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Muscarinic acetylcholine receptor and membrane dynamics: A possible factor in age related memory dysfunction

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MUSCARINIC ACETYLCHOLINE RECEPTOR AND MEMBRANE DYNAMICS: A POSSIBLE FACTOR IN AGE RELATED MEMORY DYSFUNCTION

by

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Abstract

MUSCARINIC ACETYLCHOLINE RECEPTOR AND MEMBRANE DYNAMICS: A POSSIBLE FACTOR IN AGE RELATED MEMORY DYSFUNCTION

by

Joyce A. Schoenheimer

Adviser: Professor Arnold S. Lippa

The demonstrated involvement of the hippocampal muscarinic acetylcholine receptor (mAChR) in several cognitive functions (e.g. learning and memory) has precipitated numerous efforts to characterize the mAChR. Four mAChR subtypes have been identified, all of which contain seven membrane spanning alpha-helical segments. Acetylcholine binding sites are thought to be deep within segmengts two and three. The proposed structure of the mAChR indicates that a large percentage of the receptor is imbedded within the neuronal membrane. This model suggests a dynamic relationship between membrane microviscosity and receptor conformation.

Membrane fluidity is controlled by a process that has been termed homeoviscous adaptation; a process by which the cell monitors and modulates its own membrane viscosity. The goal of these experiments was to characterize mAChR binding and determine whether manipulating membrane viscosity alone or in combination with membrane composition would affect receptor density, affinity or the ability of ligands to induce conformational in the receptor.

Interconversion of the mAChR can be studied using class A and class B agonists. While both classes of agonists can stimulate hippocampal cell firing equally well, they differ in several measures. Class A agonists (e.g. ACh and carbachol) bind to the receptor in a complex manner, induce a conformational change in the receptor, are very efficacious stimulators of PI hydrolysis and produce desensitization with prolonged application. In contrast, class B agonists (e.g. oxotremorine and pilocarpine) bind to the receptor in a simple manner, do not induce a conformational change, simulate PI hydrolysis only slightly and do not produce desensitization.

In these studies, the relationship between the mAChR and it's membrane environment was examined in two different ways. First, membrane rigidity alone was increased by decreasing the incubation temperatures in receptor binding assays. Second, membrane rigidity and membrane cholesterol levels were decreased with the drug AL721, which extracts cholesterol from the membrane. This treatment decreased the rigidity of the membrane, as measured by fluorescence polarization. The effect of these manipulations on antagonist and agonist binding was observed.

The first series of experiments demonstrated that decreasing membrane fluidity affected ligand-receptor interactions. Increases in the affinity of the receptor for the mAChR antagonist atropine, the class A agonist carbachol and the class B agonist oxotremorine were observed. The K_D for ^{3}H-QNB was unaltered and a small significant increase in the Bmax was observed at 15^{o}C. These observations could suggest that rigidifying the membrane may make the receptor more accessible to the

ligand. The ability of the ligands to induce a conformational change in the receptor appeared to be unaltered by the increased microviscosity.

In contrast, AL721 appeared to affect the conformation of the receptor. This change was demonstrated by the data showing oxotremorine binding to a single state of the mAChR in the control condition and to more than one state of the mAChR following AL721 treatment. This change from a simple binding profile to a complex one has also been observed following the increase of membrane cholesterol. It could be that deviations from an optimal membrane fluidity in either direction, have similar affects on receptor binding.

The concept of homeoviscous adaptation allowing the maintenance of normal cell functioning and deficits in this homeostatic process causing chronic membrane fluidity changes may have potential clinical applications. One of the consequences of aging is an increase in the cholesterol to phospholipid ratio in neuronal membranes, and a decreased ability of aged membranes to respond to fluidizing agents, such as ethanol.

AL721 was therefore used to fluidize hippocampal membranes from aged rats and ^{3}H-QNB binding and oxotremorine binding was measured. The goal was to characterise the aged mAChR and the flexibility of the aged membrane. No significant effects of AL721 on binding were observed. A decreased sensitivity of aged membranes to AL721 in combination with a decreased effect of fluidizing on oxotremorine binding suggests that these two events were related in the young animals. A comparison between the young and aged systems further suggests that the difference lies in the flexibility of the aged system, since the control values for ^{3}H-QNB and oxotremorine binding were essentially equal in both systems.

In summary, altering the microenvironment of the receptor does affect the receptor. Manipulation of membrane microviscosity with temperature causes a change in receptor affinity, while AL721 causes a change in the receptor-ligand interaction as measured by oxotremorine.

Finally data from aged tissue suggests the possibility that aged membrane is less responsive to its environment and is perhaps less affected by changes in membrane composition. This would support the view that aged membranes may demonstrate a deficit in homeoviscous adaptation.

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The support and inspiration that I received from my family throughout my education enabled me to begin and complete my graduate studies. I would therefore like to acknowledge my gratitude by dedicating this work to my grandparents, Robert and Dina Gersten, to my parents, Pierre and Janet Schoenheimer, to my sister Linda and to my brother Daniel. I would also like to dedicate this work to the memory of my grandparents Fritz and Ellen Schoenheimer. The memories of their strength and love are a blessing to me and to everyone who knew them.

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1.10 Characterization of the muscarinic acetylcholine receptor

1.11 Receptor Binding

1.11a Antagonists

A large amount of information concerning muscarinic receptors has been obtained using radioligand receptor binding techniques. These studies have provided many insights into receptor conformation and function. The availability of a large number of tritiated muscarinic agonists and antagonists has enabled researchers to pharmacologically characterize mAChR binding either directly using the radiolabeled antagonists and agonists in binding experiments or indirectly in competition experiments.

Classical muscarinic antagonists which have been radiolabeled and used in binding studies include quinuclidinyl benzilate (QNB) (Yamamura and Snyder, 1974), atropine (Farrow and O'Brien, 1973), N-methylscopolamine (Hulme, Birdsall, Burgen and Mehta, 1978), benzetimide (Soudijn et al., 1973), benzilylcholine (Birdsall, Burgen, Haley and Hulme, 1976), benzilylcholine mustard (Fewtrell and Rang, 1973) and N-methyl-4-piperidinyl benzilate (Kloog and Sokolovsky, 1977). These antagonists bind to the receptor in conformance with mass action for a single class of receptors. The binding is saturable and stereospecific and it is inhibited by mAChR specific compounds. The ability of the classical antagonists to bind to all receptors with equal affinity is demonstrated by Scatchard analysis (Scatchard, 1949) and Hill plots of the binding data. A Scatchard analysis of

the data results in a linear plot, and inhibition data yields a Hill coefficient not significantly different from unity. The affinity constants for the classical antagonists tend to be the same across brain regions and peripheral tissues.

In contrast to the classical antagonists, non classical antagonists, such as pirenzepine (PZ), recognize a heterogeneous population of receptors (Hammer, Berrie, Birdsall, Burgen and Hulme, 1980; El-Fakahany, Cioffe, Abdellatif and Miller, 1986; Watson, Roeske and Yamamura, 1982, 1986) the affinity constants for PZ varying greatly among tissues. PZ binds with high affinity to muscarinic receptors in the cerebral cortex, hippocampus, striatum (Watson, Roeske, Vickroy et al., 1986). This population of receptors with a high affinity for PZ has been called M1 receptors. M2 receptors are characterized by a low affinity for PZ and are found in the cholinergic nerves, superior colliculus, brainstem, cerebellum, heart and smooth muscles. (Watson, Roeske, Vickroy et al., 1986). Areas rich in M1 receptors generally exhibit low affinity for agonists, and those rich in M2 exhibit a high affinity for agonists. The terms M1 mAChR and mAChR with a low affinity for agonists cannot be used interchangeably. These two populations seem to be distinct. The same holds true for the mAChRs described as M2 or having a high affinity for agonists. A comparison of the inhibition of ^{3}H-ACH by PZ and the inhibition of ^{3}H-QNB by PZ shows that PZ inhibits both in a similar fashion. The two inhibition curves are not different despite the fact that ^{3}H-ACH labels SH and H affinity sites and ^{3}H-QNB labels SH, H and L affinity sites. This type of data indicates a lack of correlation between the sites labelled by ^{3}H-ACH and either M1 or M2 sites as defined by PZ. These data do not support the suggestion that

subclassifications based on agonist binding and affinity for PZ identify the same subset of receptors (Gurwitz, Kloog and Sokolovsky, 1985).

1.11b Agonists

Early agonist binding data suggested the existence of multiple receptor states (Birdsall, Burgen and Hulme, 1978; Ehlert, Roeske and Yamamura, 1981; Amitai, Avissar, Balderman and Sokolovsky, 1982; Birdsall, Burgen and Hulme, 1979a; 1979b). Subsequent studies concerning the multiplicity of agonist binding states, or sites , of the muscarinic receptor have identified two classes of agonists, class A and class B (Fisher, Klinger and Agranoff, 1983). Class A agonists inhibit binding in a complex manner (Fisher et al., 1983; Fisher, Figueiredo and Bartus, 1984; Lippa et al., 1985; Lippa, Critchett and Joseph, 1986; Vauquelin, Andre, DeBacker, Laduron and Strasberg, 1982), and induce a conformational change in the binding site (Vauquelin et al., 1982; Lippa et al., 1986). Examples of class A agonists are acetylcholine, carbamylcholine (carbachol), oxotremorine-M, muscarine, methacholine and cis-methyldioxolane. When class A ligands interact with the receptor, they produce an increase in input resistance of the membrane and neuronal depolarization. In addition they stimulate the hydrolysis of phosphotidylinosititol (PI) (Fisher et al., 1983, 1984; Fisher and Bartus, 1985) and also result in desensitization of hippocampal pyramidal cells (Lippa et al., 1986). Class B agonists like oxotremorine, bethanachol, pilocarpine, arecoline and pentyl-trimethylammonium recognize, more frequently, a single state of the receptor (Fisher et al., 1983;1984; Lippa et al., 1985, 1986; Vauquelin et al., 1982). Binding of these ligands also produces an increase

in input resistance and neuronal depolarization. However, unlike class A agonists, binding of class B agonists produces negligible stimulation of PI breakdown (Fisher et al., 1983, 1984), no desensitization (Lippa et al., 1986) and no conformational change (Lippa et al., 1986; Vauquelin et al., 1982).

Classification of agonist binding is difficult to carry out directly. The high affinity muscarinic compounds that are available are predominantly antagonists. The above classification of class A and B agonists was derived primarily from agonist inhibiton of antagonist binding experiments. Using the high affinity antagonists such as QNB, all receptor states can be labelled with very low concentrations of the radioligand. A direct measure of super high and high affinity states can be obtained by using low concentrations of tritiated agonists in binding assays. ^{3}H-cis-methyldioxolane (CD) and ^{3}H-oxotremorine-M (Oxo-M) have been widely used in this regard. A comparison of the density of super high affinity sites identified by ^{3}H-CD binding with the total receptor density identified by ^{3}H-QNB binding indicates that super high affinity binding sites account for 5% of the total binding sites in the rat forebrain (Ehlert, Dumont, Roeske and Yamamura, 1980) and 3-6% of the total in the hippocampus. The proportion of high and low affinity sites varies greatly among tissue regions. In the hippocampus approximately 70% - 90% of the receptors reside in the low affinity state. The percentage of SH affinity sites also varies as a function of the agonist used. This suggests that the proportion of SH affinity sites is not fixed and that ligands can induce the affinity state. This is in accord with the idea that class A agonists can induce a conformational change in the receptor, whereas class B agonists cannot.

1.12 Primary structure

In recent years a considerable amount of data has been published concerning the elucidation of muscarinic receptor structure and conformation. While pharmacological studies have enabled investigators to characterize several aspects of receptor function, in many respects they have been inconclusive. The early characterization of the muscarinic receptor based on classical antagonist binding suggested a homogeneous population of receptors (Birdsall et al., 1976a, 1976b; Hulme et al., 1978; Yamamura and Snyder, 1974), varying little across tissues. Agonist inhibition of antagonist binding, however, could not be explained in terms of a single receptor site (Birdsall et al., 1978; Ehlert et al., 1981). This led to the postulation of receptor subtypes having super high, high or low affinity states for agonists, with a single affinity for antagonists. These subtypes were thought to represent different conformations of a single receptor protein (Amitai et al., 1982; Birdsall, 1979a; 1979b).

The discovery of antagonist binding site heterogeneity and the lack of correlation with agonist binding heterogeneity suggested the possible existence of distinct muscarinic receptor proteins. Initial studies using purified muscarinic receptors identified a single band when proteins were separated based on molecular weight using gel electrophoresis techniques (Birdsall et al.,1979a, Dadi, Batteiger, Keen and Morris, 1986). These data suggested the presence of a single receptor protein. (Birdsall et al., 1979a; Dadi et al., 1986; Vetner, Eddy, Hall and Fraser, 1984; Lilly, Eddy, Schaber, Fraser and Vetner, 1984) and generally concluded that the differences between receptor subtypes was probably due to environmental and post-translational differences. This technique, however, was not able to

differentiate receptor proteins whose molecular weight varied little, but whose amino acid sequences, although homologous, were distinctly different.

However, multiple receptor subtypes have recently been identified. As many as four unique receptor proteins have been identified, cloned and expressed to date. Shosaku Numa's group (Kubo et al., 1986) was the first to clone and sequence a mAChR subtype, the M1 receptor. The clone was derived from porcine cerebrum tissue, where the M1 receptor subtype is plentiful. The M2 receptor subtype was cloned and sequenced by Peralta et al. (1987) using the porcine atrial mAChR (mRNA). Bonner, Buckley, Young and Brann (1987) later isolated complementary cDNAs for three different cortical muscarinic receptors which have been called m1, m3 and m4. M2 corresponds to the cardiac receptor.

Interestingly, Bonner et al. (1987) found that the four mRNA's for the receptor subtypes were differentially distributed. The m1, m3 and m4 mRNA species were all more abundant in the brain than in the heart. M1 and m4 were uniformly distributed throughout the layers of the cortex, whereas the m3 mRNA was localized primarily in the inner and outer layers. High concentrations of m1 and m3 mRNA were found in the hippocampus, with m1 being more abundant in the dentate gyrus. Moderate concentrations of m4 mRNA were found in the hippocampus with very low concentrations in the dentate gyrus. Bonner et al. (1987) have thus demonstrated that the subclass known pharmacologically as M1 may consist of three different receptor types, m1, m3 and m4. The human mAChR polypeptides (HM1, HM2, HM3 and HM4) have recently been cloned and further similarites and differences between the subtypes have been reported (Peralta, Winslow, Ashkenazi,

Smith, Ramachandran and Capon,1988). HM1 shares the greatest sequence identity with HM4, and HM2 shares the greatest identity with HM3.

The family of muscarinic receptors identified by Kubo et al. (1986), Peralta et al. (1987) and Bonner et al. (1987) have a high degree of homology (approximately 20-30%) with the beta-adrenergic receptor, the rhodopsin receptor and the bacteriorhodopsin receptor (Gocayne et al., 1987). Each of these receptors consist of a single protein molecule containing seven transmembrane segments. Hydropathicity studies have shown that the muscarinic receptor has a hydrpathicity profile similar to that of the beta-adrenergic and rhodopsin family receptors, suggesting that it too may be a single protein molecule containing seven transmembrane segments (Kubo et al., 1986). The highest degree of homology occurs in the transmembrane segments where the binding sites are thought to reside (See sections 1.42, 1.42a and 1.42b below).

1.20 Physiological and biochemical consequences of mAChR activation

Muscarinic receptor activation has been shown to have several intracellular consequences. Receptor activation can result in A) an increase or decrease in potassium conductance, B) an increase in phosphoinositide (PI) hydrolysis, C) a decrease in adenylate cyclase levels and D) an increase in guanylate cyclase levels.

1.21 Potassium Currents

With respect to potassium currents, there are two types of responses that have been observed in response to ACh, or exogenous muscarinic agonists; an increase in potassium conductance (Egan and North, 1986) and a decrease in potassium conductance (Segal, 1982a; Bernardo and Prince, 1982). The former response has been attributed to activation of the M2 receptor based on the concentration of PZ necessary to inhibit the response, the ability of gallamine to inhibit the response, and the tissues in which this response is observed. The functional consequence of this muscarinic response is the eflux of potassium and the hyperpolarization of the cell, which can decrease the frequency and duration of action potentials, neurotransmitter release and perhaps contribute to presynaptic inhibition. The potassium flux can also influence the movement of chloride ions and calcium cations.

The second type of mAChR activation response is the decrease in potassium conductance (Segal, 1982a; Bernardo and Prince, 1982). The mAChR subtype involved in this response is apparently the M1 subtype. The response has been characterized by tissue distribution and PZ dissociation equilibrium constant. This response is a complex response. Four sources of potassium conductance have been identified following mAChR activation. There is a leak channel that contributes to the resting membrane potential (Madison, Lancaster and Nicoll, 1987). A second conductance, which has been extensively studied, is the M-conductance (Madison et al, 1987; Halliwell and Adams, 1982). The M-channel is inactive at membrane potentials of approximately -60 mV. This channel is activated, however, within tens of milliseconds during depolarization. A third source of potassium conductance is the afterhyperpolarization conductance, which is a calcium

dependent potassium conductance (Madison et al, 1987; Pennefather, Lancaster, Adams and Nicoll, 1985). The final potassium conductance that has been identified with respect to the muscarinic receptor is the transient (or A) potassium conductance that is normally activated when the membrane is depolarized from about -100mV to about -60mV (Cassell and McLachlan, 1987). Activation of the M1 mAChR results in a decrease of these potassium conductances. The net effect is an increase in the excitability of the cell. This increased excitability can be caused by depolarization, increased sensitivity or responsiveness to synaptic inputs, decreased interspike interval, or an increase in burst responding (For review see North, 1986; Christie and North, 1988 and references therein)

1.22 Receptor Stimulated Phosphoinositide Hydrolysis

One of the major biochemical consequences of mAChR activation in the brain is the parallel increase in intracellular concetrations of inositol 1,4,5-trisphosphate (1,4,5-IP_3), which mobilizes calcium from intracellular stores in the endoplasmic reticulum, and diacylglycerol (DAG), which activates the calcium activated protein kinase C (Fisher and Agranoff, 1987). The cascade of events that takes place between the binding of the ACh, or mAChR agonists, molecule and the increase in 1,4,5-IP_3 and DAG levels has been extensively studied in the muscarinic acetylcholine system. The two second messengers are the products of the phophodiesteratic cleavage by phospholipase C of phosphotidylinositol 4,5-bisphosphate (PIP_2), a membrane associated inositide (Downes, 1982,1983). The metabolic interconversions of inositol phosphates and the probable sequence of events

following mAChR activation are shown in Appendix 1. The magnitude of the response is positively correlated with the density of mAChR in the brain (Fisher and Agranoff, 1987), indicating that the two events, receptor stimulation and increased DAG and 1,4,5-IP_3, are coupled. Additional support is provided by the observation that this process is inhibited by mAChR antagonists (Smith and Yamamura, 1985; Akiyama et al., 1986).

MAChR agonists do not all stimulate PIP_2 hydrolysis equally well. In the cerebral cortex and the hippocampus, where the majority of mAChRs are M1 receptors, class A agonists (such as acetylcholine, carbamyl choline (carbachol), oxotremorine-M, muscarine, methacholine and cis-methyldioxolane) are very efficacious stimulators of PIP_2 hydrolysis, whereas class B agonists (such as oxotremorine, bethanachol, pilocarpine, arecoline and pentyl-trimethylammonium) are not (Fisher et al., 1983; Gonzales and Crews, 1984). It has been suggested that the different biochemical consequences of mAChR activation by these two groups of agonists is related to their ability to induce a conformational change in the mAChR (Fisher and Bartus, 1985; Lippa et al., 1986, 1985). Increasing potassium ion concentration facilitates the class A agonist response and if the potassium concentration is increased to 12 mM, class B agonists will partially stimulate PIP_2 hydrolysis (Eva and Costa, 1986). Eva and Costa postulate that the potassium effect is the result of more effective receptor transducer coupling.

