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Localization of a Family of Muscarinic Receptor mRNAs in Rat Brain

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A family of 4 rat muscarinic receptors (m1, m2, m3, and m4) have recently been cloned and sequenced (Bonner et al., 1987). Since pharmacological probes that are presently available do not appear to distinguish among 3 of these muscarinic receptors, we constructed oligonucleotide probes corresponding to the N-terminal sequences of the muscarinic receptors and used them to specifically localize m1, m2, m3, and m4 mRNA in sections of rat brain using *in situ* hybridization histochemistry. Northern analysis demonstrated a 3.1 kilobase (kb) m1 mRNA, a 4.5 kb m3 mRNA, and a 3.3 kb m4 mRNA in cerebral cortex, striatum, hippocampus, and cerebellum. *In situ* hybridization histochemistry indicated a prevalence of m1 mRNA in the pyramidal cell layer of the hippocampus, the granule cell layer of the dentate gyrus, the olfactory bulb, amygdala, olfactory tubercule, and piriform cortex. Caudate putamen and cerebral cortex showed moderate levels of labeling. m2 mRNA was detectable in the medial septum, diagonal band, olfactory bulb, and pontine nuclei. m3 and m4 mRNA were also prevalent in the olfactory bulb and pyramidal cell layer of the hippocampus but were present only in low levels in the dentate gyrus. m3 mRNA was present in superficial and deep layers of the cerebral cortex, whereas m4 mRNA was more evenly distributed with a slightly more intense labeling evident in the midcortical layer. In addition, m3 mRNA was present in a number of thalamic nuclei and brain-stem nuclei, while m4 mRNA predominated in the caudate putamen. These data offer a new basis on which to interpret the heterogeneity of muscarinic actions in the CNS.

Muscarinic receptors mediate many of the actions of ACh in the CNS (see Nathanson, 1987). Radioligand binding and autoradiographic studies have demonstrated widespread distribution of these receptors throughout the nervous system (Rotter et al., 1979; Wamsley et al., 1981; Cortes et al., 1984, 1986; Nonaka and Moroji, 1984; Cortes and Palacios, 1986; Spencer et al., 1986). A number of studies have proposed heterogeneity among muscarinic receptors on the basis of agonist and antagonist selectivity (Birdsall et al., 1978; Goyal and Rattan, 1978; Hammer et al., 1980, 1986) and have ultimately led to the widely accepted classification based upon the selective muscarinic antagonist, pirenzepine, which distinguishes 2 or 3 receptor subtypes (Hammer et al., 1980). At the time that this classification scheme was proposed, it was not clear whether these subtypes reflected distinct gene products or different modifications of the same protein. It is only recently that molecular cloning of a number of muscarinic receptors has demonstrated heterogeneity due to the presence of a number of related but distinct gene products (Kubo et al., 1986a, b; Bonner et al., 1987; Peralta et al., 1987). In a previous study, we reported the cloning and sequencing of 4 muscarinic receptors (m1, m2, m3, and m4), cloned from rat cerebral cortex cDNA and human genomic libraries (Bonner et al., 1987). Sequence comparison indicates that the m1 and m2 receptors are rat and human homologs respectively of the porcine muscarinic receptors cloned previously from cortex and atria, respectively (Kubo et al., 1986a, b; Peralta et al., 1987). Expression of the muscarinic receptor cDNAs in COS-7 cells revealed m1, m3, and m4 receptors to display higher affinities toward the selective muscarinic antagonist, pirenzepine, than the m2 receptor (Bonner et al., 1987; Brann et al., 1987). Autoradiographic studies using ^{3}H-pirenzepine have demonstrated a prevalence of high-affinity pirenzepine sites in the cerebral cortex, corpus striatum, and hippocampus, with relatively few receptors in the hindbrain or cerebellum (Wamsley et al., 1984; Cortes and Palacios, 1986; Cortes et al., 1986; Mash and Potter, 1986; Spencer et al., 1986). Parallel radioligand binding studies indicated that, under the assay conditions employed, only a single class of high-affinity pirenzepine binding sites could be distinguished. Since our own studies demonstrated the existence of at least 3 muscarinic receptors in rat cortex that could express a high affinity toward pirenzepine, it is clear that autoradiographic studies using ^{3}H-pirenzepine potentially label a multiplicity of sites, thereby revealing a composite picture of the distribution of at least 3 muscarinic receptor subtypes. Since it is presently impossible to specifically label the m1, m3, or m4 gene products, we were interested to use the technique of *in situ* hybridization histochemistry to examine the distribution of the m1, m2, m3, and m4 mRNAs in sections of rat brain. The value of these studies lies in the ability to localize cell bodies that express muscarinic receptor subtypes as opposed to mapping the distribution of the receptor itself.

