Chapter 7

Subcellular and Synaptic Localization of Muscarinic Receptors in Neurons Using High-Resolution Electron Microscopic Preembedding Immunogold Technique

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Abstract

The function of a G protein-coupled receptor in the modulation of neuronal activity is highly dependent on its availability on the cell surface, on its distribution among different subcellular compartments and in relationship with the presynaptic afferents. Therefore, investigation of the precise localization of GPCRs is required to clarify their contribution to neuronal function, and can be achieved only by immunoelectron microscopy. Here, we describe the high-resolution electron microscopic preembedding immunogold technique that we have developed to analyze the subcellular and synaptic distribution of two acetylcholine muscarinic receptors (MR), M_2 and M_4 MRs in neurons in vivo. We have shown that M_2MR and M_4MR are mostly located at the plasma membrane where they are in a right position to interact with acetylcholine to modulate neuronal function. The synaptic and extrasynaptic localization of M_2MR suggests that the effect of acetylcholine might be mediated through a synaptic as well as diffuse type of transmission. The demonstration that M_2MR are present at the postsynaptic membrane beneath glutamatergic terminals provides a direct argument in favor of a co-release of ACh and glutamate. Finally, we have shown that muscarinic receptors are subject to an intraneuronal trafficking when they are stimulated and that this trafficking is different according to the duration of the stimulation (acute versus chronic).

Key words M_2 muscarinic receptor, M_4 muscarinic receptor, Acetylcholine, Immunogold, Synapse, Subcellular localization, Confocal microscopy, Electron microscopy

1 Introduction

Neuronal functions are determined by the highly precise arrangements of presynaptic and postsynaptic elements. G protein-coupled receptor (GPCRs) including MRs, are located to specific presynaptic or postsynaptic sites. The location of MRs within different neuronal compartments has a variety of functional implications, i.e., neurotransmitter release regulation at axonal level [1] or modulation of neuronal excitability at somatodendritic level [2]. Therefore, the precise localization and density of GPCRs on the cell surface seems to be a critical factor for specificity of signaling within and between

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neurons, which can be determined only using high-resolution morphological approaches.

The development of morphological approaches at high-resolution provides a wonderful tool to locate the sites of acetylcholine action, i.e., acetylcholine receptors in normal neurochemical environment and the redistribution of these sites when the cholinergic tone is modified. Especially, this procedure allowed us to identify the localization with a high resolution of two muscarinic receptors, M_2MR and M_4MR and to analyze their trafficking in different subcellular and synaptic neuronal compartments.

We will describe in this chapter how to prepare samples to analyze subcellular and synaptic localization of muscarinic receptors and more generally membrane bound proteins.

2 Materials

2.1 Buffers and Solutions

0.2 M Phosphate Buffer (PB), pH 7.4.

Solution A: 0.2 M Na_2HPO_4-$2H_2O$ (35.6 g/l in distilled water)	800 ml
Solution B: 0.2 M NaH_2PO4-$2H_2O$ (31.2 g/l in distilled water)	200 ml

Adjust at pH = 7.4.
Store at room temperature for up to 2 months.

2.1.1 Phosphate Buffered Saline (PBS)

0.2 M PB	50 ml
NaCl	8.76 g
KCl	0.2 g
Distilled water	up to 1 l

Store at room temperature for up to 2 months.

2.1.2 2 % Paraformaldehyde

Hot distilled water (60 °C, not more)	500 ml
Add paraformaldehyde (Ref: 145.004005.60, Merck)	20 g

Stir until the solution is clear.

Add PB 0.2 M, pH 7.4	500 ml

Store at 4 °C for 1 week.

2.1.3 2 % Paraformaldehyde + 0.2 % Glutaraldehyde

2 % paraformaldehyde	1 l
25 % glutaraldehyde	8 ml

(Glutaraldehyde 25 % for electron microscopy, Ref: 49626, Fluka).
Stir and use immediately.

2.2 Cryoprotectant

0.2 M PB pH = 7.4	25 ml
Glycerol (Ref: 24397296, Prolabo)	10 ml
Sucrose (Ref: S7903, Sigma)	25 g
Distilled water	75 ml

Stir to dissolve and store at 4 °C for 1 week.

2.2.1 0.1 M Sodium Acetate Buffer pH = 7

Sodium acetate trihydrate (Ref: S8625, Sigma)	13.6 g
Distilled water	1 l

Adjust at pH = 7 with acetic acid.
Store at 4 °C for up to 1 month.

2.2.2 Tris Buffer

Tris Base (Ref: T1503; Sigma)	6 g
Distilled water	1 l

Adjust at pH = 7.6 with HCl.
Store at room temperature for up to 2 months.

2.3 Pioloform (for Support Film on EM Grids)

Pioloform powder (Ref: R1275; Agar Scientific)	1 g
Chloroform	100 ml

Stir to dissolve.
Store at 4 °C for up to 2 months.

2.4 Resin Durcupan

Single component A (M epoxy resin; Sigma 44611; Sigma-Aldrich)	10 g
Single component B (hardener 964; Sigma 44612; Sigma-Aldrich)	10 g
Single component C (accelerator 960; Sigma 44613; Sigma-Aldrich)	0.3 ml
Single component D (Sigma 44614; Sigma-Aldrich)	0.2 ml

In a fume hood, combine all components in a disposable beaker. Stir until well mixed. Pour in small weighing cup and store until use.

2.5 Materials

- Perfusion stand.
- Vibrating microtome.
- 6 or 12-well plates.
- Mesh baskets fitting with the 6 or 12-well plates.

- Thin paint brush.
- EM copper grids one 2 mm × 1 mm slot (Ref: G2010-Cu; Electron Microscopy Science, Hatfield, USA).
- Thin tweezers.
- Ultramicrotome.
- Antibodies:
  - Anti-Muscarinic Acetylcholine Receptor m2 Antibody (rat), clone M2-2-B3 Ref: MAB367, Millipore.
  - Anti-Muscarinic Acetylcholine Receptor m4 Antibody (mouse), clone 17F10.2, Ref: MAB1576, Millipore.
- Nanogold® Conjugates, Nanoprobes, Yaphank, NY, USA.
  - Nanogold-Anti rat (m2R); Raised in goat, Ref: 2007: IgG molecule.
  - Nanogold-Anti mouse (m4R); Raised in goat, Ref: 2001: IgG molecule.
  - Nanogold-Streptavidin; Ref: 2016: Streptavidin.
- HQ Silver™ Enhancement Kit, Ref: 2012; Nanoprobes, Yaphank, NY, USA.

