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Distribution of m1–m4 Muscarinic Receptor Proteins in the Rat Striatum: Light and Electron Microscopic Immunocytochemistry Using Subtype-Specific Antibodies

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Muscarinic ACh receptors mediate complex and clinically important effects in the striatum. To better understand the roles of the different muscarinic receptor subtypes (m1–m4), we have determined the cellular and subcellular distribution of the m1–m4 receptor proteins in the rat neostriatum using subtype-specific antibodies and avidin–biotin–peroxidase immunocytochemistry for light and electron microscopy. m1 receptor protein is expressed in 78% of neurons and is enriched in spiny dendrites and at postsynaptic densities. A small number of m1-immunoreactive axon terminals were observed, all forming asymmetrical synapses. About 2.5% of striatal neurons express m2 receptor protein with reaction product evident, by light microscopy in scattered large oval neurons with enfolded nuclei and long aspiny dendrites. By electron microscopy, m2 immunocytochemistry labeled somata, aspiny dendrites, and many axon terminals. Most axon terminals containing m2 make symmetrical synapses with somata, and dendritic shafts and spines. In addition, many m2-immunoreactive axon terminals formed asymmetrical synapses with spines or dendrites. m3 receptor protein was not evident in somata by light microscopy but was present in a distinct population of small-caliber spiny dendrites as well as in axon terminals forming asymmetrical synapses with spines. m4 receptor protein was heterogeneously distributed in the neostriatum and localized to 44% of striatal cells. m4-positive neurons had the ultrastructural features of medium spiny neurons with reaction product particularly concentrated in spines, often at postsynaptic densities. Axon terminals containing m4 form asymmetrical synapses, primarily with spines. These findings indicate that the muscarinic receptor proteins occur in distinct populations of striatal neurons; that the receptor proteins concentrate postsynaptically at synapses, including many considered to be noncholinergic; that m2 is the predominant muscarinic autoreceptor in the striatum; and that each receptor subtype may be a presynaptic heteroceptor in the striatum modulating extrinsic striatal afferents.

[Key words: muscarinic ACh receptors, striatum, basal ganglia, electron microscopy, antibodies]

ACh has complex and clinically important actions in the striatum that are mediated predominantly by muscarinic receptors. Based largely upon physiologic and pharmacological studies, several specific actions of ACh in the striatum have been suggested. For example, ACh regulates its own release from cholinergic interneurons (via presynaptic autoreceptors); noncholinergic striatal neurons are directly affected by ACh (via postsynaptic receptors and presynaptic heteroceptors); and the release of excitatory amino acids and dopamine by extrinsic striatal afferents may be under presynaptic control of ACh (via presynaptic heteroceptors). Muscarinic receptor subtypes may mediate different muscarinic cholinergic actions in the striatum. Receptor binding studies have distinguished at least four pharmacologic muscarinic sites, M1, M2, M3, and M4 (Waelbroeck et al., 1986, 1990; Ehlert and Tran, 1990; Sugita et al., 1991). Molecular cloning studies have determined that these pharmacologic receptor subtypes are accounted for by an even larger family of five G protein–coupled receptor proteins, termed m1–m5 (Bonner et al., 1987, 1988). The second messenger transduction mechanisms of these receptor groups have been established with m1, m3, and m5 affecting phosphoinositol metabolism, while m2 and m4 inhibit cAMP production (Hulme et al., 1990). Binding studies of the cloned receptors expressed in CHO cells indicate that m1 and m2 are pharmacologically similar to M1 and M2, respectively; however, m3 is intermediate and m4 and m5 are not as selective for standard ligands (Buckley et al., 1989; Dörje et al., 1991). This overlap indicates that the pharmacologically defined receptors represent combinations of the molecular subtypes that have not been fully resolved using current receptor ligands.

Muscarinic pharmacology would be greatly clarified by understanding how the muscarinic receptors are distributed within the complex circuitry of the striatum. *In situ* hybridization studies have localized the subtype mRNAs in distinct subpopulations of striatal neurons. The mRNA for m1 is present in more than 80% of striatal neurons (Weiner et al., 1990), including cholinergic neurons, substance P neurons, enkephalin neurons, and somatostatin neurons (Bernard et al., 1992); m2 receptor mRNA is present in cholinergic interneurons (Weiner et al., 1990; Bernard et al., 1992); m3 is present in few striatal neurons (Weiner et al., 1990); m4 is present in up to 61% of striatal neurons including cholinergic neurons and most substance P neurons but in few enkephalin, somatostatin, and neurotensin neurons (Weiner et al., 1990; Vilaró et al., 1991, 1992; Bernard et al., 1992). Although not detected in the striatum, m5 mRNA occurs in the pars compacta of the substantia nigra and has been postulated to be the subtype present in nigrostriatal dopamine

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terminals (Vilaró et al., 1990; Weiner et al., 1990). Although the occurrence of message suggests that the respective muscarinic receptor proteins are expressed, protein levels may not match mRNA levels, and no information has been provided about where the receptor proteins may be transported. Yet, whether receptors are located presynaptically or postsynaptically, and at what morphological types of synapses, is key to understanding their potential synaptic interactions.

To enable identification of the receptor proteins themselves, we have developed a panel of subtype-specific antibodies against recombinant muscarinic receptor proteins (Levey et al., 1990, 1991). Using these highly selective antibodies for light and electron microscopic immunocytochemistry, we have examined the cellular and subcellular distribution of muscarinic receptor proteins m1–m4 in the neostriatum of the rat. We have not detected m5 protein in brain with certainty. These findings establish that muscarinic receptor proteins are expressed in characteristic neuronal populations and transported to specific and unexpected pre- and postsynaptic sites.

Materials and Methods

Antibodies. The preparation and extensive characterization of rabbit polyclonal antibodies specific for m1–m4 acetylcholine receptors have been previously described (Levey et al., 1991). Briefly, these antibodies were raised against proteins, fused to glutathione S-transferase, that had been derived from the putative third inner cytoplasmic loop (i3) of each receptor. The selected regions of each respective i3 loop have little or no sequence homology. Antibodies used in the present study were affinity purified using the respective fusion proteins. Each antiserum immunoprecipitates its respective receptor without cross-reaction to any of the five cloned muscarinic receptors. The distribution of the receptor proteins in rat brain is similar both by immunoprecipitation and immunocytochemistry, and antibody binding is blocked in these assays by addition of the homologous fusion protein. Also, the cellular localization of the receptor proteins in brain is in excellent agreement with the localization of their respective mRNAs (Buckley et al., 1988; Weiner et al., 1990; Vilaró et al., 1991, 1992; Bernard et al., 1992). Finally, as shown below, Western blotting studies demonstrate that the antibodies bind to the cloned and native receptors with complete specificity. These data rigorously establish the subtype specificity of the antibodies.

Gel electrophoresis and immunoblotting. Western blotting studies were performed to characterize further the specificity of the antibodies. Membranes from CHO-K1 cell lines stably expressing m1–m5 muscarinic receptor cDNAs (Buckley et al., 1989; Dörje et al., 1991) or rat brain regions were prepared as described previously (Levey et al., 1991). The total number of receptors/mg protein (determined by saturation binding) for each subtype is, for m1, 2518 fmol; m2, 747 fmol; m3, 1831 fmol; m4, 1778 fmol; and m5, 954 fmol. Samples (50 μg) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; with 8% acrylamide) and transferred to Immobilon membranes by electroblotting (150 mA, overnight) as described by Towbin et al. (1979). The blots were blocked in 5% nonfat dried milk at room temperature for 1 hr, and then incubated with affinity-purified antibodies (0.5–1.0 μg/ml) diluted in blocking buffer at 4°C overnight. After washing with several changes of TBS, blots were incubated with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (1:5000; Bio-Rad) for 1 hr. After several washes, immunoreactive proteins were visualized on the blots using enhanced chemiluminescence (ECL, Amersham) as recommended by the manufacturer, using Hyperfilm-ECL (Amersham) and exposure times of 1–120 min.