Materials and Methods

Oligonucleotide synthesis. 48-mer or 45-mer antisense cDNAs, complementary to bases 7–54 of the m1 receptor; ACCTCAGTGCCCCC-TGCTGTCAGTCCCAACATCACTGTCTTGGCACCA bases 91–147 of the m2 receptor; GGATCCCTCAGTTTGGTGACCATTATCGGG-

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AACATCCTAGTCATGGTT bases 4–51 of the m3 receptor; ACCTTGCACAGTAACAGTACAACCTCGCCTTTGTTTCCCAACATCAGC bases 7–54 of the m4 receptor; AACTTCACGCCTGTCAATGGCAGCTCAGCCAATCAGTCTGTGCGCCTG were synthesized on an Applied Biosystems 380A DNA synthesizer, purified on a 8% denaturing polyacrylamide gel, containing 8M urea, followed by phenolic extraction. Probes were tailed on their 3'-ends with either ^{32}P α-dATP (New England Nuclear) or ^{35}S α-dATP (New England Nuclear) using terminal deoxynucleotidyl transferase (BRL and Boehringer Mannheim) as described previously (Young et al., 1986). Average tail length was about 10 residues.

Northern analysis. Total mRNA was extracted from fresh rat cerebral cortex, hippocampus, striatum, and cerebellum using a guanidinium isothiocyanate extraction procedure (Chirgwin et al., 1979) and precipitated through a cesium trifluoracetate/EDTA gradient (1.51 gm/ml density). RNA was electrophoresed (15 μg/lane) on a 1% denaturing agarose gel (6.7% formalin) and electroblotted onto Genescreen. RNA size standards were also run (RNA ladder; BRL). Blots were prehybridized for 3 hr at 37°C in 4× SSPE, 5× Denhardts, 50% formamide, 1% SDS, 250 μg/ml yeast RNA, and 500 μg/ml herring sperm DNA. Hybridization was conducted overnight at 37°C in the same medium containing 10^6 dpm/ml ^{32}P-labeled probe. Blots were washed 4 × 15 min at 56°C in 1× SSPE containing 0.1% SDS, followed by 2 × 60 min washes at room temperature, blotted dry, and exposed with 2 enhancing screens for 1–2 weeks at −70°C.

In situ *hybridization histochemistry.* The overall procedure has been described previously (Young et al., 1986). Twelve micron frozen sections of rat brain were lightly fixed in 4% formalin, acetylated, dehydrated through a graded series of alcohol, defatted in chloroform, and slightly rehydrated. Sections were hybridized overnight at 37°C in a 50 μl aliquot of hybridization buffer containing 4× SSC, 1× Denhardts, 50% formamide, 10% dextran sulfate, 250 μg/ml yeast RNA, 500 μg/ml herring sperm DNA, 0.1 M DTT, and 2 × 10^6 dpm ^{35}S-labeled probe. Sections were washed in 1× SSC at 56°C followed by 2 × 1 hr washes at room temperature and rinsed in distilled water before exposure to Kodak XAR-5 film for 5 weeks.

Results

Since the muscarinic receptors belong to a family of proteins of related sequence and structure, we were concerned that our oligonucleotide probes were specific for their corresponding receptors. Muscarinic receptors share the same overall structure and a significant degree of sequence homology. The common structural features proposed for this receptor family consist of a short extracellular N-terminus, 7 membrane spanning domains and a cytoplasmic C-terminus. Despite their considerable homology, these receptors also display a number of salient differences, notably in their amino- and carboxy-termini and in their large cytoplasmic loops that connect the proposed fifth and sixth transmembrane spanning domains (Bonner et al., 1987). In order to ensure specific hybridization of each oligonucleotide probe to its corresponding muscarinic receptor mRNA, oligodeoxynucleotide probes specific to each receptor were generated against domains of the receptor mRNA corresponding to the N-termini of the receptors, regions that share no significant homology with any other members of the gene family. Specificity of these probes was confirmed by Northern blot analysis, which showed that each probe recognized a single unique RNA species. In some cases, further proof of specificity was provided by using probes corresponding to the unique large cytoplasmic loops of the receptors; these probes gave identical results to those obtained using the N-terminal probes described here (data not shown).

