

Expression of m1–m4 Muscarinic Acetylcholine Receptor Proteins in Rat Hippocampus and Regulation by Cholinergic Innervation

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A family of muscarinic ACh receptor genes are expressed in hippocampus, but little is known about the localization of the encoded proteins and their regulation by cholinergic innervation. Subtype-specific antibodies were used to localize m1–m4 proteins in the hippocampal formation by immunocytochemistry and to determine the alterations in the subtypes following deafferentation. Each of the receptors is differentially localized in Ammon's horn and dentate gyrus, with highly complementary distributions. m1 is widely expressed in somata and dendrites of pyramidal neurons and granule cells in dentate gyrus. m2 immunoreactivity is expressed mostly in nonpyramidal neurons, and in several discrete bands of fibers and puncta surrounding pyramidal neurons and other layers. m3 is enriched in pyramidal neurons, the neuropil in stratum lacunosum-moleculare and the outer third of the molecular layer of dentate gyrus. m4 is enriched in nonpyramidal neurons, in fiber pathways (alveus, fimbria, and hippocampal commissure), and in the inner third of the molecular layer. Fimbria-fornix lesions decreased ipsilateral m2- and m4-immunoreactive axons in the fimbria, with no apparent changes in the distribution of any of the receptors in hippocampus. 192-IgG immunotoxin lesions of the cholinergic septohippocampal projections, which spare noncholinergic projections, produced a small decrease in m2-immunoreactive fibers in the fimbria with no other major changes in the distribution of subtypes. Immunoprecipitation studies at 3–28 d following fimbria-fornix lesions revealed a 25% loss of m2 at 3 d in hippocampus, and upregulation of both m1 (20–29% at 7–14 d) and m4 (44% at 28 d). Thus, the vast majority of muscarinic receptor subtypes are intrinsic to the hippocampal formation and/or nonseptal hippocampal afferents. A subset of m2 and m4 are presynaptically localized, with m2 in cholinergic axons and m2 and m4 possibly in noncholinergic axons that comprise the septohippocampal pathway. The unique laminar and regional distributions of m1–m4 in the hippocampus reflect differential cellular and subcellular distributions of the subtypes and/or selective association of receptor subtypes with certain afferent and intrinsic con-

nections. These results indicate that each subtype likely has a different role in cholinergic modulation of excitatory and inhibitory hippocampal circuits.

[Key words: cholinergic, fimbria-fornix, deafferentation, presynaptic, postsynaptic, basal forebrain]

Muscarinic cholinergic neurotransmission in the hippocampus plays a key role in a variety of higher brain functions, including learning and memory. For example, blockade of muscarinic receptors or lesions of cholinergic septohippocampal projections produce memory and attentional deficits (Drachman and Leavitt, 1974; Bartus et al., 1982; Fibiger, 1991; Dekker et al., 1991; Nilsson et al., 1992; Callahan et al., 1993). Also, degeneration of basal forebrain cholinergic systems may contribute to memory loss and other cognitive deficits in Alzheimer's disease (Coyle et al., 1983). Indeed, the potential for cholinergic replacement therapies in dementia (McKinney and Coyle, 1991; Davis et al., 1992) has provided a great incentive for improved understanding of the roles of muscarinic receptors in normal brain functions and in disease. Although the mechanisms by which muscarinic receptors influence behavior are poorly understood, a wide variety of muscarinic effects have been identified in hippocampal neurons. These effects include activation of several second messenger cascades (McKinney, 1993), postsynaptic modulation of several potassium currents (Cole and Nicoll, 1984; Halliwell, 1990; Krnjevic, 1993) and calcium channels (Toselli et al., 1989), and presynaptic inhibition of release of acetylcholine and excitatory and inhibitory amino acid neurotransmitters (Raiteri et al., 1984; Pohorecki et al., 1988; Marchi and Raiteri, 1989; Raiteri et al., 1990; Richards, 1990; McKinney et al., 1993). Moreover, muscarinic receptors enhance the responsiveness of NMDA glutamate receptors (Markram and Segal, 1990) and modulate long-term potentiation and long term depression (Blitzer et al., 1990; Kahle and Cotman, 1989; Burgard and Sarvey, 1990; Markram and Segal, 1990; Williams and Johnston, 1990; Pang et al., 1993). Thus, diverse postsynaptic and presynaptic muscarinic receptor mechanisms are involved in cholinergic modulation of critical hippocampal functions.

A family of five muscarinic receptor genes, encoding highly related but functionally distinct receptor subtypes, are all expressed in hippocampus (Bonner et al., 1987; Buckley et al., 1988; Vilaro et al., 1993). Because of the limited selectivity of available drugs (Buckley et al., 1989; Dorje et al., 1991), it has not been possible to link the diversity of cholinergic actions in hippocampus to molecular subtypes of receptors. A more detailed understanding of the cellular and molecular mechanisms

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of cholinergic actions in hippocampus has been limited by a paucity of information about the localization and functions of the genetically defined receptor subtypes. We and others have developed highly specific antibodies for characterizing the native m1–m5 muscarinic receptor proteins (Levey et al., 1991; Wall et al., 1994). At least four receptor proteins (m1–m4) are present in cholinergic target fields in hippocampus by immunoprecipitation analysis; the m5 protein has not been reliably detected (Levey et al., 1991, 1994; Wall et al., 1994). Immunocytochemistry provides exquisite sensitivity, high resolution, and subtype selectivity for determining the precise cellular and subcellular (e.g., pre- and postsynaptic) distributions of the receptors (Levey et al., 1991; Hersch et al., 1994), but this approach has not yet been applied to the distributions of m1–m4 in hippocampus. Such information will be essential to clarify the differential roles of the muscarinic receptor family in hippocampal functions and their regulation by cholinergic innervation.

Thus, there were several goals of the present study. The first goal was to determine the light microscopic localization of m1–m4 immunoreactivity in the dorsal hippocampus of the rat. Secondly, we sought to determine the magnitude and distribution of changes in subtypes that occur following depletion of medial septal input to hippocampus by unilateral fimbria-fornix lesions using immunoprecipitation and immunocytochemistry, respectively. This lesioning model has been used extensively to determine putative pre- and postsynaptic distributions of muscarinic receptors in the septohippocampal pathway, and as an experimental model for regulatory changes that may occur in Alzheimer's disease. Finally, in order to investigate changes in muscarinic receptor subtypes after selective depletion of cholinergic input to hippocampus, we used recently developed 192-saporin immunotoxin lesions combined with immunocytochemical localization of m1–m4. This immunotoxin, directed against neurons expressing the low affinity nerve growth factor receptor, produce highly effective lesions of the cholinergic neurons, but spare the noncholinergic neurons which also contribute projections to the septohippocampal pathway.

Materials and Methods

Antibodies. Rabbit polyclonal antibodies specific for m1–m4 receptors were prepared, affinity purified, and characterized as described previously in detail (Levey et al., 1990, 1991; Hersch et al., 1994). Briefly, the antibodies were raised against recombinant proteins derived from the nonconserved third inner cytoplasmic loop of each receptor fused to glutathione S-transferase. The antisera each immunoprecipitate the homologous cloned receptor without cross-reaction to any other of the five cloned muscarinic receptors (Levey et al., 1991). On Western blots, each purified antibody also binds monospecifically to the appropriate subtype without cross-reaction to the other receptors (Hersch et al., 1994). Moreover, the distribution of m1–m4 receptor proteins in rat brain are similar by both immunoprecipitation, immunoblotting, and immunocytochemistry, and antibody binding is blockable with the homologous fusion protein (Levey et al., 1991; Hersch et al., 1994; Levey et al., 1994). These data rigorously establish the subtype specificity of the antibodies. A rat monoclonal antibody to the third inner loop of m2 was also used for immunocytochemistry; this antibody results in an identical distribution of staining as the polyclonal antibody and has been described previously (Levey et al., 1995).

Immunocytochemistry. Male Sprague–Dawley rats ($n = 20$; Charles River, Wilmington, MA), 250–450 gm, were deeply anesthetized with chloral hydrate, perfused intracardially at a rate of 20 ml/min with 0.9% saline, followed by 0.1 M phosphate-buffered 3% paraformaldehyde (200 ml), pH 7.4, and then 10% sucrose in the same buffer. Brains were immediately removed, immersed in 30% sucrose in phosphate buffer at 4°C until they sunk, frozen on dry ice, and sectioned at 40 μ m on a sliding microtome. Tissue sections were processed for immunocytochemistry using peroxidase anti-peroxidase (Sternberger Monoclonals,

Inc., Baltimore, MD) or avidin–biotin–peroxidase methods (Vectastain Elite, Vector Laboratories, Burlingame, CA) as described previously (Levey et al., 1991). Affinity purified rabbit polyclonal antibodies to the third inner loops of m1, m2, m3, and m4 were used at 0.5–1.0 μ g/ml. Monoclonal antibody to the same region of m2 was used at a dilution of 1:500. Antibody dilutions were determined empirically to optimize signal to noise ratio. Immunocytochemical controls consisted of adsorption of the antibodies with 100 μ g/ml of GST or muscarinic-GST fusion protein for 20 min prior to staining. For control experiments, additional sections were incubated in normal rabbit immunoglobulin (1.0 μ g/ml) instead of the primary antibody, or primary antibody was omitted.

Fimbria-fornix lesions and alterations of muscarinic receptor subtypes. To characterize the regulation of muscarinic receptor subtypes in hippocampus following unilateral denervation of the medial septal input, quantitative immunoprecipitation studies and immunocytochemistry were performed at varying time points after transection of the fimbria-fornix. Adult Sprague–Dawley rats ($n = 28$) were anesthetized intramuscularly with a combination of ketamine (13 mg/kg) and xylazine (87 mg/kg) following vagal blockade with atropine (0.1 mg/kg s.c.) and subjected to complete unilateral fimbria-fornix transection at a level immediately anterior to the septal pole of hippocampus as described previously (Koliatsos et al., 1991). After various survival times, animals were sacrificed by decapitation for immunoprecipitation studies or perfused for light microscopic localization of receptor immunoreactivity as described above. The efficacy of the lesions and their confinement to the fimbria fornix bundle on the side of the lesion was assessed with ChAT enzyme assays (Levey et al., 1983), histology, and acetylcholinesterase (AChE) histochemistry (Koliatsos et al., 1991).

For immunoprecipitation studies, the entire hippocampus (i.e., dorsal and ventral) was dissected on ice and the lesioned and nonlesioned sides compared for each rat. Because of the limited amount of tissue available for each animal, we measured only m1, m2, and m4 since these are the predominant subtypes in hippocampus. Tissue was pooled from two brains in some cases when individual homogenates were insufficient to permit triplicate analyses of each marker. The hippocampi were homogenized with a polytron at 4°C in 10 vol (w/v) of Tris-EDTA buffer (TE: 10 mM Tris; 1 mM EDTA, pH 7.5) additionally containing 0.2 mM phenylmethylsulfonyl fluoride, 1 μ M pepstatin A, 1 μ g/ml leupeptin, and 10 μ g/ml soybean trypsin inhibitor to retard proteolysis. Homogenates were centrifuged at 20,000 $\times g$ for 10 min and the supernatant discarded. Receptors were solubilized by brief rehomogenization of pellet aliquots at 4°C in TE with added detergents (TED: TE buffer, additionally containing 0.4% digitonin and 0.04% cholic acid) at a final protein concentration of approximately 1 mg/ml. The homogenate was incubated at 4°C for 1 hr, and then centrifuged at 12,000 $\times g$ for 30 min, and solubilized receptors were collected in the supernatant. For subtype immunoprecipitations, solubilized receptors were labeled with 1.0 nM 3 H-NMS, and parallel samples were mixed with a single antiserum specific for m1, m2, or m4 receptors (final dilution 1:50) at 4°C for 4 hr, and then goat anti-rabbit (final dilution 1:10) was added to a final volume of 285 μ l and incubated overnight to coprecipitate immune complexes (containing rabbit anti-receptor–receptor– 3 H-NMS). The immunoprecipitates were pelleted by centrifugation (1000 $\times g$), washed rapidly by resuspension in ice cold TED buffer, recentrifuged, and radioactivity in the pellets was determined by liquid scintillation spectroscopy. Control immunoprecipitates using nonimmune sera were performed in each assay to determine nonspecific trapping of 3 H-NMS, and these values were subtracted from experimental samples. The density of each subtype immunoprecipitated was calculated using the total soluble receptors added per assay, corrected for solubilization efficiency and total membrane binding. Results were expressed as the percentage of each receptor subtype in the lesioned hippocampus compared to the control unlesioned side. Statistical significance was determined by paired two-sample t tests in lesioned versus unlesioned hippocampi for each subtype at a probability level of 0.05 (two tailed).