Immunocytochemistry. Twelve young adult male Sprague–Dawley (Harlan) rats were deeply anesthetized with chloral hydrate and perfused transcardially with 240 ml of 3% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer. Brains were removed 1 hr after perfusion and sectioned at 40 μm using a vibratome (Technical Products International). Sections were collected in 0.1 M phosphate-buffered saline and rinsed in 0.05 M Tris-buffered saline (TBS, pH 7.2) for 10 min. Free-floating sections were preblocked for 1 hr in 4% normal goat serum (NGS) in TBS and then incubated at 4°C on a shaker platform for 48 hr in 1 μg/ml affinity-purified antibodies in 2% NGS-TBS (primary antibody omitted for control sections). Sections were then rinsed in six changes of cold TBS for a total of 1 hr, and then incubated overnight in biotinylated goat anti-rabbit secondary antibody (Vector ABC Elite) in TBS with 2% NGS. After rinsing in six changes of TBS for a total of 1 hr, the sections were incubated in avidin–biotin complex (Vector ABC Elite) for 4 hr followed by six further rinses with TBS. Final development was with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% hydrogen peroxide in TBS for 10–20 min. Sections were then rinsed with TBS for another hour.

Sections containing the caudate-putamen were rinsed in 0.1 M cacodylate buffer twice for 10 min, and then postfixed in 1% osmium tetroxide in cacodylate buffer for 30 min. After rinsing the sections for 10 min in two changes of cacodylate buffer, and for 10 min in two changes of 0.05 M acetate buffer, they were stained overnight in 2–5% aqueous uranyl acetate. Sections were again rinsed in acetate buffer, dehydrated in graded alcohols, then in propylene oxide for 5 min before being embedded in Durcupan resin (Fluka) between glass slides. Blocks were dissected from the dorsolateral striatum, mounted on plastic stubs, and sectioned using an ultramicrotome (Sorvall MT2-B). One micrometer sections were taken, mounted on glass slides, and stained with toluidine blue for light microscopic examination. Silver ultrathin sections were collected on mesh or slotted copper grids and left unstained for electron microscopy (Zeiss EM10). Lead staining was not performed to avoid the production of confounding staining artifact.

To determine the relative numbers of neuronal somata labeled in the dorsolateral striatum with each muscarinic receptor protein antibody, counts were made of labeled and unlabeled somata in 1–2-μm-thick plastic sections, counterstained with toluidine blue. These were accomplished using a 100× oil immersion lens mounted on a Nikon FXA microscope. For each antibody, between 400 and 700 neurons, sectioned through their nuclei, were counted in single sections from multiple tissue blocks from brains (three to five) with the most intense immunostaining. Counts were made for m1, m2, and m4 but not for m3 in which somatic labeling was not visible by light microscopy.

Results

Immunoblotting

Western blot experiments (Fig. 1) demonstrate the monospecificity of the m1–m4 antibodies. Each antibody recognizes a single cloned receptor protein expressed in CHO-K1 cells. None of the antibodies cross-reacted with untransfected cells, cells transfected with the other receptors in the muscarinic family, or brain proteins other than the putative native receptors. The cell lines contained several immunoreactive species for m3 and m4, presumably due to the presence of glycosylated receptors, catabolic products, and/or incomplete translation products. The sizes of the immunoreactive proteins correspond extremely well with the size of the receptors as determined by autoradiography of ^{3}H-propylbenzilylcholine mustard alkylation and SDS-PAGE (W. Simonds and A. Levey, unpublished observations). The cloned m2-immunoreactive protein did not enter the gel; however, a band is visible at the top surface of the gel where a small amount was retained. Thus, the native m2 proteins were not detected on immunoblots despite the established ability of this antibody to recognize the cloned and native m2 receptor in other assays (Levey et al., 1991). There was a high degree of regional specificity in brain with m1 receptor protein being most abundant in cortex, m3 most abundant in thalamus, and m4 most abundant in striatum and cortex. Longer exposures demonstrated a weak band of m3 immunoreactivity in the cortex and striatum at the same mobility as in thalamus.

Control immunocytochemistry

Control sections from each brain were examined by light microscopy. In each case, labeling of neurons or of neuronal processes were not observed (Fig. 2). Two control blocks from the striatum of each brain were also examined by electron microscopy. No DAB reaction product was observed in neuronal somata, dendrites, or axons. Particular attention was paid to syn-

apses that contained no reaction product both within axon terminals and also at postsynaptic densities.

m1 receptor protein immunocytochemistry

At the light microscopic level, there is very intense labeling in the striatum, including large numbers of neurons (Fig. 2). In semithin sections (Fig. 3), neurons can also be readily identified and most appear to contain perikaryal label. Labeled neurons are generally round, small to medium in size, contain scant cytoplasm, and do not have nuclear folds. Many of the neurons that do not contain label have the cytological characteristics of cholinergic or peptidergic neurons, being elongated, with enfolded nuclei, and rich cytoplasm (see example in Fig. 3). Puncta of reaction product are also visible in the neuropil that are not readily distinguishable as dendritic or axonal. Counts of labeled and unlabeled perikarya ($n = 454$) indicate that 78% of striatal neurons express m1 receptor (see Table 1).

By electron microscopy, labeled somata are round and small to medium in size with scant cytoplasm (Fig. 4*A*). Their nuclear envelopes do not include folds and nucleoli are not always prominent. Somatic labeling is perikaryal and diffuse and is not associated with any particular organelles, because most are surrounded by reaction product. However, perinuclear label associated with Golgi apparatus and endoplasmic reticulum is often seen. Label extends into the dendrites but not into initial axon segments (Fig. 4*B*). Unlabeled synapses with the m1-labeled somata occur and are primarily symmetrical. Dendritic labeling can be extensive (Fig. 4*C*) but was frequently localized to circumscribed submembranous patches (Fig. 4*D*), often at synapses (Fig. 4*E*). Even lightly labeled dendrites gave rise to densely labeled spines (Fig. 4*F*). However, individual spines arising from an m1-positive dendrite can be labeled or unlabeled (Fig. 4*G*). Some postsynaptic densities in labeled spines also appear highly labeled (Fig. 4*H,I*). Dense label was often visible within the spine neck, surrounding the spine apparatus (Fig. 4*I*). Labeled axon terminals contain reaction product surrounding the synaptic vesicles and other terminal organelles (Fig. 4*J*). m1 axon terminals are infrequent and make asymmetrical synapses primarily with spine heads (Fig. 4*J*). Since m1-positive axon terminals did not form symmetrical synapses, they are not likely to be derived from the axons of striatal neurons.

m2 receptor protein immunocytochemistry

At the light microscopic level, label was most evident in scattered large oval neurons with long aspiny dendrites (Fig. 2) and also in neuronal processes in the neuropil. Neuronal counts ($n = 679$) from semithin sections (Fig. 3) indicate that these neurons make up 2.7% of striatum neurons (see Table 1). Neuropil labeling was also evident as multiple puncta and short curvilinear elements suggestive of axon terminals and dendrites respectively (Fig. 3).

By electron microscopy, the labeled somata were observed to contain a large oval nucleus with an enfolded nuclear envelope and a prominent nucleolus (Fig. 5*A*). These neurons have abundant cytoplasm rich in mitochondria, stacks of rough endoplasmic reticulum, and Golgi complexes. Reaction product was typically concentrated just beneath the plasmalemma but occasionally was also visualized in more perinuclear cytoplasm associated with polyribosomes, endoplasmic reticulum, and Golgi elements. Somatic synapses, which were made exclusively by unlabeled axon terminals, were symmetrical and very uncommon with between zero and two evident on any given per-