3 Sequence of Procedures for Detection of mAChRs

3.1 Common Steps for Detection of MR at Light and Electron Microscopic Levels

- Perfuse-fix the animal using a gravity system with 10 ml of NaCl (room temperature) and then with a cold 2 % PFA and 0.2 % glutaraldehyde solution (100 ml for 15 min).
- Remove the brain from the skull and post-fix it in 2 % PFA alone overnight at 4 °C.
- Rinse the brain and store it in PBS at 4 °C until use (may be stored for some weeks).
- Cut sections on a vibrating microtome at 50–70 μm. Use small mesh *baskets* in a 6-well plate and put all the sections from the brain area of interest in a same well. Alternatively, serial sections may be cut. The baskets will allow to easily perform the first steps of freeze–thaw and washes.
- Carry out a freeze–thaw procedure to enhance penetration of immunoreagents:
  - Before freezing, the sections are equilibrated in a cryoprotection solution for 15 min.
  - Then, excess of cryoprotectant is absorbed with a paper towel and the sections are dropped for 5 s into isopentane cooled either in liquid nitrogen or in dry ice.
  - The sections are thawed in the cryoprotectant and then rinsed in PBS 3 times for 5 min.

- Subject sections to immunohistochemistry of muscarinic receptors alone or muscarinic receptors and a marker of cholinergic or non-cholinergic terminals (cholinergic or non-cholinergic [3]) or other markers [4].
  - Blockade of the nonspecific binding with 4 % normal serum for 30 min.
  - Incubate with the primary antibody overnight at RT under shaking. When two targets are co-detected, incubate in a mix of two primary antibodies.

3.2 Detection of mAChRs at Light Microscopic Level

3.2.1 Incubation with the Secondary Fluorescent Antibody

When the receptor only is detected, incubate the secondary antibody coupled to a fluorochrome (Alexa 594 anti-rat for M_2 MR or Alexa 594 anti-mouse for M_4 MR; dilution 1:1000; Life Technologies) for 1 h. Alternatively, if the signal for the receptor is weak, it may be intensified using a biotinylated secondary antibody (dilution 1:200; Vector Laboratories) for 1 h and then a streptavidin coupled to a fluorochrome (Alexa 594 streptavidin; dilution 1:1000; Life Technologies) for 1 h. When two antibodies are used, incubate in a mix of two antibodies coupled to a fluorochrome (Alexa 594 anti-rat for M_2 MR or Alexa 594 anti-mouse for M_4MR; dilution 1:1000; Life Technologies and Alexa 488 anti-species; dilution 1:1000; Life Technologies) for 1 h. Finally, rinse the sections in PBS. Mount the sections in VECTASHIELD (Vector Laboratories, Burlingame, CA, USA).

3.2.2 Observation of the Sections at the Confocal Microscope

The sections were observed using a fully automated upright Leica TCS SP5 fluorescence microscope equipped with a 63x oil immersion lens (numerical aperture, 1.25) and with a Leica SP5 scanning system equipped a Ar white-light laser that allows choosing any excitation wavelength from 470 to 670 nm. We choose 488 nm and 543 nm wavelength to detect Alexa488 and Al594 (Leica Microsystems, Deerfield, IL; USA). Images were treated using ImageJ and Adobe Photoshop softwares.

3.3 Detection of mAChRs at Electron Microscopic Level

3.3.1 Detection of MR

- Incubate with the secondary fluorescent antibody coupled either to a nanogold particle (1.4 nm, Nanoprobes, Yaphank, NY, USA) or to biotin (dilution 1:200; Vector Laboratories). When the secondary antibody is coupled to biotin, incubate the sections with a streptavidin coupled to a nanogold particle (1.4 nm, Nanoprobes, Yaphank, NY, USA).
- Rinse in PBS and post-fix with 1 % glutaraldehyde for 10 min.
- Rinse in PBS and incubate in 0.1 M sodium acetate, pH = 7.0, until silver intensification.
- Increase the diameter of the nanogold particle with silver intensification (HQ Silver™ Enhancement Kit, Nanoprobes, Yaphank, NY, USA; one drop of red, one drop of white, one drop of blue). Incubate in the mix for 3 min.
- Post-fix sections for EM in 1 % osmium tetroxide.

- Dehydrate sections (50 %, 10 min; 70 % + 0.1 % uranyl acetate, 25 min; 95 %, 10 min; 100 %, two times 10 min; propylene oxide, 10 min) and flat-embed in resin between two microscope slides coated with Sigmacote (Sigma SL2, Sigma Aldrich).
- Make the resin polymerize in an oven (60 °C) for 2 days.
- Examine the sections in the light microscope, cut and glue the areas of interest, cut ultrathin sections, and examine in the electron microscope.
- Quantify mAChR localization if needed.

3.3.2 Co-detection of MR and Another Protein of Interest

- When another protein of interest is detected, the secondary antibody against the receptor primary antibody is coupled to a nanogold particle and the secondary Ab against this protein is coupled to biotin. Both antibody are co-incubated.
- After silver intensification of the immunogold signal, incubate the sections in streptavidin coupled to horse radish peroxidase (HRP).
- Reveal HRP with diamino benzidine (DAB).
- Wash in PBS.
- Post-fix sections for EM in osmium tetroxide 1 % diluted in distilled water.
- Dehydrate sections (see above) and flat-embed in resin between two microscope slides.
- Make the resin polymerize in an oven (60 °C) for 2 days.
- Examine the sections in the light microscope, cut and glue the areas of interest (no more than 1 mm^2) at the top of a blank piece of polymerized resin, cut semi-thin and ultrathin sections and examine in the electron microscope.
- Quantify MR localization if needed.