1.23 Receptor Stimulated Formation of Guanylate Cyclase and Inhibition of the Formation of Adenylate Cyclase

The effects on adenylate and guanylate cyclase following mAChR activation have not been as clearly delineated as the hydrolysis of PIP_2. In the case of the adenylate cyclase system, it is thought that the mAChR is coupled to an inhibitory G protein (G_i). When the mAChR and G_i are activated, the G_i binds a membrane bound high affinity GTPase that apparently promotes the deactivation of adenylate cyclase (Olinianas, Onali, Schwartz, Neff and Costa, 1984) The formation of cGMP is stimulated in a similar fashion. Here the mAChR is coupled to an excitatory G protein (G_s) that activates guanylate cyclase and the subsequent formation of cGMP. This effector system has been observed in both tissues containing primarily M1 and those containing primarily M2 receptors (Akiyama et al., 1986 , in striatum- A.S. Lippa personal communication). The inhibition of adenylate cyclase has been attributed to the M2 receptor. It has been well documented in the heart (Ehlert, 1985,1987) and striatum (Olianas et al., 1984). Both regions contain predominantly M2 mAChRs. In addition, the antagonism of mAChR mediated adenylate cyclase inhibition by PZ requires a higher (40 fold higher) concentration of the antagonist than the inhibition of PIP_2 hydrolysis (Akiyama et al., 1986). The low affinity for PZ suggests that the inhibition of adenylate cyclase is an M2 receptor mediated phenomenon. The mAChR antagonist atropine, which binds with equal affinity to all mAChR subtypes is nearly equipotent in both effector systems (Akiyama et al., 1986).

The intracellular changes subsequent to mAChR activation are thought to be mediated by G proteins (Gonzales and Crews, 1985; Olinianas, 1982, 1984; Ehlert, 1985). There is some evidence which suggests that muscarinic receptor subtypes are differentially coupled to effector systems (Peralta et al., 1988). The M1 receptor, as defined by PZ binding, has been

identified with the PI effector system (Smith and Yamamura, 1985; Kanba, Kanba and Richelson, 1986; Akiyama et al., 1986) and cyclic GMP formation (Kanba et al., 1986). The M2 subtype has been identified with the inhibition of adenylate cyclase (Akiyama et al., 1986)

Interestingly, the subtypes that share the greatest sequence identity, seem to be coupled to similar effector systems. In the NG108-15 neuroblastoma x glioma cell line, only the HM3 subtype can be expressed. Once the HM3 receptor is expressed, muscarinic receptor activation inhibits adenylate cyclase activity, but has no effect on PI hydrolysis in NG108-15 cells (Peralta et al., 1988; Harden et al. 1986). HM3 is most similar to HM2 which is the "cardiac type" mAChR, and this has been shown to be coupled to the adenylate cyclase system as well (Ehlert, 1985, 1987). Similarly, HM1 and HM4 have high a degree of sequence identity and both are coupled to PI hydrolysis and calcium mobilaztion (Peralta et al., 1988).

The evidence suggesting a consistent pairing of particular receptor subtypes with particular effector systems is equivocal. Stimulation of cGMP formation and PIP_2 hydrolysis have both been attributed to the M1 subtype, however the relationship between the two effectors is not clear. In systems where both responses to mAChR (M1) activation can be observed, differing results have been reported. In murine neuroblastoma cells containing both M1 and M2 receptors, both mAChR mediated PIP_2 hydrolysis and cGMP formation can be inhibited by the addition of TPA (12-O-tetradecanoylphorbol-13-acetate) to the assay medium. The M2 response, the inhibition of cAMP formation, is unaffected by the treatment (Kanba, et al., 1986). In hippocampal slices mAChR activation results in a stimulation of PIP_2 hydrolysis with no change in cGMP levels. Inhibition of cAMP is also

observed in the hippocampal slice. In the striatum mAChR activation results in a stimulation of cGMP and an inhibition of cAMP. (A.S. Lippa personal communication). M1 and M2 both inhibited cAMP in striatum and hippocampus, but only the M2 resulted in stimulating cGMP and only M1 stimulated PIP_2. The cGMP response and the PIP_2 response, although not related in the hippocampus, were similarly affected by TPA in neuroblastoma cells. Taken together these results suggest that all receptor subtypes may not always be coupled to the same effector systems in all regions. It may also suggest that a particular receptor can be associated with an effector system under certain conditions and not others, or different receptor subtypes can be coupled to the same effector system.

1.30 Receptor modulation

1.31 The modulation of binding by guanyl nucleotides

Studying the effects of various agents on binding has been useful in generating information and models concerning the composition and functioning of the receptor-effector complex. Guanosine triphosphate (GTP) has been shown to be involved in neurotransmitter activation or receptor coupled inhibition of adenylate cyclase systems (for review see Ehlert, Roeske and Yamamura, 1983). The effects of GTP on muscarinic receptor binding may represent changes in the coupling of the receptor to the adenylate cyclase itself, or to some other membrane component involved in the inhibiton of the cyclase. Several direct agonist binding studies have

shown that treatment of membrane preparations with GTP, or the nonhydrolyzable analog Gpp(NH)p can, under certain conditions, result in a significant decrease in binding This has been shown for several agonists including; ^{3}H-ACh (Gurwitz et al., 1985), ^{3}H-Oxo-M (Hulme, Berrie, Birdsall, Jameson and Stockton, 1982), and ^{3}H-CD (Ehlert, Roeske and Yamamura, 1981). When agonist inhibition of antagonist binding was measured, the GTP effect was manifested as a 10-20 fold increase in the IC_{50} value for oxotremorine and carbachol, indicating a significant decrease in the affinity of the receptor for the ligand (Berrie, Birdsall, Burgen and Hulme, 1979). These effects are greatest in tissues containing high affinity agonist sites and M2 receptors such as the brainstem, cerebellum and the heart (Sokolovsky et al., 1986; Gurwitz, Kloog and Sokolovsky, 1985). In tissues where the low affinity agonist site predominates, such as the hippocampus or the cortex, much smaller GTP effects, (or no effect) are observed. The addition of GTP to the binding assay has no effect on the affinity of antagonists for the receptor. These type of data indicate that GTP binding regulatory proteins (Gi or Ni) may be involved in the cascade of events initiated by muscarinic receptor activation (See Ehlert, Roeske and Yamamura, 1983 for review).

1.32 The modulation of binding by ions

1.32a Divalent Cations

The addition of transition metal ions (2 mM nickel (Ni^{2+})) to the binding assay results in a 50% increase in binding sites in the striatum and the hippocampus with only a slight increase in the dissociation constant for ^{3}H-ACh. Similar results were obtained with the addition of 2 mM manganese

(Mn^{2+}) or cobalt (Co^{2+}). The same treatment resulted in only a 10-15% increase in the hypothalamus, brainstem and cerebellum (Gurwitz et al., 1985). This suggests that M1 and M2 receptors differ not only in their susceptibility to GTP effects but also in their sensitivity to cations. Interestingly, the cation-induced increase in ACh binding was fully reversible by Gpp(NH)p. In addition the low affinity agonist site could be converted into a GTP-sensitive high affinity site by coincubation with Ni^{2+}. This demonstrates a GTP effect in two tissue regions that are normally insensitive to GTP (Gurwitz et al., 1985).

Similar effects have been observed with the divalent cation magnesium (Mg^{2+}). An enhancement of agonist binding was observed in the medulla pons in the presence of Mg^{2+} (Birdsall, Hulme, Hammer and Stockton, 1980). Wei and Sulakhe (1980) reported that Mg^{2+} increased the affinity of the binding site for carbachol. In this experiment, however, saturating concentrations of QNB were used and it is therefore difficult to determine whether a decrease in the IC_{50} value for carbachol is due to an increased agonist affinity or a decreased antagonist affinty. In fact, Ehlert, Roeske and Yamamura (1981) measured antagonist binding using a fixed concentration of QNB, in the presence and absence of Mg^{2+} and found that Mg^{2+} converted 43% of the QNB binding sites to an anomalous low affinity state (K_D=0.64 nM). This suggests that either an agonist or antagonist effect, or some combination of both , could account for Wei and Sulakhe's observations (See Ehlert, Roeske and Yamamura, 1983 for review).

These data suggest that divalent cations can increase both the affinity of the agonist binding site and the number of binding sites. It also suggests that divalent cations present in the intracellular or extracellular medium, such

as calcium, could regulate mAChR function. Indeed several receptor mediated events, such as ACh release and modulation of the afterhyperpolarization current, are affected by calcium (Kuffler Nicholls and Martin,1984)

1.32b Monovalent Cations

Although divalent cations increase muscarinic binding, monovalent ions apparently inhibit both agonist and antagonist binding. The introduction of Tl^+ (thallium) into the buffer medium results in a 22-fold decrease in the affinity of the cortical low affinity agonist site (as compared to Na^+), and a 170-fold decrease in the affinity of the high affinity site (Birdsall, Burgen, Hulme and Wells 1979). It has been suggested that since Tl^+ is often regarded as similar to K^+, a K^+ conductance mechanism may be involved in muscarinic receptor complex (Birdsall et al., 1979). Ionic effects of K^+ on mAchR mediated events have been noted by some investigators (Birdsall et al., 1979). Eva and Costa (1986) found that increasing K^+ concentration to 12mM potentiated carbachol and cis-methyldioxolane stimulation of PI hydrolysis. Increasing K^+ concentration could also result in the potentiation of oxotremorine stimulated PI hydrolysis. No change was observed in antagonist binding (QNB and PZ) or agonist inhibition of antagonist binding (oxotremotine/^{3}H-QNB, carbachol/^{3}H-QNB) and antagonists (PZ/ ^{3}H-QNB). These studies could suggest that monovalent ions are important in the activation of the biochemical cascade subsequent to receptor activation, such as the activation of second messengers or kinases.

One thing the variety of findings in the literature suggests is that it is possible to devise a treatment that will make one receptor binding site perform like another. For example, although classical antagonist binding normally recognizes all receptor populations with equal affinity, it is possible to reveal antagonist receptor heterogeneity under appropriate conditions. These conditions depend primarily on the ionic composition of the incubation medium (Hulme et al.,1980). A second example is the ability to modulate M1 receptor binding with Gpp(NH)p following treatment with transition metal ions (Gurwitz et al.,1985). This type of data suggests that the environment in which the receptor resides can determine, to a large degree, the way in which it functions. This suggests that elements that are direct components of the receptor-effector complex, or any element in the receptor environment may have the ability to affect receptor conformation and function.

1.40 Receptor environment

1.41 Characterization of neuronal membranes

1.41a Composition

Neuronal membranes contain two major constituents; lipids and proteins (Hadley, 1985). Although the types and proportions of the lipids vary among tissues, the major lipid classes present are phospholipids, glycolipids and cholesterol. The phospholipids include phosphotidylethanolamine (PE), phosphotidylcholine (PC), phosphotidylserine (PS), phosphotidylinositol (PI), sphingomyelin, lysophosphotidylethanolamine and lysophosphotidylcholine. The principal glycolipids (-glycosphingolipids) are cerebrosides, which contain the sugar

galactose. The presence of this glycolipid is restricted to the myelin sheath (Hadley, N.F., 1985). The membrane contains both saturated and unsaturated fatty acids that are typically even-numbered chains containing 14 to 24 carbon atoms. Other elements contained within the membrane include water, carbohydrates and metal ions (Hadley, 1985).

One characteristic common to neuronal membranes is the amphipathic nature of the membrane lipids. An amphipathic compound is one whose molecules contain both polar and nonpolar domains (Lehninger,1982). Within the membrane the fatty acid molecules contain a polar head group at one end and a nonpolar hydrocarbon (fatty acyl chains) tail. The head group is hydrophilic and the tail is hydrophobic. These two characteristics of the fatty acid molecule gave rise to the Davson-Danielli-Robertson (cited in Hadley, 1985) model of membrane structure. This model postulated a bilayer lipid membrane with the nonpolar tails facing each other, forming the core of the membrane, and the polar heads facing outward, making contact with the extracellular and intracellular aqueous medium. The membrane proteins were originally thought to encase the lipid bilayer, forming a trilayer organization of one lipid bilayer between two layers of protein. This was later revised to include the presence of protein lined pores that could allow the passage of water and ions through the membrane (Hadley, 1985).

The above model was formulated in 1935 (Davson and Danielli, 1935 cited in Hadley, 1985) and revised by Robertson in the 1950's. Since then, more advanced analytical techniques have given rise to the "fluid mosaic model" which is generally accepted today. This model , which was proposed by Singer and Nicolson (1972), postulates that intrinsic membrane proteins are discrete particles in the bilayer rather than a uniform coating. These

proteins (which also demonstrate amphipathic properties) can span the entire membrane or permeate the membrane, with the nonpolar region of the protein contacting the lipid core and the polar region contacting the aqueous medium of the intracellular or extracellular fluid. Some proteins, such as acetylcholinesterase (AChE), are not imbedded in the membrane but are anchored to the membrane by a hydrophobic anchoring domain with the majority of the protein suspended in the intracellular or extracellular aqueous domain (Low, Ferguson, Futerman and Silman, 1986). In the case of AChE the molecule is thought to reside intracellularly and may be "solubilised" by phospholipase C cleavage between a phosphate and the 1,2, diacylglycerol moiety that anchors the AChE (Low et al., 1986).

A second characteristic of neural membranes is assymetry. The lipids contained within the membrane are assymmetrically distributed between the two leaflets. PS, PE and PI are more abundant on the inner leaflet, and shingomyelin and PC are found in higher concentrations on the outer leaflet (Hadley, 1985).

1.41b Determinants of fluidity

Membrane fluidity is a measure that reflects the mobility of membrane components. These components can move in three different ways. One possible transposition of elements is a lateral motion within the plane of the membrane. This lateral motion is particularly important for protein/protein coupling such as receptor coupling to cyclases (Hirata and Axelrod, 1980). Vertical movements of proteins within the lipid bilayer have been proposed as receptor related events. Heron, Shinitzky, Hershkowitz and Samuel,

(1980) and Heron, Israeli, Hershkowitz, Samuel and Shinitzky (1981) suggest that vertical movement of receptor proteins may underlie the phenomenon of supersensitivity by revealing cryptic seratonin and opiate receptors. Lipid molecules can also traverse the membrane and "flip-flop" from one leaflet to the other, this occurs at slower rates than does lateral movement, due to the difficulty with which the polar head group moves through the hydrophobic core of the membrane. The third type of movement is rotational movement, where the molecule rotates around the long molecular axis (Lange, 1986). Rotational movement of proteins has been postulated as a possible mechanism for ion channel opening and/or closure. An example of this would be the helical screw model of the nicotinic acetylcholine receptor, in which sodium channel gating involves the rotation of the alpha helices and a subsequent conformational change in the ion channel protein. Membrane fluidity is determined primarily by membrane composition . General characteristics of fatty acids that affect fluidity include; the degree of saturation of the acyl chains (unsaturated lipids are more fluid than saturated lipid) and the length of the hydrocarbon tail (longer fatty acid chains are more rigid than shorter chains). In addition, the nature of the polar head group and the hydration state of the membrane are also factors that affect the mobility of membrane constituents (Chapman, 1975a; 1975b). An analysis of particular lipids and their contribution to the overall fluidity of the membrane indicates that the concentration of cholesterol in neuronal membranes (expressed as the mole ratio of cholesterol to phospholipids (C/PL)) is a principal determinant of fluidity (Shinitzky and Henkart, 1979). The concentration of sphingomyelin (expressed as the mole ratio of sphingomyelin/lecithin (Barenholz and Thompson, 1980) the mole ratio of PE/lecithin (Hirata and

Axelrod,1980) and the ratio of protein/lipid (Shinitzky and Henkart, 1979) are also determining factors of overall fluidity.

1.41c Age Related Changes in Membrane Composition and Fluidity

The above discussion concerning membrane composition and fluidity suggests that a multitude of factors are involved in the maintenance of membrane composition and fluidity in the adult subject. The process by which membrane fluidity is controlled has been termed homeoviscous adaptation (Sinensky, 1974).

As discussed, membrane fluidity can be affected by manipulating any one of several chemical factors or by changing the physical parameters of the membrane environment such as pressure or temperature (Shinitzky, 1984; Shinitzky and Henkart, 1979). Chemical manipulations can include the substitution or addition of lipids. For example, when cholesterol concentration is increased in either natural or artificial membranes, the fluidity of the membrane is decreased and the permeability of the membrane is also decreased 3-5fold. (Fettiplace and Haydon,1980). Environmental manipulations, such as a change in temperature, also affect fluidity. At temperatures below approximately 25^{o}C, bilayer lipids reside in a crystalline gel state. Higher temperatures result in a change from the gel crystalline state to a liquid crystal state that is more fluid (Curtin, Gordon and Aloia, 1988). Changes in the fluidity of the membrane caused by changes in temperature, affect the mobility of proteins within the lipid bilayer. Decreased mobility of membrane proteins, resulting from increased membrane microviscosity, could decrease the liklihood of lateral motion and interactions such as receptor-

adenylate cyclase interactions (Samuel, Heron, Hershkowitz and Shinitzky, 1982; Whetton et al., 1983a, 1983b).

Aging has a dramatic effect on membrane composition and fluidity. One of the early effects of aging is a decreased ability of the of the cell's homeostatic mechanisms to maintain membrane fluidity within the appropriate range (Shinitzky, 1984). This age related deficit in homeoviscous adaptation makes the membrane less responsive to changes in the environment. The result of this deficit is that the accumulation of serum lipids (e.g. cholesterol and sphingomyelin) is not effectively counterbalanced by homeostatic processes and there is a subsequent change in the lipid composition of the membrane. The cholesterol and glycosphingolipid content of the membrane is increased, while the protein content is essentially unaffected (Frolkis et al.,1987); see Shinitzky, 1985 for review). Decreases in the total phopholipid content have also been reported (Frolkis, Tanin, Gorban, Bogatskaya and Sabko, 1987), however, not all the phospholipids change in the same manner. The content of PC decreases (Frolkis et al., 1987), while the sphingomyelin (Frolkis et al., 1987; Shinitzky, 1985) and the combined content of PI and PS tend to increase (Frolkis et al., 1987). The net result of these changes is a significant increase in the cholesterol to phospholipid mole ratio. These changes cause aged membranes to be significantly more rigid than younger membranes (Samuel et al., 1982). In aged membranes phospholipid methyltransferase activity is increased in an apparently unsuccessful attempt to compensate for this increased rigidity (Lippa et al., 1985).

An increase in membrane rigidity could probably affect cell functioning in several ways. One example of how age related increase in membrane

viscosity might affect the cell is the decreased ability of membranes from aged rats to depolarize in response to cold treatment and to treatment with oubain. Both of these conditions inhibit active transmembrane ion transport. The aged membrane is less responsive to these manipulations than adult membranes (Frolkis et al., 1987).

A change in ion transport across the membrane could in turn affect ion dependent processes. An age related decrease in calcium uptake, which involves transport of the cation across the membrane, has been demonstrated in various brain regions in mice (Gibson, Perrino and Dienel, 1986). The deficit increases with age, resulting in a 19-33% decrease at 10 months and a 41-51% decrease at 30 months as compared to 3 month old mice (Gibson et al., 1986). The magnitude of the deficit could affect calcium dependent neuronal functions. A decrease in calcium dependent ACh release has been reported (Gibson and Peterson, 1981; Pedata, Slavikova, Kotas and Pepeu, 1983), as well as a decrease in post-tetanic potentation with a prolonged afterhyperpolarization in hippocampal slices (Landfield and Pitler, 1984).

1.42 Position of the mAChR in the membrane

Both the mAChR protein and the membrane are potentially capable of responding to changes in the microenvironment of the cell. Therefore it is necessary to explore the interrelationship of these two elements and determine how changes in one might affect the functioning of the other. Several lines of research have allowed researchers to deduce the probable structure of the mAChR within the membrane. The mAChR shares a high

degree of sequence homology with the rhodopsin receptors and the beta adrenergic receptor. The structure of the bacteriorhodopsin receptor has been determined using electron-diffraction techniques, hydropathicity profiles and other methods (references within Kubo et al, 1986). The bacteriorhodopsin receptor is a single glycoprotein containing seven alpha-helical segments that span the membrane. The mAChR also has a hydropathicity profile suggesting seven alpha-helical hydrophobic segments (Kubo et al., 1986). It has been suggested that these seven alpha-helical transmembrane segments may be positioned within the membrane in such a way that they form a pore and act as an ion channel (Antonian and Lippa in press). Nevertheless, regardless of the exact conformation, the existence of several transmembrane segments suggests that any significant change in the viscosity of the membrane could affect receptor conformation or orientation within the membrane.

1.42a Hydrophilic segments of the receptor protein

The hydrophilic segments of the receptor protein consist of the amino-terminal region, the carboxy-terminal region and six segments connecting the alpha helices. According to Wheatley et al. (1988), the amino-terminal most probably lies on the extracellular side of the membrane, with the carboxy-terminal side residing on the cytoplasmic side. The inter alpha helical segments are short segments, with the exception of the segment connecting the fifth and sixth alpha helices (Kubo et al. 1986), which might be involved in receptor effector coupling.

