Aging and subcellular localization of m2 muscarinic autoreceptor in basalocortical neurons in vivo

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Abstract

By using immunohistochemical approaches at the light and electron microscopic levels, we have shown that aging modifies the subcellular distribution of the m2 muscarinic autoreceptor (m2R) differentially at somato-dendritic postsynaptic sites and at axonal presynaptic sites in cholinergic basalocortical neurons, in vivo. In cholinergic perikarya and dendrites of the nucleus basalis magnocellularis (NBM), aging is associated with a decrease of the density of m2R at the plasma membrane and in the cytoplasm, suggesting a decrease of the total number of m2R in the somato-dendritic field. In contrast, the number of substance P receptors per somato-dendritic surface was not affected. In the frontal cortex (FC), we have shown a decrease of cytoplasmic m2R density also leading to a decrease of the number of m2R per surface of varicosities but with no change of the density of m2R at the membrane. Our results suggest that the decrease of m2R in the somato-dendritic field of the NBM, but not a modification of the number of presynaptic m2 autoreceptors at the plasma membrane in the FC, could contribute to the decrease of the efficacy of cholinergic transmission observed with aging in the rat.

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1. Introduction

Over the past three decades, an exponentially growing number of investigations have focused on the relationship between cognitive deficits and alterations of basal forebrain cholinergic neurons in aging [2]. Impairments in a variety of behavioral and cognitive functions have been attributed to deteriorating forebrain cholinergic systems in aged humans [32,23] and rodents [13,14,21,25,31]. So far, numerous studies have thus focused on the effects of aging on the cholinergic basocortical pathway integrity in normal aged humans and animals. The prevalent hypothesis is that aging is associated with dysfunctions in the basal forebrain cortical cholinergic projection system. This is exemplified by the decrease of the number of cholinergic neurons in the basal forebrain of aged rats [10,17,43]. Notably by using unbiased stereological approaches and by counting ChAT immunoreactives neurons and total Nissl-stained neurons, a decrease of the total number of neurons in the basal forebrain due in part to a decrease of the cholinergic ones has been demonstrated [41]. In aged humans (90 year-old), the total number of neurons in the nucleus basalis of Meynert seems also decreased as compared to young subjects (6–29 year-old) [11]. Cholinergic receptors have been shown to play an important role in the regulation of cognitive processes since the selective blockade of both muscarinic [45,49] and nicotinic [15,30] receptors induces memory deficits in young subjects, in comparison to those observed in old subjects. However, the effects of aging on the abundance of cholinergic recep-
stored in PBS with 0.03% sodium azide until use.

crotome, collected in PBS, cryoprotected, freeze-thawed and sections from NBM and FC were cut on a vibrating mi-

perfused transcardially with a mixture of 2% paraformalde-

2.2. Tissue preparation

Neuroscience.

Scientifique and in accordance with the policy on the use of animals: young rats (6 months) and aged rats (24 months) bred in

the laboratory (male Sprague–Dawley). All experiments were

performed in accordance with the policy of the French Agri-

culture and Forestry Ministry (decree 87849, licence 01499),

all along the somatodendroaxonal tree of the basolocortical neurons; at the membrane and in the
cytoplasm. Notably, the m2 muscarinic receptor subtype is of particular interest in aging because this is the presynaptic muscarinic autoreceptor that negatively regulates the release of ACh from cortical cholinergic terminals [50]. In this con-
text, we have determined, in young (6 months) and aged (24 months) rats, the subcellular distribution of m2R at postsy-
naptic (cell bodies and dendrites in the nucleus basalis magnocellularis (NBM)) and presynaptic (terminals in the frontal cortex (FC)) sites, in cholinergic neurons of the basolocortical Ch4 pathway. In order to do this, we have used immunohis-
tochemistry at light and electron microscopic levels with a m2R subtype selective antibody. We have studied as a con-
trol, the subcellular localization of the substance P receptor (SPR) which, is colocalized with the m2R at the membrane
and affinity in the cortex of aged rats have also lead to conflict-
ing conclusions about the effect of aging on the integrity of forebrain cholinergic neurotransmission [1,5,29,42]. These discrepancies may be due to the use of different non-selective ligands for muscarinic receptors. The availability of specific tools, i.e. receptor subtype antibodies, offers new methods for the quantification and localization of muscarinic recep-
tor specific molecular subtypes. We bring here for the first time data concerning the subcellular distribution of one spe-
cific molecular type of muscarinic receptor, m2R, in one spe-
cific cell type, basolocortical cholinergic neurons, in differ-
ent neuronal compartment, including somato-dendritic and axonal fields. We have analyzed at the subcellular level the localization of m2R all along the somatodendroaxonal tree of the basolocortical neurons; at the membrane and in the
cytosol. Notably, the m2 muscarinic receptor subtype is of particular interest in aging because this is the presynaptic muscarinic autoreceptor that negatively regulates the release of ACh from cortical cholinergic terminals [50]. In this con-
text, we have determined, in young (6 months) and aged (24 months) rats, the subcellular distribution of m2R at postsynaptic (cell bodies and dendrites in the nucleus basalis magnocellularis (NBM)) and presynaptic (terminals in the frontal cortex (FC)) sites, in cholinergic neurons of the basolocortical Ch4 pathway. In order to do this, we have used immunohistochemistry at light and electron microscopic levels with a m2R subtype selective antibody. We have studied as a control, the subcellular localization of the substance P receptor (SPR) which, is colocalized with the m2R at the membrane and the distribution of neurons in the NBM.