4 Results

4.1 Subcellular Localization of M_2 MR and M_4 MR in Normal Conditions

The M_2 MR and M_4 MR are located mostly at the membrane of cell bodies and dendrites, where they are in a right position to interact with acetylcholine to modulate neuronal excitability. (Figs. 1, 2, 3, and 5a). The M_2 MR is also found at the axonal

Fig. 1 (continued) the striatum of perikarya of control rats using preembedding immunogold method with silver intensification. Proportion of immunoparticles associated with different subcellular neuronal compartments. For each neuron, the number of immunoparticles associated with each compartment was counted, and the proportion in relation to the total number was calculated. The largest portion of immunoparticles are associated with the plasma membrane [1]. In the cytoplasm, the immunoparticles are detected in association primarily with small vesicles [2] and endoplasmic reticulum [5]. A small proportion of immunoparticles are associated with the Golgi apparatus [4] and multivesicular bodies [3]. Some immunoparticles are not seen in association with any identified compartment [6]. *b* bouton, *n* nucleus, *G* Golgi apparatus. From Bernard et al. [11]

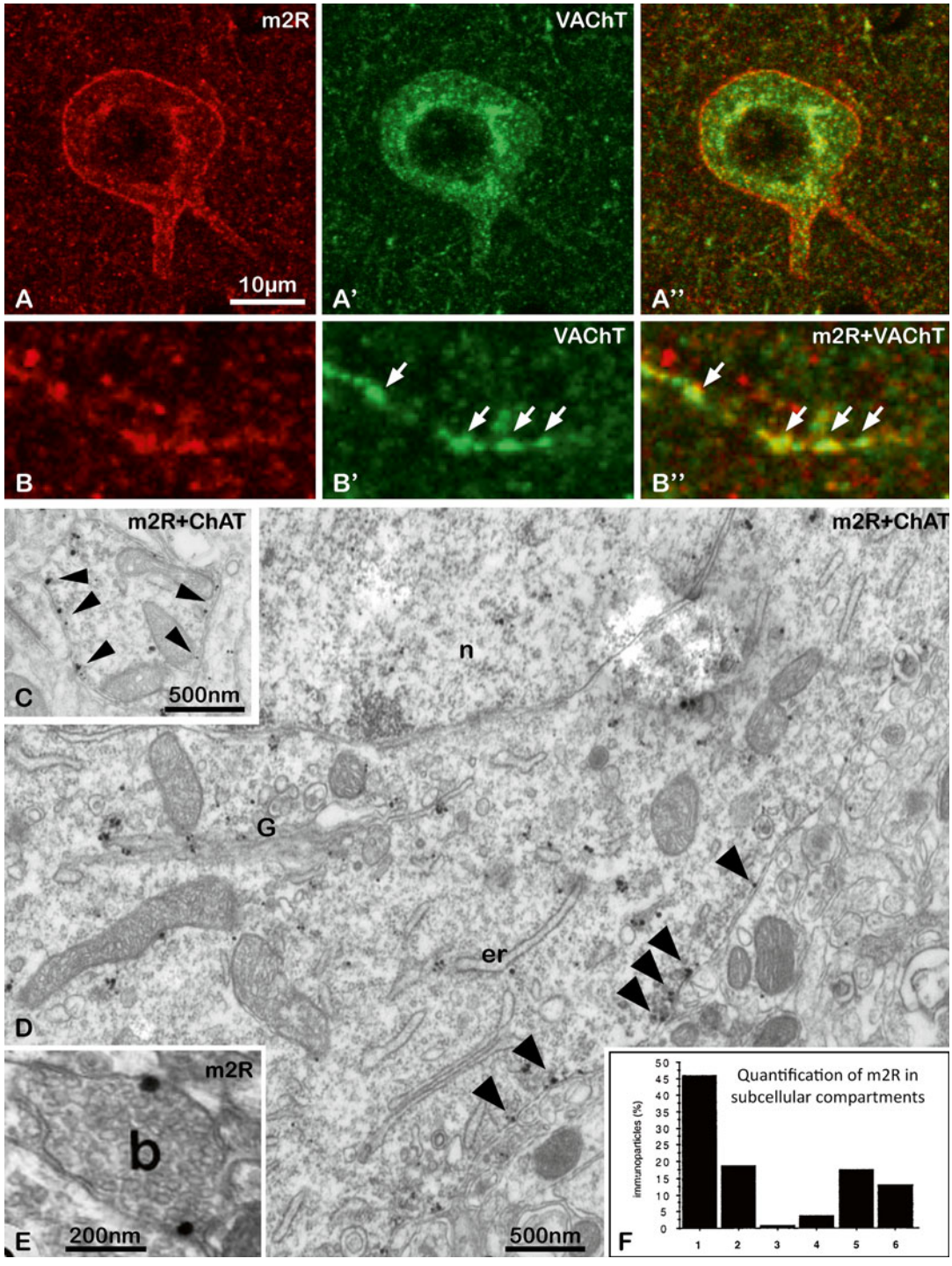

Fig. 1 Cellular and subcellular distribution of M_2 MR in the striatum of a normal animal. (**A**–**B**″) In a control mouse, the simultaneous detection of M_2 MR and VAChT immunoreactivities at confocal microscopic level shows that m2R is located at the plasma membrane of a cholinergic perikaryon (**A**–**B**″) and of cholinergic varicosities (**B**–**B**″). (**C**–**E**) Subcellular distribution of M_2 MR immunoreactivity in the striatum of rats using preembedding immunogold method with silver intensification. Detail of M_2 MR immunolabeling in the cytoplasm of cell bodies (**D**), a dendrite (**C**) and an axon (**E**). Numerous immunoparticles are associated with the plasma membrane (*arrow heads*) of the perikaryon, dendrite and axon. Some immunoparticles are associated with cytoplasmic compartments like endoplasmic reticulum (er) and Golgi apparatus (G). (**F**) Quantitative analysis of the subcellular distribution of M_2 MR in

