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The muscarinic receptor and the hippocampus: An ionic channel analysis

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THE MUSCARINIC RECEPTOR AND THE HIPPOCAMPUS : AN IONIC CHANNEL ANALYSIS

by

DEBORAH M. BENSON

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1992

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5/20/92
Date

Chair of Examining Committee

June 29, 1992
Date

Herbert D. Saltzstein
Executive Officer

Supervisory Committee

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Abstract

THE MUSCARINIC RECEPTOR AND THE HIPPOCAMPUS : AN IONIC CHANNEL ANALYSIS

by

DEBORAH M. BENSON

Sponsor: Dr. Thomas Frumkes
Advisor: Dr. Emmanuel Landau

The effects of carbachol, a cholinergic agonist, on hippocampal pyramidal neurons were studied in tissue slices of both normal and denervated hippocampal pyramidal cells, utilizing intracellular electrophysiological recording techniques. The calcium-dependent potassium current (I_{AHP}) and the voltage-dependent potassium current (I_M) were both reversibly blocked by the application of carbachol (5-10 μM). Carbachol (1-10 μM) induced a steady inward current (or depolarization) under conditions in which both I_{AHP} and I_M were inactive. This effect of carbachol was reversed by atropine and blocked by pirenzepine, indicating that it is mediated by activation of an M_1 muscarinic receptor. The carbachol effect was also blocked by potassium channel blockers cesium, tetraethylammonium and barium. Current-voltage (I-V) relationships were examined in carbachol and control conditions. In most cells, carbachol induced an inward current which reversed direction at a negative membrane potential. Furthermore, the reversal potentials induced by carbachol shifted in a

positive direction when potassium concentration in the bath was increased. Thus, a primary effect of carbachol in these cells is blockage of a potassium leak channel.

In some cells, the carbachol-induced current did not reverse direction at a negative membrane potential. To examine further this effect, the effects of carbachol were compared to those of serotonin, which increases a "pure" potassium conductance, at varying potassium concentrations. Serotonin (10 μM) produced an outward current which reversed direction in each cell near the potassium equilibrium potential. The carbachol reversal potential values were negative relative to those of serotonin at 5 and 10 mM of potassium, but were not significantly different at 25 mM of potassium. Possible explanations for the anomalous carbachol effects, including a carbachol-induced dendritic conductance increase, are discussed.

Finally, the effects of carbachol were compared in voltage-clamped hippocampal pyramidal neurons obtained from normal and fimbria-fornix-lesioned rats. A significant increase in sensitivity to carbachol was seen in denervated neurons. This supersensitivity was demonstrated on both the inward leak current and I_{AHP}. These findings provide evidence for cholinergic denervation supesensitivity in the hippocampus. Implications regarding the role of acetylcholine in learning/memory and disease are discussed.

ACKNOWLEDGEMENTS

I dedicate this dissertation to my father, for giving me my first microscope . . . and for planting the seeds of wonder and awe in my soul.

And my greatest thanks to those who helped me to go on when his passing almost stopped me in my tracks -

To my mother, for all the love and patience throughout the years, and for always being there.

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To L.L., for the gift of the I Ching... and whose spirit and poetry in motion so inspired my own movement to *Work on What Has Been Spoiled*.

And, always, to L.T., whose love, passion, and steadfast belief in my ability to succeed has meant so very much... and whose own success and ongoing journey has taught me a great deal, and given me much to aspire to.

"Decisiveness and energy [have taken] the place of the inertia and indifference that have led to decay, in order that the ending may be followed by a new beginning" [Ku]

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CHAPTER ONE : INTRODUCTION

Studies over the last decade evaluating normal and dysfunctional mechanisms of learning and memory have taken both neurochemical and physiological approaches with acetylcholine examined extensively in the former approach (see reviews: Bartus, Dean, Beer & Lippa, 1982; Beninger, Wirsching, Jhamandas & Boegman, 1989; Gray, Enz & Spiegel, 1989; Smith, 1988) and the hippocampus examined extensively in the latter approach (see reviews: Barnes, 1988; Matthies, 1989; Milner, 1989; Squire, 1987; Squire & Zola-Morgan, 1988). To appraise more precisely the underlying physiological substrates of hippocampal function, the hippocampal slice preparation (Nicoll & Alger, 1981; Schwartzkroin, 1975; Segal, 1982; Skrede & Westgaard, 1971) was conceived, allowing punctate intracellular analyses in combination with pharmacological manipulations. Using this technique, the present studies were designed to focus on two aspects of cholinergic actions in the hippocampus. First, the ionic mechanism(s) and pharmacology underlying cholinergic excitation in the rodent hippocampus were studied. Second, given the well-known understanding of hippocampal afferents and efferents (see reviews: Brown & Zador, 1990; Mesulam, Mufson, Wainer & Levey, 1983; Storm-Mathisen, 1977; Storm-Mathisen & Blackstad, 1964) and the dysfunctional effects following their transection (van der Staay, Raaijmakers, Lammers & Tonnaer, 1989; Zola-Morgan, Squire & Amaral, 1989), the response of the rodent hippocampal cholinergic system to experimentally-induced denervation was examined. The following sections of the Introduction will provide a

background and rationale for these experiments:

a) the hippocampal cholinergic system;

b) electrophysiological actions and ionic mechanisms underlying cholinergic effects in the hippocampus;

c) hippocampal denervation and transplantation - models for injury and treatment; and

d) a rationale for the present studies.

A. THE HIPPOCAMPAL CHOLINERGIC SYSTEM

The hippocampus is a bilateral structure, with a highly organized, well-defined circuitry and structure. Based on architectonic differences, it has been divided into four sequentially organized regions, CA1, CA2, CA3 and CA4 (Cajal, 1911; Lorente de No, 1934). Each of these regions is characterized by four distinct layers. The stratum pyramidale (pyramidal layer) consists of large pyramidal cell bodies, the basal dendrites of which extend to form the stratum oriens (polymorphic layer), and the apical dendrites of which extend in the opposite direction to form the stratum radiatum and the stratum lacunosum-moleculare (molecular layer). In addition, the more general terms "hippocampal formation" and "hippocampal region" include the dentate gyrus, subiculum and entorhinal cortex (Brown & Zador, 1990). As indicated in Figure 1, area CA1 lies adjacent to the subiculum, whereas area CA4 lies closest to the dentate gyrus.

The afferent and efferent connections of the hippocampus have been

well-characterized (see reviews : Amaral & Witter, 1989; Brodal, 1981; Brown and Zador, 1990). The main inputs into the hippocampus arise from the entorhinal cortex, the septal region and the contralateral hippocampus. The entorhinal cortex (itself receiving inputs from many other brain regions, including association cortices, olfactory cortex and thalamus) projects via the perforant pathway to provide a major input to the hippocampus (Powell, Cowan & Raisman, 1965). Fibers from the contralateral hippocampus (via the hippocampal commissure) represent another major input, particularly for areas CA3 and the dentate gyrus (Amaral, 1987). A third major input to the hippocampus arises from the septal region of the brain. Axons from the medial septal nucleus and nucleus of the diagonal band travel mainly via the fimbria and dorsal fornix, terminating throughout the hippocampus (Amaral & Kurz, 1985; Frotscher & Leranth, 1985). Other less extensive projections arise from the brainstem (including locus coeruleus), thalamus, hypothalamus and amygdala (Wainer & Mesulam, 1990).

Traditional descriptions of hippocampal circuitry emphasized the existence of a basic "trisynaptic circuit" (Anderson, Blackstad & Lomo, 1966), reflecting three basic types of excitatory synapses (see Figures 1 and 2a). Perforant pathway fibers from the entorhinal cortex synapse on granule cells, the principal neurons of the dentate gyrus. The dentate gyrus sends mossy-fiber axons, which are characterized by the presence of synaptic expansions at regular intervals along their length, to area CA3, where they synapse on pyramidal cells. These cells send Schaffer collateral axons which are characterized by the emergence of several axons coursing parallel to the main

Figure 1.

Schematic diagram of the neuronal elements of the hippocampal formation, including the subiculum, entorhinal cortex, dentate gyrus and areas CA1 through CA4. Also shown are stratum oriens (1), stratum pyramidale (2), stratum radiatum (3), and stratum lacunosum-moleculare (4). Perforant path input to dentate gyrus and other regions is depicted. Granule cells are shown sending mossy fibers to area CA3 pyramidal cells. Pyramidal cells in area CA3 are shown projecting via Schaffer collaterals to CA1 pyramidal cells and to other regions. (Modified from Ramon y Cajal, 1911.)

Figure 1

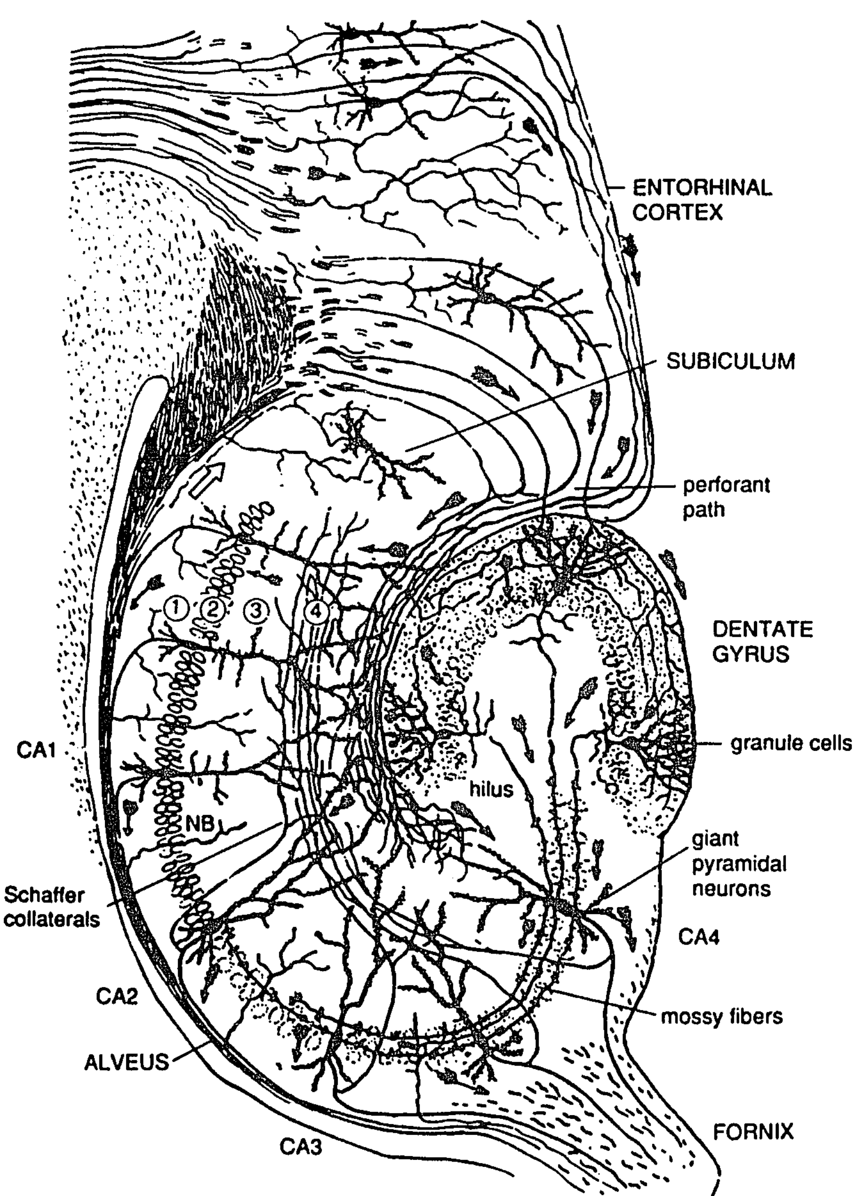
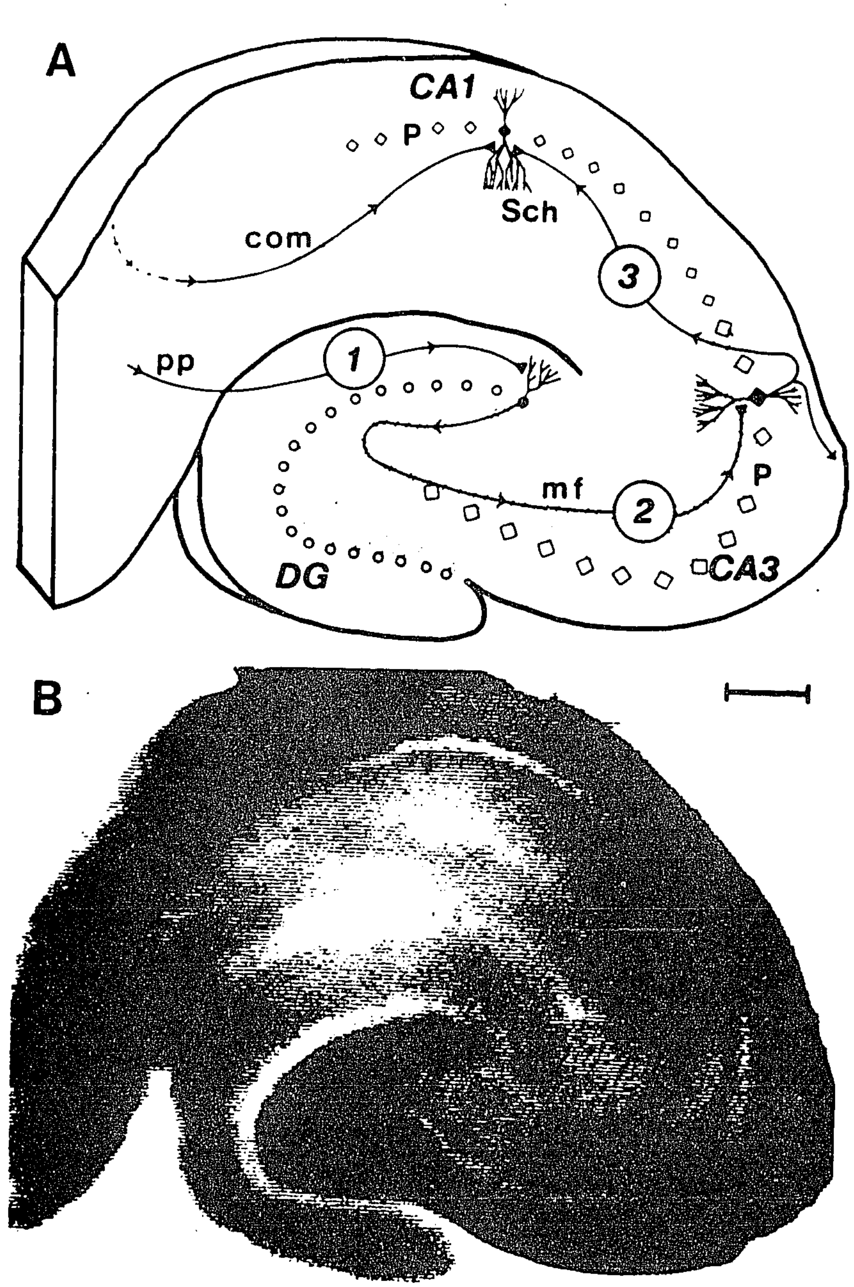


Figure 2.

(a) Schematic diagram of the tri-synaptic circuitry of the transverse hippocampal slice, showing (1) perforant path input to a granule cell in the dentate gyrus; (2) mossy fiber input to a pyramidal cell in the CA3 region; and (3) Schaffer collateral input to a pyramidal cell in the CA1 region. (b) Video microscopy of the living hippocampal slice from which A was traced. Note the clear outline (transparency) of pyramidal cell layer. Calibration bar: 55 μM. (Modified from Shepherd, 1990.)

Figure 2



fibers to area CA1, where they synapse on pyramidal cells in this region. The hippocampal pyramidal cells form the primary output from this structure, proceeding via the fimbria-fornix to innervate the lateral septal nucleus, mamillary bodies, ventromedial nucleus of the hypothalamus and anterior thalamus. Other major outputs are to the subiculum and entorhinal cortex (Amaral, Dolorfo & Alvarez-Royo, 1991; Buhl, Schwerdtfeger & Germroth, 1989; Chronister & DeFrance, 1979; Hjorth-Simonsen & Jeune, 1972), regions which provide a major source of input to the hippocampus. This trisynaptic circuit provides the possibility of a closed-loop flow of information, with the output of each region determining, in part, the input back into that region.

In addition to the single-loop, feed-forward circuitry described above, a number of additional pathways or circuits exist. For example, local circuits, characterized by pyramidal cell-interneuron interactions, also exist within the hippocampal system. These interneurons include the well-studied basket cells, and the more recently described oriens/alveus interneurons (Lacaille, Mueller, Kunkel & Schwartzkroin, 1987). These cells provide inhibitory input onto CA1 pyramidal cells, which in turn provide excitatory input back onto the interneurons. The former appear to use GABA as a neurotransmitter, while the latter may use somatostatin and/or GABA (Lacaille, et al., 1987). Thus, the circuitry and information flow through the hippocampus is considerably more complex than that implied by the simple trisynaptic notion (Amaral & Witter, 1989; Brown & Zador, 1990).

Within the hippocampus, a large number of neurotransmitters have been studied (for review, see Brown & Zador, 1990). Particularly ubiquitous are

GABA, a transmitter with widespread inhibitory as well as disinhibitory effects on various hippocampal cell types (Frotscher, Nitsch & Leranth, 1989), and glutamate, a major excitatory transmitter in the hippocampus. The actions of glutamate have been widely studied (Cotman, Flatman, Ganong & Perkins, 1986; Dingledine, 1983; Jahr & Stevens, 1987; Sah, Hestrin & Nicoll, 1989), particularly with regard to a possible involvement with long-term potentiation (see Discussion). In addition, a number of inputs from other brain regions serve as neuromodulatory influences on the hippocampus. Among these are noradrenergic projections from locus coeruleus (Storm-Mathisen, 1977), serotonergic inputs from the raphe nucleus (Moore & Halaris, 1975) and cholinergic fibers from the septal region (Mesulam, Mufson, Wainer & Levey, 1983).