1.42b Hydrophobic segments of the receptor protein - Possible binding sites

The hydrophobic segments contain uncharged amino acid residues including many nonpolar residues (Kubo et al.,1986). Charged regions would be necessary for certain molecular interactions, such as pi or sigma bonding, with these regions. Segments two and three are the only two segments that contain charged amino acid residues; aspartic acid residues are found in positions 71 and 105. Aspartic acid residue 71 in segment two is conserved in the rhodopsin and the beta adrenergic receptor. The segment three aspartic acid (105) is found in the mAChR and the beta adrenergic receptor (Kubo et al., 1986). A comparison between porcine cortical and myocardial mAChR sequences indicates four acidic amino acids that are common to both sequences which are likely binding sites, aspartic acids 71, 99, 105 and 122 (Wheatley et al., 1988). Other possible binding sites include serines 66 or 78 or threonine 76 (Antonian and Lippa, 1988a in press). The involvement of aspartic acid 71 in binding has been demonstrated by protein modification, peptide mapping and sequencing techniques (Wheatley et al., 1988) and molecular modelling studies (Antonian and Lippa,1988a in press). Molecular modelling studies have also indicated that ACh molecules could bind to the aspartate 71 and Serine 78 of segment two and the aspartate 105 and serine 109 of segment three without sterically hindering ligand/receptor interactions at either site (Antonian and Lippa, in press).

2.00 EXPERIMENT 1

2.10 Introduction

The recent developments in the characterization of the mAChR as an intramembrane protein (discussed above) increase the need to examine the inter-relationship between the mAChR and its membrane environment. It is clear that membrane composition and fluidity can affect receptor function. Several studies have shown that manipulation of the membrane environment can affect cell excitability (Crews, Camacho and Philips,1983), receptor binding (Heron et al., 1980,1981), and receptor mediated second messenger activity (Whetton et al., 1983). One way to change membrane fluidity, for a certain period of time, without affecting the lipid composition of the membrane is to maintain the tissue at different temperatures. The effect of temperature on receptor binding has been studied in several systems including the dopamine system (Zahniser and Molinoff, 1983; Kilpatrick, El Tayer, Van De Waterbeemd, Tasta and Marsden, 1986), the beta-adrenergic system (Weiland, Minneman and Molinoff, 1980; Contreras, Wolfe and Molinoff, 1986a; 1986b), and the muscarinic system (Mei, Wang, Roeske and Yamamura, 1987; Muzio, Malandrino, Ferrari and Tanon, 1986). Thermodynamic analysis of these data has yielded a considerable amount of valuable information concerning agonist induced conformational changes in these neurotransmitter receptors.

With respect to the mAChR, Muzio, Malandrino, Ferrari and Tonon (1986) found that as the incubation temperature was decreased from 37^{o}C to 10^{o}C, the affinity of the mAChR for atropine, PZ and oxotremorine increased in the rat cerebral cortex, heart and colon. All ligands were used to displace ^{3}H-QNB binding. Similar results were reported by Mei et al. (1987), decreasing incubation temperatures resulted in an increased affinity for PZ .

The observation made by Muzio et al. (1986) that agonist and antagonist binding were both sensitive to temperature changes and affected by them in the same way is not a universal finding. Gurwitz and Sokolovsky (1980) found that in the mouse medulla-pons increasing incubation temperature to 50°C caused a decrease in mAChR affinity with no change in antagonist binding, whereas in cortical tissue both agonist and antagonist binding were unaffected by the temperature change. When incubation temperatures were lowered to 0°C, agonist affinity increased and antagonist affinity remained unaltered in the cortex. Mei et al. (1987) observed a temperature dependent increase in the affinity for PZ with no change in the affinity for QNB.

The functional consequences of mAChR activation have also been reported to change after manipulation of incubation temperature. El-Fakahany and Richelson (1980) found that decreasing the incubation temperature from 37°C to 20°C resulted in a decrease in carbachol stimulated cyclic GMP formation in neuroblastoma cells. Interestingly El-Fakahany also observed that the optimal level of cyclic GMP formation occurred at 37°C, and if the temperature was increased further to 40°C, there was a subsequent decrease in cyclic GMP formation. This supports the hypothesis that there is an optimal membrane fluidity for receptor and cell function.

It is clear that a greater understanding of the effect of fluidity changes alone and compositional changes with subsequent fluidity changes, on the mAChR receptor is necessary if the role of these two factors in normal and impaired receptor function is to be determined. In the following experiments the effect of temperature on muscarinic agonist and antagonist binding was further explored. The goal of the experiments was to characterize receptor

binding and determine whether manipulating membrane viscosity alone would affect receptor density, receptor affinity, and the ability of the receptor to interconvert from a low to high affinity state or vice versa. To achieve this goal hippocampal homogenates were labelled with ^{3}H-QNB and the ability of several ligands to displace the labelled ligand were tested. The antagonist atropine was used to characterize antagonist-receptor interactions. Both the class A agonist carbachol, which induces a conformational change in the receptor under physiologic conditions, and the class B agonist oxotremorine, which does not induce a conformational change under physiologic conditions, were used to characterize agonist-receptor interactions. Ligand-receptor interactions were further characterised by a thermodynamic analysis of the receptor binding data obtained at four different temperatures (37^{o}C, 25^{o}C, 15^{o}C and 4^{o}C). This type of analysis has been used in other receptor systems (Zahniser and Molinoff,1983; Kilpatrick et al.,1986; Weiland et al.,1980; Contreras et al., 1986a; 1986b) to increase our understanding of the energetics involved in the binding of the ligand to the receptor and the changes that occur within the system as a result of receptor occupation.

2.20 Methods and Materials

2.21 Materials

^{3}H-Quinuclidinyl benzilate (^{3}H-QNB; 42.6 Ci/mmol) was purchased from New England Nuclear (Boston, Ma.). Atropine (crystalline free base), carbamylcholine chloride (carbachol), oxotremorine (1-[4-(1-Pyrrolidinyl)-2-butyryl]-2-pyrrolidinone), sodium phosphate, potassium phosphate, sucrose,

diphenylhexatriene (DPH) and tetrahydrofuran (THF) were purchased from Sigma Chemical Company (St. Louis, Mo.).

2.22 Subjects

In these studies bovine brains were used. Fresh bovine brains from mature cows were purchased from Max Insel Cohen, Inc. (Livingston, N.J.).

2.23 Tissue Preparation

Sections from the dorsal portion of each hippocampus were dissected out, pooled and homogenized on ice in 10 volumes of ice cold 50 mM sodium potassium phosphate buffer (pH 7.4) using a Polytron homogenizer (two 15 second bursts at setting 4-5, with a 30 second rest interval during which the tissue was kept on ice). The homogenate was further diluted to either 250 volumes of original weight for the saturation isotherm binding of ^{3}H-QNB or 150 volumes for the displacement binding assays.

2.24 Saturation Isotherms

One hundred µl of the hippocampal homogenate (0.3-0.5mg protein/ml) were incubated with 300 µl of ^{3}H-QNB (10 concentrations were used, ranging from 5.0 pM to 1.5 nM). One hundred µl of atropine (resulting in a final concentration of 10µM) was used to define nonspecific binding. The total assay volume was brought up to 5.4 ml with 50 mM Na-KPO_4 buffer (pH 7.4). Protein was determined using the method of Bradford (1976).

2.25 Displacement experiments

One hundred µl of hippocampal homogenate (0.5-0.7mg protein/ml) were incubated with approximate K_D concentrations of ^{3}H-QNB appropriate for each incubation temperature. At least 10 concentrations of the displacing drug were used. The final concentrations of atropine ranged from 10 pM to 50 nM , oxotremorine from 250 pM to 2.5 µM and carbachol from 50 nM to 250 µM. Ten µM atropine (final concentration) was used to define nonspecific binding. Final assay volume was 5.4 ml.

2.26 ^{3}H-QNB Binding

^{3}H-QNB saturation isotherms and displacement experiments were done at least three times at each temperature: 37°C, 25°C, 15°C and 4°C. The time necessary for ^{3}H-QNB to reach apparent equilibrium was determined at each of the four temperatures. For all saturation isotherms and displacement experiments, the incubation periods were 1h at 37°C, 3h at 25°C, 6h at 15°C and 18h at 4°C. All reactions were terminated by rapid filtration through Whatman GF/B glass fiber filters; which were then washed three times with 4 ml of ice cold Na-KPO_4 buffer. Filters were immersed in 5.0 ml of Beckman HP scintillation cocktail, and radioactivity was determined by liquid scintillation spectrometry with an efficiency of 66%. Nonspecific binding, defined as the amount of ^{3}H-QNB bound in the presence of 10 µM atropine, was usually less than 10% but was no greater than 15% at 37°C, 25°C and 15°C, and no greater than 25% at 4°C.

2.27 Microviscosity Measurement

The right and left hippocampi were dissected out from 3 rat brains. The two hippocampi from each animal were pooled and homogenized in 100

volumes ice cold 50 mM Na-KPO_4 buffer (2 15 second bursts at a setting of 4-5 on a Polytron homogenizer; the tissue was kept on ice during the 30 second rest interval). The homogenate was diluted to 1:250 (wt:vol). Aliquots of the tissue homogenate (0.5 ml) were incubated in the dark with 4.5 ml 2 μM DPH (2 mM DPH in THF was diluted 1000 fold with 50 mM Na-KPO_4 buffer, pH 7.4) for 30 min. at room temperature (20-25°C) with shaking. Samples were then brought to the appropriate temperature (37°C, 25°C, 15°C or 4°C) for 1hr. Membrane microviscosity was measured using standard fluorescence polarization techniques as described by Shinitzky and Barenholtz (1978). (Excitation wavelength was 360 nm and emission wavelength was 427 nm.) Tissue samples from each brain were tested at all four temperatures in triplicate. Polarization measurements were taken twice for each sample.

2.30 Data analysis

2.31 Saturation isotherms and displacement binding data

K_D and B_{max} values were determined by Scatchard analysis (1949). Two analyses were performed on data from inhibition of ^{3}H-QNB binding experiments. IC_{50} and n_H (Hill coefficients) values were calculated from Hill plots. IC_{50} values were converted to K_i values using the Cheng and Prusoff equation (1973). Computer-assisted iterative nonlinear analysis (Munson and Rodbard, 1980) was also used to determine IC_{50} values and whether the data could best be described by a one-site model or a two-site model of receptor occupation. If a one-site model was appropriate a single IC_{50} value was determined, if a two-site model was appropriate two IC_{50} values were determined; one for the high affinity site and one for the low affinity site.

These values were converted to K_i values (K_H and K_L when appropriate) using the Cheng and Prusoff equation (1973). All Tables show K_i values derived from computer generated IC_{50} values along with the percentage of the total sites that were in the low affinity state ($\%B_L$).

A one-way analysis of variance (ANOVA) was performed to determine whether a main effect of temperature was present and t-tests was performed to determine which of the temperature conditions differed significantly. All Statistical analyses were done using SYSTAT (Systems for Statistics) software (Wilkinson, 1987)

2.32 Thermodynamic Parameters

The Gibbs free energy change (ΔG in Kcal/mol) was calculated from the equation: $\Delta G = -RT\ln K_a$, where R=the gas constant (1.99 cal/mol x deg), T= temperature in degrees Kelvin and the association constant $K_a = 1/K_D$, for ^{3}H-QNB, or $1/K_i$, for competing ligands. The change in enthalpy (ΔH Kcal/mol) was determined from a Van't Hoff plot ($\ln K_a$ vs 1/T). The slope of the curve = $-\Delta H/R$. The entropy change ($T\Delta S$ Kcal/mol) was calculated from the equation: $\Delta G = \Delta H - T\Delta S$.

2.33 Membrane Fluidity

Microviscosity (the inverse of fluidity) is calculated from polarization values (P) using the equation $n = 2P/(0.46-P)$. Polarization values are determined from the equation $P = ((I_{//}/I_{\perp}) -1) / ((I_{//}/I_{\perp}) +1)$.

2.40 Results

Association rates of ^{3}H-QNB binding: A determination of the time necessary for ^{3}H-QNB to reach apparent equilibrium was done at 37^{o}C, 25^{o}C, 15^{o}C and 4^{o}C. The time necessary to reach equilibrium increased with decreasing temperature. Based on these data (shown in Fig. 1) the following incubation times were chosen to represent equilibrium conditions for the remaining experiments: 1h at 37^{o}C, 3h at 25^{o}C, 6h at 15^{o}C and 18h at 4^{o}C.

The effect of temperature on membrane fluidity: A one-way analysis of variance demonstrated that the effect of temperature on fluidity was significant ($p<.01$, $F=4.641$, $df= 3,32$). As incubation temperature was decreased from 37^{o}C and 25^{o}C to15^{o}C the microvisocity of the membrane increased significantly ($p<.005$, t-test). As the temperature was lowered from 15^{o}C to 4^{o}C no further increase in membrane rigidity was observed. (Data shown in Figure 2).

The effect of temperature on ^{3}H-QNB binding isotherms: Analysis of variance demonstrated that the affinity of ^{3}H-QNB for the mAChR decreased significantly ($p<.02$, $F=5.584$; $df=3,9$) as the temperature was lowered from 37^{o}C to 4^{o}C (Data shown in Table 1). The ANOVA also indicated a main effect of temperature on B_{max} ($p<.01$, $F=6.824$; $df=3,9$). The presence of a main effect was due primarily to the 15^{o}C value which differed significantly from the other three temperature conditions ($p<.05$, t-test) (Data shown in Table 1).

The effect of temperature on displacement binding: Atropine and oxotremorine both had Hill coefficients (n_H) of approximately 1.0 (Data not shown) which suggested that these data would best fit a 1-site model for

receptor occupation. Carbachol had a Hill coefficient of less than 1.0 (Data not shown) suggesting that it bound to the receptor in a complex manner and would best fit a 2-site model for receptor occupation. Accordingly, computer-assisted iterative nonlinear analysis indicated that atropine and oxotremorine binding fit a 1-site model and carbachol fit a 2-site model in the majority of cases at each of the temperatures tested (See Tables 2, 3 and 4 respectively). Each of the three drugs tested showed an increased ability to displace ^{3}H-QNB binding as the incubation temperature was lowered. This effect was most marked when the temperature was lowered from 25^{o} to 15^{o}C.

Atropine and oxotremorine affinities were temperature-sensitive ($p<.0001$, $F=16.428$; $df=3,10$, for atropine and $p<.0001$, $F=53.260$; $df=3,10$ for oxotremorine, ANOVA). K_i values for atropine progressively decreased from 37^{o}C to 15^{o}C with no further decrease when the incubation temperature was lowered to 4^{o}C. (Table 2). Similar findings were obtained for oxotremorine (see Table 3); K_i values at 37^{o}C were different from those at lower temperatures and 15^{o}C was significantly different from 37^{o}C and 25^{o}C ($p<.05$, t-test).

Although computer analysis indicated that, in the majority of cases, the mAChR antagonist atropine bound to the mAChR in a simple manner, suggesting one state of the receptor, one analysis each at 25^{o}C, 15^{o}C and 4^{o}C resulted in a statistically signicant better fit of the data points to a 2 site model (see Table 2). A similar finding was generated for the class B agonist oxotremorine. Here, one experiment at each of the four temperatures was better described by positing two states of the mAChR (see Table 3). In these few cases, the second state was of a high affinity.

Carbachol bound to the mAChR in a complex manner, suggesting two states of the mAChR in all experiments at 37°C, 25°C and 15°C (see Table 4). The high affinity state (K_H) did not demonstrate significant temperature-dependent changes ($p>.20$ $F=1.793$; $df=3,12$, ANOVA) whereas the low affinity state (K_L) was sensitive to temperature changes ($p<.03$, $F=4.641$; $df=3,10$, ANOVA). K_L values at 37°C and 25°C did not differ from each other but were significantly different from those at 15°C and 4°C ($p<.05$ t-test). At 4°C, two out of four experiments resulted in carbachol binding to a single receptor state. In these two experiments the single state was of a high affinity (see Table 4).

Thermodynamic parameters: The binding of antagonists (^{3}H-QNB and atropine) to the mAChR resulted in positive entropy changes, as evidenced by a positive $T\Delta S$ (see Table 5). ^{3}H-QNB binding also showed a positive ΔH, suggesting a positive enthalpy change, whereas atropine binding showed a negative ΔH, suggesting negative enthalpy change. The binding of ^{3}H-QNB was entropy driven and the binding of atropine was enthalpy driven.

The binding of agonists appeared to be enthalpy driven (the ΔH absolute values are greater than the $T\Delta S$ values, see Table 5). Both the carbachol (class A) and oxotremorine (class B) interactions with the mAChR resulted in negative enthalpy changes of approximately equal magnitude. These two agonists differed with respect to the entropic contribution to the receptor-ligand interaction. The negaitve entropic factor ($T\Delta S$) calculated for oxotremorine was smaller than that calculated for the carbachol. The entropic factor for oxotremorine was approximately one third

of the entropic factor for the low affinity carbachol site and one seventh of the high-affinity site (See Table 5).

2.50 Discussion

The data presented in these studies clearly indicate, as expected, that decreasing incubation temperature resulted in an increase in microviscosity. Furthermore they indicate that ligand-mAChR interactions are affected by these changes in membrane fluidity. The changes that occur are manifested primarily as changes in the affinity of the receptor for the ligands. The data, with the exception of two carbachol inhibition of ^{3}H-QNB binding experiments at 4^{o}C, do not suggest that changes in temperature, or fluidity alter the ability of a ligand to induce a conformational change in the receptor. Atropine and oxotremorine do not induce conformational changes in the receptor under physiologic conditions; the Hill coefficients were not significantly different from one at any temperature and the data was best fit by a 1-site model in all conditions. Carbachol does induce a conformational change in the receptor and its Hill coefficient was, accordingly, less than unity and the data best fit by a 2-site model in all conditions. These data suggest that minor changes in accessibility of the receptor may occur, but that the change is not great enough alter the nature of the ligand-receptor interaction. The one exception to this is the observation that two of the carbachol displacement experiments at 4^{o}C were best fit to a 1 site model. In these two cases the K_i values obtained were very close to the K_H of the other two experiments, suggesting that the observed binding was to a high affinity state of the receptor. This might suggest that rigidification of the

membrane could affect, indeed potentiate, the ability of carbachol to induce a conformational change in the receptor from a low to a high affinity state. Alternatively the changes in fluidity may themselves be producing a conformational change in the mAChR. These conclusions, while very speculative due to the small sample size, are supported by the demonstration of increased affinity of the mAChR with decreasing temperatures. Perhaps the increased rigidity of the membrane makes the receptor more accessible to the ligand by a mechanism such as vertical displacement of the receptor. This concept has been applied to the opiate and seratonin receptors to explain changes in affinity following membrane rigidification and fluidization (Heron, Shinitzky, Hershkowitz and Samuel, 1980; Heron, Israeli, Hershkowitz, Samuel and Shinitzky, 1981).

Thermodynamic characterization of the mAChR, using a representative sample of drugs, has revealed differences between antagonist and agonist binding and between class A and class B agonist binding. The binding of ^{3}H-QNB appeared to be entropy driven, due to the large entropic contribution to the Gibbs free energy. The large positive entropy along with the unfavorable increase in enthalpy is in agreement with reported findings (Mei et al., 19878). ^{3}H-QNB binding had the largest entropic factor which may be compensatory for a thermodynamically unfavorable increase in enthalpy. Atropine binding has a smaller entropy factor, with a thermodynamically favorable enthalpic contribution. The positive entropic contribution observed here with antagonist interactions and the positive enthalpic contribution seen with ^{3}H-QNB interactions agree with early preliminary studies (Sugiyama, Daniels and Nirenberg, 1977; Barlow, Birdsall and Hulme, 1979). The difference between the magnitude

of the entropic factors for ^{3}H-QNB and atropine binding could be due to differences in lipophilicity between the two drugs (Kilpatrick et al., 1986), or to the ability to induce isomerization of the receptor (Amitai, Avissar, Balderman and Sokolovsky, 1982; Kloog et al., 1979; Kloog et al., 1978).

