

**From the Neurotec Department, Division of Experimental Geriatrics
Karolinska Institutet, Huddinge, Sweden.**

**Muscarinic M₁ and M₄ receptor subtypes in normal and pathological
conditions in the central nervous system: Studies on human and
animal tissues using subtype selective ligands.**

Ezra Mulugeta



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In memory of my parents...

ABSTRACT

Snake venoms from different mamba species contain toxins that bind to muscarinic acetylcholine receptors (mAChR). The toxins MT-1 (selective for M₁ after iodination), M₁ toxin-1 (also known as MT7, selective for M₁) and M₄ toxin-1 (also known as MT3, selective for M₄ receptors) were isolated and used to study M₁ and M₄ receptor subtypes in normal and pathological conditions. The pharmacological profile of M₁ toxin-1 was determined in functional assays using cloned human M₁-M₄ receptors expressed in CHO cells. It was clearly demonstrated that M₁ toxin-1 acts as a selective noncompetitive antagonist of the muscarinic M₁ receptors by binding stably to an allosteric site.

Age-related loss of mAChRs in rat hippocampus and neocortex is still controversial. The effect of ageing on the level of M₁ and M₄ mAChR subtypes was investigated in the hippocampus and entorhinal cortex of young (21 days), adult (3 months) and old (25 months) rats. A significant increase in muscarinic M₁ receptor binding in all areas of the hippocampus and a significant loss in M₄ binding only in the CA1 region and entorhinal cortex have been observed in 25 months compared to 21-day-old rats. The increase of M₁ receptors in (old aged) rats could be due to compensatory processes as a result of the changes in M₄ receptors. The decrease in M₄ receptors in the entorhinal cortex and in the CA1 area of the hippocampus of (old aged) rats could be one of the factors leading to impaired cognitive function.

Administration of mAChR agonists or acetylcholinesterase inhibitors produces effective pain relief. However, the mAChR subtype(s) involved in the spinal cord are not fully defined. The levels of M₁ and M₄ receptor subtypes in spinal cord of acute and chronic arthritic rats were investigated. No M₁-toxin 1 binding was observed, indicating an absence of M₁ receptors. However, the binding of M₄-toxin 1 was reduced between 87-90 % in the Rexed laminae I to X of the spinal cord both in acute and chronic pain as compared to controls. These findings suggest that the mAChR M₄ subtype may have a role in cholinergic mechanisms of analgesia. Alzheimer's disease (AD) related loss of mAChR subtype has been controversial. Muscarinic M₁, M₂ and M₄ receptor subtype changes in the hippocampus of AD and control brains were evaluated. A significant decrease in M₄ receptors was observed in the dentate gyrus and CA4 regions of brain sections from AD patients compared to controls. These findings suggest that, relative to other mAChR subtype, the M₄ receptor could be the subtype that is selectively compromised in AD.

Long-term adrenalectomy (ADX) has been reported to cause significant loss of cells in the dentate gyrus and CA1-CA4 fields of the hippocampus resulting in impairment of cognitive functions. The effect of ADX on muscarinic M₁ and M₄ receptors levels in the hippocampus and entorhinal cortex of 3, 14, 30, 90 and 150 days adrenalectomized male Wistar rats was studied. A significant loss of M₄ but not M₁ receptors was observed in the different areas of the hippocampus and entorhinal cortex which varied with time after ADX. The vulnerability is in the order entorhinal cortex > dentate gyrus, CA4 > CA3 > CA2, CA1. Our results show that M₁ and M₄ receptors are differentially affected after ADX and indicate that the M₄ receptor subtype is influenced by adrenal hormones and suggest that the M₄ receptor might be the subtype linked to memory functions in the hippocampus.

In conclusion, the studies from the five papers (Papers I - V) constituting this thesis demonstrate that selective ligands like the muscarinic toxins are valuable tools to establish the roles of the different mAChR subtypes involved in normal and pathological conditions in the CNS. These findings suggest that, relative to the M₁, the M₄ receptor could be the subtype that is selectively compromised in Alzheimer's disease and in pain response. These findings might lead to new therapeutic strategies in the treatment of pain and Alzheimer's disease.

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Br. J. Pharmacol, 131 447-452, 2000.
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Manuscript.
- III. **E. Mulugeta**, N. El-Bakri, E. Karlsson, A. Elhassan and A. Adem. Loss of muscarinic M₄ receptors in spinal cord of arthritic rats: Implication for a role of M₄ receptors in pain response.
Brain Res 982, 284-287, 2003.
- IV. **E. Mulugeta**, E. Karlsson, A. Islam, R. Kalaria, H. Mangat, B. Winblad and A. Adem.
Loss of muscarinic M₄ receptors in hippocampus of Alzheimer patients.
Brain Res 960, 259-262, 2003.
- V. **E. Mulugeta**, I. Chandranath, E. Karlsson, B. Winblad and A. Adem. Temporal changes of muscarinic M₄ receptors in the hippocampus of adrenal ectomized rats.
Submitted, 2003.

LIST OF ABBREVIATIONS

A β	β -Amyloid
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholine esterase
AD	Alzheimer's disease
ADX	Adrenalectomy
AMP	Adenosine mono phosphate
APP	Amyloid precursor protein
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	Adenosine tri phosphate
BFCbS	Basal forebrain cholinergic system
CA 1-4	Ammons's horn subfields
cAMP	Adenosine 3', 5'-cyclic monophosphate
CCh	Carbachol
ChAT	Choline acetyltransferase
CHO	Chinese hamster ovary
CNS	Central nervous system
Cys	Cysteine
DAG	Diacylglycerol
DG	Dentate gyrus
GDP	Guanosine di phosphate
Gi	Inhibitory G-protein
GTP	Guanosine tri phosphate
³⁵ S-GTP γ S	³⁵ S-Guanosine-5-O-(3-thio) triphosphate
Glu	Glutamine
GPCRs	G-protein coupled receptors
IP ₃	Inositol tri phosphate
Lys	Lysine
PKC	Protein kinase C
PNS	Peripheral nervous system
mAChRs	muscarinic Acetylcholine Receptors
mRNA	Messenger ribo nucleic acid
MS-DB	Medial septal diagonal band
MT	Muscarinic Toxins
NBM	Nucleus basalis of mynert
NFTs	Neurofibrillary tangles
³ H-NMS	³ H-N-methyl scopolamine
NP	Neuritic plaques
NTs	Neuropil threads
PACAP	Pituitary Adenylate Cyclase Activating Polypeptide
³ H-QNB	³ H-Quinuclidinyl benzilate
Ser	Serine
Thr	Threonine
Tyr	Tyrosine

GENERAL BACKGROUND

The first molecule positively identified as a neurotransmitter by, Otto Loewi, was acetylcholine (ACh) (Loewi, 1921; Loewi and Navartil, 1926; Quastel JH, 1936). ACh is distributed both in the peripheral (PNS) and central nervous system (CNS). Acetylcholine is synthesized in a single step reaction from two immediate precursors, choline and acetyl coenzyme A, catalyzed by choline acetyltransferase (ChAT). Extracellularly released ACh is rapidly terminated to choline and acetate by acetylcholinesterase (AChE) (Quastel et al., 1936; Quastel JH, 1936; van der Zee and Luiten, 1999).

Acetylcholine receptors (AChRs) are divided into muscarinic and nicotinic. This has been known since Dale (Dale, 1914, 1954) investigated the effect of several chemicals on different nerve-muscle preparations. In some preparations (smooth muscles and glands) the action of ACh was mimicked by muscarine (a toxin from the mushroom *Amantia muscaria*, fly agaric) and in others (skeletal muscles) by nicotine. The action of muscarine was blocked by atropine and that of nicotine by curare. Later, when the notion of receptor was generally accepted, AChRs were classified into muscarinic and nicotinic.

This thesis focuses on the study of muscarinic acetylcholine receptor (mAChR) subtypes, M₁ and M₄ in the CNS and how they are affected in pathological conditions. This was achieved through the use of subtype selective toxins isolated from the venom of the Eastern green mamba snake (*Dendroaspis angusticeps*).

INTRODUCTION

Cholinergic pathways in the CNS

CNS cholinergic pathways are readily visualized immunohistochemically with antibodies directed against ChAT (Frotscher and Leranthe, 1986) which is restricted to cholinergic neurons. In the CNS, four major cholinergic projections exist and include the basal forebrain cholinergic areas (medial septal diagonal band MS-DB or septohippocampal pathway), nucleus basalis of Meynert (NBM), the cholinergic interneurons of the striatum and the pontomesencephalic areas (Frotscher, 1989; van der Zee and Luiten, 1999).

The NBM, provides most of the cholinergic innervation of the neocortex and the MS-DB complex innervates the hippocampus. These nuclei form a continuum of cholinergic neurons collectively termed the basal forebrain cholinergic system (Mesulam et al., 1983; van der Zee

and Luiten, 1999). The basal forebrain cholinergic system (BFChS) is among the first regions to be affected in Alzheimer's disease (Araujo et al., 1988; Altavista et al., 1990; Gaykema et al., 1992; Gallagher and Colombo, 1995; Smith and Booze, 1995; van der Zee and Luiten, 1999). The pontomecencephalic cholinergic complex are scattered throughout the pons and midbrain and project to the thalamus. Striatal cholinergic interneurons project within the striatum primarily to output neurons. The best known cholinergic routes are the NBM and MS-DB (Gallagher and Colombo, 1995; van der Zee and Luiten, 1999).

Virtually all the cholinergic input to the hippocampus is subcortical in origin (McKinney et al., 1983). Cholinergic fibers to the hippocampus have cell bodies in the medial septal nucleus and the nucleus of the diagonal band (Lewis et al., 1967). Cholinergic innervation of the hippocampus is widespread, underlying the multi-faceted effects of ACh as a modulator of hippocampal transmission (Rouse et al., 1999). The major input to the hippocampal formation comes from cortical areas through the perforant pathway (Amaral and Witter, 1995). Entorhinal cortex provides the dentate gyrus with its major input via the perforant pathway (Amaral and Witter, 1995). The dentate granule cells project to CA3 pyramidal cells via the morphologically distinctive mossy fibers (Amaral and Witter, 1995).

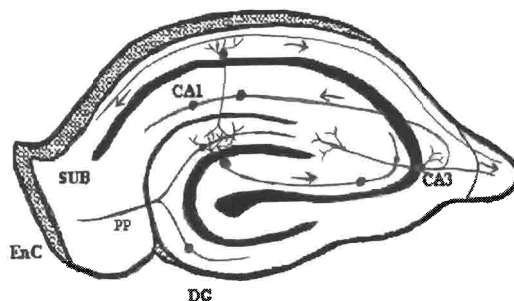


Figure. 1. Illustration of the hippocampal formation including the perforant path way (EnC-DG), the mossy fiber pathway (DG-CA3), and the schaffer collateral pathway(CA3-CA1 and CA1-Sub) Adapted from Brinton & Berger, 2000.

CA3 project to pyramidal cells of CA1 through Schaffer collateral pathway. Similarly, the CA1 pyramidal cells establish contacts with subiculum also through Schaffer collaterals (Amaral and Witter, 1995). Anatomical and physiological evidence suggests that ACh, acting via mAChRs, can differentially modulate excitatory and inhibitory transmission at many sites along the hippocampal circuit.

Muscarinic acetylcholine receptors

Muscarinic acetylcholine receptors (mAChRs) are involved in a number of important central and peripheral physiological processes. Binding studies with the muscarinic antagonist pirenzepine clearly indicated that there are subtypes of muscarinic receptors (Hammer et al. 1980). Five muscarinic acetylcholine receptor subtypes (denoted M₁ to M₅) have been cloned (Kubo et al., 1986a; Kubo et al., 1986b; Bonner et al., 1987; Peralta et al., 1987; Bonner et al., 1988).

Muscarinic AChRs belong to the superfamily of G-protein coupled receptors (GPCRs) that are structurally similar and consist of a single peptide chain which passes through the cell membrane seven times (Wess, 1993; Caulfield and Birdsall, 1998). The amino terminal part is outside the cell membrane and carboxy terminal inside the cell. The seven transmembrane domains have a helical structure and contain mostly hydrophobic amino acids. The human receptors have between 460-590 amino acids. The number of amino acid in other species differs slightly from the humans (Peralta et al., 1988; Caulfield and Birdsall, 1998). mAChRs share a common evolutionary origin with many other GPCRs, such as the rhodopsin, serotonin, and α and β -adrenergic receptors (Hulme et al., 1990; Strosberg, 1991).

Muscarinic receptors have two different types of binding sites, the *orthosteric* and the *allosteric* site. Acetylcholine and many agonists, such as carbachol, and many antagonists, such as atropine, NMS (N-methylscopolamine) and QNB (quinuclidinyl benzilate) bind to the orthosteric site. The *allosteric site* may have a regulatory function, since binding of a ligand to this site affects the binding of another ligand to the orthosteric site (Tucek and Proska, 1995). Some neuromuscular blocking agents (antagonists of nicotinic receptors), such as gallamine, pancuronium and alcuronium bind to the allosteric site, and so does the muscarinic toxin M₁ toxin-1 (Paper I). The alkaloid brucine and some of its derivatives are allosteric ligands with high selectivity for M₁ or M₃ receptors (Birdsall et al., 1997). The high selectivity of M₁ toxin-1 is due to binding to an allosteric site (Karlsson et al., 2000). There are probably several allosteric sites on each receptor, as indicated by binding studies with various radioligands (Birdsall et al., 2001; Christopoulos and Kenakin, 2002).