Total muscarinic receptor binding in the homogenates was determined by membrane filtration assay after labeling in 1 nM 3 H-NMS (82 Ci/mmol) with 1 μ M atropine sulfate to define nonspecific binding. Total solubilized muscarinic receptors were determined in aliquots of the supernatants labeled with 1 nM 3 H-NMS, and receptor-bound activity was monitored by chromatography using Sephadex G-25 columns and liquid scintillation spectroscopy. The specific activity of ChAT was determined in 25 μ l samples of the homogenates as pre-

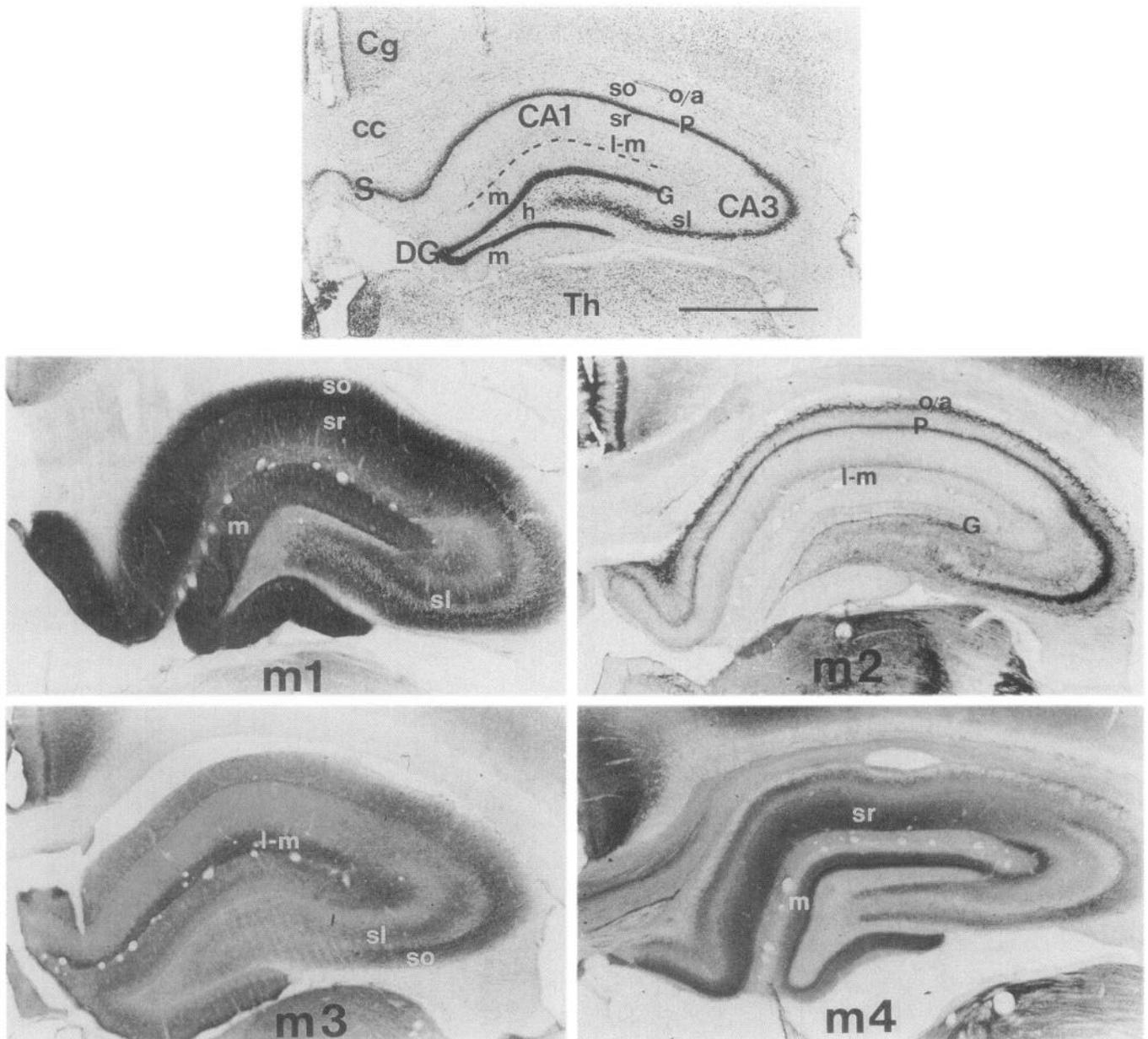


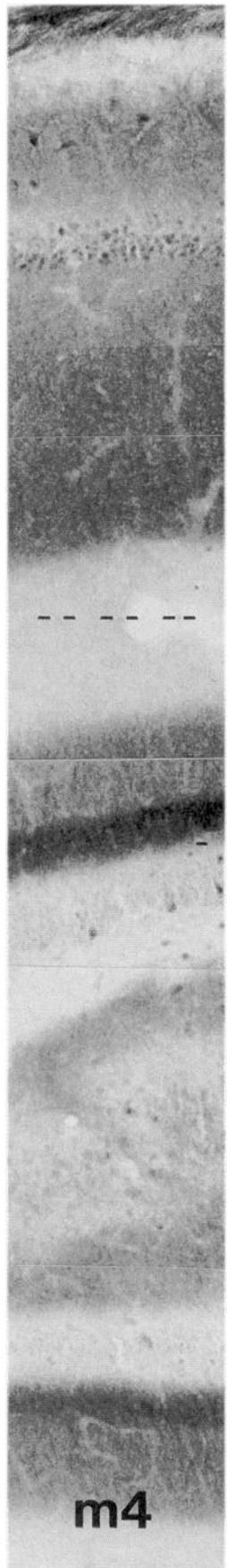
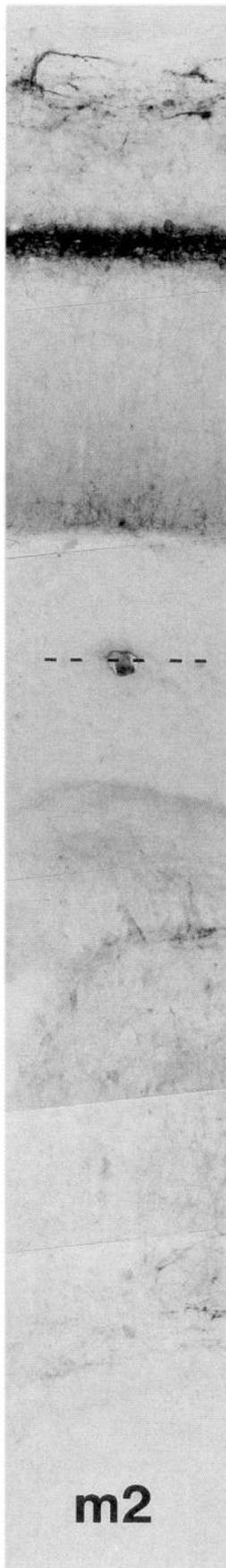
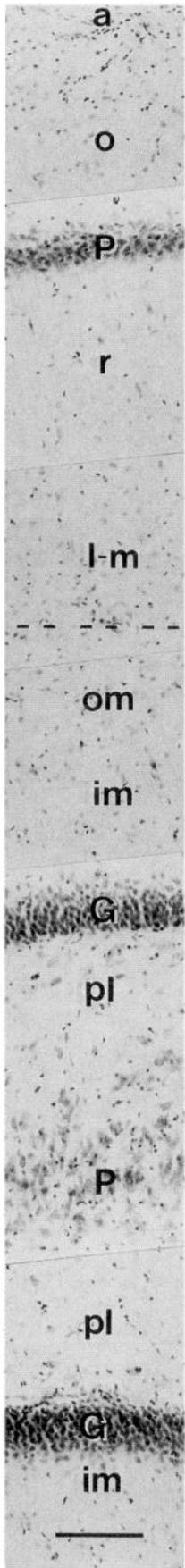
Figure 1. Light microscopic localization of m1–m4 immunoreactivity in the rat hippocampus. The *top panel* shows a Nissl-stained section to illustrate the regions and lamina of Ammon's horn and dentate gyrus. CA1 and CA3, regions of Ammon's horn; cc, corpus callosum; Cg, cingulate cortex; DG, dentate gyrus; G, stratum granulosum; h, hilus; l-m, stratum lacunosum-moleculare; m, molecular layer; o/a, oriens-alveus border; P, stratum pyramidale; S, subiculum; sl, stratum lucidum; so, stratum oriens; sr, stratum radiatum; Th, thalamus. Scale bar, 1.0 mm.

viously described (Levey et al., 1983) to assess the extent of the cholinergic lesion. Protein was determined with the bichinchonic acid method (Smith et al., 1985).

192 IgG-saporin immunotoxin lesions and alterations of muscarinic receptor subtypes. The unilateral fimbria-fornix lesions described above deplete both cholinergic and noncholinergic inputs to the hippocampus. To qualitatively assess alterations in muscarinic receptor subtypes after selective lesions of the cholinergic innervation of hippocampus, we also studied the immunocytochemical distribution of m1–m4 in adult Sprague–Dawley rats subjected to intracerebroventricular injections of 192 IgG-saporin immunotoxin ($n = 2$) or saline vehicle ($n = 1$) injections. The 192 IgG is a monoclonal antibody that binds to an extracellular epitope of the low affinity nerve growth factor receptor, and when conjugated to the saporin, a ribosome-inactivating protein, the antibody–toxin complex is internalized and results in neuronal degeneration. Because this receptor is abundantly and selectively expressed by the cholinergic

neurons in basal forebrain of normal adult rats, the 192 IgG-saporin immunotoxin produces selective loss of cholinergic neurons in this region (Book et al., 1992, 1994; Heckers et al., 1994). The 192 IgG-saporin was prepared as previously described (Wiley et al., 1991; Wiley and Lappi, 1993), and 4 μ g was pressure injected into the lateral ventricle. After a survival period of 2 weeks, animals were perfusion fixed and adjacent series of frozen sections (40 μ m) were processed for the light microscopic immunocytochemical localization of m1–m4 as described above. Additional series were processed for acetylcholinesterase histochemistry and ChAT immunohistochemistry using monoclonal antibody AB8 (Levey et al., 1983) to assess the extent of hippocampal denervation and survival of cholinergic neurons in the basal forebrain, respectively.

All animal experimentation in these studies was conducted in accordance with the policy on the use of animals in neuroscience research as approved by the Society of Neuroscience in April 1992.



Results

Immunocytochemical distribution of m1–m4 in rat hippocampus

The m1–m4 receptor immunoreactivities were differentially distributed in the rat hippocampus (Figs. 1–4). In general, receptor immunoreactivities were localized in neurons, neuritic processes, and diffusely in the neuropil, although each antibody resulted in a distinct pattern of staining. Neuronal staining typically filled the cytoplasm of the cells, except for m2 which frequently resulted in a margin of reaction product along the cell surface with much less immunoreactivity in the cytoplasm. Control sections processed with omission of antibody routinely showed only very light diaminobenzidine reaction product in occasional glial cells or processes and rarely in isolated neurons, which was unlike any muscarinic receptor immunoreactivity. Immunological specificity was also demonstrated by inhibition of staining after preadsorption of the antibodies with the i3 loop fusion proteins derived from each subtype. Further descriptions of m1–m4 immunoreactivities refer to specific staining that was not observed in controls, with the intent that the terms denote “receptor-like” immunoreactivity. As with any immunocytochemical procedure, it is not possible to be certain that the reaction product is localized only to the molecule of interest in the tissue sections despite rigorous characterization of antibody specificity by immunoprecipitation and immunoblotting.

m1 immunoreactivity was the most widely distributed subtype in hippocampus (Figs. 1–3). It was enriched in CA1 compared to CA3, but was present throughout Ammon’s horn in the soma of pyramidal neurons and their proximal apical and basal dendrites in stratum radiatum and stratum oriens, respectively. In CA3, apical dendrites of the pyramidal neurons in stratum lucidum were immunoreactive. Neuropil immunoreactivity in the hippocampal strata was generally intense and diffuse, except it was much less intense in stratum lacunosum-moleculare. The neuropil immunoreactivity was comprised of fine neuritic processes and occasional puncta, sometimes which appeared asso-

ciated with the fibers. In the dentate gyrus, the inner and outer molecular layers were diffusely immunoreactive and of similar intensity to the stratum oriens and radiatum in CA1. Granule cells and neurons in the hilus were also densely immunoreactive.