Agonist binding appeared to be enthalpy driven. Oxotremorine and carbachol binding showed thermodynamically favorable decreases in enthalpy. This decreased enthalpy was accompanied by a small, energetically unfavorable decrease in entropy for oxotremorine and a larger, energetically unfavorable decrease in entropy for carbachol. Pharmacological and physiological studies have differentiated class A and class B agonists based on their receptor binding profiles and intrinsic activity (Fisher et al., 1983; Fisher et al., 1984; Lippa et al., 1985; Lippa et al., 1986; Vauquelin et al., 1982). The present data suggest that it may be possible to differentiate the two classes of compounds on the basis of their thermodynamic profiles as well. It has been suggested that the combination of a decrease in enthalpy (indicating an exothermic reaction) in combination with a decrease in entropy (indicating an increase in the order of the system) may reflect a conformational change in the receptor protein (Weiland et al., 1979). Oxotremorine bound to the receptor in a simple manner revealing a single, temperature-sensitive, low affinity state in the majority of cases. This temperature effect has been reported for oxotremorine in cerebral cortex, colon and heart (Kloog et al., 1978). In the experiments where more than one state was revealed (one instance occurred at each temperature tested), a high affinity state became apparent. Oxotremorine binding resulted in only a very small decrease in entropy, implying a possible inability to induce conformational change, as has been

suggested previously (Weiland et al., 1979; Vauquelin et al., 1982; Lippa et al., 1986).

Complex binding was seen with carbachol in every experiment, with the exception of two experiments at 4°C, where a single, high affinity binding state was present. Both the high and low affinity interactions were enthalpy driven. Both affinity states demonstrated an energetically unfavorable decrease in entropy. The thermodynamics of carbachol binding could indicate that the low affinity state may be undergoing conformational change to the high affinity state. This would explain the large increase in the order of the system with high affinity carbachol binding. The loss of the low affinity state at 4°C may reflect an interconversion from a low to high affinity state.

These data, which suggest the ability of the low affinity carbachol state to change conformation, are compatible with recent studies involving the desensitization of intact cultured neuronal cells in response to carbachol exposure (Cioffe and El-Fakahany, 1986; Feigenbaum and El-Fakahany, 1985). In these studies brief exposure to carbachol resulted in desensitization, as measured by a decrease in the subsequent ability of agonists to stimulate cyclic GMP synthesis (Feigenbaum and El-Fakahany, 1985). This desensitization was accompanied by an approximate 50% loss of binding sites and an interconversion of low affinity carbachol state to a high affinity state (Cioffe and El-Fakahany, 1986). The concept of mAChR desensitization, as measured by a decreased ability of agonists to stimulate cyclic GMP formation, could be applied to the observation that decreased incubation temperatures result in decreased carbachol stimulated cyclic GMP formation (El-Fakahany and Richelson, 1980) and an interconversion

of the mAChR from the low to the high affinity state (Table 4). In this case, perhaps, increasing membrane rigidity by decreasing incubation temperatures resulted in a functional desensitization of the mAChR. This would suggest that a conformational change from the low affinity state to the high affinity state may be accompanied by a desensitization of the mAChR.

Parallels between thermodynamic characterization and intrinsic activity support a dynamic model of muscarinic agonist interactions (Lippa et al., 1986). According to this model, class A agonists stimulate PI hydrolysis which results in an uncoupling of the receptor from its effector mechanism(s) and allows the receptor to assume a high affinity conformation. Class B agonists do not stimulate PI hydrolysis and thus do not uncouple the receptor and are unable to convert the receptor from a low to high affinity state. Similarities between the functional consequences of lowered temperatures and Class A agonists suggest that both may involve a functional desensitization and a conversion of a proportion of the mAChRs from a low to high affinity state

3.00 EXPERIMENT 2

3.10 Introduction

Numerous investigators have studied the relationship between membrane dynamics and cellular function by manipulating both membrane composition and membrane viscosity. Indeed, manipulation of these variables affects cell function at the level of the receptor and the second messenger (Crews, 1982; Whetton, Gordon and Houslay, 1983a,1983b;

Fong and McNamee, 1985; Smith, Yamamura and Lee, 1986) and can have far reaching effects on cell function and intercellular communication. Increasing membrane viscosity by incorporating cholesterol inhibits the ability of carbachol to stimulate firing of the hippocampal pyramidal cell (Crews, Caramacho and Phillips, 1983). It has also been shown to increase serotonin (Heron, Shinitzky, Hershkowitz and Samuel, 1980) and opiate receptor binding (Heron, Israeli, Hershkowitz, Samuel and Shinitzky, 1981). Fluidizing the membranes by treatment with lecithin decreased serotonin binding (Heron et al., 1980) and opiate receptor binding (Heron et al., 1981). These two treatments, rigidifying with cholesterol or fluidizing with lecithin, have been shown to either increase or decrease the opiate receptor's susceptibility to degradation by trypsin (Heron et al., 1981). These findings suggest that changes in fluidity content may vertically displace the receptor relative to the membrane, or may induce a conformational change in the receptor protein that renders it more accessible to agents such as trypsin.

The view that incorporating additional membrane cholesterol may induce a conformational change in the receptor is supported by the observation that increasing membrane cholesterol results in a dose related change in the ability of oxotremorine to displace ^{3}H-QNB (Lippa and Bartus,1982). Oxotremorine displacement curves in the hippocampus are usually simple curves indicating binding in conformance with mass action. When hippocampal homogenates were treated with cholesterol, the binding data deviated increasingly from a one site model as the cholesterol concentration was increased. The data first represent a simple ligand-receptor interaction in the control and then change to a complex binding interaction with increased cholesterol. These data suggest that changes in

the membrane lipid content do change receptor conformation, orientation or accessibility to the ligand and subsequently change the nature of the receptor ligand interaction. (Lippa and Bartus, 1982). Similar conclusions concerning the effect of membrane cholesterol on protein-lipid interactions have been stated for the nicotinic acetylcholine receptor (nAChR). Fong and McNamee (1985) found that an optimal lipid environment existed for the ACh induced conformational change of the nAChR and subsequent ion gating activity.

Whetton, Gordon and Houslay (1983a, 1983b), studied the effect of altering membrane cholesterol content on adenylate cyclase stimulation in liver plasma membranes. Interestingly, they reported that increasing the cholesterol content (Whetton et al., 1983b) and decreasing the cholesterol content (Whetton et al., 1983a) both resulted in a decrease in glucagon stimulated adenylate cyclase. The change in adenylate cyclase activity may be due to an increased membrane rigidity, which was observed following both membrane manipulations. This explanation, however, is probably not complete. Whetton et al. (1983b) report that basal adenylate cyclase activity is rather insensitive to changes in fluidity, but is very sensitive to small changes in cholesterol content. This suggests that the mechanism by which cholesterol exerts its effect may not be simply a change in fluidity, but also may be due to a change in the membrane content and in the lipid domain structure (Whetton et al., 1983a).

Increased membrane viscosity and an increased cholesterol/phopholipid mole ratio has been observed following chronic alcohol treatment (Crews, 1982). The increased cholesterol content seen with alcohol tolerance could be a compensatory mechanism for the

disordering effects of alcohol on the membrane. Alcohol, administered acutely, decreases the order of the membrane lipids and increases membrane fluidity (Seeman, 1972). When studied in vitro, acute alcohol administration inhibits carbachol stimulated PI hydrolysis in striatal slices, but not in the hippocampal, cortical or brainstem slices (Crews, 1982). In the regions that showed no change in carbachol stimulated PI hydrolysis, inhibitory effects of alcohol on norepinephrine, potassium stimulated PI hydrolysis were demonstrated. (Crews, 1982). These results suggest that changes in the membrane environment can selectively affect brain regions and cell functions. The effect of ethanol may also be determined, in part, by the tissue preparation and method of measurement. Smith, Yamamura and Lee (1986) reported no effect of chronic ethanol treatment *in vivo*, or acute ethanol treatment *in vitro* on carbachol or norepinephrine stimulated ^{3}H-inositol 1-phosphate accumulation (a measure of PI hydrolysis) in mouse forebrain slices. In addition there was no change in the receptor density or affinity for ^{3}H-QNB or ^{3}H-PZ. They did, however, observe an alcohol inhibition of receptor mediated PI hydrolysis as measured by receptor mediated ^{32}P incorporation into synaptosomal phosphotidic acid. When synaptosomes from alcohol tolerant mice were challenged with alcohol acutely, no inhibition of PI hydrolysis was observed, suggesting that tolerance to the effects of alcohol had developed in these mice. *In vitro* alcohol challenge in control mice, resulted in an inhibition of carbachol stimulated ^{32}P incorporation. No change in the basal rate of PI hydrolysis was observed. The increased rigidity of membranes following ethanol tolerance and increased cholesterol content has widespread effects on the charcteristics of the membrane. The interaction of the cholesterol with

membrane phospholipids alters the position of intramembane proteins and limits their movement within the membrane. In addition there is an abolishment of phase transitions in the membrane phospholipids (Shinitzky and Inbar, 1976).

The studies discussed above all manipulate membrane content and membrane fluidity simultaneously and examine the effects on various receptor and cell systems. The following studies were conducted to determine the effects of altering both membrane fluidity and membrane lipid contents on mAChR binding. AL721, a mixture of egg phopholipids and neutral triglycerides, has been reported to fluidize cellular membranes due to its ability to extract cholesterol (Lyte and Shinitzky, 1985). In these studies AL721 was used to fluidize rat hippocampal membranes. The effect of this treatment on ^{3}H-QNB binding and the displacement of ^{3}H-QNB binding by the class A agonist carbachol and the class B agonist oxotremorine was studied.

3.20 Methods and Materials

3.21 Materials

^{3}H-Quinuclidinyl benzilate (^{3}H-QNB; 42.6 Ci/mmol) was purchased from New England Nuclear (Boston, Ma.). Atropine (crystalline free base), carbamylcholine chloride (carbachol), oxotremorine (1-[4-(1-Pyrrolidinyl)-2-butyryl]-2-pyrrolidinone), sodium phosphate, potassium phosphate, sucrose, diphenylhexatriene (DPH) and tetrahydrofuran (THF) were purchased from Sigma Chemical Company (St. Louis, Mo.). AL721 was obtained from Matrix Research Laboratories (Fort Lee, N.J.).

3.22 Subjects

In these studies rat brains were used. Mature Sprague Dawley and/or Wistar rat brains were obtained from Pel Freez Biologicals (Rogers, Arkansas).

3.23 Tissue preparation

Brains were thawed and the right and left hippocampi were dissected out and pooled. The tissue was homogenized in 10 volumes ice cold Na-KPO_4 buffer (pH 7.4) and diluted to 1:250 (wt:vol) with ice cold buffer.

3.24 AL721 treatment

3.24a Time course

An AL721 stock suspension (5 mg AL721/ml in 0.32M sucrose stirred for 2hr at room temperature) was made and stored at 4°C. In order to determine the incubation time for maximal fluidization a time course for the effect of AL721 on membrane fluidity was conducted. The AL721 stock was brought to 37°C and stirred for 10-15 minutes before diluting further with room temperature 0.32 M sucrose. One ml of tissue homogenate was incubated with 6.0 ml of 2.5 mg AL721/ml for times ranging from 0-4hr at room temperature (20-25°C) with shaking. In order to separate the AL721 from the hippocampal tissue, samples were first centrifuged at 20,000 times gravity for 10 min at 4°C. The pellet was then resuspended in 1 ml Na-KPO_4 buffer (pH 7.4), layered over 20 ml 0.32 M sucrose and centrifuged again under the same conditions. The resulting pellet was resuspended in 1 ml sucrose and stored on ice for use in the fluorescence polarization assay (described below). These experiments demonstrated maximal fluidity at 4hr

(Data not shown). Therefore in all subsequent experiments tissue was treated with AL721 for 4hr before use in binding experiments.

3.24b Microviscosity measurements

Aliquots of the tissue homogenate (0.5 ml) were incubated in the dark with 4.5 ml 2 μM DPH (2 mM DPH in THF was diluted 1000 fold with 50 mM Na-KPO_4 buffer) for 30 min. at room temperature (20-25^{o}C) with shaking. Membrane microviscosity was measured using standard fluorescence polarization techniques as described by Shinitzky and Barenholtz (1978). (Excitation wavelength was 360 nm and emission wavelength was 427 nm.) Tissue samples from each brain were tested at room temperature in duplicate. Polarization measurements were taken twice for each sample.

3.24c Dose Response Curve

Two ml of tissue homogenate (1:250, wt:vol in Na-KPO_4 buffer (pH 7.4)) was incubated with 10 ml AL721 (eight concentrations ranging from 0 to 5 mg AL721/ml 0.32 M sucrose) for 4hr at 25^{o}C with shaking. Incubation was terminated and AL721 was separated from the tissue homogenate by centrifugation (100,000 times gravity for 30 min at 4^{o}C). The centrifuge tube and pellet were gently washed with 0.32 M sucrose using a Pastuer pipette. The pellet was resuspended in buffer to the original tissue volume and used in the fluorescence polarization assay. A good dose response relationship was observed between AL721 concentration and degree of membrane fluidity. Maximal fluidity was obtained using 2.5 mgAL721/ml. Five different AL721 concentrations were used in the ^{3}H-QNB binding and displacements experiments: 0, 0.05, 0.15, 0.5 and 2.5 mg AL721/ml.

3.25 ^{3}H-QNB binding

Tissue was prepared and treated with AL721 as described above in section 3.24. ^{3}H-QNB binding experiments were performed at 25^{o}C. One hundred µl of the hippocampal homogenate (0.3-0.5mg protein/ml) were incubated with 300 µl of ^{3}H-QNB (Eight ^{3}H-QNB concentrations were used, ranging from 5.0 pM to 750 pM). One hundred µl of atropine (resulting in a final concentration of 10µM) was used to define nonspecific binding. The total assay volume was brought up to 5.4 ml with 50 mM Na-KPO_4 buffer (pH 7.4). Protein was determined using the method of Bradford (1976). Aliquots were taken from each experimental condition and microviscosity was determined. This ensured that the AL721 treatment was effective in each experiment.

3.26 Displacement Experiments

Experiments were performed at 25^{o}C. One hundred ml AL treated tissue was incubated with 300 µl approximate K_D concentrations of ^{3}H-QNB. Eight to ten concentrations of the displacing ligand were used. The final concentrations of carbachol ranged from 50 nM to 1 mM, and oxotremorine from 5 nM to 10 µM. Ten µM atropine (final concentration) was used to define nonspecific binding. Final assay volume was 5.4 ml. Membrane microviscosity determinations were made on aliquots from each experimental condition in every experiment.

3.30 Data Analysis

K_D and B_{max} values were determined by Scatchard analysis (Scatchard, 1949) of the saturation isotherm data. Hill coefficients were determined from Hill plots of the displacement data. IC_{50} values were determined from Hill plots and by computer-assisted iterative nonlinear analysis, which also indicated whether the data was best fit to a 1-site model or a 2-site model of receptor occupation. K_i, K_H and K_L values were calculated from the computer generated IC_{50} values using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). ANOVA was performed to determine whether a main drug effect was present. This was done with the SYSTAT (Systems for Statistics) software for the Macintosh computers (Wilkinson, 1987). T-tests were also performed, using the SYSTAT program, to determine significant differences between drug groups.

3.40 Results

The effect of AL721 on membrane fluidity: AL721 was a highly effective membrane fluidizer. As the concentration of AL721 was increased the viscosity of the membrane decreased markedly. This data is presented graphically in Figure 3.

The effect of AL721 on 3H-QNB binding isotherms: One-way analysis of variance revealed no significant dose related effect on K_D or B_{max} ($p>.18$, $F=1.795$, $df=4,16$ and $p>.08$, $F=2.553$, $df=4.16$ respectively). Comparisons using t-tests did demonstrate significant effects of AL721 on the Bmax at the two highest doeses ($p<.025$). A small but significant increase in the B_{max} values was observed at the two highest doses of

AL721, with no significant change in the K_D value over the dose range tested (See Table 6).

The effect of AL721 on displacement binding: The Hill coefficient was less than unity and computer-assisted iterative nonlinear analysis indicated that the data was best fit to a 2-site model, in the majority of cases, at each concentration (Data shown in Table 7). Carbachol binding was not significantly altered by treatment with AL721 ($p>.05$, $F=.231$, $df=5,14$ for the high affinity site and $p>.35$, $F=1.232$, $df=5,14$ for the low affinity site, ANOVA). Oxotremorine binding was sensitive to changes in fluidity. The control condition yielded a Hill coefficient that was not significantly different from unity suggesting the involvement of a single receptor site, or state. These data were best fit to a 1-site model in the majority of cases. Treatment of hippocampal tissue with AL721 resulted in a significant decrease in the Hill coefficient ($p<.01$, t-test) that was evident at the lowest dose tested. As was expected, the significant decrease in the Hill coefficient was accompanied by a change in the model of receptor occupation from a 1-site model to a 2-site model (Table 8) suggesting a change in the conformation, or orientation of the receptor. No significant effect of AL721 on the affinity for oxotremorine was demonstrated by a one-way analysis of variance ($p>.83$, $F=.417$, $df=5,16$ for the high affinity site and $p>.76$, $F=.525$, $df=5,32$ for the low affinity site).

3.50 Discussion

AL721 is a mixture of egg phopholipids and neutral triglycerides. It has been suggested that AL721 fluidizes membranes by removing

cholesterol from the membrane (Lyte and Shinitzky, 1985). This proposed mechanism of action makes the drug well suited for studying the effect of changes in membrane composition and fluidity on the mAChR. This series of experiments suggests that increasing membrane fluidity by decreasing membrane cholesterol can affect the mAChR by interconverting a proportion of these receptors from a low to a high affinity state. This change was measured with the class B agonist oxotremorine, for this ligand does not induce a conformational change and normally binds to a single site which appears to be a low affinity site. The class A agonist carbachol, which induces a conformational change in a proportion of mAChR and which recognizes both high and low affinity states of the receptor was not an effective measure of AL721 induced changes. The lack of additivity of AL721 and carbachol suggests that those mAChRs which were interconverted by AL721 maybe the same mAChRs affected by carbachol.

Fluidization with AL721 appears to produce a change in the ligand-receptor interaction and not an absolute, fixed change in the conformation of the receptor protein, or the exposure of sequestered receptors. This view is supported by a number of observations. 1) The proportion of low affinity sites seen in carbachol binding does not change significantly with AL721 treatment. Carbachol binding results in 50-70% of the sites demonstrating low affinity for the ligand in the control and experimental conditions. 2) Oxotremorine binding, which in the control condition results in 100% low affinity sites, results in 80% low affinity sites with AL721 treatment. This suggests that with fluidization oxotremorine is still not as effective as carbachol at inducing a conformational change in the receptor from a low to a high affinity state. 3) Finally, the effect of AL721 cannot be explained by

the exposure of sequestered receptors since the full effect on oxotremorine binding is already observed at the lowest doses of AL721 which show no change in receptor affinity or receptor density as measured by ^{3}H-QNB binding.

The observations made in these experiments are reminiscent of the changes in the mAChR following cholesterol treatment of hippocampal membranes (Lippa and Bartus, 1982). After either AL721 or cholesterol treatment a decrease in the Hill coefficient for oxotremorine with the appearence of a high affinity state was observed. This indicates that an optimal level of cholesterol content and membrane fluidity may exist and deviations from this optimum could produce similar changes in the ligand-receptor interaction. Similar observations have been made concerning mAChR mediated cyclic GMP formation (Whetton et al., 1983a, 1983b) and nAChR mediated ion flux (Fong and McNamee, 1985). However, it should not be assumed that the very same mechanism is responsible in both cases. Whetton et al. (1983b) suggested that, although both an increase and a decrease in membrane fluidity affected adenylate cyclase activity in the same manner, the mechanism by which cholesterol exerted it's effect was probably not a change in membrane fluidity alone.

4.00 EXPERIMENT 3

4.10 Introduction

A well, although, not universally, documented consequence of aging is an increased cholesterol content, cholesterol to phospholipid ratio

(Armbrecht, Wood, Wise, Walsh, Thomas and Strong, 1983; Shinitzky, 1984; Kessler, Kessler and Yehuda, 1985) and an increased membrane microviscocity (Freund, Brophy and Scott ,1986; Shinitzky, 1984) in neuronal and other membranes. Studies concerning the effects of membrane fluidization or rigidification on receptors and second messengers in young and mature animals have shown that these processes can be greatly affected by rather small changes in their membrane environment (see Experiment 2). Increasing the cholesterol content of neuronal membranes can result in an increased microviscosity, a decreased ability of muscarinic agonists to maximally stimulate hippocampal pyramidal cell firing (Crews et al., 1983), a decrease in the Hill coefficient for oxotremorine (Lippa and Bartus, 1982), and a change in the binding characterizations of the serotonin and opiate receptors (most notably an increase in the Bmax, or number of receptors accessible to the ligand) (Heron et al., 1980; Heron et al., 1981). These observations have lead to an increased interest in how the lipid milieu and its ability to respond to environmental manipulations changes with age.