It is believed that the ligand binding site of these receptors lies embedded within the outer half of the membrane and is at least partly exposed to the extracellular side (Peralta et al., 1987; Galron and Sokolovsky, 1988). Ligand binding to these receptors occurs through an ionic interaction between the positively charged amine group of acetylcholine and the negatively charged aspartic acid (Asp) residue conserved in the third transmembrane domain of the

receptor protein (Caulfield and Birdsall, 1998; Hulme et al., 2001). The position of the ligand binding site was determined from high resolution electron diffraction data obtained with bacteriorhodopsin (Henderson et al., 1990; Hulme et al., 1990). This finding was further supported by a mutagenesis study of the rat M₁ receptor in which substitution of the aspartic acid residue (Asp) at position 147 for asparagine (Asn) was found to eliminate binding of the muscarinic receptor antagonist ³H-QNB (Fraser et al., 1989). Similarly, mutational analysis revealed that β₂-adrenergic receptor binding occurs at the same site. This shows that the third transmembrane Asp residue is conserved not only among the muscarinic receptor family, but also among other seven transmembrane proteins (Hulme et al., 1990). The specificity of the ligand for these particular receptor subtypes is not solely dependent upon the ionic interaction between the amine moiety of the ligand and the aspartic acid residue of the receptor (Hulme et al., 1990; Wess et al., 1991). The transmembrane domains of mAChRs also contain a series of conserved serine (Ser), threonine (Thr), and tyrosine (Tyr) residues whose hydroxyl groups interact with the ester group of acetylcholine via hydrogen bonding.

mAChRs are coupled to heterotrimeric G-proteins and mediate extracellular signals to the cell interior (Hulme et al., 1990). Upon agonist binding, these receptors undergo conformational changes that enable the receptor to interact with a specific class of G-proteins that are attached to the cell membrane (Hepler and Gilman, 1992). Heterotrimeric G-proteins consist of three subunits; denoted α, β, and γ which interact with the guanine nucleotides GTP and GDP. G_{αs} stimulates adenylyl cyclase while G_{αi} inhibits it and G_{αq} activates phospholipase C (Gilman, 1987). G_{αo} has no effect on adenylyl cyclase (Wickman and Clapham, 1995). When G-protein coupled receptors are activated, GTP is exchanged for the GDP bound to the G_α subunit, leading to the dissociation of the α subunit from the Gβγ complex (Hulme et al., 1990; Hepler and Gilman, 1992; Clapham and Neer, 1993). The G_α subunit then binds to downstream effector molecules such as adenylyl cyclase and phospholipase C, which are in turn involved in several enzymatic reactions. Activation of adenylyl cyclase leads to the synthesis of Cyclic-AMP (cAMP) from ATP. cAMP mediates its effects by activating various protein kinases (Gilman, 1987). Phosphatidylinositols (Ptd Ins) are a family of membrane phospholipids that are also important in cellular signal transduction. PIP₂, a specific phosphatidylinositol with a phosphate group attached to its inositol ring, plays a key role. Phospholipase C splits PIP₂ into diacylglycerol (DAG) and inositol (1, 4, 5)-IP₃, both of which act as second messengers. IP₃ is involved in the release of calcium (Ca²⁺) from intracellular stores, which in turn leads to smooth muscle contraction, glandular secretion, and

neurotransmitter and hormone release. DAG effects the activity of membrane bound protein kinase C (PKC), which controls the phosphorylation of serine (Ser) and threonine (Thr) residues in a variety of intra cellular proteins (Hepler and Gilman, 1992).

The mAChR subtypes are associated with different G-proteins and second messenger systems mentioned above (Hulme et al., 1990). The M₁, M₃, and M₅ subtypes preferentially couple to the G α_q family and stimulate phospholipase C production and the breakdown of phosphatidylinositol which in turn leads to an increase in the level of inositol triphosphate (IP₃) and diacylglycerol (DG) (Hulme et al., 1990; Caulfield, 1993). M₂ and M₄ receptors are primarily linked to adenylyl cyclase inhibition. (Hulme et al., 1990; Caulfield and Birdsall, 1998).

Distribution of mAChRs

mAChRs are found in both the peripheral and central nervous systems, as evidenced by immunological localization of muscarinic M₁-M₅ receptors with subtype specific antibodies, receptor binding and autoradiography by subtype selective ligands (Levey, 1993; Eglén and Watson, 1996; Caulfield and Birdsall, 1998) in rabbit and rat (Vogt, 1988; Levey, 1993).

The distinct distribution of each of the subtypes in the PNS has facilitated their classification. M₁ and M₃ receptors were found abundantly in salivary glands (36% and 42% respectively). M₂ receptors were found mostly in cardiac muscle (88%), in a lesser amount in the posterior part of the small intestine (62%) and in the lungs (40%). M₄ receptors were found to be as equally abundant in the lungs (41%) as the M₂ receptors, but less abundant in the posterior part of the small intestine (Dorje et al., 1991a; Caulfield, 1993; Levey, 1993; Eglén and Watson, 1996; Caulfield and Birdsall, 1998).

mAChRs are widespread in the CNS. Both cholinergic and non-cholinergic neurons express mAChRs. The location of these receptors has been determined with immunocytochemistry using specific antibodies, in situ hybridization to locate the receptor mRNA and receptor autoradiography (Dorje et al., 1991a; Levey et al., 1991; Caulfield, 1993; Levey, 1993; Yasuda et al., 1993; Levey et al., 1995; Eglén and Watson, 1996). The five mAChR subtypes are heterogeneously distributed in the brain. The most abundant subtypes of mAChRs in the CNS are M₁, M₂, and M₄. Immunological studies performed in different laboratories with different antibodies have been in close agreement with respect to the composition of mAChR subtypes in various regions of the rat CNS (Levey et al., 1991) and human (Flynn et al., 1995). The M₁ receptor is a major post synaptic receptor that is present in cell bodies and

neurites of the forebrain, particularly in the hippocampus and to a lesser degree in the neocortex (Levey et al., 1991). M₁ receptors are localized in hippocampal pyramidal cells and striatal spiny neurons.

M₂ receptors are widely distributed throughout the brain but are most abundant in the brainstem and cerebellum. M₂ receptors are present in cholinergic, non-cholinergic, and non-pyramidal neuronal cell types in cortex and hippocampus (Levey, 1993; Rouse et al., 2000). Presynaptic M₂ receptors control the release of different transmitter substances from non-cholinergic presynaptic terminals (Caulfield, 1993; Rouse et al., 2000).

M₃ receptors are found in very low levels throughout the brain (Levey, 1996). The synaptic localization of M₃ receptors is unknown.

M₄ receptors are located in striatal spiny neurons, the olfactory tubercle and associational commissural hippocampal projections (Levey et al., 1991; Rouse et al., 1999). They are both pre- and post- synaptic, are abundant in the neostriatum and are found at moderate levels in hippocampal and cortical regions (Levey, 1996). The M₅ receptor is the only mAChR subtype that was not detected by immunocytochemistry (Levey, 1996). In situ hybridization revealed that M₅ receptor mRNA is present in a fairly abundant amount in substantia nigra suggesting that M₅ receptors may regulate dopamine release at terminals within the striatum. M₅ receptor mRNA was also found in hippocampal pyramidal neurons (Weiner et al., 1990; Vilaro et al., 1993).

Physiological role of mAChRs

mAChRs regulate many physiological processes. In peripheral neurons, mAChRs regulate smooth muscle contraction, heart rate and force, glandular secretion and the release of neurotransmitters (Eglen and Watson, 1996). In the central nervous system, mAChRs regulate arousal, attention, control of sleep-wake cycles, control of body temperature, modulation of stress and cognitive functions as learning and memory (Burgen, 1995; Eglen et al., 1999). In addition, mAChRs in the spinal cord are implicated in mediating antinociception at spinal and supraspinal sites (Eisenach, 1999).

MUSCARINIC RECEPTOR LIGANDS

The pharmacological identification of the mAChRs subtypes responsible for the various physiological functions and central effects has been complicated because of lack of specific ligands with high subtype selectivity. There are no selective agonists which differentiate

individual muscarinic subtypes. However, there are a number of natural, synthetic and peptide antagonists of varying selectivity that have been used as tools to classify mAChRs. In table 1 some of the ligands frequently used as pharmacological tools including ten peptide ligands known as muscarinic toxins are presented. Cloning of five mammalian genes encoding mAChRs has also advanced pharmacological classification of mAChRs (Kubo et al., 1986a; Kubo et al., 1986b; Bonner et al., 1987; Peralta et al., 1987; Bonner et al., 1988). The use of cell lines that express individual muscarinic receptor subtypes offers the advantage that each subtype can be studied separately. Information about the pharmacological characteristic of the antagonists described in Table 1 has been acquired by the use of Chinese hamster ovarian (CHO) cell lines transfected with human mAChRs genes and stably express the five different receptor proteins (Dorje et al., 1991b).

Classical antagonists

There are several muscarinic antagonists available which are derived from natural toxins such as muscarine, atropine, scopolamine and pilocarpine and several other ligands as shown in Table 1. The majority of these ligands bind with about equal affinity to all subtypes. Many of the attempts to synthesize selective ligands were focused on compounds that have an accessible quaternary amino group that display some affinity to the ligand binding site. Thus, many substances were developed along these line, but the success has been limited so far. The majority of these antagonists are not used as therapeutic agents with the exception of pirenzepine, which has been used for the treatment of peptic ulcers.

Muscarinic toxins

About fifteen years ago (Adem et al., 1988) isolated two toxins from the green mamba (*Dendroaspis angusticeps*) that inhibited the binding of the non-selective muscarinic antagonist ^3H -QNB to cortical synaptosomes. The new ligands were called “muscarinic toxins (MT)”, MT1 and MT2. These toxins inhibited the binding of ^3H -QNB by about 50%. This was the first indication that the two toxins display partial selectivity for the mAChRs subtypes. To date 10 muscarinic toxins have been isolated and sequenced (Table 1) (Karlsson et al., 2000). Muscarinic toxins are the most selective ligands for muscarinic M_1 , M_2 , and M_4 receptors with high or very high subtype selectivity (up to 10 000-fold). For instance, MT1, MT4 and MT5 bind with high affinity to both muscarinic M_1 and M_4 subtypes but bind with very low affinity to the rest: M_2 , M_3 and M_5 (Table 1) (Karlsson et al., 2000). M_1 -toxin 1 also

called MT7 ($K_i = 0.1 \text{ nM}$) and MT1 ($K_i \approx 20 \text{ nM}$) showed selectivity for M_1 receptor (Table 1). Thus, M_1 toxin 1 is the first highly selective ligand for M_1 receptor. MT1 displayed an overlapping selectivity both for M_1 and M_4 subtypes. M_4 toxin1 on the other hand showed high affinity for M_4 subtype but about 40 times lower affinity for M_1 and very low (about 500 times) affinity for M_2 , M_3 and M_5 (Jolkkonen et al., 1994). M_2 -toxin has been found to be selective for M_2 receptor subtype (Carsi et al., 1999). This toxin blocked the binding of ^3H -NMS by about 77% without having any effect on M_4 receptors.

Since they were discovered muscarinic toxins have been used with great success in the study of mAChRs in the CNS (Adem and Karlsson, 1997). These toxins are peptides, consist of 63-66 amino acids and have structural similarity, possesses high sequence homology and consists of four disulphides that form four loops (Fig 2).

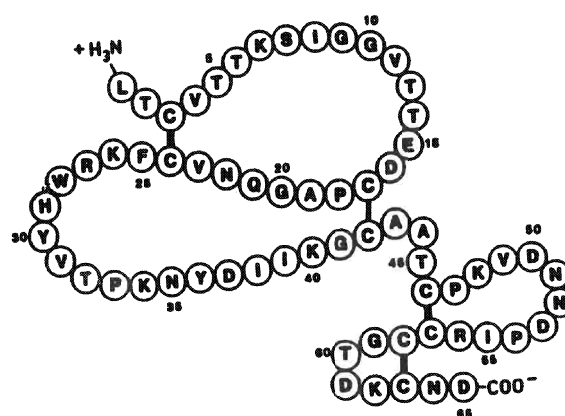


Figure 2. Amino acid sequence of muscarinic toxin 2 (MT2).

Sequence data reveal 30 identical residues between the toxins described in Table 1. Carsi et al. compared the sequence of 4 muscarinic isotoxins (m1-toxin 1, m1-toxin 2, m1-toxin 3 and m1-toxin 4) all binding to M_1 receptor with other mamba toxins (MT1, MT2, M_4 toxin 1, MT4, MT5 MT α and MT β) and found aminoacids: K5, WF10-11, Q29, F38, V55, N57 and K66 present in the four M_1 isotoxins but were absent in the homologous mamba toxins described in Table 1 (Carsi and Potter, 2000). It is possible that these amino acids are important for subtype selectivity but they do not seem to be critical for the binding to M_1 receptors (Carsi and Potter, 2000). M_2 -toxin which has 65 aminoacids and four disulfides, differs from other muscarinic toxins in that it has a structure like that of anti-nicotinic toxins.

Sequence alignment between M₂-toxin and the other muscarinic toxins given in Table 1 showed only 10-14 amino acids at identical positions but was found very similar to short α -neurotoxins (Karlsson et al., 2000).