2. Materials and methods

2.1. Experimental groups

The experiments were performed on two groups of ani-
mals: young rats (6 months) and aged rats (24 months) bred in
the laboratory (male Sprague–Dawley). All experiments were
performed in accordance with the policy of the French Agri-
culture and Forestry Ministry (decree 87849, licence 01499),
with the approval of the Centre National de la Recherche Scientifique and in accordance with the policy on the use of
animals in neuroscience research issued by the Society for Neurosciences.

2.2. Tissue preparation

The rats were deeply anesthetized with sodium chloral hy-
drate (150 mg/kg) and at least four animals per group were
perfused transcardially with a mixture of 2% paraformalde-
yde and 0.2% glutaraldehyde [41]. Fifty micrometer-thick sections from NBM and FC were cut on a vibrating mi-
crotome, collected in PBS, cryoprotected, freeze-thawed and stored in PBS with 0.03% sodium azide until use.

2.3. Immunohistochemistry

The m2R was detected by immunohistochemistry using a monoclonal antibody raised in rat against a fusion pro-
tein including a part of the third intracytoplasmic loop of the receptor (Chemicon, Temecula, CA). The specificity of the m2R antibody has previously been described in detail [3,22]. The m2R was analyzed specifically in cholinergic perikarya and dendrites in the nucleus basalis magnocellu-laris and in cholinergic varicosities in frontal cortex (FC) by combining immunodetection of m2R and choline acetyl-
transferase (ChAT) or m2R and vesicular ACh transporter (VACht), respectively, as previously described [12]. ChAT and VACht were detected using polyclonal antibodies both raised in rabbit (Chemicon, Temecula, CA and a gift of Dr. R. Edwards, University of California at San Francisco, USA, respectively). The SPR was detected using a polyclonal anti-
body raised in rabbit against a fusion protein that includes the C terminal domain of the receptor (a gift of Dr. R. Shigemoto, University of Kyoto, Japan) [40].

2.3.1. Immunofluorescence experiments

In young rats, the m2R distribution in the cholinergic neu-
rons of the NBM was analyzed at light microscopic (LM) level by combining the simultaneous detection of m2R and
ChAT. The m2R and SPR relative distributions were also an-
alyzed by combining the simultaneous detection of m2R and
SPR by immunofluorescence.

For the simultaneous detection of m2R and ChAT or m2R
and SPR, after perfusion-fixation as described above, sec-
tions were incubated in 4% normal goat serum (NGS) for 30 min and then in a mixture of m2R (1:500) and ChAT
(1:500) or m2R (1:500) and SPR (1:10,000) antibodies sup-
plemented with 1% NGS for 15 h at room temperature (RT). The sections were then incubated in a mixture of goat anti-rat
IgGs coupled to cyanine 3 (CY3) (Jackson ImmunoResearch,
West Grove, USA) and goat anti-rabbit IgGs coupled to fluo-
resceine isothiocyanate (FITC) (Jackson ImmunoResearch).
All fluorochrome-conjugated antibodies were used at a dilu-
tion of 1:200 in PBS for 1 h at RT. After washing, the sections
were mounted in Vectashield mounting medium (Vector Lab-
oratories, Burlingame, CA) and examined in a fluorescence
microscope (Zeiss).

2.3.2. Immunogold experiments

The m2R was analyzed at the electron microscopic (EM)
level specifically in cholinergic neurons of the NBM or in
cholinergic varicosities of the FC by combining m2R and
ChAT or m2R and VACht immunodetection, respectively, as
previously described [12]. The m2R was detected by the pre-
embedding immunogold technique and ChAT and VACht by
the pre-embedding immunoperoxidase using the peroxidase
anti-peroxidase (PAP) technique. Sections were incubated in
4% NGS for 30 min and then in a mixture of m2R (1:500) and
ChAT (1:500) or m2R (1:500) and VACht (1:10,000) anti-
bodies supplemented with 1% NGS for 15 h at RT. For the si-
multaneous detection of m2R and ChAT in the NBM, sections were incubated in goat anti-rat IgGs coupled to biotin (Amer sham, UK; 1:200) and goat anti-rabbit IgGs (Amersham; 1:200) for 1 h 30. After washing, the sections were incubated in PAP-rabbit (Sigma, France; 1:200) and in goat anti-biotin IgGs conjugated to gold particles (0.08 nm in diameter; Au rion, The Netherlands; 1:100 in PBS/BSA-C) for 2 h. The sections were then washed and post-fixed in 1% glutaraldehyde for 10 min. After washing in acetate buffer (0.1 M, pH 7), the signal of the gold immunoparticles was increased us ing a silver enhancement kit (HQ silver; Nanoprobes, NY , USA) for 2 min at RT in the dark. Finally, after washing in acetate buffer and in Tris buffer (TB; 0.05 M, pH 7.6), the immunoreactive sites for ChAT were revealed by incuba tion in 3,3-diaminobenzidine (DAB; Sigma, France; 0.05% in TB) in the presence of H2O2 (0.0048%). The reaction was stopped by several washes in TB. For the simultaneous detec tion of m2R and VACHT in cortex, m2R was detected using the tyramide signal amplification system (TSA; New England Nuclear, Boston, USA). Sections of FC were incubated in a mixture of goat anti-rat IgGs coupled to biotin (Amersham; 1:200) and goat anti-rabbit IgGs (Amersham; 1:200) for 1 h 30. The sections were incubated in streptavidin-HRP (New England Nuclear; 1:100; 30 min), then in biotinyl tyramide (New England Nuclear; 1:50; 7 min), and finally in a mixture of goat anti-biotin IgGs (0.8 nm in diameter; Aurion; The Netherlands, 1:100) conjugated to gold particles and PAP rabbit (DAKO, Denmark; 1:200) in PBS/BSA-C, for 2 h. The immunogold (m2R) and immunoperoxidase (VACHT) signals were revealed as described above. The sections were then stored in acetate buffer 0.1 M and processed for electron microscopy.

