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Primary structure of porcine muscarinic acetylcholine receptor III and antagonist binding studies

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The complete amino acid sequence of porcine muscarinic acetylcholine receptor III has been deduced by cloning and sequencing the genomic DNA. The antagonist binding properties of muscarinic acetylcholine receptor III expressed from the cloned DNA in *Xenopus* oocytes correspond most closely to those of the pharmacologically defined M_2 glandular (III) subtype.

Muscarinic acetylcholine receptor subtype; genomic DNA cloning; Nucleotide sequence; DNA expression; Selective antagonist; (*Xenopus* oocyte)

1. INTRODUCTION

Pharmacologically distinguishable forms of the muscarinic acetylcholine receptor (mAChR) occur in different tissues and have been classified into M_1 (I), M_2 cardiac (II) and M_2 glandular (III) subtypes on the basis of their difference in apparent affinity for antagonists [1–3]. The primary structures of two mAChR species, designated as mAChR I and mAChR II, have been elucidated by cloning and sequence analysis of DNAs complementary to the porcine cerebral and cardiac mRNAs, respectively [4–6]. Microinjection of *Xenopus* oocytes with mRNAs synthesized by transcription in vitro of the cloned cDNAs has generated functional mAChR I and mAChR II, which differ from each other both in acetylcholine-induced response and in antagonist-binding properties [4,7]. These results, together with the differential tissue location of the two mAChR mRNAs [4,5], have indicated that distinct gene products underlie pharmacologically different mAChR subtypes. The primary structures of two additional mAChR species, mAChR III and mAChR IV (defined as in [8]), have subsequently been predicted from the nucleotide sequences of the cloned cerebral cDNAs or genomic DNAs from rat and man [8,9]. The present paper describes the complete amino acid sequence of porcine mAChR III deduced from the cloned genomic DNA. mAChR III which has been produced in *Xenopus* oocytes by microinjection of mRNA synthesized by transcription in vitro of the cloned DNA is compared with the three other mAChR species produced similarly with respect to binding affinities for selective antagonists.

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Abbreviations: mAChR, muscarinic acetylcholine receptor; QNB, quinuclidinyl benzilate

2. MATERIALS AND METHODS

Rat genomic DNAs encoding mAChR III and mAChR IV (clones λrmACR14 and λrmACR38) were obtained by screening libraries constructed in λCharon 4A (Clontech) [10] and λEMBL3 [11], respectively, using oligodeoxyribonucleotide probes prepared with an automatic DNA synthesizer (Applied Biosystems) on the basis of rat cDNA sequences (nucleotides 931–989 and 1197–1226 for mAChR III; nucleotides 700–752 and 1053–1080 for mAChR IV) [8]. Porcine genomic DNA en-

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coding mAChR III was isolated by screening a library constructed in λEMBL3 using the nick-translated 1.3-kilobasepair (kb) *Pvu*II fragment from λrmACR14 as a probe. From ~7 × 10^5 plaques, two positive clones were obtained. The ~2.9-kb *Bam*HI fragment hybridizable with the probe, derived from one of these clones (λpmACR2), was subcloned in plasmid pUC18 [12] to yield plasmid pSpmACR7, which was subjected to nucleotide sequence analysis [13,14].

