohippocampal cholinergic activity by DA. In addition, the occurrence of such an interaction in other brain areas as well as the cholinergic regulation of central DA systems were discussed. Although much has been learned about DA-ACh interactions in the last decade, number of outstanding issues remain. These include the specific cholinergic / dopaminergic receptor subtypes involved, the existence and nature of this interaction in species other than rodents such as humans and the fate of these interactions in age and disease. Given the functional importance of this interaction, rapid progress concerning these issues is expected in the near future.

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> Elements in the thesis that are to be considered as **contributions to original knowledge** must be clearly indicated in the preface or by a separate statement at the beginning or at the end of the thesis. This requirement is **mandatory for the PhD thesis**

CHAPTER 2

The possible modulation of hippocampal cholinergic release by dopamine D1-like receptors has been suggested by earlier studies. The experiments presented in this chapter, in addition to confirming this possibility, pinpoint the loci of this dopamine-acetylcholine interaction to be within the hippocampus, possibly via receptors located on cholinergic terminals.

CHAPTER 3

The dopamine D5 receptor, a member of the D1-like family, has a unique and restricted distribution in the mammalian brain. By using a combined antisense-in vivo dialysis approach we show that this receptor might be involved in modulating hippocampal acetylcholine release. To our knowledge, this is the first time that a putative function has been demonstrated for this receptor.

CHAPTER 4

In this chapter, we show that the dopaminergic modulation of hippocampal cholinergic activity is preserved as the animal ages. More importantly, age-associated deficits in the spatial memory task, Morris Water Maze, can be attenuated by stimulating D1-like receptors.

CHAPTER 5

The data reported in this section of the thesis confirm and extend previous studies demonstrating the differential distribution of dopamine D1-like receptors in the hippocampal formation of the rat as compared to monkeys and humans.

Appendix

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Local Modulation of Hippocampal Acetylcholine Release by Dopamine D1 Receptors: A Combined Receptor Autoradiography and *in vivo* Dialysis Study

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The modulation of *in vivo* hippocampal ACh release by dopaminergic D1 and D2 receptors was examined in this study. Additionally, in an attempt to ascertain the location of these receptors in relation to hippocampal cholinergic terminals, fimbriaectomy and quantitative autoradiography were used. Following unilateral fimbriaectomy, whereby at least 50% of hippocampal cholineacetyltransferase (ChAT) activity was lost, a significant ipsilateral decrease in D1/^{3}H SCH23390 binding was observed in the molecular layer of the dentate gyrus while hippocampal D2/^{3}H raclopride binding was unaffected. The effects of prototypical D1 and D2 receptor agonists and antagonists on hippocampal ACh release were examined next using *in vivo* dialysis in freely moving rats. The D1 agonist SKF 38393 (10 μM to 100 μM) administered directly into the hippocampus via the dialysis probe stimulated ACh release in a concentration dependent manner. The effect of the agonist was blocked by the coadministration of the D1 receptor antagonist SCH 23390 (1 μM), which by itself failed to modulate ACh release. In contrast, neither the D2 agonist quinpirole (1–10 μM) nor the D2 antagonist sulpiride (1–10 μM) had any direct effect on hippocampal ACh release. Additionally, the infusion of these D1 and D2 drugs in the septal area failed to affect hippocampal ACh release. Taken together, these results suggest that a proportion of hippocampal D1 receptors are located on cholinergic nerve terminals and that dopamine, acting via D1 receptors, can locally stimulate hippocampal ACh release.

[Key words: ACh, dopamine, hippocampus, in vivo dialysis, receptor autoradiography, D1 receptor]

Dopamine (DA) is thought to modulate the activity of the septo-hippocampal cholinergic pathway (Robinson et al., 1979; Costa et al., 1983). Infusions of dopamine antagonists into the septum increase the firing rate of this pathway (Robinson et al., 1979) and elevate ACh turnover (Robinson et al., 1979) and high affinity choline uptake (Durkin et al., 1986) in the hippocampus. Similar effects are also observed following either intraseptal 6-hydroxydopamine injection (Robinson et al., 1979) or the destruction of the ventral tegmental A10 dopaminergic neurons (Robinson et al., 1979; Galey et al., 1985). It has been suggested that A10 dopaminergic neurons projecting to the lateral septum interact with cholinergic fibers originating from the medial septal nucleus, possibly via septal GABAergic interneurons, to bring about this inhibitory influence (Wood, 1985). On the other hand, a variety of wide ranging studies including morphological (Scatton et al., 1980; Verney et al., 1985; Simon et al., 1989), electrophysiological (Gribkoff et al., 1984; Stanzione et al., 1984; Smialowski et al., 1987) and biochemical (Bischoff, 1979; Ishikawa, 1982) approaches provide evidence suggesting that the hippocampus receives direct dopaminergic innervation. This innervation apparently originates mainly from the ventral tegmental area (VTA; and to a lesser extent from the substantia nigra) and ascends in the medial forebrain bundle to innervate various limbic structures, including the hippocampus (Verney et al., 1985).

Dopamine receptors have been classified in two broad families (D1-like and D2-like) on the basis of the activity of various agonists and antagonists (Kebabian and Calne, 1979; Seeman, 1980). More recently, molecular cloning techniques have shown that the D1 family comprises two receptors (d1 and d5) while the D2 family consists of at least three different receptor proteins (d2, d3, and d4) (for a recent review, see Niznik and Van Tol, 1992). D1 receptors activate adenylate cyclase whereas members of the D2 receptor class have been shown to couple to numerous effector systems, including the inhibition of adenylate cyclase and the activation of potassium channels, among others (Monsma et al., 1990; Sibley and Monsma, 1992). Both the D1 and the D2 receptor subtypes have been localized in the septum as well as the hippocampus of various mammalian species including the rat (Bischoff et al., 1980; Bruink et al., 1986; Dawson et al., 1986; Grilli et al., 1988; Tiberi et al., 1991; Mengod et al., 1992).

Given this background, it is thus likely that dopamine may act as a modulator of the septo-hippocampal cholinergic pathway both at the level of the cell bodies in the septal area and at the nerve terminals within the hippocampus. In the present study, these two possibilities were investigated directly by examining the effects of local administration of selective dopamine D1 and D2 receptor agonists and antagonists on hippocampal ACh release using *in vivo* dialysis in freely moving rats as well as by evaluating the effects of unilateral fimbriaectomy on these

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receptors in the hippocampus as monitored by quantitative autoradiography. Our results suggest that a certain proportion of pharmacologically defined D1 receptors are located on cholinergic nerve terminals and that dopamine may act, via D1 receptors, as a stimulatant of ACh release at level of the cholinergic nerve terminals within the hippocampus.

Materials and Methods

Materials. Male Sprague–Dawley rats (250–350 gm) obtained from Charles River Canada (St. Constant, Quebec, Canada) were maintained on a 12 hr light–dark cycle (light on at 7:00 A.M.) in temperature and humidity controlled rooms for at least 3–4 d prior to surgery. Animals were fed standard laboratory chow and had access to tap water ad libitum. Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care (CCAC).

The dialysis probes were made from AN69 Hospal fibers (molecular weight cut off < 60,000 i.d. = 220 μm, o.d. = 310 μm). SCH23390 HCl, (+)SKF38393 HCl, (−)SKF 38393 HCl, quinpirole HCl, sulpiride, and eticlopride HCl were obtained from RBI (Watick, MA). Neostigmine Bromide was purchased from BAS (West Lafayette, IN). ^{3}H SCH23390 (80.7 Ci/mmol), ^{3}H raclopride (70.0 Ci/mmol), ^{3}H-Hyperfilms and microscale standards were purchased from Amersham Canada (Oakville, Ontario, Canada). The deuterated variant of ACh, $^2H_4$ ACh bromide [$(CH_3)_3NBrCD_2CD_2OC$-(0)CH_3], used as internal standard for ACh determination was obtained from Merck, Sharp and Dohme Isotopes (Montreal, Quebec, Canada). Developer (D-19) and fixer (Rapid Fix) were obtained from Kodak Chemical Inc. (Montreal, Quebec, Canada). All other reagents and chemicals were of HPLC or GC-MS grade and purchased from either Fisher Scientific Co. (Montreal, Quebec, Canada) or Aldrich Chemicals (Chicago, IL).

Fimbriaectomy. The fimbria fornix of male rats was unilaterally interrupted by a knife cut lesion under sodium nembutal anesthesia (50 mg/kg). Briefly, at a 90° angle and coordinates of 1 mm behind bregma and 3.0 mm lateral to the midline suture (Paxinos and Watson, 1982), a leukotome knife (Kopf Instruments) was lowered via an opening in the skull to a depth of 4.0 mm below dura. The wire in the knife was then extended under the fimbria and the leukotome slowly brought back to the dura. For sham operated animals, the leukotome was lowered and brought back to the surface as above but without extending the wire. The animals were allowed to recover from anesthesia under a warm light and individually housed according to CCAC guidelines. Two weeks postsurgery, the animals were sacrificed and the efficacy of the lesions was assessed by determining hippocampal ChAT activity.