Northern blot analysis revealed hybridizing bands in cerebral cortex, hippocampus, corpus striatum, and cerebellum using the m1, m3, and m4 probes (Fig. 1). The approximate sizes of the bands were 3.1 kb (m1), 4.5 kb (m3), and 3.3 kb (m4). No hybridization was seen using the m2 probe. m1 mRNA was most abundant in cortex and hippocampus, m3 mRNA was relatively abundant in hippocampus, with lesser amounts in cerebral cortex and cerebellum and very little in striatum, while m4 mRNA predominated in striatum and hippocampus with relatively little in cerebral cortex. No hybridization of any probe was observed to mRNA extracted from liver or kidney—tissues that express no muscarinic receptors (data not shown).

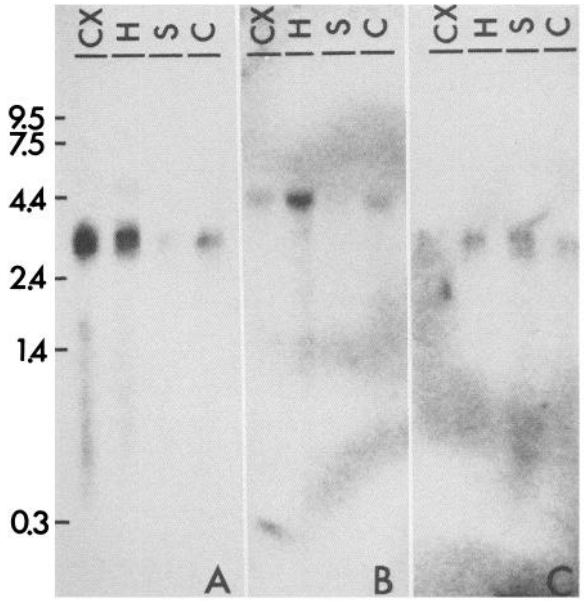

Figure 1. Northern blot analysis of mRNA extracted from rat cerebral cortex (*CX*), hippocampus (*H*), striatum (*S*), and cerebellum (*C*). RNA, 15μg, was applied to each lane and electrophoresed, blotted and hybridized as described in Materials and Methods. Molecular weight standards (BRL RNA ladder) were run in parallel. *A–C,* Blots hybridized to the m1, m3, and m4 probes, respectively.

In situ hybridization histochemistry revealed each muscarinic receptor mRNA to be uniquely distributed throughout the regions of the CNS examined (Fig. 2).

m1 mRNA was prevalent in the pyramidal layer of the hippocampus, the granule cell layer of the dentate gyrus, the olfactory nuclei and plexiform layers of the olfactory bulb, the olfactory tubercule, basolateral amygdaloid nuclei, and the piriform cortex. Lesser amounts were also seen in the cerebral cortex and the caudate putamen. A slight laminar distribution was evident in cerebral cortex, where m1 mRNA was predominantly in the more superficial and deeper cortical layers. No hybridization to hindbrain structures was observed.

m2 mRNA was observed in the pontine nuclei, plexiform layers of the olfactory bulb, medial septal nuclei, and diagonal band. A very low hybridization signal was also seen in thalamic structures, including the habenulae and anteroventral thalamic nuclei upon prolonged exposure of the autoradiograph. No hybridization to cerebral cortex or striatum was evident. This restricted localization may not be a true reflection of m2 mRNA distribution in rat brain since the probe used in this study was based upon the sequence of the human m2 cDNA reported earlier (Bonner et al., 1987). Since we do not know the degree of sequence similarity between rat and human m2 receptors, then the relative dearth of m2 mRNA may be a consequence of a poor match between the human m2 probe and the rat m2 mRNA. However, the fact that the m2 probe used in this study recognizes a 6.2 kb band in Northern blot analysis of rat atrial