There are notable similarities between the effects of increased cholesterol content and aging on cell function . Aged membranes have been described as more rigid than young membranes (Shinitzky, 1984; Freund et al., 1986). Aging also results in a decreased ability of acetylcholine, or muscarinic agonists, to stimulate firing in the hippocampal pyramidal cell, which is parallelled by a decrease in the Hill coefficient for oxotremorine (Lippa et al., 1985), and an increase in the binding of ligands to serotonin receptors (Antonian et al., 1987). These findings suggest that perhaps fluidizing aged membranes could restore certain physiological and

biochemical parameters to the level of young conspecifics (for a detailed discussion of the cholinergic hypothesis of aging, please see Appendix 1).

Freund et al.(1986) found that fluidization of young brain membranes with ethanol resulted in an increase in the percentage of low affinity sites as measured by carbachol binding. In addition they found that aged membranes demonstrated an increased baseline viscocity and a decreased senitivity to the fluidizing effects of aliphatic alcohols. In the aged membranes, alcohol treatment resulted in a significantly smaller increase in the percentage of low affinity sites. Freund's results may suggest that, in the aged membranes, some proportion of the binding sites are already in a high affinity, desensitized state. It is possible that the decreased effect of ethanol on carbachol binding observed by Freund et al., was due to the inability of the ethanol to disorder the membrane from the old subjects as effectively as the membrane from the young subjects (Armbrecht et al., 1983).

Not all fluidizing agents are less effective in aged subjects; conflicting results have been reported. Freund et al. (1986) found that old membranes are less affected by ethanol administered in vitro. Wood, Williamson, Rocco and Strong (1986) reported no age difference in the effect of chronic ethanol administered in vivo on membrane phopholipids, or on the ability of the subjects to adapt to in vivo chronic ethanol indicating that aged membranes and young membranes were equally responsive to the ethanol. Centrophenoxine which may fluidize the membrane by perturbing the lipids (although the mechanism by which it increases fluidity is not understood) has also been reported to fluidize membranes from young and old mouse liver equally well (Wood, Gorka, Armbrecht, Williamson and Strong, 1986). It is possible that fluidizing the membrane using agents such as ethanol may

not be an effective way of countering the increased membrane microviseosity that occurs with aging. This could be due to the generalised effect alcohol has on the membrane; administered acutely alcohol perturbs the mebrane lipids without affecting the membrane lipid composition.

If the changes in receptor function seen with aging are a consequence of increased membrane cholesterol, it may be necessary to manuipulate cholesterol levels, thereby affecting membrane viscosity, to achieve a restoration of the receptor binding profile seen in young membranes. This series of experiments was performed to determine the effects of fluidization with AL721 on mAChR in aged membranes. Because AL721 reportedly fluidizes membranes by extracting cholesterol, it may be particularly effective at fluidizing aged membranes. In these experiments, hippocampal homogenates from aged female rats were treated with AL721. In young hippocampal homogenates, AL721 treatment resulted in a change in ^{3}H-QNB binding and in oxotremorine displacement of ^{3}H-QNB binding, with no change in carbachol displacement of ^{3}H-QNB binding. The aged membranes were therefore characterized by ^{3}H-QNB saturation isotherms and displacement experiments using oxotremorine displacement of ^{3}H-QNB.

4.20 Methods and Materials

4.21 Materials

^{3}H-Quinuclidinyl benzilate (^{3}H-QNB; 42.6 Ci/mmol) was purchased from New England Nuclear (Boston, Ma.). Atropine (crystalline free base), carbamylcholine chloride (carbachol), oxotremorine (1-[4-(1-Pyrrolidinyl)-2-butyryl]-2-pyrrolidinone), sodium phosphate, potassium phosphate,

sucrose, diphenylhexatriene (DPH) and tetrahydrofuran (THF) were purchased from Sigma Chemical Company (St. Louis, Mo.). AL721 was obtained from Matrix Research Laboratories (Fort Lee, N.J.).

4.22 Subjects

In these experiments rat brains were used. Female Wistar rats from the Gerontology Research Center colony in Bethesda MD, were obtained from Dr. Donald Ingram, National Institute of Aging, Bethesda, MD. All rats were at least 24 months of age.

4.23 Tissue preparation

Female rats (24 mos. old) were sacrificed and the brains extracted. The right and left dorsal hippocampi from each rat were dissected out, pooled and homogenized in 3.0 ml Na-$KHPO_4$ buffer (pH 7.4) and stored at -70°C. For use in polarization and binding experiments, tissue was thawed and diluted to 6.0 ml with buffer and briefly homogenized. Protein concentration was determined using the BioRad colorimetric assay and the tissue pool was then further diluted with buffer to a concentration of approximately 0.7 mg protein/ml.

4.24 AL721 treatment

Dose Response Curve: Two ml of tissue homogenate (1:250, wt:vol in Na-KPO_4 buffer (pH 7.4)) was incubated with 10 ml AL721 (eight concentrations ranging from 0 to 5 mg AL721/ml in 0.32 M sucrose) for 4hr at 25°C with shaking. Incubation was terminated and AL721 was separated

from the tissue homogenate by centrifugation (100,000 times gravity for 30 min at 4°C). The centrifuge tube and pellet were gently washed with 0.32 M sucrose using a Pastuer pipette. The pellet was resuspended in buffer to the original tissue volume and used in the fluorescence polarization assay. A good dose response relationship was observed between AL721 concentration and degree of membrane fluidity. Maximal fluidity was obtained using 2.5 mgAL721/ml. Four different AL721 concentrations were used in the ^{3}H-QNB binding and displacements experiments: 0, 0.05, 0.5 and 2.5 mg AL721/ml.

4.25 Saturation Isotherms

^{3}H-QNB binding: Tissue was prepared and treated with AL721 as described above in section 4.24. ^{3}H-QNB binding experiments were performed at 25°C. One hundred µl of the hippocampal homogenate (0.3-0.5mg protein/ml) were incubated with 300 µl of ^{3}H-QNB (Eight ^{3}H-QNB concentrations were used, ranging from 5.0 pM to 750 pM). One hundred µl of atropine (resulting in a final concentration of 10µM) was used to define nonspecific binding. The total assay volume was brought up to 5.4 ml with 50 mM Na-KPO_4 buffer (pH 7.4). Protein was determined using the method of Bradford (1976). Aliquots were taken from each experimental condition and microviscosity was determined. This ensured that the AL721 treatment was effective in each experiment.

4.26 Displacement Experiments

Experiments were performed at 25°C. One hundred ml AL treated tissue was incubated with 300 µl approximate K_D concentrations of ^3H-QNB. Eight to ten concentrations of the displacing ligand were used. The final concentrations of oxotremorine ranged from 5 nM to 10 µM. Ten µM atropine (final concentration) was used to define nonspecific binding. Final assay volume was 5.4 ml. Membrane microviscosity determinations were made on aliquots from each experimental condition in every experiment.

4.27 Microviscosity measurements

Aliquots of the tissue homogenate (0.5 ml) were incubated in the dark with 4.5 ml 2 µM DPH (2 mM DPH in THF was diluted 1000 fold with 50 mM Na-KPO_4 buffer) for 30 min. at room temperature (20-25°C) with shaking. Membrane microviscosity was measured using standard fluorescence polarization techniques as described by Shinitzky and Barenholtz (1978). (Excitation wavelength was 360 nm and emission wavelength was 427 nm.) Tissue samples from each brain were tested at room temperature in duplicate. Polarization measurements were taken twice for each sample.

4.30 Data Analysis

K_D and B_{max} values were determined by Scatchard analysis (Scatchard, 1949) of the saturation isotherm data. Hill coefficients were determined from Hill plots of the displacement data. IC_{50} values were determined from Hill plots and by computer-assisted iterative nonlinear analysis, which also indicated whether the data was best fit to a 1-site model

or a 2-site model of receptor occupation. K_i, K_H and K_L values were calculated from the computer generated IC_{50} values using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). ANOVA was performed to determine whether a main drug effect was present. This was done with the SYSTAT (Systems for Statistics) software for the Macintosh computers (Wilkinson, 1987). T-tests were also performed, using the SYSTAT program, to determine significant differences between drug groups.

4.40 Results

The effect of AL721 on membrane fluidity: AL721 fluidized the membranes from old rat hippocampus in a dose dependent fashion. The data are presented in Figure 4. However, a comparison of the effect of AL721 on the microviscosity of young and old membranes, suggests that the fluidizing agent was more potent and had a greater effect on the young tissue than the old (see Figure 5).

The effect of AL721 on ^{3}H-QNB binding isotherms: A main effect of AL721 on K_D and B_{max} was not demonstrated by one-way analysis of variance ($p>.067$, $F=2.730$, $df=3,23$ for K_D and $p>.69$, $F=.528$, $df=3,23$ for B_{max}). No significant change in the B_{max} was observed (Table 9) even at the highest doses which resulted in a significant increase in the B_{max} in the hippocampal tissue from young subjects.

The effect of AL721 on the ability of oxotremorine to displace ^{3}H-QNB: No significant change in the affinity for oxotremorine or the Hill coefficient was observed over the AL721 concentrations tested . The K_i values for untreated hippocampal homogenates was virtually the same in the

young and old tissue (compare Tables 8 and 10). The minimum effective dose for AL721 was higher in the old tissue than the young tissue. A change in the model of best fit from a 1-site to a 2-site model was observed following treatment with 0.05 mgAL/ml in the young tissue as compared to 0.5 mgAL/ml in the aged tissue (see Tables 8 and 10). In addition, although the control condition in both the young hippocampal tissue (see Table 6) and the old hippocampal tissue (Table 7) were characterised by a 1-site model, the Hill coefficient for oxotremorine in the old tissue is significantly lower than that for the young tissue ($t=1.909$, $p<.05$ one-tailed t-test).

4.50 Discussion

In this study AL721 treatment fluidized membranes from aged rats. However, the results from these experiments demonstrate that membranes from aged tissue were not as sensitive to the fulidizing effects of AL721 as membranes from young tissue. By way of example old membranes appear to be less able to respond to changes in the microenvironment (Shinitzky, 1984 and Figure 5). This observation is in accord with results reported by Freund et al. (1986) and Armbrecht et al. (1983) who used alcohol to fluidize membranes. These results support the view that aging is accompanied by a deficit in homeoviscous adaptation.

The decreased fluidization of aged membranes was paralleled by a decreased effect of AL721 on oxotremorine binding, the minimum effective dose of AL721 on oxotremorine binding in young tissue was 0.05 mgAL721/ml and the minimum effective dose in old tissue was 0.5 mgAL721/ml. This suggests that the degree of fluidization and the change in

oxotremorine binding were probably related. A comparison of the data from young and aged hippocampal homogenates, suggests that the difference between the two lies in the flexibility of the aged system, rather than a decreased number or affinity of the receptors. There was no difference between the two receptor populations as measured by ^{3}H-QNB binding; the K_D and B_{max} values were the same in the control conditions for both tissues (see Tables 6 and 9). In addition the affinity for oxotremorine was the same in both populations. The significant differnce between the Hill coefficients for oxotremorine in the young and aged tissue suggests the possibility that the two populations may not be identical.

This reduced flexibility on the part of the mAChR is also evident when looking at the ^{3}H-QNB binding data. At the two highest doses AL721 treatment of young tissue resulted in an increase in the Bmax suggesting, perhaps that cryptic receptors were revealed, no such increase was observed following treatment of aged tissue.

5.00 Summary and Conclusions

The series of exeperiments presented here allow several interesting points to be made concerning the mAChR and membrane dynamics. First, it is clear that altering the microenvironment of the receptor does have an effect on the receptor. In addition, altering membrane fluidity with temperature and AL721 affect the receptor in two very different ways. The temperature experiments indicated that increasing the microviscosity of the membrane resulted in an increased affinity of the receptor for the ligand, with no conformational change. Similar results have been reported for the

mAChR by Muzio et al. (1986) and Mei et al. (1987). These data also suggest that if microviscosity were decreased by increasing temperature a decrease in the affinity of the receptor would result. These results have, in fact, been reported by EL-Fakahany. In contrast to the temperature dependent characteristics of mAChR binding, fluidization with AL721 does not affect the affinity of the receptor. In each of the experiments affinity of the receptor for all ligands tested remained largely unaffected by AL721, while the conformation, or orientation, of the receptor within the membrane appeared to be altered. This was demonstrated in the oxotremorine displacement experiments by a significant decrease in the Hill coefficient and a change in the model of best fit from a 1-site to a 2-site model. These observations indicate that small changes in the microviscosity of the membrane can have particular effects on the mAChR depending on the nature of the fluidity change.

The studies discussed above address the question of what are the effects of changes in the membrane state on the receptor. It is also possible to adopt another perspective and ask what are the effects of receptor activation on the microenvironment, or microviscosity of the membrane. Reports in the literature indicate that transient fluidity changes do occur following numerous cellular events. Membrane fluidity changes have been documented following diverse processes such as the creation of transmembrane electrical potential (Corda, Pasternak and Shinitzky, 1981), small changes in pH (Schacter and Shinitzky, 1977), the binding of neurotransmitters (Schneeweiss, Naquira, Rosenheck and Schneider, 1979) and transmethylation of phospholipids (Hirata and Axelrod, 1978). All of these processes are potentially involved in the activation of the

muscarinic receptor. Although the nature of the fluidity change may not be understood in all these processes, some of them certainly affect the lipid composition of the membrane. One example of a naturally occurring, transient change in the lipid composition of the microenvironment is receptor stimulated hydrolysis of phosphoinositide (PI). PI is found in the cytoplasmic facing leaflet of neuronal membranes (Curtin, Gordon and Aloia, 1988 in press). The synthesis and metabolism of membrane bound lipids which result in changes in fluidity could thereby affect the affinity or conformation of the mAChR, which is an intramembrane protein.

The interaction between membrane state and receptor function indicates a dynamic relationship between the mAChR and its microenvironment. The variety of physiologic processes that alter the membrane environment in combination with the multiplicity of effects that changes in the membrane environment can have on the mAChR suggests that this dynamic receptor-membrane relationship is one of enormous complexity. A greater understanding of the different factors involved in the receptor- membrane interaction may yield important information concerning cell and receptor function, information transduction and pathology.

It is possible that one factor could be a reciprocal, feedback system in which membrane dynamics and receptor activation modulate one another. Reciprocal modulation could be involved in the phenomenon of desensitization. Desensitization of mAChR-mediated events have been observed following application of class A agonists. These agonists are characterised by their ability to induce a conformational change in the receptor and stimulate PI hydrolysis. If both of these processes produce changes in the microviscosity of the surrounding membrane, the altered

fluidity might then affect the conformation of the recptor protein and thereby produce a functional desensitization. This change in fluidity is a transient effect that could occur over a period of seconds to minutes and revert back to the basal state shortly after cessation of agonist stimulation.

Lippa et al., (1986) found that Class A agonists produce desensitization of hippocampal pyramidal cells, which is manifested as a decrease in the frequency of burst responding when the duration of agonist application exceeded 30 seconds. El-Fakahany and Richelson (1980) reported a similar response pattern in neuroblatoma cells. Application of the Class A agonist carbachol initially stimulated cyclic GMP formation, whereas continued application of the agonist resulted in a decrease in the magnitude of the response.

Diacylglycerol, one of the two principle second messenger metabolites of PI hydrolysis, activates protein kinase C. Phorbol esters, which are protein kinase C activators, have been shown to mimic the effect of mAChR on certain electrophysiological parameters (Malenka, Madison, Andrade and Nicoll (1986), and potentiate desensitization in sympathetic ganglion neurons (Downing and Role, 1987). Is is possible that one of the substrates of the protein kinase C is the mAChR. This would represent a feedback system in which mAChR activation is, in a sense, self regulated. Both receptor activation and protein phosphorylation can be modulated by changes in fluidity (Hershkowitz, Heron, Samuel and Shinitzky,). These observations further support the view that changes in fluidity subsequent to receptor activation and PI hydrolysis may be a factor in desensitization.

Desensitization appears to be a phenomenon that regulates many receptor mediated events. It has also been suggested that desensitization

may play a role in pathological conditions such as age-related memory dysfunction (Lippa et al., 1985). The studies presented here support and extend the view that age related mAChR dysfunction may involve a desensitization-like process. Aging has been associated with a conformational change in the mAChR (Lippa et al., 1985) and the studies presented her indicate an age related decrease in the adaptability of the receptor-membrane complex to changes in the microenvironment. If functional desensitization in a normal system is defined as a decreased ability to respond maximally to a given stimulus, then the aged mAChR and the aged membrane could be viewed as residing in a desensitized state.

Although the model presented here of a dynamic mAChR-membrane interaction is clearly an oversimplification of the interrelated processes that occur in vivo, it can be a useful model with which neurotransmitter receptor functions and their involvement in pathological states can be studied. The data presented here indicate that the receptor cannot be thought of as a protein that exists in isolation, unaffected by it's microenvironment. Although this point is an obvious one, it is a difficult one to include in research strategies for the development of effective pharmacotherapies. If we are able to increase our understanding of this dynamic relationship between the mAChR and the broader membrane environment it may be possible to apprehend the aging process and develop more refined pharmacotherapies for age related disorders.

6.00 APPENDIX 1

6.10 Background

Aging is a poorly understood, ubiquitous phenomenon that can affect every level of normal organismic functioning. One of the most commonly observed consequences of aging in humans and in animals is memory impairment (Dean, Goas, Regan, Scozzafava and Bartus, 1981; Reisberg, Ferris and de Leon, 1982; Reisberg, Ferris, de Leon and Crook, 1985). This cognitive deficit can be either "normal", in the sense that the individual's ability to function is not significantly impaired, or "pathological", meaning the individual is no longer an independent, functional member of his community (Reisberg et al., 1982; Reisberg et al., 1985). Memory deficits that occur with normal aging have been termed Benign Senescent Forgetfulness (BSF) (Kral, 1962). Pathological memory deficits have been associated with the onset of Alzheimer's disease. Indeed the prevalence of pathological aging, or Alzheimer's disease, is increasing. The percentage of the U.S. population over 65 years of age has increased from 4% at the beginning of the 20th century to 11% today. It is expected to reach 20% in the next 50 years (this prediction indicates that by the year 2030 fifty million individuals will be over the age of 65). Although the majority of this population does not suffer from Alzheimer's, approximately 6% is afflicted and this represents a very large number of disabled individuals both presently and in the future (for review see Crook, Bartus, Ferris and Gershon, 1986).

The enormity of these statistical projections has given rise to numerous research efforts to understand both memory and aging. Consequently, our

understanding of memory processes, aging processes and their interrelationship at the molecular, cellular, physiological and behavioral levels has increased. From a wide body of literature the etiology of Alzheimer's disease is associated with the degeneration of central cholinergic neurons (Crook et al., 1986), just as Parkinson's disease is associated with the degeneration of central dopamine neurons (Kandel and Schwartz, 1985). However, the etiology of BSF remains unknown. Several pharmacological interventions have been tested in the hope that discovery of an effective therapy will alleviate the disorder as well as provide greater understanding of its etiology. No pharmacological agent(s) has been developed that singly, or in combination, will reliably, and safely over prolonged periods improve the condition of patients with BSF or Alzheimer's.

It seems evident that research must continue to address the problem of the exact nature of the aging process. Aging appears to involve a deterioration of homeostatic functions, or an imbalance among central nervous system (CNS) processes. This type of dysfunction would result in greater and greater deficits over time. It is possible that very subtle changes in cellular function may have far reaching effects on cellular communication, and subsequent cognitive functions. For this reason, it is necessary to determine 1) which pharmacosystems in the CNS might be critically involved in normal memory 2) what are the characteristics of this system in the normal individual and 3) how do the characteristics of this (these) systems change with age.

While several neurotransmitter and hormonal systems have been implicated in aging and memory dysfunction (see Crook et al., 1986), the

involvement of the cholinergic system in memory has long been recognized both anecdotally and empirically (Bartus, Dean, Beer and Lippa, 1982).

6.20 The cholinergic hypothesis

The cholinergic hypothesis of geriatric memory dysfunction has provided a framework within which normal and pathological memory processes can be studied (Bartus et al., 1982). The hypothesis states that specific disruptions in the central cholinergic systems occur to some degree in normal aged individuals and to a much greater degree in the demented elderly. These disruptions are functionally similar to those observed following pharmacological blockade of the cholinergic system in healthy young subjects, and play an important role in the cognitive and memory deficits seen in aging and dementia. Finally it suggests that the enhancement, or restoration, of cholinergic function may alleviate the clinical disorder (Bartus, Dean, Beer and Lippa, 1982).