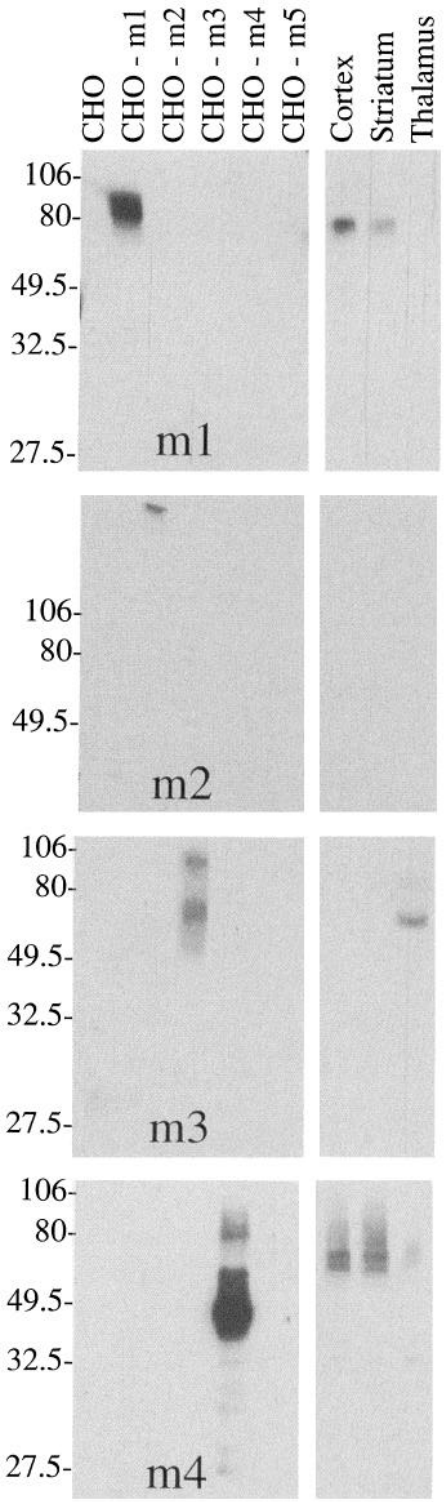
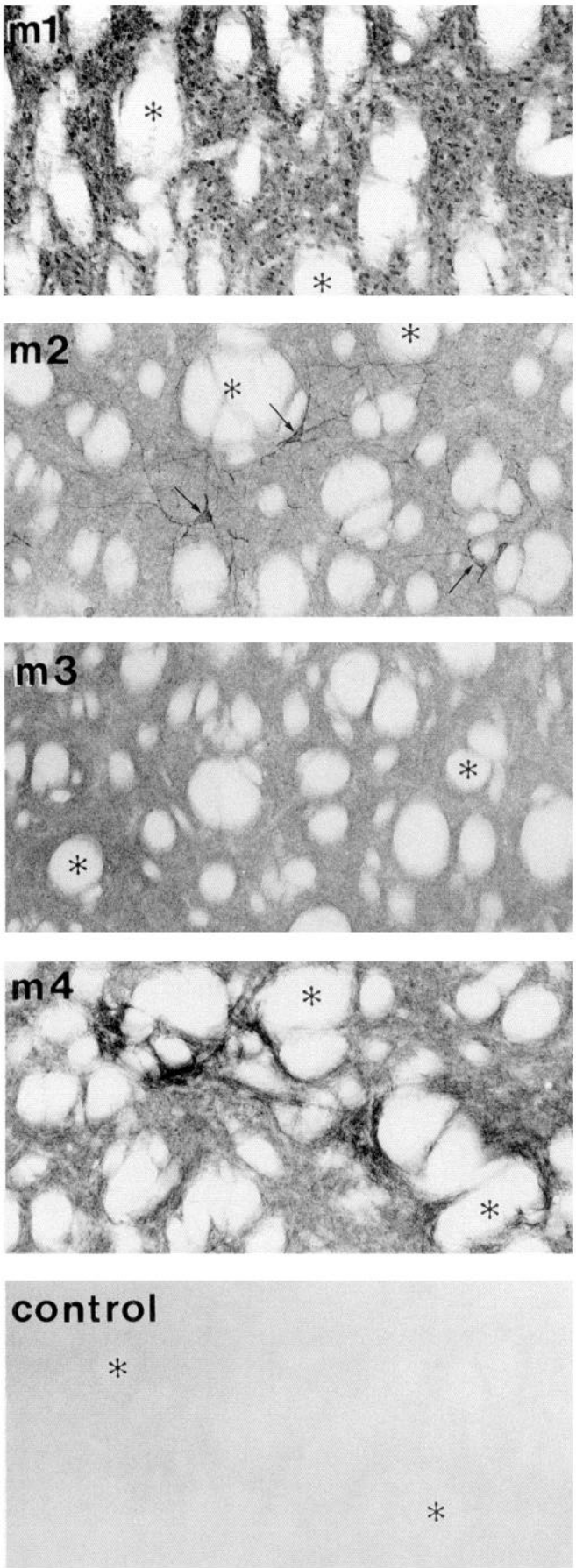


Figure 1. Western blots demonstrating the specificity of m1–m4 antibodies. Aliquots (50 μg) of membranes were prepared, subjected to SDS-PAGE, and immunoblotted. The *left column* contains membranes from control (*CHO*) or stably transfected CHO-K1 cell lines individually expressing m1–m5 receptors (*CHO-m1* through *CHO-m5*) while the *right column* includes rat cerebral cortex, striatum, and thalamus. Each antibody reacts with a single cloned receptor on the blots. Although the m2-immunoreactive protein did not penetrate the gel, it is visible in a band at its top surface. Note the subtype specificity of each antibody for the respective cloned receptor, and the high degree of regional specificity in brain.



ikaryal profile (Fig. 5*B*). Well-labeled dendrites were evident in the neuropil with reaction product filling them diffusely, outlining cytoskeletal elements and organelles (Fig. 5*C*). Appositions between labeled dendrites exhibited receptor-positive junctional specializations similar in appearance to postsynaptic densities (Fig. 5*D*). Labeled dendritic shafts were postsynaptic at both symmetrical and asymmetrical synapses (Fig. 5*C*). There were dense bands of reaction product underlying the postsynaptic membranes at some of these synapses (Fig. 5*E*).

Numerous preterminal axons and axon terminals (Fig. 5*F*) were evident as were occasional myelinated axons (Fig. 5*G*). m2 axon terminals forming symmetrical synapses contacted spines, dendrites, and somata. Single sections through somata typical of medium spiny cells demonstrated that some received multiple m2-positive symmetrical synapses (Fig. 5*H,I*) while others received none. Dendrites postsynaptic to m2 axon terminals were of all sizes and many could be identified as spiny (Fig. 5*J,K*). Labeled axon terminals were frequently observed synapsing with spine heads receiving an unlabeled asymmetrical synapse (Fig. 5*L,M*). A smaller population of m2-positive axon terminals were evident making asymmetrical synapses with spines (Fig. 5*N,O*) and dendritic shafts (Fig. 5*P*). Occasionally, these axodendritic synapses were with m2-positive dendrites (Fig. 5*Q*).

m3 receptor protein immunocytochemistry

By light microscopy, there was diffuse staining but m3 could not be localized to any structural elements (Fig. 2). However, in semithin sections (Fig. 3), m3 receptor protein immunoreactivity was visible as scattered puncta in the neuropil. Perikaryal labeling was either nonexistent or too light to permit definitive identification of m3-positive somata. By electron microscopy, labeled processes were much less frequent compared to the other muscarinic receptors. Perikaryal reaction product was minimal but occasionally observed in neurons with the cytological features of medium spiny cells (Fig. 6*A*). Dendritic label occurred only in very discrete patches (Fig. 6*B*) and was primarily within spiny dendrites. Many of the spines arising from labeled dendrites were themselves unlabeled (Fig. 6*B*). Furthermore, labeled spines frequently arose from dendrites with little or no apparent reaction product (Fig. 6*C*). The most intense labeling was present in spine heads and axon terminals. Some labeled spines contained postsynaptic densities that also appeared labeled (Fig. 6*D*). However, most spines contained reaction product peripheral to the synaptic active zone (Fig. 6*E*). Labeled myelinated axons (Fig. 6*F*), preterminal axons (Fig. 6*G*), and axon terminals (Fig. 6*H,I*) were observed. m3-labeled terminals were observed to form asymmetrical synapses exclusively and predominantly with spine heads. The reaction product contained within labeled axon terminals was usually located at a distance from the active zone (Fig. 6*H,I*).

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Figure 2. Light micrographs of 50 μm sections of rat striatum following muscarinic receptor immunocytochemistry (100× magnification). A control section is shown below for comparison. The large bundles of myelinated axons (*asterisks*) appear unstained. The m1 receptor antibody labels large numbers of neurons and diffusely stains the neuropil. The m2 receptor antibody stains large neurons (*arrows*) as well as neuronal processes. With m3 immunocytochemistry, some diffuse background staining is appreciable compared to controls. With m4 receptor immunocytochemistry, there is intense and heterogeneous staining of the striatal neuropil but individual neurons are not well visualized.

