# Surface and Intracellular Distribution of a Putative Neuronal Nicotinic Acetylcholine Receptor

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*Abstract.* Chick ciliary ganglion neurons have a membrane component that shares an antigenic determinant with the main immunogenic region (MIR) of nicotinic acetylcholine receptors from skeletal muscle and electric organ. Previous studies have shown that the component has many of the properties expected for a ganglionic nicotinic acetylcholine receptor, and that its distribution on the neuron surface in vivo is restricted predominantly to synaptic membrane. Here we report the presence of a large intracellular pool of the putative receptor in embryonic neurons and demonstrate that it is associated with organelles known to comprise the biosynthetic and regulatory pathways of integral plasma membrane proteins. Embryonic chick ciliary ganglia were lightly fixed, saponin-permeabilized, incubated with an anti-MIR monoclonal antibody (mAb) followed by horseradish peroxidase-conjugated secondary antibody, reacted for peroxidase activity, and examined by electron microscopy. Deposits of reaction product were associated with synaptic membrane, small portions of the pseudodendrite surface membrane, most of the rough endoplasmic reticulum, small portions of the nuclear envelope, some Golgi complexes, and a few coated pits, coated vesicles, multivesicular bodies, and smooth-membraned vacuoles. No other labeling was present in the neurons. The labeling was specific in that it was not present when the anti-MIR mAb was replaced with either nonimmune serum or mAbs of different specificity. Chick dorsal root ganglion neurons thought to lack nicotinic acetylcholine receptors were not labeled by the anti-MIR mAb. Substantial intracellular populations have also been reported for the muscle acetylcholine receptor and brain voltage-dependent sodium channel α-subunit. This may represent a general pattern for multisubunit membrane proteins during development.

LITTLE is known about the distribution and regulation of nicotinic acetylcholine receptors (AChRs)[1] on neurons because methods have not been available for unambiguously identifying the receptor protein. Chick ciliary ganglion neurons have nicotinic AChRs that mediate chemical synaptic transmission through the ganglion (Martin and Pilar, 1963*a*, *b*). Recently monoclonal antibodies (mAbs) to the main immunogenic region (MIR) of AChR α-subunit from muscle and electric organ have been used to identify a membrane component on chick ciliary ganglion neurons that has many of the properties expected for a neuronal nicotinic AChR. On the neuron surface, the cross-reacting component is located predominantly in synaptic membrane (Jacob et al., 1984). It is an integral membrane component that in detergent extracts has a sedimentation coefficient similar to AChR from muscle and electric organ; it binds to concanavalin A, a lectin known to block AChR function on the neurons; the component has the tissue distribution expected for a ganglionic AChR in that it is present in ciliary and sympathetic ganglia but is not detectable in heart, liver, spinal cord, retina, or dorsal root ganglia; and the component reaches peak levels per ganglionic protein at a time in development when synapses are being consolidated on the neurons (Smith et al., 1985). The amount of cross-reacting component on the neurons in culture can be modulated by cholinergic agents, and in most cases, the levels of acetylcholine (ACh) sensitivity associated with the neurons are modulated in parallel (Smith et al., 1986). A similar component that cross-reacts with mAbs to the MIR of muscle and electric organ AChR can be isolated from chick brain (Whiting and Lindstrom, 1986). Antisera to the component from chick brain block the function of AChRs on ciliary ganglion neurons in culture (Stollberg et al., 1986). These results taken together strongly suggest that the component on ciliary ganglion neurons that is recognized by mAbs to the MIR of muscle and electric organ AChR is, in fact, a neuronal nicotinic AChR.

A membrane component on neurons that binds α-bungarotoxin has previously been proposed as a neuronal nicotinic

1. *Abbreviations used in this paper*: AChR, acetylcholine receptor; HRP, horseradish peroxidase; mAb, monoclonal antibody; MIR, main immunogenic region; PBS-glycine, phosphate-buffered saline containing 0.75% glycine; PG, phosphate-buffered saline plus 10% (vol/vol) normal goat serum; RER, rough endoplasmic reticulum.

AChR. Recently such a component has been purified from chick brain, and partial amino acid sequence data has revealed homology with chick muscle AChR (Conti-Tronconi et al., 1985). Chick ciliary ganglion neurons also have α-bungarotoxin binding sites but the toxin binding component is unlikely to represent the functional, synaptic AChR on the neurons in this case because it is not located at synapses (Jacob and Berg, 1983; Loring et al., 1985), and the ACh response of the neurons is neither inhibited by α-bungarotoxin nor altered in parallel with changes in the number of α-bungarotoxin binding sites on the cells (Smith et al., 1983). Moreover, in detergent extracts of ciliary ganglia it can be shown that the component recognized by mAbs to the MIR of muscle and electric organ AChR is clearly distinct from the component that binds α-bungarotoxin (Smith et al., 1985).

We have used a mAb to the MIR of muscle and electric organ AChR to examine the ultrastructural distribution of the cross-reacting component in saponin-permeabilized embryonic ciliary ganglia. The ganglion contains two populations of neurons: ciliary and choroid cells. Both neuron types receive nicotinic cholinergic transmission from the accessory oculomotor or Edinger-Westphal nucleus (Martin and Pilar, 1963a,b; Cowan and Wenger, 1968; Landmesser and Pilar, 1978). Ciliary and choroid cells can be distinguished on the basis of their size, shape, location in the ganglion, cytological organization, and synaptic morphology (Hess, 1965; Landmesser and Pilar, 1972, 1978; Pilar et al., 1980). We report here that both ciliary and choroid neurons in embryonic ganglia have substantial amounts of intracellular putative neuronal AChR identified by the anti-MIR mAb. The intracellular component is associated with organelles known to comprise the biosynthetic and regulatory pathways for integral surface membrane proteins. A preliminary account of some of these results has appeared (Jacob et al., 1985).

## Materials and Methods

### Antibody Preparation

Hybridoma cell lines were obtained by fusing myeloma cell lines with spleen cells from rats immunized with purified AChR protein. MAb 35 was raised against receptor from *Electrophorus* electric organ (Tzartos et al., 1981). It recognizes the MIR present on the extracellular portion of the α-subunit of muscle and electric organ AChR (Tzartos and Lindstrom, 1980; Tzartos et al., 1981, 1983). MAbs 43 and 140 were raised against AChR from *Electrophorus* and *Torpedo* electric organ, respectively (Tzartos et al., 1981; Gullick and Lindstrom, 1983). They are of the same antibody subclass as mAb 35 but of different specificity. MAb 140 recognizes the δ-subunit and mAb 43 is of unknown subunit specificity; neither mAb recognizes the MIR. MAb stocks were prepared by ammonium sulfate precipitation of hybridoma culture media. Horseradish peroxidase (HRP)-conjugated affinity-purified goat anti-rat IgG was prepared using a periodate oxidation conjugation method as described (Hurn and Chantler, 1980).