m2 immunoreactivity was present in a striking network of neurons with long varicose processes distributed along the stratum oriens/alveus border which encapsulated the entire hippocampus (Figs. 1–3). Within the oriens, many of the fibers were parallel to the alveus, but some were also perpendicular. Scattered multipolar neurons with varicose processes were also present deeper in the stratum oriens and along the pyramidal layer, and occasionally in the stratum radiatum (Fig. 3). These neurons often exhibited punctate immunoreactivity on their soma. The pyramidal neuron layer displayed a dense plexus of fine fibers and puncta outlining the perikarya, in basket-like formations, with the density of immunoreactivity greater in CA3 than in CA1. However, the pyramidal neurons themselves showed little or no immunoreactivity in the soma (Fig. 3). Fine neurites were oriented radially in the neuropil of the stratum radiatum and were more dense in the most distal aspect of this layer, forming a discrete band of immunoreactivity at the transition with the more lightly immunoreactive lacunosum-moleculare (Fig. 2). Within this band, varicose fibers were oriented parallel to the hippocampal fissure. In dentate gyrus, the inner third of the molecular layer displayed more m2 immunoreactivity than the outer two-thirds and there was a discrete narrow band of increased immunoreactivity between these regions. A similar band of densely immunoreactive fibers, many of them coarse with varicosities and large puncta, was marginated just beneath the granule cell layer. The perikarya of the granule cells showed little or no immunoreactivity. In the hilus, scattered multipolar neurons with long processes formed a relatively dense plexus of immunoreactivity. Occasional blood vessels also exhibited light to moderate m2 immunoreactivity which appeared to be associated with the endothelial cells. A minor population of fine

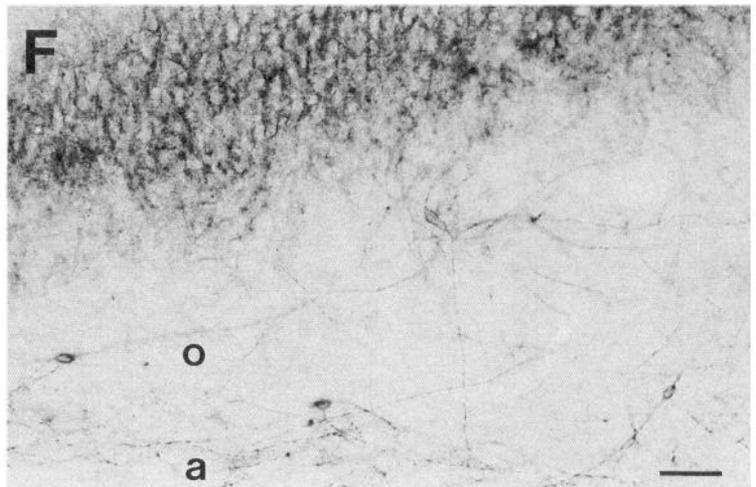
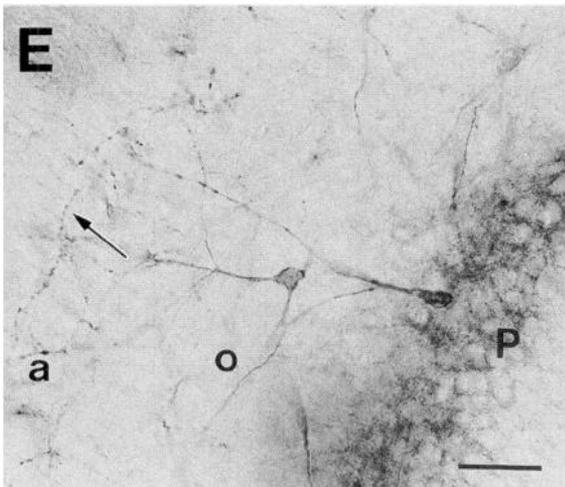
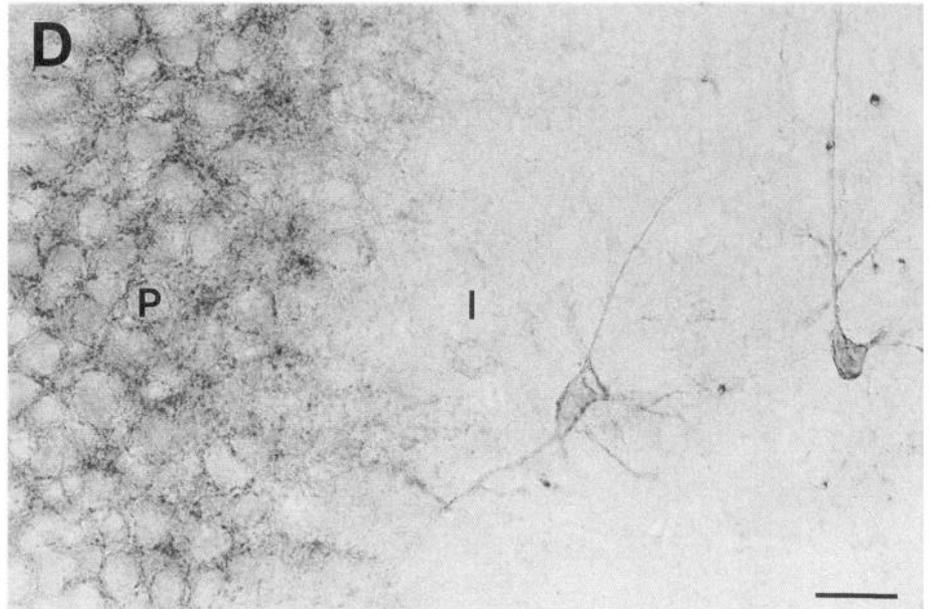
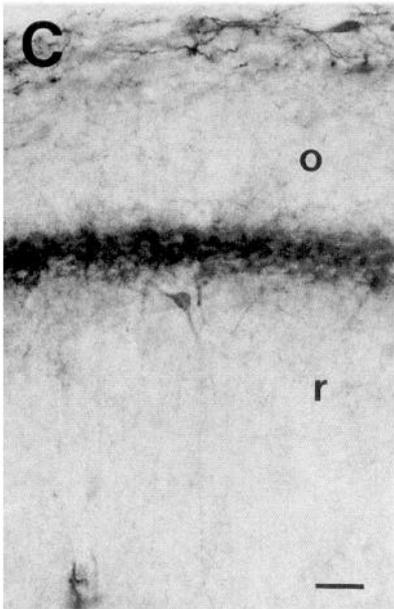
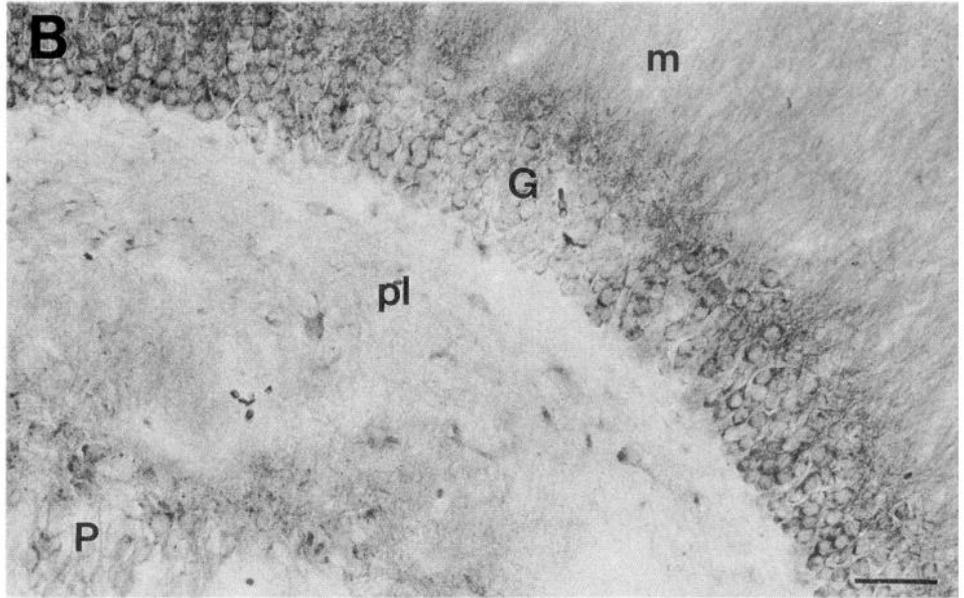
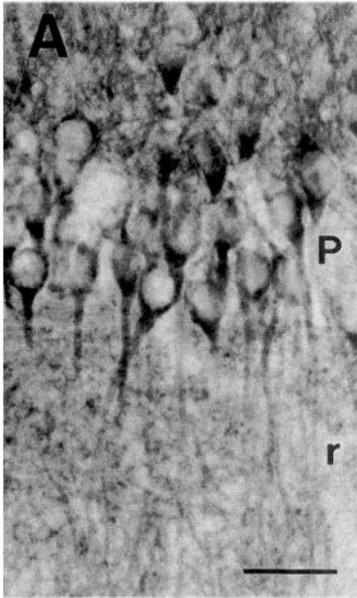
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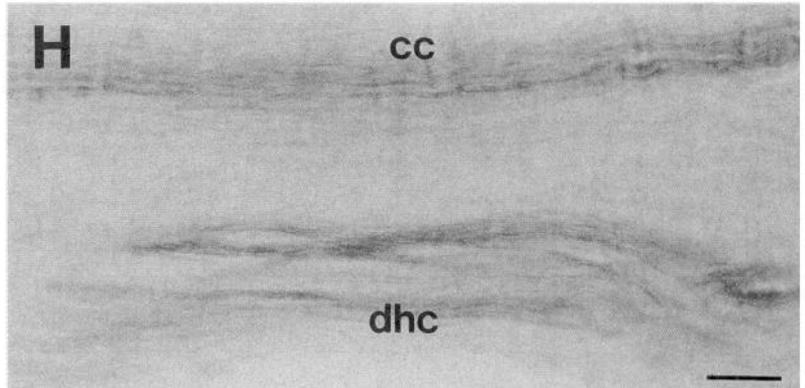
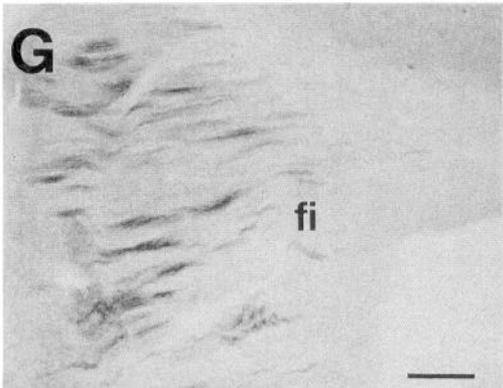
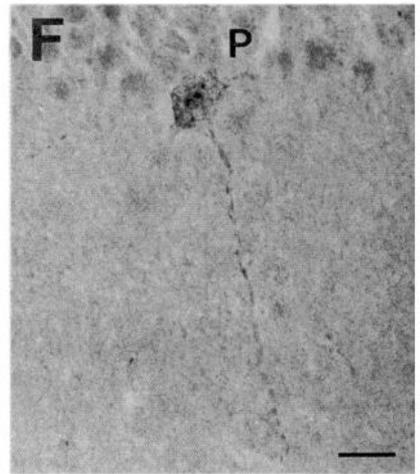
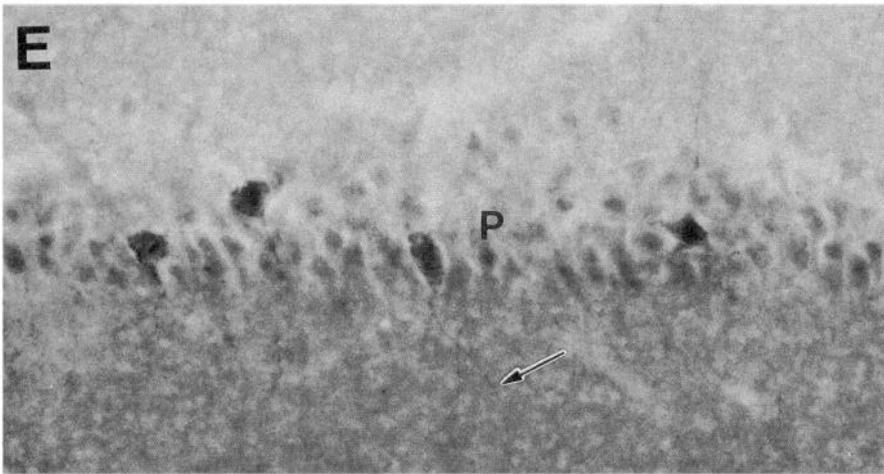
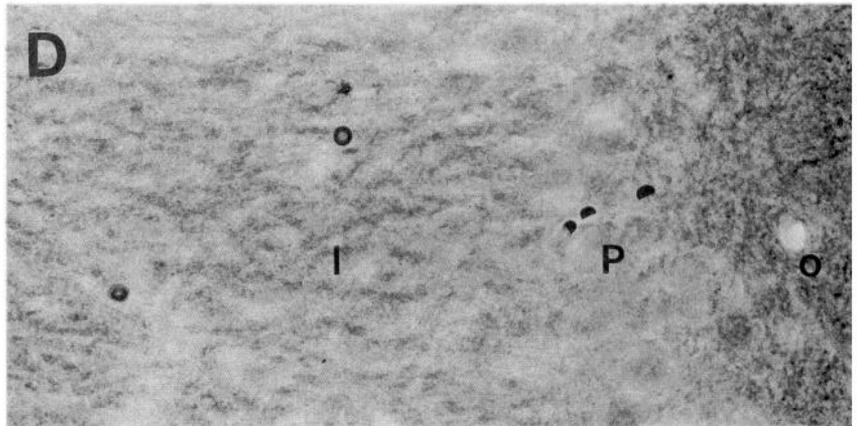
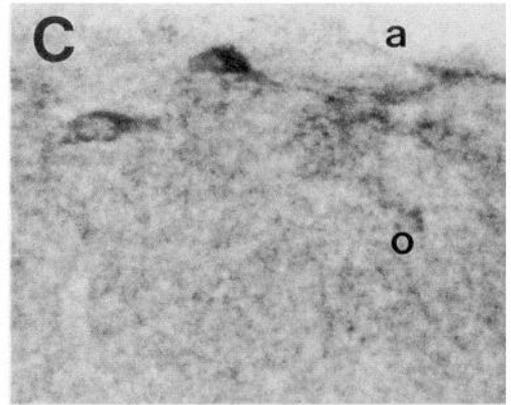
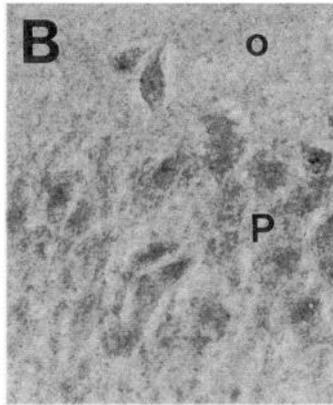
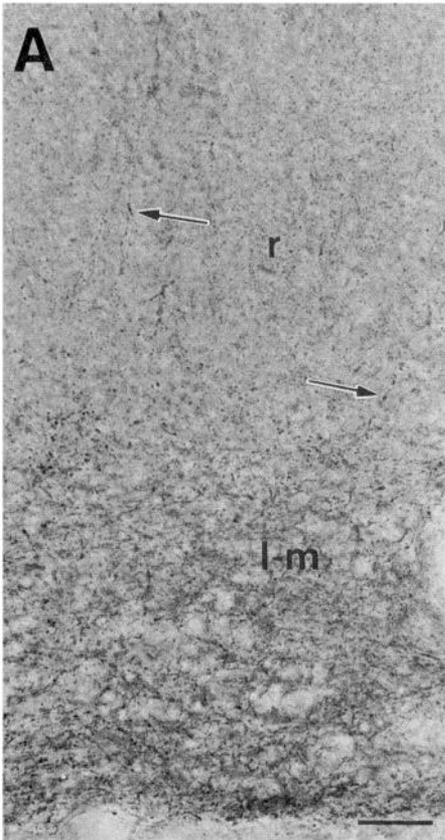
Figure 2. Laminar distributions of m1–m4 immunoreactivity in CA1 and dentate gyrus. Each panel shows a similar column from coronal sections of dorsal hippocampus processed for Nissl (far left) or the muscarinic receptor immunoreactivities. The hippocampal fissure is marked by the *hatched lines*, with CA1 above the line and the dentate gyrus below the line. *a*, alveus; *G*, stratum granulosum; *im*, inner molecular layer; *l-m*, stratum lacunosum-moleculare; *o*, stratum oriens; *om*, outer molecular layer; *P*, stratum pyramidale; *pl*, polymorph layer; *r*, stratum radiatum. Scale bar, 0.1 mm.