Muscarinic toxins are homologous to a large number of other snake toxins, such as short chain neurotoxins, fasciculins, calciseptines, mambins and cardiotoxins/cytotoxins. The structures of many of these toxins have been resolved by X-ray crystallography and NMR spectroscopy (Menéz A, 1992; Segalas et al., 1995). Their structure (Fig 3) resembles the so called three-finger toxins, a name which refers to the structure where loops I, II and III are outstretched like the middle fingers of a hand with the disulfides and loop IV in the palm of the hand.

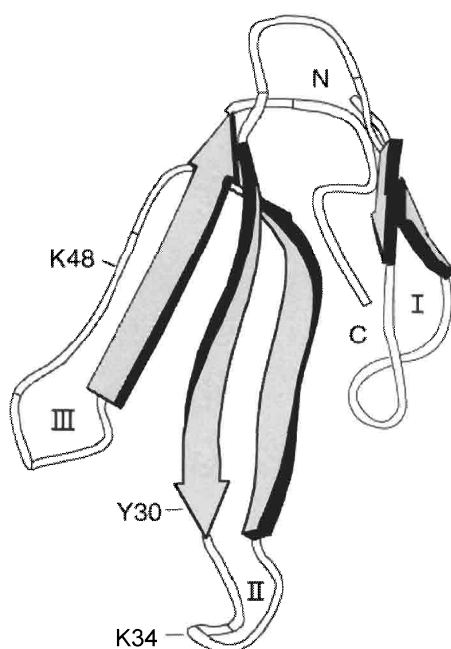


Figure 3. Schematic figure of the three-dimensional structure of MT2 showing the peptide backbone. The three-finger structure is shown loops I, II and III outstretched as the three middle fingers of a hand. N (C) = N (C)-terminus. The positions of Tyr 30 (invariant), Lys 34, (not 38 as indicated) (or Arg, positive charge) and Lys 48 (in two toxins I) are indicated. The part between Tyr 30 and Lys/Arg 34 (31-33) and Lys/Ile 48 can be important for the selectivity

The toxins does not have α -helix but consist of three adjacent loops rich in β -sheets that are connected to a core containing four disulfide bridges. For instance, MT2 toxin shows structural similarity with other snake toxins. Based on similarities on the position of cystein residues in the sequence and circular dichroism spectra of many of the snake toxins, Karlsson and Ducancel (Ducancel et al., 1991; Karlsson et al., 1991) proposed that the disulfide pairing and global folding of MT2 toxin might be similar to other snake toxins. To confirm this Ségalas et al. investigated the solution structure of MT2 toxin by 2 dimensional NMR

spectroscopy and molecular modeling and found that, similar to many of the snake toxins whose structures were already determined, the MT2 toxin structure was also composed of four disulfides, organized in double- and triple-stranded β -sheets. Thus, on the basis of this NMR data and the common sequential organization of this toxin, it was suggested that the structure of MT2 toxin share a typical three-finger structure and is directly associated with the three-finger fold of snake toxins (Segalas et al., 1995).

In the structure of muscarinic toxins, the second loop between Cys 24 and Cys 42 (Fig 2) seem to be the sites that are critical for subtype specificity since chemical modification resulting from iodination and mutation of the toxins changed their specificity. A comparison of muscarinic toxins with sequences of 175 other toxins made by Mebs et al. (Mebs D, 1991) showed that Tyr 30 is unique for muscarinic toxins. For instance, MT1 binds normally to M_1 and M_4 receptors but after iodination with ^{125}I , MT1 binds only to M_1 with a higher affinity than the native toxin (Karlsson et al., 1994; Jolkkonen et al., 1995; Waelbroeck et al., 1996; Adem et al., 1997). It was suggested that the invariant Tyr 30 (Fig. 2) which has a protruding side-chain that is exposed to the solvent (Segalas et al., 1995) is seemingly the most exposed Tyr residue that is easily iodinated (Fig 2). There are also other residues that are believed to play significant role in binding to the active site of the receptor. There is for example a lysine residue (Lys 34) in the tip of Loop II that is common to most of the toxins. All of the toxins shown in Table 1 have also a cationic group (Lys or Arg) in position 34, which may be involved in receptor binding (Karlsson et al., 2000). This positive charge may bind to an amino acid with a negatively charged side-chain (Asp or Glu) or by π -electron interactions with an aromatic amino acid (Trp, Tyr or Phe). The number of hydrophobic amino acids in the second loop is significantly higher in muscarinic toxins compared to other toxins, suggesting that hydrophobic residues might contribute to selectivity.

The pharmacological activity of MT1, MT2, M_4 toxin 1 and M_1 toxin 1 was investigated in biochemical, functional and behavioral studies. The *in vivo* effect of some of the muscarinic toxins after injection in discrete brain regions has been investigated. For instance, injection of MT1 or MT2 into dorsal hippocampus of rats after training in an inhibitory avoidance test improved memory as compared to control rats injected with buffer. The non-selective agonist, oxotremorine, has a similar effect, while the antagonist scopolamine had the opposite effect and deteriorated the memory (Jerusalinsky et al., 1993; Jerusalinsky et al., 1995; Jerusalinsky et al., 1998). Based on these findings MT1 and MT2 were categorized as muscarinic agonists at the M_1 subtype. This was later corroborated by studying MT1 and MT2 toxins in peripheral

tissues, in which agonist like action of these toxins were observed in the rabbit vas deferens (Jerusalinsky et al., 2000).

Table 1. Affinity values (Ki) for some muscarinic antagonists and for muscarinic toxins isolated from green and black mamba snake venom.

Ligand	Relative subtype selectivity	Pharmacological profile	Affinity (Ki) nM					Ref.
			M ₁	M ₂	M ₃	M ₄	M ₅	
pirenzepine	M ₁ , M ₄	antagonist	3-15	200-500	80-200	8-80	80-640	[1, 2]
Himbacine	M ₂ , M ₄	antagonist	63-100	5-10	40-120	1.6-10	500-800	[1, 2]
Tripitramine	M ₂ , M ₁ , M ₄	antagonist	1.6-4	0.2-0.4	40-80	6.3-16	30-50	[1, 2]
AFDX 384	M ₂ , M ₄	antagonist	32-50	1-6	16-63	2-10	>500	[1, 2]
pFHHSiD	M ₃	antagonist	32-63	120-1000	13-16	32	100	[1, 3]
Atropine	NS	antagonist	0.2-1	0.5-1	0.2-1.3	0.3-0.8	0.2-1.3	[2]
4-DAMP	NS	antagonist	0.6-2.5	4-16	0.5-1.3	0.4-4	1-1.3	[1, 2]
Muscarinic toxins from the venom of green mamba snake (<i>Dendroaspis angusticeps</i>)								
M ₁ -toxin 1	M ₁	Antagonist	0.1	>2000	>2000	>2000	>2000	[4, 5]
MT1	M ₁ , M ₄	Agonist	22-49	>1000	>1000	29-58	>1000	[4, 5]
MT2	NS	Agonist	630	>2000	>2000	1900	>1000	[4, 5]
MT4	M ₁ , M ₄	NA	62	>1000	>1000	87	>1000	[4, 5]
MT5	low M ₁		180	>1000	>1000	540	>1000	[4, 5]
M ₂ -toxin	M ₂		nb	150	nb	nb	nb	[6]
M ₄ -toxin 1	M ₄ , M ₁	Antagonist	78-300	>1000	>1000	1.4-2	>1000	[4, 5]
M ₄ -toxin 2	M ₂		190	>425	>425	3.6	>425	[4, 5]
Muscarinic toxins from the venom of black mamba snake (<i>Dendroaspis polylepis</i>)								
MT α	NS		23	44	3	5	8	[4, 5]
MT β	NS		>1000	>2000	140	120	350	[4, 5]

[1]. (Caulfield, 1993)

[2]. (Caulfield and Birdsall, 1998)

[3]. (Eglen and Watson, 1996)

[4]. (Adem and Karlsson, 1997)

[5]. (Karlsson et al., 2000)

[6]. (Carsi et al., 1999)

The pharmacological profiles of M₁-toxin 1 and M₄-toxin 1 was also studied utilizing the cloned human muscarinic receptors stably expressed in CHO cells. M₄ toxin-1, antagonized

the acetylcholine inhibition of AC stimulated by forskoline or dopamine D₁ receptor. M₄ toxin-1 was also found to be a much weaker antagonist of the cardiac M₂ coupled to cyclic AMP and of cerebral cortical M₁ receptor coupled to stimulation of phosphoinositide hydrolysis (Olianas et al., 1996). Based on this evidence M₄ toxin-1 was acknowledged as a potent antagonist at central M₄ receptors. There is also experimental evidence implicating M₄ muscarinic receptors in memory. Injection of M₄-toxin-1 into the dorsal hippocampus of rats after inhibitory avoidance training produced amnesia (Jerusalinsky et al., 1998). The pharmacological activity of M₁-toxin 1 was investigated by examining its effect on the agonist-induced [³⁵S]-GTPγS binding mediated by cloned muscarinic receptor subtypes expressed in CHO cells and by the native M₁ and M₂ receptors present in N1E-115 neuroblastoma cells (Olianas et al., 2000). In CHO cells expressing the M₁ receptor, low concentrations of M₁-toxin 1 (1-30 nM) counteracted the acetylcholine-induced [³⁵S]-GTPγS binding in a non-competitive manner, whereas in CHO cells expressing the M₂, M₃ or the M₄ receptor subtype the toxin was completely inactive at concentrations up to 100 nM binds stably to the M₁ receptor and blocks it persistently. In N1E-115 neuroblastoma cells, M₁-toxin 1 (0.3–3 nM) antagonized the M₁-mediated stimulation of phosphoinositide hydrolysis non-competitively, but failed to affect the M₄ receptor-mediated inhibition of AC, displaying high discriminative capacity even in cell systems expressing both receptors (Olianas et al., 2000).

MUSCARINIC RECEPTORS IN NORMAL AGING AND PATHOLOGICAL CONDITIONS

Age-dependent changes of mAChRs

The effect of normal aging on the cholinergic system is controversial. Physiological changes that occur during aging which affect brain regions involved in memory function have been demonstrated neurochemically (Giannakopoulos et al., 1997) and morphologically (Small, 2001). Several in vitro studies report conflicting results regarding the subtypes of mAChRs that are affected. Age-dependent change in muscarinic acetylcholine receptors in different regions of rat brain have been studied by several workers (Mangano MR, 1988; Biegon et al., 1989; Lee et al., 1994; Amenta et al., 1995; Tayebati et al., 2002) .

A significant decrease in M₁ binding in CA1 and CA4 was detected by autoradiography in 27 months old rats with no change in M₂ binding (Amenta et al., 1995). The same group (Tayebati et al., 2002) used a combination of kinetic and equilibrium binding and found the following changes in 22 month old rats as compared to 6 month old rats; in the granular layer

of dentate gyrus a decrease in M₁ and M₂ and in the molecular layer a decrease in M₂ and an increase in M₃; in the pyramidal neuron layer of CA1 a decrease in M₁ and M₂ and in the stratum radiatum (radial layer) a decrease in M₂; in the pyramidal layer of CA3 a decrease in M₅ and in the stratum radiatum a decrease in M₂. After solubilisation of receptors with a detergent and precipitation with subtype specific monoclonal antibodies M₁-M₄ receptors were determined in several regions of rat brain (Tice et al., 1996). In hippocampus M₄ receptors increased significantly by about 20 % from 16 to 90 days old rats, whereas M₁, M₂ or M₃ remained constant. The cause for the conflicting information might depend on several factors such as rat strain, age of the animals, selectivity of ligand and the accuracy of the technique employed.

Muscarinic receptor changes in Alzheimer's disease

Alzheimer's disease is the most well documented neuropathological disorder affecting elderly people. The disease is characterized by progressive loss of memory and other cognitive functions (reasoning, judgement, planning, sequencing, calculation and organizing) resulting in a profound dementia. The average survival time of AD is 8-10 years between onset and death with a variable course of development.

One of the major neuropathological features of AD is the presence of neurofibrillary formations (Glenner and Wong, 1984; Grundke-Iqbal et al., 1986) which can appear in three forms: neurofibrillary tangles (NFTs), neuropil threads (NTs) (Braak et al., 1986) and neuritic plaques (NPs) (Wisniewski, 1973; Braak and Braak, 1991). These formations are often observed in cell bodies and proximal dendrites. These formations are caused by abnormalities in a neuronal cytoskeletal protein known as "tau" which is a microtubule binding protein (Wisniewski and Terry, 1968). Thus, tangle formation is a result of hyperphosphorylated tau (Kosik, 1990).

Brain regions affected by AD also contain senile plaques. Plaques consist of aggregated fibrillary peptide the main constituent of which is Amyloid Precursor Protein (APP). APP can occur in three different isoforms: APP₆₉₅, APP₇₅₁ and APP₇₇₀ (Kang et al., 1987; Tanzi et al., 1987). Abnormal phosphorylation of tau and abnormal formation of A β ₁₋₄₂, A β ₁₋₄₃ gives rise to a deposition of proteinaceous substance leading to interrupted neurotransmission which subsequently results in neuronal cell death (Kandel et al., 2000; Price, 2000).