### Fluorescence Microscopy

Sections of embryonic chick ciliary ganglia were labeled by indirect immunofluorescence using a modification of the method of Swanson et al. (1983) and were examined by fluorescence microscopy. Ciliary ganglia from 16-d-old White Leghorn chick embryos were dissected and fixed by immersion in freshly prepared 0.1% paraformaldehyde in phosphate-buffered saline (PBS) for 0.5 h. The ganglia were rinsed three times for 5 min each with PBS containing 0.75% glycine (PBS-glycine), infused with 2.3 M sucrose in PBS as a cryoprotectant (Tokuyasu et al., 1984), and embedded in optimum cutting temperature compound in an embedding mold which was then frozen on dry ice and trimmed for sectioning. 10-μm sections were cut on a cryostat at −18°C and picked up on subbed glass slides (Rogers, 1973) at room temperature. The sections were air dried at room temperature for 30 min, rinsed three times for 5 min each with PBS-glycine, and incubated for 1 h at room temperature in a humidity chamber with ~150 μl of a solution containing 0.1 μM mAb in PBS. The sections were rinsed three times with PBS-glycine, incubated in 0.1 μM biotinylated rabbit anti-rat antibody in PBS for 0.5–1.0 h at room temperature, rinsed three times with PBS-glycine, incubated in 0.1 μM rhodamine-labeled avidin in PBS for 0.5–1.0 h at room temperature, and rinsed three times with PBS-glycine. The sections were then partially dried, mounted in glycerol, and examined with a Leitz Diavert fluorescence microscope as previously described (Ravdin and Berg, 1979).

### Electron Microscopy

Ciliary ganglia were lightly fixed and detergent-permeabilized to gain access to intracellular sites according to a modification of the method of Atsumi (1981). The ganglia were dissected from 16-d-old chick embryos and fixed by immersion for 1 h at room temperature in freshly prepared 0.75% paraformaldehyde in 0.02 M sodium phosphate buffer (pH 7.2) containing 0.06 M sucrose. The ganglia were rinsed three times for 15 min each in 0.05 M ammonium chloride in PBS to quench the unreacted aldehyde groups (Brown and Farquhar, 1984), and rinsed once in 0.02 M sodium phosphate buffer containing 0.06 M sucrose. The ganglia were permeabilized with 0.5% (wt/vol) saponin in PBS containing 10% (vol/vol) normal goat serum (PG) for 0.5 h, rinsed once in PG and incubated in 0.1 μM mAb in PG for 2.5 h at room temperature. The ganglia were rinsed three times for 10 min each in PG and incubated in 1 μM HRP-conjugated goat anti-rat antibody in PG for 2 h. The ganglia were then processed according to a modification of the method of Brown and Farquhar (1984). The ganglia were rinsed for 45 min with several changes of 0.1 M sodium cacodylate buffer (pH 7.4) containing 7.5% sucrose, fixed for 1 h in 1.6% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 5% sucrose, and rinsed several times during a 45-min period with sodium cacodylate buffer containing 7.5% sucrose, all at room temperature. The ganglia were stored overnight in this buffer at 4°C, and then rinsed three times for 15 min each with 0.05 M Tris-HCl buffer, (pH 7.4) containing 7.5% sucrose, incubated for 1.5 h in the dark in 0.05% 3,3'-diaminobenzidine in Tris-HCl buffer containing sucrose plus 0.03% hydrogen peroxide, and rinsed three times for 5 min each with Tris-HCl buffer containing sucrose. The ganglia were postfixed for 1 h at room temperature in 1% osmium tetroxide reduced with 1% potassium ferricyanide in 0.1 M sodium cacodylate buffer containing 5% sucrose. The ganglia were then briefly rinsed with distilled water, dehydrated in a graded series of alcohols, and embedded in Epon 812. Thin sections were stained lightly with methanolic uranyl acetate and examined with a Philips 300 electron microscope.

### Materials

Biotinylated rabbit anti-rat antibody and rhodamine-labeled avidin were purchased from Vector Laboratories (Burlingame, CA). Saponin, normal goat serum, 3,3'-diaminobenezidine, hydrogen peroxide, and HRP were purchased from Sigma Chemical Co., St. Louis, MO. OCT compound was purchased from American Scientific Products Div., American Hospital Supply Corp., McGaw Park, IL.

## Results

### Fluorescent Light Microscopic Analysis

The distribution of the putative AChR in ciliary ganglia was first examined at the light microscopic level using indirect immunofluorescence. Sections of ciliary ganglia from 16-d-old chick embryos were incubated with mAb 35, an mAb to the MIR of muscle, and electric organ AChR α-subunit. Embryonic ganglia at this stage of development were used because their chemical synaptic contacts are well differentiated (Landmesser and Pilar, 1972) and the contacts have previously been demonstrated to bind mAb 35 (Jacob et al., 1984). Binding of the mAb to the sections was detected by using a biotinylated rabbit anti-rat antibody followed by rhodamine-labeled avidin. The avidin-biotin system was ap-

plied to achieve an amplification of the fluorescent signal. The interiors of neuronal somas were intensely labeled except for, when visible, the nuclei (Fig. 1 *a*). Both choroid and ciliary neurons appeared to have large amounts of intracellular labeling. On some neurons, asymmetric labeling could be distinguished along the soma surface. Neither the processes extending from the cell bodies nor the small glial cells that encapsulate the neuron somas were labeled. Substitution of nonimmune serum at a comparable concentration of immunoglobulin for the mAb did not result in significant labeling of the neurons (Fig. 1 *b*).

### *Ultrastructural Distribution in Embryonic Ganglia*

Ultrastructural analysis of the intracellular labeling was carried out to identify the subcellular locations of putative AChR in the neurons. Ciliary ganglia from 16-d-old chick embryos were lightly fixed and permeabilized by saponin treatment to give the anti-MIR mAb access to intracellular sites. After labeling with mAb 35, the permeabilized ganglia were incubated with an HRP-labeled secondary antibody, reacted for peroxidase activity, and examined with the electron microscope. The pattern of mAb binding on the neuron surface in the lightly fixed, permeabilized ganglia resembled the distribution previously observed for anti-MIR mAb binding on neurons in freshly dissected, unfixed ciliary ganglia (Jacob et al., 1984). In both cases HRP reaction product was predominantly associated with the specialized synaptic membranes present both on the smooth portion of the cell