Hippocampal ChAT activity. Hippocampal punches from 300 μm brain slices of the lesioned and sham operated animals were assayed for ChAT activity. Homogenates from these punches were incubated for 15 min in a buffer containing ^{14}C acetyl CoA as previously described in detail (Araujo et al., 1988) using the method of Fonnum (1969) as modified by Tucek (1978). The animals that were used in subsequent receptor autoradiographic studies showed hippocampal ChAT activity losses of 51 ± 4% on the lesioned side as compared to the contralateral hippocampi.

Dopamine receptor autoradiography. The status of hippocampal dopaminergic receptors following fimbriaectomy was assessed as described in detail elsewhere (Debonnel et al., 1990). In brief, following sectioning at −17°C, 20 μm hippocampal slices were incubated for 60 min at room temperature in 50 mM Tris HCL buffer (pH 7.4) containing 120 mM NCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.0 nM ^{3}H SCH 23390 for D1 receptors or 5.7 nM ^{3}H raclopride for D2 receptors. Serial sections were incubated in this buffer but with the addition of 1 μM SCH 23390 or 1 μM (+) butaclamol to ascertain the specificity of the D1 or D2 radioligand binding, respectively. The sections were then rinsed five times (2 min each) in fresh ice-cold buffer. Buffer salts were removed by a rapid dip in ice-cold distilled water and the sections rapidly air dried. Autoradiograms were generated by apposing the sections alongside with tritium standards to tritium sensitive films for 4 weeks. The films were then developed as described before (Quirion et al., 1981) and ^{3}H SCH 23390 and ^{3}H raclopride binding quantified (fmol/mg tissue wet weight) using computer assisted microdensitometric image analysis system (MCID System, Imaging Research Inc., St. Catherines, Ontario, Canada). Although, the D1 radioligand, ^{3}H SCH 23390, also binds 5HT2 receptors (Bischoff et al., 1986), under the conditions used in this study, this ligand is likely preferentially labeling D1 receptors (Dawson et al., 1986). Anatomical areas were identified according to the Paxinos and Watson's atlas (1982). Significant differences between experimental groups (n = 4 in each group) were determined by a one-way analysis of variance (ANOVA).

Probe implantation and hippocampal in vivo dialysis. Male Sprague–Dawley rats (250–350 gm) were anesthetized with sodium nembutal (50 mg/kg). Transverse probes (Damsma et al., 1987) were stereotaxically implanted in the dorsal hippocampus and for septally manipulated animals also in the lateral septum (Giovannini et al., 1994) at coordinates of 3.8 mm posterior to bregma and 3.5 mm below the skull for the hippocampus and 0.7 mm anterior to bregma and 4.5 mm below the skull for the septum (Paxinos and Watson, 1982). The animals were individually housed and allowed to recover from surgery for 2 d prior to their use in the *in vivo* dialysis experiments. Each animal was dialyzed only once.

At the beginning of each dialysis experiment, animals were placed in lidless cages and connected to a BAS microliter syringe pump in a manner as to allow them to freely move in the cages. The probes were perfused for a one hour wash out period at a flow rate of 2.34 μl/min with an Ungerstedt-Ringer solution (127 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.3 mM $CaCl_2$, pH 7.4) containing 5 μM neostigmine bromide, a cholinesterase inhibitor. Twenty five minute dialysate fractions were collected into a 1ml glass vials containing 46 μl of 0.1 *N* HCl and 50 pmol of deuterated ACh as internal standard. Following about 3 hr of baseline hippocampal ACh release, drugs of interest were tested by inclusion in the perfusion Ringer's solution for the remainder of the dialysis experiment. For septally manipulated animals, the drugs of interest were perfused into the septum in a Ringer's solution lacking neostigmine. The samples were frozen immediately and stored at −80°C until assayed by gas chromatography/mass spectroscopy (GC/MS). Following most experiments, probe location was verified by standard histological examination of the brain.

In the experiment designed to evaluate the potential diffusion of the locally applied drugs, the *in vivo* dialysis setup was as described above for hippocampally manipulated animals except that ^{3}H SCH 23390 (1 μM) was used and no samples were collected. The animals were sacrificed immediately following the termination of the experiment. Twenty micrometer slices of the brains of these animals were exposed to tritium sensitive Hyperfilms for 10 d.

GC/MS analysis of ACh. ACh content of the dialysate fractions was determined by GC-MS as described in detail by Marien and Richard (1990). Briefly, frozen samples were lyophilized overnight, reconstituted in 250 μl acetonitrile, capped, heated at 80°C for 30 min, and dried under a gentle stream of nitrogen gas. Quaternary amines present in the samples were demethylated by adding 250 μl sodium benzene thiolate solution (160 mg in 18 ml of redistilled methyl ethyl ketone and 35 μl glacial acetic acid) under a flow of nitrogen and reacting at 80°C for 45 min. Samples were then extracted into 35 μl citric acid and washed twice with 250 μl pentane. Finally the samples were extracted into 80 μl of ethyl acetate and concentrated down to 3–5 μl volume before being injected in to the GC-MS (Hewlett-Packard 5987b). The demethylated derivatives of ACh were analyzed by selective ion monitoring of 132 atomic mass units (amu) for endogenous and 136 amu for the internal standard, $^2H_4$-ACh.

The amount of endogenous ACh in each dialysate was calculated (Jenden and Hanin, 1974) from the peak area ratio of endogenous versus deuterated internal standard. Calculations were not corrected for the recovery of ACh by each dialysis probe. Sample ACh content was expressed as a percentage of average baseline (eight sample collections preceding drug infusion). Significant differences between experimental groups were determined by a one-way analysis of variance (ANOVA).

Results

Effect of fimbriaectomy on hippocampal D1 and D2 receptor binding sites

A significant loss (18.2%, p = 0.0038) of D1/^{3}H SCH23390 binding was observed in the molecular layer of the dentate gyrus of the ipsilateral hippocampus in lesioned animals (29.9 ± 0.8 vs 36.6 ± 1.1 fmol/mg tissue wet weight) (Fig. 1*A,B*). No detectable differences were observed in ^{3}H SCH 23390 binding in CA1, CA2, and CA3 subfields between the ipsi- and contralateral hippocampi of fimbriaectomized rats (Fig. 1*A,B*). ^{3}H SCH 23390 binding levels were similar in the hippocampus of sham