The hippocampus and cerebral cortex are the main parts of the brain where notable nerve degeneration occurs including a loss of cholinergic functions (Bowen et al., 1976; Araujo et

al., 1988). Neurochemical analyses have suggested that deficits in presynaptic cholinergic markers such as ChAT activity, choline uptake and acetylcholine synthesis and release as some of the main factors contributing to the progression of the disease. A cholinergic hypothesis for AD is also generally accepted (Bowen et al., 1976; Coyle et al., 1983; Perry, 1986). Disturbances in the cholinergic pathway, connecting the basal forebrain region to the hippocampus, amygdala and cerebral cortex, which are vital for memory formation and retention has been reported comprehensively (Whitehouse et al., 1982). There are several reports on decrease in cholinergic parameters, such as activity of ChAT, acetylcholine levels, acetylcholinesterase, nicotinic and muscarinic receptors and neurons in some brain areas (Coyle et al., 1983; Mash et al., 1985; Nordberg and Winblad, 1986; Reinikainen et al., 1987). Thus, mAChRs receptors have a central function for memory, learning and cognitive processes and their role in the progression of Alzheimer's disease have been documented extensively (Levey, 1996; van der Zee and Luiten, 1999).

Role of mAChRs in pain response

Pain impulses (nociception) are transmitted through two classes of fibers known as, A δ and C fibers (Kandel et al., 2000). These fibers terminate predominantly in the dorsal horn of the spinal cord. The dorsal horn is divided into six layers known as Rexed laminae (I-VI). Each laminae is innervated by distinct types of fibers (neurons). A δ fiber is a myelinated fiber which is about 2-5 μ m in diameter with a conducting rate of 5-30 m/s. C fibers conduct slowly (< 1m/s) and are unmyelinated with a diameter of < 1.0 μ m (Kandel et al., 2000). Pain and temperature signals are conveyed by these two fibers to the superficial layers of the dorsal horn (Kandel et al., 2000). Acetylcholine is one of the neurotransmitters present in descending supraspinal fibers as well as in primary afferent fibers (Hartvig et al., 1989; Abelson and Høglund, 2002). Thus, ACh is considered to be one of the major neurotransmitters in pain modulation. ACh acts as an analgesic at the level of the spinal cord as indicated by the spinal administration of muscarinic receptor agonists or acetylcholinesterase inhibitors which attenuate pain (Iwamoto and Marion, 1993a). However, the mechanisms and the site of actions of cholinergic agents in the spinal cord are not well defined (Hartvig et al., 1989; Eisenach, 1999). Muscarinic receptors are localized in the superficial layers of the dorsal horn of the spinal cord, thus the relief of pain (analgesia) achieved by spinal injection of cholinergic agonists reflects primarily muscarinic receptor activation (Eisenach, 1999). The role of mAChRs in modulating antinociception and mAChRs

mediated analgesia through both spinal and supraspinal mechanisms has been demonstrated by a number of groups (Hartvig et al., 1989; Iwamoto and Marion, 1993a; Naguib and Yaksh, 1997; Abelson and Høglund, 2002). Although the analgesia is similar in magnitude to morphine, it is less likely to lead to tolerance and addiction. It has been reported that the intraspinal administration of muscarinic agonists like oxotremorine or carbachol induces the intraspinal release of acetylcholine, suggesting a possible connection between pain threshold and acetylcholine release (Abelson and Høglund, 2002). It is unclear which of the subtypes (M₁-M₅) are involved. Some investigators suggest M₁ (Ghelardini et al., 2000), M₂ (Gomez et al., 1999), M₁ and/or M₂ (Iwamoto and Marion, 1993b), M₁ and possibly M₃ (Naguib and Yaksh, 1997) and M₄ and/or M₅, (Sheardown et al., 1997) receptors to be involved. Other results indicate; M₁ (Høglund and Baghdoyan, 1997; Shannon et al., 1997; Sheardown et al., 1997) and M₁ and M₃ subtypes (Sauerberg et al., 1995) are not involved.

AIMS OF THE PRESENT STUDY

ACh regulates several important physiological actions through its various receptors. The G-protein coupled mAChR subtypes mediate their effects by regulating various effector systems involved in the second messenger systems, while the ligand-gated nicotinic receptor subtypes act directly on the ion channels mediating fast transmission. The focus of this thesis lies on the G-protein coupled mAChR subtypes. In this thesis selective muscarinic toxins were used to investigate muscarinic M₁ and M₄ receptor subtypes in normal and pathological conditions.

The specific aims of this thesis were:

1. To characterize the pharmacological profile of M₁ toxin-1 (MT7). The activity of the toxin was investigated in functional assays of the cloned human M₁- M₄ receptors expressed in CHO cells and of the native M₁ and M₄ present in murine NIE-115 neuroblastoma cells.
2. To investigate age related changes of muscarinic M₁ and M₄ receptor subtypes in brains of young, adult and aged rats.
3. To study the effect of acute and chronic pain on the level of M₁ and M₄ muscarinic receptors in spinal cords of arthritic rats.
4. To investigate if the levels of M₁, M₂ and M₄ muscarinic receptors in the hippocampus of post mortal Alzheimer brains.
5. To examine the effect of short and long term adrenalectomy on the level of M₁ and M₄ receptors in the hippocampus of adrenalectomized rats.

MATERIALS AND METHODS

In this section a brief description of the methods used in this thesis is presented. The detailed description of the methods can be found in papers I-V.

Tissues

Paper I

In this study characterization of the pharmacological profile of M₁ toxin 1 was studied in a cell culture model. We examined the activity of M₁-toxin 1 (MT7) in functional assays of the cloned human M₁-M₄ receptors expressed in CHO cells and of the native M₁ and M₄ receptors present in murine neuroblastoma cells (NIE-115).

Paper II

Male Sprague-Dawley rats were used to examine age dependent changes in muscarinic M₁ and M₄ receptors levels in the hippocampus. The rats were obtained from ALAB (Stockholm, Sweden) and they were housed in cages (60 x 35 x 20 cm) in a noise reduced, humidity and temperature (21°C) controlled room with 12-hour light/dark illumination schedule with light on at 7.00 a.m. Twenty-one day old (n = 4), 3 and 25 months old (n = 5) were brought to the laboratory one hour before sacrifice. The brains were removed and frozen immediately with dry ice. All experimental procedures and housing conditions followed the guidelines and recommendations of the Swedish animal protection legislation and were approved by the Animal Ethics Committee, Huddinge Sweden.

Paper III

To investigate the effect of acute and chronic pain on the level of M₁ and M₄ muscarinic receptors arthritic rats were used as a model. The study included 15 female Lewis rats weighing 160-180 g. Arthritis was induced by intradermal injection of a suspension (50 µl) of heat-killed *Mycobacterium butyricum* in paraffin oil (10 mg/mL) into the base of the tail (n = 10) according to the method developed by Pearson and Wood (Pearson M, 1959). Controls received 50µl paraffin oil by the same route (n = 5). The arthritic rats were divided into two groups, an acute group (n = 5) which was sacrificed 12 days after inoculation with mycobacteria and a chronic group (n = 5) which were sacrificed 30 days after inoculation. The control group (n = 5) was sacrificed after 30 days. The animals were housed one group in

each cage at 21 °C in a 12-hour light/dark cycle and given water and food pellets *ad libitum*. The animals were killed by decapitation and the sacro-lumbar joint was cut and the whole spinal cord was pushed out by a syringe filled with saline, immediately frozen on dry ice and stored at -70 °C until used. The regional ethical committee (Huddinge, Sweden) approved this study.

Paper IV

To check if the levels of M₁, M₂ and M₄ muscarinic receptors are affected in the hippocampus of post mortal Alzheimer's brains, we assessed a series of brains from the Brain Bank at the Department of Neuropathology, Case Western Reserve University (Cleveland, Ohio, USA). Brain tissue was obtained from 4 controls and 7 AD cases. The mean ages of the subjects in the groups were 73 ± 3.6 and 74 ± 2 years for AD and controls respectively. The brains were sampled at autopsy and removed within 3 to 8 hours after death. The post-mortem intervals between death and tissue retrieval were not different for the two groups. The diagnosis of AD, suspected and assessed clinically, was confirmed by histopathological examination which revealed the presence of the usual hallmarks including neuritic plaques in the cortex and neurofibrillary tangles (NFT) in the hippocampus meeting the criteria set by the consortium to establish a registry for AD (CERAD, 1992). Control subjects had no evidence of dementia and were also without neurological or psychiatric disorder. The majority of subjects both controls and AD, had died from similar causes which was bronchopneumonia. Hippocampii from control and AD were frozen at -80°C for receptor autoradiographic studies.

Paper V

The effect of short and long term adrenalectomy on the level of M₁ and M₄ receptors in the hippocampus of adrenalectomized rats were examined. One hundred fifty adult male Wistar rats weighing from 180 to 200 grams were obtained at the age of 70 days from (Al Ain, UAE university). The rats were anesthetized with pentobarbital (50 mg/Kg body weight) and either sham operated or adrenalectomized. They were placed in different cages, 4-5 rats per group and cage at 21°C in a 12-hour light/dark cycle. The rats received food pellets and water *ad libitum*. Adrenalectomized rats received saline in the drinking water. The regional ethical committee of the Faculty of Medicine and Health Sciences (Al Ain, UAE) approved this study..

EXPERIMENTAL PROCEDURES

Isolation of Toxins

Three toxins MT1 (muscarinic toxin 1), M₄ toxin-1 and MT7 (M₁ toxin-1) were used in this thesis. The previous methods for isolation of MT1 (Adem et al., 1988) and of MT3 (Jolkkonen et al., 1994) were improved considerably. M₁ toxin-1 was isolated as described earlier (Jolkkonen, 1996) or a recombinant toxin was used (Nasman et al., 2000). For isolation of biological substances, several methods which utilized different parameters are normally used. For the isolation of muscarinic toxins, a combination of three chromatographic methods were used; gel filtration (utilizes differences in molecular size), ion exchange chromatography (differences in charge) and reversed phase chromatography (differences in hydrophobicity). Several factors determine the degree of purity. For instance, adsorption to the matrix of the chromatographic support due to hydrophobic interactions or hydrogen bonding can influence the separation.

Ammonium acetate (AmAc) buffers were used in all separation steps. The main reason for using ammonium acetate is that it is volatile and can be removed directly by freeze-drying where it decomposes into ammonia and acetic acid. With buffers containing non-volatile salts, such as phosphates or NaCl, these have to be removed by dialysis prior to freeze-drying or by gel filtration which requires extra steps and reduces the yield.

Fig. 4 shows gel filtration of crude venom on Sephadex G-50. The total volume of the column $V_t = V_o + V_i + V_g$ [1] where V_o = void volume (volume between gel particles), V_i = internal volume (volume inside particles) and V_g = volume of gel.

Molecules which are too large to penetrate into the gel particles elute with an elution volume $V_e = V_o$. On Sephadex G-50 proteins with a molecular weight 30,000 or larger elute with $V_e = V_o$. Thus, it is not possible to separate proteins with molecular weights 30, 000 and 50,000 on Sephadex G-50. Small molecules which penetrate into the smallest cavities of the gel particles elute with $V_e = V_o + V_i$. Separation according to size occurs only when $V_o < V_e < V_o + V_i$ and the molecular weight can be estimated from the elution volume. Molecules with $V_e > V_o + V_i$ are adsorbed to the gel.

The first peak elutes with $V_e = V_o$, in this case 113 ml (See legend). Some material elutes before the maximum because of broadening of peaks due to diffusion. V_i can be determined

from the elution volume of a small molecule which does not adsorb to the gel, for instance tritiated H₂O. It can also be estimated from the V_g; 1 gram dry Sephadex G-50 gives about 10 ml gel, the column volume is 358 ml and it contains 36 g Sephadex G-50, density 1.5 g/ml which corresponds to V_g = 24 ml. Thus, from equation [1]

$V_o + V_i = 334$ ml. 100 ml of eluate was collected in a graduate before fraction collection was started. Fraction size 5.3 ml and fraction 44 had $V_e = 100 + 45 \times 5.3 = 333$ ml.

Thus, material eluting after fraction 44 was adsorbed to the gel and the elution volume cannot be used to estimate the size of the molecules.

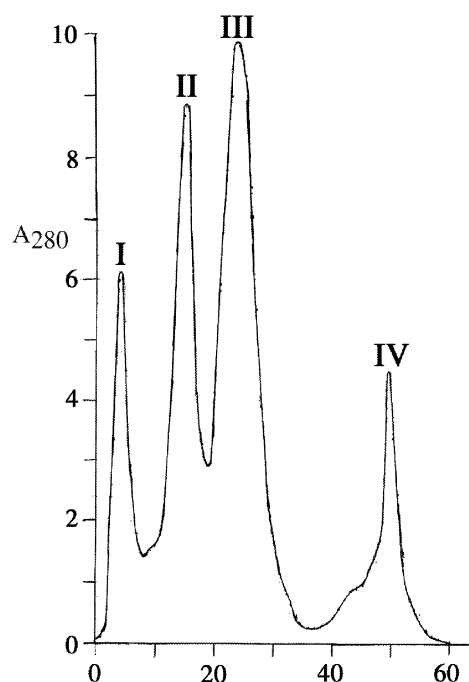


Fig. 4. Gel filtration of *Dendroaspis angusticeps* (green mamba) venom on Sephadex G-50. Column: 2.0 x 114 cm, V_t = 358 ml, buffer 0.10 M AmAc (pH 6.7). 1 g venom dissolved in 0.10 M AmAc and clarified by centrifugation (25,000 g, 30 min), 9.7 ml applied to column. 100 ml effluent was discarded before fraction collection was started. Fractions 5.3 ml per 17 min. V_o (at fraction 4, maximum of peak) = $100 + 4 \times 5.3 - 4.9$ (half of sample volume) - 3 (volume of tubings) = 113 ml. Peak III which contained the various toxins was freeze-dried.