Table 1. Proportions of striatal neurons expressing individual muscarinic receptor subtypes

	Percentage of striatal neurons expressing muscarinic receptors		
		mRNA	
Subtype	Protein	Weiner et al.	Bernard et al.
m1	77.8 ± 3.2 (n = 454)	81	85
m2	2.7 ± 2.6 (n = 669)	3.5	Large neurons
m3	Somata undetected	Somata undetected	Not done
m4	44.2 ± 1.4 (n = 395)	42	61

Data show comparison of receptor protein immunocytochemistry in the present study with *in situ* hybridization studies detecting mRNA (Weiner et al., 1990; Bernard et al., 1992).

m4 receptor protein immunocytochemistry

By light microscopy, m4 receptor protein immunocytochemistry produced intense labeling of the neuropil in a patchy distribution throughout the striatum (Fig. 2). Labeled perikarya were difficult to visualize within the densely stained neuropil in thicker sections but were easily identified in semithin plastic sections (Fig. 3). Counts of labeled and unlabeled perikarya (n = 395) in semithin sections revealed that about 45% of dorsal striatal neurons are m4 positive (see Table 1). It is notable that some somata with the cytological appearance of striatal interneurons were labeled.

Many m4-positive somata could be identified by electron microscopy and most were small to medium-sized neurons with scant cytoplasm, round nuclei, and no nuclear envelope folds (Fig. 7*A*). A small number of larger somata with oval nuclei, prominent nucleoli, and richer cytoplasm were also observed (Fig. 7*B*), suggesting localization to striatal interneurons. Somatic label was present throughout the cytoplasm surrounding all types of organelles, though more concentrated peripherally. Large- and small-caliber m4-positive dendrites were evident in the neuropil (Fig. 7*C,D*). The most intense label was present in small (distal) spiny dendrites, which were diffusely labeled and contained reaction product surrounding cytoskeletal elements and organelles (Fig. 7*C,D*). Reaction product was often concentrated just beneath dendritic postsynaptic membrane (Fig. 7*D*). Spines were also filled with reaction product, often concentrated within the spine necks and at postsynaptic densities (Fig. 7*D,E*). Axon terminals labeled by m4 receptor immunocytochemistry were relatively frequent. Many were observed making asymmetric synapses with spine heads (Fig. 7*F,G*), including some that are m4 positive (Fig. 7*H,I*). Axon terminals forming symmetrical synapses were not seen.

Discussion

The results of the present study include several major findings related to the cellular and subcellular distribution of muscarinic receptor proteins. First, at a cellular level, the muscarinic receptor proteins were found to be present in distinct populations of striatal neurons. Second, at the subcellular level, for each receptor protein, reaction product was found to concentrate postsynaptically at synapses, many of which are usually considered to be noncholinergic. Third, m2 localization suggests it to be the predominant muscarinic autoreceptor in the striatum. Fourth, each receptor subtype was localized presynaptically in axon terminals forming asymmetrical synapses, suggesting roles as presynaptic heteroceptors modulating extrinsic striatal afferents.

The validity of these findings is based upon the specificity and sensitivity of the antibodies. Their specificity and lack of cross-reactivity have previously been established (Levey et al., 1990, 1991) and are now even further demonstrated by immunoblotting experiments utilizing CHO cells stably transfected with and expressing the receptor cDNAs as well as rat brain tissue. The sensitivity of the muscarinic receptor protein antibodies is demonstrated by the close correspondence between the numbers of cells they label and the numbers labeled by *in situ* hybridization as well as by the exquisite labeling of subcellular sites visualized by electron microscopy.

Cellular localization of muscarinic receptors

The present results have localized four muscarinic receptor subtypes to postsynaptic locations in the dorsal striatum of the rat. The majority of striatal neurons express m1 receptors; m2 receptors are restricted to a population of large interneurons; m3 receptors are present in a subset of spiny dendrites; and m4 is expressed in about half of all neostriatal neurons. The proportions of striatal neurons expressing these receptor protein are in excellent agreement with the numbers of neurons expressing each mRNA as determined by *in situ* hybridization studies (Table 1). This suggests that the presence of mRNA accurately predicts muscarinic receptor expression in the striatum. Small differences between the immunocytochemistry and *in situ* hybridization may reflect some mismatch between the detection of mRNA and the production of significant quantities of receptor proteins or minor differences in the sensitivity and/or specificity of each method. Striatal neurons expressing significant mRNA for m3 receptor have not yet been reported. However, using immunocytochemistry, we have localized m3 receptor protein to distal spiny dendrites, indicating that it is expressed in at least a subset of striatal neurons. Since neuronal elements are completely unlabeled in control tissue, we believe that the m3 labeling represents expressed receptors. Since m3 immunocytochemistry is the least intense and labels the fewest processes, its mRNA may be present at levels that have not permitted ready detection.

Light and electron microscopy of neuronal somata immunoreactive for the muscarinic receptors provides information about which striatal neuronal types express them. Since most striatal neurons are medium spiny cells and about three-quarters of striatal neurons are m1 immunoreactive, it seems clear that most medium spiny cells express m1. Indeed, by electron microscopy, most m1-immunoreactive neurons have the cytological features of medium spiny cells (DiFiglia et al., 1980; Dimova et al., 1980; Wilson and Groves, 1980; Bolam, 1984; Izzo et al., 1987). This is supported by Bernard et al. (1992), who