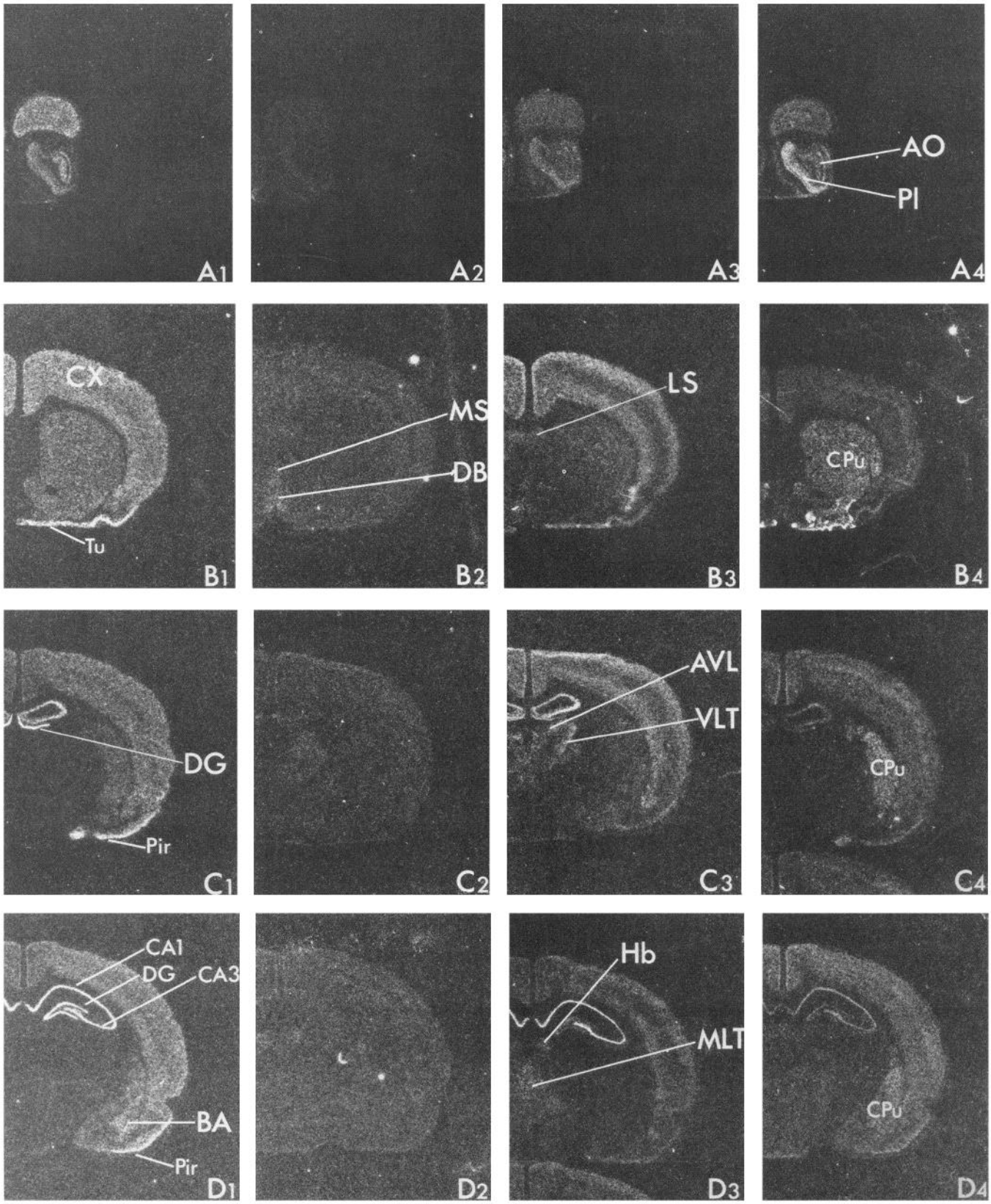

Figure 2. *In situ* hybridization histochemistry. Coronal sections of rat brain were prepared and hybridized to ^{35}S-tailed probes as described in Materials and Methods. Columns 1–4 show autoradiographs of m1, m2, m3, and m4 mRNA distributions respectively in 7 planes of section (*A–G*). Abbreviations: *AO,* anterior olfactory nuclei; *AVT,* anteroventral thalamic nuclei; *BA,* basolateral amygdaloid nuclei; *CA1, CA2,* and *CA3,* regions of hippocampus; *CG,* central gray; *CX,* cerebral cortex; *CPu,* caudate putamen; *DB,* diagonal band; *DG,* dentate gyrus; *Hb,* habenulae; *LS,* lateral septal nuclei; *MLT,* medial thalamic nuclei; *MS,* medial septal nuclei; *Pir,* piriform cortex; *Pl,* plexiform layers of olfactory bulb; *Pn,* pontine nuclei; *SuC,* superior colliculus; *Tu,* olfactory tubercle; *VLT,* ventrolateral thalamic nuclei.