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Figure 3. Cellular and subcellular localization of m1 (*A, B*) and m2 (*C–F*) immunoreactivity in hippocampus. *A*, m1 immunoreactivity in the soma of CA1 pyramidal neurons (*P*) and in their apical dendrites in stratum radiatum (*r*). *B*, m1 immunoreactivity in the soma of dentate granule cells (*G*), their apical dendrites in the molecular layer (*m*). Also note m1-immunoreactive nonpyramidal neurons in the polymorph layer as well as pyramidal neurons (*P*) in the hilus (CA3c). *C*, m2 immunoreactivity in CA1 is localized in a band of neurons and processes at the oriens/alveus border, in a dense band of puncta and scattered neurons along the pyramidal cell layer, and more diffusely in fine neuritic processes in stratum oriens (*o*) and stratum radiatum (*r*). *D*, Higher magnification illustrating granular punctate m2 immunoreactivity around the soma of pyramidal neurons (*P*) in CA3, and in the nonpyramidal neurons in stratum lucidum (*l*). Note the dense immunoreactivity in the plasmalemma of nonpyramidal neurons, including the soma and neurites. *E* and *F*, Examples of m2 immunoreactivity expressed in nonpyramidal neurons in stratum oriens (*o*) and varicose axons in the alveus (*a*), one of which is marked by the *arrow*. Scale bars: *A, C, and D*, 25 μ m; *B, E, and F*, 50 μ m.

Figure 4. Cellular and subcellular localization of m3 (*A–D*) and m4 (*E–H*) immunoreactivity in hippocampus. *A*, m3 immunoreactivity is enriched in puncta in the stratum lacunosum-moleculare (*l-m*) and occasional fine varicose fibers (*arrows*) in stratum radiatum. *B*, m3-immunoreactive pyramidal neurons (*P*) in CA1. *C*, m3-immunoreactive nonpyramidal neurons at the alveus (*a*)/oriens (*o*) border. *D*, m3 immunoreactivity is enriched in CA3, appearing finely granular and punctate in the dendritic fields of the pyramidal neurons (*P*) in stratum lucidum (*l*) and stratum oriens (*o*). The red blood cell in the stratum lucidum, above the “1,” is in sharp focus, highlighting the typical diffuse and granular distribution of m3. *E*, m4 immunoreactivity is localized in pyramidal neuron layer (*P*), with more intense staining of scattered somata and the thin varicose fibers (*arrow*) emanating from the cells. *F*, Higher magnification of an m4-immunoreactive neuron and its process. *G*, m4-immunoreactive axonal fibers are prominent in the fimbria. *H*, A subset of crossing fibers in the dorsal hippocampal commissure (*dhc*) and corpus callosum (*cc*) are m4-immunoreactive fibers. Scale bars: *A–F*, 25 μ m; *G*, 100 μ m; *H*, 50 μ m.





immunoreactive axons was present in the alveus, fimbria, and fornix.

m3 immunoreactivity was enriched in the stratum oriens and stratum lucidum in CA3 and in the stratum lacunosum-moleculare in CA1 (Fig. 1). The soma of pyramidal neurons were light to moderately immunoreactive, but scattered neurons in this layer were more darkly stained. Occasional nonpyramidal neurons in the stratum oriens were also immunoreactive. However, much of the immunoreactivity was finely punctate and diffuse in the neuropil (Figs. 2, 4). In the stratum lacunosum-moleculare this neuropil immunoreactivity was most dense and appeared associated with a plexus of fibers and puncta (Fig. 4). The stratum lucidum neuropil immunoreactivity was more finely granular than in the lacunosum-moleculare, and appeared associated with the dendrites of pyramidal neurons. In the dentate gyrus, the most outer aspect of the molecular layer displayed dense neuropil immunoreactivity, particularly in the inferior blade. In the superior blade, there was a poorly distinguished band of increased immunoreactivity in the middle third of the molecular layer, as well as a band in the most outer aspect (see Figs. 1, 2, 8). Neurons in the granule cell layer and hilus were moderately immunoreactive.

m4 immunoreactivity was enriched in CA1 and dentate gyrus compared to CA3 (Fig. 1). Pyramidal neurons were lightly immunoreactive and a few scattered nonpyramidal neurons with fine neuritic processes that entered the stratum radiatum were more intensely immunoreactive (Fig. 4). Fine processes, oriented in all directions, were also present in the stratum oriens. Neuropil immunoreactivity in hippocampus proper was most dense in the stratum radiatum and stratum oriens, with much less immunoreactivity in the lacunosum-moleculare and stratum lucidum. In the dentate gyrus, the neuropil in molecular layer showed extreme variations in the immunoreactivity. The inner third of the molecular layer was the most intensely stained region of the hippocampus, with a moderately intense immunoreactivity in the middle third of the molecular layer, and little or no immunoreactivity in the outer molecular layer (Fig. 2). The somata in the granule cell layer showed little immunoreactivity. Scattered immunoreactive multipolar neurons were present in the hilus. Prominent fiber bundles with m4 immunoreactivity were present in the hippocampal commissure, alveus, fimbria, and fornix, but only a subset of fibers were immunoreactive (Fig. 4). In addition, the cells lining the ventricles around the hippocampus exhibited m4 immunoreactivity.

Fimbria-fornix lesions and alterations in muscarinic receptor subtypes

To characterize the regulation of muscarinic receptor subtypes in hippocampus following unilateral denervation of septal input, quantitative immunoprecipitation studies and qualitative light microscopic immunocytochemistry were performed at varying time points after transection of the fimbria-fornix. Only m1, m2, and m4 were evaluated in these animals since this set of studies was performed prior to development of methods for characterization of m3 (Levey et al., 1994).

In the unlesioned hippocampus, total membrane binding of $^3\text{H-NMS}$ averaged 966 ± 216 (SEM) fmol/mg protein. The percentages of the individual subtypes determined by immunoprecipitation were m1, $36 \pm 3\%$ (SEM); m2, $33 \pm 2\%$; and m4, $27 \pm 1\%$, with a total recovery of $96 \pm 5\%$ of the solubilized receptors in the sum of the immunoprecipitates. In the lesioned hippocampus, $^3\text{H-NMS}$ binding was increased at most time

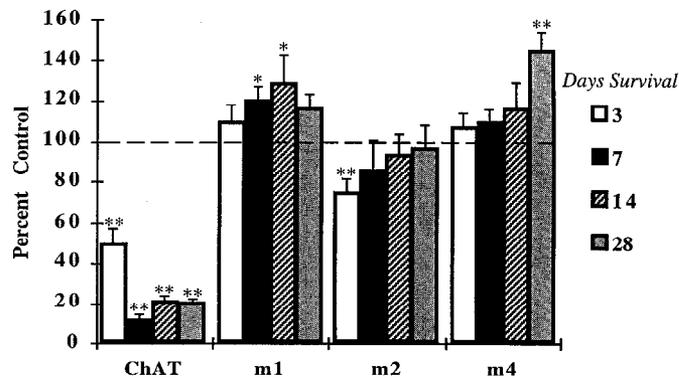


Figure 5. Immunoprecipitation analysis of muscarinic receptor proteins in the hippocampus at varying time points following ipsilateral fimbria-fornix lesions. The values shown are the percentages of control levels in the contralateral hippocampus. ChAT was assayed enzymatically in the same tissues used for receptor subtype determination by immunoprecipitation. Error bars represent SEM. Statistically significant differences between the ipsilateral lesioned and contralateral unlesioned (control) hippocampi were determined with paired *t* tests; *, $P < 0.05$; **, $P < 0.01$.

points, averaging $110 \pm 4\%$ (SEM) of the unlesioned side at 3 d, $115 \pm 8\%$ at 7 d, $99 \pm 9\%$ at 14 d, and $118 \pm 5\%$ at 28 d. The recovery of solubilized receptors in the sum of the immunoprecipitates from lesioned hippocampus (from all survival periods) averaged $91 \pm 3\%$, and this difference was not statistically significant from recovery from unlesioned hippocampus (paired *t* test, two tailed, $P = 0.40$).

Alterations in the abundance of m1, m2, and m4 following unilateral fimbria-fornix lesions are shown in Figure 5. The efficacy of the lesions was assessed in these animals by radiochemical measurement of ChAT activity, which averaged 0.48 ± 0.04 nmol $^3\text{H-ACh}$ formed/min/mg protein on the unlesioned side. On the lesioned side, specific activity of ChAT was reduced to 47% of the activity of the contralateral hippocampus at 3 d postlesion, and was further reduced to only 10–20% of normal levels at 7 d, 2 weeks, and 4 weeks survival; all statistically significant reductions ($p < 0.01$, paired *t* test). The m1 receptor was significantly increased by 20–29% in the lesioned hippocampus at 7 and 14 d ($p < 0.05$, paired *t* test). The m2 receptor was significantly decreased by 25% at 3 d postlesion, and then progressively returned to baseline levels. The m4 receptor progressively increased post lesion, reaching 144% of the levels on the unlesioned side at 28 d ($p < 0.01$, paired *t* test).