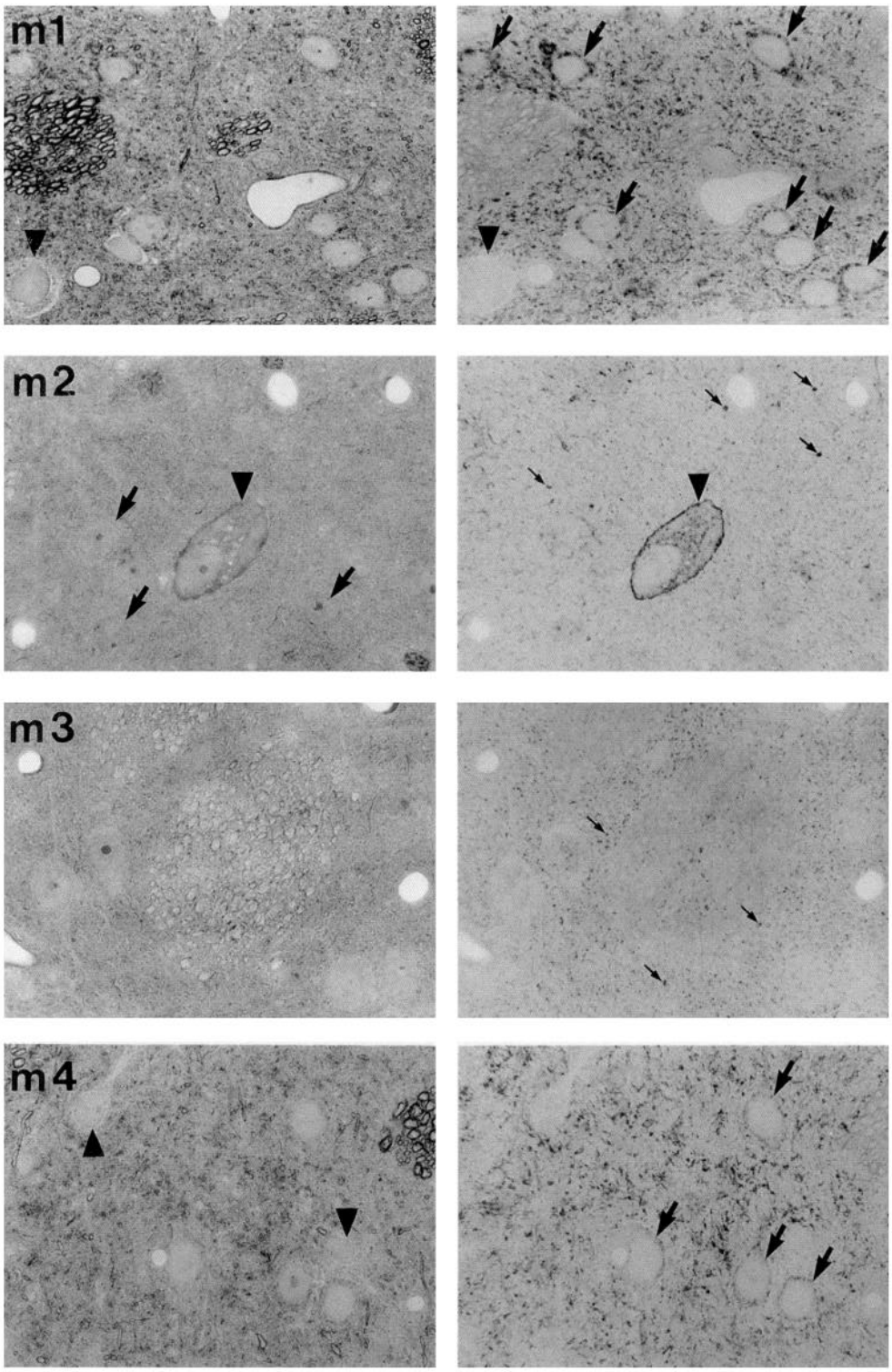

Figure 3. Paired light micrographs of 2 μm plastic sections following receptor immunocytochemistry and counterstaining with toluidine blue (710× magnification). The *left column* consists of unfiltered photographs that show tissue morphology well. The *right column* consists of the same sections photographed through a dark blue filter (Wratten 47B), which makes the DAB reaction product more visible and masks the counter stain. m1 receptor immunocytochemistry reveals intense labeling of the neuropil and most neurons (*arrows*). A single large neuron is unlabeled (*triangle*). With m2 receptor immunocytochemistry, the majority of neurons are unlabeled (*arrows*). However, one large, well-stained neuron is evident (*triangle*) as are puncta of label within the neuropil (*small arrows*). With m3 receptor immunocytochemistry, neuronal somata appear unlabeled; however, small puncta of reaction product are visible in the neuropil (*arrows*). m4 receptor immunocytochemistry reveals labeling of a subset of striatal neurons (*arrows*) and of many puncta throughout the neuropil. Examples of unlabeled neurons are marked by *triangles*.

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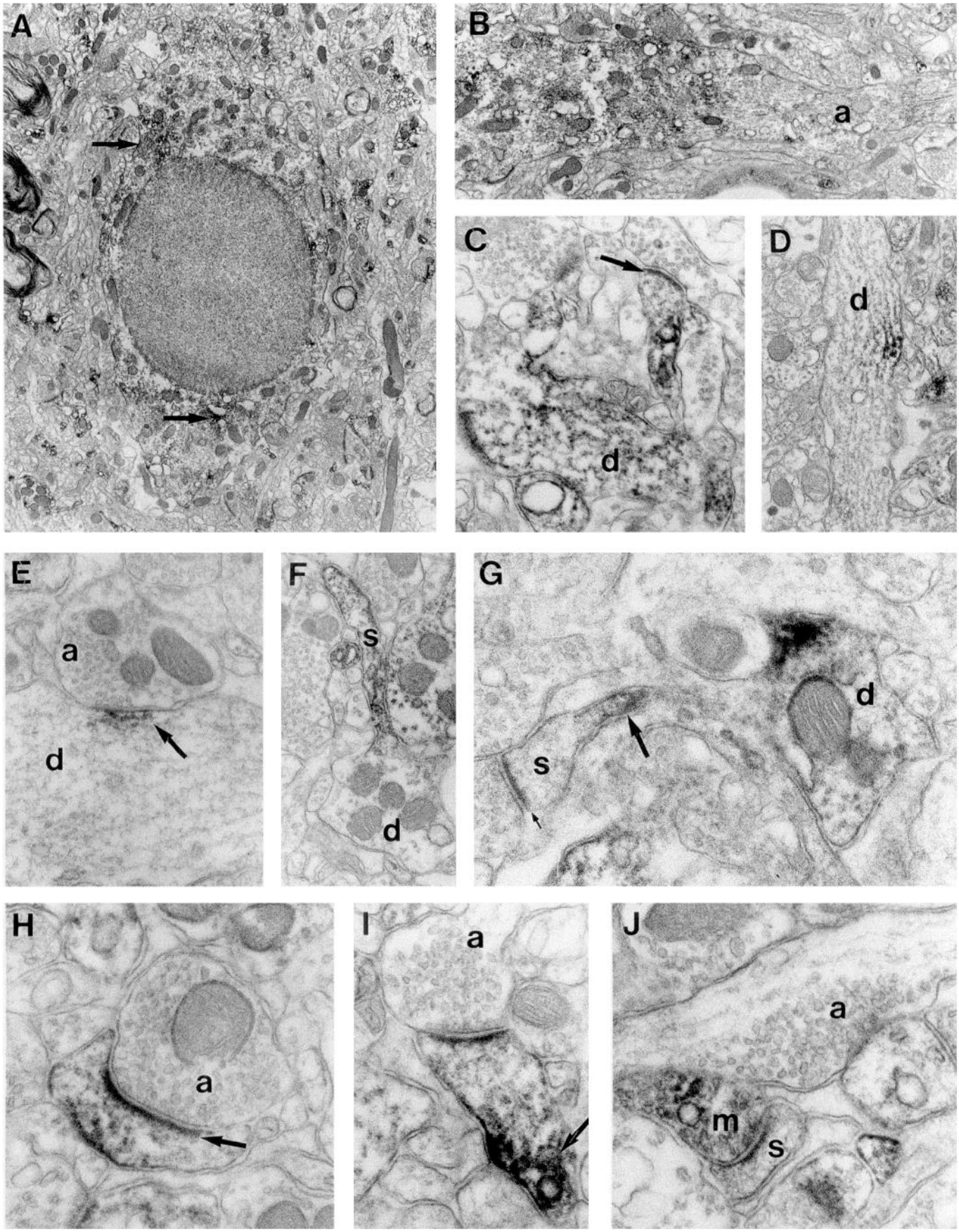
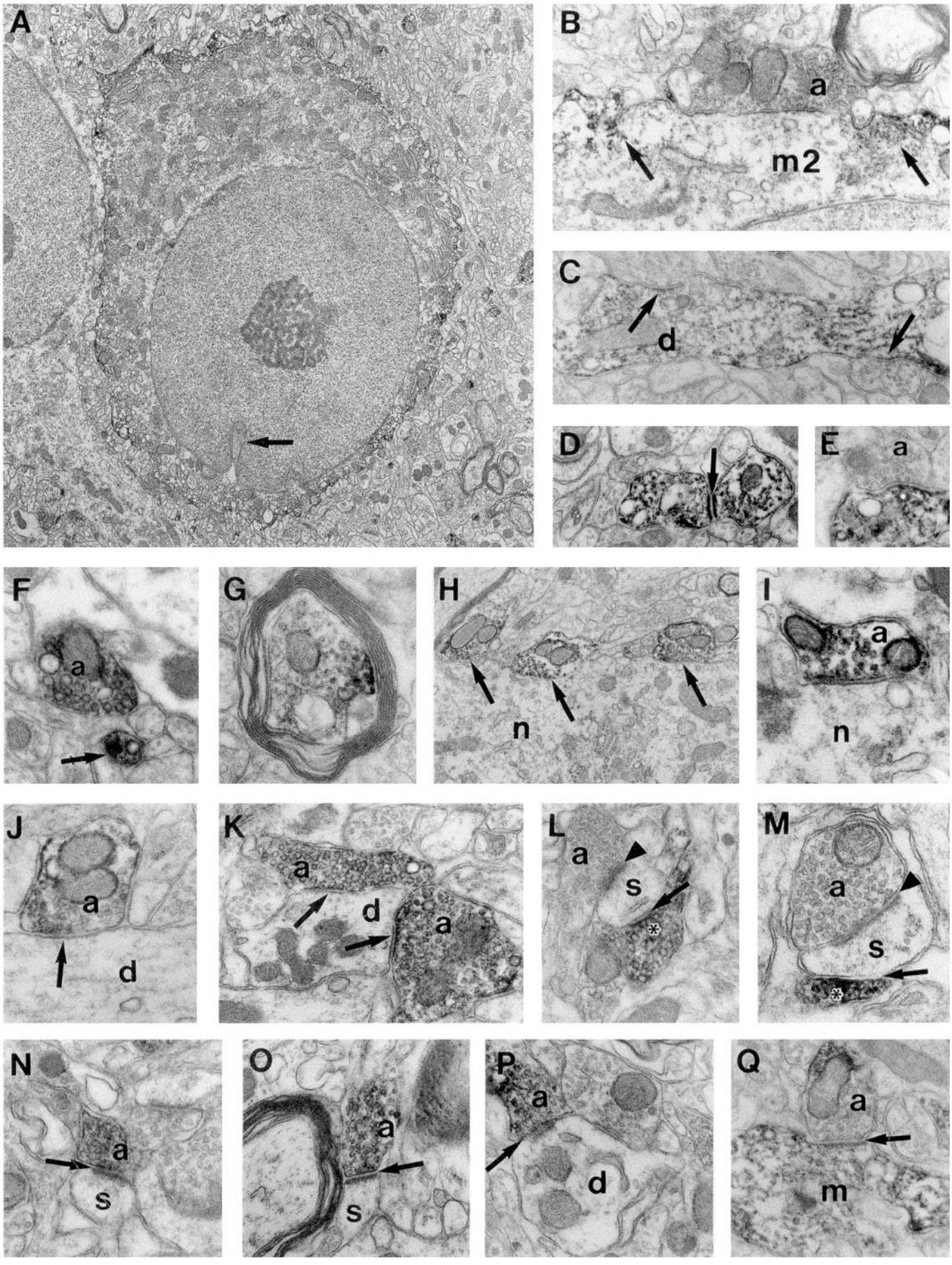
Figure 4. Electron micrographs demonstrating the subcellular localization of m1 receptor protein. *A*, Section through an m1-immunoreactive neuron. DAB reaction product is especially evident in the regions containing Golgi apparatus and endoplasmic reticulum (*arrows*). Note the many immunoreactive processes in the surrounding neuropil. *B*, A section through the initial axon segment (*a*) of an m1-positive neuron showing that reaction product does not enter the axon. *C*, Electron micrograph of a spiny dendrite (*d*) illustrating dispersal of reaction product amid the microtubules and a spine with dense label in its neck and also at the postsynaptic density (*arrow*). *D*, A more proximal dendrite (*d*) with more localized reaction product. *E*, A dendrite (*d*) containing a deposit of reaction product (*arrow*) localized postsynaptically at a synapse with an axon terminal (*a*) forming a symmetrical synapse. *F*, Electron micrograph of an unlabeled dendrite (*d*) giving rise to a labeled spine (*s*). *G*, A well-labeled dendrite (*d*) giving rise to a spine with reaction product within its neck (*large arrow*) and postsynaptic density (*small arrow*), but not within the spine head (*s*). *H*, Axospinous synapse between an unlabeled axon terminal (*a*) and an m1-positive spine head. The postsynaptic density (*arrow*) appears intensely labeled with DAB reaction product. *I*, Another axospinous synapse between an unlabeled axon terminal (*a*) and an m1-positive



