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# A NOVEL SUBTYPE OF MUSCARINIC RECEPTOR IDENTIFIED BY HOMOLOGY SCREENING

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A new member of the protein superfamily of G-protein coupled receptors has been isolated by homology screening. By virtue of its homology with other muscarinic acetylcholine receptors and its ability to bind muscarinic specific antagonists, this muscarinic receptor subtype is designated M4. The M4 mRNA is preferentially expressed in certain brain regions. The existence of multiple receptor subtypes encoded by distinct genes in the brain has functional implications for the molecular mechanisms underlying information transmission in neuronal networks. 

Acetylcholine plays a key role in the neuronal mechanisms underlying memory, learning, arousal and control of movement. The majority of cholinergic synapses in the vertebrate central nervous system are muscarinic. Muscarinic acetylcholine receptors (mAChR) are widely distributed, being present in neurons of the central and peripheral nervous systems, in cardiac and smooth muscle tissue and in exocrine glands. Their activation results in a variety of G-protein mediated events including the inhibition of adenylate cyclase, activation of phosphoinositide turnover, stimulation of cGMP synthesis and regulation of potassium channels (for a review, see ref. 1).

Existence of at least two different subtypes of the mAChR was predicted on the basis of differential binding affinities for selective muscarinic receptor antagonists (2-4). Recently, cDNAs for mAChRs have been isolated from either porcine cerebral cortex or heart and were shown by functional expression to encode two different subtypes M1 and M2, respectively (5-7). Pharmacological evidence supports the possibility that additional mAChR

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subtypes contribute to the observed diversity in mAChR responses (8). Such other mAChR subtypes may share structural homologies with the M1 and M2 subtypes, thus providing a basis for the isolation of cDNAs encoding receptor subtypes by screening for homologous clones.

Sequence comparisons of the two mAChR subtypes M1 and M2 and of other G-protein coupled receptors (e.g. the opsins and β-adrenergic receptor) show that all G-protein coupled receptors possess a similar tertiary structure (5, 9). Most characteristically, they all possess seven hydrophobic membrane spanning regions. For each of these receptors, a particularly well conserved sequence motif occurs between the transmembrane regions (TM) 1 and 2. We have utilized this intrinsic homology for screening a cDNA library derived from rat forebrain mRNA and have isolated a novel subtype of the mAChR.

## MATERIALS AND METHODS

A λgt10 rat forebrain cDNA library (10) was screened with two 50-mer oligonucleotide pools RSβ1 (5' CTGCAGACRGTCACCAAMTACTTYATCACC TCCYTGGCCTGTGCTGATCT 3') (IUPAC-IUB ambiguous base code) and RSM2 (5' CTCCAGACRGTCAACAAYTACTTYYTGTTCAGCYTGGCCTGTGCTGACCT 3') and rescreened with shorter more degenerate pools RS3a-f (5' WSCYTVGCYTGYGCYGAYYT 3') and RS4a-f (5' CARACWGTBAMYAAYTAYTTYMT 3'). Filters were washed in 1xSSC or 5xSSC at 42°C for both 50-mers and for the RS4a-f pool, respectively and in 3M TMACl (11) at 50°C for the RS3a-f pool. DNA sequences were obtained by the chain-termination method using M13 vectors (12).

Cellular expression data were obtained using the host vector system and transformation procedures described by Eaton et al. (13). Membrane fractions of transfected cells were assayed for $^{3}$H-QNB (quinuclidinylbenzilate) binding as described (7). In situ hybridization with $^{32}$P-labelled cRNA prepared using T7 RNA polymerase utilized a subtype-specific (TM5-6) 358bp StuI-PstI DNA fragment (nt 936 - 1294) subcloned into vector pGEMII (Promega). Hybridization was performed as described (14). Following RNase digestion the sections were washed 2 times in 2xSSC, 0.05% inorganic sodium pyrophosphate for 10 minutes each at room temperature, followed by 2 washes at 50°C in 0.1xSSC, 0.05% inorganic sodium pyrophosphate for 15 minutes each prior to film autoradiography.

## RESULTS AND DISCUSSION

The superfamily of G-protein coupled receptors (opsins, β-adrenergic and muscarinic receptors) contain a highly conserved sequence between TM 1 and 2. The consensus amino acid sequence for this region is L-Q-T-V-N/T-N-Y-F-L/I-L/T-S-L-A-C-A-D-L. We used two degenerate 50-mer oligonucleotides (RSβ1 and RSM2) spanning this entire region to screen $2x10^{6}$ clones of a rat forebrain cDNA library. Two cDNA clones with a 3.8kb insert were obtained. These clones also hybridized to the two other highly degenerate oligonucleotide pools (RS3a-f and RS4a-f) which are based on either the 5' or 3' sequences of the longer probes. DNA sequence analysis (Fig. 1)

Figure 1. Nucleotide sequence of the rat mAChR M4 cDNA. The 589 amino acid M4 polypeptide within the sequenced 2341bp region is indicated. Potential N-linked glycosylation sites are boxed and hydrophobic transmembrane domains are underlined. An inframe 5' stop codon (TAA) is indicated by asterisks.

indicated a single long open reading frame encoding a polypeptide of 589 amino acids with an Mr of 66,151 daltons. The initiation codon is assigned to the first ATG triplet encountered (nt 132) which also appears downstream of an in-frame nonsense codon, TAA (nt 87-89).