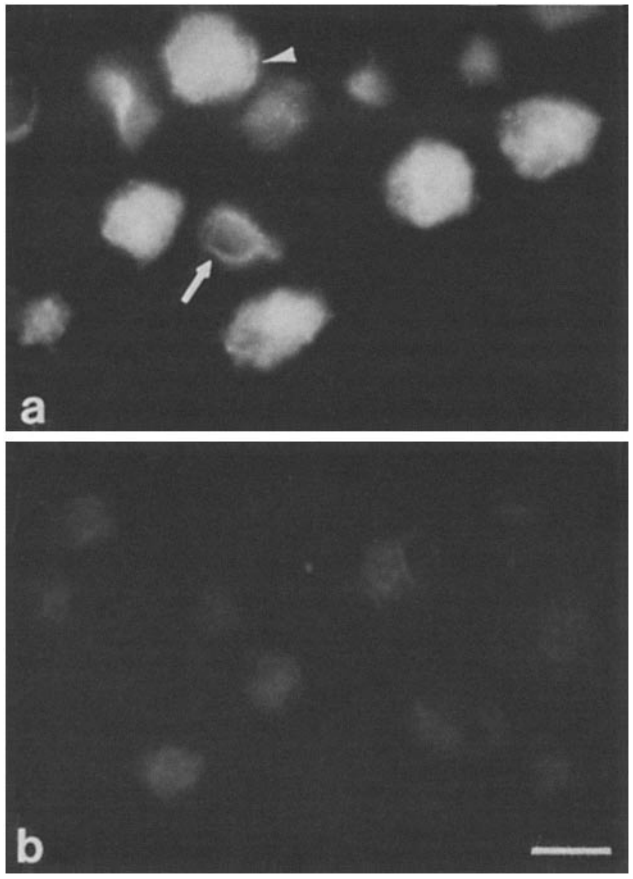


*Figure 1.* Embryonic ciliary ganglion sections labeled by indirect immunofluorescence for anti-MIR mAb binding. Sections from 16-d-old embryonic chick ciliary ganglia were incubated either with (*a*) mAb 35 or (*b*) nonimmune rat serum at an equivalent concentration of immunoglobulin, and then stained with biotinylated secondary antibody and rhodamine-labeled avidin to detect bound antibody, and examined by fluorescence microscopy. In *a* the interiors of the neuron somas are intensely labeled with the exception of the nuclei (*arrow*). Asymmetric labeling can be distinguished on the surface of some neuron somas (*arrowhead*). Neuronal processes are not labeled and neither are the small glial cells that encapsulate the neuron cell bodies. In *b* there is no significant fluorescent staining of the neurons. Bar, 30 μm.

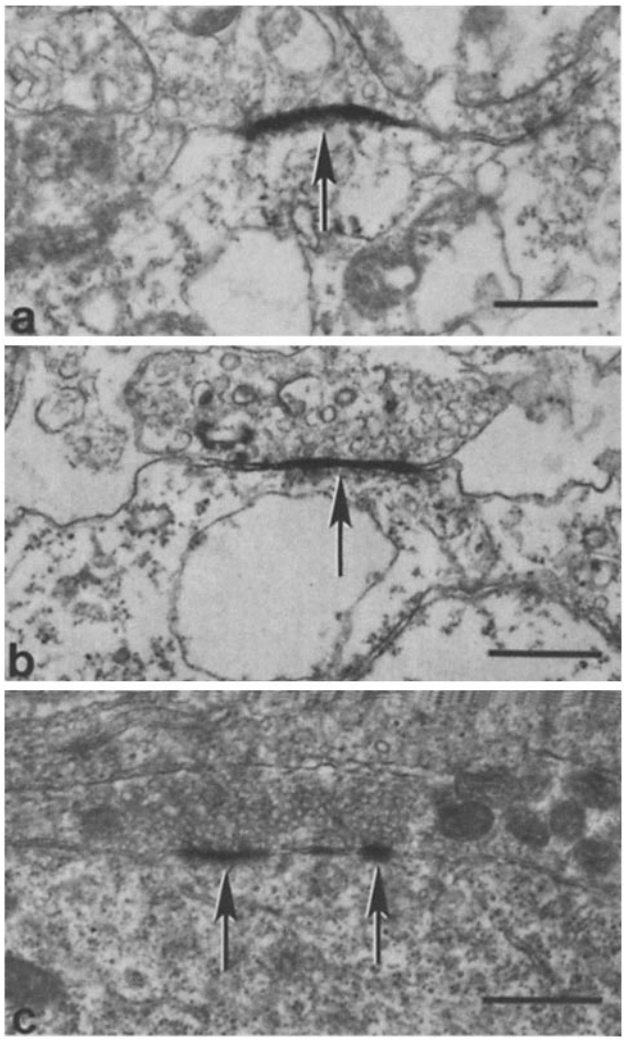


*Figure 2.* Ultrastructural distribution of anti-MIR mAb binding sites on the surface of permeabilized embryonic ciliary ganglion neurons. Ciliary ganglia from 16-d-old chick embryos were lightly fixed, saponin-permeabilized, incubated with mAb 35, labeled with HRP-conjugated secondary antibody, and stained for HRP reaction product to detect mAb. Dense deposits of HRP reaction product fill the clefts of synapses (*arrows*) present on the smooth portion of the neuronal somata (*a* and *b*). The label is restricted to the specialized synaptic zones (see text) in the areas of pre- and postganglionic cell apposition. For comparison, synaptic labeling (*arrows*) is demonstrated on a neuron (*c*) in a freshly dissected, unfixed ciliary ganglion after the binding of HRP-conjugated mAb 35 and HRP reaction. In fresh unfixed ganglia the ultrastructural characteristics of the pre- and postsynaptic cells are better preserved due to the absence of the membrane disruption which is caused by exposure to saponin in permeabilized ganglia. (*a–c*) Bars, 0.5 μm.

soma (Fig. 2) and on the short processes or pseudodendrites that extend from the postsynaptic cell in the region of preganglionic innervation. Synapses on ciliary ganglion neurons are characterized by a parallel arrangement and thickening of the pre- and postsynaptic membranes, a widened synaptic cleft, an enhanced postsynaptic density, and an accumulation of synaptic vesicles adjacent to the presynaptic membrane (De Lorenzo, 1960; Hess, 1965; Landmesser and Pilar, 1972). The label filled the clefts of the synapses but it did not extend beyond the specialized synaptic contact zones in the regions of pre- and postganglionic cell apposition. The restriction of the reaction product to the specialized synaptic sites was preserved in the permeabilized ganglia (Fig. 2, *a* and *b*) even in the presence of the membrane disruption caused by exposure to detergent (see Fig. 2 *c* for comparison). In both permeabilized and unfixed ganglia labeling was also observed on small portions of the surface membrane of the numerous pseudodendrites that emerge from the postsynaptic cell in the vicinity of the presynaptic terminals. HRP reaction product was not present elsewhere on the neuron surface membrane and was not associated with the surfaces of other cell types in the ganglion.