Immunocytochemical studies of m1, m2, and m4 were also performed at the same time points following unilateral fimbria-fornix lesions to determine the distribution of the alterations in receptor subtypes (Fig. 6). The efficacy of the lesions was assessed in these animals by AChE histochemistry. In most cases the lesion selectively involved one side of the fimbria-fornix and portions of the overlying frontal and cingulate cortex. In a few cases the most rostral tip of the hippocampus was also lesioned. In all lesioned animals there was a major reduction in AChE positive fibers in the hippocampus proper and dentate gyrus. There were no dramatic changes in the distribution of m1, m2, and m4 in the hippocampus or dentate gyrus at any time point, as shown in the examples from an animal at 28 d postlesion. There appeared to be small increases in the neuropil immunoreactivity for m4 in CA1 and dentate gyrus on the lesioned side, and small decreases in m2-immunoreactive fibers in the hilus and infragranule cell layer. There were no changes in

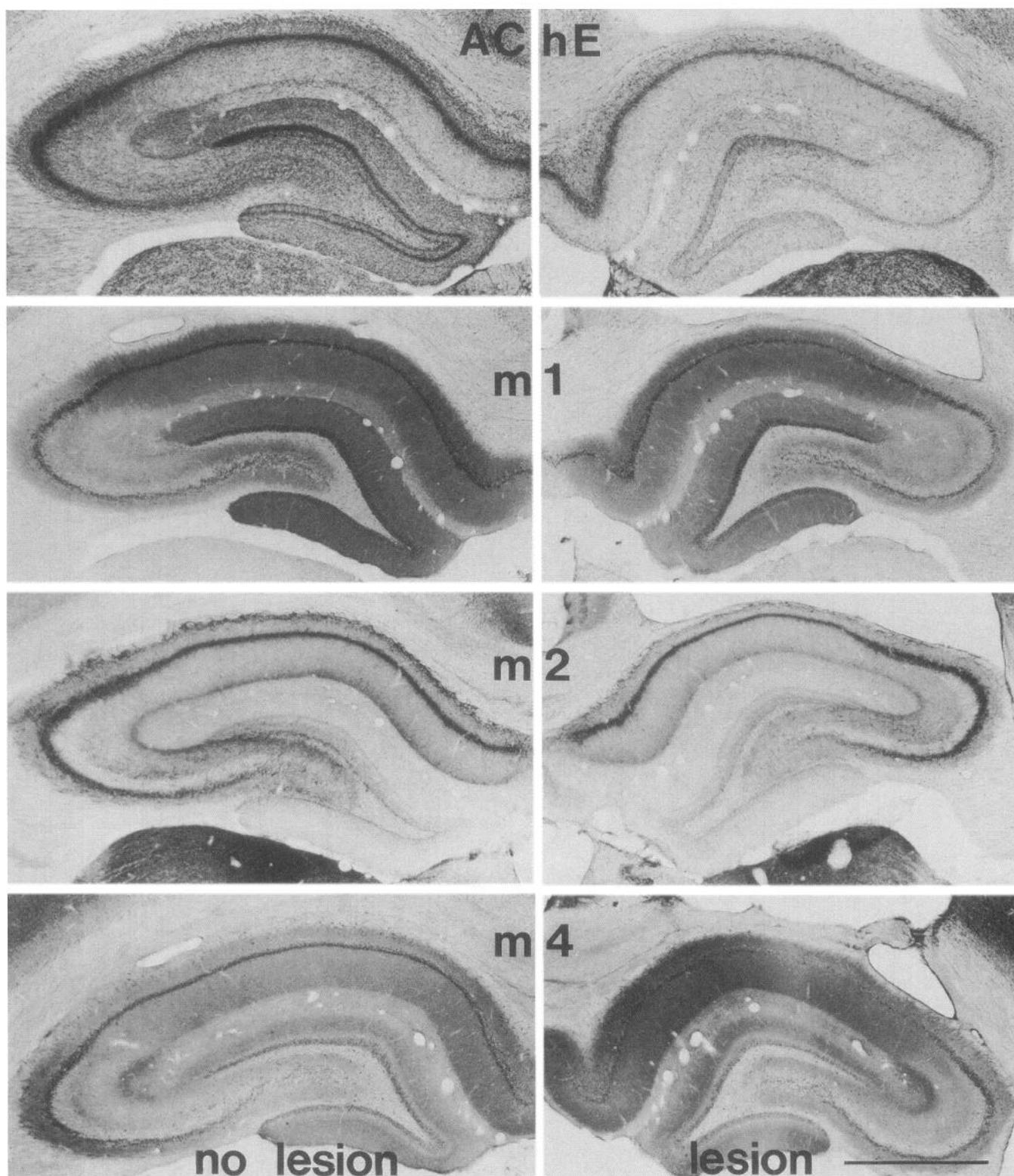


Figure 6. Immunocytochemical localization of muscarinic receptor subtypes in the dorsal hippocampus 28 d after fimbria-fornix lesions. The *left column* shows coronal sections from the contralateral hippocampus, and the *right column* shows coronal sections ipsilateral to the lesion. All sections are from the same animal at approximately the same rostrocaudal level. The photomicrographs were all taken with the same exposures and the right and left sides developed in the same manner. Note the large reduction in AChE activity in the lesioned hippocampus (*top panel*). In contrast, there are no major differences in the distributions of m1, m2, and m4. The m3 immunoreactivity was not localized in these animals. Variations in intensity between unlesioned and lesion sides are difficult to interpret because the immunocytochemical methods are not quantitative, although the increased intensity of m4 on the lesioned side (in the dendritic fields of CA1 and dentate gyrus) is consistent with immunoprecipitation results at this time point. Scale bar, 1.0 mm.

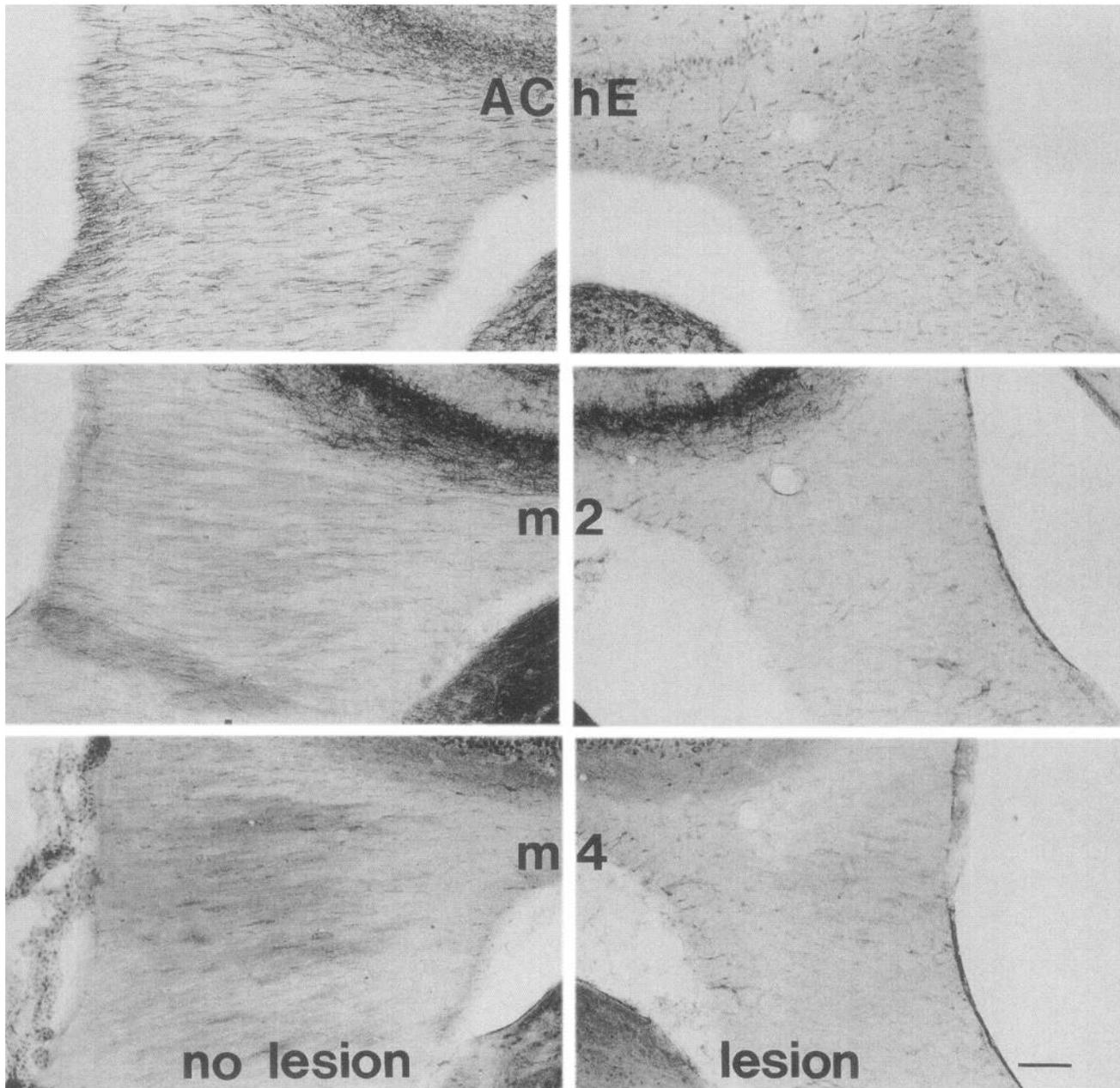


Figure 7. Immunocytochemical localization of m2 and m4 muscarinic receptor subtypes in the fimbria 28 d after fimbria-fornix lesions. The *top panel* shows virtually complete loss of AChE-positive fibers in the shrunken fimbria ipsilateral to a more rostral lesion. The normal pattern is shown on the left. Note the similarly extensive loss of m2 and m4 fibers ipsilateral to the lesion. Scale bar, 100 μ m.

the distribution of neurons expressing the receptors. In the fimbria, which contains hippocampal afferent and efferent fibers from septum and other regions, there was a significant loss of AChE-stained fibers and also loss of m2 and m4 immunoreactivity (Fig. 7).

192 IgG-saporin immunotoxin lesions and alterations of muscarinic receptor subtypes

The fimbria-fornix lesions described above result in loss of both cholinergic and noncholinergic septal inputs to the hippocampus. To determine alterations in the distributions of m1–m4 receptor subtypes in hippocampus following selective cholinergic denervation, qualitative light microscopic immunocytochemistry was

performed at 2 weeks following 192 IgG-saporin immunotoxin lesions. In the medial septum and diagonal band, there was nearly complete loss of ChAT-immunoreactive neurons, as described elsewhere (Levey et al., 1995), confirming the efficacy of the lesion. Since ChAT immunoreactivity is technically difficult to visualize in terminal fields, AChE histochemistry was used to examine the loss of innervation in the hippocampus. As shown in Figure 8, there was nearly complete bilateral loss of AChE positive fibers in animals injected with the immunotoxin compared to the saline injection or to control animals from the other studies. The loss of AChE was throughout the hippocampus proper, dentate gyrus, and in the fimbria-fornix. As with the fimbria-fornix lesions, there were no dramatic alterations in the

distributions of m1–m4. There were a few changes in the relative intensity of subtypes, although these should be interpreted cautiously because they are potentially a result of interanimal variations inherent to the procedures. Nonetheless, in both animals receiving the 192 IgG-saporin injections there was a generalized increase in m1 immunoreactivity compared to the vehicle injected animal. The m2-immunoreactive neurons and fibers did not change, with the exception of an incomplete loss in m2-immunoreactive fibers in the fimbria-fornix. The relative intensity of m4 immunoreactivity was possibly increased in stratum radiatum of CA1. In the fimbria, there was no apparent change in m4-immunoreactive fibers despite a marked loss of AChE-stained fibers (not shown).

Discussion

The present study provides the first direct localization of m1–m4 proteins in the hippocampal formation of the rat by light microscopic immunocytochemistry, and identifies the distribution and magnitude of regulatory changes in the subtypes following deafferentation of septal innervation by fimbria-fornix lesions and following selective immunotoxin lesions of the cholinergic component of the septohippocampal pathway. There are several major findings: (1) m1–m4 immunoreactivities are differentially localized in neuronal populations and in the neuropil in dentate gyrus and hippocampus proper, with highly complementary distributions; (2) deafferentation of the septohippocampal projection by unilateral fimbria-fornix transection produced a loss in a fraction of m2 receptors and m4 receptors in axons in the fimbria, although each of the subtypes were intrinsic to the hippocampal region (and/or were located on non-septal hippocampal afferents) and upregulated following the lesions with no dramatic changes in their light microscopic distributions; (3) immunotoxin lesions selective for the cholinergic component of the septohippocampal projection resulted in a small loss in m2-immunoreactive axons in the fimbria, with no substantial changes in the distributions of this or other subtypes in the dentate gyrus and hippocampus proper. Based on the differential cellular and subcellular distributions of the receptors and their alterations following lesions, we suggest various roles for the subtypes in the diverse modulation of hippocampal circuits by ACh.