The hydropathy profile of this protein predicts seven membrane spanning regions within which the homologies to the other G-protein coupled receptors is greatest. The predicted amino acid sequence is most homologous to the muscarinic receptor class possessing 45.5% and 39.3% overall amino acid identity with the porcine M1 (5) and M2 (6, 7) subtypes, respectively (Fig. 2). Following the nomenclature of Peralta and Capon who have characterized by functional expression the human genomic mAChR subtypes (15), the mAChR in this report was designated M4.

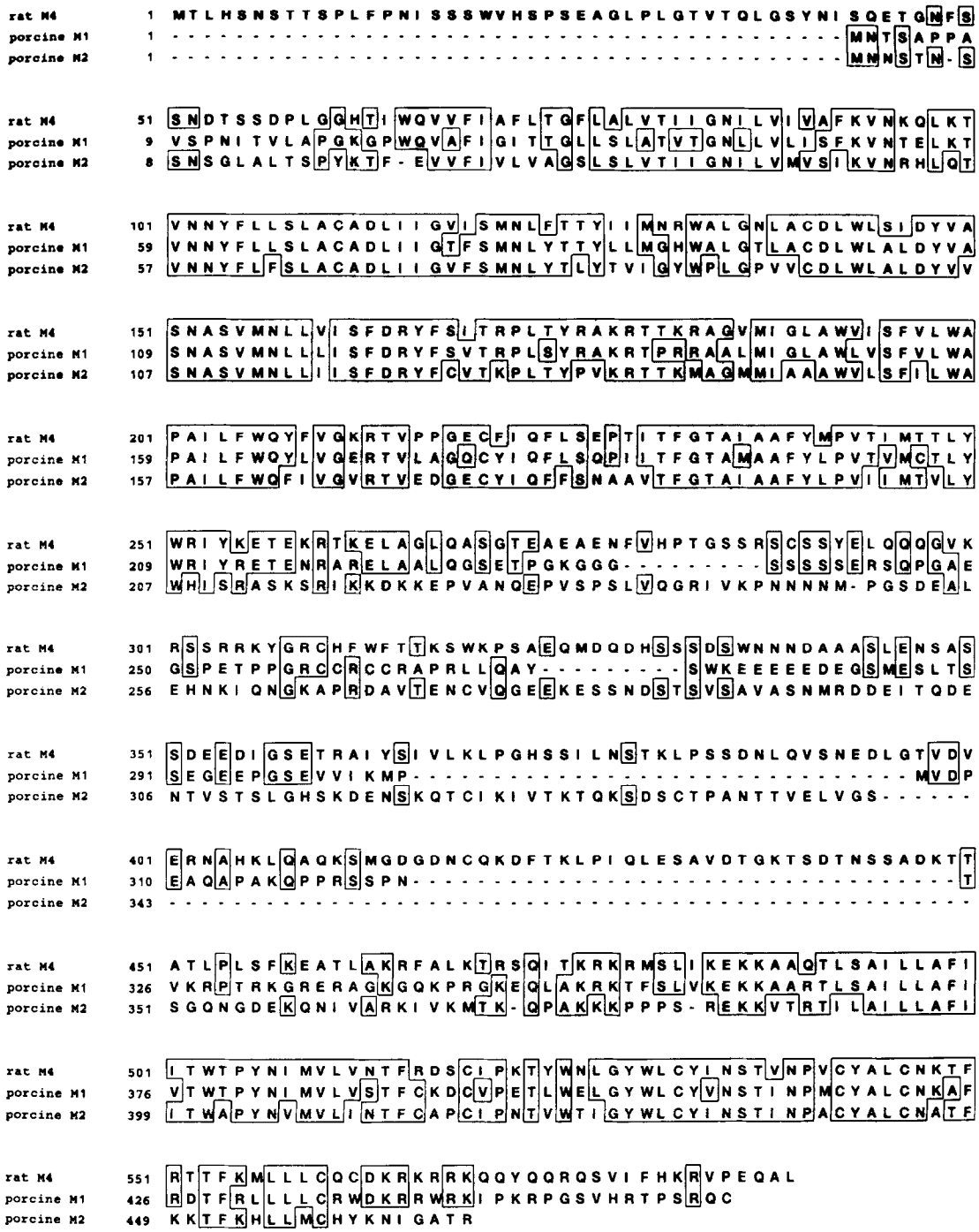


Figure 2. Comparison of muscarinic receptor subtype protein sequences. Identical amino acids in the sequences are boxed. The M4 sequence has 45.5% identity with the porcine M1 sequence (5) and 39.3% identity with the porcine M2 sequence (6, 7). Gaps have been introduced to maximize the homology.