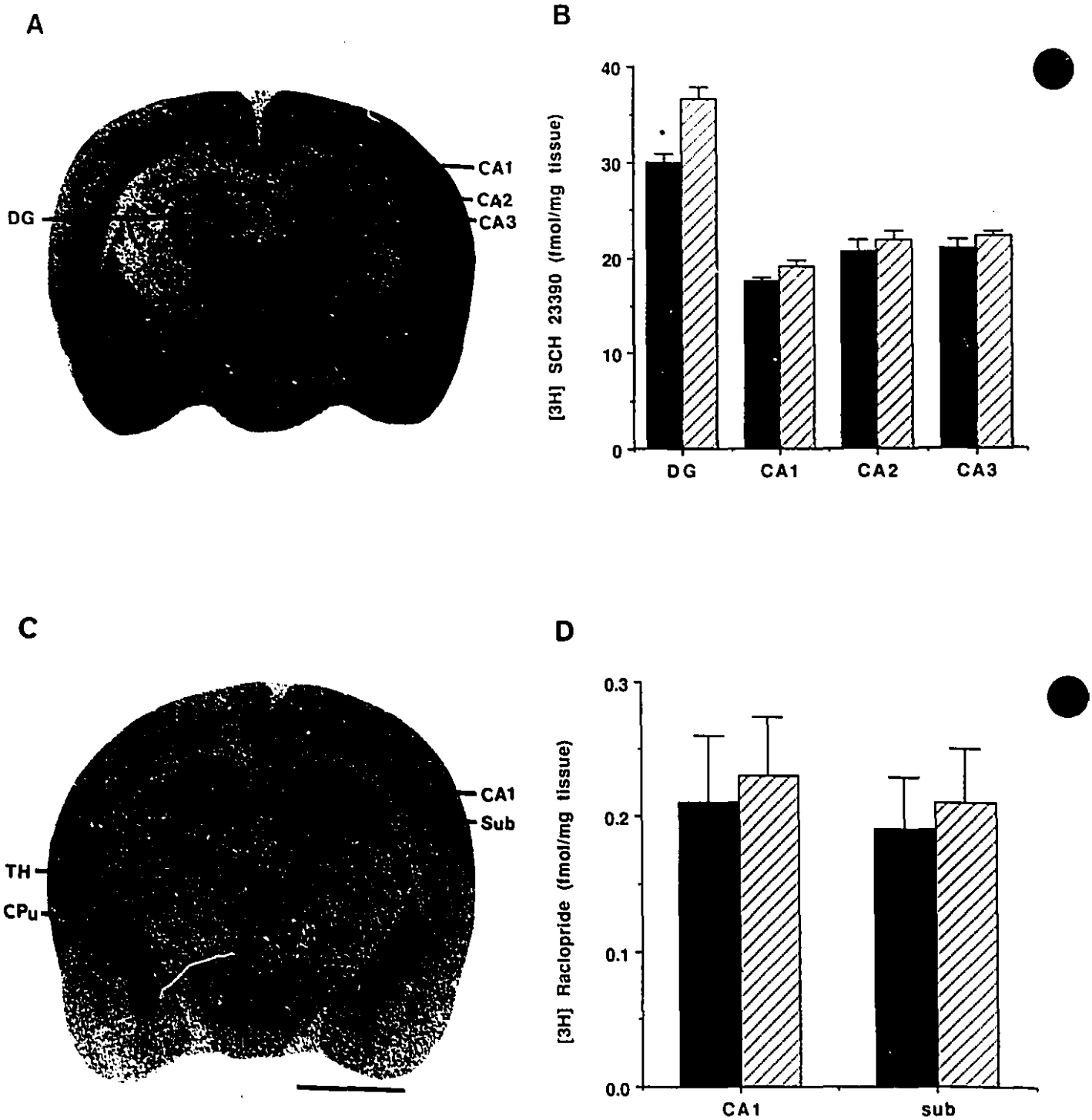

Figure 1. Effect of fimbriaectomy on hippocampal D1/^{3}H SCH 23390 and D2/^{3}H raclopride binding. Data represent mean ± SEM from four different animals expresses in fmol/mg tissue, wet weight. Sections from unilaterally fimbriaectomized rats were incubated with 1.0 nM ^{3}H SCH 23390/D1 (*A, B*) or ^{3}H raclopride/D2 (*C, D*) as described in Materials and Methods. Nonspecific binding was defined in the presence of 1 μM SCH 23390 or 1 μM (+) butaclamol for D1 and D2, respectively. Autoradiograms (*A, C*) generated by apposing the sections against tritium sensitive films were subsequently quantified (*B, D*) using computer assisted image analysis system. The histograms represent specific labeling obtained by subtracting nonspecific from total binding. *Solid* and *shaded bars* represent the ipsi- and contralateral, respectively. Statistical analysis was evaluated by one-way analysis of variance (ANOVA). *, $p < 0.05$; significantly different from nonlesioned contralateral side. The bar equals 2 cm. Abbreviations: *CA*, Ammon's horn; *CPu*, caudate-putamen; *DG*, dentate gyrus; *Sub*, subiculum.

operated animals as compared to the contralateral hippocampi of the fimbriaectomized animals (data not shown).

Hippocampal D2/^{3}H raclopride binding was considerably lower than that of D1/^{3}H SCH 23390. Using ^{3}H raclopride, the only detectable amount of specific binding in the dorsal hippocampus was observed in the CA1 and subicular regions (Fig. 1*C*). No differences were seen between ipsi- and contra-lateral hippocampi of unilaterally fimbriaectomized animals (Fig. 1*D*). Additionally, no detectable changes of either ^{3}H SCH 23390 or ^{3}H raclopride binding levels were observed in the septum or cortical regions as a result of unilateral fimbriaectomy. In all the animals used here, ChAT activity in the ipsilateral hippocampi decreased by 51 ± 4% as compared to the contralateral hippocampi or the hippocampus of sham operated animals.

Local dopaminergic receptor modulation of hippocampal ACh release

The average basal efflux of ACh from the dorsal hippocampus was 4.8 pmol/25 min (n = 52). Moreover, there were no appreciable differences in basal ACh release between the various experimental groups studied here. Hippocampal intraprobe administration of the active enantiomer of the selective D1 agonist (+)SKF 38393 (10–100 μM), but not the inactive enantiomer (−)SKF 38393 (10 μM), increased ACh release in a concentration dependent manner (Fig. 2*A*). The D1 antagonist SCH 23390 (1–10 μM), by itself, had no appreciable effect on hippocampal ACh release in freely behaving animals (Fig. 2*B*). However, the coinfusion of the D1 antagonist (1 μM) with the D1 agonist (10 μM) blocked the stimulatory effect of the latter (Fig. 2*C*).

Under the conditions used here, neither the D2 agonist quinpirole HCl (1–10 μM) (Fig. 3*A*) nor the D2 antagonists sulpiride (1–10 μM) (Fig. 3*B*) or eticlopride (10 μM, data not shown) significantly modified hippocampal ACh release. Interestingly, when infused in to the lateral septum neither the D1 nor D2 drugs had an effect on hippocampal ACh release (Fig. 4). No appreciable obvious behavioral sequelae (locomotion, sniffing, grooming, etc.) were induced by these dopaminergic drugs administered via the probe.

Diffusion of hippocampally infused ^{3}H SCH23390

^{3}H SCH 23390 (1 μM, 1 μCi) infused via the probe for 4 hr showed virtually no diffusion beyond the hippocampal area immediately in contact with the dialysis probe (Fig. 5). Considering that this concentration of the unlabelled D1 antagonist blocked the stimulation of hippocampal ACh release induced by the D1 agonist SKF 38393, it suggests that this effect is occurring locally.

Discussion

A variety of studies have demonstrated the presence of direct dopaminergic innervation into the hippocampus. This innervation is concentrated in the ventral subiculum, in particular in the presubiculum (Verney et al., 1985). Hippocampal D1 receptors, on the other hand, are mainly located in the molecular layer of the dentate gyrus and the dorsal hippocampus (Dawson et al., 1986; Grilli et al., 1988; Tiberi et al., 1991). In the present study, we observed that a significant portion of D1 receptors in the molecular layer of the dentate gyrus was lost following fimbriaectomy. This suggests that a proportion of hippocampal D1 receptors are located presynaptically on afferent terminals. Moreover, a concomitant reduction in hippocampal ChAT activity was observed as a result of the lesion. Considering that a

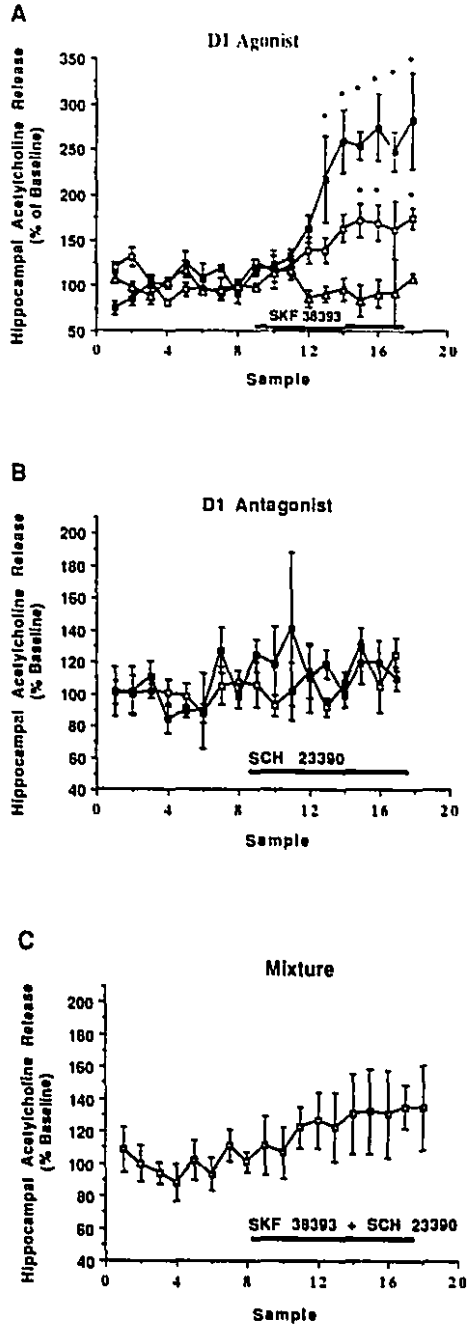

Figure 2. Effects of the infusion of the D1 agonist (+)SKF 38393 [*A*: 10 μM, □; 100 μM, ■; or its inactive enantiomer (−)SKF 38393 10 μM, Δ], the D1 antagonist SCH 23390 (*B*: 1 μM, □; 10 μM, ■), or their combination (*C*: 10 μM (+)SKF 38393 and 1 μM SCH 23390) on hippocampal ACh efflux. Data represent mean ± SEM (n = 4 for each concentration). Following baseline determination, Ringer's solution containing the drug of interest was perfused through the probe implanted in the hippocampus for the period indicated by the black bars (upto 4 hr). Dialysate ACh content, measured by GC-MS, is expressed as a percent of baseline. Baseline was calculated from the average of eight samples preceding drug infusion. Statistical evaluation was evaluated by one-way analysis of variance (ANOVA). *, $p < 0.001$; significantly different from baseline. Only the D1 agonist induced significant effects (*A*) that were blocked by the a coinfusion of the antagonist (*C*).