In addition to anti-MIR mAb binding sites on neuronal surfaces, there were substantial amounts of intracellular binding revealed in the permeabilized ganglia. Dense deposits of HRP reaction product were associated with specific organelles distributed throughout the neuronal cytoplasm (Fig. 3). The bulk of the intracellular labeling was present on the Nissl bodies or rough endoplasmic reticulum (RER) of the neurons where deposits of HRP reaction product lined the

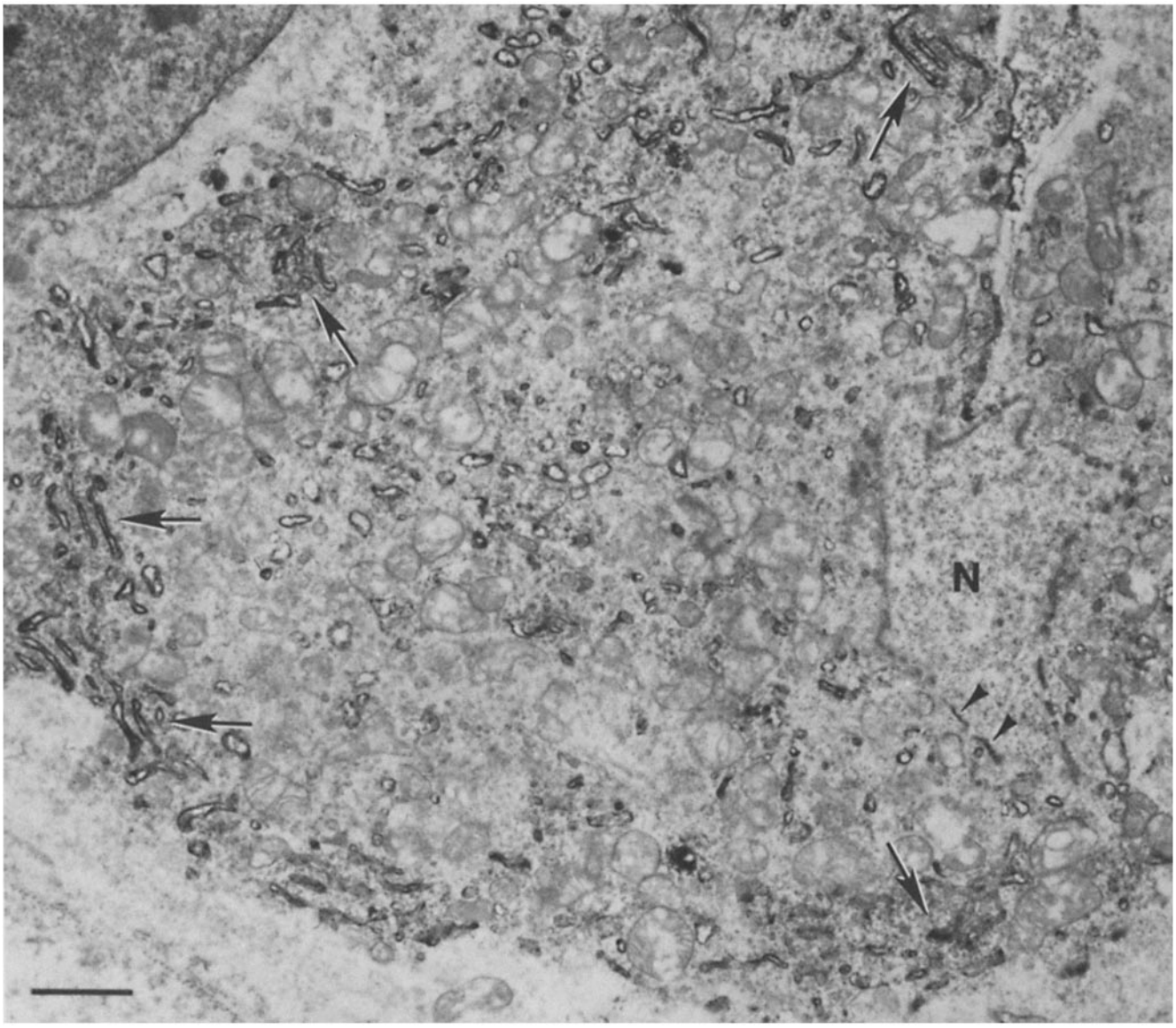


*Figure 3.* Ultrastructural distribution of intracellular anti-MIR mAb binding sites in embryonic ciliary ganglion neurons. Lightly fixed, saponin-permeabilized ciliary ganglia from 16-d-old embryos were incubated with mAb 35, followed by HRP-conjugated secondary antibody and HRP reaction. Label is associated with specific organelles distributed throughout the ciliary neuron soma. Most of the label is present on the Nissl substance or RER which is concentrated in a layer around the ciliary cell circumference (*arrows*). A few labeled individual cisternae of RER are scattered throughout the center of the cell. Small portions of the nuclear envelope (*arrowheads*) are also labeled. The nucleus itself (*N*) is not labeled and neither are the mitochondria. Bar, 1 μm.

ribosome-studded membranes of the RER cisternae (Fig. 3 and 4). In ciliary cells of 16-d-old chick embryos, distinct granules of Nissl substance often appear to be concentrated in a layer around the cell circumference and in a perinuclear ring. In the choroid cells individual cisternae of the Nissl substance and numerous ribosomes in polysomal arrays are densely scattered throughout the cytoplasm (Landmesser and Pilar, 1974; Pilar and Landmesser, 1976). In permeabilized ganglia the Nissl substance was observed in its characteristic cytological organization in both neuron types, and most of the individual cisternae in all of these distributions were labeled (e.g., Fig. 3).

Reaction product was also present on small portions of the nuclear envelope of some of the neuronal nuclei (Fig. 3–5). In neurons as well as in other cell types, the perinuclear cisterna is continuous in several places with the cisternae of the RER, and occasionally ribosomes are associated with the cytoplasmic surface of the nuclear envelope (Peters et al., 1976; Alberts et al., 1983). In ciliary ganglion neurons, a few ribosomes appear to be attached to or in close proximity to small portions of the outer membrane of the nuclear envelope of some nuclei (Fig. 5, *inset*). Labeled cisternae of RER were present in the vicinity of the labeled regions of the neuronal nuclear envelope.