spine. The postsynaptic density appears labeled and there is also especially intense label in the spine neck, beneath the cell membrane and surrounding the spine apparatus (*arrow*). The spine neck is a potential site for receiving cholinergic synapses. *J*, Electron micrograph demonstrating presynaptic localization of m1 receptor protein in an axon terminal (*m*) forming an asymmetrical synapse with an m1-immunoreactive spine (*s*). An adjacent unlabeled axon terminal (*a*) is visible. Magnification: *A*, 6000×; *B*, 10,300×; *C*, 31,700×; *D*, 17,500×; *E*, 32,000×; *F*, 25,400×; *G*, 44,000×; *H–J*, 46,700×.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combined ISH with immunocytochemistry, and found m1 message in neurons expressing met-enkephalin and substance P. They also found m1 message in striatal interneurons, containing ChAT, neurotensin, and somatostatin. While we have seen some m1-positive somata with the cytology of interneurons, they have not been neurochemically identified. Large striatal interneurons express m2 and have the same cytology as cholinergic neurons identified by ChAT immunocytochemistry (Bolam et al., 1984; Wainer et al., 1984; Phelps et al., 1985; DiFiglia, 1987; Dimova et al., 1993) as well as AChE staining (Satoh et al., 1983; Bolam et al., 1984). In addition, m2 mRNA and protein have been colocalized with ChAT in the majority of these neurons in the rat (Bernard et al., 1992; A. I. Levey, S. M. Edmunds, and S. M. Hersch, unpublished observations).

These patterns of receptor expression suggest possible functional consequences. For example, it seems certain that some medium spiny cells express both m1 and m4, while others express only m1. The m1 receptor stimulates phosphoinositide metabolism while m4 inhibits cAMP production (Bonner, 1989; Hulme et al., 1990). Thus, striatal neurons expressing one or both subtypes in spiny dendrites may respond very differently to ACh. It is unclear, however, whether neurons containing both subtypes transport them to the same or different synapses. The functioning of multiple receptors may help explain the complex physiological responses of striatal neurons to ACh. At hyperpolarized resting potentials, muscarinic agonists alter the A-current in striatal neurons such that the effects of excitatory inputs are attenuated while, at depolarized resting potentials, muscarinic agonists increase excitability by inactivating the A-current (Akins et al., 1990). These responses may occur in different neurons (e.g., with or without m4). However, if both responses occur within individual neurons expressing both receptors, perhaps the effects of one muscarinic subtype may predominate at hyperpolarized states while the other predominates at more depolarized states. Distinct physiologic responses may also relate to distinct striatal circuits. Colocalization studies indicate that a subset of neurons expressing m4 mRNA (Weiner et al., 1990) and protein, as shown here, correspond to substance P neurons (Bernard et al., 1992). Applying the current findings to the known predominant connections of substance P neurons (Gerfen, 1992), striatal neurons projecting directly to the basal ganglia output nuclei (globus pallidus, pars interna or entopeduncular nucleus along with the substantia nigra, pars reticulata) are likely to have a mixed m1 and m4 response while neurons projecting to the globus pallidus, pars externa may primarily have an m1 response.

Postsynaptic muscarinic receptors

The present results have shown that the receptors are not only expressed, but also are selectively transported to relevant postsynaptic sites. It was anticipated that muscarinic receptors would be primarily enriched at putative cholinergic synapses which, based upon ChAT immunocytochemistry, are predominantly symmetrical synapses occurring on spines necks, dendrites, and somata. Concentrations of reaction product were indeed visualized in spine necks and in somatodendritic regions postsynaptic at symmetrical synapses.

We were most surprised, however, to find that the muscarinic receptors frequently appeared to be enriched at the postsynaptic densities of asymmetrical synapses. This was especially true of m1 and m4 and, to a lesser extent, m3. Since striatal asymmetrical synapses are known to originate primarily from glutamatergic cortical, thalamic, and subthalamic afferents, this localization suggests the presence of postsynaptic muscarinic receptors at synapses using excitatory amino acids as their primary neurotransmitter. It is possible that muscarinic receptor immunoreactivity postsynaptic at noncholinergic synapses represents diffusion of DAB reaction product from nearby receptors. Other techniques, such as immunogold, may provide improved spatial resolution. However, the postsynaptic density labeling described in the present study does not occur in neurons that are retrogradely filled with HRP visualized with DAB (e.g., White et al., 1980). This fact, and the selectivity and intensity of the immunostaining of postsynaptic densities, suggests that this localization may be functionally significant. We hypothesize that these receptors are strategically positioned to modulate excitatory synapses under the influence of parasynaptic ACh. We have noted a similar phenomenon with localization of muscarinic m1 and m2 receptors at excitatory amino acid synapses in the cerebral cortex (Mrzljak et al., 1993) and dopamine D1 and D2 receptors at excitatory synapses in the striatum (Levey et al., 1993). These results suggest that individual postsynaptic elements containing receptors for multiple neurotransmitters may be a general feature of CNS synapses. In particular, receptors for modulatory neurotransmitters, such as ACh and dopamine, may be common residents of the postsynaptic membranes of excitatory synapses. It will be very interesting to determine, through future double labeling experiments, whether excitatory amino acid receptors are present at these synapses and also whether neurons expressing multiple muscarinic and dopamine receptors segregate these receptors to different intracellular regions, such as different spines, or intermingle them.