pSpmACR7 was cleaved by *Tth*111I, treated with T_4 DNA polymerase in the presence of the four deoxyribonucleoside triphosphates (blunt-ended), ligated with the synthetic *Xba*I linker 5′-CTCTAGAG-3′ and cleaved by *Xba*I and *Sma*I. The resulting ~2.1-kb fragment, the ~320-basepair (bp) *Nae*I(2255)/*Eco*RI(on vector) fragment from plasmid pSPBM1 [7] and the 3.0-kb *Eco*RI/*Xba*I fragment from plasmid pSP64 [15] were ligated to yield plasmid pSPPM3; the *Nae*I site is specified by the number indicating the 5′-terminal nucleotide generated by cleavage [4]. The 2.5-kb *Tth*111I/*Bam*HI fragment from λrmACR14 was blunt-ended, ligated with the *Xba*I linker and cleaved by *Xba*I and *Fnu*DII. The resulting ~1.8-kb fragment, the ~320-bp *Nae*I(2255)/*Eco*RI(on vector) fragment from pSPBM1 and the 3.0-kb *Eco*RI/*Xba*I fragment from pSP64 were ligated to yield plasmid pSPRM3. The ~3.8-kb *Bam*HI/*Sal*I fragment from λrmACR38 was blunt-ended, ligated with the synthetic *Hin*dIII linker 5′-ACAAGCTTGT-3′ and cleaved by *Pvu*I and *Hin*dIII. The resulting ~1.2-kb *Pvu*I/*Hin*dIII fragment was isolated. The ~0.8-kb *Hin*fI fragment excised from the ~3.8-kb *Bam*HI/*Sal*I fragment from λrmACR38 was blunt-ended, ligated with the *Hin*dIII linker and cleaved by *Hin*dIII and *Pvu*I. The resulting ~0.5-kb *Hin*dIII/*Pvu*I fragment, the above-mentioned ~1.2-kb *Pvu*I/*Hin*dIII fragment and the ~3.7-kb *Hin*dIII fragment from pSPBM1 were ligated to yield plasmid pSPRM4 carrying the protein-coding sequence in the same orientation as the SP6 promoter. mRNAs specific for porcine mAChR III, rat mAChR III and rat mAChR IV were synthesized by transcription in vitro [15,16] using *Eco*RI-cleaved pSPPM3, *Eco*RI-cleaved pSPRM3 and *Xba*I-cleaved pSPRM4 as templates, respectively. The resulting mRNAs were shown by electrophoresis on 1.5% agarose gel to be homogeneous and to have expected sizes of ~2.4, ~2.1 and ~2.4 kilobases, respectively. mRNAs specific for porcine mAChR I and mAChR II were synthesized as in [7]. Each mRNA was injected into *Xenopus laevis* oocytes (mRNA concentration, 1 μg/μl; average volume injected per oocyte, ~40 nl). The injected oocytes were incubated at 19°C for 3 days as in [4] before cell extracts from them were prepared for ligand binding assays. (−)-[^{3}H]Quinuclidinyl benzilate-binding activity was assayed as in [4], except that 0.42 nM (−)-[^{3}H]QNB (spec. act. of 30 Ci/mmol) was used.

3. RESULTS AND DISCUSSION

Nucleotide sequence analysis of the ~2.9-kb *Bam*HI fragment derived from the porcine genomic DNA clone λpmACR2 (see section 2) reveals an open reading frame of 1770 nucleotides (fig.1). It is concluded that this reading frame encodes porcine mAChR III because the predicted sequence of 590 amino acid residues (calculated M_r 66074) shows 94% and 96% identity with the rat [8] and human mAChR III sequences [9], respectively. The protein-coding region of the porcine mAChR III gene contains no introns as is the case for the other mAChR genes hitherto reported [6,8,9].

Porcine mAChR III shares characteristic structural features with the other mAChR species documented [4–6,8,9,17]. Its hydropathy profile [18] suggests the presence of seven putative transmembrane α-helices (segment I, amino acid residues 68–91; II, 105–125; III, 143–164; IV, 185–207; V, 230–252; VI, 493–513; VII, 528–547); segment VII is less hydrophobic than the other segments. Porcine mAChR III also has the two conserved aspartic acid residues (residue 114 in segment II and residue 148 in segment III), and its amino-terminal region preceding segment I contains several potential sites of *N*-glycosylation [19] (asparagine residues 6, 7, 15, 41, 48 and 53); *N*-glycosylation of residues 6 and 7 may be mutually exclusive. Its carboxy-terminal region contains several potential sites of phosphorylation (threonine residues 550, 553 and 554 and serine residue 578).