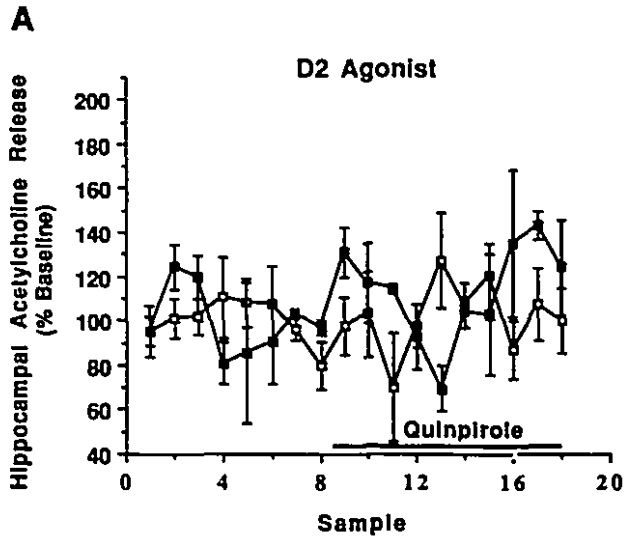

Figure 3. Effects of the infusion of the D2 agonist quinpirole (*A*: 1 μM, □; 10 μM, ■) and the D2 antagonist sulpiride (*B*: 1 μM, □; 10 μM, ■) on hippocampal ACh efflux. Data represent mean ± SEM (n = 4 for each concentration). Following baseline determinations, Ringer's solution containing the drug of interest was perfused through the probe implanted in the hippocampus for the period indicated by the *solid bars* (up to 4 hr). Dialysate ACh content, measured by GC-MS, is expressed as a percent of baseline. Baseline was calculated from the average of eight samples preceding drug infusion.

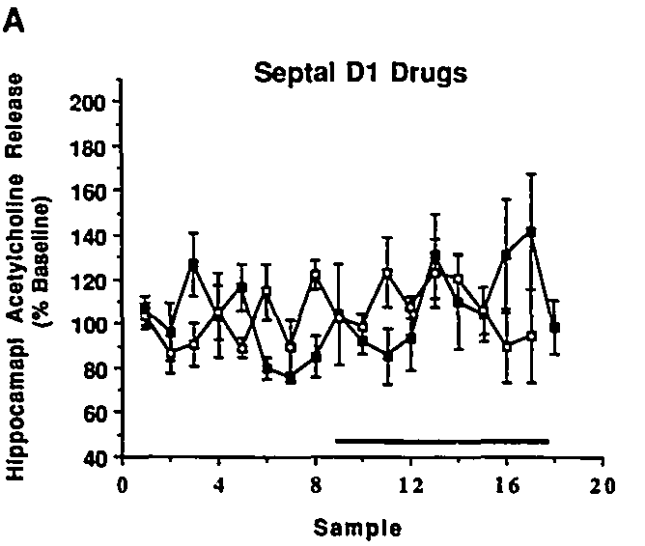

Figure 4. Effects of the septal infusion of the D1 ligands (*A*: SKF 38393 10 μM, □; SCH 23390 10 μM, ■) and the D2 ligands (*B*: quinpirole 10 μM, □; sulpride 10 μM, ■) on hippocampal ACh efflux. Data represent mean ± SEM (n = 4 for each ligand). Following baseline determinations, Ringer's (minus neostigmine) solution containing the drug of interest was perfused through the probe implanted in the septum for the period indicated by the black bars (up to 4 hr). Samples were collected from the probe implanted in the dorsal hippocampus. Dialysate ACh content, measured by GC-MS, is expressed as a percent of baseline. Baseline was calculated from the average of eight samples preceding drug infusion.

et al., 1985), it is logical to assume that at least some of the lost D1 receptors are located on cholinergic nerve terminals. Interestingly, in senile dementia of the Alzheimer's type (SDAT), a marked reduction in hippocampal D1 receptors has been reported (Cortes et al., 1988), with the highest loss (89%) seen in the molecular layer of the dentate gyrus. It is well known that one of the hallmarks of SDAT is the destruction of basal forebrain cholinergic neurons which, in part, project to the hippocampus (Davies and Maloney, 1976; Whitehouse et al., 1982; Coyle et al., 1983). Therefore, in agreement with these studies, the reduction in hippocampal D1 receptors observed here could be due to the loss of presynaptically located receptors resulting from the cholinergic denervation. Nevertheless, the possibility that some of these D1 receptors are located on afferents other than cholinergic that are contained within the fimbria-fornix projection cannot be excluded solely on the basis of the present experiments.

D2 receptors, on the other hand, are found in the hippocampus

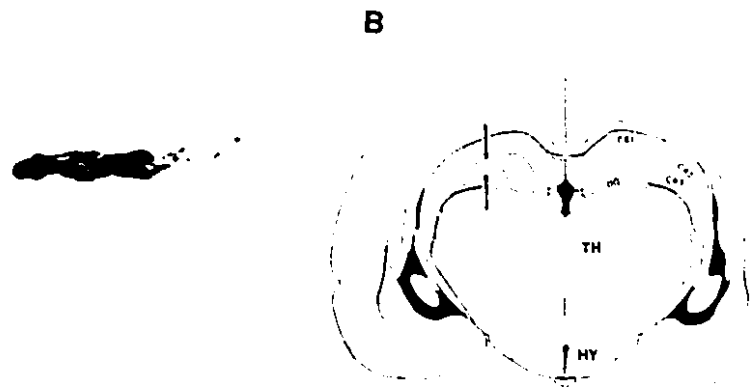

Figure 5. Diffusion of trans-hippocampally infused ^{3}H SCH 23390 in brain parenchyma. ^{3}H SCH23390 (1 μM, 1 mCi/mmol) was perfused for 4 hr in the dorsal hippocampus as described in Materials and Methods; 20 μm brain sections from these animals were exposed to tritium sensitive films and photomicrographs taken. *A* shows the extent of diffusion of the radioactive material; *B*, a schematic representation of the section in *A*. The *arrows* mark the location of the dialysis probe. It is rather evident that diffusion was minimal and the infused radioactivity remained in close proximity to the dialysis probe. Abbreviations: *CA*, Ammon's horn; *DG*, dentate gyrus; *HY*, hypothalamus; *TH*, thalamus.

in very low amounts compared to D1 receptor levels (Fig. 1*D*). In the dorsal hippocampus, D2/^{3}H raclopride binding sites are mainly located in the CA1 and subiculum regions (Fig. 1*C*). In the present study, no alteration in hippocampal D2 receptor densities were observed following fimbriaectomy. It would thus appear that this dopamine receptor subtype is not directly associated with the septo-hippocampal cholinergic nerve projection.

Recently, the technique of *in vivo* dialysis has been used to study the possible regulation by dopaminergic drugs of the septo-hippocampal cholinergic pathway. Systemic administration of dopaminergic drugs has shown that dopamine potentially stimulates hippocampal ACh release via both D1 and D2 receptor subtypes (Imperato et al., 1993) or the D1 subtype alone (Day and Fibiger, 1994) in young animals, and at least via D1 receptors in aged-memory impaired rats (Hersi et al., 1994). A number of possible loci exist for this apparent interaction between DA and ACh. Given the direct dopaminergic innervation of the hippocampus and the putative localization of D1 receptors on hippocampal terminals (see above), a direct modulation of hippocampal ACh release by DA drugs is a likely possibility.

The D1 receptor agonist (+)SKF 38393 applied directly into the hippocampus via the dialysis probe stimulated, in a concentration dependent, ACh release. This effect was blocked by the D1 antagonist SCH 23390 while the inactive stereoisomer (−)SKF 38393 had no effect by itself, attesting to the specificity of the effect for the D1 receptor family. SCH 23390 infused alone in the dialysis probe did not alter hippocampal ACh levels. These findings suggest that the D1 receptors located in the dorsal hippocampus can enhance ACh release and that this action is likely phasic in nature, in view of the lack of effect of the D1 antagonist alone.

A question arises, however, as to the extent of diffusion of locally applied drugs in the *in vivo* dialysis paradigm used here. This is especially pertinent considering the relatively long period of drug infusion. Administration of ^{3}H SCH 23390 under conditions identical to those used for the nonradioactive drugs, showed that diffusion is rather minimal and that the ligand is mostly, if not exclusively, found in the area surrounding the dialysis probe (Fig. 5). Thus, it would appear that local interaction can account for the effects of D1 drugs on hippocampal ACh release as observed in the present study or following systemic administration (Imperato et al., 1993; Day and Fibiger, 1994; Hersi et al., 1994).