The muscarinic receptor M4 protein sequence is longer than the other muscarinic receptor proteins at both the N-terminal region, where five

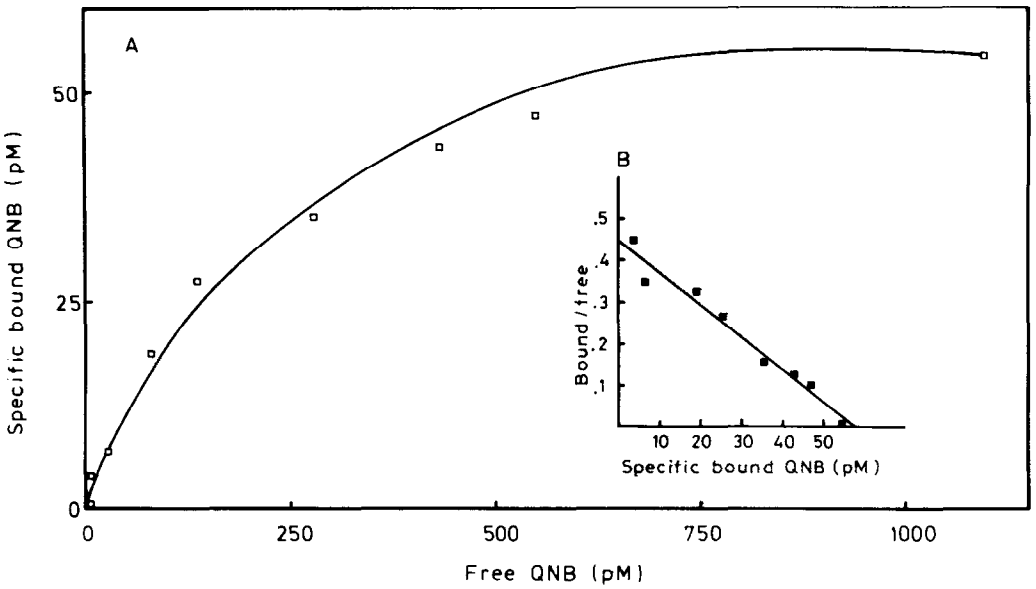


Figure 3. $^{3}$H-QNB binding to the M4 mAChR subtype. A) Specific $^{3}$H-QNB binding was determined from the difference between total binding of the labelled antagonist in the presence or absence of 10μM atropine. B) Scatchard analysis (using least-squares linear regression) of the binding data gives a $K_D$ of 68 pM (n=4). Untransfected cells did not bind $^{3}$H-QNB. $B_{max}$ values varied depending upon the transfection efficiency with transfected cells expressing between 4,000-20,000 receptors. The data shown are from a representative experiment.

potential N-linked glycosylation sites are present, and also in the intracellular loop region connecting transmembrane domains 5 and 6. This loop region has very low homology among all G-protein coupled receptors as well as between different subtypes of a receptor family. The functional significance of these highly variable and therefore unique sequences in each receptor subtype, is unknown. However, their intracellular location suggests that they may represent regulatory sites for signal transduction.

To demonstrate that the cDNA we isolated is a muscarinic receptor subtype we transiently expressed the M4 cDNA in mammalian cells (13). Membrane fractions of these cells were analyzed for $^{3}$H-QNB (a specific muscarinic receptor antagonist) (1-3) binding. The presence of a high affinity binding site for QNB on these membranes demonstrated that the M4 receptor subtype is indeed a muscarinic receptor (Fig. 3). The affinity constant ($K_D$= 68pM; n=4) is of the same range as that determined for either the M1 or M2 subtypes (1, 5, 7, 15).