This septo-hippocampal projection is of particular importance to this dissertation, and has been widely studied using anatomical, physiological and neurochemical techniques across species (Amaral & Eckenstein, 1986; Bakst & Amaral, 1984; Dutar, Lamour, Rascol & Jobert, 1986; Geneser, 1986; Green & Mesulam, 1989; Lewis & Shute, 1967; Mesulam, Mufson, Levey & Wainer, 1983; Mesulam, Mufson, Wainer & Levey, 1983; Shute & Lewis, 1967; Storm-Mathisen & Blakstad, 1964; Wainer & Mesulam, 1990). Considerable evidence exists that the medial septal nucleus (MSN) and diagonal band regions of the basal forebrain provide a major component of cholinergic projections to the hippocampus. In the macaque monkey (Mesulam, Mufson, Levey & Wainer, 1983) and rat (Mesulam, Mufson, Wainer & Levey, 1983), the ascending cholinergic system is subdivided into six basal forebrain and upper brainstem

sectors, which correspond to different connectivity patterns (pathways) and cytoarchitectonic characteristics. The "Ch1" and "Ch2" sectors consisted of choline acetyltransferase (ChAT)-positive neurons in the MSN and vertical limb of the diagonal band, respectively. The Ch1 cells are small, and intermingled with ChAT-negative cells. The ChAT-positive cells are concentrated along the midline and outer edges of the MSN, and constituted approximately 30-50 % of the cells in that region. Significant labelling of cholinergic neurons using acetylcholinesterase in this area occurred after hippocampal injection of the tracer (horseradish peroxidase (HRP)-wheat germ agglutinin (WGA)). Ch2 cells are larger than those in Ch1, and are also intermingled with ChAT-negative cells in the diagonal band. In this region, ChAT-positive cells constitute approximately 50-75% of identifiable cells. Again, significant labelling in this area occurred after injections of HRP-WGA into the hippocampus. Other sectors (Ch3-Ch6) were found to project to other, non-hippocampal targets (including olfactory bulb, globus pallidus, thalamus, hypothalamus). Also, a substantial proportion of retrograde labelling was found within acetylcholinesterase-negative perikarya in the MSN and diagonal band following hippocampal injections of HRP-WGA, raising the possibility that other, non-cholinergic, septo-hippocampal projections exist. This possibility has since been confirmed by others (Wainer, Levey, Rye, Mesulam & Mufson, 1985; and see below).

The physiological and pharmacological properties of septo-hippocampal neurons have been studied in the rat (Bland, 1986; Dutar, et al., 1986). In the latter study, nearly half (43.5 %) of the cells projecting from the medial septal nucleus to the hippocampus displayed a rhythmic, bursting pattern of

spontaneous activity. This rhythmic bursting pattern is consistent with evidence that septal neurons are partially responsible for the hippocampal theta rhythm (Andersen, Bland, Myhrer & Schwartzkroin, 1979; Kramis, Vanderwolf & Bland, 1975; Rawlins, Feldon & Gray, 1979; Stewart & Fox, 1990). Furthermore, the majority of these cells were excited by cholinergic agonists, with muscarinic agonists being more effective than nicotinic agonists.

The fact that the septo-hippocampal pathway contains non-cholinergic projections has been confirmed. Freund and Antal (1988) demonstrated the existence of GABA-containing neurons in the MSN and, using anterograde tracing techniques, showed that these neurons innervate the hippocampus, synapsing on GABA-ergic interneurons in all, but the pyramidal, regions of the hippocampal formation. Peptidergic projections are also present in the pathway from the MSN to the hippocampus (Senut, Menetrey & Lamour, 1989). While cholinergic neurons were the most numerous, galanin-positive neurons represented 22% of septo-hippocampal cells labelled, with additional, but lesser, labelling for luteinizing hormone-releasing hormone, calcitonin gene-related peptide and enkephalin in this pathway.

In sum, substantial evidence exists documenting the preponderance of acetylcholine in the hippocampus, and confirming the primary origins of hippocampal acetylcholine - that is, the MSN, through the septo-hippocampal pathway. While not exclusively cholinergic, this pathway provides the major source of acetylcholine in the hippocampus. Further characterization of the septo-hippocampal cholinergic pathway has demonstrated that these fibers terminate in all layers of the hippocampus (Frotscher & Leranth, 1985).

Recently, specific innervation of the CA1 region by septohippocampal cholinergic fibers has been demonstrated by choline acetyltransferase (ChAT) immunocytochemistry and Golgi impregnation techniques, with evidence of these fibers terminating on cell bodies, spines and dendritic shafts of CA1 pyramidal neurons (Frotscher, Nitsch & Leranth, 1989).

With regard to cholinergic receptor distribution, autoradiographic techniques have revealed the presence of both muscarinic (Cortes & Palacios, 1986; Frey, Ehrenkaufer & Agranoff, 1985) and nicotinic (Clarke, Pert & Pert, 1984; Clarke, Schwartz, Paul, Pert & Pert, 1985) receptors in the hippocampus. Highest concentrations of muscarinic receptors have been found in the dentate gyrus and CA1 regions in both rat and human hippocampus (Cortes & Palacios, 1983; Lang & Henke, 1983; Palacios & Mengod, 1989). Receptors of the M_1 subtype are the most numerous, with a much lower density of M_2 receptors. These latter receptors were localized primarily in CA1 and CA2 regions (Cortes & Palacios, 1986; Potter, Flynn, Hanchet, Kalinoski, Luber-Narod & Mash, 1984). Highest densities of nicotinic receptor binding have been found in the dentate gyrus, with low densities observed in other areas (Clarke, Pert & Pert, 1984; Clarke, Schwartz, Paul, Pert & Pert, 1985). Similar findings have been obtained using immunohistochemical and in situ hybridization techniques (for review, see Palacios & Mengod, 1989).

B. ELECTROPHYSIOLOGICAL ACTIONS AND IONIC MECHANISMS UNDERLYING CHOLINERGIC EFFECTS IN THE HIPPOCAMPUS.

It has long been established that neuronal signalling is controlled by the gating (opening and closing) of ionic channels within the cell membrane (for reviews, see Kandel, 1985; Koester, 1985). These ion channels are selective; that is, they allow primarily one type of ion (typically, Na+, K+, Cl- or Ca++) to pass through. Classically, a distinction has been made between passive channels (also known as leak channels), which are always open and are important in determining the resting membrane potential, and active, or gated, channels (Eccles, 1964; Hille, 1992). Active channels have gating mechanisms which provide the ability to open or close in response to various conditions or stimuli, and it is this class of channels which is largely responsible for the generation of action potentials and synaptic potentials. Gated (active) channels can be further subdivided into two categories: those that are voltage-sensitive, and open or close in response to changes in the membrane potential, and chemically-gated channels, which are activated or inactivated by the coupling of neurotransmitters to membrane receptors. More recently, it has been discovered that a number of channels are both voltage-gated and chemically-gated (for reviews, see Hille, 1992; Kolb, 1990; Nicoll, 1988).

Among the diversity of voltage and/or chemically gated channels, potassium (K+) channels have been among the most widely studied, particularly with regard to their role in controlling cellular excitability. A number of distinct potassium channels (and associated currents) have been described

(for reviews, see Hille, 1992; Kolb, 1990; and see Table 1 for summary). One of the earliest characterized potassium channels, known as the delayed rectifier, carries a voltage-sensitive outward current (I_K) which is largely responsible for the membrane repolarization following an action potential (Lancaster & Pennefather, 1987). This channel is blocked by tetraethylammonium (TEA), cesium and 4-amino-pyridine (Hille, 1992). A fast, transient potassium current, termed I_A, has been described in cultured rat hippocampal neurons (Nakajima, Nakajima, Leonard & Yamaguchi, 1986). It is also voltage-sensitive, becoming activated when a cell is depolarized following a period of hyperpolarization, and is thought to mediate the interspike interval in repetitively firing cells (Hille, 1992). It can be blocked by TEA and 4-amino-pyridine. A slow, voltage-sensitive potassium current (I_M) has been well-studied in hippocampal pyramidal neurons (Adams, Brown & Constanti, 1982; Benardo & Prince, 1982a,b; Halliwell & Adams, 1982). This outward current is inactive at the resting membrane potential, becoming active upon depolarization. It contributes to the accommodation of action potential discharge, and its inhibition by muscarinic agonists allows for prolonged firing (Jones, 1985). I_M can be blocked by barium (Adams et al., 1982). Two voltage-sensitive and calcium-dependent potassium currents have been described. One such current, termed I_C (Madison et al., 1987), is a fast, outward current activated upon depolarization, and serves to terminate periods of calcium entry by hyperpolarizing the cell (Hille, 1992). A much slower, calcium-dependent potassium current, I_{AHP}, is also voltage-dependent, becoming activated by calcium entry during action potential discharge. It is also an outward current,

Table 1 : Summary of Known Voltage-Dependent Potassium Channels

Current Carried	Channel's Response to Depolarization	Sensitivity to ACh	Blocking Agents
I_K (delayed rectifier)	opens	no	TEA, Cs+, 4-ap
I_A	opens	yes	TEA, 4-ap
I_M	opens	yes	Ba++
I_C*	opens	no	TEA, Cs+, charybdotoxin
I_{AHP}**	opens	yes	Cs+, apamin
anomalous (inward) rectifier	closes	no	TEA, Cs+, Ba++

* , ** - calcium-dependent K+ channels; therefore, also blocked indirectly by Ca+ channel blockers (e.g., cadmium)

** - also blocked indirectly by cAMP

and responsible for the afterhypolarization seen following repetitive firing in hippocampal pyramidal cells (Hotson & Prince, 1980). I_C can be blocked directly by TEA, cesium and charybdotoxin, and indirectly by calcium channel blockers such as cadmium (Hille, 1992). I_{AHP} can be blocked directly by cesium and apamin, and indirectly by calcium channel blockers and cAMP (Nicoll, 1988). Unlike the channels described thus far, which carry outward currents and become activated (opened) upon depolarization (thus serving to pull the membrane potential back towards the resting state), an additional potassium channel, the inward, or anomalous, rectifier, is activated during hyperpolarization and closes during depolarization (Hille, 1992; Kolb, 1990). This channel is blocked by TEA, cesium and barium ions.

In addition to being distinguished in terms of their voltage-sensitivities, kinetics and sensitivities to various blocking agents, several of these potassium channels are often indirectly influenced by neurotransmitters through activation of second messengers. In the hippocampus, a number of neurotransmitter receptors are coupled to potassium channels (for review, see Nicoll, 1988). For example, norepinephrine prevents the accommodation of action potentials by blocking I_{AHP} (Madison & Nicoll, 1982). This action is mediated by B-adrenergic receptors, and involves the production of cyclic AMP (Madison & Nicoll, 1986). GABA exerts its inhibitory effects in the hippocampus by two mechanisms. First, coupling to GABA-A receptors results in activation of chloride channels (Bormann, Hamill & Sakmann, 1987). However, an additional effect of GABA is activation of potassium channels (inward rectifier), through coupling to GABA-B receptors (Gahwiler & Brown, 1985; Newberry &

Nicoll, 1985). Serotonin exerts its inhibitory effects by activating the same potassium channels, and the coupling of both GABA-B and serotonin receptors to these channels are thought to be mediated through G-proteins (Andrade, Malenka & Nicoll, 1986).

Acetylcholine has also been thought to exert its effects in the hippocampus through coupling to potassium channels. The primary effect of acetylcholine in the hippocampus is excitatory; that is, acetylcholine causes a slow depolarization of hippocampal pyramidal cells, typically with an associated increase (over the resting state) in input resistance (Benardo & Prince, 1982a, 1982b; Cole & Nicoll, 1983, 1984; Dodd, Dingledine & Kelly, 1981; Segal, 1982), reflecting potassium channel closure. This depolarization is sometimes preceded by a rapid, short-lasting hyperpolarization (Benardo & Prince, 1982; Segal, 1982). It has been suggested that this hyperpolarizing effect involves presynaptic actions of acetylcholine; namely, excitation of inhibitory (probably GABA-ergic) interneurons (Benardo & Prince, 1982; McCormick & Prince, 1986). However, since the response can be found in the presence of tetrodotoxin (which blocks synaptic activity), it is more likely to be due to a transitory increase in potassium (K+) conductance (Segal, 1982). Similar actions of acetylcholine have been reported in cortex (McCormick & Prince, 1986) and sympathetic ganglion cells (Adams, Brown & Constanti, 1982), although acetylcholine has been shown to be entirely inhibitory in other brain regions, including the pons (Egan & North, 1986). Other excitatory actions of acetylcholine on hippocampal pyramidal neurons include increased firing rates due to blockage of the accomodation of cell discharge that normally occurs

(Cole & Nicoll, 1983), and enhancement of the slow excitatory post-synaptic potential (EPSP) characteristic of these cells (Cole & Nicoll, 1984; Madison, Lancaster & Nicoll, 1987).

The excitatory actions of acetylcholine appear to be due to actions on muscarinic receptors. First, most studies report that while muscarinic agonists mimic the effects of acetylcholine, nicotinic agonists typically fail to produce effects (Cole & Nicoll, 1983, 1984; Nicoll, 1985), although rare (1 out of 7 cells studied) excitatory responses to nicotine have been reported (Benardo & Prince, 1982). Second, such effects as depolarization, increased membrane resistance, increased firing rates and effects on membrane currents have been reversed with muscarinic antagonists, but not with nicotinic antagonists (Benardo & Prince, 1982; Cole & Nicoll, 1983, 1984; McCormick & Prince, 1986). Furthermore, nicotinic receptor activation is related to much faster cellular events than those described here, which have a relatively slow time course (Brown, 1983).

Several different subtypes of muscarinic receptors have now been described (Birdsall & Hulme, 1983; Christie & North, 1988), with at least two subtypes characterized in the hippocampus; the M_1 receptor, which is sensitive to pirenzepine and the M_2 receptor, which is insensitive to pirenzepine (Muller & Misgeld, 1986). These different subtypes appear to mediate different components of the muscarinic response.

Muscarinic-induced excitation of hippocampal pyramidal cells appears to occur through the reduction of the potassium (K^+) conductance of the cell membrane (Dodd, Dingledine & Kelly, 1981). This action is quite complex,

however. Rather than a direct coupling between muscarinic receptors and potassium channels, it has been suggested (Dodd et al., 1981) and recent evidence has confirmed (see Nathanson, 1987; Nicoll, 1988 for reviews) that activation of the muscarinic receptor leads indirectly to changes in potassium membrane permeability, through activation of various intracellular second messenger pathways. Among those implicated are protein kinase C (Nicoll, 1988), G-proteins (Brown, 1988), cyclic-GMP (Richelson & El-Fakahany, 1981) and inositol triphosphate (Dutar & Nicoll, 1988a,b). This is consistent with the observations that muscarinic effects typically evolve and dissipate slowly (Dodd et al. 1981).

The mechanism(s) of excitatory actions triggered by muscarinic receptor coupling is even more complex, however. As stated previously, this action involves, through intermediary processes, a blockade of potassium conductance. However, more than one potassium channel is involved. Muscarinic agonists have been found to block I_M in hippocampal pyramidal neurons (Adams, Brown & Constanti, 1982; Benardo & Prince, 1982a,b; Halliwell & Adams, 1982), an effect mediated through activation of a pertussis-toxin-insensitive G-protein (Brown, 1988). Muscarinic agonists have also been shown to block I_{AHP} in the hippocampus (Benardo & Prince, 1982a,b; Cole & Nicoll, 1983, 1984a,b; Madison, et al., 1987), This blockade, in addition to I_M blockade, is responsible for the loss of neuronal accommodation and increased firing rates observed with muscarinic activation. Muscarinic effects on I_{AHP} are potent; indeed, I_{AHP} is about ten times more sensitive to carbachol, a muscarinic cholinergic agonist, than I_M (Madison et al., 1987), and are most

likely mediated through activation of protein kinase C (Nicoll, 1988). Finally, cholinergic receptor activation has been shown to block I_A in cultured rat hippocampal neurons (Nakajima, Nakajima, Leonard & Yamaguchi, 1986), although this action does not contribute to acetylcholine's significant long-lasting excitatory effects on pyramidal cells.

It has been suggested that muscarinic-induced depolarization of hippocampal pyramidal cells is due to blockade of I_M (Adams, et al., 1982) or to a combination of I_M and I_{AHP} blockade (Benardo & Prince, 1982). If either of these were the case, however, then no acetylcholine-induced depolarization should be observed under conditions when all of the above-mentioned channels are inactive. Such conditions would include the presence of increased intracellular cAMP, which blocks I_{AHP}, and when the cell is held at potentials negative to -60mV, where both I_{AHP} and I_M are blocked. Recent evidence, however, indicates that, even under conditions where both I_M and I_{AHP} are blocked, a depolarization, or inward current shift, can still be obtained with muscarinic agonists (Jones, 1985; Landau, Blitzer, Benson & Davis, 1986; Madison et al., 1987). Thus, the depolarizing effects appear to be due to actions on an additional current(s). However, the nature of this current is as yet unknown and requires further study. Additionally, mechanisms underlying the responsiveness of hippocampal cells to muscarinic stimulation following injury have yet to be fully explored.