In contrast to D1 drugs, neither the stimulation nor the blockade of D2 receptors by local, intraprobe infusion of prototypical drugs into the dorsal hippocampus had any effect on ACh release.

Another possible locus for ACh/DA interaction is the lateral septal area. Dopaminergic projections arising from the VTA and terminating in the lateral septum were postulated to interact with the cholinergic cell bodies of the medial septum that give rise to the cholinergic septo-hippocampal pathhway (Wood, 1985). In the present study, the manipulation of neither D1 nor D2 receptors in the septal area had any effect on hippocampal ACh release. Thus, it would appear that dopamine stimulates hippocampal ACh release by acting on D1 receptors located in the hippocampus. However, although the lesion data presented here suggests that these D1 receptors are located on cholinergic terminals, the possibility of transsynaptic action involving interneurons or other noncholinergic afferents cannot be ruled out at the present time.

In the classical view of the synapse, a close juxtaposition must exist between the nerve fiber terminals enriched with a given neurotransmitter and its postsynaptic receptors. However, this is clearly not always the case and may even be the exception (Beaudet and Descarries, 1978). Discrepancies between the localization of receptors and the distribution of the relevant neurotransmitter has been referred to as the mismatch issue (Kuhar, 1985; Herkenham, 1987). In the present situation, the hippocampal dopaminergic innervation, arising mainly from the VTA and the substantia nigra, is mostly restricted to the ventral hippocampus (Verney et al., 1985). In contrast, hippocampal dopaminergic receptors are predominantly found in the dorsal hippocampus (Dawson et al., 1986; Grilli et al., 1988; Tiberi et al., 1991; this study). Interestingly, however, more than 40% of the dopamine present in the hippocampus has been proposed to be located within dorsal noradrenergic terminals (Bischoff et al., 1979). It may be conceivable that, under certain conditions, dopamine and noradrenaline may be coreleased from the terminals of the latter present in the dorsal hippocampus. It is also tempting to suggest that crossover between neurotransmitters may exist to partly account for the apparent mismatches. For instance, under excessive cell firing conditions, the release of noradrenaline in quantities large enough to saturate its own receptors may lead to the binding of this neurotransmitter to other related receptor families such as those for dopamine. In this way, a secondary level of complexity would be added to code for signal strength and more effective transmission.

Another intriguing possibility to account for apparent ligand–receptor mismatches is known as volume transmission (VT). In

contrast to the classical synaptic mode of signal transmission. VT refers to the diffusion of chemical signals in the extracellular fluid. A neurotransmitter could thus act at a considerable distance from its site of release (for a recent review, see Benfenati and Agnati, 1991). For example, it has been reported that when transient parkinsonism is induced in cats following MPTP administration, dopamine released from spared ventral striatum terminals can diffuse over a distance of 5.5–7.0 mm to the more extensively denervated dorsolateral striatum (Schneider et al., 1994). Similarly, dopamine released from fiber terminals located in the ventral hippocampus could diffuse to the dorsal part where the dopaminergic D1 receptors are mostly found. However, direct evidence in support of this possibility have yet to be provided. Interestingly, D1 receptors have, in the past, been linked with volume transmission in areas such as the median eminence and globus pallidus (Fuxe et al., 1988). In any case, it is evident from the data reported here that the activation of D1 receptors present in the dorsal hippocampus can modulate ACh release and are hence fully functional.

Finally, it should be added that the physiological significance of the dopaminergic system in the hippocampus is not yet clear. Intrahippocampal injections of dopamine receptor agonists evoke theta rhythms in hippocampal electroencephalograms (Smialowski, 1985). This type of rhythmic oscillation is known to occur during periods of learning (Winson, 1978). Moreover, during theta oscillation, hippocampal synapses are in a state of heightened plasticity and the stimulatory requirements for the induction of long term potentiation (LTP) are markedly reduced (Huerta and Lisman, 1993). Interestingly, a well established role of the hippocampal cholinergic nerve terminals is to elicit theta rhythms (Bland, 1986). Most recently, dopamine was reported to modify LTP in the Schaffer collateral pathway of the rat hippocampus via D1 receptors (Huang and Kandel, 1995). In addition, mnemonic deficits in aged rats can be attenuated by D1 receptor agonists, and this effect was proposed to be mediated by the release of ACh in the hippocampus (Hersi et al., 1994). Therefore, it appears that a role for DA in the hippocampus could, at least in part, be associated with learning and memory, likely via the modulation of hippocampal cholinergic functions.

In summary, a certain proportion of hippocampal D1 receptors appears to be located directly on septo-hippocampal cholinergic nerve terminals. Moreover, *in vivo* hippocampal ACh release is facilitated by the local stimulation of D1 but not D2 receptors. Recent data have clearly shown that the D1 receptor family comprises both the d1 and d5 subtypes (see introductory section). At present, it is unclear which of these two subtypes is involved in the modulation of hippocampal ACh release as selective probes have yet to be developed to discriminate between these members of the D1 receptor family. Other strategies such as the use of functional receptor antibodies or oligonucleotide antisenses would have to be used.

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DOPAMINE D_1 RECEPTOR LIGANDS MODULATE COGNITIVE PERFORMANCE AND HIPPOCAMPAL ACETYLCHOLINE RELEASE IN MEMORY-IMPAIRED AGED RATS

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Abstract—The possible modulation by D_1 drugs of learning abilities of a population of aged memory-impaired animals was investigated in the present study. The level of D_1 [^{3}H]SCH 23390 receptors was first examined by quantitative autoradiography to ascertain if cognitive deficits seen in these animals could be related to alterations in the levels of these receptors. No significant differences in [^{3}H]SCH 23390 binding were observed in any of the brain areas examined between young, and aged memory-unimpaired and aged memory-impaired animals. However, the cognitive deficits of the aged-impaired rats were modulated by D_1 drugs. The D_1 agonists SKF 38393 and SKF 81297 (3.0 mg/kg, i.p.) significantly reduced the latency period to find a hidden platform in the Morris Water Maze, reflecting improved cognitive functions, while the D_1 antagonist SCH 23390 (0.05 mg/kg, i.p.) had no overall significant effect. Moreover, the D_1 agonist SKF 38393 increased, whereas the antagonist inhibited, *in vivo* hippocampal acetylcholine release.

Taken together, these results suggest that functional hippocampal acetylcholine dopamine interactions exist in aged memory-impaired rats. More importantly, the cognitive deficits seen in the aged-impaired rats can be attenuated by stimulations of D_1 receptors, hence suggesting an alternative approach to alleviate the cognitive deficits seen in the aged brain.

Key words: spatial memory, aging, hippocampus, *in vivo* dialysis.

A substantial body of evidence has implicated the central cholinergic system as an integral component of the neural circuitry associated with learning and memory (for recent reviews see Refs 14, 42 and 49). For example, the systemic administration of muscarinic cholinergic receptor antagonists disrupts the performance of animals in a variety of tasks such as operant matching/non-matching to sample[5,46] and spatial memory tasks including the radial arm maze[6,41,54] and Morris Water Maze.[9,55,57] In addition, lesion studies have, in general, highlighted the importance of the cholinergic system in learning and memory. For instance, lesions of the cholinergic neurons of the nucleus basalis magnocellularis or the medial septum/diagonal band of Broca disrupt the performance in memory tasks sensitive to cholinergic blockade.[4,18,38,56] Besides acetylcholine (ACh), however, several other neurotransmitter systems likely play a role in learning and memory (for review see Ref. 14). One such neurotransmitter is dopamine (DA).

There is a well characterized reciprocal interaction between ACh and DA in the control of normal motor activity at the level of the basal ganglia (for recent review see Ref. 50). More recently, it has become evident that complex interactions also exist between ACh and DA in the normal functioning of memory and cognition. Mnemonic deficits brought on by denervation or pharmacological blockade of central cholinergic systems can be modulated by dopaminergic drugs. For example, memory deficits induced by muscarinic receptor antagonists are attenuated by DA antagonists[32] acting on D_1 receptors,[28] whereas cognitive impairments resulting from nicotinic receptor blockade are exacerbated by D_2 receptor antagonists.[33,34] Conversely, impairments in avoidance learning,[1] operant responding[30] and conditioned avoidance[2] caused by the systemic administration of

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Abbreviations: ACh, acetycholine; AI, aged-impaired; AU, aged-unimpaired; DA, dopamine; GC-MS, gas chromatogruphy-mass spectroscopy SCH 23390, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzapine hydrochloride; SKF 38393, R(+)-2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-benzazepine hydrochloride; SKF 81297, (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide.

the non-specific DA receptor antagonist haloperidol are attenuated by the muscarinic antagonist scopolamine, the latter at doses that do not have effects by themselves.