mRNAs specific for porcine and rat mAChR III were synthesized by transcription in vitro of the respective cloned DNAs and were injected into *Xenopus* oocytes. The resulting mAChR III species were examined for antagonist-binding properties, using oocyte extracts, in comparison with mAChR I, mAChR II and mAChR IV produced similarly. The apparent dissociation constant (K_d) for (−)-[^{3}H]QNB was similar for all the mAChR species (84–130 pM) (table 1). The apparent K_d values for selective antagonists were obtained by measuring displacement of (−)-[^{3}H]QNB binding by increasing concentrations of the antagonists (fig.2 and table 1). Hexahydrosiladifenidol, selective for the M_2 glandular (III) subtype relative to the M_2 cardiac (II) subtype [3,21,22], showed the highest binding affinity for mAChR III (K_d = 4.0–4.4 nM). The affinity for this antagonist decreased in the order of mAChR IV (K_d = 20 nM), mAChR I (K_d = 51 nM) and mAChR II (K_d = 280 nM). Pirenzepine, selective for the M_1 (I) subtype [1], exhibited the highest affinity for mAChR I (K_d = 18 nM). mAChR III and mAChR IV showed intermediate affinities for pirenzepine (K_d = 120–180 nM), and mAChR II the lowest af-

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                                5'-----GGATACAATGCAAAGAACAAATCCAATGATGACAGAAATTTTCCTGACTCCATCTTTTCTCTTTTCTCCCCAGACTATGTCAGAGAGTCACC    -1
  1                                10                                  20                                  30
Met Thr Leu His Asn Asn Asn Thr Thr Ser Pro Leu Phe Pro Asn Ile Ser Ser Ser Trp Ile His Gly Pro Ser Asp Ala Gly Leu Pro
ATG ACC TTG CAC AAT AAC AAT ACA ACC TCA CCT TTG TTT CCA AAC ATC AGC TCT TCC TGG ATT CAC GGC CCT TCC GAT GCA GGG CTG CCC     90
                                40                                  50                                  60
Pro Gly Thr Val Thr His Phe Gly Ser Tyr Asn Ile Ser Gln Ala Ala Gly Asn Phe Ser Ser Pro Asn Gly Thr Thr Ser Asp Pro Leu
CCA GGA ACG GTT ACT CAT TTT GGC AGC TAC AAC ATT TCT CAG GCA GCT GGG AAT TTC TCC TCT CCA AAT GGC ACC ACC AGT GAC CCT CTG    180
                                70                                  80                                  90
Gly Gly His Thr Ile Trp Gln Val Val Phe Ile Ala Phe Leu Thr Gly Ile Leu Ala Leu Val Thr Ile Ile Gly Asn Ile Leu Val Ile
GGA GGT CAC ACC ATC TGG CAA GTG GTG TTC ATT GCA TTC TTA ACA GGC ATC CTG GCC TTG GTG ACT ATC ATC GGC AAT ATC CTG GTG ATC    270
                                100                                 110                                 120
Val Ala Phe Lys Val Asn Lys Gln Leu Lys Thr Val Asn Asn Tyr Phe Leu Leu Ser Leu Ala Cys Ala Asp Leu Ile Ile Gly Val Ile
GTG GCA TTC AAG GTC AAC AAG CAA CTG AAG ACA GTC AAC AAC TAC TTC CTC TTA AGT CTG GCC TGT GCT GAC CTG ATT ATC GGG GTC ATT    360
                                130                                 140                                 150
Ser Met Asn Leu Phe Thr Thr Tyr Ile Ile Met Asn Arg Trp Ala Leu Gly Asn Leu Ala Cys Asp Leu Trp Leu Ser Ile Asp Tyr Val
TCA ATG AAT CTG TTT ACT ACC TAC ATC ATC ATG AAT CGA TGG GCT TTA GGG AAC TTG GCC TGT GAC CTC TGG CTT TCC ATT GAC TAT GTG    450
                                160                                 170                                 180
Ala Ser Asn Ala Ser Val Met Asn Leu Leu Val Ile Ser Phe Asp Arg Tyr Phe Ser Ile Thr Arg Pro Leu Thr Tyr Arg Ala Lys Arg
GCT AGC AAT GCC TCG GTC ATG AAT CTT CTG GTC ATT AGC TTT GAC AGG TAC TTT TCC ATC ACG AGG CCG CTC ACA TAC CGA GCC AAA AGA    540
                                190                                 200                                 210
Thr Thr Lys Arg Ala Gly Val Met Ile Gly Leu Ala Trp Val Ile Ser Phe Ile Leu Trp Ala Pro Ala Ile Leu Phe Trp Gln Tyr Phe
ACA ACA AAG CGA GCT GGT GTG ATG ATA GGT CTG GCT TGG GTC ATC TCC TTC ATC CTT TGG GCT CCT GCC ATC TTG TTC TGG CAA TAC TTT    630
                                220                                 230                                 240
Val Gly Lys Arg Thr Val Pro Pro Gly Glu Cys Phe Ile Gln Phe Leu Ser Glu Pro Thr Ile Thr Phe Gly Thr Ala Ile Ala Ala Phe
GTT GGG AAG AGA ACT GTC CCT CCA GGA GAG TGT TTC ATC CAG TTC CTC AGT GAG CCC ACC ATC ACC TTC GGC ACG GCC ATC GCT GCC TTT    720