Fig. 5 shows ion-exchange chromatography on Bio-Rex 70 of the gel filtration fraction containing the various toxins present in the venom of *Dendroaspis*. The chromatogram shown was actually obtained in the first isolation of muscarinic toxins (Adem et al. 1988). It is included here to illustrate the complexity of snake venoms, for instance mamba venoms contain 70-80 proteins in the molecular weight range 6000-8000, i.e. substances that elute in peak III (Fig. 4). In this case, after the non-retarded fraction with muscarinic toxins had come out, the column was eluted with 1.25 M AmAc and the material still on the column was eluted

in one single peak. This was done to facilitate the work, since only one fraction had to be recovered and freeze-dried instead of 14 if the complete gradient elution was done.

Bio-Rex 70 is a cation-exchanger consisting of polymerised methacrylic acid cross-linked with divinylbenzene. It has been used much for fractionation of snake venoms. It has a very high resolving power, for instance the two fasciculins differ only by one uncharged amino acid. At position 46 of totally 61 amino acids fasciculin 1 has Tyr and fasciculin 2 Asn. The separation is believed to depend on increased hydrogen bonding to the ion-exchanger, Tyr can form one and Asn three hydrogen bonds and consequently fasciculin 2 is more retarded.

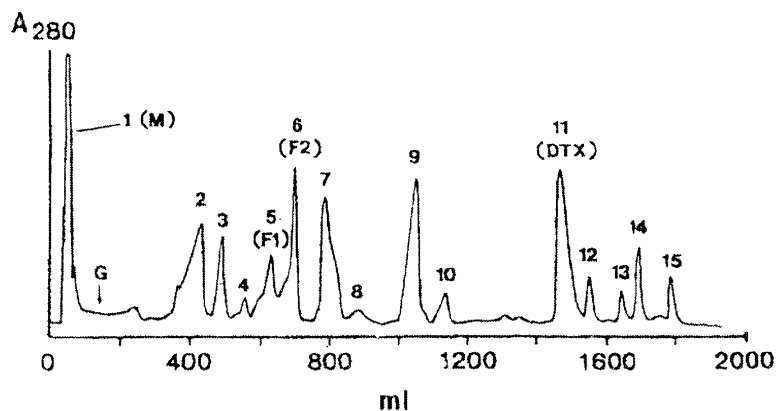
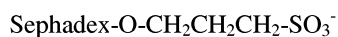


Fig. 5. Ion-exchange chromatography of peak III (Fig. 4) on Bio-Rex 70 (2.0 x 30.8 cm) equilibrated with 0.20 M ammonium acetate (pH 7.3). The sequence of operations: Elution with 200 ml AmAc, application of sample in 20 ml 0.01 M AmAc, elution with 120 ml 0.03 M AmAc and elution with a concave gradient of 0.03 M AmAc vs. 1.25 M AmAc (pH 7.3).

G indicates start of gradient elution. Gradient volume 2.0 litre and formed by two connected cylinders of 9 cm (with 0.03 M buffer) and 6 cm (1.25 M). Flow rate 38 ml/h. Fraction size 8.1 ml. The non-retarded fraction 1(M) contained muscarinic toxins, peaks 5 (F1) and 6 (F2) contained the two fasciculins, inhibitors of acetylcholine esterase, peaks 11, 12, 14 and 15 contained four different dendrotoxins, blockers of voltage dependent potassium channels and peak 13 contained calcicludine, blocker of L-type calcium channels.

Fig. 6. The fraction containing the muscarinic toxins was chromatographed on the cation-exchanger Sulphopropyl-Sephadex C-25. The matrix is Sephadex G-25 to which sulphopropyl groups are substituted.



A more shallow gradient was used than previously (Adem et al. 1988) and more peaks were obtained. The molecular weights of the substances in the various peaks were determined by mass spectrometry (MALDI TOF). The molecular weight in peak MT3 was 7379.6 which agrees very well with that calculated from the sequence 7379.0 (Jolkkonen et al. 1994). The first peak contained the two toxins MT1 and MT2 in agreement with previous results (Adem et al. 1988). This was confirmed by mass spectrometry which indicated two toxins with molecular weights 7509 (MT1) and 7040 (MT2) being present. Hydrophobic interactions and hydrogen bonding effects seem to be insignificant for the separation on Sulphopropyl-Sephadex in contrast to Bio-Rex 70 and the HPLC ion-exchanger (Fig. 8) where these interaction can influence the separation very much.

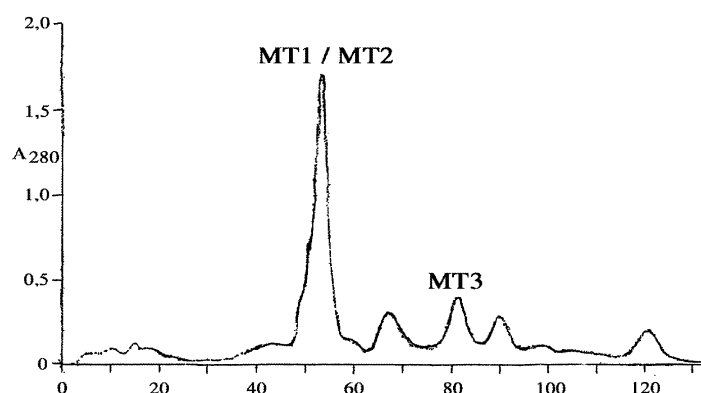


Fig.6. Ion-exchange chromatography on Sulphopropyl-Sephadex C-25 of fraction 1 (M) from chromatography on Bio-Rex 70 (Fig. 5). The column (2.0 x 30 cm) was equilibrated with 0.05 M AmAc (pH 5.2). The freeze-dried sample was dissolved in 20 ml 0.05 M AmAc (pH 5.2) and after sample application the column was eluted with a concave gradient of 1000 ml 0.05 M AmAc (pH 5.2) and 450 ml 1.0 M AmAc (pH 6.5). As in Fig. 2 the gradient was formed by two connected cylinders. The cylinder with 9 cm diameter contained 0.05 M buffer, the 6 cm diameter cylinder 1.0 M buffer. 4.6 ml fractions per 10 min were collected. The peaks denoted M1/MT2 and MT3 contained respective toxins identified by mass spectrometry.

Fig.7. A new chromatography on Sulphopropyl-Sephadex C-25 under slightly different conditions than in Fig. 3 separated the toxins MT1 and MT2. Only MT1 was used, since MT2 can be easily inactivated. Native MT1 binds both to M_1 and M_2 receptors, but after iodination only to M_1 .

MT1 was homogeneous when analysed by reversed phase HPLC on a cyano-propyl column and only one peak was seen.

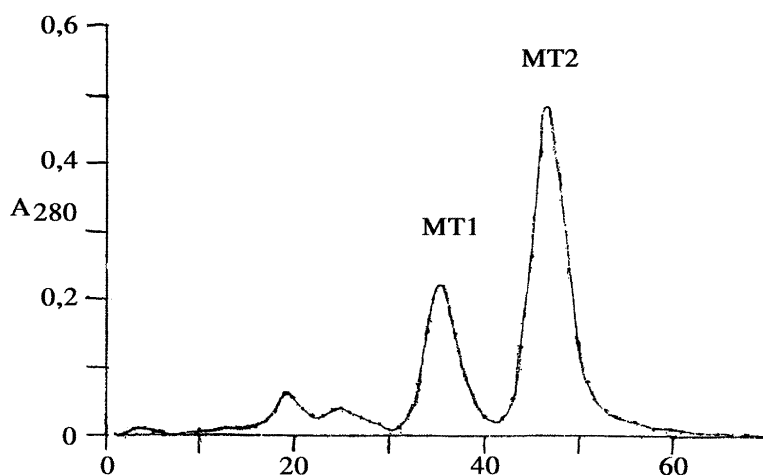


Fig. 7. Chromatography on Sulphopropyl-Sephadex of fraction MT1/MT2 from Fig. 6. The same column as in Fig. 3, but equilibrated with 10 mM AmAc (pH 6.7). The freeze-dried sample was dissolved in 20 ml 10 mM AmAc and after sample application the column was eluted with a concave gradient of 900 ml 10 mM AmAc (pH 6.7) vs. 400 ml 1.00 M AmAc (pH 6.7). The gradient was formed as explained for Fig. 3. Fractions 5.6 ml per 12 min. Only MT1 was recovered.

Fig. 8 shows the final chromatography of MT3 obtained as explained in Fig. 6. The chromatography was done by HPLC on the cation-exchanger BioGel TSK SP-5-PW (SP = sulphopropyl). The main peak contained MT3. The two first eluting peaks seemed to contain toxins that bind to M5 muscarinic receptors. The matrix of the ion-exchanger is hydroxylated vinyl polymer to which sulphopropyl groups are substituted. The matrix is rather hydrophobic and therefore hydrophobic interactions can influence the separation, for instance the two fasciculins elute in reversed order compared to Bio-Rex 70. Fasciculin 2 with the hydrophobic amino acid Tyr-46 elutes after fasciculin 1 with the hydrophilic Asn-46. MT3 was homogeneous when analysed by reversed phase HPLC.

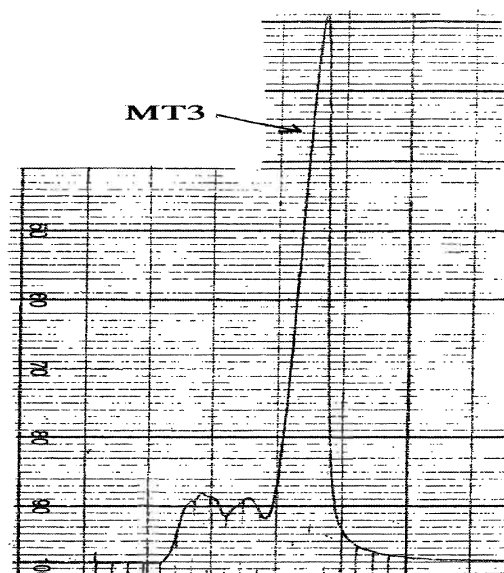


Figure 8. HPLC ion-exchange chromatography of fraction MT3 from Fig. 6. Column. BioGel TSK SP-5-PW equilibrated with 20 mM AmAc (pH 6.7). Buffer A. H₂O. buffer B 1.00 M AmAc (pH 6.7). Programme: 0-2 min 2 % B, 2-16 min a linear gradient from 2 to 16 % B. Flow rate 4 ml per min. 2 ml fractions. .

Preparation of synaptosomes

Male Sprague Dawely rats (150-200 g) were decapitated, the brains were rapidly removed, the cortices collected and homogenised in 20-30 volumes of ice-cold 0.32 M sucrose and centrifuged at 1000 x g for 10 min. The supernatant was recovered and centrifuged at 17 000 x g for 15 min. The pellet containing the crude synaptosomal fraction was resuspended in the original volume of ice cold 0.32 M sucrose, homogenized and kept frozen at -70° C until used. The protein concentration was determined by the method of Lowry with bovine serum albumin as standard.

Muscarinic receptor binding assay

Displacement of ³H-NMS by ligands (purified toxins) to membrane receptors was measured by a rapid filtration technique on glass fiber filters using 24-well cell harvester. Briefly, aliquots of (0.1 ml) of rat synaptosome preparation or CHO cells stably expressing muscarinic receptor M₁, M₂, M₃, M₄, or M₅ subtypes, were incubated with ³H-NMS and purified toxins in a final volume of 1.0 ml of 50 mM Na-K phosphate buffer, pH 7.4. After incubation for 60 min at 25°C, the mixture was filtered through Whatman GF/B filters pre treated with 0.05% polyethyleneimine (PEI), which reduces non-specific binding to glass fiber filters and washed with 50 mM Na-K phosphate buffer, pH 7.4. Filter bound radioactivity was then determined

by liquid scintillation spectrometry. 1 μ M atropine was used to measure non-specific binding. Specific binding was obtained by subtracting the non-specific binding in the presence of 10^{-4} M atropine from the total binding in the absence of atropine.

Iodination of purified toxins

The selective toxins were iodinated for receptor autoradiographic studies according to standard procedures using chloramine T as an oxidizing agent. Excess free radioactive iodine-125 was removed by gel filtration on Sephadex G-25 containing 1-2 mg/ml serum albumin to diminish adsorption of the iodinated toxin to Sephadex.