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Figure 5. Electron micrographs demonstrating the subcellular localization of m2 receptor protein. *A*, Section through the soma of a large aspiny striatal neuron immunoreactive for m2. This is a large-sized neuron with rich cytoplasm and nuclear folding (*arrow*). Reaction product is primarily distributed beneath the plasmalemma. *B*, Electron micrograph of a rare somatic synapse between an unlabeled axon terminal (*a*) and an m2-immunoreactive neuron (*m2*). Reaction product is not present postsynaptically but is plentiful outside the region of this synapse (*arrows*). *C*, m2-immunoreactive aspiny dendrite (*d*) with reaction product surrounding the organelles and microtubules. Synapses occurring on this dendrite are visible (*arrows*). *D*, Junctional complex (*arrow*) binding two m2-immunoreactive dendrites. The junctional densities appear intensely immunoreactive. *E*, Synapse between an unlabeled axon terminal (*a*) and an m2-immunoreactive dendrite illustrating concentration of reaction product in a dense band under the postsynaptic membrane. *F*, m2-positive axon terminal (*a*) and preterminal axon (*arrow*). Synaptic vesicles appear to be surrounded by reaction product. *G*, Myelinated axon containing m2 receptor protein. *H*, Segment of the soma of a medium-sized neuron (*n*) receiving synapses from multiple m2-positive terminals (*arrows*). *I*, Higher magnification view of an m2 axon terminal (*a*) synapsing with a neuronal soma (*n*). *J* and *K*, Symmetrical synapses (*arrows*) between m2-immunoreactive axon terminals (*a*) and unlabeled dendritic shafts (*d*). *L* and *M*, Probable symmetrical synapses (*arrows*) occurring between m2-positive axon terminals (*asterisks*) and spine heads (*s*) that are also postsynaptic to unlabeled axon terminals (*a*) forming asymmetrical synapses with them (*triangles*). *N–Q*, m2-immunoreactive axon terminals (*a*) were also observed to form asymmetrical synapses (*arrows*) with spines (*s*), dendrites (*d*), and with m2-immunoreactive dendrites (*m*). Magnification: *A*, 6500×; *B–D*, 21,400×; *E*, 28,600×; *F, G, I, J*, and *M–P*, 35,800×; *H*, 12,300×; *K* and *L*, 27,200×; *Q*, 27,000×.

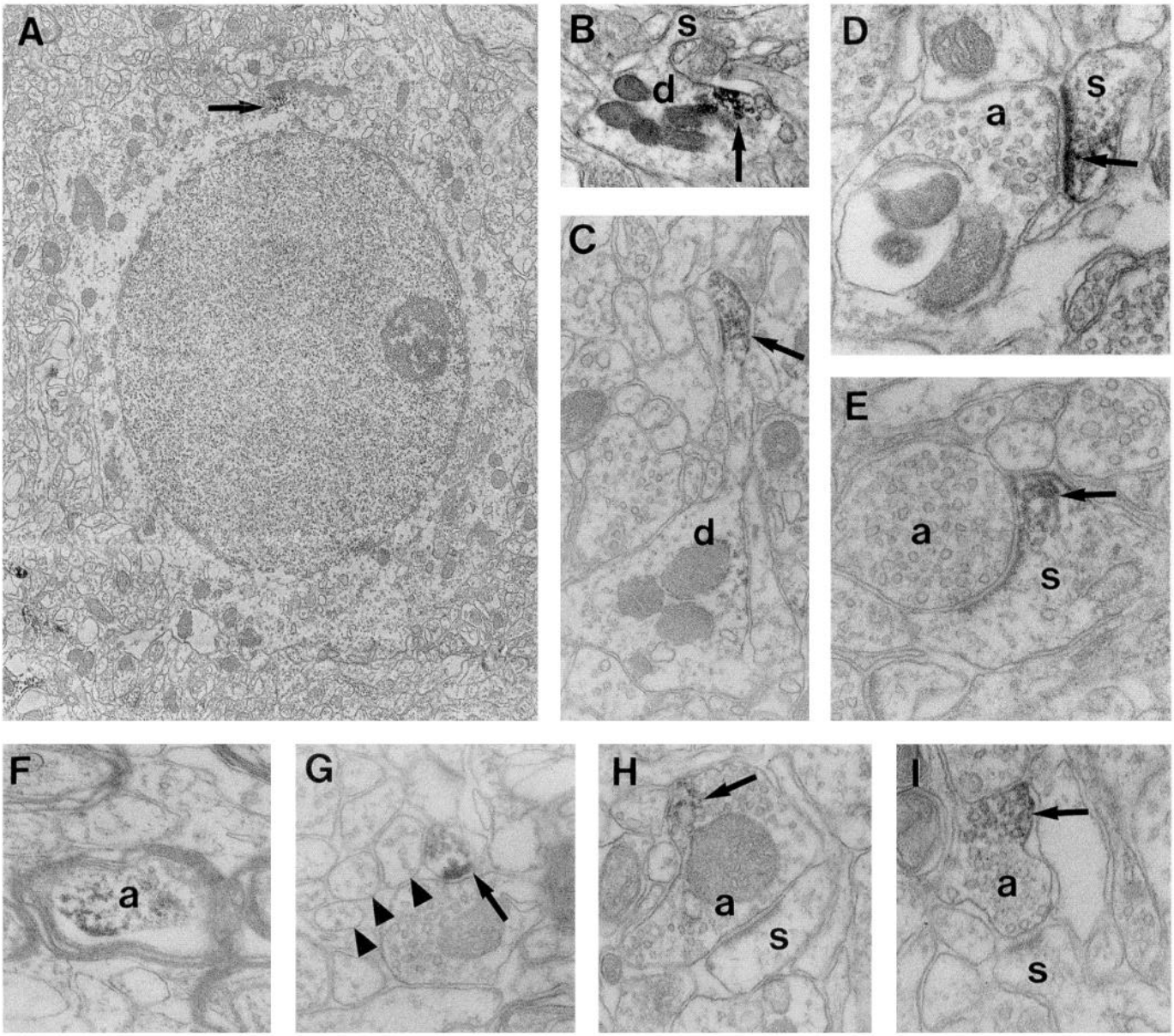

Figure 6. Electron micrographs demonstrating the subcellular localization of m3 receptor protein. *A*, Section through a medium-sized neuron with a single small deposit of reaction product (*arrow*). Note the few labeled processes in the surrounding neuropil. *B*, m3-immunoreactive dendrite (*d*) with a submembranous deposit of reaction product (*arrow*) and an unlabeled spine (*s*). *C*, m3-immunoreactive dendrite (*d*) with minimal dendritic label but much more in a spine head (*arrow*) arising from it. *D* and *E*, m3-positive dendritic spines (*s*) forming asymmetrical synapses with unlabeled axon terminals (*a*). The upper spine (*D*) demonstrates intense labeling of the postsynaptic density (*arrow*). The lower spine (*E*) contains label that is primarily adjacent to the postsynaptic density (*arrow*). *F* and *G*, m3-immunoreactive myelinated axon (*a*) and unmyelinated preterminal axon (*arrow*). Adjacent, unlabeled preterminal axons are evident for comparison (*triangles*). *H* and *I*, m3-positive axon terminals (*a*) forming asymmetrical synapses with unlabeled dendritic spines (*s*). In contrast to the other muscarinic subtypes, reaction product (*arrows*) is distant from the active zones of the synapses. Magnification: *A*, 9200×; *B*, 21,900×; *C*, 31,100×; *D* and *E*, 44,700×; *F*, 34,400×; *G*, 41,600×; *H*, 34,600×; *I*, 47,000×.

Presynaptic autoreceptors and m2 neurons

Previous studies utilizing ChAT immunoelectron microscopy have demonstrated cholinergic axon terminals to form symmetrical synapses with spines, dendrites, and neuronal somata. Pharmacologic experiments have indicated that the major muscarinic presynaptic autoreceptor in the striatum is M2-like, possibly corresponding to either the m2 or m4 receptor proteins (Buckley et al., 1988; Lapchak et al., 1989; Weiler, 1989; Dörje et al., 1991; Vilarö et al., 1991, 1992). The present results bridge