C. HIPPOCAMPAL DENERVATION AND TRANSPLANTATION - MODELS FOR INJURY AND TREATMENT

Experimental denervation has been used as a model for injury to various neuronal systems. Denervation studies are often performed to explore the extent to which the brain is altered, structurally or functionally, following injury or damage, as well as its potential for plasticity, in terms of recovery following damage. One of the most widely studied regions concerning the effects of denervation is the hippocampus; particularly, the septo-hippocampal pathway, which provides the major source of cholinergic input to the hippocampus (Amaral & Eckenstein, 1986; Bakst & Amaral, 1984; Dutar, et al., 1986; Geneser, 1986; Green & Mesulam, 1989; Lewis & Shute, 1967; Mesulam, et al., 1983; Shute & Lewis, 1967; Storm-Mathisen & Blakstad, 1964). Due to its involvement in learning and memory processes, and dysfunction associated with a number of disease states (e.g., Alzheimer's Disease, medial temporal amnesia), the ability of the hippocampus to re-organize, or compensate, for damage is of particular theoretical and clinical significance. To date, studies addressing this issue have focused primarily on analyses of muscarinic receptor binding following denervation, and have produced conflicting results. Some authors report decreases in muscarinic receptor numbers following cholinergic denervation (deBelleroche, Gardiner, Hamilton & Birdsall, 1985; Mash, Flynn & Potter, 1985), while others report increases (Dawson, Gage, Hunt & Wamsley, 1989; Joyce, Gibbs, Cotman & Marshall, 1989; Tayrien & Loy, 1984; Westlind, Grynfarb, Hedlund, Bartfai & Fuxe, 1981) or no change

(Overstreet, Speth, Hruska, Ehlert, Dumont & Yamamura, 1980) in receptor numbers. Results from these studies are difficult to interpret, since the relative contributions of pre-synaptic, post-synaptic and non-neuronal cells cannot be separated, and denervation may have various effects on these cell types. In fact, it has been proposed (Nathanson, 1987) that ultrastructural microscopic analysis may be the only means of resolving this issue. Functional studies have not shown receptor supersensitivity (Bird & Aghajanian, 1975), but utilized an iontophoretic method, in which drug concentration and presynaptic effects cannot be controlled. Thus, the question of whether altered sensitivity following septo-hippocampal denervation occurs is as yet unresolved.

Another approach to studying plasticity following denervation is to study consequent modifications in neuronal circuitry. Lesion-induced changes in cholinergic innervation to the hippocampus have been described, and there is substantial evidence for sprouting of intact afferent axons into deafferented regions following denervation (Cotman & Nieto-Sampedro, 1984; Geddes, Monaghan, Cotman, Lott, Kim & Chui, 1985; Nyakas, Luiten, Balkan & Spencer Jr., 1988). The patterns of sprouting were studied in rats after experimentally-induced denervation, as well as in Alzheimer's Disease (AD) brains (Geddes, et al., 1985), and sprouting responses were found in both the AD and rat hippocampi. Thus, the remaining cholinergic neurons in AD patients are capable of a sprouting response; however, the influence of such a response on behavior is unknown.

The development of neural transplantation techniques has provided another approach to explore plasticity, as well as recovery of function, following

damage to various brain regions. Transplantation of neuronal tissue following disruption of normal functioning (e.g., denervation) has been widely demonstrated in the hippocampus (Anderson, Gibbs & Cotman, 1988; Bjorklund, Segal & Stenevi, 1979; Buzsaki, Gage, Kellenyi & Bjorklund, 1987; Dunnett, Low, Iversen, Stenevi & Bjorklund, 1982; Segal, Greenberger & Pearl, 1989), with resultant changes in structural (including survival and growth of transplanted tissue, establishment of afferent and efferent connections with host brain) and functional activity. Most studies (see above) have utilized a denervation followed by transplantation paradigm, although other techniques have been used. For example, the effects of unilateral transplantation of suspended embryonic septal neurons into the hippocampus of adult rats were studied following administration of AF64A, a specific cholinergic toxin (Ikegami, Nihonmatsu, Hatanaka, Takei & Kawamura, 1989). The grafted cells provided extensive cholinergic reinnervation to the host hippocampus, as measured by increased acetylcholinesterase-staining and ChAT levels on the grafted side. Similar results were reported following grafting of fetal hippocampal neurons into the CA1 region of the hippocampus of adult rats after ischemic occlusion lesions (Tonder, Sorensen, Zimmer, Jorgensen, Johansen & Diemer, 1989).

Reinnervation of the hippocampus following damage has been demonstrated for a number of neurotransmitter systems, from various brain regions. Embryonic locus coeruleus (LC) neurons were implanted into adult rats following 6-OHDA or 5,7-DHT intraventricular injections, causing significant, bilateral adrenergic denervation of the hippocampus (Bjorklund, et al., 1979. The pattern formed by ingrowing LC neurons was similar to that of

normal LC afferents, with fairly specific reinnervation to regions normally innervated by adrenergic afferents. They also performed electrophysiological experiments, in which stimulation of the LC implants caused inhibitory responses in host neurons similar to that observed after stimulation of the LC of normal rats. Fetal raphe cells have been transplanted into the serotonin-depleted hippocampus, and been shown to survive and successfully reinnervate the host hippocampus (Segal & Azmitia, 1986).

The majority of transplantation research in the hippocampus has focused on investigation of cholinergic reinnervation patterns. Successful cholinergic reinnervation has been demonstrated using cholinergic-rich grafts from a number of brain regions, including hippocampus (Buzsaki et al., 1987; Tonder et al., 1989), septum (Dawson, et al., 1989; Dunnett et al., 1982; Gage & Bjorklund, 1986; Gage et al., 1984; Hisanaga et al., 1988; Ikegami et al., 1989; Kaseda et al., 1989; Nilsson et al., 1988), habenula (Anderson et al., 1988), nucleus basalis of Meynert (Nilsson et al., 1988; Clarke et al., 1990), and striatum (Anderson et al., 1988; Nilsson et al., 1989; Clarke et al., 1990). Thus, grafted cholinergic neurons which do not normally innervate the hippocampus can send axons and form synaptic contacts with the host hippocampus. However, recent findings indicate that grafts from different brain regions can show markedly different patterns of growth and innervation. The patterns of reinnervation of cholinergic-rich fetal cell suspensions obtained from the septal region, nbM, striatum, brainstem and spinal cord grafted into the hippocampi of adult rats showed considerable variation in the patterns of connectivity of surviving ChAT and acetylcholinesterase-positive cells from different regions

(Nilsson et al., 1988; Clarke et al, 1990). Interestingly, these patterns were not related to the growth of the grafts. The septal grafts produced the most cholinergic reinnervation, followed by the nbM, brainstem, spinal cord and, finally, striatum. Electron microscopic analysis revealed that, although all grafts formed some synaptic contacts with host cells, the septal grafts produced the highest number of contacts, whereas the spinal cord and striatal grafts produced the fewest. The innervation observed following brainstem and spinal cord grafts was abnormal both morphologically (e.g., containing large varicosities) and with respect to innervation patterns. It was concluded that neuronal properties other than simply transmitter type are important in the formation of appropriate graft-host connections.

Another issue regarding the effectiveness of transplanted tissue is the role of trophic factors. It has been proposed that these trophic factors are released by the damaged brain following injury, and may act to facilitate neuronal sprouting and/or graft outgrowth into host tissue (Cotman & Nieto-Sampedro, 1984; Gage & Bjorklund, 1986). Gage & Bjorklund (1986) implanted fetal septal cell suspensions into the hippocampal formation of both normal rats and those with prior fimbria-fornix lesions. They found that the size of the transplants in denervated animals was more than twice the size of grafts in non-lesioned controls. Furthermore, the number of acetylcholinesterase-positive cells in the transplant and hippocampal ChAT activity in the denervated group was 2-3 times higher than in non-lesioned animals. Thus, the lesion appeared to enhance the effectiveness of the grafts. They concluded that this was due to the release of trophic factors upon denervation, which facilitated the

survival and growth of implanted grafts.

Furthermore, there is evidence that even exogenously applied trophic agents can influence neuronal survival following brain injury. Exogenously applied ß-nerve growth factor (ß-NGF) and ganglioside GM1 prevented retrograde degeneration of nucleus basalis of Meynert axons in decorticated rats, with a concomitant increase in choline acetyltransferase activity (Cuello, Garofalo, Kenigsberg & Maysinger, 1989). While the addition of trophic factors may enhance survival of neuronal tissue, it is not necessary, however. Hisanaga et al. (1988) compared the survival and growth of implanted fetal rat septal neurons which were cultured prior to grafting in either a ß-NGF-containing or ß-NGF-free medium. While ChAT activity in the culture was higher in the ß-NGF than non-ß-NGF containing medium, this difference failed to affect the grafts' survival and growth once implanted.

In addition to evidence that transplanted neurons can successfully grow and replenish cholinergic input into the denervated hippocampus, numerous reports have demonstrated that this reinnervation translates into functional recovery. Thus, septal grafts into the hippocampus of rats following septal or fimbria-fornix lesions have been shown to restore spatial learning deficits observed in these animals following such lesions (Dunnett et al., 1982; Segal et al., 1989). However, Segal et al. (1989) observed these restorative effects only following physostigmine. Others have reported that septal grafts can ameliorate learning deficits in memory-impaired aged rats (Dunnett et al., 1988; Gage et al., 1984).

While studies of neural transplantation may provide a better

understanding of mechanisms underlying plasticity, there are a number of unresolved issues which hinder both conclusive interpretation of results obtained, as well as the potential clinical usefulness of this technique. While much success has been reported in terms of graft survival and growth, perfect (100%) survival rates have not been obtained in any of the studies cited. Furthermore, several studies have reported incidences of abberrant patterns of reinnervation (Bjorklund et al., 1979; Clarke et al., 1990; Nilsson et al., 1988; Tonder et al., 1989). The extent of functional (behavioral) recovery reported is variable, and is sometimes only evident upon further manipulation of the graft-host system (see Segal et al., 1989). The relevance of findings obtained from hippocampal transplantation work to theories of cholinergic involvement in learning and memory processes is also difficult to interpret. As stated previously, hippocampal denervation, while effectively eliminating much of the cholinergic input to the hippocampus, also alters other, non-cholinergic pathways. Furthermore, behavioral improvements observed after transplants are not necessarily related to cholinergic reinnervation specifically (Gage et al., 1984). Finally, the clinical utility of these techniques, particularly within the hippocampal system, is highly questionable, as most disorders of learning and memory associated with hippocampal damage also involve damage to other brain regions, such as the neocortex (as in AD), amygdala or medial temporal region (as in various amnesic syndromes).

D. RATIONALE.

The present studies were designed to focus on two of the aforementioned, as yet unresolved, issues regarding cholinergic, and particularly, muscarinic actions in the hippocampus. In the first series of experiments, the mechanism(s) underlying muscarinic excitation of hippocampal pyramidal cells were investigated in rodents using intracellular electrophysiological techniques. As reviewed above, none of the established electrophysiological effects of muscarinic receptor activation can fully explain the depolarizing action of muscarinic agonists. Thus, we examined the ionic mechanism and the pharmacology of the depolarization (or inward current) induced by carbachol, a typical muscarinic agonist. In accord with previous findings, it was hypothesized that the carbachol-induced depolarization would be dose-dependent and muscarinic in nature. To test these hypotheses, we studied the effects of various doses of carbachol, and the effects of atropine (a muscarinic antagonist) and pirenzepine (an M_1 receptor antagonist). It was also hypothesized that carbachol would block an additional potassium current (other than I_M and I_{AHP}) which could account for its excitatory effects on hippocampal pyramidal cells. To test this hypothesis, we examined the effects of various potassium channel blockers (cesium, tetraethylammonium and barium), and current-voltage relationships before and after carbachol application.

The second series of experiments attempted to account for a secondary and unpredicted effect of carbachol which was found in a number of cells. To rule out methodological problems, carbachol effects were compared to those of

serotonin, a transmitter which alters the current-voltage relationships in these cells in a predictable manner, due to its well-established activation of potassium channels (Andrade, Malenka & Nicoll, 1986). To determine if the secondary effect was potassium-mediated, the effects of carbachol and serotonin were examined following alteration of bathing potassium concentrations.

In the third series of experiments, the response of the rodent hippocampal muscarinic system to experimentally-induced denervation was studied electrophysiologically. Results from studies to date regarding this issue are, at best, inconclusive. Procedures typically involve analysis of muscarinic receptor binding following denervation (Dawson, Gage, Hunt & Wamsley, 1989; deBelleroche, Gardiner, Hamilton & Birdsall, 1985; Joyce, Gibbs, Cotman & Marshall, 1989; Mash, Flynn & Potter, 1985; Overstreet, Speth, Hruska, Ehlert, Dumont & Yamamura, 1980; Tayrien & Loy, 1984; Westlind, Grynfarb, Hedlund, Bartfai & Fuxe, 1981), and are difficult to interpret since contributions of pre-synaptic, post-synaptic and non-neuronal cells cannot be determined. In an earlier electrophysiological study, Bird & Aghajanian (1975) failed to demonstrate supersensitivity following denervation; but again, could not rule out presynaptic or non-specific effects due to the methodology employed (iontophoretic application). Thus, the important question of whether altered sensitivity to muscarinic agents occurs following septo-hippocampal denervation is as yet unresolved.

The present study was designed to address this issue by studying the effects of known concentrations of carbachol, a cholinergic agonist, on the intracellular activity of normal and denervated hippocampal pyramidal cells. It

was hypothesized that carbachol would produce a greater response in denervated as compared to normal pyramidal cells, providing evidence for functional denervation supersensitivity within this system. The purpose of these studies was to contribute to the literature a detailed account of muscarinic cellular actions in the hippocampus, and explore the effect of denervation damage to the hippocampal cholinergic system on responses to muscarinic agents. The findings obtained may provide further insights into the role of acetylcholine in learning and memory processes, as well as implications for treatment of disorders involving the hippocampal cholinergic system.

CHAPTER TWO: AN ANALYSIS OF THE DEPOLARIZATION/INWARD CURRENT PRODUCED IN RODENT HIPPOCAMPUS BY MUSCARINIC RECEPTOR STIMULATION.

Muscarinic-induced depolarization of hippocampal pyramidal cells has been explained by blockade of the potassium curents, I_M and I_{AHP} (Halliwell & Adams, 1982; Madison et al., 1987). However, it has recently been suggested that additional current(s) may be involved (e.g., Jones, 1985; Landau, Blitzer, Benson & Davis, 1986). These series of experiments were designed initially to confirm previous findings that carbachol does block both I_{AHP} and I_M, and second, to investigate the existence and nature of an additional effect (beyond that on I_M and I_{AHP}), by studying its pharmacology and ionic dependence (Benson, Blitzer & Landau, 1988).

Methods

Adult male guinea pigs (200-350 grams) supplied by Charles River Laboratories were anaesthetized with chloral hydrate (300 mg/kg) and decapitated. The brain was rapidly removed and chilled (to prevent cell death), and the hippocampi removed. Transverse slices, 400-500 μM thick, were cut using a Sorvall TC-3 tissue slicer. Slices were transferred to a holding chamber and kept at an interface between artificial cerebrospinal fluid (ACSF) and an oxygenated (95% O_2/5% CO_2) atmosphere, at room temperature (22-25°C) for at least 1 hr prior to recording. Single slices were then transferred to a recording chamber similar to that described by Nicoll & Alger (1981). Slices

were placed on a nylon net, immobilized with a few pieces of silver wire and submerged beneath a continuously superfusing medium. The medium flowed continuously over the slice, and was drawn off from a side well. Experiments were performed either at room temperature or at 30-32°C. No differences were found between carbachol effects obtained in these temperature ranges. The medium was constantly bubbled with a 95% O_2/5% CO_2 mixture, and had the following composition (mM): NaCl, 130; KCl, 5; NaH_2PO_4, 1.25; $NaHCO_3$, 24; $CaCl_2$, 2.5; $MgCl_2$, 0.5; dextrose, 15; pH, 7.4.

In the voltage clamp experiments described below, bathing solutions contained 0.5 μM tetrodotoxin (TTX) to suppress action potentials. In some experiments, atropine sulfate (0.1 to 1.0 μM) or pirenzepine (20 nm to 1.0 μM) were used to reverse or block carbachol effects. Calcium ions were sometimes removed from the medium and replaced with magnesium ions, and cadmium (200 μM) was added, to control for any contribution of calcium ions to the observed effects. In other experiments, the potassium channel blockers barium chloride (4mM) or tetraethylammonium chloride (30mM) were added to the solutions. In the barium experiments, phosphate ions and sulfate ions were removed and replaced with the corresponding chloride salts. Carbachol was chosen as the cholinergic agonist in these experiments, as it is not subject to breakdown by acetylcholinesterase (Bird & Aghajanian, l975). It was prepared in a 1-10 mM solution and diluted to the required concentrations just prior to recording. Solutions were switched using a multi-port valve or three-way stopcock without disturbing the fluid level in the bath. Drugs and salts were obtained from Sigma Corp. or Baker Chemicals, with the exception of

pirenzepine, which was a generous gift of Karl Thomae through Boehringer Ingelheim.

Pyramidal cells in the CA_1 layer of the hippocampus (see Figure 2) were impaled with glass microelectrodes which were pulled (Sutter Instruments, Inc.) from glass tubing (outside diameter, 1.0 mm, inside diameter, 0.6 mm; WPI Instruments or A-M Systems). The pyramidal cell layer was clearly visible under low magnification, and impaled cells which were aberrant in terms of spike characteristics, resting potential or membrane resistance were either lost rapidly or discarded. Microelectrodes were filled with either potassium methylsulfate (2M, $KMeSO_4$; electrode resistance, 80-200 MΩ) or potassium chloride (3M, KCl; electrode resistance, 40-120MΩ), to control for any possible contribution of chloride ions. Since no differences were found between carbachol effects obtained with these two electrode types, these data were pooled. In some instances, TEA (2M), cesium chloride (2M) or cyclic-AMP (100-300mM, cAMP) was present in the solutions filling the micro-electrodes, to block various potassium channels.