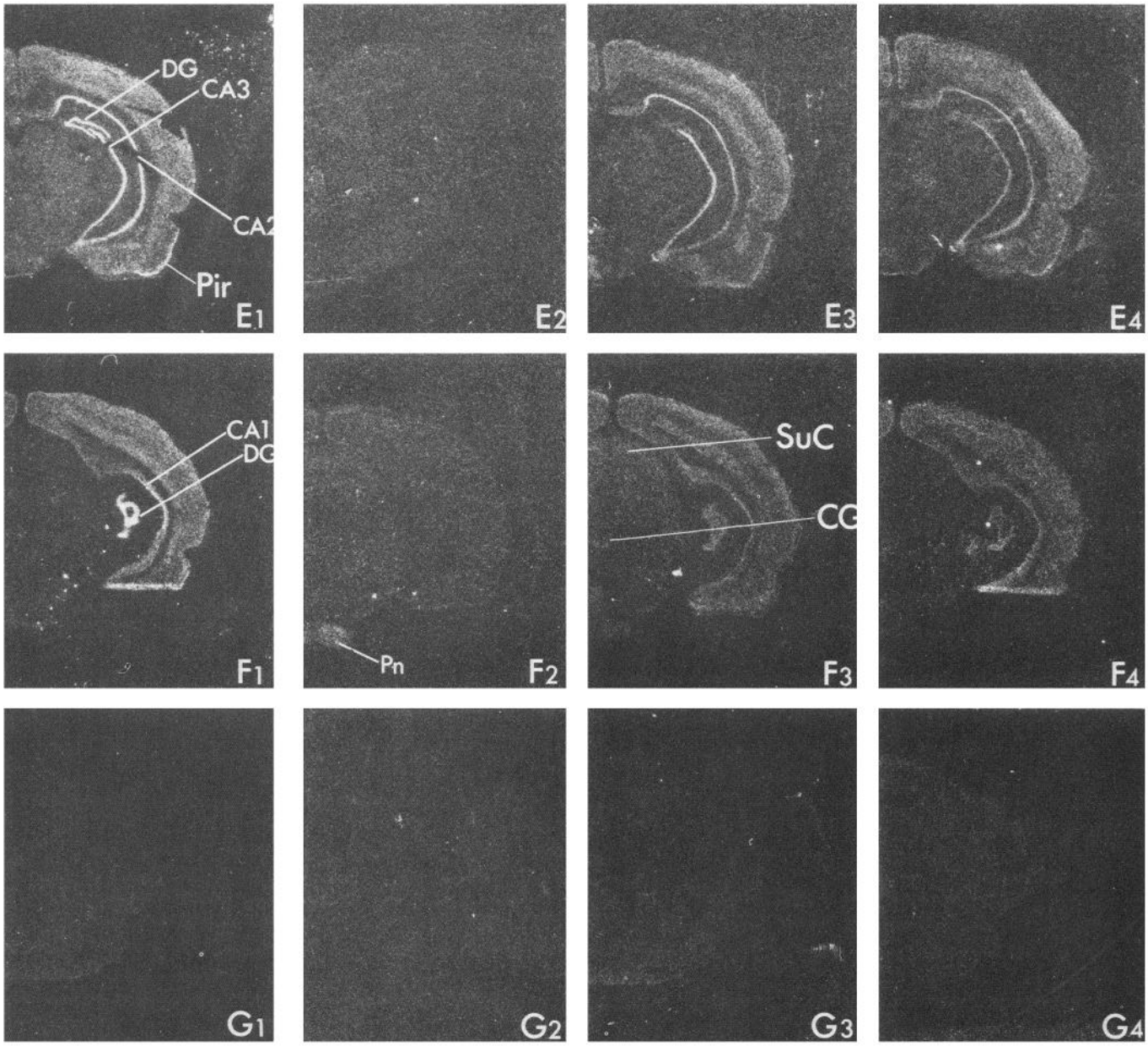

Figure 2. Continued.