6.21 Memory

The cholinergic hypothesis of geriatric memory dysfunction is a broad statement encompassing a neurotransmitter system that innervates nuclei throughout the central nervous system (CNS) and its possible involvement in complex cognitive funcitons. Clearly defining the different processes involved in normal memory is therefore necessary if the precise role of the cholinergic system is to be delineated. Memory is generally accepted to be a multi-staged process involving encoding strategies, immediate, short and

long term memory stores, and retrieval capabilities (Drachman and Leavitt, 1974). Immediate memory allows the maintenance of a small amount of information for brief periods of time; access to this information is usually lost if the subject is distracted. Short-term memory represents an intermediate storage capacity. It allows for the accumulation of information and is necessary for the acquisition of procedural and declarative information. Short term memory, particularly the acquisition of declarative knowledge, appears to be dependent on the integrity of the hippocampal complex and the septo-hippocampal pathway. (Olton, Becker and Handelmann, 1980). Consolidation processes transfer information from short term memory into long term memory stores. These long term memory stores are presumably distributed throughout the cerebral cortex. Retrieval enables the subject to access and use information stored at any level to guide behavior (Drachman and Leavitt, 1974).

6.22 Clinical symptomatology

Short term memory impairments are commonly reported in the aged population (Reisberg et al., 1982; Reisberg et al., 1985). Both aged individuals with normal forgetfulness and those who later become demented initially report similar subjective complaints (Reisberg et al., 1982; Reisberg et al., 1985). The difference between the two populations is that one experiences a mild memory deficit and the other, for some reason, develops a severe, progressive pathology.

Clinical symptomatology of normal aging and dementia differ in the magnitude of cognitive impairment. Cognitive changes that accompany

normal aging have been termed Benign Senescent Forgetfulness (BSF) (Kral, 1962) and as the name implies these changes do not interfere with normal social or professional functioning. BSF is characterized by subjective complaints of cognitive deficit which include a decreased ability to recall names of places and objects or to remember where objects have been placed. These symptoms are quite common and there is no significant difference in the death rates of individuals exhibiting BSF and aged individuals with preserved memories.

Reisberg and his colleagues (1982; 1985) have devised a seven stage Global Deterioration Scale (GDS) for age-related cognitive decline and Alzheimer's disease. In their scale, BSF is stage 2, the Forgetfulness stage. Stage 3, the Early Confusional stage, represents a borderline condition between normal aging and senile dementia of the Alzheimer's type (SDAT). It is characterized by an increased deficit in recall of names, places and objects' locations. In addition some deficit in the recall of recent events and activities can be present. Concentration deficits are easily elicited in a clinical evaluation setting. This is the first stage at which occupational and social functioning may be compromised.

Four stages of dementia are described in the GDS. The earliest phase (GDS 4) is characterized by a clear functional deficit in which the patient has difficulty handling daily living activities such as financial matters and shopping. Concentration and calculation deficits are clearly evident and the memory deficit, by this stage, has increased considerably to include major recent events and past memories. Orientation deficits may result in the inability of the patient to correctly identify the current month or season. The deficits increase in magnitude with each stage. A variety of other symptoms

may appear; for example personality changes such as delusional behavior, obsessive behaviors, anxiety, and cognitive abulia may become evident. Other symptoms that sometimes appear as the disease progresses, include aphasia, apraxia, agnosia and akinesia. A large degree of heterogeneity is seen in the clinical profile of affected individuals. In the latest stage (GDS 7), verbal ability is lost, the patient is incontinent, requires assistance in toileting and feeding and basic psychomotor skills are lost. Patients classified in GDS stages 4-7 exhibit increased mortality rates as compared to aged matched controls.

6.23 Age related biochemical changes in humans

Any attempt to understand the cognitive changes that accompany aging must involve an investigation of observable biochemical events in the central nervous system (CNS) of young and aged subjects. This view has revealed extensive information concerning age related changes in the CNS, and is embodied in the cholinergic hypothesis. The cholinergic hypothesis states that disruptions of the cholinergic system occur in the normal aged and to a larger extent in the demented. It is somewhat surprising, given the breadth of the cholinergic system, that age-related changes are fairly focussed and specific. The largest and most reliable changes are seen in the hippocampus and the cortex. This has been demonstrated by a significant decrease in the levels of choline acetyltransferase (CAT) (Perry, Perry, Blessed and Tomlinson, 1977; Perry, Perry, Gibson, Blessed and Tomlinson, 1977; Reisine, Yamamura, Bird, Spokes and Enna, 1978) and the appearance of senile plaques, neurofibrillary tangles and cortical neuronal

degeneration (Tomlinson, Blessed and Roth, 1968; Tomlinson, Blessed and Roth,1970; Tomlinson and Henderson, 1976) in these regions. Neurofibrillary tangles, senile plaques and neuronal degeneration have been observed in frontal and medial-temporal cortex (Reisine et al., 1978; Perry, Perry, Blessed et al., 1977; Tomlinson et al., 1968, 1970). Decreased CAT levels have been documented in other areas including the caudate nucleus (Reisine et al., 1978; Tomlinson and Henderson, 1976; Scheibel and Scheibel, 1975), amygdala (Tomlinson and Henderson, 1976; Scheibel and Scheibel, 1975) and putamen (Reisne et al., 1978) where the effect of age is usually more variable. In the caudate nucleus some studies have reported significant decreases (Perry, Perry, Gibson et al., 1977; Reisine et al., 1978), while others have not (Perry, Perry, Gibson et al., 1977). Thus it appears that the hippocampus and cortex, both major relays in the cholinergic system, are more vulnerable to age-related degeneration than other structures within the system.

The susceptibility of the hippocampus to degeneration suggests that cognitive functions that rely on the integrity of this structure would be among the first to succumb to the aging process and indeed this is the case. The hippocampus is necessary for normal memory (Scoville, 1954; Scoville and Milner, 1957; Olton et al., 1980; Olton and Papas, 1979; O'Keefe and Nadel, 1979; Zola-Moran, Squire and Amaral, 1986) and memory processes are the first cognitive functions to become impaired with increasing age (Dean et al., 1981; Reisberg et al.,1982 &1985). The degree of cognitive loss in elderly patients has been positively correlated with the degree of neuronal degeneration (Perry, Tomlinson, Blessed, Bergmann, Gibson and Perry, 1978). Structural changes seen in normal aging are also seen in the brains of

demented patients, and the difference appears to be largely quantitative. CAT levels are significantly decreased, and a significantly larger number of senile plaques are present in demented subjects relative to age matched controls (Perry, Perry, Gibson et al., 1977; Tomlinson et al., 1970). Neurofibrillary tangles have been reported in both nondemented and demented aged in the hippocampus with a significantly higher concentration of tangles in the brains of demented patients. In the cortex Tomlinson et al. (1970) found that tangles were only widespread in the demented patients; generalized neurofibrillary change was not observed in control brains. Cortical neuronal degeneration was also more extensive in demented individuals.

6.24 Similarity between aging and cholinergic blockade in humans

Drachman and Leavitt (1974) were among the first to elucidate the similarities between the memory and cognitive deficits produced by normal aging and those induced by pharmacological blockade of the central cholinergic system. In their studies, they showed that in young, healthy subjects the anticholinergic scopolamine produced short-term memory deficits as measured by ordered recall of digits and free recall of word lists, while sparing immediate recall. Mild long-term memory impairments were observed which could largely be attributed to an attentional deficit. These data replicated and extended findings on scopolamine induced short-term memory deficits (Safer and Allen, 1971). The nonmemory cognitive functions impaired following scopolamine administration included the WAIS performance IQ (Wechsler, 1955) and Drachman's organicity index (1974;

1981), a calculated measure relating performance and verbal IQ to the norm as defined by normal, young (21 years old) healthy individuals. The verbal IQ was unaffected by scopolamine. This pattern of deficits paralleled the deficits seen in normal aged subjects who exhibit impairments in short-term memory, performance IQ and the organicity index with mild attentional deficits resulting in lower long-term memory scores (Drachman, 1977, 1981; Drachman and Hughes, 1971).

6.25 Animal studies of memory and aging

The continuous nature of the GDS highlights the view that normal aging and dementia may represent a continuum and differ quantitatively. This view is supported by the biochemical data discussed above, which also demonstrate a quantitative difference between these two populations, and the positive correlation between the degree of cognitive deficit and the degree of neuronal degeneration (Perry et al., 1978). Thus it appears that biochemical and psychological changes that occur with aging can be represented along two interrelated continua. Numerous animal studies have been conducted in an attempt to further understand the relationship between these two classes of processes, and to develop pharmacological therapies to alleviate the clinical symptoms.

The demonstration of a possible link between cholinergic blockade and geriatric memory dysfunciton has led to extensive research that has attempted to 1) characterize the cholinergic system, with respect to its biochemical, pharmacological and behavioral parameters 2) determine how these parameters change in normal and pathological aging and 3) explore

pharmacological interventions that might ameliorate the clinical manisfestations of these conditions.

The efforts that have been made to understand the interrelationship among the cholinergic system and biochemical and cognitive changes that occur with normal and pathological aging have been complicated by the difficulty of finding suitable animal models for dementia. Since man is the only species known to exhibit SDAT (Rupniak and Iversen, 1987), a naturally occurring, closely related animal model is therefore unavailable. It is possible, however, to study memory processes and the changes that occur as a consequence of age and correlate these with biochemical and physiological changes. In addition, it is possible to produce lesions to specific cholinergic pathways (Olton, 1983) and/or pharmacologically interfere with cholinergic transmission (Bartus et al., 1982) and produce behavioral effects in animals commensurate to what one would expect in an animal model of senile dementia. By using such a broad approach it should be possible to use animal data to make inferences about human pathology.

Human studies, such as those done by Drachman and Leavitt (1974), have indicated that short term memory is preferentially impaired with aging, while immediate memory and long term memory are spared. Animal studies, using rats, have replicated and extended these findings. In learning situations that require long term reference memory young and aged rats learn and retain the task equally well (Lowy, Ingram, Olton, Waller, Reynolds and London, 1985; Goodrick, 1968). Short term memory tasks, or working memory tasks, differentiate these two populations (Ingram, London and Goodrick, 1981). Two variables that highlight the decreased capacity of the aged short term memory are the length of time information must be held in

short term memory and degree of difficulty, as defined by the number of choice points in a maze.

When immediate memory is tested significant differences between young and aged rats are not evident. Over time, however, the difference between the two groups becomes apparent (Lippa, Pelham, Beer, Critchett, Dean and Bartus, 1980). This can be seen as an increased rate of forgetting in the aged animals in a passive avoidance paradigm. Retention of the simple passive avoidance task is one of the behaviors that shows the greatest decrement as a function of aging (Dean et al., 1981) No difference between young (approximately 7-9 months old) and old (approximately 22-26 months old) rats is observable when retention is tested one hour after training and the old rats are only moderately impaired four hours after training (Bartus, Flicker and Dean, 1983; Lippa et al., 1980). However, old rats demonstrate little retention of learning 24 hours after training, whereas young rats show excellent retention of the training session (Lippa et al., 1980; Strong, Hicks, Hsu, Bartus and Enna, 1980; Brizee and Ordy,1979-maximum retention time tested was 6h). The performance of middle aged rats (approximately 15 months old), which generally has a higher degree of variance, has a mean falling between those of the other two groups (Lippa et al., 1980; Strong et al., 1980).

The differentiation of young and old animals by the difficulty of the task and the demand placed on short term memory is well illustrated in a maze learning task. Aged rats do not differ significantly from young rats in their ability to learn mazes containing up to four choice points (Ingram et al., 1981; Goodrick, 1972). However, when the memory load is increased by using mazes containing eight, twelve or fourteen choice points, performance in the

aged group is significantly impaired (Ingram et al., 1981). This impairment is evident whether the learning is food or shock motivated, and has been observed in both sexes of several strains and species of laboratory rodents (for review see Ingram, 1985). Radial arm maze performance shows a similar pattern of age related impairment. Throughout training old rats continue to make many errors, showing little improvement across trials in a task that is usually mastered by young adult rats in relatively few trials (Wallace, Krauter and Campbell, 1980; Ingram et al., 1981). It has been suggested that success in the radial arm task depends on short term memory (Olton et al., 1980).

The above discussion clearly indicates that a rodent model can serve as a useful tool in the study of age related changes in behavior and cholinergic function. Another model that has been utilized is the nonhuman primate model. The phylogenetic proximity of monkeys to man makes cross species comparisons more scientifically valid. Indeed, behavioral parameters that seem to be affected by aging in humans can easily be studied in monkeys. Learning, memory, reaction time and attention are examples of these. The memory deficits that have been well documented in humans and rodents (see above discussion for refs) also occur in primates. This has been demonstrated reliably using an automated delayed response task in an Automated General Experimental Device (AGED); an aparatus specifically designed to test several behavioral parameters in a well controlled stimulus environment (Bartus, 1979).

Aged monkeys (older than eighteen years) demonstrate short term memory deficits, with other memory functions remaining intact (Bartus et al., 1983). This pattern is similar to the age related memory impairments

described above in humans. When tested in a delayed response task in which there is little or no demand on short term memory, no significant difference is found between young (5-7 years) and aged monkeys. When continual information is available or with 0 second delays aged monkeys do not differ significantly from young monkeys (Bartus, Fleming and Johnson, 1978). As the delay between stimulus presentation and opportunity to respond is increased, aged monkeys are increasingly impaired, relative to young monkeys, in their ability to remember the stimulus location. This has been demonstrated for intervals of 2, 5, 10, 15 and 20 seconds. (Bartus, Dean and Beer, 1980; Bartus et al., 1978). As would be expected, the performance of middle aged monkeys (10-15 years old), falls between that of the young and aged monkeys at each of the delay intervals tested.

Other similarities between human and monkey data are noteworthy. For example, the attentional deficit that Drachman and Leavitt (1974) observed in their elderly subjects has also been demonstrated in monkeys. Tasks requiring the attentional inhibition of irrelevant stimuli differentiated young and aged monkeys (Bartus and Dean,1979), while young and aged monkeys were not statistically different in their ability to form stimulus-reinforcement associations. Both groups were able to form and retain the associations for weeks indicating no age related impairment of reference, or long term, memory (Bartus et al., 1979).

6.26 Animal studies: biochemical changes that occur with aging

The continuous decline in performance with increasing age observed in animals is parallelled by physiological and biochemical changes in the

hippocampal cholinergic system. As the animal ages, there is a decrease in the ability of acetylcholine (ACh) to maximally excite hippocampal pyramidal cells. (Lippa et al., 1980; Lippa, Loullis, Rotrosen, Cordasco, Critchett and Joseph,1985; Segal, 1982; Haigler, Cahill, Crager and Charles, 1985). Other age related changes in electrophysiology that could contribute to the decreased ACh response include a prolonged afterhypepolarization and a reduced post-tetanic potentiation in hippocampal slices (Landfield and Pitler, 1984). These physiological changes may reflect age related changes in underlying biochemical parameters. An age related decrease in muscarinic receptor density has been reported in the hippocampus (Lippa et al., 1980; Lippa, Critchett, Ehlert, Yamamura, Enna and Bartus, 1981; Bartus et al., 1982; Gurwitz, Egozi, Henis, Kloog and Sokolovsky, 1987) the cortex (Strong et al., 1980; Gurwitz et al., 1987) the striatum (Strong, Rehwaldt and Wood, 1986; Strong et al., 1980; Gurwitz et al., 1987; Morin and Westerlain, 1980), cerebellum (Morin and Westerlain, 1980) and the olfactory bulb (Gurwitz et al., 1987). This decrease in muscarinic receptor density is, however, a controversial finding with several studies reporting no change in some regions (Morin and Westerlain, 1980) or slight increases (Gurwitz et al., 1987; Springer, Tayrien and Loy, 1986; for review see Bartus et al., 1982).

In the cases where a decrease in receptor density has been reported, the magnitude of the decrease (approximately 20%) would probably not be sufficient to account for the striking cognitive changes. It has been suggested that a conformational change in the receptor protein, rather than a change in receptor number, could contribute to the observed age related behavioral deficits (Lippa et al, 1985). Two additional measures that have been correlated with age related behavioral deficits are a significant loss of

neurons and an increase in the lipofuscin in the CA1 field of the hippocampusand in visual area 17 (Brizee and Ordy,1979).

The physiological changes observed within the cholinergic system could be explained by a decrease in ACh synthesis. The rate limiting factor in ACh synthesis is the availability of the precursor choline. High affinity choline uptake has been reported to decrease with age in the striatum (Strong et al.,1986). The availability of another component of ACh synthesis, CAT has also been studied across age groups. Strong et al. (1980;1986) reported decreased CAT levels in the cerebral cortex and corpus striatum. Springer et al (1986) reported a 24-38% decrease in CAT and acetylcholinesterase (AChE) activity in the hippocampus of 40 month old rats as compared to 12 month old rats. A decrease of similar magnitude was observed in the septum and the nucleus basilis (Springer et al., 1986), two nuclei which send major cholinergic fiber pathways to the hippocampus and cortex respectively. Again this is not a universal finding, Ingram et al. (1981), reported no significant difference between groups of young and old rats when comparing CAT levels in the cortex or hippocampus. This measurement replicated the findings of Lippa and his colleagues (1980) who also found no change in CAT levels in the hippocampus as a consequence of age.

Although the magnitude of any single reported cholinergic deficit would be an unlikely explanation for the observed cognitive deficits, when summated these changes represent widespread alterations within the cholinergic system and they may be a factor in memory dysfunction.

6.27 Animal studies: cholinergic blockade

It is clear from the above discussion of preclinical studies that animal models can reliably demonstrate age related behavioral changes, and that these changes are correlated with physiological and biochemical changes in the cholinergic system. Blockade of the central cholinergic system produces a constellation of cognitive deficits that resembles that seen in aged conspecifics (see discussion below for references); short term memory is impaired, some attentional deficit can be present and immediate and long term memory are unaffected. These observations support the cholinergic hypothesis and provide models with which the effect of cholinergic supplementation and blockade on behavior can be studied.

In a series of studies, Bartus and his colleagues (Bartus, 1978; Bartus, 1979; Bartus, Dean and Beer, 1980; Bartus, Dean Sherman, Friedman and Beer, 1981; Bartus and Johnson, 1976) have looked at the effect of manipulating the central cholinergic system on behavior in nonhuman primates. All the studies were conducted in the AGED apparatus using a delayed matching to sample task, thereby increasing the validity of cross study comparisons. When young monkeys were administered scopolamine or atropine, muscarinic acetylcholine receptor (mAChR) antagonists, performance on this task was impaired in a dose response fashion. Furthermore, as the retention interval was increased, performance was increasingly impaired (Bartus and Johnson,1976; Bartus1978). This pattern of results parallels that seen in aged monkeys; with both increasing age and increasing retention interval, performance is impaired in a dose response fashion (Bartus et al.,1980; Bartus et al., 1978). The similarity between the effects of age and scopolamine on short term memory further supports the cholinergic hypothesis and suggests that, while other neurotransmitter

systems are probably involved, the cholinergic system does play a necessary and specific role in short term memory processes. Further, it appears that age related cholinergic dysfunciton may contribute to age related memory dysfunction.

6.28 Pharmacological intervention in animals and humans

The results from behavioral and pharmacological studies in primates indicate that they can serve as a possible predictor for pharmacological intervention in humans. The age related memory dysfunction exhibited by both humans and monkeys can be produced in young conspecifics by administering cholinergic antagonists (Drachman and Leavitt, 1974; Drachman, 1977; Safer and Allen, 1971; Bartus and Johnson, 1976; Bartus 1978). In an attempt to develop possible pharmacological intervention strategies, the efficacy of various cholinergic agents at reversing, or preventing, either scopolamine induced memory dysfunction or age related memory dysfunction have been evaluated. Strategies frequently used in this effort include: 1) the prevention of memory dysfunction by the concurrent or subsequent administration of an acetylcholinesterase inhibitor, 2) the administration of acetylcholine agonists. 3) precursor loading with choline or lecithin (see section 1.40 below for a review of the biosynthesis and metabolism of ACh).