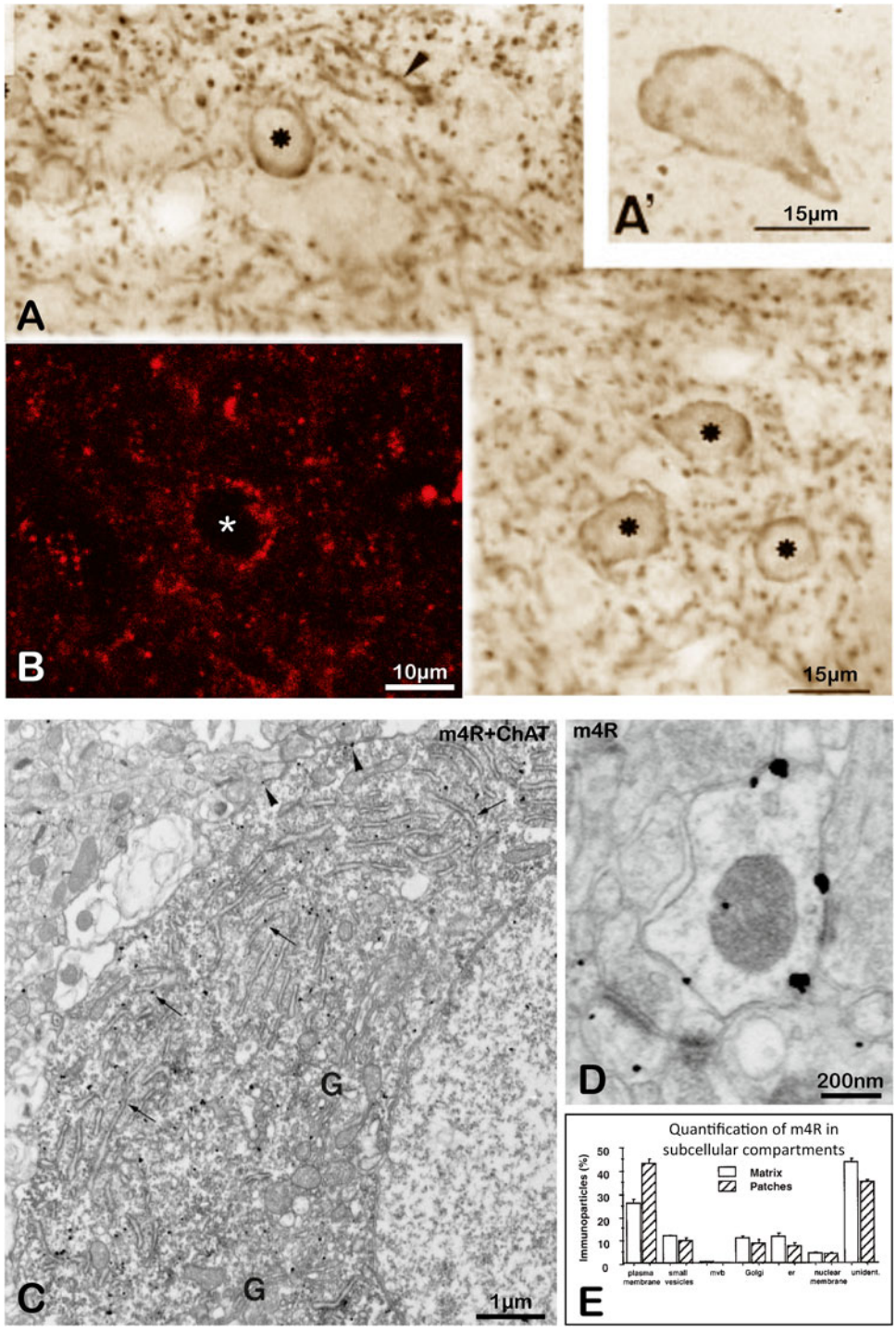

Fig. 2 Cellular and subcellular distribution of M_4 MR. Immunohistochemistry in striatal neurons in control animals (**A**, **A′**, **B**) using the immunoperoxidase (**A**, **A′**) and immunofluorescence (**B**) methods. The M_4 MR immunolabeling is detected at the membrane of some cell bodies of neurons often seen in clusters (*asterisks*). Immunolabeling for m4R is also seen in dendrites (**A**: *arrowheads*), but with reduced immunoreactivity. (**C**, **D**) Subcellular distribution of m4R immunoreactivity in the striatum of normal animals using the preembedding immunogold method with silver intensification. Detail of M_4 MR immunolabeling in the cytoplasm of a cell bodies (**C**) and a dendrite (**D**). Some immunoparticles are associated with the plasma membrane (*arrow heads*) of the perikaryon and dendrite. Immunoparticles are associated also with cytoplasmic compartments like endoplasmic reticulum (*arrows*) and Golgi apparatus (G). (**E**) Quantitative analysis of the subcellular distribution of

membrane where they are supposed to modulate acetylcholine release. Surprisingly, we were not able to detect M_4 MR at axonal varicosities, despite the fact that this receptor was clearly shown to be involved in acetylcholine release [5]. We cannot exclude that this was due to a lack of sensitivity of the immunogold technique.

4.2 Synaptic Localization of M_2 MR

The localization of the M_2 MR was analyzed in correlation with synapses by electron microscopic immunohistochemistry in the mouse trigeminal, facial, and hypoglossal motor nuclei (Fig. 3). In all nuclei, M_2 MR were localized at the membrane of motoneuronal perikarya and dendrites. The M_2 MR were concentrated at cholinergic synapses located on the perikarya and most proximal dendrites. However, M_2 MR at cholinergic synapses represented only a minority (<10 %) of surface M_2 MR. The M_2 MR were also found in abundance at glutamatergic synapses in both motoneuronal perikarya and dendrites. A relatively large proportion (20–30 %) of plasma membrane-associated M_2 MR were located at glutamatergic synapses.

The synaptic and extrasynaptic localization of M_2 MR suggests that the effect of acetylcholine might be mediated through a synaptic as well as diffuse type of transmission. The demonstration that M_2 MR are present at the postsynaptic membrane beneath glutamatergic terminals provides a direct argument in favor of a co-release of ACh and glutamate by the same terminal as suggested by different groups [6, 7].