Receptor autoradiography

For receptor autoradiography 10 μ m thick coronal brain sections from Sprague-Dawley (Paper II) or Wistar rats (Paper V), were cut [paper III, 10 μ m spinal cord sections (lumbar region) from female Wistar rats; Paper IV human hippocampal sections were used] with a microtome at -20 °C and thaw-mounted, on slides (two sections per slide) and incubated for 60 minutes with:

- a) 2 nM 125 I-MT7 (specific activity 122 Ci/mmol), or with 3 nM 125 I-M₄ toxin1 (specific activity 52 Ci/mmol) in 50 mM Na-K phosphate buffer containing 1% bovine serum albumin (**Paper II**, male Sprague –Dawley rats brain). Or
- b) 0.55 nM 125 I- M₁-toxin 1 (specific activity 41 Ci/mmol) or with 2.7 nM 125 I- M₄-toxin 1 (specific activity 82 Ci/mmol) in 50 mM Na-K phosphate buffer (pH 7.4) containing 1 % bovine serum albumin (**Paper III**, spinal cord of female Wistar rats). Or
- c) 10 nM [125 I]MT-1, M₁ selective (specific activity 37.6 Ci/mmol, K_i=22–49 nM), or with 2 nM [125 I]M₄ toxin-1, M₄ selective (specific activity 21.8 Ci/mmol, 1.4–2 nM) in 50 mM Na,K phosphate buffer containing 1% BSA. Since we had no toxin for M₂ receptors, sections were incubated with low concentration (2.4 nM) [3 H]AF-DX-384 (NEN specific activity 120 Ci/mmol, K_i=6.0 nM for M₂ and 10 nM for M₄) in 50 mM Na,K phosphate buffer, pH 7.4. At this concentration, [3 H]AF-DX-384 displays a 1.5-fold selectivity for M₂ versus M₄ receptors (**Paper IV**, human Alzheimer’s brain). Or

- d) 2 nM ^{125}I -M₁-toxin 1, (specific activity 122 Ci/mmol), or with 3 nM ^{125}I -M₄ toxin-1 (specific activity 52 Ci/mmol) in 50 mM Na-K phosphate buffer containing 1% BSA (**Paper V**, Wistar rats brain).

After incubation for 60 minutes the sections were exposed to Hyperfilm (Amersham, UK) for 6 days along with calibrated radioactive standards (^{125}I -microscales, Amersham). Specific binding was obtained by subtracting binding in the presence of 10^{-4} M atropine (nonspecific) from the binding in the absence of atropine (total binding) and it was expressed as femtomol/mg tissue wet weight (fmol/mg wet weight).

Image analysis

Quantification of autoradiograms was performed by measuring and comparing the optic density of different brain regions by computerized image analysis systems: IBAS II, Contron, Germany for paper IV, image analyzer Bio-Rad Multianalyst Version 1.1, Biorad Laboratories Inc., Life Sciences Group, CA, USA) for **papers II, V** and Image gauge 2.5.3. Science Lab 97 for **paper III**. The areas analyzed were hippocampus (dentate gyrus, CA4, CA3, CA2, CA1) and in some cases thalamus and hypothalamus areas.

CHO cell culture and membrane preparation (Paper I)

CHO cells stably expressing the cloned human M₁-M₄ receptors (CHO/M₁-M₄ cells) were grown as a monolayer in Ham's F-12 medium (GIBCO – BRL) supplemented with 10% foetal calf serum (GIBCO – BRL) in a humidified atmosphere (5% CO₂) at 37°C. Cells were grown to 80 % confluency in plastic Petri dishes (Falcon), the medium was removed, and the cells were washed with ice-cold phosphate-buffered saline (PBS). The cells were then scraped into an ice-cold buffer containing 25 mM sodium phosphate buffer (pH 7.4) and 5 mM MgCl₂ and lysed with an Ultra-Turrax homogenizer. The cell lysate was centrifuged at 32,500xg for 30 min at 4°C and the pellet was resuspended in the same buffer at a protein concentration of ~3 mg ml⁻¹. The membrane preparations were either used immediately or stored at -75°C.

Assay of guanosine -5'-O-(3-[^{35}S]-thio) triphosphate binding (Paper I)

CHO cell membranes were diluted 10 fold in an ice-cold buffer containing HEPES/NaOH (10 mM), EDTA (1 mM) (pH 7.4), centrifuged and resuspended in the same buffer supplemented with 0.1% bovine serum albumin (BSA). The binding of [^{35}S]-GTP γ S was assayed in a reaction mixture (final volume, 100 μ l) containing (mM) HEPES/NaOH 25 (pH 7.4), MgCl₂

10, EDTA 1, GDP (0.1 μ M) for M₁ and M₃ and GDP (1 μ M) for M₂ and M₄ receptor activities (Lazareno *et al.*, 1993), NaCl 100 mM, 10 kallikrein inhibitor units (KIU) of aprotinin. The membranes (1.5 – 2.0 μ g of protein) were preincubated in the presence of the indicated concentrations of ACh and MT-7 at 30°C for 30 min. The samples were then placed on ice and the reaction was initiated by the addition of 10 μ l of [³⁵S]-GTP γ S (final concentration, 1.0 – 1.5 nM). The samples were incubated for 45 min at 30°C. The reaction was stopped by the addition of 5 ml of ice-cold buffer containing HEPES/NaOH (10 mM) (pH 7.4) and MgCl₂ (1 mM), immediately followed by rapid filtration through glass fibre filters (Whatman GF/C) presoaked in the same buffer. The filters were washed twice with 5 ml of buffer and the radioactivity retained was determined by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 100 μ M GTP γ S. Assays were performed in duplicate.

[³H]-NMS binding assay (Paper I).

The binding of [³H]-NMS to CHO/M₁ cell membranes was measured in a buffer containing sodium phosphate (25 mM, pH 7.4), MgCl₂ (5 mM), 0.1% BSA, and 10 – 12 μ g of membrane protein. The final assay volume was 1.0 ml. In competition experiments, the MT-7 concentration ranged from 50 pM to 30 nM and the ³H-NMS concentrations were 0.05, 3.0 and 15 nM. The incubation was carried out at 30°C for 90 min. When the rate of dissociation of ³H-NMS was studied, the membranes were incubated with 1.0 nM ³H-NMS for 60 min before the addition of either vehicle or MT-7 (3 nM). After 20 min, atropine (10 μ M) was added to each sample and the incubation was stopped at different time intervals after the atropine addition over a total period of 60 min. To investigate the stability of MT-7 binding to M₁ receptors, CHO/M₁ cell membranes were preincubated with either vehicle or MT-7 (100 nM) for 45 min at 30°C. Thereafter, the samples were centrifuged at 32,500 \times g for 20 min at 4°C and resuspended in fresh buffer. Aliquots of the membrane suspension were incubated in the presence of 1.5 nM [³H]-NMS for the indicated times over a total period of 8 h. The incubation was stopped by adding 4 ml of ice-cold buffer without BSA to each sample followed by immediate filtration through glass fibre filters presoaked in 0.1% polyethylenimine for at least 18 h. The filters were washed twice with the same buffer, dried and the bound radioactivity was counted by liquid scintillation spectrometry. Nonspecific binding was determined in the presence of 10 μ M atropine. Assays were performed in triplicate.

N1E-115 neuroblastoma cell culture (Paper I)

Cells were obtained from European Collection of Cell Cultures (U.K.). The cells were grown in Dulbecco's modified Eagle's medium containing 2 mM glutamine and 10% foetal calf serum in 75-cm² flasks (Falcon). The medium (20 – 30 ml) was changed on day 2 of subculture and every subsequent day. Confluent cell cultures (6 – 8 days postpassage) were used for the experiments.

Assay of [³H]-inositol phosphates ([³H]-IPs) accumulation (Paper I)

N1E-115 cells were prelabelled with myo-[³H]-inositol (1 µCi ml⁻¹) in Dulbecco's modified Eagle medium for 24 h at 37°C in an incubator. The medium was then removed and the cells were washed twice with PBS. The cells were detached from the tissue culture flask by incubation in PBS containing EDTA (0.5 mM) for 5 min at 37°C followed by gentle agitation of the flask. The cell suspension was aspirated, mixed with an equal volume of PBS containing MgCl₂ (1 mM) and centrifuged at 300xg for 1 min. The cells were resuspended in a freshly oxygenated Krebs-HEPES buffer containing (mM) HEPES/NaOH 25, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 10, NaCl 110, KCl 3.8, CaCl₂ 1.2 and LiCl 10. Aliquots of the cell suspension were distributed into Bio-vials (Beckman, Ireland) and incubated for 30 min at 37°C in the presence and in the absence of MT-7. Thereafter, CCh was added as indicated and the incubation was continued for 45 min. The final incubation volume was 300 µl. The incubation was terminated by adding 940 µl of chloroform-methanol (1 : 2 v v⁻¹). After the samples were shaken for 10 min, 310 µl aliquots of chloroform and water were added. The samples were centrifuged at 1000xg for 10 min and the upper aqueous phase was applied to a column of Dowex 1x8 in the formate form. The column was washed with 20 bed volumes of H₂O, 20 bed volumes of 5 mM myo-inositol and 16 bed volumes of 5 mM sodium tetraborate in 60 mM sodium formate. [³H]-IPs were eluted by adding 6 bed volumes of 1 M ammonium formate in 0.1 M formic acid ((Berridge et al., 1983). The radioactivity present in the eluate and in the organic phase was determined by liquid scintillation counting. For each sample the accumulation of [³H]-IPs was corrected for the amount of myo-[³H]-inositol incorporated in the organic phase. Assays were performed in triplicate.

Assay of [³H]-cyclic AMP accumulation (Paper I)

N1E-115 cells grown in 36-mm plastic dishes were incubated in Dulbecco's modified Eagle medium containing 2 µCi ml⁻¹ of [³H]-adenine for 1 h at 37°C in an incubator. Thereafter, the medium was removed and the cells were incubated in an oxygenated Krebs-HEPES buffer

containing 3-isobutyl-1-methylxanthine (1 mM) in the absence and in the presence of MT-7 for 30 min at 37°C. Pituitary adenylate cyclase activating polypeptide (PACAP) 38 and CCh were then added as indicated and the incubation was continued for 10 min. The incubation was stopped by the aspiration of the medium and the addition of an ice-cold solution containing 6% (w v⁻¹) perchloric acid and 0.1 mM [¹⁴C]-cyclic AMP (~3000 c.p.m.). After 30 min at ice-bath temperature, the solution was neutralized by the addition of ice-cold 0.6 M KOH and left on ice for additional 30 min. Following centrifugation at 15,000xg for 5 min, the supernatant was collected, and [³H]-cyclic AMP was isolated (Salomon, 1979). The recovery of [³H]-cyclic AMP from each sample was corrected on the basis of the recovery of [¹⁴C]-cyclic AMP. Assays were performed in triplicate. Protein content was determined by the method of Bradford (Bradford, 1976) using BSA as a standard.

Adrenalectomy (Paper V)

Bilateral adrenalectomy was performed as described (Adem et al., 1994). Briefly, male Wistar rats (n = 100) were adrenalectomized under Pentobarbital (60 mg/ml) anaesthesia. As controls sham operated (n = 50). The adrenalectomised (ADX) rats received 0.9% NaCl in their drinking water to maintain salt balance. The rats were weighed weekly until the treatment period set (e.g. 20 weeks for the group of rats undergoing 20 weeks treatment) and their weight was documented to follow the changes of weight after adrenalectomy. The rats were sacrificed by decapitation 3, 14, 30, 90 and 150 days after adrenalectomy. At each time point 20 ADX and 10 sham operated rats were sacrificed. The brains were taken out immediately and frozen on dry ice and stored at -70°C until used for receptor autoradiography studies.

Electrophoresis of proteins and Western blot analysis (Paper V).

For Western blott studies, the whole brain of ADX (n = 5) and Sham operated rats (n = 5), one month after surgery were removed and the hippocampii were dissected on ice. The hippocampii were homogenized with a polytron at 4°C in homogenization buffer (50 mM HEPES, 150 mM NaCl, 50 nM NaF, 1 mM Na₃VO₄, 10% glycerol, 10% Triton X-100, 1 µg/ml Leupeptin, 1 mM PMSF, pH 7.2). The homogenate was centrifuged at 17000 x g for 15 minutes at 4°C. An aliquot of the supernatant was kept for protein determination and SDS sample buffer containing; beta-mercaptoethanol was added to the remainder. The samples were boiled for three minutes and after cooling, the equivalent of 30 µg, of protein/lane was loaded on to a 12% polyacrylamide gel. The samples were electrophoresed under constant

voltage, transferred on to nitrocellulose membranes which had been blocked with 50% non-fat milk. To determine whether neuronal cell death after adrenalectomy was apoptotic, levels of PARP, p53 and Bcl 2 were analysed by Western blott using specific polyclonal antibodies (Santa Cruz Biotechnology, USA). Antibodies were applied at a dilution of 1:1000 or 1:2000 (PARP) and incubated at room temperature for 2 hours, washed and then incubated with the secondary antibody (1:2000) conjugated to horseradish peroxidase. Anti-PARP, p53 and Bcl 2 antibody binding, was visualized by enhanced chemiluminescence. Qualitative determination of the abundance of muscarinic M₁ and M₄ receptor subtypes in the whole hippocampus of sham operated and adrenalectomized rats was performed by Western blotting using M₁ and M₄ subtype specific antibodies (Research and Diagnostic Antibodies, California, USA). For M₁ and M₄ receptors, hippocampal membrane homogenates were incubated with M₁ (1:100) and M₄ (1:100) polyclonal antibodies and then incubated with the secondary antibody conjugated to horseradish peroxidase. Anti- M₁ and M₄ antibody binding was visualized by enhanced chemiluminescence as described above.

Statistical analyses

Statistical analyses were performed according to Fisher's least significant difference method for multiple comparisons after a one-way analysis of variance (ANOVA).

Results from cell culture studies (Paper I) were given as mean±standard error of the mean (s.e.mean). Concentration – response curves were analysed by a least squares curve-fitting computer programme (GraphPAD Prism, San Diego, CA, U.S.A.). Statistical significance of the difference between means was determined by Student's *t*-test.