                                250                                 260                                 270
Tyr Met Pro Val Thr Ile Met Thr Ile Leu Tyr Trp Arg Ile Tyr Lys Glu Thr Glu Lys Arg Thr Lys Glu Leu Ala Gly Leu Gln Ala
TAT ATG CCT GTC ACC ATT ATG ACT ATT TTA TAC TGG AGG ATC TAT AAG GAA ACT GAA AAA CGT ACC AAA GAG CTT GCC GGG CTG CAA GCC    810
                                280                                 290                                 300
Ser Gly Thr Glu Ala Glu Ala Glu Asn Phe Val His Pro Thr Gly Ser Ser Arg Ser Cys Ser Ser Tyr Glu Leu Gln Gln Gln Ser Leu
TCT GGG ACA GAG GCA GAG GCA GAA AAC TTT GTT CAC CCC ACA GGT AGT TCT CGG AGC TGC AGC AGC TAT GAG CTT CAG CAG CAA AGC CTG    900
                                310                                 320                                 330
Lys Arg Ser Ala Arg Arg Lys Tyr Gly Arg Cys His Phe Trp Phe Thr Thr Lys Ser Trp Lys Pro Ser Ala Glu Gln Met Asp Gln Asp
AAA CGC TCA GCC AGG AGG AAG TAT GGA CGC TGC CAC TTC TGG TTC ACA ACC AAG AGC TGG AAG CCC AGT GCT GAG CAG ATG GAC CAA GAC    990
                                340                                 350                                 360
His Ser Ser Ser Asp Ser Trp Asn Asn Asn Asp Ala Ala Ala Ser Leu Glu Asn Ser Ala Ser Ser Asp Glu Glu Asp Ile Gly Ser Glu
CAC AGC AGC AGT GAC AGC TGG AAT AAC AAT GAT GCT GCT GCC TCC CTG GAA AAC TCC GCC TCC TCC GAT GAG GAG GAC ATT GGC TCA GAA   1080
                                370                                 380                                 390
Thr Arg Ala Ile Tyr Ser Ile Val Leu Lys Leu Pro Gly His Ser Thr Ile Leu Asn Ser Thr Lys Leu Pro Ser Ser Asp Asn Leu Gln
ACA AGA GCC ATC TAC TCC ATC GTG CTC AAG CTT CCA GGT CAC AGC ACC ATC CTC AAC TCC ACC AAG TTA CCG TCT TCA GAC AAC CTG CAG   1170
                                400                                 410                                 420
Val Pro Glu Glu Glu Leu Gly Thr Val Asp Leu Glu Arg Lys Ala Ser Lys Leu Gln Ala Gln Lys Ser Met Asp Asp Gly Gly Ser Phe
GTG CCC GAG GAG GAG CTG GGG ACA GTG GAC TTG GAG AGA AAA GCC AGC AAA CTG CAA GCC CAG AAG AGC ATG GAC GAT GGA GGC AGT TTT   1260
                                430                                 440                                 450
Gln Lys Ser Phe Ser Lys Leu Pro Ile Gln Leu Glu Ser Ala Val Asp Thr Ala Lys Ala Ser Asp Val Asn Ser Ser Val Gly Lys Thr
CAA AAA AGC TTC TCC AAG CTT CCC ATC CAG TTA GAG TCA GCC GTG GAC ACA GCC AAG GCC TCT GAT GTC AAC TCC TCA GTG GGT AAG ACC   1350
                                460                                 470                                 480
Thr Ala Thr Leu Pro Leu Ser Phe Lys Glu Ala Thr Leu Ala Lys Arg Phe Ala Leu Lys Thr Arg Ser Gln Ile Thr Lys Arg Lys Arg
ACG GCC ACT CTA CCT CTG TCC TTT AAG GAA GCT ACT CTG GCC AAG AGG TTT GCT CTG AAG ACC AGA AGT CAG ATC ACC AAG CGG AAA CGG   1440
                                490                                 500                                 510
Met Ser Leu Ile Lys Glu Lys Lys Ala Ala Gln Thr Leu Ser Ala Ile Leu Leu Ala Phe Ile Ile Thr Trp Thr Pro Tyr Asn Ile Met
ATG TCG CTC ATC AAG GAG AAG AAA GCG GCC CAG ACC CTC AGC GCC ATC TTG CTT GCC TTC ATC ATC ACC TGG ACC CCC TAC AAT ATC ATG   1530
                                520                                 530                                 540
Val Leu Val Asn Thr Phe Cys Asp Ser Cys Ile Pro Lys Thr Tyr Trp Asn Leu Gly Tyr Trp Leu Cys Tyr Ile Asn Ser Thr Val Asn
GTT CTG GTG AAC ACC TTT TGT GAC AGC TGC ATA CCC AAA ACC TAT TGG AAT CTG GGC TAC TGG CTG TGC TAC ATC AAC AGC ACC GTG AAC   1620
                                550                                 560                                 570
Pro Val Cys Tyr Ala Leu Cys Asn Lys Thr Phe Arg Thr Thr Phe Lys Met Leu Leu Leu Cys Gln Cys Asp Lys Arg Lys Arg Arg Lys
CCC GTG TGC TAT GCC CTG TGC AAC AAA ACA TTC AGA ACC ACT TTC AAG ATG CTG CTG CTG TGC CAG TGT GAC AAA AGG AAG AGG CGC AAG   1710
                                580                                 590
Gln Gln Tyr Gln Gln Arg Gln Ser Val Ile Phe His Lys Arg Val Pro Glu Gln Ala Leu
CAG CAG TAT CAG CAA AGA CAG TCA GTC ATT TTC CAC AAG CGG GTG CCC GAG CAG GCC TTG TAG AAGGAGGCGTTGTCCATAGCAGTCACCAAACGCACA   1809