4.3 Subcellular Redistribution of M_2 MR and M_4 MR After Acute and Chronic Activation

Our data show that muscarinic receptors are subject to an intraneuronal trafficking when they are activated and that this trafficking is different according to the duration of the stimulation (acute versus chronic). When MR are acutely stimulated, few M_2 MR immunoparticles are detected in association with the plasma membrane of the somatodendritic compartment (Figs. 4b, b′, e, e′ and 5c). In parallel, M_2 MR and M_4 MR immunoreactivity is seen in the cytoplasm in association with small endosome-like vesicles (Figs. 4b′, ev and 5c). Muscarinic receptors are thus endocytosed and then either recycled to the plasma membrane or degraded in lysosomes [8]. When ACh receptors are chronically stimulated like in acetylcholinesterase knockout mice (AChE−/−), trafficking of

Fig. 2 (continued) M_4 MR in the striatum of control rats using the pre-embedding immunogold method with silver intensification in two striatal regional compartments, matrix and patches. Proportion of immunoparticles associated with different subcellular neuronal compartments in perikarya of medium spiny neurons. For each cell body, the number of immunoparticles associated with each compartment was counted, and the proportion in relation to the total number was calculated. In medium spiny neurons, of the immunoparticles that are associated with an identified compartment, most of them are preferentially located at the plasma membrane. The proportion of immunoparticles at the membrane is much higher in patches than in matrix. In the cytoplasm, the immunoparticles are mostly detected in association with small vesicles, the Golgi apparatus, and the endoplasmic reticulum. A small proportion of immunoparticles are associated with multivesicular bodies (*mvb*) and the outer nuclear membrane. Some immunoparticles are not seen in association with any of an identified compartment. (**D**–**E**) Reproduced with permission, from Bernard et al. [4]