Physostigmine, an acetylcholinesterase inhibitor, has been reported to have some positive effects on scopolamine induced memory dysfunction. Beneficial effects occur under controlled conditions over a narrow dose range, with a large degree of variance between subjects. In monkeys

physostigmine significantly reduced the severity of the scopolamine induced short term memory deficit by approximately 50% (Bartus 1978). A similar improvement has been noted in humans (Drachman, 1981, 1977). The possibility that the improvement could be due only to an overall increase in arousal has been ruled out by control studies that fail to show improvement following d-amphetamine (Drachman 1977) , methylphenidate (Bartus, 1978) or piracetam (Bartus et al., 1981) administration. Arecoline, a mAChR agonist, has also been reported to improve memory under certain experimental conditions (Sitaram, Weingartner and Gillin, 1978). The advantage of this compound is that the effective dose range between subjects and the magnitude of the beneficial effects across subjects are generally less variant.

Precursor loading using choline or lecithin, a dietary source of choline, has proven to be an ineffective way of improving memory function in aged rats (Bartus et al, 1981), aged monkeys (Bartus, 1980), and aged humans (Mohs, Davis, Tinklenberg and Hollister, 1980; Thal et al., 1981; Drachman, 1981; for review of clinical studies see Bartus et al., 1982). The negative result in aged subjects and demented humans could perhaps be explained by 1) an inability of the aged brain to synthesize ACh, 2) an inability of the aged brain to use the additional ACh due to some other deficit further down the response cascade or 3) the inability of the remaining functional neurons to fully compensate for lost neurons in the affected brain regions.

Suggestive results have been reported with choline in combination with piracetam (Bartus et al., 1981). Choline and piracetam (a drug that reportedly increases the ability of the central nervous system to function more

effectively under hypoxic conditions by potentiating the conversion of ADP to ATP (see Bartus et al., 1981 for refs)) were administered alone and in combination and the effect on one trial passive avoidance in aged rats was determined. Neither compound had a significant effect on retention in aged subjects. When administered in combination, however, retention of the passive avoidance task was significantly improved. Chronic administration (1 week) was superior to acute administration. Following behavioral assessment, brain regions were assayed for choline and ACh contents. Interestingly, choline treatment resulted in significant increases in choline levels in the cortex and striatum, with a nonsignificant increase in the hippocampus. Piracetam treatment significantly increased choline levels in the hippocampus with no effect in the cortex or striatum. The behavioral effect observed in the piracetam plus choline condition could indicate that moderation of the cortex and striatum or the hippocampus is necessary but not sufficient to modify behavior. The hippocampus and the cortex may both have to be altered to exert an effect. While choline levels were significantly altered by the treatment, ACh levels changed little. In fact ACh levels in the hippocampus were reduced slightly (19%). These results suggest that aged brains may not be able to synthesize additional ACh even when choline levels are increased. The slight decrease in ACh levels in the hippocampus could be due to variation across subjects, or perhaps the piracetam potentiated the release of ACh from the hippocampus and thereby reduced the overall levels slightly.

6.29 Implications of the Cholinergic Hypothesis

The three methods of supplementing the cholinergic system discussed above (precursor loading, acetylcholinesterase inhibition, and receptor stimulation), have not been as effective at improving age related memory dysfunction as investigators had hoped they would be. It is interesting to note that the closer the drug's target site was to the receptor, the greater the effect of the treatment on memory was. Choline treatment, which attempts to modify neurotransmitter production presynaptically, was less effective than physostigmine, which prevents neurotransmitter degradation in the synaptic cleft. Both of these treatments were less effective than mAChR agonists which target the receptor directly, thereby affecting postsynaptic events.

The disappointing results obtained with cholinergic treatments do not refute the cholinergic hypothesis. The data presented here clearly implicate the central cholinergic system in memory and aging processes. It is possible that pharmacological supplementation of the cholinergic system alone may not be sufficient compensation for the diffuse changes that occur with aging. This idea is supported by the fact that compounds like piracetam, which do not affect the cholinergic system specifically, do have synergistic effects when administered with choline. It may be important to target pharmacosystems in combination to achieve significant clinical improvement in the aged.

A second factor to consider is the kinetic properties of the cholinergic compounds that have been tested. Physostigmine, arecoline and other compounds are easily and quickly degraded; the very short half life (several minutes long) may indicate that potential benefits cannot be realized during the time the drug is active. The beneficial effects that have been noted, in humans and animals, following cholinomimetic therapy have occured over a

small and variable range of drug doses. Beyond this range, the high incidence of adverse side effects also makes widespread administration of cholinergic drugs difficult. Perhaps, when cholinergic drugs with higher specificity are available that are degraded more slowly empirical results may be more favorable.

In the above discussion, evidence has been presented that indicates a parallel appearance of age related memory dysfunction and cholinergic dysfunction. However, it is not clear that the cholinergic system is exclusively involved in this disorder. Memory deficits can be observed following manipulation of the cholinergic system, but the reverse case is not as easily demonstrated. The improvement in memory functions that follow manipulation of the cholinergic system are subtle, and occur over small dose ranges under tightly controlled empirical conditions. The weak point in the cholinergic hypothesis appears to be the fact that supplementing the cholinergic system does not result in the predicted amelioration of clinical symptoms. As mentioned above, however, this could be due to the pharmacokinetic properties of the presently available compounds. Another possibility is that the modest effects of cholinomimetics on age related memory disorders may be due to the etiology of the underlying deficit. Supplementation of the cholinergic system may not be able to compensate for the degeneration that has already taken place. Another alternative is that it may be necessary to develop combination therapies before significant clinical improvement is reliably observed. In summary, research that has been conducted to test the cholinergic hypothesis has revealed a wealth of information concerning the role of the cholinergic system in different memory processes, the effects of age on memory and the cholinergic system, and

possible methods for the treatment of age related memory dysfunction. Although treatment of age related memory disorders is not yet available, empirical data does suggest that further research is warranted, and the development of effective pharmacotherapies is possible.

The above discussion has established a clear connection between the cholinergic system and memory function and how these two change with aging. A better understanding of the cholinergic system in unimpaired adult subjects is necessary if the hope of developing effective therapeutics is to be realized in the future. The remainder of the discussion and the subsequent experiments will therefore strive to present and extend currently available data concerning the characterization of the muscarinic cholinergic system and the microenvironment within which it resides. The mAChR was chosen because it has been implicated in age related memory disorders. First, the topography of the cholinergic system in the CNS and the biosynthesis of ACh will be briefly reviewed, followed by a characterization of the mAChR and its microenvironment.

6.30 The anatomy of the cholinergic system

The central cholinergic system is comprised of several pathways that innervate cortical, midbrain and brainstem regions (Shute and Lewis, 1967). The ascending cholinergic reticular system contains two pathways, the dorsal tegmental pathway and the ventral tegmental pathway. The former pathway originates in the nucleus cuneiformis and terminates in the tectum, metathalamus and thalamus, supplying cholinergic fibers to the superior and inferior colliculi, dorsal and deep pretectal nuclei, the medial and lateral

geniculate bodies and several thalamic nuclei. Two groups of fibers leave the dorsal tegmental pathway. These tracts are the ventral supra-optic decussation and the medial strial bundle. Cholinergic fibers travelling via the ventral supra-optic decussation supply the superior colliculus and the pretectal nuclei. Additional fibers via the medial strial bundle terminate on the lateral geniculate body (Shute and Lewis, 1967).

The ventral tegmental pathway runs from its origin in the ventral tegmental area and the substantia nigra to the subthalamus, hypothalamus and basal forebrain areas. Cholinergic fibers terminate in the oculomotor nucleus, the mammillary bodies, the subthalamic nucleus, the entopeduncular nucleus and the globus pallidus. Additional fibers terminate in the posterior and lateral hypothalamic areas, the lateral preoptic area, the paraventricular and supraoptic hypothalamic nuclei and the olfactory tubercle. Additional cholinergic fibers arising from these nuclei are corticopetal fibers. The neocortex is supplied by fibers originating in the globus pallidus and the preoptic area. The olfactory bulb and cortex are supplied by the olfactory tubercle. These fibers also supply subcortical structures such as the caudate-putamen, nucleus accumbens, amygdaloid nuclei and the nucleus of lateral olfactory tract. Fibers leaving the globus pallidus join the internal capsule, enter the external capsule and are distributed to the lateral cortex above the rhinal fissure. Fibers that terminate in the inferolateral cortex, below the rhinal fissure, originate in the lateral preoptic and anterior amygdaloid areas. These fibers also supply the amygdaloid nuclei. A group of fibers from the dorsal end of the lateral preoptic area give rise to the lateral strial bundle. The lateral strial bundle forms part of the bed nucleus of the stria terminalis, and may represent a

feedback system for the efferent projection from the olfactory portion of the amygdala (Shute and Lewis, 1967). The latero-ventral aspect of the hemisphere below the rhinal fissure (olfactory cortex) is supplied by fibers originating in the lateral preoptic area that run rostrally in the olfactory tract and peduncle. Additional fibers from the lateral preoptic area supply the cortex on the superior aspect of the hemisphere. These fibers ascend anterior to the genu of the corpus collosum and run caudally in the cingulum. There are also connections, via the lateral preoptic area with the diagonal band and the septum (Shute and Lewis, 1967).

Fibers arising from the medial septal nucleus and the nucleus of the diagonal band innervate the hippocampus proper, dentate gyrus and the subiculum via the medial supracollosal stria of Lancisi, the dorsal fornix , the alveus and the fimbria. A more recently defined cholinergic pathway runs from its origin in the nucleus basilis of Meynert, which is located beneath the globus pallidus, to frontal and parietal cortex (Angevine and Cotman, 1981). This pathway accounts for approximately half of the acetylcholine found in these regions. (Angevine and Cotman, 1981).

Histochemical staining techniques show that acetylcholinesterase (AchE), a marker for cholinergic neurons, is aggregated in the base of the hippocampal pyramidal cell layer, and is heavier in the CA3 field than elsewhere. AChE is also present in the mossy fiber region, in association with the bases of the apical pyramidal dendrites, suggesting that cholinergic fibers are found throughout the hippocampus with the highest concentration in the pyramidal layer of CA3 field. Several midbrain and forebrain nuclei form a cholinergic network in connection with the hippocampus. These pathways project to the medial and frontal cortex and connect with the

ascending cholinergic reticular system. Clearly the cholinergic system is a diffuse network of pathways contacting numerous structures in several brain regions.

6.40 Biosynthesis, metabolism and release of ACh

ACh is synthesized in the cytoplasm of cholinergic terminals and stored in synaptic vesicles. Two precursors are involved in the synthesis of ACh, choline and acetyl-coenzyme A (acetyl- CoA). The transfer of the acetyl group to the choline is catalyzed by the enzyme choline acetyl transferase (ChAc). Acetyl-CoA is formed in all cells during the metabolism of fats and carbohydrates. Choline cannot be synthesized endogenously. It is obtained through the diet and transported to neurons in the bloodstream. Choline is transported into cholinergic neurons by a high affinity choline uptake mechanism. The choline uptake process is voltage dependent and depends on the presence of sodium (Na). The result is that when the cell is depolarized and ACh is released, vesicular stores are depleted and the high affinity choline uptake mechanism is activated (Iversen and Iversen, 1981; Kandel and Schwartz, 1985).

The ACh stored in synaptic vesicles is released into the synaptic cleft when the neuron is stimulated. This process is calcium dependent. When the cell is depolarized there is an influx of calcium, this is thought to facilitate the fusion of the vesicle to the presynaptic membrane. Following fusion of the vesicles, which contain a fixed number of ACh molecules (one quantum, or several thousand molecules), the entire contents of the vesicle is released into the cleft by an exocytotic process. Decreasing the calcium

concentration decreases the probability that a given quantum will be released. It has not been determined whether calcium acts directly or indirectly, by acitvating a calcium binding protein like calmodulin, to promote fusion. Following release from the vesicles into the synaptic cleft, ACh is degraded by the enzyme acetylcholinesterase (AChE), which can be found in both the presynaptic and postsynaptic membrane. AChE hydrolyzes ACh to acetate and choline (Kandel and Schwartz, 1985).

Table 1

Temperature-Dependent Changes in ^{3}H-QNB Binding†

Temperature (°C)	K_D (pM)	B_{max} (pmol/mg protein)
37	15.6 ± 0.9	0.35 ± 0.03
25	16.3 ± 0.6	0.34 ± 0.01
15	23.4 ± 0.2	0.46 ± 0.03 a£,b£,d¥
4	44.5 ± 0.1 a+,b+,c+	0.32 ± 0.02

† All data reported as mean ± standard error of the mean
a= Significantly different from 37°C, t-test
b= Significantly different from 25°C, t-test
c= Significantly different from 15°C, t-test
£= Probability level $p < .05$
+= Probability level $p < .01$
¥= Probability level $p < .005$

Table 2

Temperature-Dependent Changes in the Affinity of Atropine†

Temperature (°C)	K_i (pM)	Best Fit*
37	916.3 ± 63.1	1 site (3/3)**
25	486.9 ± 24.3[a¥]	1 Site (2/3)
15	271.3 ± 43.737[a¢,b£]	1 Site (3/4)
4	405.1 ± 93.5[a¢]	1 Site (3/4)

† All data reported as mean ± standard error of the mean
* Determined by computer-assisted iterative nonlinear analysis
** The numerator represents the number of experiments that best described by the model indicated (1 site or 2 sites). The denominator represents the total number of experiments performed.
a= Significantly different from 37°C, t-test
b= Significantly different from 25°C, t-test
£= Probability level $p < .05$
¥= Probability level $p < .005$
¢= Probability level $p < .001$

Table 3

Temperature-Dependent Changes in the Affinity of Oxotremorine†

Temperature (°C)	K_i (nM)	Best Fit*
37	717.7 ± 61.9	1 site (2/3)**
25	317.4 ± 31.0[a¢]	1 Site (2/3)
15	164.5 ± 19.3[a¢,b+]	1 Site (3/4)
4	196.5 ± 22.9[a¢b£]	1 Site (3/4)

† All data reported as mean ± standard error of the mean
* Determined by computer-assisted iterative nonlinear analysis
** The numerator represents the number of experiments that best described by the model indicated (1 site or 2 sites). The denominator represents the total number of experiments performed.
a= Significantly different from 37°C, t-test
b= Significantly different from 25°C, t-test
£= Probability level $p < .05$
+= Probability level $p < .01$
¢= Probability level $p < .001$

Table 4

Temperature-Dependent Changes in the Affinity of Carbachol†

Temperature (°C)	K_H (μM)	K_L (μM)	n_H	Best Fit*	$\%B_L$*
37	4.8 ± 1.8	151.9 ± 17.6	0.58 ± 0.04	2 (5/5)**	62.3
25	2.6 ± 0.6	180.5 ± 72.4	0.60 ± 0.02	2 (3/3)	44.3
15	0.6 ± 0.2	44.4 ± 4.4a£b£	0.52 ± 0.05	2 (4/4)	49.7
4	4.9 ± 2.1	32.4 ± 5.3a£b£	0.58 ± 0.06	2 (2/4)	24.2***

† All data reported as mean ± standard error of the mean

* Determined by computer-assisted iterative nonlinear analysis

** The numerator represents the number of experiments that were best described by the model indicated (1 site or 2 sites). The denominator represents the total number of experiments performed.

*** In 2 out of 4 experiments carbachol displacement best fit a 1 site model and 0% B_L was detected

a= Significantly different from 37°C, t-test

b= Significantly different from 25°C, t-test

£= Probability level $p < .05$

Table 5

Thermodynamic Parameters* of Antagonist and Agonist Binding

	ΔG (Kcal/mol)	ΔH (Kcal/mol)	TΔS (Kcal/mol)
ANTAGONISTS:			
^{3}H-QNB	-15.4	3.2	18.6
ATROPINE	-12.8	-10.0	2.8
AGONISTS:			
OXOTREMORINE	-8.9	-10.3	-1.5
CARBACHOL (K_H)	-7.6	-17.5	-9.9
CARBACHOL (K_L)	-5.4	-9.5	-4.1

* Thermodynamic parameters calculated from mean K_i values obtained at 37°C.

Table 6

The Effect of AL721 on ^{3}H-QNB Binding in Young Rat Hippocampus†

AL721 (mg/ml)	K_D (pM)	B_{max} (pmol/mg protein)
Control	19.0 ± 2.1	0.67 ± 0.04
0.05	23.7 ± 1.3	0.75 ± 0.06
0.15	33.5 ± 8.8	0.83 ± 0.05
0.5	20.9 ± 3.2	0.85 ± 0.04a£
2.5	17.2 ± 3.3	0.85 ± 0.05a£

† All data reported as mean ± standard error of the mean
a= Significantly different from Control, t-test
£= Probability level $p < .05$

Table 7

The Effect of AL721 on the Affinity of Carbachol in Young Rat Hippocampus†

AL721 (mg/ml)	K_H (μM)	K_L (μM)	n_H	Best Fit*	$\%B_L$*
Control	0.6 ± 0.5	40.6 ± 12.7	0.55 ± 0.06	2 (4/4)**	64.3
0.05	0.2 ± 0.1	30.1 ± 11.6	0.47 ± 0.02	2 (3/3)	66.9
0.15	0.3 ± 0.2	34.4 ± 2.6	0.42 ± 0.04	2 (3/3)	57.6
0.25	0.5 ± 0.2	52.2 ± 24.1	0.46 ± 0.04	2 (3/3)	52.8
0.5	0.6 ± 0.2	77.4 ± 35.1	0.48 ± 0.03	2 (3/4)	57.4
2.5	0.5 ± 0.4	21.5 ± 5.7	0.54 ± 0.07	2 (3/4)	72.2

† All data reported as mean ± standard error of the mean

* Determined by computer-assisted iterative nonlinear analysis

** The numerator represents the number of experiments that were best described by the model indicated (1 site or 2 sites). The denominator represents the total number of experiments performed.

Table 8

The Effect of AL721 on the Affinity of Oxotremorine in Young Rat Hippocampus†

AL721 (mg/ml)	K_H (nM)	K_L (nM)	n_H	Best Fit*	$\%B_L$*
Control		338.3 ± 42.9	0.95 ± 0.04	1 (5/7)**	100.0
0.05	29.1 ± 23.0	410.4 ± 91.3	0.62 ± 0.05[a]	2 (5/6)	70.9
0.15	9.8 ± 5.6	471.9 ± 99.9	0.63 ± 0.06[a]	2 (5/6)	78.7
0.5	17.7 ± 13.4	369.5 ± 64.1	0.73 ± 0.04[a]	2 (4/7)	80.9
2.5	7.2 ± 4.6	368.1 ± 55.5	0.67 ± 0.04[a]	2 (4/6)	79.5

† All data reported as mean ± standard error of the mean

* Determined by computer-assisted iterative nonlinear analysis

** The numerator represents the number of experiments that were best described by the model indicated (1 site or 2 sites). The denominator represents the total number of experiments performed.

a= Significantly different from control, $p<.01$, t-test

Table 9

The Effect of AL721 on ^{3}H-QNB Binding in Old Rat Hippocampus†

AL721 (mg/ml)	K_D (pM)	B_{max} (pmol/mg protein)
Control	17.8 ± 1.5	0.67 ± 0.09
0.05	21.7 ± 1.4	0.70 ± 0.06
0.5	26.5 ± 2.4	0.65 ± 0.08
2.5	34.7 ± 12.3[a]	0.55 ± 0.07

† All data reported as mean ± standard error of the mean
a= Significantly different from Control, $p < .05$, t-test

Table 10

The Effect of AL721 on the Affinity of Oxotremorine in Old Rat Hippocampus†

AL721 (mg/ml)	K_H (nM)	K_L (nM)	n_H	Best Fit*	$\%B_L$*
Control		411.4 ± 87.7	0.83 ± 0.07	1 (4/5)**	100.0
0.05		636.1 ± 123.2	0.90 ± 0.07	1 (3/4)	100.0
0.5	6.5 ± 5.3	386.9 ± 51.9	0.86 ± 0.09	2 (3/5)	81.3
2.5	22.0 ± 12.6	503.8 ± 42.3	0.72 ± 0.11	2 (3/5)	76.3

† All data reported as mean ± standard error of the mean

* Determined by computer-assisted iterative nonlinear analysis

** The numerator represents the number of experiments that were best described by the model indicated (1 site or 2 sites). The denominator represents the total number of experiments performed.

Figure Legend

Figure 1: Time course of ^{3}H-QNB binding to the mAChR receptor as a function of temperature.

Bovine hippocampal homogenates were incubated with approximate K_D concentrations of ^{3}H-QNB for the times indicated.