RESULTS AND DISCUSSIONS

Paper I

In membranes of CHO/M₁ cells, MT-7 caused a concentration-dependent reduction of the maximal stimulation of [³⁵S]-GTPγS binding obtained by ACh. At 1.0, 10 and 30 nM, MT-7 inhibited the maximum agonist response by 15.7±1.5 (*P*<0.01), 50.5±3.1 and 72.8±1.7% (*P*<0.001), respectively. The EC₅₀ values of ACh were 6.0±0.8, 6.8±0.5, 13.0±1.2 and 7.3±1.0 μM in the absence and in the presence of 1.0, 10 and 30 nM MT-7, respectively. However MT-7 did not affect the ACh- induced stimulation of [³⁵S]-GTPγS binding to M₂, M₃, and M₄ receptors. Thus, at nanomolar concentrations MT-7 significantly inhibited the stimulation of [³⁵S]-GTPγS binding obtained by ACh at M₁ receptors but did not affect the response to the agonist when M₂, M₃ and M₄ receptors were stimulated. At each receptor subtype, the toxin failed to stimulate basal [³⁵S]-GTPγS binding, indicating a lack of agonist activity.

Carbachol (CCh) increased [³H]-IPs accumulation by approximately 5 fold with an EC₅₀ value of 33.8±4.0 μM, in N1E-115 cells. Addition of MT-7 at 0.3, 1.0 and 3.0 nM reduced the maximal agonist stimulation by 35.1±3.5, 69.3±2.1 and 84.9±1.3% (*P*<0.001), respectively. The agonist EC₅₀ values were 54.0±6.0, 100±9.0 and 89.9±7.0 μM at 0.3, 1.0 and 3.0 nM MT-7, respectively. This shows as that as in neuroblastoma cells, MT-7 potently inhibited the CCh stimulation of [³H]-IPs accumulation, a response previously proposed to be mediated by endogenous M₁ receptors (Kamba et al., 1990). In these cells, the toxin did not affect the CCh inhibition of PACAP 38-stimulated cyclic AMP accumulation, an effect mediated by M₄ receptors (McKinney et al., 1991; Olanas et al., 1999). Collectively, these functional data indicate that MT-7 is an antagonist of the M₁ receptor and confirm the subtype selectivity previously observed in radioligand binding studies (Adem and Karlsson, 1997).

In neuroblastoma cells, while CCh caused a concentration-dependent inhibition of PACAP 38 (10 nM)-stimulated [³H]-cyclic AMP accumulation with a maximal effect corresponding to a 35.5±2.1% (*n*=5, *P*<0.001) reduction and an EC₅₀ value of 5.6±0.8 μM. Addition of MT-7 (5 nM) failed to affect the CCh inhibitory effect in these cells. This shows that, the toxin did not affect the CCh inhibition of PACAP 38-stimulated cyclic AMP accumulation in these cells, an effect mediated by M₄ receptors (McKinney et al., 1991; Olanas et al., 1999).

To further characterize the mode of toxin action on M₁ receptors, radioligand binding studies were conducted in CHO/M₁ cell membranes.

In competition experiments, MT-7 caused a concentration-dependent reduction of specific [³H]-NMS binding at different radioligand concentrations. However, the toxin consistently failed to cause a complete inhibition of [³H]-NMS binding. This behaviour was particularly evident at 3 and 15 nM [³H]-NMS, where the maximal inhibitions elicited by 25 nM MT-7 corresponded to ~93 and 82% of total specific binding. The toxin IC₅₀ values were 0.26±0.02, 0.27±0.03 and 0.48±0.05 nM at 0.05, 3 and 15 nM [³H]-NMS, respectively.

This shows that the toxin was not able to completely inhibit the binding of the radioligand and the fraction of [³H]-NMS not displaced by the toxin increased with increasing concentrations of the radioligand. In addition, the IC₅₀ values of the toxin were little affected by large increases in the concentration of the radiolabelled ligand.

In dissociation experiments, MT-7 (3 nM) markedly decreased the rate of atropine-induced dissociation of [³H]-NMS from M₁ receptors. The estimated dissociation rate constants were 0.05 and 0.01 min⁻¹ in control and toxin-treated membranes, respectively. This finding indicates that MT-7 is capable of binding to the M₁ receptor also when the primary binding site is occupied by a competitive ligand and, by acting on the secondary allosteric site, can sterically regulate the accessibility of the primary binding site to competitive ligands.

To investigate the reversibility of MT-7 binding, CHO/M₁ cell membranes were pretreated with either vehicle or MT-7 (100 nM), washed by centrifugation and resuspension and assayed for [³H]-NMS binding. In membranes pretreated with MT-7 there was a reduction in radioligand binding, which remained constant for at least 8 h. The binding withstood washing and resuspension of the membranes and was not reversible for at least 8 h at 30°C, as judged by the lack of recovery of [³H]-NMS binding to control values.

Overall the results obtained from these studies indicate that MT7 behaves as an allosteric antagonist which binds to a secondary site to decrease the affinity of the radioligand.

In conclusion, the present study shows that MT-7 behaves as a selective and noncompetitive antagonist of the cloned and native muscarinic M₁ receptor subtype. These properties make MT-7 an unmatched tool for the identification and characterization of M₁ receptors in different biological systems.

Paper II

Age-related changes in the status of muscarinic cholinergic receptors have been reported in human and animal brain. Both a decrease and no change in muscarinic receptors in human hippocampus with increasing age has been reported (Giacobini, 1990). In the hippocampus of aged rats large, modest or no decreases in muscarinic binding sites have been described (Decker, 1987; Gilad and Gilad, 1987; Biegon et al., 1989). Data on the influence of aging on hippocampal muscarinic cholinergic receptors are conflicting because all these studies were performed using non-selective ligands

In this study we assessed muscarinic M₁, and M₄ receptors in the hippocampus and entorhinal cortex of young (21 days), adult (3 months) and old (25 months) rats by autoradiography using selective ligands, ¹²⁵I-M₁ toxin-1 and ¹²⁵I-M₄ toxin-1.

In the hippocampal regions dentate gyrus and CA4 to CA1 the level of M₁ receptors increased with age (Fig 1, paper II). The increase in 25-month-old rats as compared to 21-day-old rats was significant in dentate gyrus (p<0.05), CA4 (p<0.01), CA3 (p<0.01) , CA2 (p<0.01) and CA1 (p<0.001). Furthermore, as compared to adult rats, (3 months old), 25 months old rats showed significant increase (p < 0.05) of M₁ receptors in CA1. In entorhinal cortex M₁ receptors were constant from 21 days to 25 months. M₁ receptors do not seem to be involved in memory functions, because knock out mice lacking M1 receptors did not show any signs of memory impairment (Miyakawa et al., 2001).

M₄ receptors decreased significantly in CA1 (p<0.001), entorhinal cortex (p<0.001) and were constant in dentate gyrus, CA4, CA3 and CA2 (Fig. 2, Paper II) of 25 months old rats compared to 21 days old rats. Significant loss (p< 0.001) of M₄ receptors was also observed in entorhinal cortices of 25 months old rats as compared to 3 months old rats. However, no loss of M₄ receptors was observed in dentate gyrus, CA1, CA2 and CA4 areas of 25 months old rats as compared to 3 months old rats.

The increase of M₁ receptors in old aged rats compared to 21-days old rats could be due to neural changes occurring during brain development. neural changes occurring during brain development. However, using M1 and M4 selective toxins no changes were observed in the level of M₁ (except in CA2) or M4 (except in entorhinal cortex) in the hippocampus and entorhinal cortex of 25-months old rats compared to adult rats. Thus, the M₄ receptor subtype which is suggested to be involved in memory functions was not changed in the hippocampus of aged rats compared to adult rats. In order to have reliable data on the status of muscarinic receptor changes relevant to memory function in old rats, the aged rats should be divided by behavioural tests into those with impaired and those with normal cognitive functions..

Paper III

A major site of analgesic action of cholinergic agents is the spinal cord. ACh is considered to be one of the major neurotransmitters in pain modulation. It is therefore believed that ACh plays a role as an analgesic substance in the spinal cord, this was subsequently confirmed by spinal administration of muscarinic receptor agonists or acetylcholinesterase inhibitors that produced effective pain relief (Hartvig et al., 1989; Eisenach, 1999). However, the analgesic mechanisms and the site of actions of cholinergic agents in the spinal cord are not fully defined (Eisenach, 1999). Muscarinic receptors are present in the superficial and deep dorsal horn of the spinal cord, areas of nociceptive information transmission and modulation (Barber et al., 1984; Eisenach, 1999). Spinal injection of cholinergic agonists results in analgesia, which primarily reflects muscarinic receptor activation. However, which muscarinic receptor subtype is involved is still a matter of controversy. Thus in this study we investigated, using muscarinic receptor subtype selective toxins, the levels of M₁ and M₄ muscarinic receptor subtypes in acute and chronic arthritic rats.

The first signs of inflammation (arthritis) in rats inoculated with mycobacterium appeared in the ankle joints starting between days 9-11. There was symmetrical hind paw swelling, increased warmth, and redness, which persisted until the end of the experiment. Histological analysis (hemotoxylin-eosin staining) showed synovial hypertrophy and round cell infiltration. On day 30, X-rays of the inflamed joints of chronic rats showed soft tissue swelling, decreased bone density (osteoporosis), cartilage loss and bone destruction. Rats in the control group showed no macroscopical, histological, or radiographic signs of joint inflammation (results not shown).

The binding of M₁ and M₄ selective toxins was measured in the lumbar region of the spinal cord. Using M₁ specific toxin ¹²⁵I- M₁-toxin 1 we showed only background levels of radioactivity in the dorsal horns of control, acute and chronic arthritic rats. However, the binding of ¹²⁵I- M₄-toxin 1 was significantly reduced both in acute and chronic arthritic rats as compared to controls (Fig. 1, Paper III) indicating loss of muscarinic M₄ receptors. As seen in Fig.1 in Paper III the loss of M₄ receptors in the different Rexed laminae was: In Rexed laminae (I-II) 89 % in acute and 87 % in chronic arthritis (p<0.0001). In Rexed laminae (III-V) 89 % in acute and 88 % in the chronic (p<0.0001), in (VI) 89 % in both cases (p<0.0001) and in (VII-IX) 88 % in both acute and chronic arthritis. Finally the reduction in Rexed laminae X was 87% in the acute and 88% in the chronic rats (p<0.0001). No significant difference was observed in the binding of ¹²⁵I- M₄-toxin 1 between the acute and

chronic rats. Figures 2a and 2b of Paper III show representative autoradiograms of ^{125}I - M_4 -toxin 1 binding in the spinal cord of control and chronic rats respectively along with non specific binding represented in Fig. 2c of Paper III.

Although it is difficult to measure and demonstrate persistent pain in laboratory animals, many studies indicate that rats with adjuvant arthritis hyperventilate and self-administer opiates, suggesting that arthritis in rats offers may be an animal model of pain (Walker et al., 1999; Colpaert et al., 2001). ACh is released in response to physiologic signals such as pain (Eisenach, 1999). This hypothesis of pain induced activation of spinal ACh release is further supported by functional studies, in which spinal neostigmine in sheep produces antinociception which is greatest in the first day after laminectomy surgery, less the following day and absent 5 days after surgery (Bouaziz et al., 1995; Eisenach, 1999). Spinally released ACh activates muscarinic receptors. It has been demonstrated that acute and chronic stimulation of G-protein coupled receptors for classical neurotransmitters by cholinesterase inhibitors induces a dramatic decrease of the density of muscarinic receptors at the plasma membrane probably by increasing extra neuronal content of endogenous acetylcholine (Koenig and Edwardson, 1996, 1997). Thus the decrease in muscarinic M_4 receptors observed in our study indicates that these receptors were subjected to long-term stimulation by high levels of endogenous acetylcholine which is released to inhibit the acute and chronic pain.

These findings suggest that the muscarinic M_4 receptor subtype may be involved in cholinergic mechanisms of analgesia.

Paper IV

In the present study, mAChR changes were investigated in postmortem hippocampal brain sections from AD patients and controls using ^{125}I -MT-1 (M_1 selective), ^{125}I - M_4 toxin-1 (M_4 selective) and ^3H -AFDX-384 (M_2 partial selective). The main finding of this study was a significant decrease in M_4 receptor binding in the dentate gyrus and CA4 regions of the hippocampus of AD patients compared to controls. Our findings are in contrast to those obtained by immunoprecipitation using monoclonal antibodies in which a 25% increase in M_4 receptors was reported (Flynn et al., 1995; Levey, 1996). Our results showed no change in the levels of muscarinic M_1 receptors in the hippocampus of AD patients compared to controls indicating that hippocampal M_1 receptors are not affected in AD. In contrast to previous reports, which showed decreases in M_2 receptors (Flynn et al., 1995; Levey, 1996) we found no significant differences in the level of M_2 receptor binding between control and AD brains.

However, AFDX-384 even at such a low concentration is not a selective ligand to assess M₂ receptor changes.

mAChRs can modulate hippocampal function through at least two different ways: a) Presynaptic inhibition of the release of various transmitter substances (Russo et al., 1993; Levey, 1996; van der Zee and Luiten, 1999).. b) Postsynaptic excitation of central synapses (Levey, 1996). Although the functional role of the M₄ receptor is not yet clear, it has been suggested that one effect of acetylcholine on this receptor subtype is to modulate the release of neurotransmitters. Injection of the M₄ toxin-1 into hippocampus of rats causes amnesia (Jerusalinsky et al., 1998). If M₄ receptors also have a similar role in humans, the decrease in M₄ receptors may be responsible for memory deficits in Alzheimer's disease.