Some of the Golgi complexes in the neurons were labeled. Deposits of HRP reaction product were present on individual cisterna and on the distended vacuolar portion of the Golgi complexes (Fig. 6). Label was also present on coated pits that were associated with the plasma membrane in the vicinity of labeled synapses and labeled pseudodendrite surface membrane (Fig. 7, *a* and *b*). A few of the coated vesicles in the neuron somas were filled with reaction product. Some of the labeled coated vesicles were associated with membranous structures that can be tentatively identified as Golgi ele-

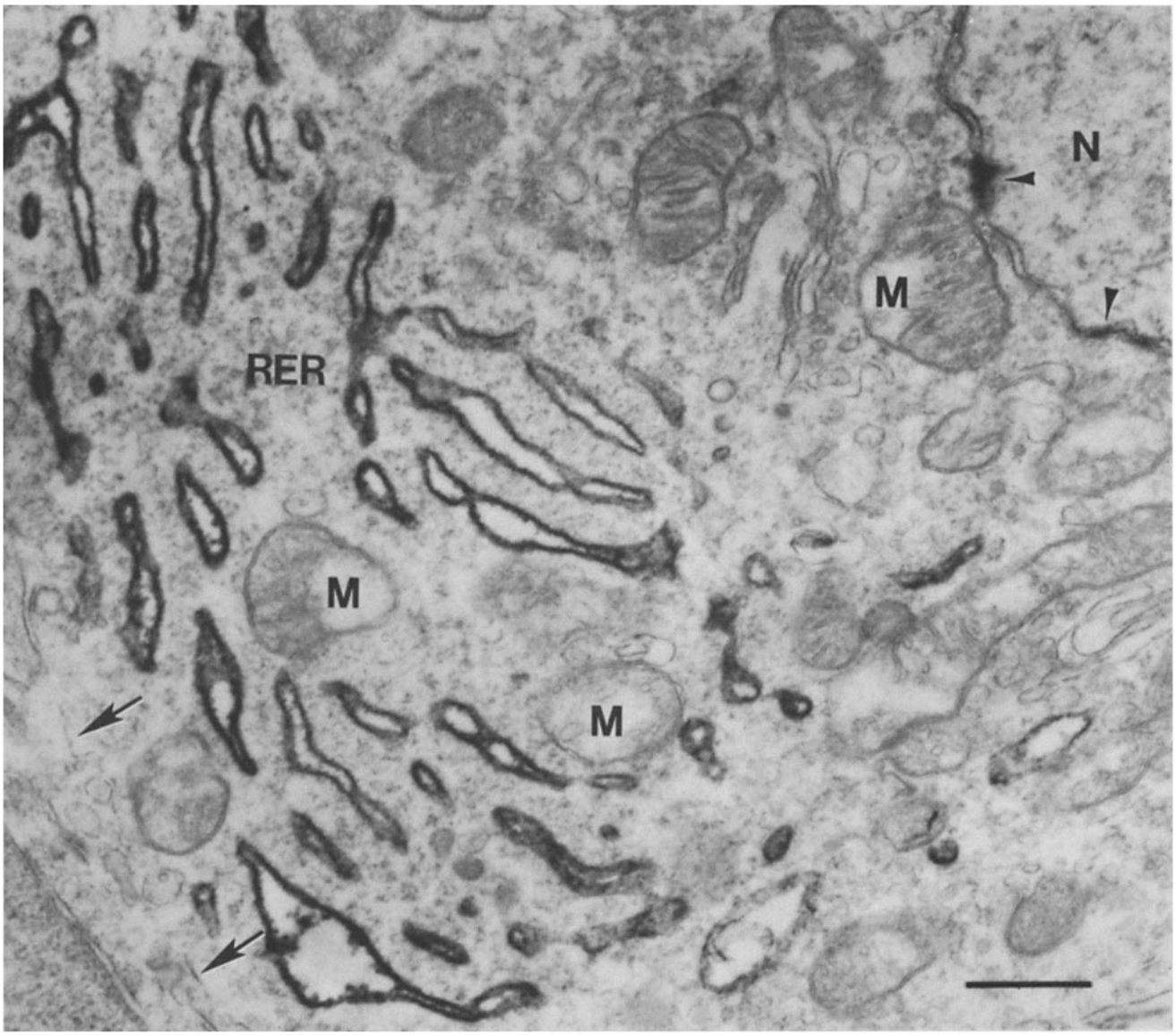


*Figure 4.* Anti-MIR mAb binding sites on the RER. Ganglia processed as described in Fig. 3 are shown at higher magnification to illustrate labeling associated with the RER of the neuron soma. HRP reaction product lines the ribosome-studded membranes of the RER cisternae. Small portions of the nuclear envelope (*arrowheads*) are also labeled. No label is associated with the nucleus (*N*), mitochondria (*M*), free ribosomes, polysomes, and the inner cytoplasmic leaflet of the neuron surface membrane (*arrows*). Bar, 0.5 μm.

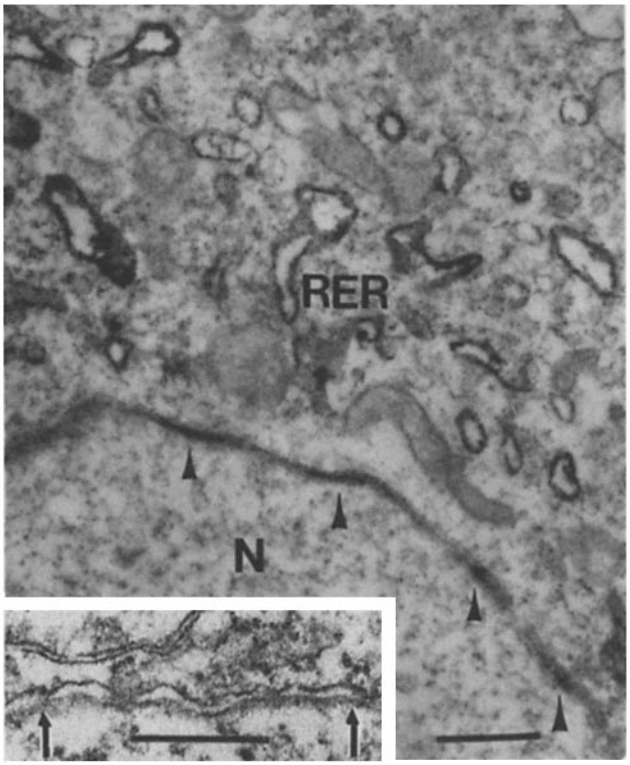


*Figure 5.* Anti-MIR mAb binding sites on the nuclear envelope. Ganglia processed as described in Fig. 3 are shown at higher magnification to illustrate HRP labeling associated with small portions of the nuclear envelope (*arrowheads*). Labeled cisternae of RER are present in the vicinity of the labeled portions of the nuclear envelope. The nucleus (*N*) is not labeled. The inset shows that occasionally a few ribosomes (*arrows*) appear to be attached to or in close proximity to small portions of the outer membrane of the nuclear envelope of the neurons. Bar, 1 μm. (*inset*: Bar, 0.5 μm).