mRNA (unpublished observations) suggests that it does recognize rat m2 mRNA, although possibly not as efficiently as a probe based on the rat m2 sequence would.

m3 mRNA was prominent in the pyramidal cell layer of the hippocampus, but, in contrast to m1 mRNA, only low levels were detectable in the dentate gyrus. A similar but more pronounced laminar distribution to that seen with the m1 probe was seen in the cerebral cortex, where m3 mRNA was predominantly expressed in the superficial and deep layers. Piriform cortex and olfactory tubercule were also heavily labeled. Other labeled areas included the plexiform layers and nuclei of the olfactory bulb and numerous thalamic structures, including the habenulae, the anteroventral, ventrolateral, and midline thalamic nuclei. Low levels of m3 mRNA were also detected in the lateral septal nuclei and caudate putamen, as well as hindbrain nuclei including the superior colliculus, pontine nuclei, and central gray.

m4 mRNA predominated in the caudate putamen, olfactory bulb, piriform cortex, and pyramidal cell layer of the hippocampus and, as with the m3 mRNA, showed relatively little hybridization in the dentate gyrus. Cerebral cortex was relatively evenly labeled, although the midcortical layer appeared more intensely labeled than surrounding cortex. Very low levels of m4 mRNA were detectable in the thalamus and cerebellum.

Discussion

The results of this study clearly show that at least 4 different muscarinic receptor genes are expressed in rat brain. Furthermore, each muscarinic receptor mRNA has a unique distribution within the CNS.

Numerous workers have mapped muscarinic receptors in the CNS using radioligands to label the receptor. Comparison of the data derived from the use of *in vitro* autoradiography with *in situ* hybridization studies is difficult. Since mRNA is present

only in cell bodies and proximal dendrites, *in situ* hybridization identifies cell bodies that make the receptor, while *in vitro* autoradiography localizes the receptor itself. The latter may be on the cell body or on processes distal to the cell body. Comparison is further hindered by the failure of currently available radioligands to specifically label individual muscarinic receptor subtypes. From our pharmacological studies of the m1, m2, m3, and m4 receptor cDNAs transfected into COS cells and L cells it is clear that pirenzepine does not distinguish clearly between m1, m3, and m4 receptors (Bonner et al., 1987; Brann et al., 1987). Clearly, mapping studies using nonselective ligands such as ^{3}H-QNB or ^{3}H-NMS actually localize at least 4 receptors, while ^{3}H-pirenzepine labels at least 3 receptors. The overall pattern of receptor distribution as revealed by radioligand binding studies and *in vitro* receptor autoradiography is that of a predominantly telencephalic location of high-affinity pirenzepine receptors in cerebral cortex, hippocampus, dentate gyrus, and corpus striatum. In contrast, low-affinity pirenzepine receptors are more widespread, being present in a number of hindbrain structures in addition to the forebrain. However, the distribution of muscarinic receptors is in broad agreement with the prevalence of m1, m3, and m4 mRNA in forebrain and the presence of m2 mRNA in brain-stem structures such as the pontine nuclei.

Autoradiographic localization studies alone cannot directly tell us anything of the functions subserved by each receptor subtype. However, the fact that such a multiplicity of responses to muscarinic receptor activation is known to occur in the CNS (North, 1986; Nathanson, 1987) and the current demonstration of a heterogeneous expression of 4 different muscarinic receptor genes warrants of comparison of the known responses with the receptor mRNA distributions described in this study. However, it is worth pointing out that there is no evidence that the relative abundances of receptor proteins are reflected in the relative abundances of their mRNAs since individual mRNAs could differ in their translation efficiencies. Furthermore, the data presented here may be incomplete since other muscarinic receptor subtypes may exist that have not yet been identified. Nevertheless, such comparisons may provoke novel insights into the responses that may be transduced by each muscarinic receptor.