Muscarinic receptor localization in hippocampus: pharmacological versus molecular subtypes

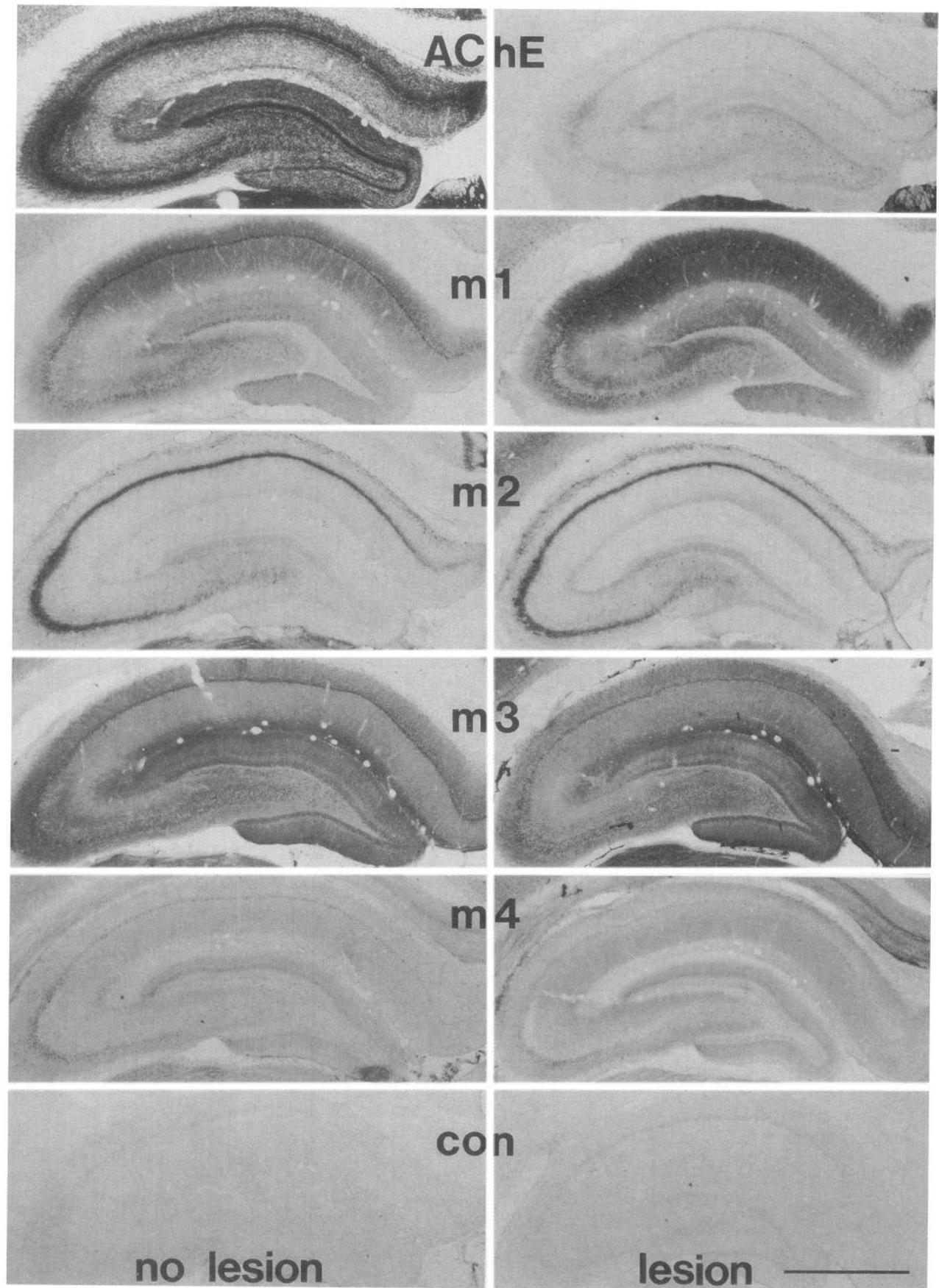
Our immunocytochemical studies demonstrating differential regional, laminar, and cellular localization of m1–m4 receptor subtypes extend many previous localization studies using receptor autoradiography and *in situ* hybridization, and help clarify the relationship between the pharmacological and molecular classification systems for the receptor subtypes. Subclasses of muscarinic receptor binding sites are distinguished pharmacologically using antagonists, and are designated by upper case letters, M1–M4. The molecular subtypes, distinguished by their primary nucleotide or amino acid sequences using complementary nucleotide probes or subtype-specific antibodies, respectively, are designated by lower case letters, m1–m5. Because available ligands do not distinguish among each of the molecular subtypes (Buckley et al., 1989; Dorje et al., 1991), there is no simple correspondence between pharmacological and molecular subtypes. In hippocampus, recent homogenate binding studies have distinguished all four subclasses of muscarinic binding sites, M1–M4 (Waelbroeck et al., 1990), although most autoradiographic studies have distinguished only the M1 and M2 sites.

M1 binding sites, typically defined using pirenzepine, are most abundant in dentate gyrus and hippocampus proper (Wamsley et al., 1984; Cortes and Palacios, 1986; Mash and Potter, 1986; Spencer et al., 1986; Dawson et al., 1989; Joyce et al., 1989), closely resembling the distribution of m1, which is the most abundant molecular subtype in hippocampus (Levey et al., 1994; Wall et al., 1994). However, m4 also has a relatively high affinity for pirenzepine (Buckley et al., 1989; Dorje et al., 1991), and its regional distribution overlaps with m1 with highest levels in dentate gyrus and CA1 (see Fig. 1). The m4 receptor is also abundant, accounting for about 20–25% of hippocampal muscarinic receptors (Levey et al., 1994; Wall et al., 1994). Thus, M1 binding sites in hippocampus are probably comprised of m1 and also possibly m4.

M2 binding sites have been visualized autoradiographically in hippocampus using several ligand binding strategies (Cortes and Palacios, 1986; Mash and Potter, 1986; Spencer et al., 1986; Regenold et al., 1989; Wang et al., 1989; Quirion et al., 1993; Vilaro et al., 1993). Although most M2 sites are heterogeneous and probably consist of at least m2 and m4 proteins (Dorje et al., 1991; Vilaro et al., 1992b, 1993; Quirion et al., 1993), those M2 sites directly labeled with ACh and other agonists (Spencer et al., 1986; Regenold et al., 1989; Vilaro et al., 1993) are most similar to the distribution of m2 protein shown here, with several well demarcated bands of increased receptor density along stratum pyramidale, stratum oriens, at the border between stratum radiatum and lacunosum-moleculare, and in the infragranular region. Thus, under some conditions, agonist binding may be selective for the m2 molecular subtype in the hippocampus.

The autoradiographic distribution of M3 has been investigated less than M1 and M2 because of greater problems with the selectivity of ligands. Methods for visualization of M3 have been developed using ³H-4-DAMP, an antagonist with nearly identical affinities to m1–m5 proteins (Dorje et al., 1991), in combination with various antagonists to occlude non-M3 receptors (Flynn and Mash, 1993; Vilaro et al., 1993; Zubieta and Frey, 1993). We recently directly compared the distributions of such M3 binding sites with m3 immunoreactivity, and although they are very similar in many regions of brain (Levey et al., 1994), there are marked laminar differences in hippocampus. Although the basis for the discrepancies between M3 and m3 is unknown, differences in the sensitivity and/or specificity of each method are likely (Levey et al., 1994). Autoradiographic methods for M4 and M5 binding sites have yet to be developed.

Localization of m1–m4 proteins is in closer agreement with the mRNAs than with the binding sites. For example, m1 protein and m1 mRNA (Buckley et al., 1988; Vilaro et al., 1993) are both present in pyramidal neurons throughout Ammon's horn, in granule cells, and in scattered interneurons. The m2 protein and mRNA (Vilaro et al., 1993) are both expressed in inhomogeneous distributions, with higher levels of each in CA3 than in CA1. In dentate gyrus, they are both found at highest levels in the hilus with little or no detectable signal in granule cells. The m3 protein and mRNA (Buckley et al., 1988; Vilaro et al., 1993) are both most abundant in hippocampal pyramidal neurons in CA3. The m4 protein and mRNA (Vilaro et al., 1993) also have similar distributions in Ammon's horn, with CA1 and CA3c greater than CA3. However, there are also some differences. While m2 mRNA is reportedly expressed at high levels in pyramidal neurons at the rostral pole of hippocampus and in few scattered neurons in the pyramidal layer elsewhere (Vilaro et al.,



1993), m2 immunoreactivity is in nonpyramidal neurons in the oriens/alveus (O/A) border and scattered along the pyramidal layer (see Fig. 3C). Since O/A neurons appear relatively dense and laminar in coronal sections at the rostral pole of hippocampus, they may have been misidentified as pyramidal neurons in previous *in situ* hybridization studies (Vilaro et al., 1993). Perhaps the greatest discrepancy is for m5, since the mRNA is expressed in CA1 pyramidal neurons (Vilaro et al., 1993) and the protein has yet to be detected in hippocampus (Levey et al., 1994; Wall et al., 1994). Thus, m5 protein is either present at extremely low levels and/or the antibodies are insensitive. The laminar mismatches between the respective mRNA transcripts and proteins are expected. Whereas the mRNA for each subtype is restricted largely to neuronal somata, the proteins are transported to specific postsynaptic sites on dendrites and presynaptic sites on axons where they are functionally relevant. In fact, the highly selective and differential laminar distributions of m1–m4, suggests that each subtype is preferentially associated with discrete hippocampal connections via pre- or postsynaptic mechanisms.

Alterations in muscarinic receptor subtypes following cholinergic deafferentation

Lesions of septohippocampal projections have been used previously to determine the putative pre- or postsynaptic distribution of muscarinic receptors in hippocampus (Yamamura and Snyder, 1974), and only recently has this approach been applied to muscarinic receptor subtypes (Dawson et al., 1989; Joyce et al., 1989; Dawson and Wamsley, 1990; Bauer et al., 1992; Wall et al., 1994). Three days after bilateral ibotenic acid lesions of the septum, M1 sites are unchanged while M2 sites are reduced 15–20% in CA3 and in the hilus (Bauer et al., 1992), consistent with a presynaptic localization of M2 on septal inputs. At longer survival periods (2–8 months), M1 and M2 binding sites are both increased, with regional and laminar differences in Ammon's horn and dentate gyrus (Dawson et al., 1989; Joyce et al., 1989). A recent immunoprecipitation study (Wall et al., 1994) of m1–m4 proteins at 10 and 24 d post fimbria-fornix transection demonstrated a 22% decrease in m2 and increases in m1 (14%), m3 (77%), and m4 (29%). Our immunoprecipitation studies using different antibodies and survival times closely match these changes. However, we did not quantify m3 in these animals because we were unable to reliably measure this subtype at the time the experiments were performed. Our results further indicate that the changes in molecular subtypes are dynamic, as suggested by previous ligand binding studies (Dawson et al., 1989; Joyce et al., 1989; Bauer et al., 1992). For instance, the loss of m2 is most evident at 3 d postlesion and then is compensated by progressive upregulation of m2 at later survival times. This suggests that following a loss of presynaptic m2 receptors on septohippocampal projections, there is upregulation

of a separate pool of m2 receptors which is intrinsic to hippocampus, similar to the upregulation of m1, m3, and m4.

Our immunocytochemical studies following deafferentation of hippocampus provide far better spatial resolution than immunoprecipitation studies or autoradiographic binding techniques. The strikingly similar distributions of m1–m4 before and after extensive lesions of either the septal or cholinergic innervation of hippocampus indicates that the vast majority of muscarinic receptors, including all molecular subtypes, are intrinsic to hippocampus, and/or are located on non-septal hippocampal afferents. This finding also suggests that upregulation of the subtypes, as detected by immunoprecipitation, is a generalized phenomenon, probably occurring in all hippocampal elements that normally express m1–m4 subtypes. Persistence or upregulation of receptors after septal deafferentation has typically been interpreted as evidence for postsynaptic localization of muscarinic receptors (Yamamura and Snyder, 1974; Dawson et al., 1989; Joyce et al., 1989; Dawson and Wamsley, 1990; Bauer et al., 1992; Wall et al., 1994). However, presynaptic receptors could also upregulate if they are located on the terminals of intrinsic hippocampal projections, or on the terminals of nonseptal afferents projections, such as from hypothalamus, thalamus, brainstem, and entorhinal cortex. In fact, the cellular and subcellular resolution provided by immunocytochemistry provides strong evidence for both pre- and postsynaptic localization of the m1–m4 subtypes, as discussed below.