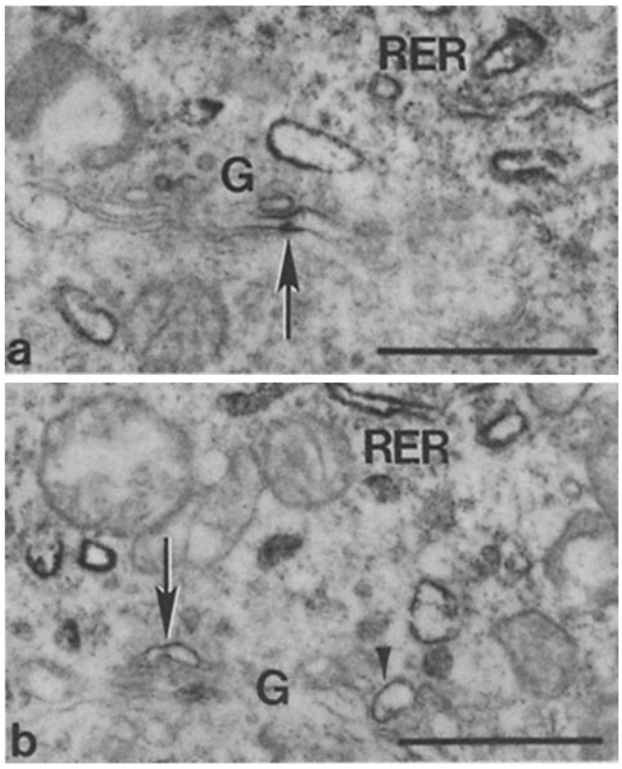


*Figure 6.* Anti-MIR mAb binding sites on Golgi complexes. Ganglia processed as described in Fig. 3 are shown at higher magnification to illustrate HRP reaction product on Golgi complexes (*G*). The label coats the smooth-surfaced membrane of individual cisterna (*arrows*, *a* and *b*) and distended vacuolar portions (*arrowhead, b*) of the Golgi complexes. Label is also present lining the ribosome-studded membranes of the RER cisternae. Mitochondria are not labeled. Bars, 1 μm.

ments (Fig. 7, *c* and *d*). The presence of a dense, fuzzy coat along the surface of one of the membranous structures and its association with numerous similarly coated vesicles suggests that this structure may be a *trans*-most Golgi cisterna (Griffiths et al., 1981, 1985). Both the cisterna and some of the associated coated vesicles were labeled (Fig. 7 *c*). However, most of the coated vesicles, particularly those present in Golgi regions, were not labeled. HRP reaction product was also present on some of the vesicular components of some multivesicular bodies (Fig. 7, *e* and *f*) and on occasional smooth-membraned vacuoles.

No labeling was present in the nucleus, and none was associated with mitochondria, cytoskeletal elements, free ribosomes, polysomes, or the inner cytoplasmic leaflet of the surface membrane. No labeling was present in nonneuronal cells in the ganglion.

The surface and intracellular labeling of neurons represented specific binding of the anti-MIR mAb. Substitution of nonimmune serum or different mAbs of the same immunoglobulin subclass as mAb 35 at an equivalent concentration of immunoglobulin for the anti-MIR mAb failed to generate labeling in ciliary ganglion neurons (Fig. 8). No labeling of chick dorsal root ganglion neurons was obtained when dorsal root ganglia were processed by the same experimental protocol that results in substantial surface and intracellular labeling of ciliary ganglion neurons (Fig. 9).

## *Discussion*

In chick ciliary ganglion neurons anti-MIR mAbs recognize a putative nicotinic AChR. Ultrastructural studies of fresh unfixed ganglia (Jacob et al., 1984) and of the permeabilized ganglia shown here reveal the surface distribution of the putative AChR to be predominantly restricted to the specialized synaptic membrane. Both indirect immunofluorescent staining of ciliary ganglion sections and ultrastructural studies on permeabilized ganglia indicate that the neurons also have substantial numbers of intracellular binding sites for the anti-MIR mAb. Analysis of the mAb binding site distribution in permeabilized embryonic ciliary ganglia at the ultrastructural level demonstrates that the intracellular binding is associated with organelles known to function in the biosynthesis, intracellular processing, and transport of integral plasma membrane proteins. Labeled organelles included most of the RER, small portions of the nuclear envelope, some Golgi complexes, and a few coated pits, coated vesicles, multivesicular bodies, and smooth-membraned vacuoles. The intracellular binding is clearly specific since (*a*) it occurs only with mAbs of the appropriate specificity, (*b*) the distribution of intracellular binding is restricted and reproducible, (*c*) the predominantly synaptic location of surface binding is preserved with the techniques used here to reveal intracellular binding, and (*d*) the intracellular and surface binding is seen only in neurons known to have AChRs.