Lately, however, questions have been raised about the validity of equating mnemonic deficits induced by acute pharmacological manipulations or discrete mechanical or chemical lesions with those occurring during the normal aging process.[16,22] For instance, the cholinergic deficits observed in aging occur within a context of age and/or disease-related pathologies in other neurotransmitter systems. Even more relevant here is the observation that the well characterized reciprocal antagonistic interaction between ACh and DA in the basal ganglia is altered in senescent animals.[27] For example, the dopaminergic agonist apomorphine inhibited K^+-evoked ACh release from striatal slices of young and middle-aged, but not old, animals. Similarly, the muscarinic agonists, oxtremorine and carbachol, inhibited DA-stimulated adenylate cyclase in cell preparations obtained from young and middle-aged, but not from old rats.[27]

Therefore, the purported ACh–DA interactions in cognitive processes seen in young animals must be reinvestigated in a model that better approximates the conditions of the aged brain. Memory loss in the aged human population varies widely from the so-called successful aging, whereby an individual performs most memory tasks as well as young cohorts to the severe deficits seen, for example, in Alzheimer's disease. Analogously, about 30% of aged (24–25 months) Long–Evans rats exhibit impairments in spatial memory as evidenced by a poor performance in tasks such as the Morris Water Maze.[19,25,45] It is believed that these aged memory-impaired rats better represent the global dynamics of cognitive alterations occurring in aging in certain subgroups. The neuroanatomical structure most implicated in spatial memory is thought to be the hippocampal formation.[8,36] Interestingly, the majority of DA receptors in the hippocampus are of the D_1 (d1/d5) subtype.[10,40] Accordingly, in this study, we examined (i) the level of D_1 receptor as a function of age and cognitive status, (ii) whether the memory deficits that characterize the aged-impaired (AI) animals can be modulated with D_1 drugs, and (iii) the effect of D_1 receptor agonist and antagonist on *in vivo* hippocampal ACh release.

EXPERIMENTAL PROCEDURES

Animals and materials

Male Long–Evans rats (three to six months old) were obtained from Charles River Canada (St Constant, Quebec, Canada) and aged in our animal facilities. The animals were maintained on a 12 h light–dark cycle (lights on at 7.00 a.m.) in temperature and humidity controlled rooms. Animals were fed standard laboratory chow and had access to tap water *ad libitum*. Any animals showing signs of respiratory ailment were removed from the study. Animal care was according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council for Animal Care (CCAC).

The concentric dialysis probes (molecular weight cut-off 20,000 and a surface of 2 mm in length and 0.5 mm in diameter) were from BAS (West Lafayette, IN, U.S.A.) and the transverse probes were made from AN69 Hospal fibres (molecular weight cut-off <60,000, i.d. = 220 μm, o.d. = 310 μm). Physostigmine sulphate was from Sigma Chemical Co. (St Louis, MO, U.S.A.), while SCH 23390 hydrochloride, SKF 81297 hydrobromide and SKF 38393 hydrochloride were obtained from RBI (Natick, MA, U.S.A.). [^{3}H]SCH 23390 (80.7 Ci/mmol), ^{3}H-Hyper-films and microscale standards were purchased from Amersham Canada (Oakville, Ontario, Canada). The deuterated variant of ACh, [$^2H_4$]ACh bromide, $[(CH_3)_3NBrCD_2CD_2OC{-}(O)CH_3]$, which was used as internal standard for ACh determination, was obtained from Merck, Sharp and Dohme Isotopes (Montreal, Quebec, Canada). Developer (D-19) and fixer (Rapid Fix) were obtained from Kodak Chemical Inc. (Montreal, Quebec, Canada). All other reagents were of high-performance liquid chromatography or gas chromatography–mass spectroscopy (GC–MS) grade and purchased from either Fisher Scientific Co. (Montreal, Quebec, Canada) or Aldrich Chemicals (Chicago, IL, U.S.A.).

Behavioural screening

Young and 24–25 month old rats (n = 132) were evaluated for their learning capacities using the well established Morris Water Maze task,[19,37,45] as described previously by our group.[25,48] Briefly, animals are required to find a submerged platform (2 cm) in a pool (1.6 m diameter) of water that has been made opaque by the addition of powdered skimmed milk. The animals solve the task using only distal spatial cues provided in the testing room. Rats were given 15 trials over five successive days (three trials per day; maximum trial duration of 2 min; inter-trial interval about 20 min) with the platform submerged. At the end of this five day period, the aged animals are categorized as aged-impaired (AI) or aged-unimpaired (AU) based on their performance with respect to the young cohorts. As reported earlier,[25] aged animals demonstrate great individual differences in their learning capabilities (Fig. 1). Except for those used in D_1 drug behavioural testing, all animals were immediately run on probe trials on the fifth day with the platform elevated 2 cm above the surface of the water. These trials were conducted to ensure that the impairments were not related to any visual defects or to the animal's inability to perform the motor demands of the task.[19,37,45] For D_1 drug testing, the animals categorized as AI were subdivided into groups of overall equal performance and given three trials a day for three days starting at day 6, preceded by i.p. injection of the drug of interest (see footnote to Table 1 for further details). These animals were given probe trials immediately following termination of testing on the last day. In these probe trials, first the platform was removed altogether to determine the length of time that the animal spends in the training quadrant for a trial length of 30 s. Then the platform was reinstated and also made visible to the animal to check for any visual or motor deficits. The drug employed here were chosen based on earlier studies that showed the effectiveness of such doses in similar behavioural paradigms.[35]

D_1 receptor autoradiography

The status of D_1 ([^{3}H]SCH 23390) receptors in AI, AU and young animals was assessed using a method described in detail elsewhere.[12] In brief, 20-μm brain sections were incubated for 60 min at room temperature in 50 mM Tris–HCl buffer (pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1.0 nM [^{3}H]SCH 23390. Consecutive sections were also incubated in the presence of 1 μM SCH 23390 to ascertain the specificity of

the labelling. The sections were then rinsed five times (2 min each) in fresh ice-cold buffer. The buffer salts were removed by a rapid dip in ice-cold distilled water and the sections air dried. Autoradiograms were generated by apposing the sections alongside tritium standards to tritium-sensitive films for four weeks. The films were then developed as described before[47] and [^{3}H]SCH 23390 specific labelling quantified (fmol/mg tissue wet weight) using a computer-assisted microdensitometric image analysis system (MCID System, Imaging Research Inc., St Catherines, Ontario, Canada). Anatomical areas were identified and named according to Paxinos and Watson.[44] Significant differences between experimental groups were determined by a one-way analysis of variance (ANOVA), $P < 0.05$ being considered significant.

Probe implantation and hippocampal in vivo *dialysis*

AI rats were anaesthetized with nembutal (50 mg/kg, i.p.). Guide cannulae (BAS, West Lafayette, IN, U.S.A.) were stereotaxically implanted in the dorsal hippocampus, as described elsewhere.[48] The animals were individually housed and allowed to recover from surgery for two to three days prior to their use in the *in vivo* dialysis experiments. Sixteen to twenty hours before dialysis, a vertical probe was implanted via the guide cannula so as to give a final depth of 3 mm below the dura. Young (five months old) and some AI rats were implanted with transverse probes[48] in the dorsal hippocampus, as described elsewhere.[23] Each animal was dialysed only once. At the beginning of each dialysis experiment, animals were placed in lidless cages and connected to a BAS microlitre syringe pump in a manner as to allow them to freely move in the cages. The probes were perfused for a 1 h washout period at a flow rate of 2.34 μl/min with an Ungerstedt–Ringer solution (125 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.5 mM NaH_2PO_4, pH 7.4) containing 75 μM physostigmine.[48] Twenty-minute dialysate fractions were collected into 1-ml glass vials containing 46 μl of 0.1 N HCl and 50 pmol of deuterated ACh as internal standard. The samples were frozen immediately and stored at −80°C until assayed by GC–MS. SKF 38393 (3.0 mg/ml) and SCH 23390 (0.05 mg/ml) were dissolved in 0.9% saline solution and injected i.p. in a volume of 1 ml/kg. Following most experiments, probe location was verified by standard histological examination of the brain.

Gas chromatography–mass spectroscopy acetylcholine

ACh content of the dialysate fractions was determined by GC–MS, as described by Marien and Richard.[31] Briefly, frozen samples were lyophilized overnight, reconstituted in 250 μl acetonitrile, capped, heated at 80°C for 30 min and dried under a gentle stream of nitrogen gas. Quaternary amines present in the samples were demethylated by adding 250 μl sodium benzene thiolate solution (160 mg in 18 ml of redistilled methyl ethyl ketone and 35 μl of glacial acetic acid) under a flow of nitrogen and reacting of 80°C for 45 min. Samples were then extracted into 35 μl of citric acid and washed twice with 250 μl of pentane. Finally, the samples were extracted into 80 μl of ethyl acetate and concentrated down to 3–5 μl volume before being injected into the GC–MS (Hewlett–Packard 5987b). The amount of endogenous ACh in each dialysate was calculated from the peak area ratio of endogenous vs deuterated internal standard.[26] Calculations were not corrected for the recovery of ACh by each dialysis probe. Sample ACh content was expressed as a percentage of average baseline (six sample collections preceding drug administration). Significant differences between experimental groups were determined by a one-way analysis of variance (ANOVA), $P < 0.05$ being considered significant.