Paper v

Long-term adrenalectomy induces a dramatic loss of cells in the dentate gyrus and CA1-CA4 fields of the hippocampus resulting in impairment of cognitive functions such as spatial learning, memory and exploratory behaviour. The present study was designed to study the effect of corticosteroid hormone deprivation (as a result of adrenalectomy) on the level of entorhinal and hippocampal M₁ and M₄ muscarinic receptor binding. Efficiency of adrenalectomy was confirmed by analysis of plasma corticosterone level using radioimmunoassay method. Adrenalectomized rats showing level of corticosterone below 20 ng/ml (below the level of detection) were regarded as completely adrenalectomized. Neuronal changes of M₁ and M₄ receptor levels were investigated using receptor autoradiography in the entorhinal cortex and the hippocampus of sham operated and adrenalectomized rats 3, 14, 30, 90 and 150 days after adrenalectomy. Moreover, the level of hippocampal M₁ and M₄ muscarinic receptors were evaluated 1 month after adrenalectomy by immunoblot analysis with anti M₁ and anti M₄ antibodies respectively. We also investigated apoptotic markers as well as the involvement of other proteins such as p53 and BCL-2, which modulate apoptosis. Nissl staining of hippocampal sections from adrenalectomized and sham-operated rats are shown in Figure 1 (Paper V). Loss of hippocampal granule cells 30, 90 and 150 days after adrenalectomy is illustrated in Fig. 1 H-J (Paper V). Cell loss appeared first in the dorsal blade of the granule cells and almost at the lateral end of the blade.

Densitometric analysis of Western blots from sham operated and adrenalectomized rats hippocampal homogenates 1 month postoperative showed a decrease in the level of M₄ receptors in adrenalectomized rats as compared with sham groups using M₄ specific antibody (Fig 2, Paper V). Quantitative autoradiography was employed to determine the changes of

receptor levels. Binding results showed significant change in the levels of muscarinic M₄ but not M₁ receptors (Fig 3, 4, , Paper V), 120 in the entorhinal cortex, the dentate gyrus and in the different CA fields of the hippocampus of adrenalectomized rats 3, 14, 30, 90 150 days after bilateral adrenalectomy compared to sham operated rats. The loss of M₄ receptors in the different areas of the hippocampus and entorhinal cortex varied with time after adrenalectomy. The vulnerability is in the order entorhinal cortex > dentate gyrus, CA4 > CA3 > CA2, CA1. Taken together our Western blot and receptor autoradiography results indicate ¹²⁵I-M₄ toxin 1 is binding to the muscarinic receptor protein. Our results show that M₁ and M₄ receptors are differentially affected by adrenalectomy. These results indicate that each subtype likely has a different role in the hippocampus. The M₄ receptor subtype loss might lead to dysfunction in hippocampal synaptic transmission, ultimately affecting memory process.

The perforant pathway originates in the entorhinal cortex, where layer II pyramidal cells provide a massive innervation of dentate granule cell dendrites in the molecular layer, and where layer III pyramidal cells project to the CA 1 region of the hippocampus proper (Steward, 1976; Steward and Scoville, 1976). These pyramidal cells in layer II and III of the entorhinal cortex receive cholinergic input from the basal forebrain. The primary postsynaptic action of Ach on perforant pathway cells is excitation via muscarinic receptors (Klink and Alonso, 1993; Klink R, 1994). Cholinergic stimulation in these cells is antagonized by low concentrations of pirenzepine (Klink R, 1994) indicating the involvement of receptors of the M₁- family which include M₁, M₃, and/or M₄ receptors (Buckley et al., 1989; Dorje et al., 1991a). Rouse and Levey (Rouse and Levey, 1996) suggested that M₁ and M₃ receptors are the best candidates for mediating these postsynaptic effects.

In contrast to Rouse and Levey (Rouse and Levey, 1996) our results using selective ligands indicate that receptors in the entorhinal cortex are of the M₄ and M₁ subtype. However, the presence of M₂, M₃ and/or M₅ receptors cannot be ruled out. Our results showed that cells displaying M₄ but not M₁ muscarinic receptors in the entorhinal cortex are highly vulnerable to adrenalectomy. Following the entorhinal cortex, the areas most vulnerable were the dentate gyrus, an area which receives massive innervation from layer II pyramidal cells of the entorhinal cortex, and CA4 of the hippocampus. However, in the CA1, which receives innervation from layer III of the entorhinal cortex, the M₄ receptors were less vulnerable to adrenalectomy. It remains to be seen if there is a link between the loss of muscarinic receptors in the entorhinal cortex and the dentate gyrus. The loss of these receptors could be, in addition to being from the dentate gyrus cells also from afferent terminals in the dentate gyrus such as

the perforant pathway, septohippocampal pathway, and commissural and associational pathways. The CA4 area similar to the dentate gyrus also showed significant loss of M₄ receptors 14 days after adrenalectomy. Similarly the loss of muscarinic receptors in the CA3, CA2 and CA1 could be in addition to being from cells in these areas also from afferent terminals in these areas. The M₄ receptor subtype lost in the dentate and CA fields of adrenalectomized rats could either be pre- or post- synaptically localized and that in either case lead to dysfunction in hippocampal synaptic transmission, ultimately affecting memory process.

Recently a study with mice lacking the M₁ muscarinic receptor indicate that the M₁ receptor plays an important role in the regulation of locomotor activity but appear to be less critical for cognitive processes (Miyakawa *et al.*, 2001). Jerusalinsky *et. al* reported injection of M₄-toxin 1, a selective M₄ antagonist, into hippocampus of rats causes amnesia (Jerusalinsky *et al.*, 1998). Muscarinic transmission is also implicated in disease states in which cholinergic systems are disturbed and deficits in memory and attention result (Drachman and Leavitt, 1974; Bartus *et al.*, 1982; Dekker *et al.*, 1991). We have recently reported a specific loss of muscarinic M₄ receptors in the dentate and CA4 region of the hippocampus of post mortem Alzheimer's brains (Mulugeta *et al.*, 2003). Based on our findings (El-Bakri *et al.*, 2002; Mulugeta *et al.*, 2003) we have suggested that the M₄ muscarinic receptor subtype might be involved in memory functions. We and others have shown that long-term adrenalectomy results in memory impairment (Islam *et al.*, 1995, (McNaughton *et al.*, 1989; Sapolsky *et al.*, 1991; Armstrong *et al.*, 1993; Conrad and Roy, 1993). The memory impairment observed in long-term adrenalectomized rats could be due to loss of the M₄ receptor subtype. Thus our present findings that the M₄ receptor is significantly reduced in the hippocampus of adrenalectomized rats further supports the suggestion that the M₄ receptor subtype might be involved in memory functions.

The adrenalectomized rat model has shown damage of the hippocampal granule and pyramidal cells in different neurodegenerative conditions (Sloviter *et al.*, 1989; Sapolsky *et al.*, 1991; Adem *et al.*, 1994). It is known from morphological studies that bilateral adrenalectomy in adult rats results in substantial decrease in dentate granule cells through apoptosis (Sloviter *et al.*, 1993; Islam *et al.*, 1999). Apoptosis might be a crucial event in the progression of the neurodegeneration seen after adrenalectomy. The activation of caspase-3 triggers a series of events. Caspase-3 mediated apoptosis was confirmed in our study by detection of PARP cleavage in adrenalectomized rat hippocampal homogenates. Our results also demonstrate increased expression levels of p53 in adrenalectomized rat hippocampal

homogenates. Induction of p53 is yet another mechanism of cellular response to DNA damage and cellular distress signals (Schuler and Green, 2001) resulting in apoptosis.

BCL-2 is a suppressor of apoptosis (Sun et al., 2002). The 1 month adrenalectomized rats showed a significant decrease in the expression levels of BCL-2 in their hippocampal homogenates as compared to controls. Generally, under these conditions of reduced BCL-2 expression, there is a shift in the balance towards apoptosis that was evident in the adrenalectomized rat hippocampii. Our findings are in line with those of Greiner et al. 2001 which demonstrated reduced expression of bcl-2 mRNA in male adult Sprague Dawley rats five days after adrenalectomy. Although our previous studies have shown that apoptotic cell death is increased in hippocampii of long-term adrenalectomized rats [Islam et al., 1999], the evidence was based on morphological observations and did not define the mechanisms through which the apoptosis occurred. It could be speculated that the biochemical changes seen in 5 days (Greiner et al. 2001) and 1 month (this study) adrenalectomized rat hippocampii could be the basis of the morphological changes observed after long-term adrenalectomy. Our findings suggest that adrenalectomy induces apoptosis in part through the activation of a caspase cascade.

Our data on time and region-dependent decreases in hippocampal M₄ receptor binding indicate that the M₄ receptor subtype is influenced by adrenal hormones and suggest that the M₄ receptor might be linked to memory function in the hippocampus.

CONCLUSIONS

Paper I

The pharmacological profile of M₁-toxin 1 (MT 7) was presented. M₁-toxin 1 behaves as a selective and noncompetitive antagonist of the cloned and native muscarinic M₁ receptor subtype by binding stably to an allosteric site. These properties make MT-7 an unmatched tool for the identification and characterization of M₁ receptors in different biological systems.

Paper II

The effect of ageing on the level of M₁ and M₄ mAChR subtypes was demonstrated. A significant increase in muscarinic M₁ receptor binding in all areas of the hippocampus and a significant loss in M₄ binding only in the CA1 region and entorhinal cortex have been observed in 25-month-old compared to 21-days-old rats. The increase of M₁ receptors in old aged rats compared to 21-days old rats could be due to developmental changes. However, compared to adult rats no changes were observed in the level of M₁ (except in CA2) or M₄ (except in entorhinal cortex) in the hippocampus and entorhinal cortex of 25-months old rats. Thus, the M₄ receptor subtype which is suggested to be involved in memory functions was not changed in the hippocampus of aged rats compared to adult rats. In order to have reliable data on the status of muscarinic receptor changes in old rats relevant to memory function, the aged rats should be divided into those with impaired and those with normal cognitive functions by behavioural studies.

Paper III

M₄ receptors loss was demonstrated for the first time in the spinal cord of animal models of pain. Down-regulation of muscarinic M₄ receptors in the dorsal horn is the result of prolonged stimulation by high levels of endogenous acetylcholine released in response to the pain stimuli. It could be speculated that the muscarinic M₄ receptor is involved in cholinergic mechanisms of analgesia. Thus muscarinic M₄ selective agonists could be useful as analgesics.

Paper IV

Loss of muscarinic M₄ receptors in the dentate gyrus and CA4 regions of the hippocampus of Alzheimer brains was demonstrated. This finding suggests that, relative to other muscarinic

receptor subtypes (M_1 and M_2), M_4 could be the subtype that is selectively compromised in Alzheimer's disease.

Paper V

Temporal and region-dependent changes in muscarinic M_4 but not M_1 receptor were demonstrated in the hippocampus and entorhinal cortex of adrenalectomized rats. M_1 and M_4 receptors are differentially affected by ADX and indicate that the M_4 receptor subtype is influenced by loss of adrenal hormones. The M_4 receptor subtype loss might lead to dysfunction in hippocampal synaptic transmission, ultimately affecting memory processes.

FUTURE PERSPECTIVES

Venoms and toxins of natural origin have long been important because of their pharmacological effects. Toxins have been used to elucidate physiological mechanisms. They can also be used therapeutically, or as the starting point in the design of new therapeutic agents. For example, tubocurarine is a muscle relaxant accompanying general anaesthetics. It is isolated from the South American arrow poison curare which contains several alkaloids. It has also been used as a lead compound in the development of new drugs, e.g. pancuronium and alcuronium. The acetylcholinesterase inhibitor physostigmine from the seeds of the West African plant calabar bean *Physostigma venenosum* is widely used and is also a lead compound for other anticholinesterase agents, such as neostigmine and edrophonium. Omega-conotoxin from the fish hunting snails *Conus* blocks one subtype of voltage-dependent calcium channels and is used as an analgesic drug. In contrast to morphine, prolonged use of omega-conotoxin does not give rise to addiction.

Mamba snake venoms contain several selective toxins. A number of muscarinic toxins were isolated from the venom of the Eastern green mamba *Dendroaspis angusticeps* and the black mamba *D. Polylepis*. To date, 10 muscarinic toxins have been isolated and their sequences determined. Some of these toxins, M_1 -toxin 1, M_2 toxin, and M_4 -toxin1, are highly selective for M_1 , M_2 , and M_4 subtypes of muscarinic receptors, respectively. A future challenge is the isolation of the M_3 and M_5 subtype selective toxins. Muscarinic receptors are widely distributed in the body, and are involved in the regulation of many physiological processes including pain, heart rate and memory.

Subtype selective toxins are important tools to determine the level and distribution of the different subtypes as well as to understand the role of the different subtypes in diseases associated with dysfunction of the cholinergic system. Conventional drugs that act via muscarinic receptors do not discriminate sufficiently between the different subtypes of receptors and consequently cause unwanted side-effects. Studies on the structure-activity relationships of the muscarinic toxins may provide leads to future design of more specific drugs with fewer side-effects.

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