Cerebral cortex

Activation of muscarinic receptors in rat cortex stimulates phosphoinositide hydrolysis (Downes, 1982) and give rise to both a synaptic inhibition and a slow excitation (Krnjevic et al., 1971; Krnjevic, 1974). In guinea pig cortex pyramidal neurones, slow excitation occurs by inhibition of a voltage-dependent K^+ current (M current; Brown and Adams, 1980) and is mediated by a high affinity pirenzepine receptor (McCormick and Prince, 1985). In contrast, inhibition is believed to occur by excitation of muscarinic receptors on inhibitory interneurons that have a low affinity for pirenzepine (McCormick and Prince, 1985). Stimulation of phosphoinositide hyrolysis in cerebral cortex is also mediated by a high-affinity pirenzepine receptor (Brown et al., 1984; Lazareno et al., 1985). Inhibition of the M-current by muscarinic agonists has also been described in the piriform cortex (ffrench-Mullen et al., 1983) and olfactory bulb (Constanti and Galvan, 1983), both regions that express m1, m3, and m4 mRNAs. The coexpression of m1, m3, and m4 muscarinic receptor genes in cerebral cortex indicates that stimulation of phosphoinositide hydrolysis and inhibition of M-current could be mediated by up to 3 muscarinic receptors. Numerous localization studies have demonstrated a prevalence of high-affinity pirenzepine sites in cortical layers II, III, and VI. From the distribution of the m3 mRNA across the cortex it would appear that this laminar distribution may be largely due to a heterogeneous distribution of m3 receptors. An exception to the coexpression of m1, m3, and m4 receptor mRNA in cortex is provided by the exclusive presence of m1 mRNA in the basolateral amygdaloid nuclei. Interestingly, chemical kindling of this locus by muscarinic agonists is used as an animal model of epilepsy (McNamara, 1978; Wasterlain and Jones, 1981).

Hippocampus

Numerous responses are elicited in hippocampus by activation of muscarinic receptors. These include: inhibition of the M-current (Halliwell and Adams, 1982); inhibition of a slow after hyperpolarization caused by activation of a Ca^{2+}-activated K^+ conductance (Cole and Nicoll, 1984); a membrane depolarization (Bernardo and Prince, 1982; Cole and Nicoll, 1984); an inhibition of a fast, transient K^+ current (Nakajima et al., 1986); reduction of a Ca^{2+} current (Gahwiler and Brown, 1987) and stimulation of phosphoinositide hydrolysis (Downes, 1982; Gil and Wolfe, 1985). Clearly the expression of at least 3 different muscarinic receptor genes in hippocampus offers the potential for specific identification of these responses with individual receptor types. At present, characterization of these responses with respect to inhibition by pirenzepine has been carried out only for stimulation of phosphoinositide hydrolysis, which has been shown to be coupled to a receptor expressing a high affinity towards pirenzepine (Gil and Wolfe, 1985; Fisher, 1986). The observation that m1 receptors are the dominant subtype found in the dentate gyrus is interesting since muscarinic receptors in the dentate gyrus are specifically down-regulated in kindled animals (Dasheiff et al., 1982; Savage and McNamara, 1982; Savage et al., 1983), thus lending further support to the idea that m1 receptors play a role in seizure formation.

Striatum

Two major responses to muscarinic receptor activation have been characterized in striatum. First, a presynaptic augmentation of dopamine release from striatal nerve terminals has been demonstrated. Unlike most other presynaptic actions of ACh, this response appears to be mediated by a high-affinity pirenzepine receptor (Raiteri et al., 1984; James and Cubeddu, 1987). However, no muscarinic receptor mRNA was detected in the substantia nigra, the area that projects dopaminergic neurons to the striatum. Stimulation of muscarinic receptors also causes an inhibition of adenylate cyclase, a response mediated by a low-affinity pirenzepine receptor (Gil and Wolfe, 1985). The functions subserved by striatal m1, m3, and m4 receptors remain to be elucidated.

Thalamus

Much less is known about muscarinic receptors in thalamus, although autoradiographic and radioligand binding studies have shown most receptors to be of the low-affinity pirenzepine type (Cortes and Palacios, 1986; Cortes et al., 1986; Mash and Potter, 1986). Muscarinic stimulation of reticular thalamic neurons leads to a hyperpolarization due to an increase in K^+ conductance that is mediated by a low-affinity pirenzepine type of receptor (McCormick and Prince, 1986). Accordingly the low levels of m2 mRNA found in the thalamic nuclei may encode the receptor responsible for this hyperpolarization. We are unaware of any thalamic response that has been linked to a high-affinity pirenzepine type receptor that may be encoded by the m3 mRNA

detected in the thalamus; however, it would appear that m3 receptors may constitute the low amounts of high-affinity pirenzepine receptors that have been described in thalamus.