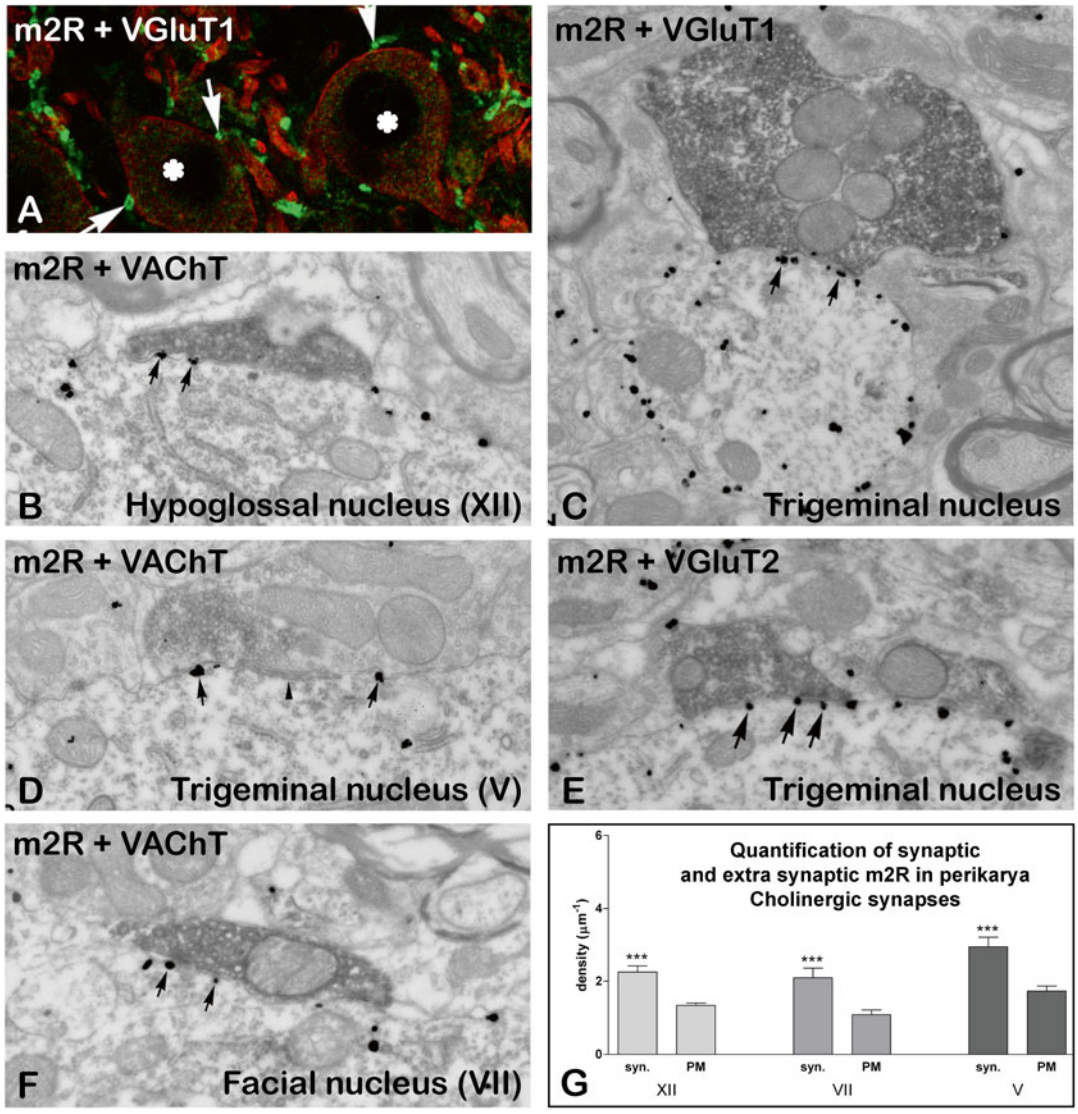

Fig. 3 Synaptic distribution of M_2 MR immunohistochemistry in brainstem motor nuclei in mouse. (**A**) Confocal microscopic illustration of the distribution of M_2 MR and the vesicular transporter of glutamate type 1 (VGLUT1) immunoreactivity in the trigeminal motor nucleus. The surface of motoneuron cell bodies (*asterisks*) is intensely M_2 MR-immunoreactive (*red*). VGLUT1 immunostaining is predominantly confined to large intensely stained varicosities (*green*). VGLUT1-immunoreactive varicosities form close contacts with M_2 MR-immunoreactive perikarya and dendrites (*arrows*). (**B**–**H**) Electron microscopic qualitative (**B**–**E**, **H**) and quantitative (**G**) analysis of the subcellular distribution of M_2 MR at cholinergic (**B**, **D**, **F**) and glutamatergic (**C**, **E**) synapses in hypoglossal nucleus (**B**), trigeminal motor nucleus (**C**–**E**) and facial nucleus (**F**) after preembedding immunogold method with silver intensification. The M_2 MR immunoparticles are detected at the plasma membrane, in association with its inner side. Some immunoparticles are found at the postsynaptic membrane (*arrows*) under VAChT (**B**, **D**, **F**) or VGluT1 (**C**) or vesicular transporter of glutamate type 2 (VGluT2) (**E**) immunoreactive presynaptic boutons (electron-dense peroxidase product). The VAChT-immunoreactive boutons form synapses on perikarya (**B**, **D**, **F**) and the VGluT1 and 2-immunoreactive boutons form synapses on dendrites. (**G**) Statistical analysis (nonparametric Wilcoxon matched pairs test) shows that the density of M_2 MR at VAChT-positive cholinergic synapses (syn.) is significantly higher than the overall density of receptors at the plasma membrane (PM) in motoneurons of the hypoglossal (XII), facial (VII) and trigeminal motor (V) nuclei (e). ***$P < 0.001$. From Csaba et al. [3]