Signals were amplified by an Axoclamp-2 amplifier and displayed on a Tektronix 5113 oscilloscope, a Nicolet 4096 digital oscilloscope, and recorded either on chart paper using a Gould Brush-2400 chart recorder or on magnetic tape using a Racal Store 4-D tape recorder. This allowed observation of the membrane potential (voltage) and spontaneous neuronal firing under various experimental conditions. To study the ionic mechanisms underlying carbachol effects, either a current clamp or voltage clamp arrangement was employed. Of interest were the ionic currents either activated or inactivated by carbachol. In

the current clamp mode, the amplifier allowed the passage of current pulses through the recording electrode, employing a bridge arrangement, thereby varying membrane potential and recording consequent changes in voltage-dependent ionic conductances which represent the opening or closing of membrane channels). In these experiments, different current steps were imposed and the resulting change in voltage was measured at its peak. This was usually 80-100 msec after the start of the pulse, when the charging of the membrane capacitance was already complete, and before anomalous rectification could cause a sag. However, activation of these voltage-dependent currents can result in further change of the membrane potential. Thus, it is difficult to achieve a stable membrane potential utilizing this technique, limiting the precision by which one can measure voltage-dependent currents.

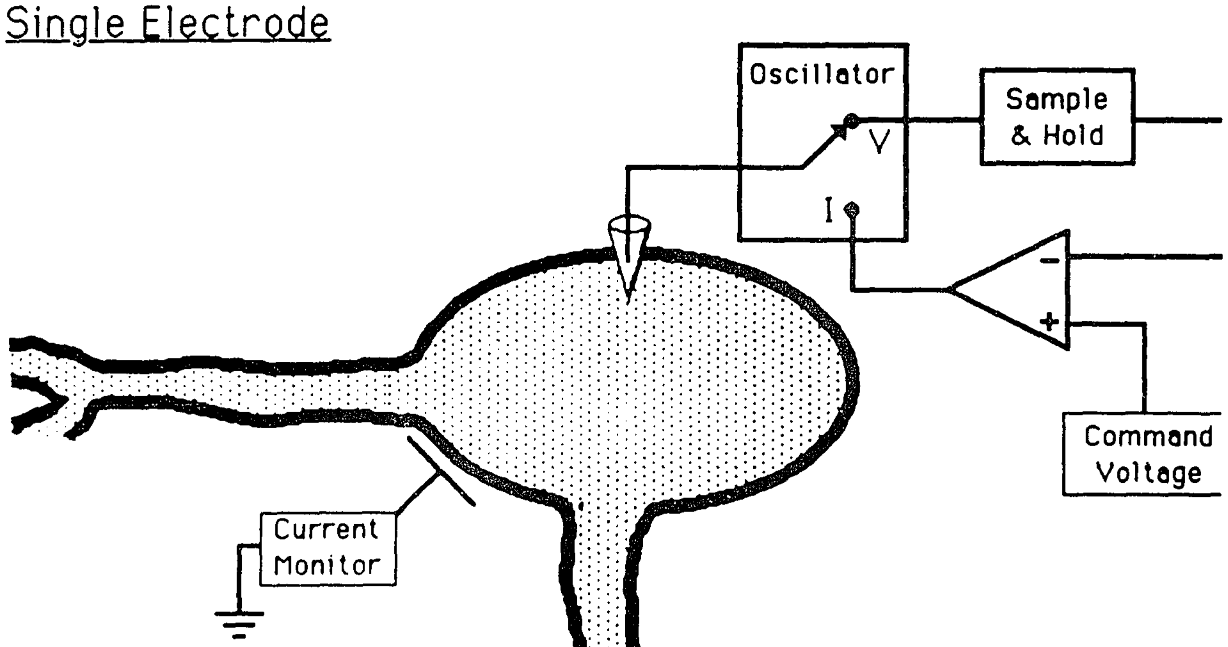
A more controlled technique for studying voltage-dependent ionic currents is the voltage clamp (Hille, 1984; Hodgkin, Huxley & Katz, 1952). In this situation, the membrane voltage can be stepped to various designated levels. The amplifier employs a negative feedback system, regularly reading the membrane potential and supplying whatever current is necessary to maintain the designated voltage level. Thus, the membrane potential remains stable, and, by recording the current that must be generated by the voltage clamp to maintain the designated voltage, membrane currents can be measured.

For voltage-clamping, a single-electrode voltage clamp mode was used (Figure 3), and the output of the voltage clamp headstage was monitored employing a 5111 Tektronix oscilloscope. A switching frequency of 2-3 kHz,

Figure 3.

Schematic illustration of single-electrode voltage-clamp technique.

Figure 3



and a 30% duty cycle were used, and cells were discarded in which the electrode voltage did not settle completely between oscillations. Errors at the beginning of the voltage steps usually were small and settled within less than 10 msec.

To determine the ionic basis for the carbachol-induced currents, Instantaneous current-voltage (I-V) plots were constructed before and after the addition of carbachol to the bathing medium. First, the slope of the I-V curves were compared in carbachol and control conditions. Changes in I-V slopes are reflective of alterations (e.g., openings/closings) in channel states (for review, see Hille, 1992). If the slope of an I-V curve increases (reflecting increased conductance), this indicates that a channel has opened; if the slope decreases, this indicates channel closure. Next, by examining the point at which the I-V curves crossed over, the nature of the ion in question was determined. This was based on the principle that the cross over point represents the membrane potential at which there is no net current flow, which would, in turn, represent the equilibrium potential for that ion (Hille, 1992).

In the voltage-clamp experiments, voltage steps were induced and the instant, "ohmic", component was studied. The current measurement was taken at the end of the capacitative transient, about 20 msec after the start of the pulse. For studying I_{AHP} and I_M, voltage steps were chosen based on earlier work of others (Adams, Brown & Constanti, 1982; Madison & Nicoll, 1982), which established the voltage-dependence and time courses of these currents. For recording, KCl electrodes coated with Sylgard were used.

Criteria for acceptance of cells for inclusion in analyses were : a resting

potential of <-55 mV, an action potential amplitude of >70mV, and a membrane resistance of >20MΩ. These values are characteristic of healthy cells, and are consistent with those reported elsewhere for hippocampal CA1 pyramidal cells (Benardo & Prince, 1982; Cole & Nicoll, 1984; Cotman et al., 1986; Halliwell & Adams, 1982; Segal, 1982). Analyses of carbachol effects and effects of various conditions (pirenzepine, atropine, cesium, TEA, barium, cadmium, potassium concentration) were performed with repeated measures analyses of variance or paired t-tests in the case of within-cell analyses, and a one-way ANOVA or independent t-tests in the case of between-cell analyses. Following ANOVA's, post-hoc Scheffe tests were used to determine the significance of differences among specific groups. Error values referred to in the text are standard error of the mean (SEM). Specific statistical tests used for each experiment are stated in the separate studies.

Results

Carbachol induces a depolarization/inward current in hippocampal pyramidal cells.

Carbachol depolarized current clamped cells (n=22), or produced an inward current in voltage clamped cells (n=21) under normal (artificial cerebrospinal fluid) conditions. The depolarization induced by 5 uM carbachol and 10 uM doses of carbachol (see Table 2) failed to differ from each other (t=.005). Carbachol (5uM) in a current-clamped cell (Figure 4a) depolarized the

cell membrane, which was accompanied by a concomitant increase in the rate of spontaneous neuronal firing. The membrane potential as well as the firing rate returned to near-baseline levels following washout of the drug. However, carbachol occasionally (n=3) depolarized cells to the point of "depolarization blockade", at which point neuronal firing ceased (see Figure 4b). Again, both the membrane potential and firing rate returned to near baseline levels following carbachol removal. The response of a voltage-clamped cell to carbachol (5 μM, Figure 4c) is reflected as an inward current, indicating the negative current supplied by the voltage clamp to prevent the membrane from depolarizing.

Carbachol-induced inward current is not I_M nor I_{AHP}.

One possible reason for the carbachol-induced inward current is its blockade of the potassium currents, I_M and I_{AHP}. This was confirmed in that carbachol blocked both the slow, calcium-dependent potassium current, I_{AHP}, and the faster potassium current, I_M (see Figure 5a). An I_{AHP} was elicited in a voltage-clamped cell by stepping the voltage from -55 to -35 mV for 3 seconds, producing a slowly evolving current and a slow outward current tail. Carbachol (5μM) produced a marked inward current shift and reversibly blocked the outward current. The size of I_{AHP} was significantly different ($F(3,8)=430.17$, $p \leq .0001$) before, during and after carbachol application (n=4), such that carbachol significantly reduced I_{AHP} (Scheffe comparison, $p \leq .05$), and a significant recovery of the current upon washout of carbachol ($p=.05$).

Table 2 : Carbachol-induced depolarizations/inward currents

Clamp Mode	Condition	Carbachol concentration (uM)	Avg. depol. (mV)/ inward curr. (nA)	N
Current clamp	Control (ACSF)	5	8.8 ± .88	13
		10	9.8 ± 1.2	9
"	barium	5	0.0*	2
"	cesium	5	.67 ± .33*	3
"	TEA	10	0.0*	6
Voltage clamp	Control	1	.07 ± .01	8
		5	.14 ± .04	6
		10	.11 ± .01	7
"	barium	5	.003 ± .003*	3
"	cadmium	10	.21 ± .07	4

* significantly different ($p < .05$) than effect obtained in control solution with corresponding carbachol concentration.

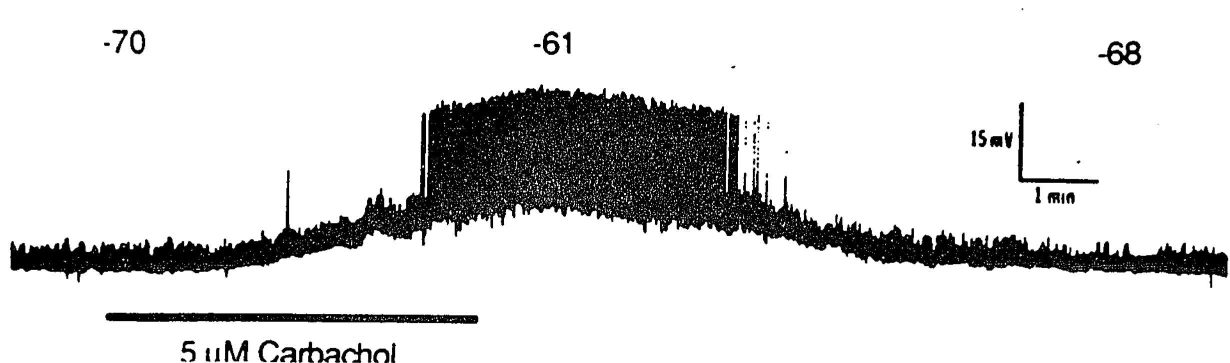
Figure 4.

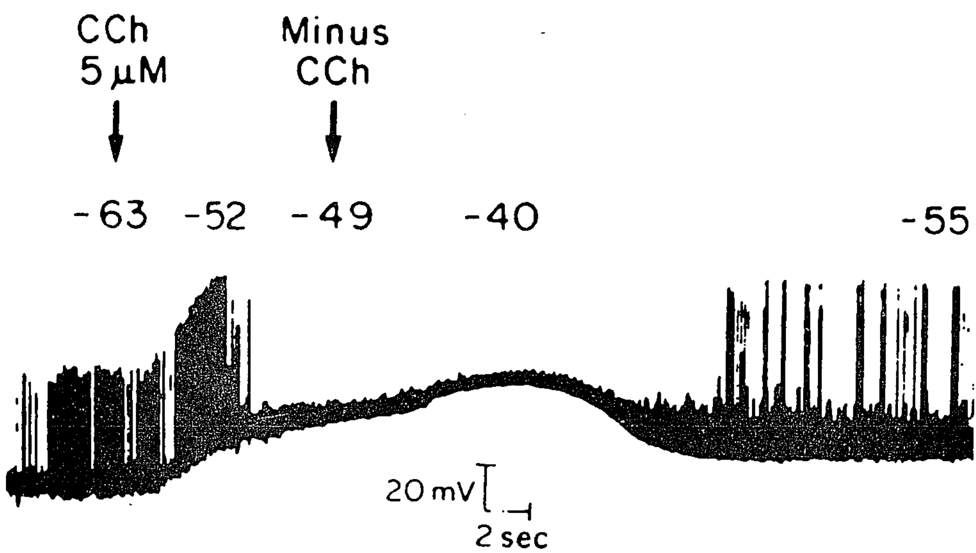
(a), Carbachol-induced depolarization and increase in spontaneous firing rate in hippocampal CA1 pyramidal cell. Recording electrode contained KCl.

(b), Carbachol-induced depolarization blockade in another cell. Note partial recovery of resting membrane potential and firing frequency upon washout of the drug. Recording electrode: KCl.

(c), Carbachol-induced inward current in voltage-clamped cell. Impaling electrode contained 200mM cAMP, resulting in abolition of the spike after-hyperpolarization (left-hand panel). Resting potential, -65 mV. After addition of 0.5 μM TTX, cell was clamped at -67 mV, and exposed to 5μM carbachol for 6 min. Dotted line indicates baseline holding current. Vertical cal: 0.25 nA/20mV Horizontal cal: 400msec/1min.

Figure 4





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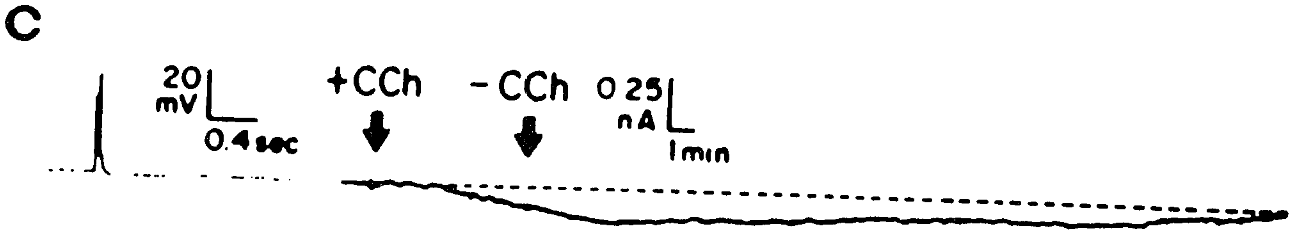


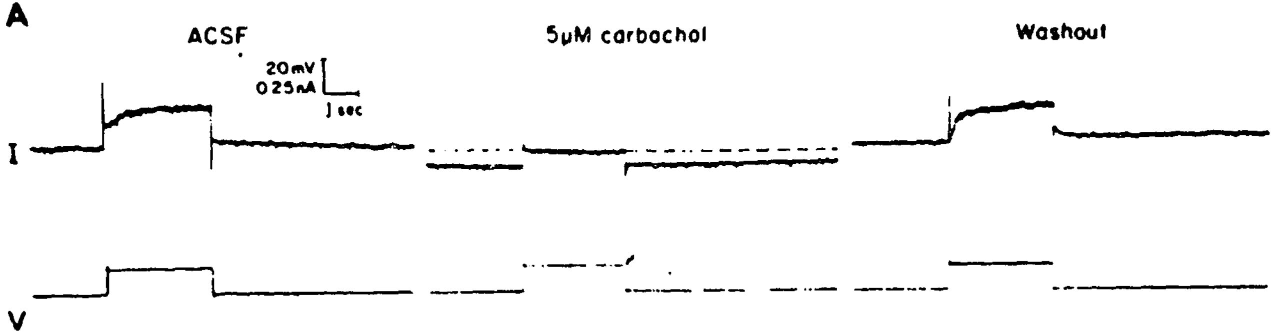
Carbachol effects on the potassium current, I_M, were studied by blocking the AHP current with cAMP (100mM to 200mM). Figure 5b illustrates a typical cell which was held at -49 mV and stepped to -69 mV. A faster inward current, I_M, was produced, and was reversibly blocked by carbachol (5μM). The size of I_M was significantly different ($F(5,12)=14.39$, $p \leq .001$) before, during and after carbachol application (n=7), with I_M significantly reduced by carbachol (Scheffe test, $p \leq .05$). Of particular importance is the observation that, in addition to blocking I_M, carbachol induced an inward current at the end of the hyperpolarizing pulse when the voltage was clamped at -69 mV. A similar inward current was seen in Figure 4c. These carbachol-induced currents cannot be attributed to a carbachol effect on I_{AHP} since this current was blocked by the cAMP (Figure 4c, left-hand panel). Furthermore, the inward currents occur at voltages where I_M is inactivated (Halliwell & Adams, 1982). Thus, in addition to its action on I_{AHP} and I_M, carbachol must induce another current in hippocampal pyramidal neurons.

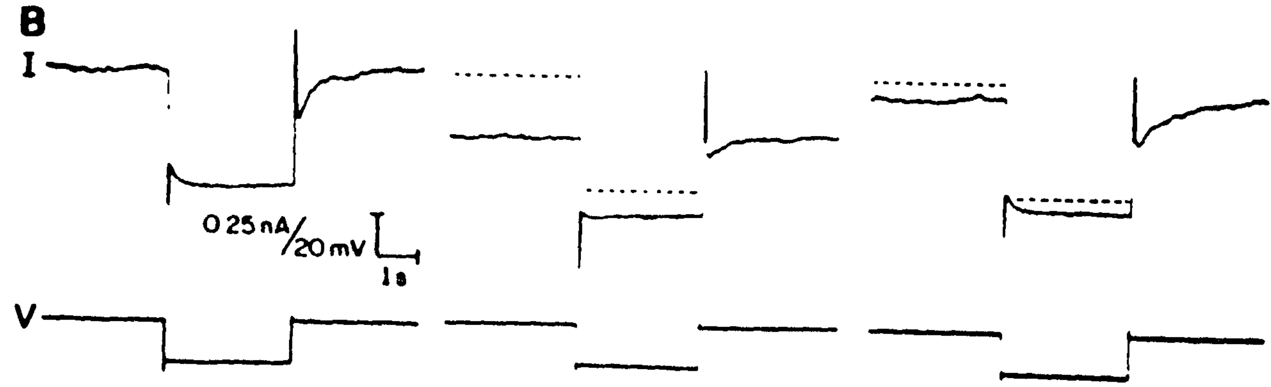
Figure 5.