The expression pattern of the M4 subtype was determined in rat brain by *in situ* hybridization using $^{32}$P-labelled antisense cRNA derived from the TM5-6 subtype specific sequences of the cDNA. M4 transcripts were highly abundant in cells in all subfields of the hippocampus, the dentate gyrus,

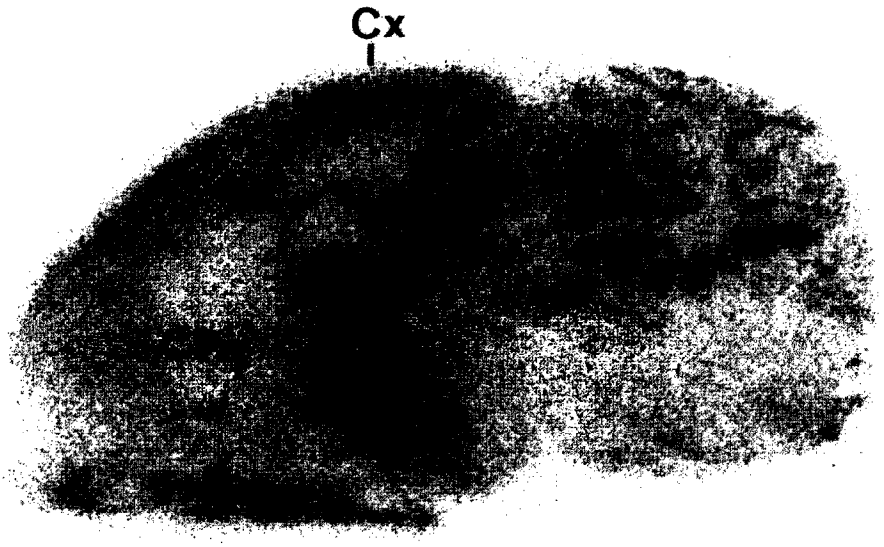


Figure 4. M4 mAChR mRNA expression in rat brain. Rat brain saggital sections were hybridized with a $^{32}$P-labelled subtype-specific cRNA probe. Brain regions showing the highest levels of expression included cerebral cortex (Cx), hippocampus (Hi), dentate gyrus (DG), thalamus (Th) and the granule cell layer of the cerebellar cortex (Cb). M4 mRNA was not highly expressed in the caudate-putamen (CPu).

the cerebral cortex and the granule cell layer of the cerebellar cortex (Fig. 4). A strong signal was also obtained in the grey matter of the spinal cord. We were not able to detect any specific signal in either heart or kidney (not shown). No signals were obtained with sense strand cRNA probes or when sections were treated with RNase before hybridization with cRNA.

The demonstration of a family of muscarinic receptor genes suggests that preferential coupling of a given type of G-protein (e.g. $G_i$, $G_o$, $G_s$ etc.) (16) to specific receptor subtypes may occur. Thus, activation of endogenous mAChRs in the cell lines NG108-15 and 1321-N1 inhibits adenylate cyclase activity and stimulates phosphoinosityl turnover, respectively (17), suggesting that these cell lines express a distinct receptor subtype that couples to a specific effector G-protein. Preferential coupling of a G-protein with a receptor subtype may occur in part by interaction with the subtype specific sequences located intracellularly between TM5 and 6. In support of this notion, deletion of part of the TM5-6 sequence in the β-adrenergic receptor abolishes its ability to regulate adenylate cyclase activity while not affecting ligand binding (18).

Our localization studies demonstrate that the M4 receptor subtype is expressed in specific regions of the central nervous system. The M1 subtype

has been localized to the cerebral cortex and corpus striatum (5) while the M2 subtype is expressed in the medulla-pons and heart atrium (6). Thus, not only does the neuronally expressed M4 subtype display a higher degree of protein identity to the cerebral M1 subtype but it also has a similar regional distribution in brain (e.g. cerebral cortex). The existence of distinct genes encoding mAChR subtypes, in addition to permitting tissue-specific gene expression, could also allow for differential gene regulation within the same tissue or cell.

Hippocampal and cerebral cortex cholinergic systems (where the M1 and M4 receptor subtypes are expressed) are particularly involved in the processes of learning and memory (1). These cognitive processes are believed to rely upon the phenomenon of synaptic plasticity. We propose that receptor subtype heterogeneity is one mechanism by which such plasticity is achieved. More explicitly, by controlling the relative levels of expression of two (or more) receptor subtypes, a single cell could potentially alter quantitatively as well as qualitatively its response to the synaptic release of a given neurotransmitter. The availability of molecular probes for these receptor subtypes will allow the examination of this hypothesis.

The full extent of mAChR subtype diversity is unknown but, based on the methods outlined here, can be addressed. Whether protein sequence similarities between mAChR and other classes of G-protein coupled receptors are sufficient to allow their isolation remains to be seen.

Subsequent to the completion of this work, Bonner et al. (19) reported a similar rat muscarinic receptor subtype designated m3. Our results are consistent with theirs except that we note three amino acid differences between our predicted protein sequence and theirs, repectively: $A_{184}$ > R, $R_{516}$ > C and $M_{556}$ > T. These workers (19) also demonstrated higher levels of expression of this subtype in the dentate gyrus relative to the hippocampus as compared to our finding of similar levels of expression in both the hippocampus and the dentate gyrus.

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