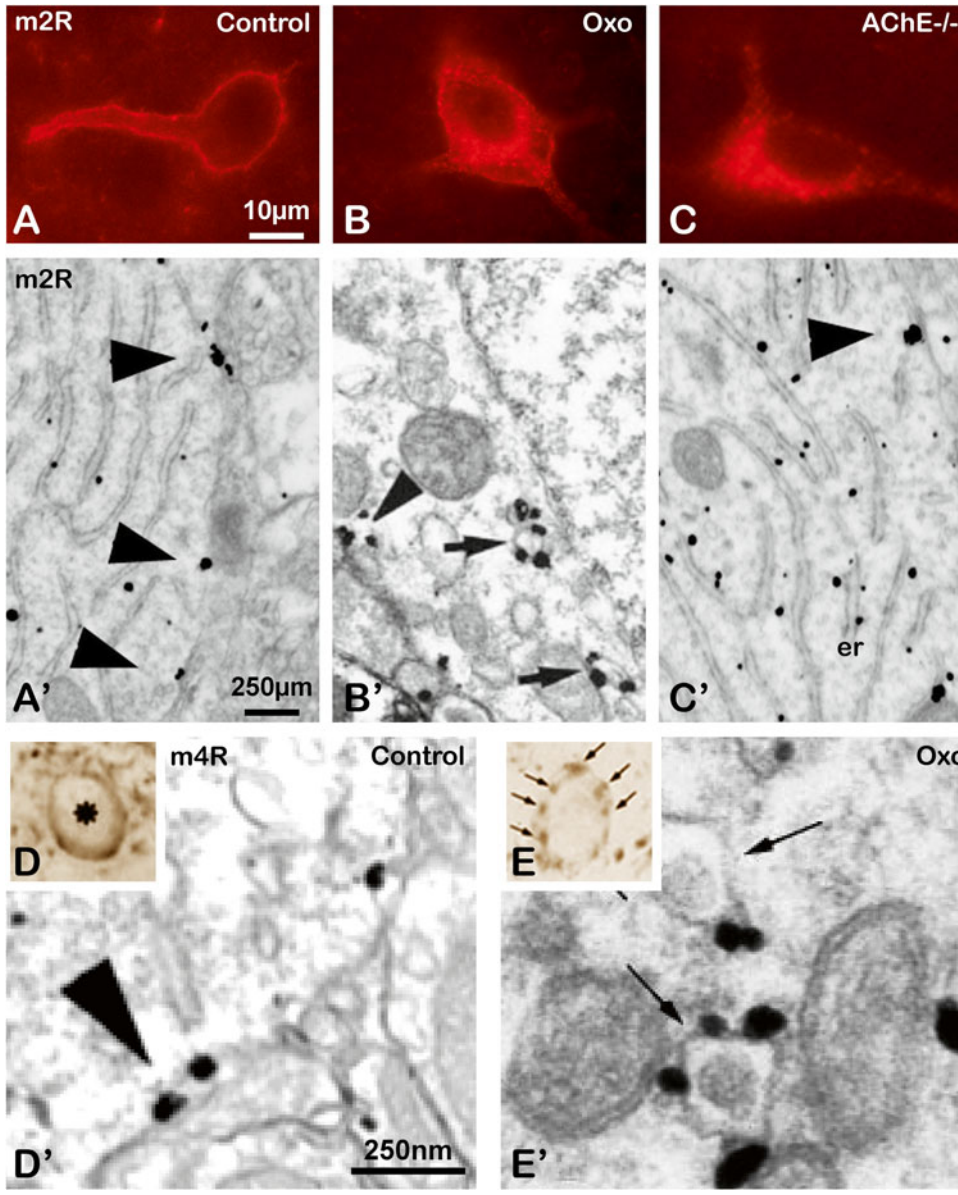

Fig. 4 Effect of acute and chronic modifications of ACh levels on the cellular and subcellular distribution of M_2 MR and M_4 MR in neurons of the striatum in vivo. Images were collected under epifluorescence (**A**–**C**), and electron microscopy (**A**′–**C**′, **D**′, **E**′) using immunofluorescent (**A**–**C**), and peroxidase (**D**, **E**) histochemistry and a pre-embedding immunogold method (**A**′–**C**′, **D**′, **E**′). (**A**, **A**′, **D**, **D**′) In control mice, M_2R and M_4R immunoreactivity are mostly detected at the plasma membrane. Immunoparticles are associated mostly with the internal side of the plasma membrane (*arrowheads*). (**B**, **B**′, **E**, **E**′) After acute treatment with oxotremorine ('Oxo'; 0.5 mg/kg subcutaneously for 1 h), M_2 MR and M_4 MR immunoreactivities are seen in the cytoplasm. (**C**, **C**′) After chronic stimulation of ACh receptors in acetylcholinesterase knockout mice (AChE–/–), no staining is observed at the membrane, whereas strong immunoreactivity is detected in the cytoplasm. Few immunoparticles are detected in association with the plasma membrane (*arrowheads*). By contrast, numerous particles are seen in the cytoplasm associated with the endoplasmic reticulum (er) and Golgi apparatus. This suggests that, when ACh receptors are chronically stimulated, targeting of M_2 receptors is blocked in the intraneuronal compartments of synthesis and maturation, and thus they are no longer targeted to the membrane. Scale bars, 10 mm in (**A**–**E**); 500 nm in (**F**, **G**); 50 nm in (**H**). Reproduced, with permission, from Bernard et al. [8]

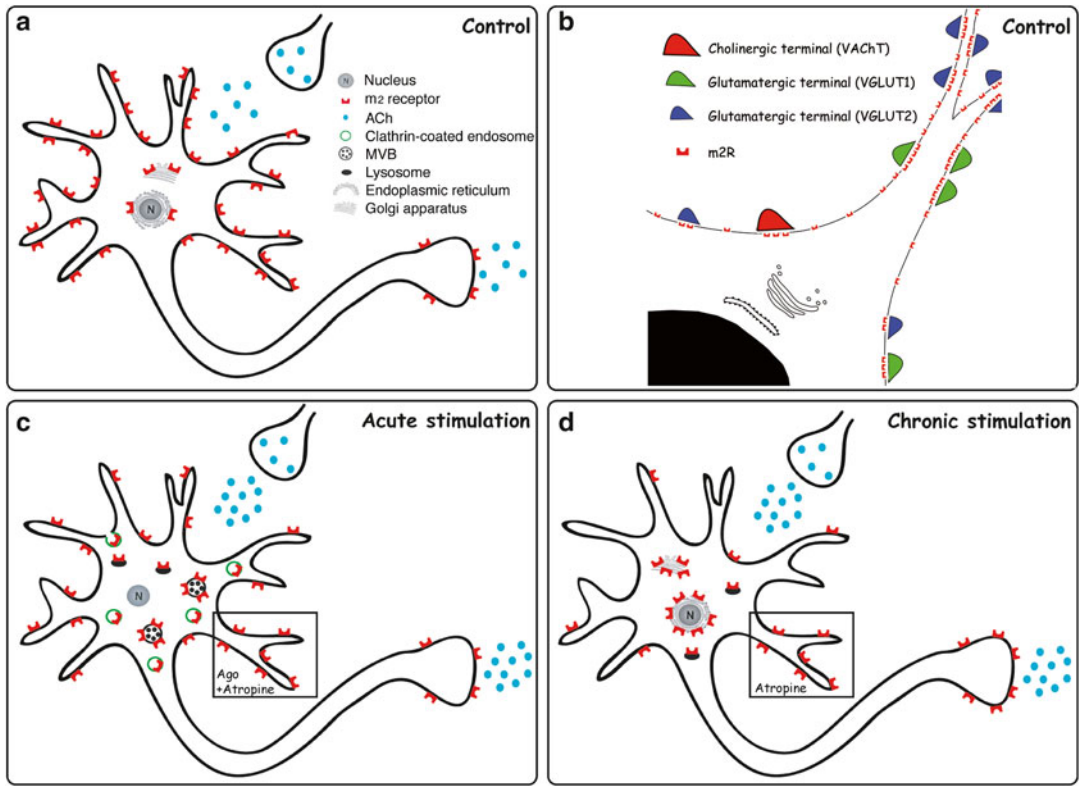

Fig. 5 Schematic representation of the subcellular and synaptic distribution of M_2 MR in neurons. (**a**) In control mice, most M_2 MR are at the plasma membrane of the somatodendritic compartment and at axonal varicosities. (**b**) In motoneurons in the brainstem, the M_2 MR are localized at the plasma membrane of perikarya and dendrites, at higher density in dendrites than in cell bodies. Cholinergic terminals (VAChT-immunopositive) form synapses on perikarya. VGLUT1-labeled glutamatergic terminals form synapses on perikarya and large-caliber dendrites, whereas small VGLUT2-labeled glutamatergic terminals form synapses on perikarya and small-caliber dendrites. The density of both VGLUT1- and VGLUT2-labeled synapses is higher in dendrites than in perikarya. Note the enrichment of M_2 MR at VAChT-labeled cholinergic as well as VGLUT1- and VGLUT2-labeled glutamatergic synapses. (**c**) After acute stimulation of cholinergic neurons, M_2 MR density decreases in the somatodendritic plasma membrane and M_2 MR accumulate in association with endosomes and multivesicular bodies (MVBs). The decrease in M_2 MR density at the membrane is blocked when the muscarinic-receptor antagonist atropine is injected. In cholinergic varicosities, localization of M_2 MR at the plasma membrane is similar to that in control animals. (**d**) After chronic stimulation of ACh receptors, M_2 MR are almost absent from the somatodendritic plasma membrane but accumulate in the cytoplasm in association with the endoplasmic reticulum and Golgi apparatus. The decrease in M_2 MR at the plasma membrane is blocked after atropine injection in a chronically stimulated animal. In cholinergic varicosities, M_2 MR density at the plasma membrane is increased. Intracellular GPCRs are those that have been retrieved from the plasma membrane (acute stimulation) or blocked on their way out to the membrane (chronic stimulation)

muscarinic receptors ends in the intraneuronal compartments of synthesis and maturation, and thus they are no longer targeted to the membrane of the somatodendritic compartment (Figs. 4c, c′ and 5d). Conversely, M_2 MR density increases at axonal varicosities (Fig. 5d).