(a) Carbachol effect on I_{AHP}. Chart records from a voltage-clamped cell are shown. 0.5 μM tetrodotoxin (TTX) was present throughout the experiment to prevent spiking. Top row: current, bottom row: voltage. Leftmost panel: Cell was clamped at -55 mV and stepped for 3 sec to -35 mV. Note the slowly developing current (I_{AHP}) during the pulse, and the subsequent outward "tail" current. Middle panel: the same step, 3 min into 5μM carbachol. Note the inward current shift, and the abolition of both the developing and tail currents by carbachol. The dotted line in this trace indicates control holding current. Rightmost panel: 15 min after washout of carbachol. Note the recovery of I_{AHP} (both phases) and also of the current shift. Recording electrode: KCl. Vertical cal: 0.25 nA/20mV. Horizontal cal: 1 sec.

(b). Carbachol effect on I_M. Chart records from a different voltage-clamped cell are shown. 0.5 μM TTX present to avoid spikes. 200mM cAMP present in the recording electrode to block I_{AHP}. Top row: current, bottom row: voltage. Leftmost panel: Cell was clamped at -49 mV and stepped to -69 mV for 3 sec. Note the small inward relaxation (I_M) produced. Middle panel: Application of 5 μM carbachol (5.5 min) produces a large inward current shift, and blocks I_M. Dotted lines in this trace and in next panel indicate baseline current levels at holding and at end of pulse. Rightmost panel: one hour after washout of carbachol (in the presence of 1 μM atropine). Both current shift and I_M recover to near baseline levels. Recording electrode: 200mM cAMP, 3M KCl. Vertical cal: 0.25 nA/20mV. Horizontal cal: 1 sec.

Figure 5





Pharmacology of the carbachol-induced depolarization or inward current.

a) Carbachol dose-response function.

The dose-response relationship for carbachol in voltage-clamped cells (n=21; Figure 6a) was studied with cAMP-filled microelectrodes (100-300mM) to block I_{AHP} and voltage was held between -60 and -70 mV to inactivate I_M. The inward currents produced by 1, 5 and 10 uM carbachol are shown in Table 2. These effects approached but failed to reach statistical significance ($F(2,18)=2.61$, $p \leq .10$). The apparent dissociation constant (K_D) for carbachol was 1.2 μM. Carbachol concentrations greater than 1μM were utilized because currents generated from lower doses were too small to measure accurately.

b) The effects of atropine and pirenzepine.

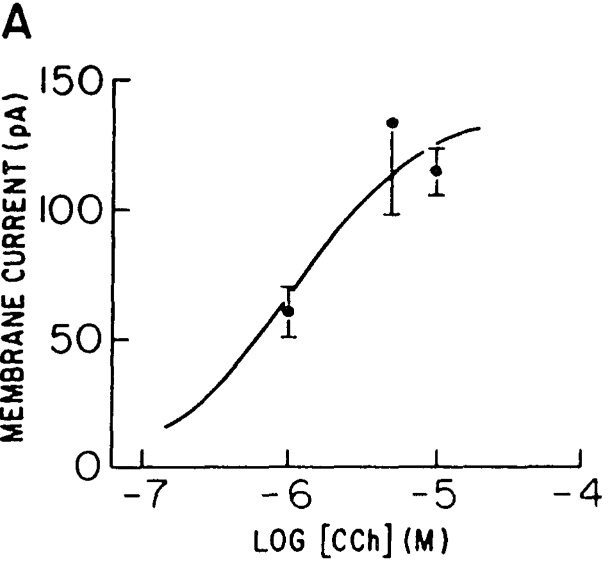
The inward current produced by carbachol (1 μM) was significantly ($t(6)=4.84$, $p \leq .003$; n=4) reversed by adding atropine (0.1 μM) into the bathing medium. Pirenzepine, an M_1 receptor antagonist, reversibly blocked the depolarization induced by carbachol (5 μM) in 10 current clamped cells containing cAMP (300 mM) and held between -60 and -70 mV. Pirenzepine doses of 20 nM (n=2), 50 nM (n=2), 200 nM (n=2), 500 nM (n=3) and 1μM (n=1) lowered the carbachol-induced depolarization (Figure 6b) non-significantly ($F(4,5)=4.47$, $p \leq .07$). The apparent dissociation constant, K_i, for the pirenzepine effect was 18 nM, suggesting M_1 cholinergic receptor activation.

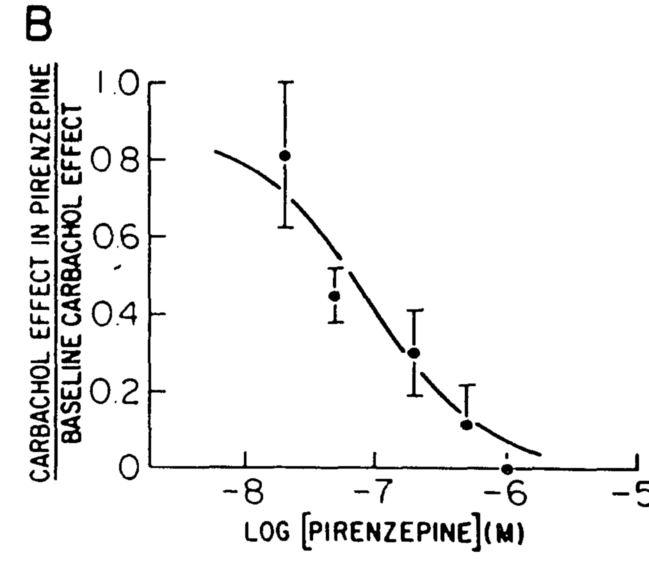
Figure 6

(a). Dose-response relationship for carbachol. Cells were voltage-clamped between -60 and -70 mV in the presence of 0.5 μM TTX. Impaling electrode contained 100-300mM cAMP and 3M KCl. The inward current generated by 1,5 and 10μM carbachol was plotted. Duration of carbachol application: 3-6 min. The curve was fitted using the equation $I=142C/(K_D+C)$, where I was the carbachol-induced current in pA, C was the carbachol concentration and K_D was the apparent dissociation constant. The K_D was 1.2 μM.

(b). Pirenzepine effect on the carbachol-induced depolarization. Cells were held between -60 and -70 mV. Recording electrodes contained 300 mM cAMP in each case. Carbachol, 5μM, was applied before and after pirenzepine application, and pirenzepine effect is plotted as the ratio of the carbachol responses after and before pirenzepine. Duration of carbachol application: 2-3 min. The theoretical curve was fitted to the equation : $E = 5/(5+1.2(1+I/K_i))$, where E was the relative effect of carbachol in the presence of a given dose, I, of pirenzepine. K_i was the apparent pirenzepine dissociation constant. The fitting was done with a non-linear regression curve-fitting program. The K_i for the pirenzepine effect was 18 nM.

Figure 6





d) The effects of cesium injection.

Three cells were impaled with micro-electrodes filled with CsCl (2M), current-clamped in the presence of TTX (0.5 μM) and held between -60 and -75 mV to eliminate cesium-induced depolarizations and spontaneous spikes. The depolarization induced by carbachol was significantly reduced ($F(2,15)=15.71$, $p \leq .0002$) in cells impaled with cesium electrodes (see Table 2).

e) The effects of barium ions.

In current clamped cells, the effect of carbachol was significantly ($F(2,15)=15.71$, $p \leq .0002$) blocked when $BaCl_2$ (4mM) was present in the bathing medium (Table 2). In voltage-clamped cells, the inward current produced by carbachol was similarly blocked by barium (Table 2; $t(8)=2.53$, $p \leq .035$). The effect of Ba^{2+} could be reversed by washing out the drug in one cell tested. Occasionally a fast, transient inward current was observed in the presence of Ba^{2+} shortly after starting the carbachol application. The Ba^{2+}-induced inhibition of the carbachol effect was significantly slower than its blocking of I_M. Thus, Ba^{2+} blocked the M current after an average of 4.0 ± 1.0 min (n=4) of exposure, whereas it took 51.5 ± 13.9 min (n=6) to block the carbachol-induced depolarization or inward current ($t(8)=2.73, p \leq .026$). This provides further evidence that the depolarization is not the result of M channel blockade.

f) The effects of tetraethylammonium (TEA).

TEA was applied either in the bath (30 mM, 2 cells) or in the micropipette

(2 M, 4 cells) and TTX was present throughout the experiments. With both extracellular and intracellular TEA, prolonged depolarizing shifts and long-lasting spontaneous spikes were observed. Cells were held in current clamp (between -60 and -80 mV) and carbachol (10μM) applied. TEA blocked carbachol-induced depolarizations relative to control cells (Table 2; $t(13)=6.57$, $p \leq .0001$).

g) The effect of cadmium.

To evaluate the involvement of Ca^{2+} ions in generating carbachol-induced inward current, carbachol (10 μM) was applied in the presence of the calcium channel blocker cadmium (200 μM). The inward currents induced by carbachol in voltage-clamped cells tested with cadmium failed to differ significantly from the inward current produced by the same concentration of carbachol in control cells (Table 2). Thus, it appears that external calcium ions are not strictly necessary for generating the carbachol-induced depolarization.

The carbachol-induced inward current typically reverses near the potassium equilibrium potential, E_k.

To study the ionic mechanism of the carbachol effect, current-voltage (I-V) relationships were obtained for hippocampal pyramidal cells before and after the application of carbachol in both voltage and current-clamped neurons. Figure 7 illustrates a voltage-clamped cell which was held at -70 mV, and subjected to negative and positive voltage steps. Carbachol produced a net

inward current at -60 mV which changed direction at -78 mV (reversal potential, Erev). The carbachol effect was partially reversed when the drug was washed out.

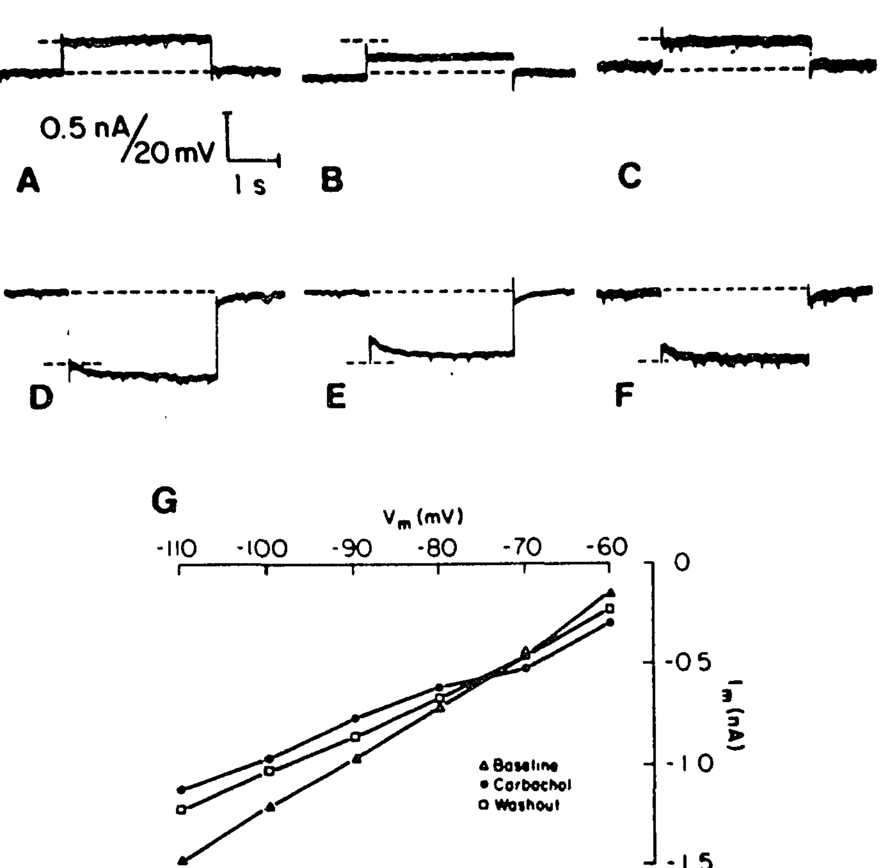
Carbachol's (1-10μM) effect was observed in 22 voltage-clamped cells, inducing an inward current which reversed direction at negative voltage steps, thereby crossing the control I-V curve. Similar results were obtained in 16 current-clamped cells, and no significant differences between voltage clamp and current clamp cross-over points was observed. The average reversal potential for carbachol in pooled voltage- and current-clamped cells in potassium (5mM) was -91.9 ± 2.5 mV (n=38), with a range of -68 to -140 mV. The I-V plots sometimes exhibited an inward going rectification. Usually, when carbachol I-V plots were subtracted from control I-V plots, linear drug-induced I-V relationships were obtained, indicating that the carbachol-sensitive conductance is not voltage-sensitive.

In summary, the carbachol-induced depolarization/inward current cannot be explained by blockage of either I_M or I_{AHP}, since it was demonstrated under conditions when both these currents were blocked. However, the fact that the potassium channel blockers cesium, TEA and barium completely blocked the carbachol effect, coupled with the finding that the effect is reversed at a membrane potential which is near to the potassium equilibrium potential, indicates that the carbachol-induced inward current/depolarization is due to inactivation (blockage) of an additional potassium current. This effect is separate from the previously documented muscarinic effects on I_M or I_{AHP}, and appears to represent a "leak", or non-voltage-dependent, conductance.

Figure 7.

Carbachol produces a net inward current which reverses at about E_K. Chart records (current traces) and I-V plot from a single voltage-clamped cell in 5 mM potassium are shown. 0.5 μM TTX and 200 μM $CdCl_2$ present throughout the experiment. In (a-c) the cell was held at -70 mV and stepped to -60 mV for 3 sec. In (d-f) the same cell was held at -70 mV and stepped to -100 mV. Note the developing current, I_Q, which, typically, was not affected by carbachol. (b,e) 7 min into 10 μM carbachol. Note the inward current shift at -60 mV which is reversed in direction at -100 mV. Baseline levels (holding and instantaneous currents) are indicated by dotted lines. (c,f) 45 min after washout of carbachol into 0.1 μM atropine. (Serotonin was applied briefly between b and c). Note the partial reversal of the carbachol effect. Vertical cal: 0.5 nA. Horizontal cal: 1 sec. (g) Complete instantaneous current-voltage relationship for the carbachol effect in this cell. Note the reversal potential of -78 mV, and the partial reversal of the carbachol effect. Recording electrode: KCl. Open triangles refer to control, filled circles refer to carbachol, and open squares refer to washout conditions.

Figure 7



CHAPTER 3: EVIDENCE FOR AN ADDITIONAL CONDUCTANCE ACTIVATED BY MUSCARINIC RECEPTOR STIMULATION.

While the results described in Chapter 2 indicate that muscarinic receptor activation decreases a potassium leak (non-voltage-sensitive) conductance, this simple explanation is unlikely to account for all carbachol effects on these cells. This next series of experiments were designed to examine the nature of a secondary, unpredicted, effect of carbachol; namely, a carbachol-induced inward current which did not reverse direction near the potassium equilibrium potential. This phenomenon was found in a number of cells (Benson, Blitzer & Landau, 1988).

Methods

The methodology employed in these experiments was the same as described in Chapter 2.

Results

In some cells, carbachol produces an inward current with no clear reversal potential.

In a number of cells, control and carbachol I-V curves never crossed in the range of voltages studied (-50 to -140 mV). Thus, carbachol produced an inward current at all voltages tested, failing to reverse direction as in those cells described previously (Chapter 2). This effect was found in 17 voltage-clamped

cells and 9 current-clamped cells (e.g., Figure 8). No differences in cell characteristics (e.g., resting membrane potential, membrane resistance) were observed in crossing vs. non-crossing cells. In the cell in Figure 8, carbachol caused an inward current at all voltages studied, which disappeared upon carbachol washout.

This phenomenon was considered to be an actual effect of carbachol because : a) it reversed upon washout of the drug in 18 of the 19 cells in which recovery could be assessed, and b) it was reversed by adding atropine (0.1 μM, n=4). The I-V plots before and after carbachol appeared qualitatively to converge in a negative direction in 9 cells, in a positive direction in 7 cells and failed to converge in 10 cells.

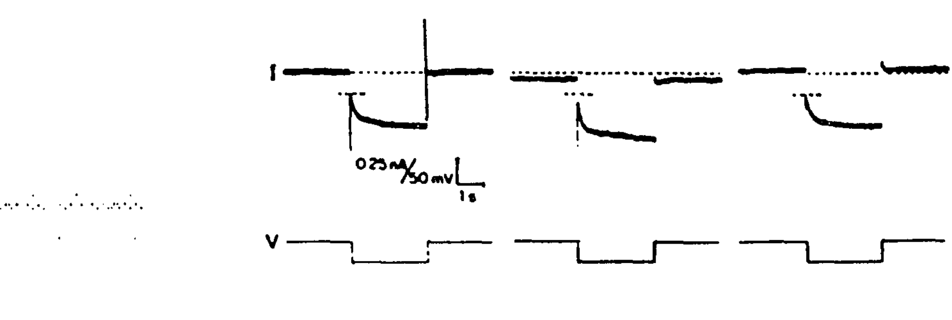
A comparison with the effects of serotonin.