CATCAGCCCACA-----3'                                                                                                    1821
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Fig.1. Nucleotide sequence of the porcine genomic DNA encoding mAChR III. The nucleotide sequence of the message strand, together with the deduced amino acid sequence, is shown. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the ATG triplet encoding the initiating methionine, and the nucleotides on the 5′-side of residue 1 are indicated by negative numbers; the number of the nucleotide residue at the right end of each line is given. Amino acid residues are numbered beginning with the initiating methionine.

Table 1

Apparent dissociation constants (K_d) of molecularly defined mAChR species for antagonists

Antagonist	K_d (M)				
	mAChR I (porcine)	mAChR II (porcine)	mAChR III (porcine)	mAChR III (rat)	mAChR IV (rat)
(−)-[^{3}H]QNB	8.4×10^{-11}	1.3×10^{-10}	1.2×10^{-10}	1.0×10^{-10}	9.5×10^{-11}
Hexahydrosiladifenidol	5.1×10^{-8}	2.8×10^{-7}	4.4×10^{-9}	4.0×10^{-9}	2.0×10^{-8}
Pirenzepine	1.8×10^{-8}	6.6×10^{-7}	1.8×10^{-7}	1.3×10^{-7}	1.2×10^{-7}
AF-DX 116	2.5×10^{-6}	7.3×10^{-7}	2.3×10^{-6}	3.1×10^{-6}	2.3×10^{-6}

The apparent K_d values for (−)-[^{3}H]QNB (means of 3 experiments) were obtained by Scatchard analysis, and those for hexahydrosiladifenidol, pirenzepine and AF-DX 116 were calculated from the IC_{50} values derived from fig.2 [20]

finity (K_d = 660 nM). These three classes of binding affinity for pirenzepine are similar to those reported originally for mAChRs in brain (cerebral cortex and hippocampus), glands and heart [1]. AF-DX 116, selective for the M_2 cardiac (II) subtype [2], exhibited a higher affinity for mAChR II (K_d = 0.73 μM) than for the other mAChR species (K_d = 2.3–3.1 μM). The apparent K_d values of mAChR I and mAChR II for (−)-[^{3}H]QNB, pirenzepine and AF-DX 116 are in agreement with those obtained in our previous studies [4,7]. Differential binding affinities for pirenzepine and AF-DX 116 have also been reported for different rat and human mAChR species expressed from the cloned DNAs in mammalian cells [8,9].