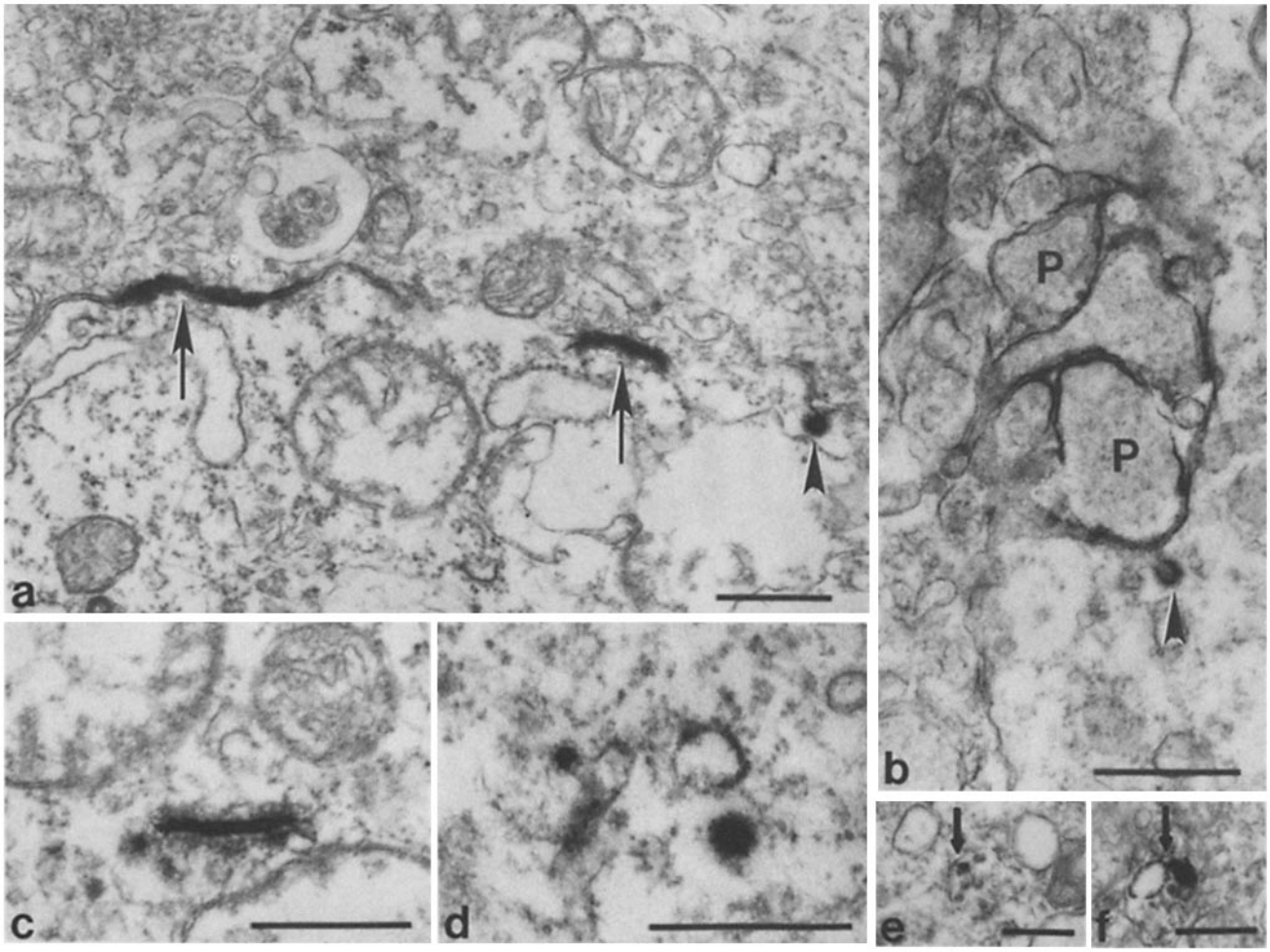


*Figure 7.* Anti-MIR mAb binding sites in coated pits, coated vesicles, and multivesicular bodies. Ganglia processed as described in Fig. 3 are shown at higher magnification to illustrate labeling of coated pits, coated vesicles, and multivesicular bodies. Labeled coated pits (*arrowheads*) are associated with the plasma membrane in the vicinity of labeled synapses (*arrows, a*) and labeled pseudodendrite (*P*) surface membrane (*b*). A few labeled coated vesicles are present in the neuron somas (*c* and *d*). Some of the labeled coated vesicles are associated with membranous elements. A dense fuzzy coat lines the outer surface of a thin membranous element and its numerous associated coated vesicles (*c*). Both the membranous cisterna and some of the associated coated vesicles are labeled. Labeled coated vesicles are also associated with an unlabeled, smooth-membraned cisterna (*d*). One reactive coated vesicle appears to be in continuity with the cisterna. In the neuron somas, the vesicular components (*small arrows*) of some multivesicular bodies are also labeled (*e* and *f*). Bars: (*a–d*) 0.5 μm; (*e* and *f*) 0.3 μm.

Substantial amounts of intracellular binding for anti-MIR mAbs have also been observed in chick lateral spiriform nucleus (Swanson et al., 1983) and rat hypothalamic magnocellular neurons (Mason, 1985) following indirect immunocytochemical labeling.

The anatomical approaches used to identify intracellular binding of anti-MIR mAbs in neurons do not permit a quantitative estimate of the proportion of sites that is intracellular. Studies on embryonic ciliary ganglion neurons maintained for 1 wk in dissociated cell culture, however, indicate that about two-thirds of the total anti-MIR mAb binding sites associated with the neurons are inaccessible to antibody until the cells are permeabilized, implying that the majority of sites is internal to the plasma membrane (Stollberg, 1985; Stollberg, J., and D. Berg, unpublished observations). The relative amounts of surface and internal HRP staining seen in labeled, permeabilized embryonic ciliary ganglion neurons are consistent with a similar proportion of anti-MIR mAb binding sites being intracellular in vivo.

Preliminary biochemical characterization suggests that a substantial portion of the internal sites are associated with components similar to those on the neuronal surface. Scatchard analyses of anti-MIR mAb binding in detergent extracts of embryonic ciliary ganglia, which includes both surface and internal components, reveal a single class of high affinity binding sites. All of the binding sites in ganglion homogenates are associated with integral membrane proteins that can be solubilized in Triton X-100, and most of the solubilized component adsorbs to DEAE cellulose at neutral pH, binds to concanavalin A, and sediments in sucrose gradients predominantly as a species of ~10 S (Smith et al., 1985). Some of the intracellular binding component, perhaps the portion sedimenting slower than 10 S (Smith et al., 1985), may represent unassembled precursor subunits.

Similar results have been reported for AChR in muscle. As much as half of the total AChR associated with rat skeletal myotubes in cell culture or with young adult rat muscle in vivo appears to be intracellular (Pestronk, 1985). Intracellu-