The non-crossing I-V curves observed in carbachol might have resulted from such methodological problems as an inadequate space clamp. Therefore, to evaluate methodological validity, the I-V characteristics of serotonin were studied since serotonin increases potassium conductance (Andrade, Malenka & Nicoll, 1986; Andrade & Nicoll, 1987; Colino & Halliwell, 1987), and should produce an outward current which would cross (i.e., reverse direction) close to the true potassium equilibrium potential (E_K). This expectation was confirmed (see Figure 9) in 12 voltage-clamped and 14 current-clamped cells which failed to differ in cross-over values (unpaired t-test, $p>.30$). The pooled reversal potential for serotonin in voltage and current-clamped cells in potassium (5

Figure 8.

Non-crossing carbachol-induced inward current in I-V curve. Shown are chart records and I-V plot from a voltage-clamped cell. 0.5 μM TTX present throughout. Top row: current, bottom row: voltage. a) Cell was clamped at -60 mV and stepped for 3 sec to -100 mV. Dotted lines in this and subsequent traces indicate holding current level and instantaneous current level. b) 20 min into 1 μM carbachol. Note the small inward current shift, present at holding voltage and also upon step to -100 mV. c) 34 min after washout of carbachol (in 0.1 μM atropine). Both the holding current and instantaneous current recovered to baseline levels after washout of carbachol. Vertical cal: 0.25 nA/50mV. Horizontal cal: 1 sec. d) I-V plot for carbachol effect in this cell. An inward current was present at all voltages studied (no cross-over), and recovered after washout. Recording electrode: 100 mM cAMP, 3M KCl. Open triangles refer to baseline, filled circles refer to carbachol, open squares refer to washout conditions.

Figure 8



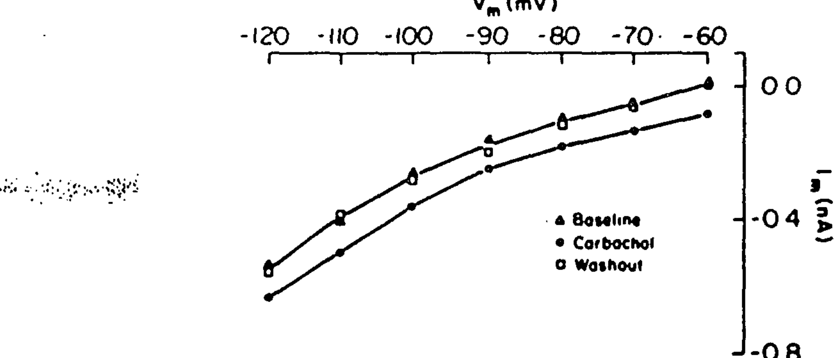
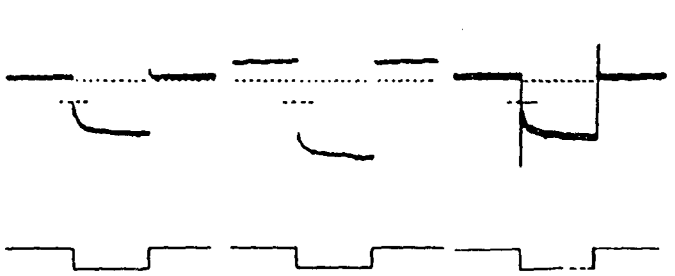
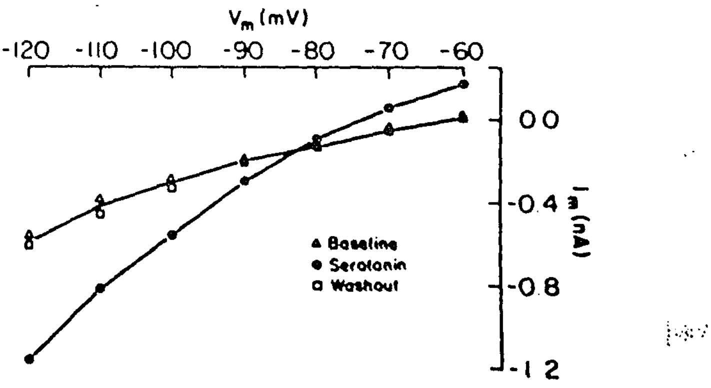


Figure 9.

Serotonin (10 μM)-induced outward current (same cell as in Fig. 8). Shown are chart records and I-V plot from a voltage-clamped cell. 0.5 μM TTX present throughout. Top row: current, bottom row: voltage. (a), cell was clamped at -60 mV and stepped to -100 mV for 3 s. Note outward current produced (b), and recovery after washout (c). Vertical cal: 0.25 nA/50mV. Horizontal cal: 1 sec. (d), the complete I-V relationship for the serotonin effect. Note the cross-over at -83 mV, and recovery after washout. Serotonin was applied for 3 min and recovery obtained after 7 min of washout. Recording electrode: 100 mM cAMP, 3M KCl. Open triangles refer to baseline, filled circles refer to serotonin, open squares refer to washout condition.

Figure 9





mM) was -85.0 ± 1.4 mV (n=26), with a range of -68 mV to -97 mV. The theoretical value for E_K, derived from the Nernst equation, was -88 mV. Thus, the reversal potential for serotonin lies close to the theoretical E_K. However, the reversal potential for serotonin was significantly more positive than that for carbachol (unpaired t-test; $t(62) = 2.31$, $p \leq .02$), and was easily reversible in all cells studied.

In some experiments, carbachol was applied in the same cells as serotonin and produced a non-crossing I-V curve (Figure 8) in 4 of 12 voltage-clamped cells and in 5 of 14 current-clamped cells. There were no cells in which serotonin produced a non-crossing I-V curve. Hence, the non-crossing curves observed for carbachol were not accompanied by similar curves for serotonin, and the former non-crossing curves could not be due to a clamp artifact. Indeed, space clamp conditions are typically worse for serotonin, which decreases membrane resistance, than for carbachol, which increases membrane resistance.

<u>The effect of increasing the bathing potassium concentration ($[K+]_o$) on the carbachol and serotonin-induced currents.</u>

To investigate further the occasionally-occurring phenomenon of non-crossing I-V curves following carbachol, experiments were performed at higher bathing potassium concentrations (10 and 25 mM) to confirm further that the carbachol-induced inward current was potassium-mediated. If, as expected, both the serotonin and carbachol effects are potassium-mediated (Andrade,

Malenka & Nicoll, 1986; see Chapter 2), the reversal potentials for these effects would be expected to shift with varying potassium concentrations.

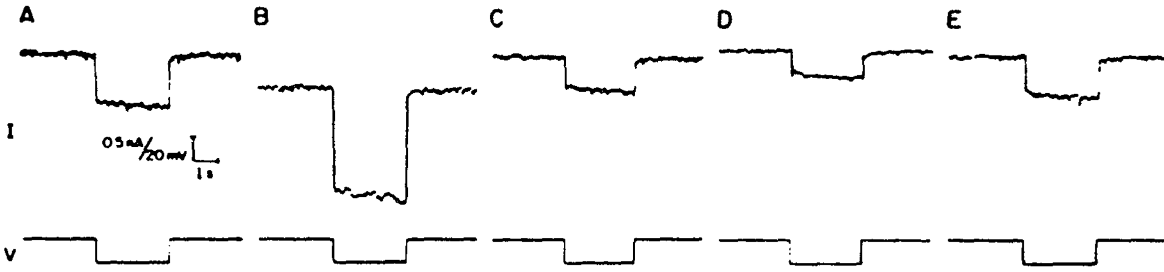
Predictably, significant shifts in the reversal potentials were found for both carbachol and serotonin upon varying bathing potassium concentrations ($F(2,105)=100.96$, $p \leq .0001$). A voltage-clamped cell in potassium (25mM, Figure 10) was held at -60 mV and either serotonin or carbachol applied. Whereas serotonin increased the membrane conductance and carbachol decreased it, the reversal potentials were similar (-46mV). Carbachol was applied in the same cell (n=6) in both 5 and 25mM of K^+. In each case, there were predictable shifts in the carbachol reversal potential with increasing potassium. Notably, in one cell, in which there was a carbachol-induced non-crossing inward current in 5mM K^+, the inward current reversed direction when carbachol was later applied in 25mM K^+, and crossed the control I-V curve at -44mV.

Figure 11 and Table 3 summarize carbachol and serotonin effects at bathing potassium concentrations of 5, 10 and 25 mM from voltage and current-clamped cells. Both carbachol and serotonin exhibited positive shifts in reversal potentials with increasing potassium concentration. The reversal potential for carbachol is significantly lower than the reversal potential for serotonin and the predicted reversal potential at potassium (5mM). Whereas the reversal potential for carbachol is also significantly lower than the reversal potential for serotonin at 10 mM potassium ($t(23)=2.79$, $p \leq .01$), the reversal potentials for both carbachol and serotonin failed to differ at a higher (25mM) potassium concentration ($p > .50$). A substantial percentage (48% and 25%, respectively)

Figure 10.

Carbachol and serotonin in 25 mM K^+. Chart records and I-V plots are shown from a voltage-clamped cell. 0.5 μM TTX present throughout the experiment. Top row: current, bottom row: voltage. a) Cell was held at -60 mV and stepped to -80 mV for 3 sec. b) 4 min into 10 μM serotonin. Note the inward current seen at holding voltage, and even larger current observed at -80 mV. c) 25 min after washout of serotonin, current recovers to baseline level. d) 9 min after application of 10 μM carbachol. Note the outward current seen at holding and larger current observed at -80 mV. e) 31 min after washout of carbachol, current recovers to baseline level. Vertical cal: 0.5 nA/20mV. Horizontal cal: 1 sec. f-g) Complete I-V relationships for serotonin and carbachol, respectively. Note the crossover for both serotonin and carbachol at ~-46 mV, and recovery of both curves after washout. Recording electrode: KCl.

Figure 10



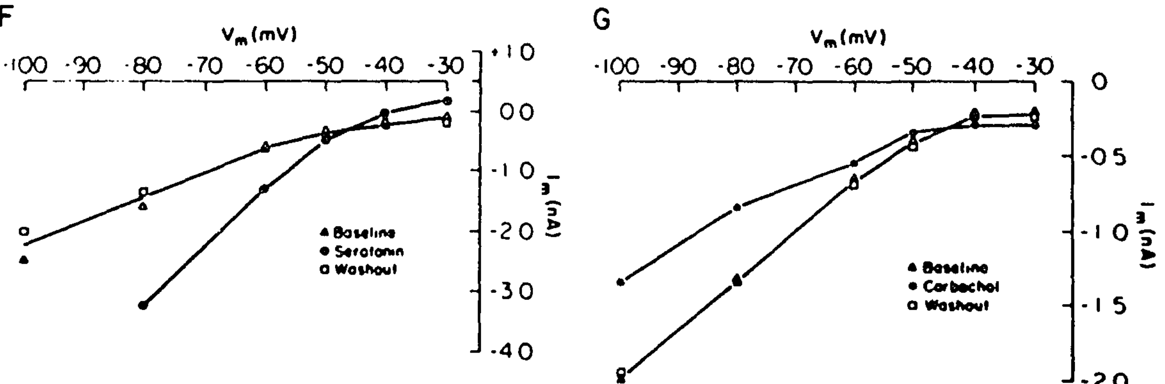


Figure 11.

Reversal potentials for carbachol and serotonin shift with increasing $[K^+]_o$. Reversal potentials (E_{rev}) for carbachol (closed squares and dotted line) and serotonin (open squares) effects are plotted at 5, 10 and 25 mM bathing potassium concentrations. The theoretical Nernstian shift over these concentrations is also shown (solid line), computed according to the equation : $E_k=58 \log ([K]_{out}/[K]_{in})$. Numbers refer to number of cells in each condition. Numbers in parentheses refer to number of cells at each potassium concentration in which a non-reversing inward current was obtained in carbachol. The solid line denotes the theoretical Nernst relationship for this range of potassium concentrations. The slope of the line was 58mV per 10-fold change in $[K^+]_o$, and the intercept was chosen so that the line would cross at the average E_{rev} for serotonin and carbachol in 25 mM potassium. This point was selected because the values for serotonin and carbachol had the least variability, and were not significantly different (see above). Note that while the E_{rev} values for serotonin lie close to the predicted Nernstian values, there is a deviation in carbachol at both 5 and 10 mM potassium.

Figure 11

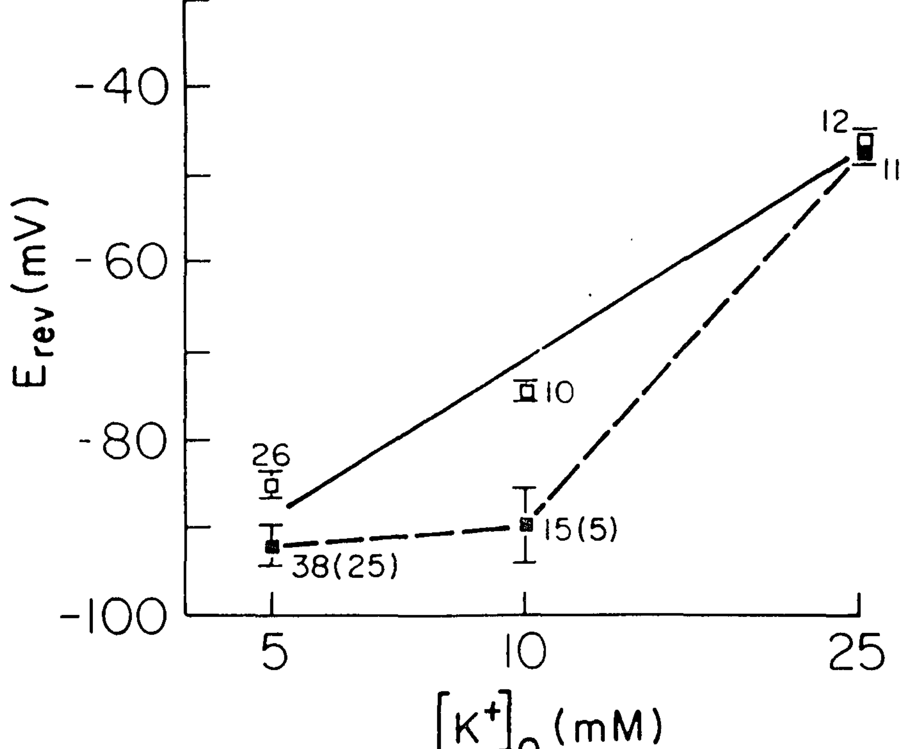


Table 3 : Reversal potentials for carbachol and serotonin at 5, 10 and 25 mM bathing potassium concentrations

Condition	Bathing K+ concentration (mM)	Avg. reversal potential (mV)	N	Number of non-crossing cells
Carbachol	5	-92.0 ± 2.5*	38	25
"	10	-90.0 ± 4.4*	15	5
"	25	-47.8 ± 1.2	11	0
Serotonin	5	-85.0 ± 1.4	26	0
"	10	-74.5 ± 1.2	10	0
"	25	-46.9 ± 0.8	12	0

* significantly different ($p<.02$) than value for serotonin at corresponding K+ concentration.

of cells at both 5 and 10 mM concentrations of potassium yielded non-crossing I-V curves in carbachol, but none were obtained at 25mM potassium, and none were observed for serotonin.

In summary, there was a sub-population of cells in which carbachol induced an inward current which did not reverse direction near the potassium equilibrium potential. This indicates that there is a mechanism in addition to blockade of a potassium leak channel (see Chapter 2) invoked by carbachol. The non-crossing I-V curves which were observed in some carbachol experiments at bathing potassium concentrations of 5 mM occurred less frequently at 10 mM potassium and failed to occur in 25 mM potassium. This suggests that this additional mechanism is influenced, and indeed, nullified, by increasing bathing potassium concentrations. One explanation is that the observed anomalous carbachol effects (non-crossing I-V curves) are due to the existence of a dendritic conductance which is activated by carbachol.

CHAPTER 4: FUNCTIONAL MUSCARINIC SUPERSENSITIVITY IN DENERVATED RAT HIPPOCAMPUS.

The results from Chapters 2 and 3 provided a detailed analysis of the inward current induced by carbachol, which was shown to be mediated by M_1 receptor activation. The next series of experiments were designed to study the carbachol-induced inward current under abnormal conditions; namely, experimentally-induced hippocampal denervation (Benson, Blitzer, Haroutunian & Landau, 1989).

Methods

Hippocampal slices were prepared (according to methods described in Chapter 2) from normal adult rats (control group) and from rats with lesions of the fimbria-fornix (denervated group). For the denervated group, animals were anesthetized with pentobarbital (60 mg/kg i.p.), fixed in a stereotaxic device, and fimbria-fornix lesions were made by aspiration of the fimbria-fornix (Bregma -1.0) and the overlying cortex. This procedure produces reliable cholinergic denervation, although non-cholinergic inputs are also affected (Tayrien & Loy, 1984). Once the animals had returned to within 80% of their pre-surgery body weight (3-6 weeks following surgery), they were sacrificed and electrophysiological recordings obtained. The control group consisted of normal adult rats which had undergone no surgical or other intervention prior to sacrifice and electrophysiological recordings.

Electrophysiological recordings were obtained from CA1 pyramidal

neurons in tissue slices using standard intracellular recording techniques (see Chapter 2). Cells were impaled with KCl (3M)-containing microelectrodes and voltage-clamped using a single-electrode voltage-clamp apparatus (Axoclamp-2, Axon Instruments; switching frequency: 2-3 kHz, 30% duty cycle). All experiments were performed at 30-32°C and in the presence of 0.5 μM tetrodotoxin (TTX) to suppress action potentials. Following recording, acetylcholinesterase histo-chemistry was performed on all slices, to confirm the reduction in hippocampal cholinergic activity presumed to occur following the experimental denervation. A modification of the procedure of Commins and Yahr (1984) was used for acetylcholinesterase histochemistry with tetraisopropyl-pyrophosphoramide as the inhibitor of non-specific acetylcholinesterase. Sections were mounted on slides and were processed identically. The sections were "over"-incubated for 24 hours to maximize staining of the lesioned sections. Stained slices were blindly rated by an experimenter, based on appearance under light microscope, as coming from either a denervated or control animal. These ratings were subsequently confirmed.