RESULTS

Behavioural screening of aged animals

Cognitive performances of young (six months; $n = 10$) and aged (24–25 months; $n = 132$) male Long–Evans rats in the Morris Water Maze were examined. An animal was designated as AI if the latency to find the platform on each of test days 2–5 was >2 S.D. higher than the mean of the young animals. AU animals showed latencies over this period that were <0.5 S.D. higher than the mean for the young animals. A significant portion (33%) of aged animals from our colony demonstrated significantly impaired performance, whereas 27% of the aged animals were clearly unimpaired. An analysis of variance (with Scheffe *post hoc* test) shows that, as a group, the AI animals differ significantly from both the young and AU animals on test days 2–5 (Fig. 1). No differences were observed between young and AU animals. The performance of these animals has also been examined in terms of the distance travelled before locating and climbing onto the platform. Analysis of these data shows the same pattern of differences as the latency figures (data not shown). When these same animals are provided an opportunity to swim towards a platform that has been raised above the level of the water (visually-cued condition), there are no differences in latency or distance measures (data not shown), indicating that the differences between groups are not related to swimming abilities.[25]

Dopaminergic D_1/[^{3}H]SCH 23390 receptors and aging

No clear differences in D_1/[^{3}H]SCH 23390 binding densities were observed between the AI and AU groups in any of the brain regions examined (Fig. 2).

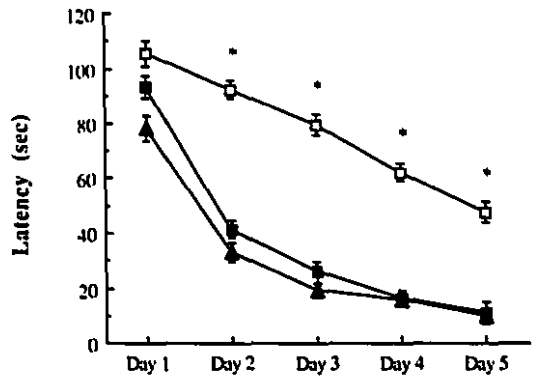

Fig. 1. Behavioural screening of aged animals. Cognitive performances of male young (six months, $n = 10$) and aged (24–25 months, $n = 132$) Long–Evans rats in the Morris Water Maze task were examined as described in the Experimental Procedures and Results section. A significant portion (33.2%) of aged animals from our colony demonstrated significantly impaired performance whereas 27.5% of the aged animals showed clearly unimpaired performance. *$P < 0.01$ compared to young animals. (□) AI; (■) AU; (▲) young.

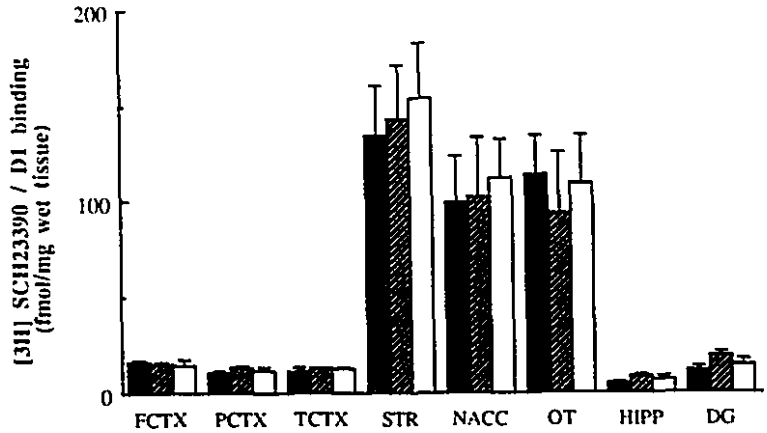

Fig. 2. Effect of aging and spatial memory impairment on D_1 receptor binding levels. Sections from AI, AU and young animals were incubated with 1.0 nM [^{3}H]SCH 23390 as described in the Experimental Procedures section. The histograms represent specific labelling obtained by subtracting non-specific from total binding. Data represent mean ± S.E.M. from six animals per group. No significant difference was observed in [^{3}H]SCH 23390 binding in any of the regions studied. DG, dentate gyrus of dorsal hippocampus; FCTX, frontal cortex; HIPP, CA1/CA2/CA3 subfields of dorsal hippocampus; NACC, nucleus accumbens; OT, olfactory tract; PCTX, parietal cortex; STR, striatum; TCTX, temporal cortex. (□) AI; (▨) AU; (■) young.

Similarly, aging *per se* does not seem to alter D_1/[^{3}H]SCH 23390 binding site levels as evidenced by comparable binding densities between the aged and young cohorts (Fig. 2).

Behavioural effects of SKF 38393, *SKF* 81297 *and SCH* 23390 *in the aged-impaired rats*

Administration of the D_1 agonists SKF 38393 and SKF 81297 (3.0 mg/kg, i.p.) to AI animals 15 min before trial resulted in a significant reduction (44% and 51%, respectively; $P < 0.05$) in the latency to find the submerged platform compared to saline-treated AI controls (Table 1). The D_1 antagonist SCH 23390 (0.05 mg/kg, i.p.) induced an average 33% increase in latency over the three days of testing (Table 1). This overall increase failed to reach statistical significance. However, when performance trend across the three days of testing was examined for the antagonist, a worsening of the deficit of the AI animals, which reached statistical significance on the last day of testing, was observed (Table 1). In probe trials wherein the platform was removed and the percentage of time spent in the training quadrant was examined, no significant differences were observed between groups (data not shown). There were no apparent differences in swim speed between experimental groups following the administration of either the D_1 agonists or the antagonist (data not shown). Moreover, during probe trials wherein the submerged platform was made visible to the animals, there were no differences in latency to find the platform following drug administration (data not shown).

Effect of SKF 38393 *and SCH* 23390 *on hippocampal acetylcholine release*

Administration of the selective D_1 agonist SKF 38393 (3.0 mg/kg, i.p.) increased hippocampal ACh release by about two-fold over baseline in both young and AI rats (Fig. 3A, B). The maximal increase in ACh release was observed approximately 40 min after the administration of the D_1 agonist. On the other hand, the D_1 antagonist SCH 23390 (0.05 mg/kg, i.p.)

Table 1. Effect of SKF 38393, SKF 81297 and SCH 23390 on latency period/learning abilities of aged-impaired animals in the Morris Water Maze task

	Latency (s)			
Group	Day 1	Day 2	Day 3	Overall
Saline	43.3 ± 11.06	26.58 ± 4.16	24.8 ± 11.06	33.20 ± 5.7
SKF 38393	23.07 ± 4.01	20.64 ± 5.26	11.49 ± 3.17	18.80 ± 2.50*
SKF 81297	24.42 ± 11.20	11.28 ± 3.76	10.93 ± 2.10	16.09 ± 4.21*
SCH 23390	44.7 ± 10.73	36.78 ± 8.46	48.87 ± 10.85*	44.40 ± 5.80

Animals that were designated as AI as described in Fig. 1 were utilized. These AI rats were injected (i.p.) with either SKF 38393 (3.0 mg/kg), SKF 81297 (3.0 mg/kg), SCH 23390 (0.05 mg/kg) or vehicle (0.9% saline) 15 min prior to the first testing of the day. Three trials separated by 10 min rest period were administered per animal. Each animal underwent a total of nine trials over a three day period. Data represent group mean ± S.E.M. of five different animals, except for the SKF 81297 group, where $n = 3$. *$P < 0.05$.