5 Notes

5.1 Perfusion

5.1.1 Perfusion System

We use a gravity system to perfuse the animals. Though such a system may take longer time to achieve organ perfusion, the results are reproducible and perfusion is thorough. Gravity systems allow consistent pressure and controlled flow rates, providing good perfusion of the major organs, especially brain. Excessive pressure using other methods, like a pump, may cause artifacts in the neuronal ultrastructure.

5.1.2 Fixative

Glutaraldehyde is an essential compound of the fixative solution. The concentration of glutaraldehyde has to be carefully chosen. The percentage has to be high enough to preserve the ultrastructure of the tissue for analysis at EM level and low enough to avoid interference with antibody binding that may cause considerable decrease of intensity of labeling or even nonspecific binding. In our hands, 0.5–0.2 % of glutaraldehyde in addition to 2–4 % paraformaldehyde gave us good results on labeling intensity and ultrastructure preservation for the majority of primary antibodies, including anti-MR antibodies.

5.2 Tissue Permeabilization

The section freezing allows to produce small ice crystals that mechanically disrupt the tissue causing only limited damages and thus enhance the penetration of reagents. Detergents like Triton X-100 must not be used because of the irreversible damages they cause to neuronal ultrastructure.

5.3 Specificity of Primary Muscarinic Receptor Antibodies

We and others have tested different anti-muscarinic receptors antibodies by immunohistochemistry [9]. Only anti-M_2 MR and M_4 MR antibodies commercialized by Millipore (Ref: MAB367 and MAB1576) have been found to selectively label the receptors, i.e., the immunohistochemical M_2 MR and M_4 MR signals were abolished in the corresponding knock-out mice. Anti-M_2 MR antibodies bind rat and mouse M_2 MR. Surprisingly, if we found labeling with anti-M_4 MR antibodies in rats, we were able to detect M_4 MR only in one strain of mouse, e.g., control mice of M_2 KO mice from Taconic farm (Germantown, NY).

5.4 Adjustment of Primary Antibody Concentration

It is important to be aware that the immunogold technique is less sensitive compared to immunofluorescence or immunoperoxidase. Increase of the concentration of the antibody may be necessary to obtain a good signal.

5.5 Gold Coupled Secondary Antibodies or Streptavidin?

When possible, we prefer to use biotinylated secondary antibodies and streptavidin coupled to gold particles instead of secondary antibodies directly coupled to gold beads. First, it allows to use the same gold coupled compound in experiments detecting first

antibody produced in different species. Second, we found a more important variability in labeling obtained with secondary antibodies coupled to gold particles compared to the same labeling obtained with biotinylated secondary antibodies and streptavidin coupled to gold particles.

5.6 Coating Grids with Pioloform

Samples for transmission electron microscopy must be supported on a thin electron transparent film, to hold the specimen in place while in the objective lens of the TEM. We use pioloform as a support film. For the detailed procedure, see [10].

5.7 Ultrathin Sections Cutting Procedure

Semi-thin sections (1 μm thick) are first cut and observed under a light microscope. When the labeling correspond to the area of interest, ultrathin sections (5 nm thick) are cut. Since the reagent do not penetrate deeply into the tissue, ultrathin sections have to be cut at the surface of the tissue in the few first micrometers, as parallel as possible to the surface.

The use of single slot grids instead of mesh grids makes the observation more comfortable. It allows for example to analyze the labeling on a whole structure like a cell body and not be hindered by bars of the mesh grid.

5.8 Quantification

Counting immunoparticles at the ultrastructural level is important for comparing the abundance of receptor in each compartment in basal and experimental conditions. For that, sections from control or wild type animals and treated animals or KO mice must be processed for immunohistochemistry in the same time. The analysis are performed on EM at a final magnification of about 4000×, using the Metamorph software (Universal Imaging Corporation, Paris, France) [11] or Image-Pro Plus image analysis software (Media Cybernetics, Bethesda, MD) [3] on a personal computer. In cell bodies, the immunoparticles are identified and counted in association with different subcellular compartments: the plasma membrane, endosome-like vesicles, multivesicular bodies, the Golgi apparatus, endoplasmic reticulum, the nuclear membrane. Some immunoparticles are classified as associated with an unidentified compartment, because they were associated with either no detectable organelles or an organelle that could not be identified as one of the five previous ones. The results are expressed as:

1. The percentage of immunoparticles associated with the different subcellular compartments in normal animals.
2. The number of immunoparticles per membrane length (micrometers), cytoplasmic surface (square micrometers), multivesicular body, or Golgi apparatus in normal and treated rats (mice).

For the analysis of the localization of M_2 MR in relationship with the presynaptic terminals, immunoparticles are identified and

counted in association with the plasma membrane and at the postsynaptic membrane beneath the VAChT-, VGLUT1-, and VGLUT2-immunoreactive presynaptic boutons. The latter area included the synaptic complexes and also adjacent plasma membrane in apposition with the VAChT-, VGLUT1-, and VGLUT2-immunoreactive presynaptic boutons. Results are expressed as density of M2 MR at the postsynaptic membrane beneath VAChT-, VGLUT1-, and VGLUT2-labeled boutons and overall at the plasma membrane (number of immunoparticles per 1 μm membrane length). We assume that the number of immunoparticles is proportional to the absolute number of the receptor. The values from control and treated animals are compared using a suitable statistical test.

6 Conclusions

Despite the tricky aspect of the post-embedding immunohistochemistry, this chapter demonstrates the extraordinary power of the high-resolution electron microscopic immunohistochemistry to locate muscarinic receptors in the different neuronal compartments, including synapses and to analyze the trafficking of these receptors in neurons. These data give new keys to understand the mode of transmission of ACh through muscarinic receptors.

Such experimental approaches may be adapted to the analysis of other neurotransmitter receptors localization or other proteins in the brain or in other tissues and may help to better understand the link between localization of GPCRs and their intraneuronal trafficking and neuronal responses induced by GPCR activation. This might enable the development of new strategies for treating neurological diseases associated with altered GPCR signaling, such as Parkinson's disease.

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