Pons

As in the thalamus, activation of muscarinic receptors in the pontine reticular nucleus has been demonstrated to cause a hyperpolarization due to a rise in K^+ conductance mediated by a low-affinity pirenzepine receptor (Egan and North, 1986). A low- to intermediate-affinity pirenzepine receptor is also coupled to phosphoinositide hydrolysis in the pons (Lazareno et al., 1985). As suggested in the case of the thalamus, the m2 receptor could be responsible for this action.

Septum

Cells of the medial septum, diagonal band of Broca and Meynerts nucleus give rise to the chief cholinergic projection to the cerebral cortex and hippocampus (Lewis et al., 1967; Shute and Lewis, 1967). Inhibition of ACh release by muscarinic autoreceptors on cholinergic nerve terminals occurs in both cerebral cortex and hippocampus (Marchi et al., 1981; Marchi and Raiteri, 1984; Raiteri et al., 1984; Meyer and Otero, 1985). The receptor mediating this action has been characterized as a low-affinity pirenzepine type. mRNA encoding presynaptic receptors would be anticipated to be found in cholinergic nerve cell bodies that project to the target areas. Hence, m2 mRNA detected in the medial septum and diagonal band may thus encode the receptors that are subsequently transported to nerve terminals in the cortex and hippocampus, where they act as inhibitory presynaptic autoreceptors. This is of potential clinical importance since Alzheimer's disease is accompanied by a degeneration of septal nuclei (Coyle et al., 1983) and a decline of low-affinity pirenzepine receptors in cerebral cortex (Mash et al., 1985).

Cerebellum

The results obtained with the cerebellum remain enigmatic in that Northern analysis shows the presence of m1, m3, and m4 mRNAs, yet *in situ* hybridization reveals little labeling in the sections examined. A likely possibility is that muscarinic receptor mRNA is present in discrete nuclei that contributed to the total RNA extracted from the cerebellum but were not present in the sections examined in this study. Further experiments are underway to examine the possibility.

Our data illustrate the differential expression of 4 muscarinic receptor genes in the CNS. It should be noted, however, that these data may not provide a complete description of muscarinic receptor expression in the CNS since there is evidence for the existence of additional muscarinic receptors. First, hybridization of rat and human genomic DNA with m1 receptor cDNA indicates the presence of at least one more closely related gene in the rat and human genomes (Bonner et al., 1987) and potentially several others. Second, pharmacological studies using a novel antagonist, AF-DX 116 (Hammer et al., 1986) have indicated the presence of 2 low-affinity pirenzepine receptors subtypes; a low- to intermediate-affinity pirenzepine/high-affinity AF-DX 116 type (cardiac type) and a low-affinity pirenzepine/low-affinity AF-DX 116 type (glandular type). The m3 receptor has the pharmacological properties of glandular receptors (unpublished observations). Also, it is clear that each muscarinic receptor is linked to a specific subset of effector mechanisms and this may underlie the heterogeneous distribution of receptor mRNA observed here (Jones et al., 1988). Answers to these questions must await derivation of pharmacological probes capable of specifically interacting with only one receptor subtype.

Note added in proof. Subsequent to the submission of this manuscript, Peralta et al. (1987) reported the cloning of human genes for the same 4 muscarinic receptors but used a different nomenclature. Their M1, M2, M4 and M3 correspond to our m1, m2, m3, and m4, respectively, i.e., M4 = m3 and M3 = m4 (Bonner et al., 1987).

The recent cloning of a rat m2 cDNA (Gocayne et al., 1987) has allowed us to compare the human m2 and the rat m2 sequences corresponding to the oligonucleotide probe used in this study. Out of 48 nucleotides there are only 5 mismatches (i.e., 90% homology) that are distributed fairly evenly throughout the length of the probe. These mismatches will clearly destabilize the hybrid between the human probe and the rat mRNA, but the extent to which this occurs may only be determined empirically. The fact that this probe hybridizes to a 6.2 kb m2 RNA in rat atria is the most direct evidence of its fidelity.

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