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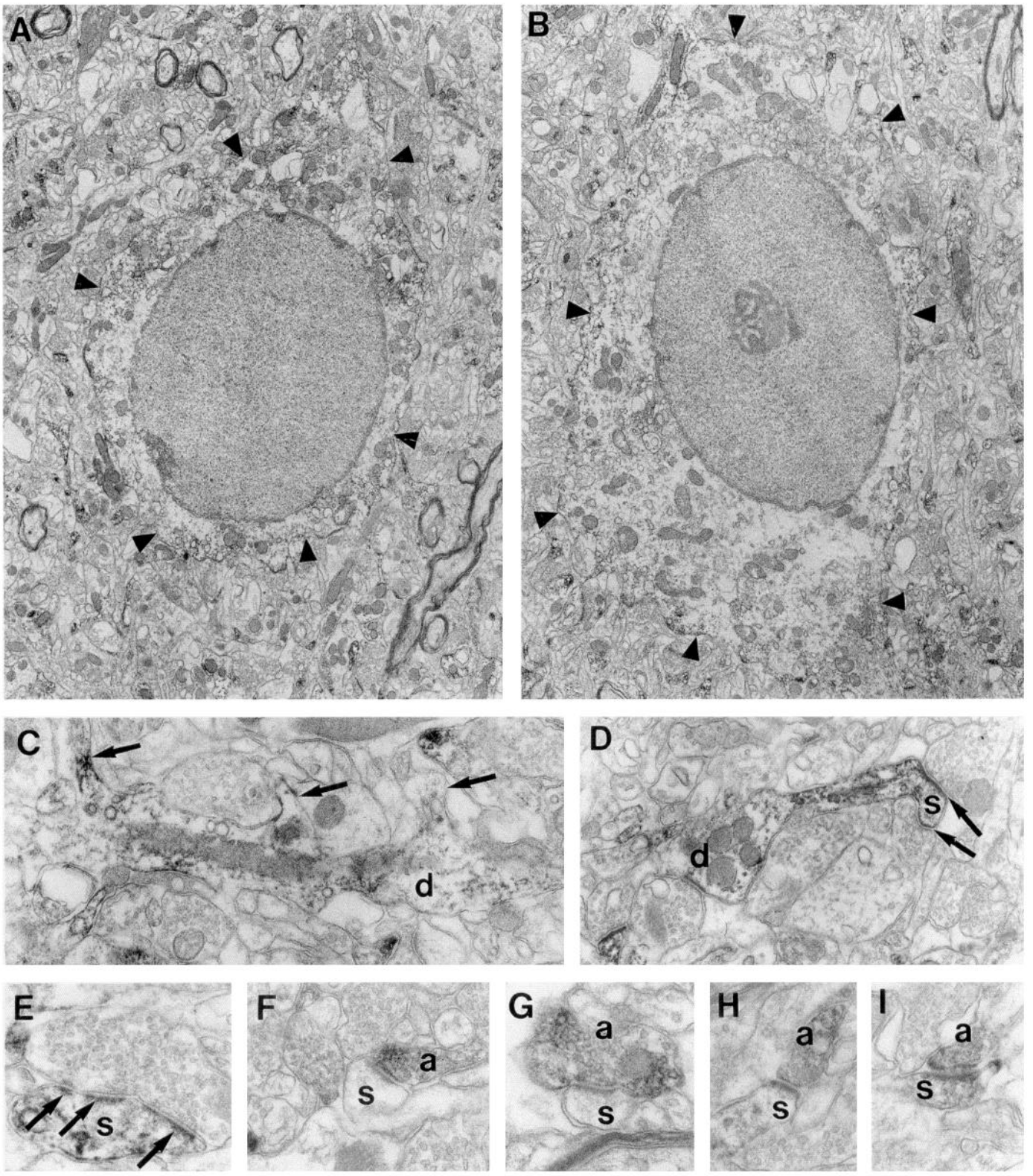
Figure 7. Electron micrographs demonstrating the subcellular localization of m4 receptor protein. *A*, Section through an m4-immunoreactive, medium-sized neuron with the cytological features typical of medium spiny cells. Reaction product is dispersed through the cytoplasm but somewhat more concentrated beneath the plasmalemma, which is marked by *triangles*. Note the large number of labeled processes in the surrounding neuropil.



B, Electron micrograph of a larger neuron with more abundant cytoplasm, and rare somatic synapses, indicating that it is probably a striatal interneuron. Reaction product is most visible beneath the plasmalemma, which is marked by *triangles*. *C*, m4-immunoreactive dendrite (*d*) giving rise to multiple labeled spines (*arrows*). Reaction product is dispersed through the cytoplasm, surrounding microtubules and organelles. *D*, m4-positive spiny dendrite (*d*). Asymmetrical synapses (*arrows*) are present on the dendritic shaft and spine head (*s*). Note the concentration of reaction product in the spine neck and at the postsynaptic densities. *E*, Higher-magnification view of a large m4-immunoreactive spine (*s*) with apparent accumulation of reaction product at the postsynaptic densities (*arrows*). Compare this to the unlabeled spine heads (*s*) in *F* and *G*. *F* and *G*, Asymmetrical synapses between m4-labeled axon terminals (*a*) and unlabeled spine heads (*s*). Reaction product appears to coat the synaptic vesicles. *H* and *I*, Asymmetrical synapses between m4-labeled axon terminals (*a*) and m4-labeled dendritic spines (*s*). Magnification: *A* and *B*, 7300×; *C* and *D*, 22,200×; *E–I*, 34,200×.

these findings by localizing m2 to large cholinergic interneurons and to numerous axon terminals with the same morphology as those containing ChAT. Although m4 was detected in the somata of possible cholinergic interneurons, this subtype, as well as m1 and m3, was not localized to axon terminals making symmetrical synapses. Thus, m2 appears to be the primary cholinergic autoreceptor in striatum.

This study suggests that m2 receptor proteins may also function as postsynaptic receptors, since much of the intense m2 immunoreactivity occurs in somatodendritic compartments. Of course, we are likely to be detecting m2 receptor protein at stages of its synthesis and transport during which it may not be functional. However, the possibility of functional postsynaptic m2 receptors is supported by m2 immunoreactivity often being concentrated at postsynaptic membrane regions (see Fig. 5*E*). There may also be parasynaptic or volume transmission for m2 as well as the other subtypes.

Axon terminals containing m2 receptor proteins also displayed evidence of specificity in their terminations. For example, it appeared that some medium-sized neurons receive large numbers of m2-labeled, and presumable cholinergic, synapses while others receive few or none. Perhaps m4 immunoreactive neurons constitute one of these neuronal types. This possibility can be further explored using double labeling experiments to identify m2 terminals and their postsynaptic neurons. Reconstruction of neuronal somata from serial thin sections would be required to precisely quantify their cholinergic input. Synapses between m2-positive axon terminals and m2-positive dendrites were also observed, suggesting a potential mechanism for cholinergic neurons to integrate their actions with other cholinergic neurons.

Presynaptic muscarinic heteroceptors

Each muscarinic receptor was localized to axon terminals forming asymmetrical synapses, indicating their origin extrinsic to the striatum. These findings suggest that each of these muscarinic receptors may be presynaptic heteroceptors in the striatum. Their functional significance is suggested by pharmacological evidence that an M3-like binding site acts presynaptically in the striatum to potently inhibit glutamate release (Sugita et al., 1991). There are a number of striatal afferents and even receptor subtypes that are candidates for mediating these presynaptic effects. Corticostriatal and corticothalamic axon terminals form many of the asymmetrical synapses occurring in striatum (Kemp, 1968). Infragranular neocortical pyramidal cells, possibly including corticostriatal neurons, are known to express m1 and m3 mRNA (Buckley et al., 1988). Thalamic neurons, possibly including those projecting to the striatum, express m2 and m3 receptors (Buckley et al., 1988; Levey et al., 1991). Additional sources may include excitatory afferents from the subthalamic nucleus (Canteras et al., 1990; Smith et al., 1990), which contains neurons expressing m3 and m4 (Weiner et al., 1990), and the midbrain tegmentum (Hallanger and Wainer, 1988) as well as a subset of nigrostriatal afferents (Hattori et al., 1991). Immunocytochemistry and *in situ* hybridization studies have demonstrated that neurons expressing several muscarinic receptor subtypes also occur in these regions (Buckley et al., 1988; Weiner et al., 1990; Levey et al., 1991). Thus, it may be predicted that m1 and/or m3 receptors may be present in corticostriatal terminals, m2 or m3 receptors in thalamostriatal terminals, and m3 or m4 in subthalamostriatal terminals. Studies combining receptor immunocytochemistry with lesions or anterograde tracers will enable a detailed understanding of which extrinsic afferents contain the muscarinic receptors.

Conclusion

At least four of the five cloned muscarinic receptor subtypes have now been demonstrated to occur in the striatum. They are differentially distributed in subsets of neurons and in a variety of highly specific pre- and postsynaptic sites. These findings indicate that there must be exquisitely precise gene regulation governing the cellular distribution of receptor expression, as well as highly specific transport mechanisms that direct the proteins to a variety of pre- and postsynaptic sites in brain. ACh muscarinic effects are manifold but specific cholinergic and noncholinergic synapses are likely to be mediated by different subtypes. Further knowledge may enable the development of therapeutic agents designed to affect specific circuits and synapses using m1–m5 receptor subtypes.

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