Dose-response analyses of carbachol effects in control and denervated cells were performed using ANOVA with post-hoc contrasts. Comparison of carbachol effects on I_{AHP} in control vs. denervated cells was performed using an independent t-test. Error values referred to in the text are standard error of the mean (SEM).

Results

Carbachol (0.5 - 5 uM) was applied, and the resulting inward currents generated were measured in control and denervated pyramidal cells. Responses to brief applications of carbachol are illustrated in Figure 12, which shows the inward current produced by carbachol (0.5 and 5 μM) in a control (panels a and b) and denervated (panels c and d) cell. The carbachol-induced current was larger in the denervated compared to the control cell at both carbachol concentrations.

The inward currents induced by carbachol (0.5uM, 1uM, 3uM, 5uM) in control vs. denervated cells were significantly different between groups ($F(1,5)=11.76$, $p \leq .02$) and across doses ($F(3,15)=60.23$, $p \leq .0001$), but not for the interaction between groups and doses ($p > .40$). Thus, carbachol induced a larger inward current in denervated cells in a dose-dependent manner (contrast analysis, $.0001 < p < .03$). The currents induced by carbachol (0.5, 1, 3 and 5 uM) in control and denervated cells are summarized in Table 4. The carbachol-induced inward current was reversible following washout of the drug in all cells tested. The apparent dissociation constant (K_D) for carbachol decreased from 2.1 μM in control to 0.9 μM in denervated cells; there was a corresponding increase in the maximum current, I_{max}, from 185 pA in control to 231 pA in the denervation condition.

The ability of carbachol to block the calcium-dependent potassium current, I_{AHP}, was also examined (Figure 13). Whereas carbachol (0.5 uM) reduced (70%), but did not abolish the I_{AHP} in this and all (n=7) control cells, the I_{AHP} was completely blocked by carbachol in this and all (n=6) denervated

Figure 12.

The effect of carbachol on the inward leak current is enhanced in denervated cells. Sample records (current traces) are shown in a-d. Cells were voltage-clamped at -65 mV and carbachol applied for 2 minutes. Note the resulting inward leak current, which reversed completely after washout of carbachol. a) Control cell, 0.5 μM carbachol; b), same cell as in a, 5μM carbachol. c) Denervated cell, 0.5 μM carbachol; d) same cell as in c, 5 μM carbachol. Note break in records, which indicates period of increased chart speed (100x, not included). e) graph of average current (pA) vs. carbachol concentration (μM). Bars indicate standard error of the mean (SEM). Open circles, control; filled circles, denervated condition. Numbers under points indicate number of cells in each condition. Lines fitted according to the equation $I = I_{max} C/(K_D+C)$, where I is the inward current (pA), C is the carbachol concentration (μM) and K_D is the apparent dissociation constant. For the control curve, K_D was 2.1 μM and I_{max} was 185 pA. For the denervated condition, K_D was 0.9 μM and I_{max} was 231 pA.

Figure 12

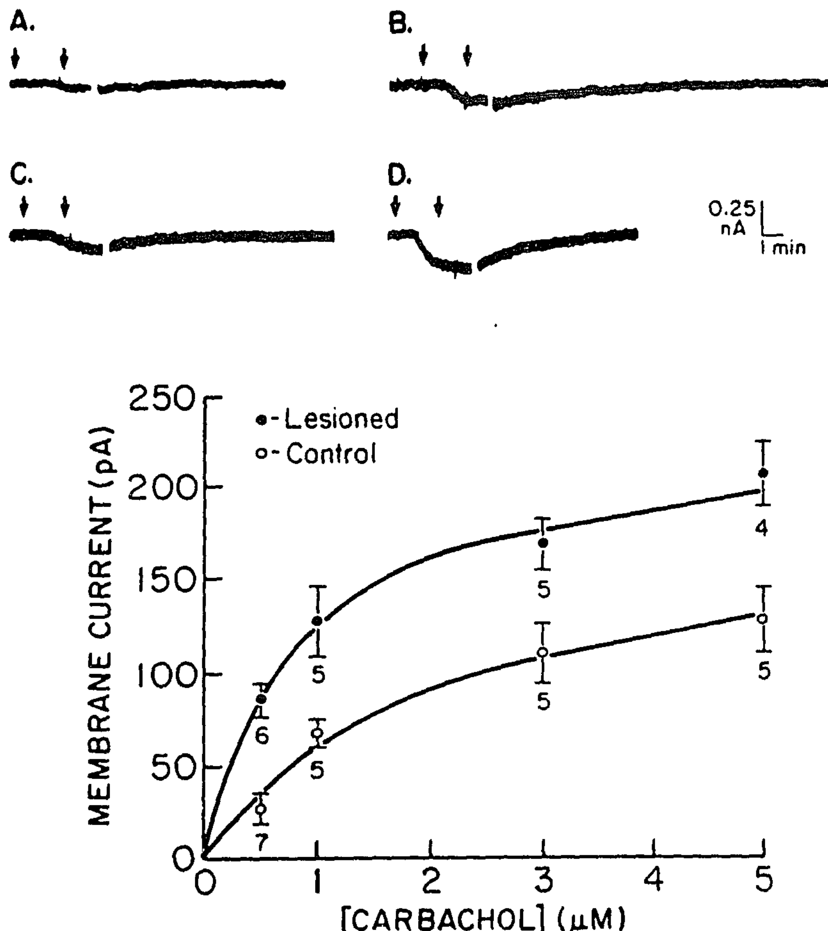


Table 4 : Carbachol-induced inward currents in control and denervated cells

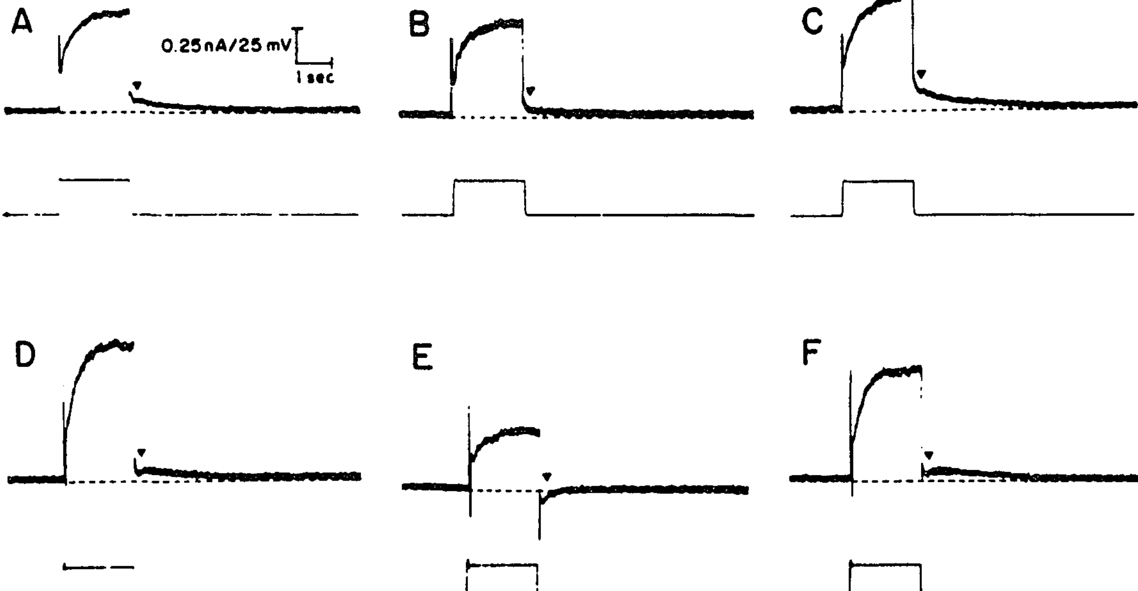
Condition	Carbachol concentration (uM)	Avg. inward current produced (nA)	N
Control	0.5	.02 ± .006	3
"	1	.07 ± .007	3
"	3	.13 ± .010	3
"	5	.14 ± .019	4
Denervated	0.5	.09 ± .009*	4
"	1	.14 ± .021*	4
"	3	.18 ± .016*	4
"	5	.21 ± .018*	4

* significantly different ($p<.02$) than effect in control cells for corresponding dose of carbachol.

Figure 13.

Carbachol effect on I_{AHP} is enhanced in denervated cells. Cells were voltage-clamped as described in Fig. 12, and I_{AHP} elicited by imposing a 20 mV, 2 second long depolarizing step. Dotted lines indicate holding current level for each condition. Arrow denotes where AHP current was measured (200 msec following end of pulse). Response of a control cell (top row) and a denervated cell (bottom row) to 2 second, 20 mV depolarizing steps are shown. Top row: control cell: Note the slowly developing current during the pulse and the outward "tail" current immediately after return to holding voltage (-65 mV). Leftmost panel, baseline; middle panel, in 0.5 μM carbachol; rightmost panel, 10 minutes after washout of carbachol. Note diminution, but not complete disappearance of I_{AHP} tail current in carbachol. Bottom row: denervated cell (holding voltage:-60 mV). Leftmost panel, baseline; middle panel, in 0.5 μM carbachol; rightmost panel, 9 minutes after washout of carbachol. Note the complete abolition of I_{AHP} by carbachol in the denervated cell, and also the fast inward current at the end of the depolarizing pulse.

Figure 13



cells ($t(11)=4.31$, $p \leq .001$). Higher concentrations of carbachol (1-5 μM) completely abolished I_{AHP} in both conditions. In all denervated cells, a fast inward current was observed at the end of the depolarizing pulse, which was more pronounced in the presence of carbachol, when the I_{AHP} tail current was completely abolished (see Fig.13e). This current was also seen in 1 control cell.

Slices were stained for acetylcholinesterase following the period of recording. All slices obtained from fimbria-fornix lesioned rats (Figure 14, left panel) showed markedly decreased acetylcholinesterase staining compared to slices obtained from control animals (Figure 14, right panel).

In summary, the results presented demonstrate muscarinic supersensitivity in hippocampal pyramidal cells following experimental cholinergic denervation. This supersensitivity was reflected in an enhanced blockade of the previously described leak current (see Chapter 2), and was dose-dependent. An enhanced blockade of I_{AHP} was also observed at one dose tested (0.5uM carbachol). Such denervation supersensitivity could have implications regarding the responsiveness of these cells to various pharmacological agents following injury or disease.

Figure 14.

Acetylcholinesterase activity in 40 micron coronal sections through the anterior hippocampus of a control (left panel) and a fimbria-fornix-lesioned rat (right panel). Both sections were mounted on a single slide and were processed identically. The sections were "over"-incubated for 24 hours to maximize staining of the lesioned section. Nevertheless, staining in the lesioned hippocampus section was so light as to force photographic over-exposure to demonstrate the section outline. Calibration: bar indicates 1mM.

Figure 14

CHAPTER 5: DISCUSSION

The present series of experiments provided evidence regarding the mechanisms underlying muscarinic excitation (depolarization/inward current) of hippocampal pyramidal cells, as well as the responsiveness of these cells to muscarinic receptor activation following denervation. Specific results will be discussed individually, followed by a general discussion of possible implications of the present results for the role of acetylcholine in learning/memory processes.

The carbachol-induced inward current is due to a reduction in potassium leak conductance.

Carbachol induces a net inward current under conditions where neither the AHP nor the M currents are active. This would most notably comprise the experiments in which the cell was held between -60 and -70 mV, loaded with cAMP and exposed to carbachol (1uM, see Chapter 2). At this membrane potential both the M and AHP conductances are inactive (Halliwell & Adams, 1982, Lancaster & Adams, 1986). Furthermore, loading the cells with cAMP blocks I_{AHP} and applying carbachol (1uM) should leave the M current largely unaffected (Madison & Nicoll, 1986, Madison et al, 1987). Thus, the inward current produced by carbachol cannot be accounted for by blockage of I_M or I_{AHP}. Since carbachol-induced depolarizations occur at voltages more negative than -60 mV, it is clear that this inward current is responsible for the

depolarizing effect of carbachol. A similar conclusion has been reached by Madison, et al. (1987), concerning the mechanism of the slow EPSP in hippocampal pyramidal neurons and is consistent with the earlier results of Dodd et al. (1981).

The carbachol effect was blocked by the potassium channel blockers, cesium, TEA and barium. Also, in the majority of cells studied, the I-V characteristic in carbachol crossed the control I-V curve at a voltage close to or negative to the potassium equilibrium potential. Furthermore, the crossover points for carbachol shifted in a positive direction when the bathing potassium concentration was increased. Thus, it appears that the carbachol effect is potassium-mediated.

The carbachol-induced inward current observed in the present studies does not appear to be a universal phenomenon, however. Gahwiler & Brown (1985) found no net inward currents produced by carbachol in cultured hippocampal cells held at voltages negative to -60 mV. A similar negative result has also been found by McCormick & Prince (1986) in cingulate cortex. Thus, it appears that the additional inward current described above may not be expressed in some experimental situations (i.e., cultured neurons) or in some brain areas other than the hippocampus.

The inward current produced by carbachol was sometimes difficult to reverse completely following washout of the drug. This resembles the finding by Andrade & Nicoll in hippocampal pyramidal neurons (1987), that serotonin often produces a small inward current which is only partially reversible. This phenomenon might involve receptor desensitization, which results in a long-

term inactivation of receptors (Katz & Thesleff, 1957; Boyd, 1987). Muscarinic receptor desensitization following prolonged exposure to agonists has indeed been shown to occur in hippocampus (Muller & Misgeld, 1986).

A second effect of carbachol may be an increase in dendritic membrane conductance.

A sub-population of cells yielded carbachol I-V curves which did not cross the control I-V curves. Similar non-crossing I-V curves have been reported previously following drop application of acetylcholine (Segal, 1982). Furthermore, carbachol crossover values were more variable, and were significantly lower than for serotonin at 5 and 10, but not 25 mM potassium concentrations. One possibility is that the non-crossing curves are artifactual (e.g., due to poor space clamp). This is not likely, however, because: a) the non-crossing curves were reversed by atropine; b) serotonin, when applied in the same cells in which such curves were observed in carbachol, produced predictable cross-overs in the I-V curves; and c) non-crossing I-V plots were seen in 5 and 10mM concentrations, but never in 25 mM concentrations of potassium. Thus, the phenomenon of non-crossing I-V curves in carbachol appears to be a genuine effect.

It would be possible to explain the observed effects of carbachol by postulating the existence of a dendritic conductance which is activated by carbachol. In high potassium, the membrane resistance should decrease and changes in dendritic conductance would contribute less to measurements taken at the soma. The effect of the dendritic conductance increase would not be

discernible, and the cross-over voltage for carbachol would be similar to that for serotonin, and close to E_K. Under normal circumstances (e.g., 5mM bathing potassium concentration), however, the dendritic conductance would summate with the potassium conductance decrease caused by carbachol. The variability in carbachol I-V characteristics would then result from different contributions of the dendritic conductance across cells. This difference could be a function of the magnitude of this conductance in the dendrites, or the distance of such dendrites from the soma. Further insight into the nature of this conductance is suggested by the finding that the carbachol effects are completely blocked by potassium channel blockers. This may indicate that the dendritic conductance is blocked directly by these agents, or that it is voltage-dependent and becomes activated as a consequence of carbachol blocking the potassium leak channel. The ionic nature of this conductance is unclear. It could not be carried by chloride or calcium ions, since the depolarizing effect of carbachol was insensitive to the chloride content of the recording electrode or to the presence of cadmium ions in the bathing medium. Therefore, the most likely ion contributing to this conductance would be sodium. It is noteworthy that muscarinic agonists induce an inward current possibly carried in part by sodium ions in bullfrog sympathetic ganglion cells (Jones, 1985). Thus, it is possible that a similar non-specific ion channel mediates a conductance increase in the dendrites in hippocampal pyramidal cells.

Finally, one might consider the possibility that such a dendritic conductance is induced by another transmitter released by interneurons. However, this possibility is unlikely since carbachol induced a non-crossing I-V

curve in three cells studied in solutions containing cadmium, which should prevent transmitter release by blocking calcium channels. However, studying the effects of bicuculline might be of value to more directly explore contributions of GABA-ergic interneurons. An interesting consequence of the dendritic conductance change might be that acetylcholine enhances a dendrite to soma differential, since it will tend to enhance synaptic effects in the soma and decrease them in the dendrites.

The muscarinic nature of the carbachol effects

The carbachol effects were reversed by atropine, a muscarinic antagonist. In addition, the depolarization was blocked by pirenzepine, an M_1 receptor antagonist. Thus, the cholinergic actions described in these experiments are likely to be mediated via activation of muscarinic, particularly, M_1, and not nicotinic, acetylcholine receptors. This is consistent with other reports of primarily muscarinic-mediated actions of cholinergic agents in the hippocampus. Thus, muscarinic agonists have typically been found to mimic the effects of acetylcholine, whereas nicotinic agonists typically fail to produce effects (Cole & Nicoll, 1983, 1984; Nicoll, 1985). Furthermore, all excitatory effects such as depolarization, increased membrane resistance, increased firing rates and effects on membrane currents have been reversed with muscarinic antagonists, but not with nicotinic antagonists (Benardo & Prince, 1982; Cole & Nicoll, 1983, 1984; McCormick & Prince, 1986). Finally, the time-course of events related to nicotinic receptor activation are significantly faster than those described in the present study (Brown, 1983).