The results described above indicate that mAChR I, mAChR II and mAChR III correspond most closely to the pharmacologically defined M_1 (I), M_2 cardiac (II) and M_2 glandular (III) subtypes, respectively. In support of this view is the differential tissue location of the mRNAs encoding

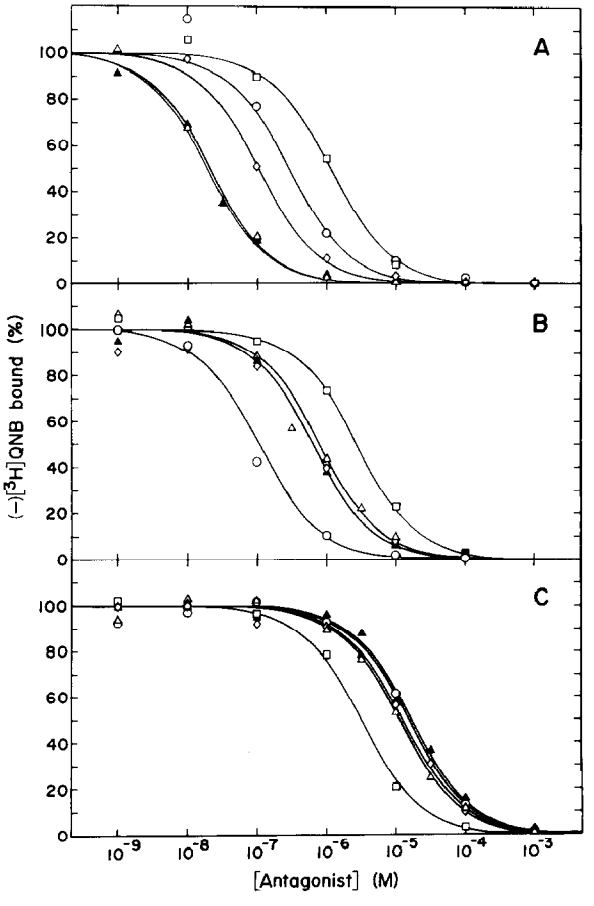

Fig.2. Effects of hexahydrosiladifenidol (A), pirenzepine (B) and AF-DX 116 (C) on (−)-[^{3}H]QNB binding in extracts from *Xenopus* oocytes injected with the mRNA specific for porcine mAChR I (○), porcine mAChR II (□), porcine mAChR III (△), rat mAChR III (▲) or rat mAChR IV (◇). Data are from 2–4 experiments. Values for 100% and 0% binding were determined by measurements in the absence of antagonists and in the presence of 10 μM atropine, respectively. The 0% values [59–153 dpm (○), 51–154 dpm (□), 75–176 dpm (△), 67–165 dpm (▲) or 47–143 dpm (◇)] were 5–26% (○), 0.4–1% (□), 0.7–2% (△), 0.8–2% (▲) or 0.7–3% (◇) of the 100% values. The theoretical curves have been drawn by nonlinear least-squares analysis as in [4]. The IC_{50} values of hexahydrosiladifenidol, pirenzepine and AF-DX 116 were 302 nM, 105 nM and 15.5 μM for mAChR I; 1.17 μM, 2.75 μM and 3.16 μM for mAChR II; 21.4 nM, 813 nM and 11.0 μM for porcine mAChR III; 20.9 nM, 646 nM and 16.6 μM for rat mAChR III; and 107 nM, 646 nM and 12.6 μM for mAChR IV, respectively.

these mAChR species, revealed by blot hybridization analysis [4,5,9]. Thus, the mAChR heterogeneity in tissues with respect to antagonist binding can be accounted for by the presence of individual molecularly distinct mAChR species or various combinations of them.

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