Presynaptic muscarinic receptors in hippocampus

Our studies provide anatomical evidence for the molecular identity of muscarinic autoreceptor subtype(s) in hippocampus. The pharmacology of the receptors that inhibit ACh release has been described variously as M2 (Raiteri et al., 1984; Pohorecki et al., 1988; Lapchak et al., 1989), M2-cardiac like (Richards, 1990), M2-noncardiac like (Marchi and Raiteri, 1989), and as M4 (McKinney et al., 1993). The high resolution of immunocytochemistry enabled direct detection of m2 and m4 in the unlesioned fimbria and fornix, providing direct evidence for a "presynaptic" or at least axonal localization of these subtypes. Interestingly, m2 and m4 have nearly identical affinities for M2-selective ligands (Buckley et al., 1989; Dorje et al., 1991) and share signal transduction mechanisms (Peralta et al., 1988). The loss of m2- and m4-immunoreactive fibers after fimbria-fornix transection suggests that both subtypes may originate in the septal region and terminate in hippocampus, although these lesions disrupt other hippocampal afferent and efferent connections that also travel in this pathway. The immunoprecipitation studies demonstrate that quantitatively the afferents in the fimbria-fornix provide the hippocampus with only a small minority of either subtype.

The 192-saporin immunotoxin lesions provided a unique opportunity to further identify the origin of m2- and m4-immu-

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Figure 8. Immunocytochemical localization of m1–m4 muscarinic receptor subtypes in the hippocampus at 14 d after intracerebroventricular injection of vehicle (no lesion) or 192 IgG-saporin (lesion). The *left column* shows coronal sections from the same vehicle-treated animal, and the *right column* shows comparable sections from a single immunotoxin-treated animal. The photomicrographs were taken with the same exposure times for each receptor in order to facilitate comparisons. Note the nearly complete loss of AChE-positive fibers throughout the hippocampus proper and dentate gyrus in the lesioned animal (*top panels*). The dorsal thalamus is barely visible in these same panels, showing preserved AChE activity in this structure as a positive control for the histochemical reaction. In contrast, there is little or no change in the distributions of m1–m4 after lesions. Subtle increases in m1 immunoreactivity throughout hippocampus and dentate gyrus and in m4 in CA1 (stratum radiatum) can be seen, but are of uncertain significance because of potential interanimal variation and nonquantitative nature of the method. The control sections from each animal (*con. bottom panels*) were processed with omission of the primary antibodies. Scale bar, 1.0 mm.

noreactive axons in the fimbria-fornix. The immunotoxin lesions depleted ChAT immunoreactivity in the medial septum and diagonal band (Levey et al., 1995) as well as acetylcholinesterase-positive fibers in the hippocampus (Fig. 8), yet spared GABAergic septal neurons (Levey et al., 1995) which also project to hippocampus (Amaral and Kurz, 1985; Wainer et al., 1985). The loss of ChAT immunoreactivity has been shown previously to result from neurodegeneration of cholinergic projection neurons (Book et al., 1992; Book et al., 1994; Heckers et al., 1994). In our lesioned animals there was a small decrease in m2 fibers in the fimbria, with no apparent change in m4. Thus, m2-immunoreactive fibers in the fimbria are at least in part cholinergic, while most m4-immunoreactive fibers appear to be noncholinergic. Since the reduction in m2 was less with the immunotoxin lesions than with fimbria-fornix transections, m2 may also be expressed in noncholinergic axons. This possibility is supported by with recent studies confirming expression of m2 mRNA (Vilaro et al., 1992a) and protein (Levey et al., 1995) in both cholinergic and noncholinergic neurons in the medial septum and diagonal band. Together, our combined anatomical-lesioning studies provide strong evidence that m2 is the molecular identity of a presynaptic muscarinic autoreceptor on cholinergic septohippocampal terminals. These studies do not exclude the possibility that other muscarinic receptor subtypes may also function as autoreceptors in hippocampus, several of which are expressed in medial septal neurons (Vilaro et al., 1994; Levey et al., 1994) in addition to the more abundant m2 subtype (Levey et al., 1995). For example, other autoreceptors might be expressed at levels too low to be detected with our methods or might be masked by upregulation that occurs in hippocampus following cholinergic denervation. Moreover, both lesioning methods used in this study spare the cholinergic neurons which are intrinsic to hippocampus and which potentially also express muscarinic autoreceptors.

The sparing of m4 axons in the fimbria-fornix after 192-saporin immunotoxin lesions indicates that these fibers are noncholinergic. Observations about the immunocytochemical distribution of m4 in hippocampus provide additional clues to identity of m4-immunoreactive axons in the fimbria. One possibility is that m4 is localized in hippocampal associational and commissural projections. This subtype is expressed in neurons in the hilus and in numerous axons in the fimbria, fornix, and dorsal hippocampal commissure (Fig. 4). It is also strikingly enriched in the neuropil in the inner third of the molecular layer of dentate gyrus (Fig. 2). Mossy cells in the hilus give rise to associational connections which terminate on the inner third of the ipsilateral molecular layer (Lynch et al., 1976; Ribak et al., 1985). These neurons also send commissural projections to the contralateral hippocampus, via axons which enter the fimbria, collect in the fornix, cross in the dorsal hippocampal commissure, and then terminate on the inner third of molecular layer (Lynch et al., 1976; Ribak et al., 1985). Interestingly, lesion studies (Nadler et al., 1976) and immunocytochemical studies (Soriano and Frotscher, 1994) suggest that aspartate and/or glutamate are the neurotransmitters released in these pathways. Furthermore, the pharmacology of the muscarinic subtype mediating aspartate (Raiteri et al., 1990) and glutamate (Marchi and Raiteri, 1989) release in hippocampus is similar to that of m4, with relative insensitivity to pirenzepine and high affinity for AF-DX 116 (Buckley et al., 1989; Dorje et al., 1991). Thus, ACh could potentially regulate the activity of excitatory hippocampal associ-

ational and commissural connections via presynaptic m4 receptors.

Presynaptic muscarinic receptors also depress inhibitory responses in hippocampus (Valentino and Dingledine, 1981; Segal, 1989; Pitler and Alger, 1992). The m2 molecular subtype is a particularly good candidate for mediation of these effects, since the protein is expressed abundantly in many nonpyramidal neuron populations, including probable O/A interneurons, basket cells, and other interneurons, and it is localized in many presumptive axons and puncta. Indeed, preliminary electron microscopic studies indicate that m2-immunoreactive puncta surrounding pyramidal neurons correspond to axon terminals that are typical of those derived from GABAergic basket cells (Levey et al., 1992). Other GABAergic terminals, for example, septohippocampal, may also express m2 for the reasons described above. It should be emphasized that these data do not exclude the presynaptic localization of other muscarinic subtypes on GABA terminals since all of the proteins may be expressed in some nonpyramidal neuron populations. Future immunoelectron microscopic studies will be necessary to confirm the identities of presynaptic receptor subtypes at various hippocampal synapses.

Postsynaptic muscarinic receptors in hippocampus and functional significance

The immunocytochemical and lesion studies indicate that m1–m4 proteins are all postsynaptically distributed in somata and dendrites of different neuronal populations in hippocampus. ACh exerts powerful atropine-sensitive excitations of pyramidal neurons by suppression of several distinct potassium currents, including the voltage-independent leak conductance, the voltage-dependent I_M , the fast-inactivating I_A , and the calcium-activated slow afterhyperpolarization current I_{AHP} (Cole and Nicoll, 1984; Halliwell, 1990; Krnjevic, 1993). Other postsynaptic actions include activation of a potassium current (Wakamori et al., 1993), inhibition of voltage-dependent calcium channels (Toselli et al., 1989), uncoupling of a calcium-dependent potassium conductance (Muller and Connor, 1991), and enhanced NMDA receptor responsiveness (Markram and Segal, 1990). Surprisingly little is known about the pharmacology of these diverse effects, but multiple subtypes are clearly involved (Muller and Misgeld, 1986; Dutar and Nicoll, 1988; Halliwell, 1990). The leak conductance responsible for membrane depolarization has been attributed to M1 (Muller and Misgeld, 1986; Benson et al., 1988; Dutar and Nicoll, 1988) or M3 (Pitler and Alger, 1990), whereas the blockade of the I_{AHP} has been linked to M1 (Dutar and Nicoll, 1988; Segal and Fisher, 1992) or M2 (Muller and Misgeld, 1986; Halliwell, 1990). These pharmacologies are virtually compatible with any of the molecular subtypes, although m1, m3, or m4 are the predominant subtypes in pyramidal somata and dendrites. In addition, multiple subtypes could be coexpressed in some pyramidal neurons. Further complexities are likely to result from localization of muscarinic receptor subtypes in various nonpyramidal neuron populations (Reece and Schwartzkroin, 1991). Clearly, additional anatomical, physiological, and molecular studies will be necessary to delineate the postsynaptic actions of individual muscarinic receptors at various hippocampal synapses.

References

- Amaral DG, Kurz J (1985) An analysis of the origins of the cholinergic and noncholinergic septal projections to the hippocampal formation of the rat. *J Comp Neurol* 240:37–59.