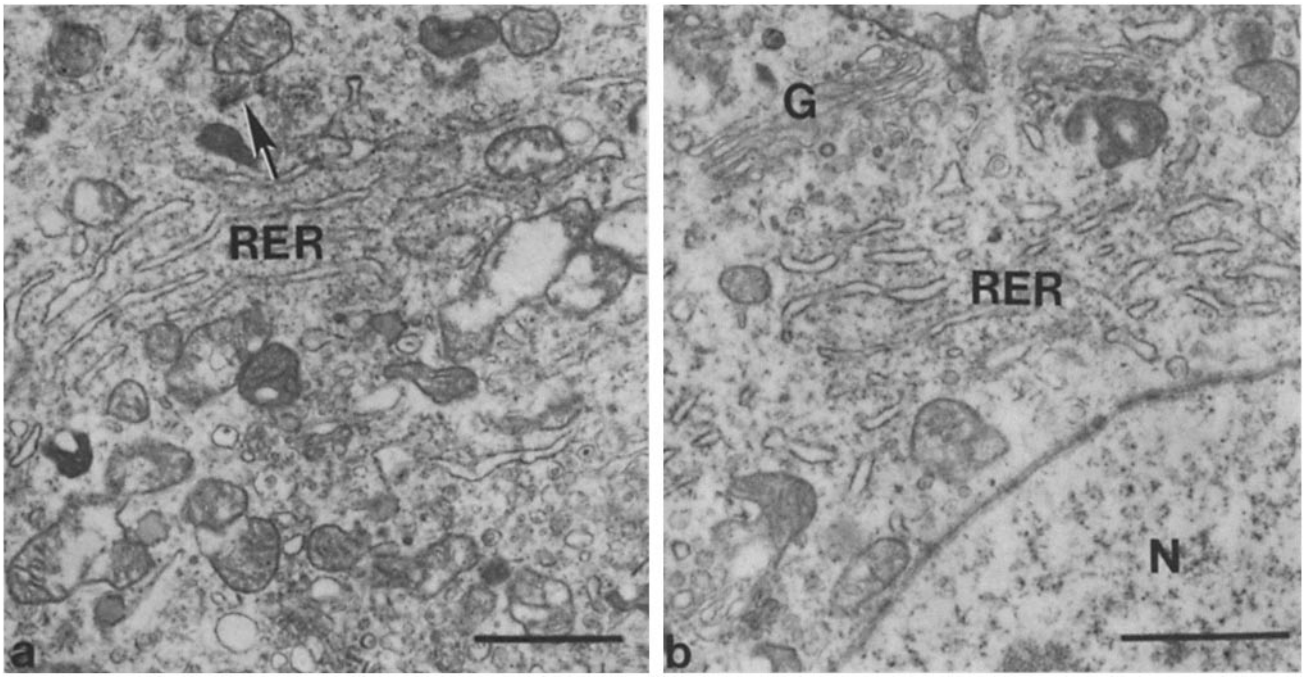


*Figure 8.* Specificity of labeling with anti-MIR mAbs. No significant labeling is present on the surface or in the interiors of neuron somas when ganglia are processed as described in Fig. 3 with mAb 35 being replaced by either nonimmune serum (*a*) or mAb 43 (*b*), a mAb of the same immunoglobulin subclass as mAb 35 but different specificity. Indicated structures include a synapse (*arrow*), RER, Golgi complex (*G*), and nucleus (*N*). Bars, 1 μm.

lar AChR has also been identified in embryonic chick skeletal muscle in situ and in cell culture. Ultrastructural studies on lightly fixed, saponin-permeabilized chick myotubes indicate that the intracellular AChR is associated with Golgi complexes, multivesicular bodies, perinuclear membranous elements, coated vesicles, and smooth-membraned and small vesicles (Fambrough and Devreotes, 1978; Atsumi, 1981; Bursztajn and Fischbach, 1984). That little RER labeling has been reported for chick myotubes probably reflects the fact that little RER can be found in the myotubes (Fambrough and Devreotes, 1978; Atsumi, 1981). RER would be expected to contain AChR since cell-free mRNA translation studies have demonstrated that the individual subunits of AChR from *Torpedo* electric organ and the muscle cell line BC3H-1 are synthesized on polyribosomes and cotranslationally inserted into the membrane of the RER via a signal sequence–signal recognition particle interaction (Anderson and Blobel, 1981; Merlie et al., 1981; Anderson et al., 1982; Sebbane et al., 1983). Recently, precursor forms of AChRs lacking certain subunits have been identified in rat skeletal myotubes in culture (Carlin et al., 1986).

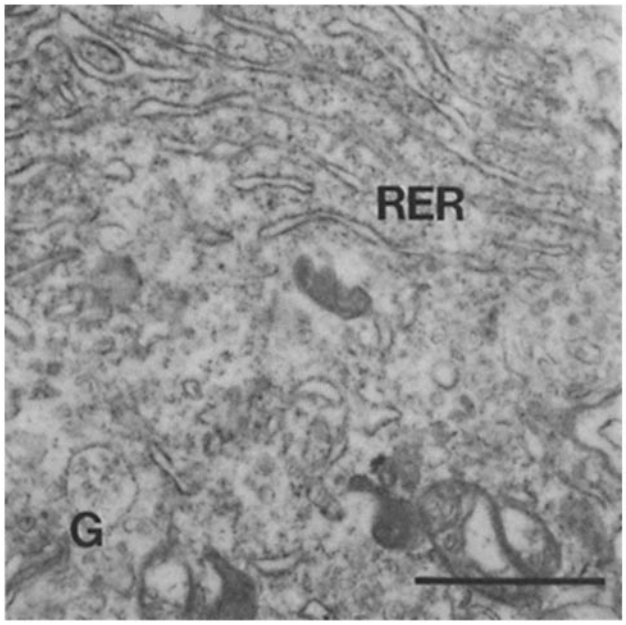


*Figure 9.* Lack of anti-MIR mAb binding sites in dorsal root ganglion neurons. Dorsal root ganglia from 16-d-old chick embryos were lightly fixed, saponin-permeabilized, incubated with mAb 35 followed by HRP-conjugated secondary antibody and HRP reaction as described for ciliary ganglia in Fig. 3. No specific labeling is apparent in the ganglion. Indicated structures include the RER and Golgi complex (*G*). Bar, 1 μm.

The implication that putative intracellular AChR, represented by anti-MIR mAb binding sites in chick ciliary ganglion neurons, is necessarily destined for expression on the cell surface, however, is not supported by experiments in cell culture where it appears that only a small proportion of the total intracellular component is actually inserted into the surface membrane (Stollberg, 1985; Stollberg and Berg, unpublished studies). Some of the intracellular sites, e.g., those associated with a few of the coated pits, coated vesicles, and multivesicular bodies, may represent endocytosis and turnover of the surface membrane component. When intact, freshly dissected ciliary ganglia are stained for anti-MIR mAb binding sites with an HRP-conjugated mAb, specific labeling is found in some coated pits in the vicinity of labeled synaptic or pseudodendritic surface membrane, in the vesicular components of some multivesicular bodies, and in a few

smooth-membraned and coated vesicles (Jacob et al., 1984). Surface AChRs on skeletal myotubes and BC3H-1 cells in culture are also thought to be internalized possibly via coated vesicles and degraded in secondary lysosomes (Fambrough et al., 1978; Libby et al., 1980; Hyman and Froehner, 1983).

Many of the intracellular anti-MIR mAb binding sites in ciliary ganglion neurons, however, are likely to represent neither AChR in transit to the surface nor AChR being internalized from the surface. Chick and rat myotubes and BC3H-1 cells in culture have been shown to have pools of AChR or individual subunits that are synthesized and stored or degraded intracellularly without being transported to the cell surface (Devreotes et al., 1977; Fambrough and Devreotes, 1978; Merlie and Lindstrom, 1983; Merlie, 1984; Pestronk, 1985). Similarly, about two-thirds of the total voltage-dependent sodium channel α-subunits that can be identified in developing rat brain cells appear to be intracellular (Schmidt et al., 1985). In view of this precedence for substantial internal pools of plasma membrane components, the findings reported here of intracellular anti-MIR mAb binding sites in neurons are not inconsistent with their representing AChRs. Substantial internal pools of assembled or partially assembled multisubunit molecules may be a general pattern for plasma membrane proteins in developing cells. The biological significance of intracellular receptor that fails to be inserted into the surface membrane, however, remains obscure. Some of the intracellular component may represent the overproduction of individual subunits that either fail to assemble properly or form "abortive" multisubunit complexes in some respect. Alternatively, the internal sites may represent a reserve of receptor for developmental contingencies.

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