However, the experiments performed here were limited to investigations of muscarinic agents, and nicotinic actions were not directly studied. Thus, it is not possible to rule out other cholinergic effects on hippocampal pyramidal cells which may be due to nicotinic receptor activation. Nicotinic acetylcholine receptors have been identified and characterized in the hippocampus (Aracava, Deshpande, Swanson, Rapoport, Wonnacott, Lunt & Albuquerque, 1987), and rare excitatory responses to nicotine have been reported (Benardo & Prince, 1982). Thus, future research should investigate more explicitly the role of nicotinic agents in cholinergic actions in the hippocampus.

Carbachol effects in denervated hippocampus

The findings described in Chapter 4 represent the first demonstration of central muscarinic denervation supersensitivity at the single cell level. Thus, the difficulties inherent in receptor binding studies, which analyze large, unspecified populations of cells, were circumvented. The present results were obtained in voltage-clamped cells in the presence of TTX. Therefore, the effects cannot be due to presynaptic activation, which may have influenced the results of Bird & Aghajanian (1975). Furthermore, the supersensitivity cannot be explained by changes in acetylcholinesterase activity, since carbachol is not sensitive to breakdown by acetylcholinesterase (Bird & Aghajanian, 1975). Thus, these effects must be due to changes in the cholinergic receptor. This supersensitivity was reflected by both an increase in leak current, which is mediated by M_1 receptors (Muller & Misgeld, 1986), and an enhanced blockade of I_{AHP}, which is an M_2 receptor mediated effect (Muller & Misgeld, 1986). The

dose-response analysis for the leak current indicates both an increase in I_{max} and a decrease in the apparent K_D. The former may indicate an increase in the total number of M_1 receptors, whereas the latter points to a qualitative change in receptors in denervated cells. A possible explanation for the decrease in K_D may involve a change in coupling of the receptor to effector molecules. If the receptors were coupled through G-proteins, their affinity for the agonist might be modulated by changes in such proteins (Stryer & Bourne, l986).

Still, the findings presented with regards to the effects of denervation are somewhat preliminary, and future research could address a number of additional points. First, the effects of fimbria-fornix lesions are not specific to the cholinergic system (Sara, 1989; Tayrien & Loy, 1984), and other transmitter systems may have been altered due to the procedure. It is not clear what effects these other alterations may have had on the denervated cells. In this regard, it would be interesting to compare these results with the responses of CA1 cells to cholinergic agents following treatment with AF64A (a specific cholinergic neurotoxin), which would yield exclusive cholinergic denervation.

Second, only qualitative estimates were obtained regarding the extent of cholinergic denervation by observing gross changes in acetylcholinesterase staining in denervated vs. control hippocampal slices. It would be of value to quantify the extent of cholinergic depletion (e.g., by measuring ChAT activity), and assess the magnitude of the correlation between the amount of cholinergic depletion and the size of the response to cholinergic agents. In this regard, it would be interesting to see whether the nicotinic as well as muscarinic component of the hippocampal cholinergic system demonstrates denervation-

induced supersensitivity.

Finally, it would be useful in future research to study the time-course of the supersensitivity effect. It is unclear at this time whether the supersensitivity demonstrated (evidenced at 3-6 weeks post-lesion) is a temporary response to the insult, or whether it represents a long-lasting, permanent change in the functioning of the system as a result of injury.

Speculations regarding the role of acetylcholine in learning and memory

The role of the hippocampal cholinergic system in learning and memory processes has been well-documented (see reviews: Barnes, 1988; Eichenbaum, Otto & Cohen, 1992; Matthies, 1989; Milner, 1989; Squire, 1987; Squire & Zola-Morgan, 1988). Evidence linking this system with learning and memory has come from a number of sources, including studies of disease states (e.g., Alzheimer's Disease), neurochemical and behavioral changes associated with aging, animal lesion studies and pharmacological studies. Numerous animal lesion studies have confirmed that hippocampal lesions can produce long-lasting impairments in new learning (Zola-Morgan, Squire & Amaral, 1989; Zola-Morgan, Squire, Amaral & Suzuki, 1989; van der Staay, Raaijmakers, Lammers & Tonnaer, 1989). Electrophysiological recordings have demonstrated changes in hippocampal cellular activity associated with learning (O'Keefe & Nadel, 1978; O'Keefe & Speakman, 1987; Wible, Findling, Shapiro, Lang, Crane & Olton, 1986). Age-related changes in hippocampal functioning have been associated with declines in memory functioning (Barnes, 1988; deToledo-Morrell, Geinisman & Morrell, 1988; Lamour, Bassant, Jobert &

Joly, 1989; Landfield, 1988; Walker, Kitt, Struble, Wagster, Price & Cork, 1988; Winocur, 1988). Alterations in the hippocampus have also been found in memory-impaired humans (Press, Amaral & Squire, 1989; Squire, 1987; Zola-Morgan, Squire & Amaral, 1986). Reductions in cholinergic markers have been reported in the hippocampi of patients with Alzheimer's Disease (Terry & Davies, 1980; Coyle, Price & DeLong, 1983; Quirion, Aubert, Lapchak, Schaum, Teolis, Gauthier & Araujo, 1989). In the hippocampus and cortex, no alteration of M_1 receptor binding sites have been reported, with markedly decreased densities of [3H]ACh sites observed, which were thought to reflect presynaptic M_2 receptors (Quirion et al., 1989). Of considerable relevance to the present studies, an individual with a severe amnesia resulting from ischemia was found on autopsy to have a complete, bilateral loss of cells confined to the entire CA1 region of the hippocampus (Zola-Morgan et al., 1986).

Pharmacological studies have involved examination of the effects of cholinergic agonists or antagonists on memory performance in normal and/or disease states. There have been a number of reports of negative effects of cholinergic antagonists on memory in animals (McDonald, Costa & Murphy, 1988; Whishaw, 1989; Wirsching, Beninger, Jhamandas, Boegman & El-Defrawy, 1984) and humans (Beatty, Butters & Janowsky, 1986). Cholinergic agonists have been found to enhance performance on learning/memory tasks in some animal studies (Haroutunian, Barnes & Davis, 1985; Ordy, Thomas, Volpe, Dunlap & Colombo, 1988). There have been numerous reports of cholinergic drug (e.g., agonist) treatments for AD patients, but results from these studies have been contradictory (Davis, Mohs, Davis, Horvath, Greenwald,

Rosen, Levy & Johns, 1983; Jotkowitz, 1983; Summers, Majovski, Marsh, Tachiki & Kling, 1986; Thal, Fuld, Masur, Sharpless & Davies, 1983; for reviews, see Gray, Enz & Spiegel, 1989; Smith, 1988). For example, not all doses investigated have been effective, and, in fact, some (higher) doses have been found to disrupt, rather than enhance, performance (Haroutunian, Barnes & Davis, 1985). It has been suggested that, since muscarinic receptor agonists also tend to reduce acetylcholine release through stimulation of presynaptic autoreceptors (probably M_2), this may counteract the positive effects of postsynaptic activation. This would reduce the therapeutic usefulness of currently available muscarinic agents, which typically have mixed activity at various receptor subtypes (Haroutunian, Barnes & Davis, 1985). This notion is similar to that proposed by Mash, Flynn & Potter (1985). Thus, the negative findings may be related to the lack of selectivity of cholinergic agents used, in addition to an incomplete understanding of the complexity of the cholinergic system, rather than to a lack of involvement of the cholinergic system in these processes. Of interest in this regard is a report that FKS-508, a novel muscarinic agonist which is highly selective for M_1 receptors ameliorated the memory impairments induced by AF64A treatment in rats (Nakahara, Iga, Saito, Mizobe & Kawanishi, 1989).

An alternative explanation for the lack of consistency of therapeutic effects of cholinergic stimulation in diseases involving cholinergic dysfunction may be related to the present findings. Denervation supersensitivity has been proposed as a recovery mechanism following central nervous system injury (Almli & Finger, 1988). Deficits following brain injury might recede if

postsynaptic receptors became supersensitive and the limited remaining inputs after damage were able to sufficiently drive the postsynaptic cells. As demonstrated in the present studies, the existence of a central muscarinic supersensitivity following hippocampal denervation might influence the responsiveness of these cells following injury, or to treatment with muscarinic agonists. Thus recovery might be expected to occur even in cases where the total number of receptors is decreased due to post-synaptic cell death (Reinikainen, Reikkinen, Halonen and Laakson l987). However, as demonstrated in Chapter 2, cholinergic stimulation of hippocampal pyramidal cells sometimes excites cells to the point of depolarization blockade, with the net effect of decreasing the ability of cells to generate action potentials. Should this effect be enhanced following denervation, the supersensitivity might result in a counter-therapeutic effect. To further explore this issue, future research could investigate the relationship between the extent of denervation supersensitivity in hippocampus and behavioral deficits/recovery. The findings would be particularly relevant if the supersensitivity was found to be long-lasting, as opposed to a transient phenomenon.

As indicated above, however, caution must be taken with regard to extending the results of the present studies to learning/memory or disease states. While only the cholinergic (muscarinic) system was studied in this dissertation, the manipulations described (e.g., hippocampal denervation) certainly affected other neurotransmitter systems as well. Sara (1989) reported a 50% reduction in hippocampal norepinephrine as well as acetylcholine following fornix lesions in rats studied. Thus, some deficits reported following

these types of lesions (particularly, septo-hippocampal) could be due to destruction of non-cholinergic cells (Mayo, 1989). Second, pharmacological manipulation of other neurotransmitter systems, including dopaminergic (Levin, 1988), opioid (Levin, 1988) and GABA-ergic (Chrobak, Stackman & Walsh, 1989) have been shown to influence memory functioning. Furthermore, changes in other transmitter systems have been reported in AD and normal aging. These include somatostatin (Beal, Mazurek, Tran, Chattha, Bird & Martin, 1985), galanin (Crawley & Wenk, 1989) and the catecholamines (McEntee & Crook, 1990). These findings, coupled with the findings of widespread neuropathological changes associated with AD (e.g., Pearson, Esiri, Hiorns, Wilcock & Powell, 1985) render the "cholinergic hypothesis" of dementia over-simplified, at best.

An additional approach towards understanding the nature of the role of the cholinergic system in learning/memory requires both further analysis and characterization of the cellular effects of cholinergic agents, and a related physiological mechanism which could underlie learning/modifiability of behavior. Long-term potentiation (LTP) has been regarded as such a mechanism, underlying information storage in the brain (Teyler & Discenna, 1984; Gustafsson & Wigstrom, 1988; Kennedy, 1989; Matthies, 1989). LTP refers to a long-lasting increase in synaptic efficacy resulting from afferent tetanic stimulation. First described in the rabbit hippocampal formation in 1973 (Bliss & Lomo, 1973), LTP was elicited by the application of brief (1s or less), high frequency stimulation of the perforant path, which produced subsequent enhancement of synaptic transmission in the dentate gyrus lasting up to several

hours (Bliss & Lomo, 1973). LTP has since been demonstrated in a number of other species and brain structures, including neocortex, amygdala, septum, superior cervical ganglion and optic tectum (see Teyler & Discenna, 1984, for review). It has been associated with both morphological (Desmond & Levy, 1990, Gustafsson & Wigstrom, 1988) and biochemical (Linden, Murakami & Routtenberg, 1986; Goh & Pennefather, 1990) changes.

The relevance of LTP to learning and memory requires both the demonstration that it is related to memory, and that the intact animal has the innate ability to elicit LTP due to naturally-occurring patterns of synaptic activity. Berger (1984) demonstrated that induction of LTP in the perforant path-dentate gyrus synapse in naive rabbits enhanced their rate of learning a two-tone discrimination task (measured by nictitating membrane response) compared to control animals. Blockade of NMDA receptors (with AP5) caused a selective impairment in spatial learning, accompanied by a suppression of LTP in vivo, whereas other types of learning (e.g., visual discrimination) were not affected (Morris, Anderson, Lynch & Baudry, 1986). Barnes (1988) reviewed a series of experiments from his laboratory demonstrating a significant correlation between forgetting rates on spatial learning tasks and decay rates of LTP in both young and old rats. Furthermore, exposure to novel, enriched environments induced a spontaneous increase in hippocampal evoked potentials which was similar in magnitude and time course to LTP.

The cellular mechanisms underlying LTP have been widely debated (Teyler & Discenna, 1984; Robinson, 1986; Gustafsson & Wigstrom, 1988; Kauer, Malenka & Nicoll, 1988; Bekkers & Stevens, 1990; Malinow & Tsien,

1990). It is still unclear whether the expression and maintenance of LTP involves primarily presynaptic or postsynaptic processes. Regarding presynaptic mechanisms, Malinow & Tsien (1990) and Bekkers & Stevens (1990) have demonstrated an increase in the probability of transmitter release during LTP, utilizing quantal analyses in either whole-cell voltage-clamped or cultured hippocampal neurons. However, other investigators emphasize post-synaptic events as critical for LTP maintenance. Triggering of LTP requires activation of NMDA receptors, a subtype of glutamate receptors which are located in large numbers in the hippocampus, particularly in regions CA1 and the dentate gyrus (Kennedy, 1989). Blockade of these receptors, while not interfering with normal synaptic transmission, prevents the induction of LTP (Harris, Ganong & Cotman, 1984). These receptors are normally blocked in a voltage-dependent manner by magnesium. Depolarization of post-synaptic neurons (such as that produced by afferent tetanus) relieves the magnesium blockade, however, and allows the entry of calcium ions into the cell. This calcium influx has been demonstrated to be a necessary occurrence for LTP to be produced (Kennedy, 1989; Muller & Lynch, 1990). Low frequency (below that typically required to induce LTP) afferent stimulation, when coupled with depolarizing current pulses on post-synaptic neurons, has been found to elicit LTP (Gustafsson, Wigstrom, Abraham, & Huang, 1987), and LTP can be preferentially induced during the positive phase of the theta rhythm (Pavlides, Greenstein, Grudman & Winson, 1988). LTP can be prevented by hyperpolarization of the post-synaptic cell during afferent tetanus (Malinow & Miller, 1986).

Neuromodulatory influences on LTP have been investigated, particularly in light of interest in the role of various neurotransmitters, particularly acetylcholine, in learning/memory. In an early review of the literature, Collingridge (1985) reported facilitatory effects of GABA antagonists, variable effects of noradrenergic agonists and the opiates, inhibitory effects of adenosine, and no effects of dopaminergic or cholinergic agents on LTP. Hopkins & Johnston (1984) provided evidence for noradrenergic facilitation of LTP, demonstrating a reversible increase in the magnitude, duration and probability of induction of LTP in the CA3 region of hippocampus by norepinephrine and the beta-adrenergic agonist, isoproterenol. The beta-adrenergic antagonists, propranolol and timolol reversibly blocked LTP; thus, noradrenergic effects on LTP appear to be mediated by beta-adrenergic receptors. They later provided evidence that noradrenergic effects on LTP depend on stimulation of cyclic-AMP (cAMP) production, and that cAMP plays a modulatory role in the induction of LTP (Hopkins & Johnston, 1988).

Recent studies of cholinergic effects on LTP have been variable (Blitzer, Gil & Landau, 1990; Chavez-Norieta, Bliss & Halliwell, 1989; Williams & Johnston, 1988). The latter investigators reported that LTP of the mossy fiber-CA3 synapse was blocked by muscarine, in a dose-dependent fashion. Muscarine (1uM) typically blocked the induction of LTP, while muscarine (10uM) produced a significant depression of synaptic transmission. They proposed that the muscarinic effect is mediated by a decrease in calcium current known to be produced by muscarine in CA3 cells. In contrast, LTP was suppressed in a dose-dependent manner in hippocampal CA1 neurons by

scopolamine, a muscarinic antagonist (Hirotsu, Hori, Katsuda & Ishihara, 1989). If applied after the establishment of LTP, however, scopolamine had no effect. They suggested that muscarinic mechanisms, therefore, are of little importance in the maintenance of LTP. While scopolamine had no effect on LTP in CA1, it significantly suppressed LTP in area CA3 (Tanaka, Sakurai & Hayashi, 1989). The cholinesterase inhibitor, HP 029, significantly enhanced LTP both in CA1 and CA3 regions. Carbachol has recently been found to enhance LTP in the CA1 region of hippocampal slices (Blitzer, Gil & Landau, 1990). This finding was obtained only when the depressive effect of carbachol on the EPSP was controlled for, however, suggesting that the facilitatory effect of cholinergic agents on LTP may be masked by it's inhibitory effect on the EPSP. Atropine blocked carbachol's effect on LTP; thus, the effect appears to be muscarinic.

Based on findings to date, conclusions regarding the role of the cholinergic system in LTP are unclear. Carbachol's facilitatory effect on LTP (Blitzer, Gil & Landau, 1990) may be related to its blockade of the potassium leak current as described in the present studies. Since carbachol induces depolarization by blocking the potassium leak current, this blockade would allow calcium entry, thought to be necessary for the induction of LTP. Furthermore, the finding that carbachol decreases membrane conductance, thereby enhancing synaptic transmission, could be another mechanism by which it exerts a facilitatory effect on LTP. While the present studies provide some basis for muscarinic effects on LTP induction, they do not necessarily suggest a role in the maintenance of LTP; thus, the functional relevance of the muscarinic system in LTP may be limited. In addition, the variability in effects of

cholinergic agents on LTP may be due to different experimental procedures, or different mechanisms underlying LTP in various brain regions.

In conclusion, the present studies have provided a detailed account of muscarinic cellular actions in the hippocampus, and examined the response of this system to experimentally-induced denervation. These findings provide a basis for further research and insights into the role of acetylcholine in learning and memory processes, as well as implications for treatment of brain injury or degenerative disorders involving the hippocampal cholinergic system.

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