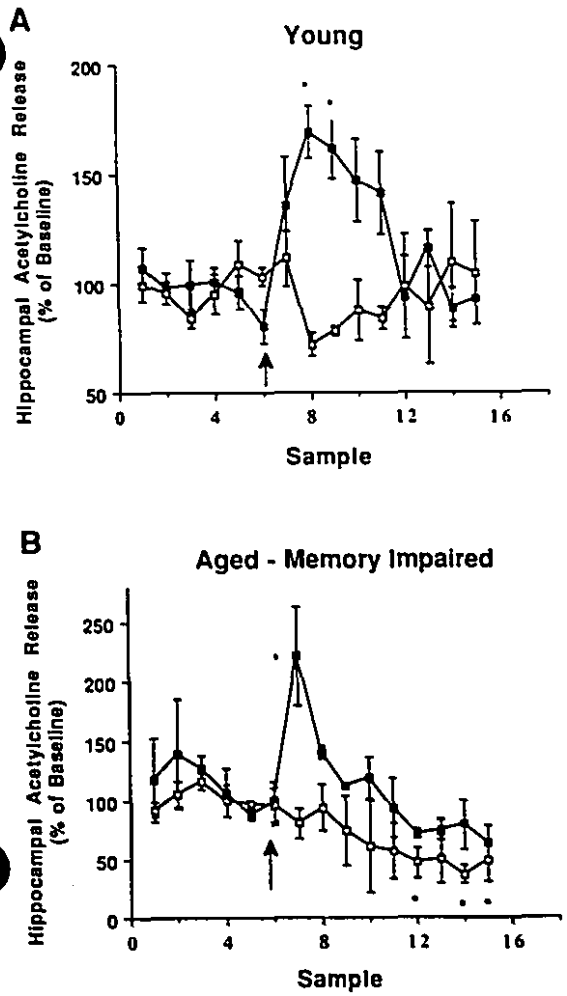

Fig. 3. Effect of D_1 receptor agonist, SKF 38393, and antagonist, SCH 23390, on *in vivo* hippocampal ACh release of young and AI rats. Data represent group mean ± S.E.M. for SKF 38393 ($n = 4$ for AI and $n = 5$ for young) and SCH 23390 ($n = 5$ for AI and $n = 4$ for young); 0.9% saline solutions containing either SKF 38393 (3.0 mg/kg, ■) or SCH 23390 (0.05 mg/kg, □) were injected i.p. following baseline collection (as indicated by the arrow). Dialysate ACh content, measured by GC–MS, is expressed as a percentage of baseline. Baseline is the average of six samples preceding drug application. $^*P < 0.05$: significantly different from baseline.

transiently inhibited ACh release in young animals, whereas in AI rats this inhibition persisted and reached statistical significance by about 2 h following drug administration (Fig. 3A, B).

DISCUSSION

Various studies using pharmacological manipulations have suggested the existence of complex interactions between ACh and DA in learning and memory processes. However, questions have been raised against equating memory deficits induced by pharmacological manipulations with those occurring during normal aging and in disease states. In our studies, we sought to address this point by examining ACh–DA interactions in the AI rat model. The majority of DA receptors in the hippocampus, a structure most implicated in learning and memory, are of the D_1 (d1/d5) subtype.[10,40] Therefore, we examined the status of brain D_1 receptor levels in AI vs AU, and in young animals. No apparent differences in the densities of D_1 binding sites were observed in any of the brain areas examined between AI and AU animals. Furthermore, no significant differences in hippocampal D_1 binding levels were observed between the young and aged cohorts. However, the D_1 agonists SKF 38393 and SKF 81297 improved the cognitive abilities of the AI rats in the Morris Water Maze task. D_1 drugs also modulated hippocampal ACh release, thus suggesting a possible mechanism for the observed behavioural effects.

Numerous studies have examined the status of various neurotransmitters in AI animals by monitoring changes in receptor levels. The most consistent finding appears to be that of rather normal densities. For example, the present consensus is that the total number of muscarinic receptors does not change in AI compared to AU and young animals (for review see Refs 13, 29 and 51, but also see Refs 7 and 20), although a specific subtype may be altered.[3] In the present study, we report that levels of dorsal hippocampal D_1 receptors are apparently unchanged during aging and in the AI rats. This finding also suggests that endogenous DA levels are not altered in these animals. In fact, it has been shown that neither the levels of DA nor those of its metabolites are altered in AI animals.[20] Nevertheless, the maintenance of normal receptor densities does not necessarily mean that these receptors are functionally intact. For instance, the efficiency of receptor transduction mechanisms could be altered with age, and in various disease processes.[17,39,43,52] In fact, although cortical D_1 receptor densities are unchanged in Alzheimer's disease, a reduction in the number of the high-affinity agonist states of these receptors has been reported.[15] The functional significance of this finding remains to be established.

The D_1 agonists SKF 38393 and SKF 81297 significantly improved the cognitive performance of AI animals in the Morris Water Maze task. On the other hand, the antagonist SCH 23390 had no overall significant effect on the memory deficits of these animals, although a worsening of the performance trend of the AI animals across days was observed. In order to demonstrate a statistically overall effect of the antagonist at the dose used, an increase in the latency between drug administration and behavioural testing would likely be required to coincide with the time of likely inhibition (2 h) of hippocampal ACh release (Fig. 3B). Alternatively, there could be a ceiling phenomenon in the magnitude of the deficit that can be demonstrated in this task. In addition to measuring spatial learning ability, the Morris Water Maze task also involves both visual and motor components.[19,37,45] However, at the doses used in the

present study, neither of the D_1 drugs had an effect on visually cued performance as evidenced by probe trials. Similarly, motor side effects were absent as the swim speeds between drug-treated and saline-treated AI animals were comparable. Thus, it seems likely that the behavioural effects of the D_1 drugs in the AI rats resulted from the modulation of cognitive abilities, although effects on attentional and/or motivational mechanisms cannot be excluded at this time.

There is a series of studies, most notably by Levin and collaborators (for a review see Ref. 28), that strongly suggest the existence of interactions between ACh and DA in learning and memory processes in young animals. With regards to D_1 receptors, these investigators found that scopolamine-induced radial arm maze choice accuracy deficits are reversible by the co-administration of the D_1 antagonist SCH 23390.[28,32] In contrast, we report here that spatial memory deficits of the AI rat are reversible by the D_1 agonists SKF 38393 and SKF 81297. One possible explanation for this apparent discrepancy relates to the type of memory being examined by the tasks used in these respective studies. Although both radial arm maze and Morris Water Maze evaluate spatial working memory, the former is the "effortful" type as compared to the latter, which examines memory of rather "automatic" nature.[8,21,36] Therefore, it is quite conceivable that these two types of mnemonic processes are subserved by distinct neuroanatomical structures or pathways that might be differentially sensitive to dopaminergic drugs. Another more likely possibility relates to the nature of the memory deficit being investigated. For instance, concerns have been raised against equating memory deficits induced by pharmacological manipulations with those occurring as the result of normal aging.[16] For example, whereas muscarinic antagonists like scopolamine produce an acute and short-lasting blockade of cholinergic receptors, aging is a slowly progressing, irreversible process that involves, in addition to changes in pre- and postsynaptic cholinergic functions,[20,48] substantial alterations in various other neurotransmitter systems.[16] Accordingly, caution must be exercised when generalizing from data based on acute pharmacological manipulation in young and healthy animals to cognitive deficits seen in aging.

A number of possible mechanisms could explain the behavioural effects of the D_1 drugs in the AI animals. Most prominent among these, however, is the septohippocampal cholinergic projection. This pathway is thought to be a crucial component of the circuitry involved in the type of memory examined by the Morris Water Maze.[8,36] Interestingly, the modulation of hippocampal ACh release by the D_1 drugs in the AI animals (Fig. 3B) mirrored the effects of these same drugs on spatial learning abilities (Table 1). This suggests that hippocampal ACh activity may underlie, at least in part, the changes in the cognitive performance of these animals. A possible site of action of the D_1 drugs in modulating hippocampal ACh release is the septal nuclei. It is well established that ventral tegmental area dopaminergic perikarya send projections to the lateral septum.[53] These dopaminergic terminals are thought to inhibit, via GABAergic interneurons, the activity of medial septal neurons which give rise to the septohippocampal cholinergic pathway.[58] However, if indeed this was the case, effects opposite to those observed here would have been expected: the D_1 agonist, via its stimulation of the release of GABA, indirectly inhibiting hippocampal ACh release. A more likely locus for the interaction between D_1 receptors and hippocampal ACh release is within the hippocampus. In fact, the existence of direct, albeit sparse, dopaminergic innervation in the hippocampus arising from the ventral tegmental area and the substantia nigra has been established.[53] The hippocampal formation is also relatively enriched with D_1 receptors and a proportion of these receptors is postulated to reside directly on dorsal hippocampal cholinergic terminals, where D_1 receptors were shown to locally modulate ACh release.[23] Apart from the hippocampus, however, the frontal cortex is also likely to be involved in the performance of the Morris Water Maze task and, interestingly, D_1 drugs have previously been shown to modulate cortical ACh release in a manner analogous to the hippocampus.[10a,b] Therefore, in addition to the hippocampus, the frontal cortex could be a possible site for the behavioural effects of the D_1 drugs utilized in our study. Moreover, the effect of these D_1 drugs on other neurotransmitters remains to be investigated.

Finally, our findings on hippocampal ACh release in AI animals are in general very similar to those seen in young animals.[11,24,this study] Thus, unlike the reciprocal interactions between striatal ACh and DA innervations which are reportedly affected in aging,[27] hippocampal ACh–DA D_1 receptor interactions generally appear to be maintained as the animal ages and becomes cognitively impaired.

CONCLUSIONS

The spatial learning deficit seen in the AI rats was significantly attenuated by the D_1 agonists SKF 38393 and SKF 81297. Our data also suggest that an underlying mechanism could relate to the modulation of hippocampal ACh release. In light of the relatively limited success of cholinesterase inhibitor-based therapies in age-associated memory deficits, an interesting alternative possibility could be the use of D_1 agonists.

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