Figure 1A and 1B Association Rate for (^{3}H)-QNB binding

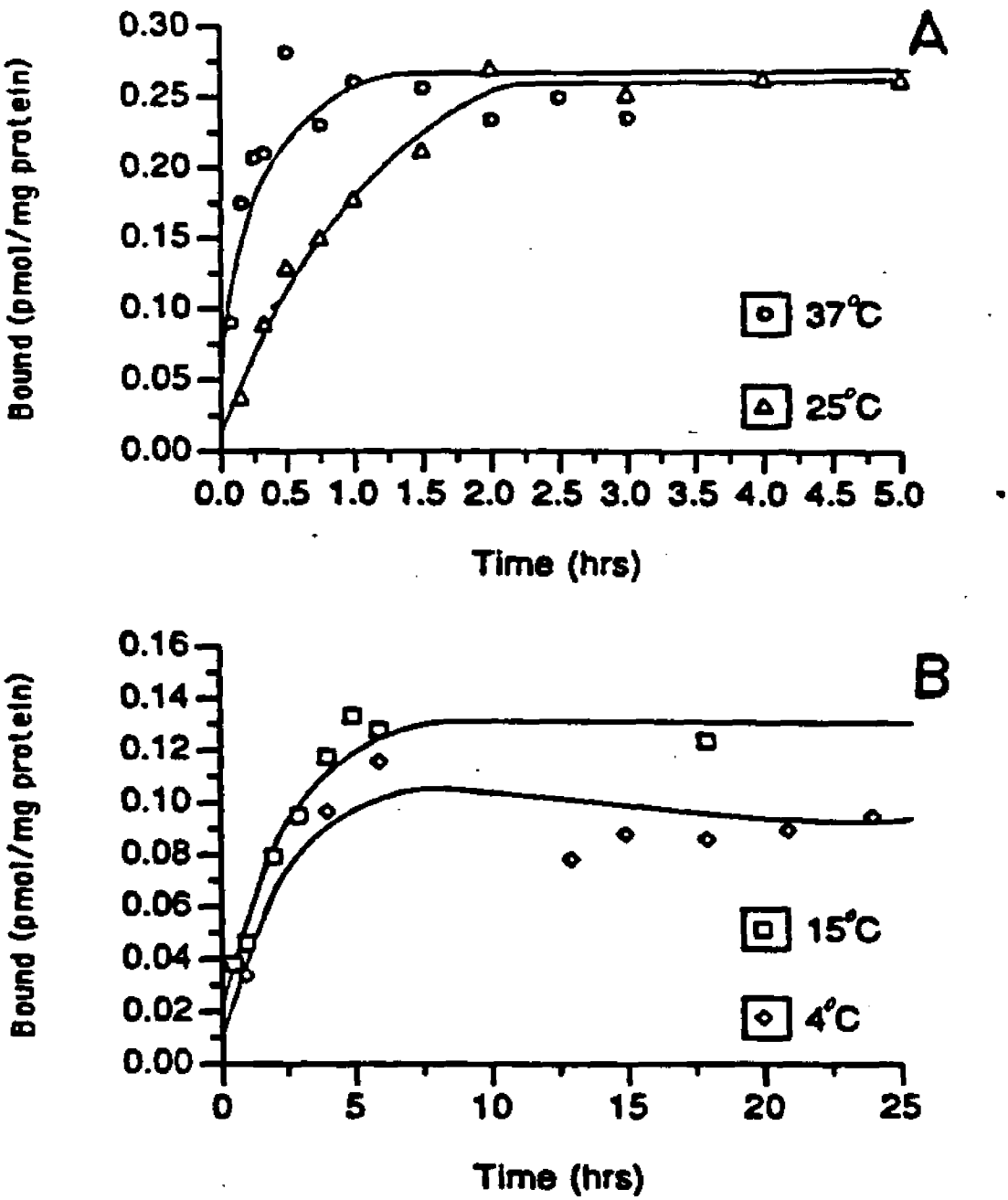


Figure Legend

Figure 2: The effect of temperature on membrane fluidity in young rat hippocampus.

Hippocampal homogenates were incubated with the fluorescent probe DPH (2μM). Microviscosity was determined according to standard procedure. Please see "Methods" for details concerning measurement (section 2.27) and data analysis (section 2.33). Measurements were taken at 37°C, 25°C, 15°C and 4°C.

Figure 2 The Effect of Temperature on Membrane Fluidity in Young Rat Hippocampus

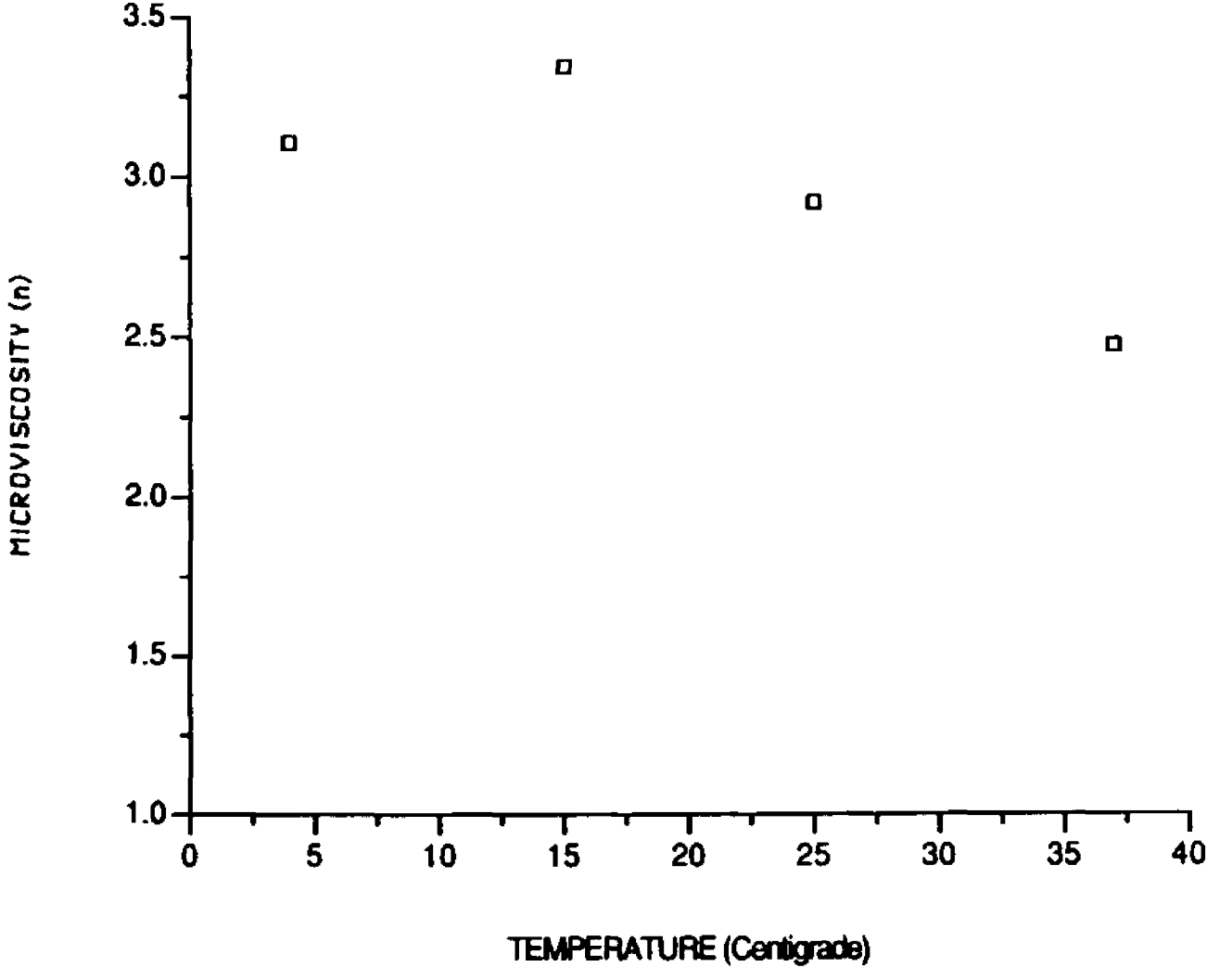


Figure Legend

Figure 3: The effect of AL721 on membrane fluidity in young rat hippocampus.

Young rat hippocampal homogenates were treated with increasing concentrations of AL721. The tissue was then washed and aliquots were incubated with the fluorescent probe DPH (2μM). Microviscosity was determined according to standard procedure. Please see "Methods" for details concerning measurement (section 3.24) and data analysis (section 3.30).

Figure 3 The Effect of AL721 on Membrane Fluidity in Young Rat Hippocampus

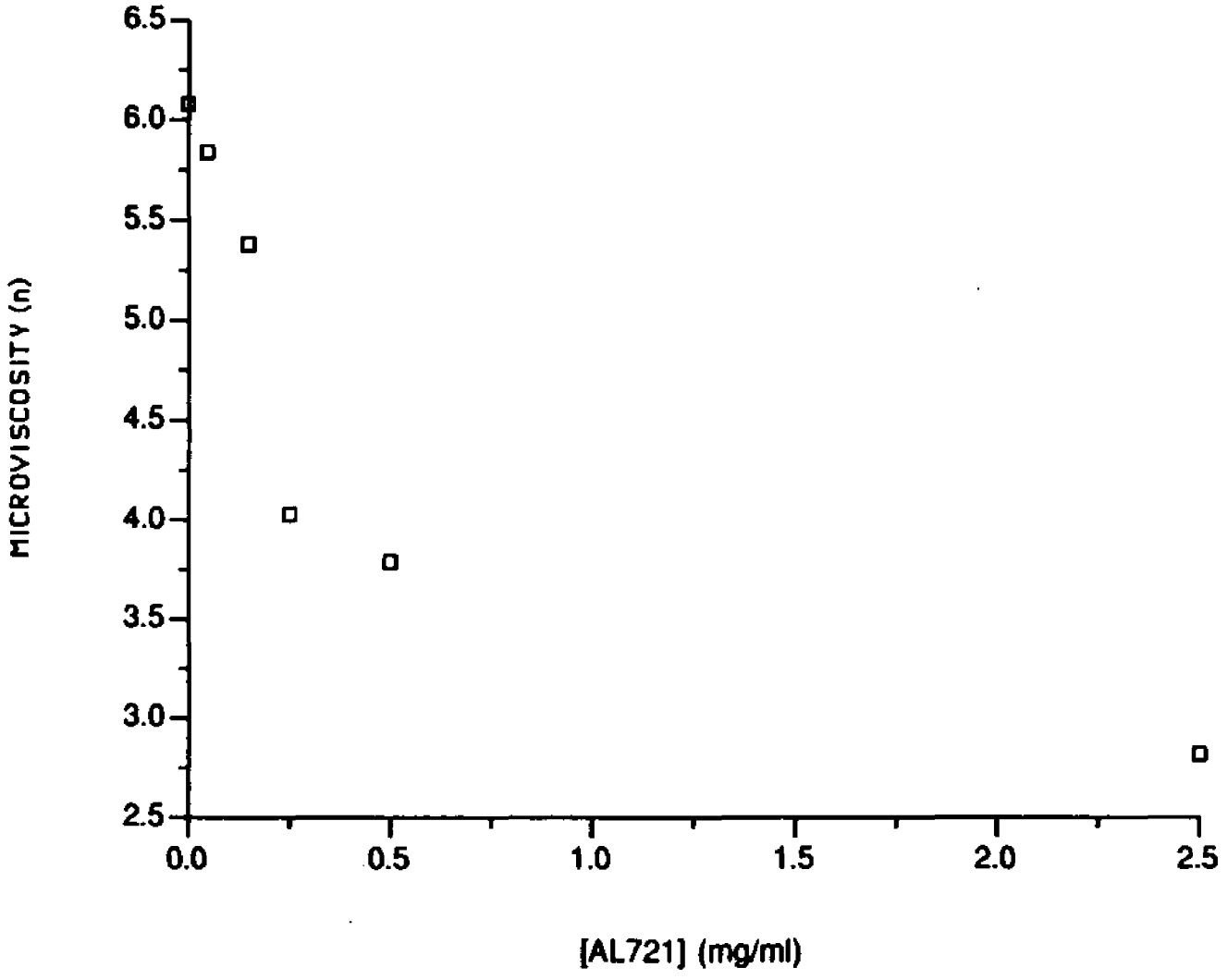


Figure Legend

Figure 4: The effect of AL721 on membrane fluidity in old rat hippocampus.

Old rat hippocampal homogenates were treated with increasing concentrations of AL721. The tissue was then washed and aliquots were incubated with the fluorescent probe DPH (2μM). Microviscosity was determined according to standard procedure. Please see "Methods" for details concerning measurement (section 4.27) and data analysis (section 4.30).

Figure 4 The Effect of AL721 on Membrane Fluidity in Old Rat Hippocampus

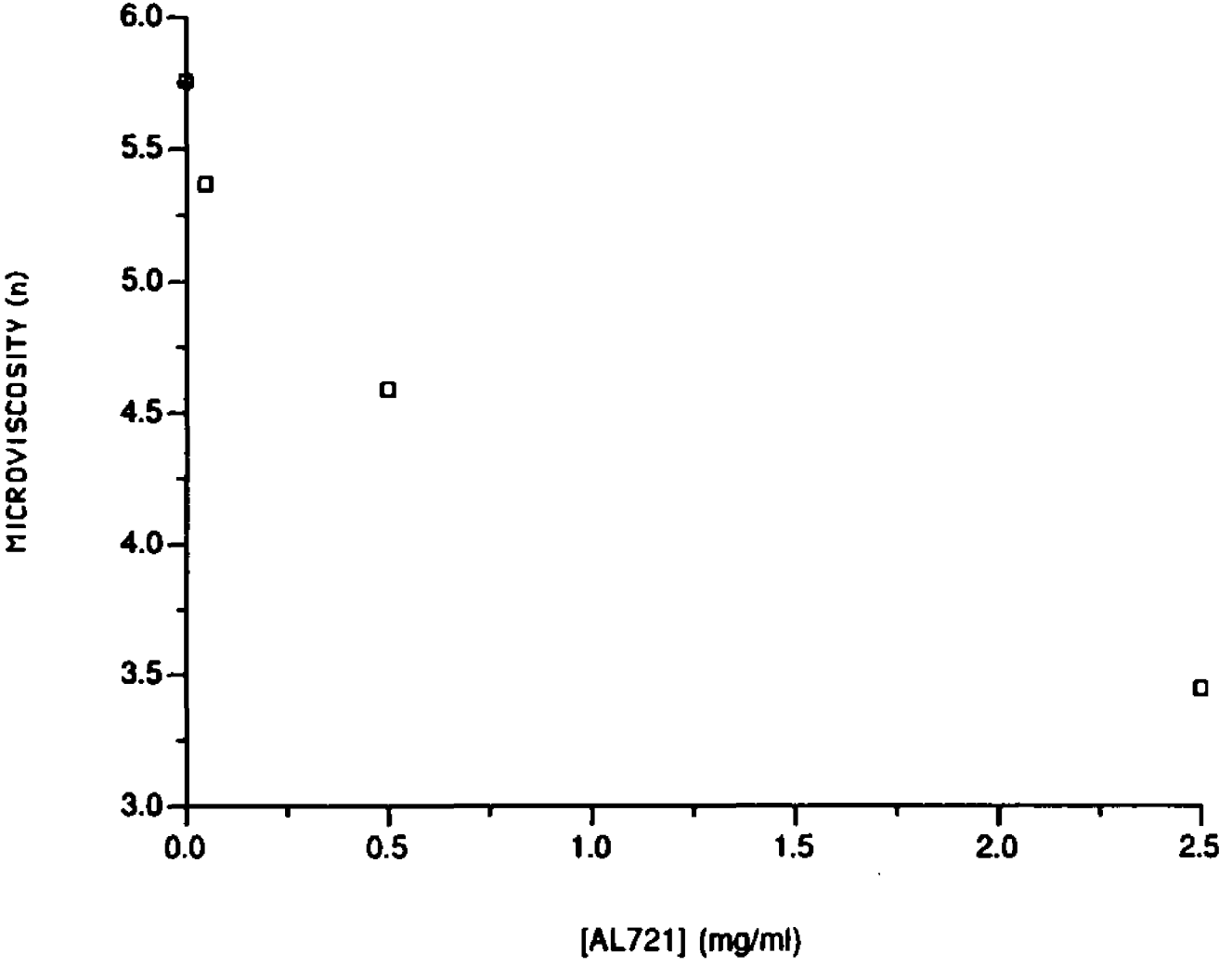
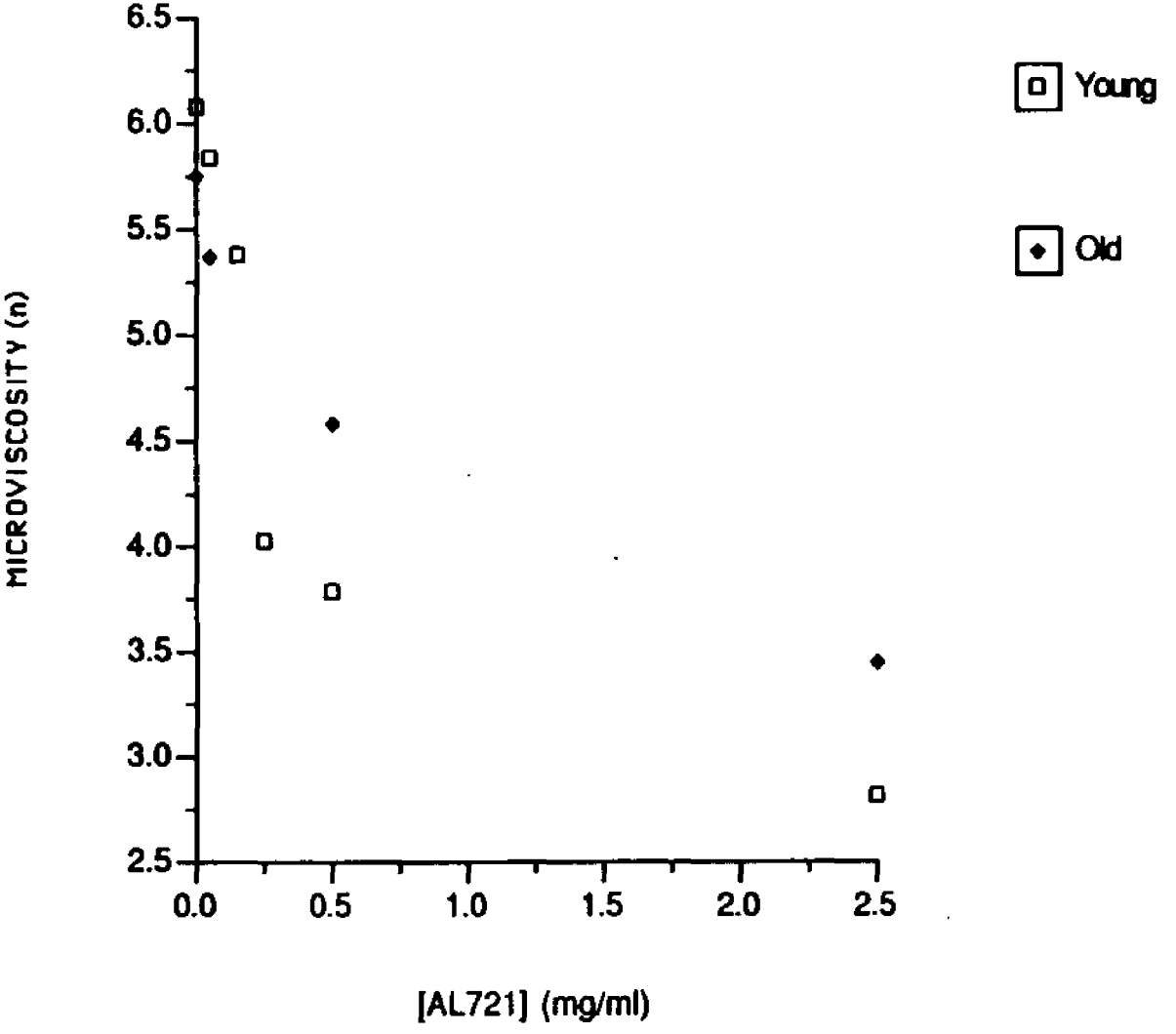


Figure Legend

Figure 5: The effect of AL721 on membrane fluidity in young and old rat hippocampus.

Young and old rat hippocampal homogenates were treated with increasing concentrations of AL721. The tissue was then washed and aliquots were incubated with the fluorescent probe DPH (2μM). Microviscosity was determined according to standard procedure. Please see "Methods" for details concerning measurement (section 3.24 and 4.27) and data analysis (section 4.30 and 3.30).

Figure 5 The Effect of AL721 on Membrane Fluidity in Young and Old Rat Hippocampus



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