- Bartus RT, Dean RLI, Beer B, Lippa AS (1982) The cholinergic hypothesis of geriatric memory dysfunction. *Science* 217:408–417.
- Bauer A, Schulz JB, Zilles K (1992) Muscarinic desensitization after septal lesions in rat hippocampus: evidence for the involvement of G-proteins. *Neuroscience* 47:95–103.
- Benson DM, Blitzer RD, Landau EM (1988) An analysis of the depolarization produced in guinea-pig hippocampus by cholinergic receptor stimulation. *J Physiol (Lond)* 404:479–496.
- Blitzer RD, Gil O, Landau EM (1990) Cholinergic stimulation enhances long-term potentiation in the CA1 region of rat hippocampus. *Neurosci Lett* 119:207–210.
- Bonner TI, Buckley NJ, Young AC, Brann MR (1987) Identification of a family of muscarinic acetylcholine receptor genes. *Science* 237:527–532.
- Book AA, Wiley RG, Schweitzer JB (1992) Specificity of 192 IgG-saporin for NGF receptor-positive cholinergic basal forebrain neurons in the rat. *Brain Res* 590:350–355.
- Book AA, Wiley RG, Schweitzer JB (1994) 192 IgG-saporin. I. specific lethality for cholinergic neurons in the basal forebrain of the rat. *J Neuropathol Exp Neurol* 53:95–102.
- Buckley NJ, Bonner TI, Brann MR (1988) Localization of a family of muscarinic receptor mRNAs in rat brain. *J Neurosci* 8:4646–4652.
- Buckley NJ, Bonner TI, Buckley CM, Brann MR (1989) Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* 35:469–476.
- Burgard EC, Sarvey JM (1990) Muscarinic receptor activation facilitates the induction of long-term potentiation (LTP) in the rat dentate gyrus. *Neurosci Lett* 116:34–39.
- Callahan MJ, Kinsora JJ, Harbaugh RE, Reeder TM, Davis RE (1993) Continuous ICV infusion of scopolamine impairs sustained attention of rhesus monkeys. *Neurobiol Aging* 14:147–151.
- Cole AE, Nicoll RA (1984) Characterization of a slow cholinergic post-synaptic potential recorded *in vitro* from rat hippocampal pyramidal cells. *J Physiol (Lond)* 352:173–188.
- Cortes R, Palacios JM (1986) Muscarinic cholinergic receptor subtypes in the rat brain. I. Quantitative autoradiographic studies. *Brain Res* 362:227–238.
- Coyle JT, Price DL, DeLong MR (1983) Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science* 219:1184–1190.
- Davis KD, Thal LJ, Gamzu ER, et al. (1992) A double-blind, placebo-controlled multicenter study of tacrine for Alzheimer's disease. *N Engl J Med* 327:1253–1259.
- Dawson VL, Wamsley JK (1990) Hippocampal muscarinic supersensitivity after AF64A medial septal lesion excludes M1 receptors. *Brain Res Bull* 25:311–317.
- Dawson VL, Gage FH, Hunt MA, Wamsley JK (1989) Normalization of subtype-specific muscarinic receptor binding in denervated hippocampus by septodiaminal band grafts. *Exp Neurol* 106:115–124.
- Dekker AJAM, Connor DJ, Thal LJ (1991) The role of cholinergic projections from the nucleus basalis in memory. *Neurosci Biobehav Revs* 15:299–317.
- Dorje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR (1991) Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J Pharmacol Exp Ther* 256:727–733.
- Drachman DA, Leavitt JL (1974) Human memory and the cholinergic system. A relationship to aging? *Arch Neurol* 30:113–121.
- Dutar P, Nicoll RA (1988) Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger responses: electrophysiological studies *in vitro*. *J Neurosci* 8:4214–4224.
- Fibiger HC (1991) Cholinergic mechanisms in learning, memory, and dementia: a review of recent evidence. *Trends Neurosci* 14:220–223.
- Flynn DD, Mash DC (1993) Distinct kinetic binding properties of *N*-[³H]-methylscopolamine afford differential labeling and localization of M1, M2, and M3 muscarinic receptor subtypes in primate brain. *Synapse* 14:283–296.
- Halliwell JV (1990) Physiological mechanisms of cholinergic action in the hippocampus. *Prog Brain Res* 84:255–272.
- Heckers S, Ohtake T, Wiley RG, Lappi DA, Geula C, Mesulam M-M (1994) Complete and selective cholinergic denervation of rat neocortex and hippocampus but not amygdala by an immunotoxin against the p75 NGF receptor. *J Neurosci* 14:1271–1289.
- Hersch SM, Gutekunst CA, Rees HD, Heilman CJ, Levey AI (1994) Distribution of m1-m4 muscarinic receptor proteins in the rat striatum: light and electron microscopic immunocytochemistry using subtype-specific antibodies. *J Neurosci* 14:3351–3363.
- Joyce JN, Gibbs RB, Cotman CW, Marshall JF (1989) Regulation of muscarinic receptors in hippocampus following cholinergic denervation and reinnervation by septal and striatal transplants. *J Neurosci* 9:2776–2791.
- Kahle JS, Cotman CW (1989) Carbachol depresses synaptic responses in the medial but not the lateral perforant path. *Brain Res* 482:159–163.
- Koliatsos VE, Applegate MD, Knusel B, et al. (1991) Recombinant human nerve growth factor prevents retrograde degeneration of axotomized basal forebrain cholinergic neurons in the rat. *Exp Neurol* 112:161–173.
- Krnjevic K (1993) Central cholinergic mechanisms and function. *Prog Brain Res* 98:285–292.
- Lapchak PA, Araujo DM, Quirion R, Collier B (1989) Binding sites for [³H]AF-DX 116 and effect of AF-DX 116 on endogenous acetylcholine release from rat brain slices. *Brain Res* 496:285–294.
- Levey AI, Armstrong DM, Atweh SF, Terry RD, Wainer BH (1983) Monoclonal antibodies to choline acetyltransferase: production, specificity, and immunohistochemistry. *J Neurosci* 3:1–9.
- Levey AI, Stormann TM, Brann MR (1990) Bacterial expression of human muscarinic receptor fusion proteins and generation of subtype-specific antisera. *FEBS Lett* 275:65–69.
- Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J Neurosci* 11:3218–3226.
- Levey AI, Hersch SM, Edmunds SM (1992) Light and electron microscopic localization of m2 muscarinic receptor protein in rat septum and hippocampus. *Soc Neurosci Abstr* 18:1143.
- Levey AI, Edmunds SM, Heilman CJ, Desmond TJ, Frey KA (1994) Localization of muscarinic m3 receptor protein and M3 receptor binding in rat brain. *Neuroscience* 63:207–221.
- Levey AI, Edmunds SM, Hersch SM, Wiley RG, Heilman CJ (1995) A light and electron microscopic study of m2 muscarinic acetylcholine receptor in the basal forebrain of the rat. *J Comp Neurol* 351:339–356.
- Lynch G, Gall C, Rose G, Cotman C (1976) Changes in the distribution of the dentate gyrus associational system following unilateral or bilateral entorhinal lesions in the adult rat. *Brain Res* 110:57–71.
- Marchi M, Raiteri M (1989) Interaction acetylcholine-glutamate in rat hippocampus: involvement of the two subtypes of M-2 muscarinic receptors. *J Pharmacol Exp Ther* 248:1255–1260.
- Markram H, Segal M (1990) Long-lasting facilitation of excitatory postsynaptic potentials in the rat hippocampus by acetylcholine. *J Physiol (Lond)* 427:381–393.
- Mash DC, Potter LT (1986) Autoradiographic localization of M1 and M2 muscarinic receptors in the rat brain. *Neuroscience* 19:551–564.
- McKinney M (1993) Muscarinic receptor subtype-specific coupling to second messengers in neuronal systems. *Prog Brain Res* 98:333–340.
- McKinney M, Coyle JT (1991) The potential for muscarinic receptor subtype-specific pharmacotherapy for Alzheimer's disease. *Mayo Clin Proc* 66:1225–1237.
- McKinney M, Miller JH, Aagaard PJ (1993) Pharmacological characterization of the rat hippocampal muscarinic autoreceptor. *J Pharmacol Exp Ther* 264:74–78.
- Muller W, Connor JA (1991) Cholinergic input uncouples Ca²⁺ changes from K⁺ conductance activation and amplifies intradendritic Ca²⁺ changes in hippocampal neurons. *Neuron* 6:901–905.
- Muller W, Misgeld U (1986) Slow cholinergic excitation of guinea pig hippocampal neurons is mediated by two muscarinic receptor subtypes. *Neurosci Lett* 67:107–112.
- Nadler JV, Vaca KW, White WF, Lynch GS, Cotman CW (1976) Aspartate and glutamate as possible transmitters of excitatory hippocampal afferents. *Nature* 260:538–540.
- Nilsson OG, Leanza G, Rosenblad C, Lappi DA, Wiley RG, Bjorklund A (1992) Spatial learning impairments in rats with selective immunolesion of the forebrain cholinergic system. *Neuroreport* 3:1005–1008.
- Pang K, Williams MJ, Olton DS (1993) Activation of the medial septal area attenuates LTP of the lateral perforant path and enhances heterosynaptic LTD of the medial perforant path in aged rats. *Brain Res* 632:150–160.
- Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J, Capon DJ

- (1988) Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. *Nature* 334:434–437.
- Pitler TA, Alger BE (1990) Activation of the pharmacologically defined M3 muscarinic receptor depolarizes hippocampal pyramidal cells. *Brain Res* 534:257–262.
- Pitler TA, Alger BE (1992) Cholinergic excitation of GABAergic interneurons in the rat hippocampal slice. *J Physiol (Lond)* 450:127–142.
- Pohorecki R, Head R, Domino EF (1988) Effects of selected muscarinic cholinergic antagonists on [³H] acetylcholine release from rat hippocampal slices. *J Pharmacol Exp Ther* 244:213–217.
- Quirion R, Aubert I, Araujo DM, Hersi A, Gaudreau P (1993) Autoradiographic distribution of putative muscarinic receptor subtypes in mammalian brain. *Prog Brain Res* 98:85–93.
- Raiteri M, Leardi R, Marchi M (1984) Heterogeneity of presynaptic muscarinic receptors regulating neurotransmitter release in the rat brain. *J Pharmacol Exp Ther* 228:209–214.
- Raiteri M, Marchi M, Costi A, Volpe G (1990) Endogenous aspartate release in the rat hippocampus is inhibited by M2 'cardiac' muscarinic receptors. *Eur J Pharmacol* 177:181–187.
- Reece LJ, Schwartzkroin PA (1991) Effects of cholinergic agonists on two non-pyramidal cell types in rat hippocampal slices. *Brain Res* 566:115–126.
- Regenold W, Araujo DM, Quirion R (1989) Quantitative autoradiographic distribution of [³H]AF-DX 116/M2 muscarinic receptor binding sites in rat brain. *Synapse* 4:115–125.
- Ribak CE, Seress L, Amaral DG (1985) The development, ultrastructure and synaptic connections of the mossy cells of the dentate gyrus. *J Neurocytol* 14:835–857.
- Richards MH (1990) Rat hippocampal muscarinic autoreceptors are similar to the M₂ (cardiac) subtype: comparison with hippocampal M₁, atrial M₂, and ileal M₃ receptors. *Br J Pharmacol* 99:753–761.
- Segal M (1989) Presynaptic cholinergic inhibition in hippocampal cultures. *Synapse* 4:305–312.
- Segal M, Fisher A (1992) AF102B, a muscarinic M1 receptor agonist, mimics some effects of acetylcholine on neurons of rat hippocampus slices. *Eur J Pharmacol* 220:103–106.
- Smith PK, Krohn RI, Hermanson GT, et al. (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76–85.
- Soriano E, Frotscher M (1994) Mossy cells of the rat fascia dentata are glutamate-immunoreactive. *Hippocampus* 4:65–70.
- Spencer DG Jr, Horvath E, Traber J (1986) Direct autoradiographic determination of M1 and M2 muscarinic acetylcholine receptor distribution in rat brain: relation to cholinergic nuclei and projections. *Brain Res* 380:59–68.
- Toselli M, Lang J, Costa T, Lux HD (1989) Direct modulation of voltage-dependent calcium channels by muscarinic activation of a pertussis toxin-sensitive G-protein in hippocampal neurons. *Pfluegers Arch* 415:255–261.
- Valentino RJ, Dingledine R (1981) Presynaptic inhibitory effect of acetylcholine in the hippocampus. *J Neurosci* 7:784–792.
- Vilario MT, Wiederhold KH, Palacios JM, Mengod G (1992a) Muscarinic m2 receptor mRNA expression and receptor binding in cholinergic and non-cholinergic cells in the rat brain: a correlative study using *in situ* hybridization histochemistry and receptor autoradiography. *Neuroscience* 47:367–393.
- Vilario MT, Wiederhold KH, Palacios JM, Mengod G (1992b) Muscarinic M2-selective ligands also recognize M4 receptors in the rat brain: evidence from combined *in situ* hybridization and receptor autoradiography. *Synapse* 11:171–183.
- Vilario MT, Mengod G, Palacios G, Palacios JM (1993) Receptor distribution in the human and animal hippocampus: focus on muscarinic acetylcholine receptors. *Hippocampus* 3:149–156.
- Vilario MT, Palacios JM, Mengod G (1994) Multiplicity of muscarinic autoreceptor subtypes? Comparison of the distribution of cholinergic cells and cells containing mRNA for five subtypes of muscarinic receptors in the rat brain. *Mol Brain Res* 21:30–46.
- Waelbroeck M, Tastenoy M, Camus J, Christophe J (1990) Binding of selective antagonists to 4 muscarinic receptors (M1 to M4) in rat forebrain. *Mol Pharmacol* 38:267–273.
- Wainer BH, Levey AI, Rye DB, Mesulam M-M, Mufson EJ (1985) Cholinergic and non-cholinergic septohippocampal pathways. *Neurosci Lett* 54:45–52.
- Wakamori M, Hidaka H, Akaike N (1993) Hyperpolarizing muscarinic responses of freshly dissociated rat hippocampal CA1 neurones. *J Physiol (Lond)* 463:585–604.
- Wall SJ, Wolfe BB, Kromer LF (1994) Cholinergic deafferentation of dorsal hippocampus by fimbria-fornix lesioning differentially regulates subtypes (m1-m5) of muscarinic receptors. *J Neurochem* 62:1345–1351.
- Wamsley JK, Gehlert DR, Roeske WR, Yamamura HI (1984) Muscarinic antagonist binding site heterogeneity as evidenced by autoradiography after direct labeling with [³H](–)QNB and [³H]pirenzepine. *Life Sci* 34:1395–1402.
- Wang J-X, Roeske WR, Hawkins KN, Gehlert DR, Yamamura HI (1989) Quantitative autoradiography of M2 muscarinic receptors in the rat brain identified by using a selective radioligand [³H]AF-DX116. *Brain Res* 477:322–326.
- Wiley RG, Lappi DA (1993) Preparation of anti-neuronal immunotoxins for selective neural immunolesioning. *Neurosci Protocols* 20:1–11.
- Wiley RG, Oeltmann TN, Lappi DA (1991) Immunolesioning: selective destruction of neurons using immunotoxin to rat NGF receptor. *Brain Res* 562:149–153.
- Williams S, Johnston D (1990) Muscarinic depression of synaptic transmission at the hippocampal mossy fiber synapse. *J Neurophysiol* 64:1089–1097.
- Yamamura HI, Snyder SH (1974) Postsynaptic localization of muscarinic cholinergic receptor binding in rat hippocampus. *Brain Res* 78:320–326.
- Zubieta JK, Frey KA (1993) Autoradiographic mapping of M3 muscarinic receptors in the rat brain. *J Pharmacol Exp Ther* 264:415–422.