The SPR was analysed at EM level using the immunogold technique. Sections were incubated in 4% NGS for 30 min and then in SPR (1:10,000) antibody supplemented with 1% NGS for 15 h at RT. After washing, sections were incubated in goat anti-rabbit IgGs conjugated to gold particles (0.8 nm in diameter; Aurion; The Netherlands, 1:100 in PBS/BSA-C). The immunogold signal was revealed as described above.

2.4. Preparation for electron microscopy

The sections were rinsed, post-fixed in 1% osmium tetrox ide and dehydrated in ascending series of ethanol dilutions, that also included 70% ethanol containing 1% uranyl acetate. They were then treated with propylene oxide, impregnated in resin overnight (Durcupan ACM; Fluka, Buchs, Switzerland), mounted on glass slides and cured at 60° C for 48 h. Areas of interest (the NBM and the layer 5 of the FC) were cut out from the sections and glued to blank cylinders of resin. The observations were performed in layer 5 of the FC because this layer was shown to receive the most dense innervation from the cholinergic neurons of the NBM [38,39]. Ultrathin sections were collected on pioloform-coated single-slot copper grids. The sections were stained with lead citrate and examined in Philips CM10 or Tecnai 20 EM.

2.5. Quantitative analysis

2.5.1. Immunofluorescence experiments

In young rats, the percentage of ChAT immunopositive cell bodies in the NBM that were also m2R immunoreactive was analyzed from immunofluorescence-treated sections double labelled for m2R and ChAT. Since some m2R immunopositive neurons were not cholinergic, the percentage of m2R immunoreactive cell bodies in the NBM that were not ChAT immunopositive was also quantified. In the NBM, all SPR-positive neurons were seen to express m2R. The percentage of m2R positive neurons that were also immunopositive for SPR was thus analysed. For these analysis, both hemispheres in two sections from four young and four old rats were used.

2.5.2. Immunogold experiments

We analyzed the variations of the subcellular localization and the density of m2R and SPR in different subcellular elements. The distribution of m2R and SPR in the NBM and of m2R in the FC was analysed from immunogold-treated sections at the electron microscopic level [12]. The analysis of the distribution of m2R in cholinergic neurons and of SPR in the NBM, was performed on negatives of micrographs at a final magnification of 2950 using the Metamorph software (Universal Imaging, Paris, France). After scanning the negative (Magic Scan, version 3.1; Umaxa), the images were converted into positive pictures and magnified to allow the identification of the subcellular element showing immunoparticles. The measures were performed on four animals per group. A mean of 10 perikarya per animal (for m2R and SPR) and 40 dendritic profiles (for m2R) were analyzed. In perikarya, the immunoparticles were identified and counted in association with six subcellular compartmets. The five compartmets are the plasma membrane, endosome-like small vesicles, the Golgi apparatus, the endoplasmic reticulum and the outer nuclear membrane. Some immunoparticles were classified as associated with a sixth unidentified compartment because they were associated either with no detectable organelles or with an organelle that could not be identified as one of the previous compartments. In dendrites, the distribution of the immunoparticles was quantified in cholinergic dendritic profiles associated with each quantified cholinergic neuron on the same micrographs. All cholinergic dendrites on the picture were used for the quantification. The percentage of ChAT immunopositive dendrites in the NBM that were also m2R immunoreactive was analyzed. A cholinergic dendrite was considered as immunopositive when at least two particles were detected. The immunoparticles were localized in association with three subcellular compartments: the plasma membrane, endosome-like small vesicles and unidentified compartments. The percentage of VACHT immunopositive varicosities in the FC that were also m2R immunoreactive was analyzed from immunogold-treated sections double labelled for m2R and VACHT. All VACHT positive varicosities from at least three ultrathin sections were counted under...
the EM and the percentage of the varicosities that were also m2R positives was calculated. The analysis of the distribution of m2R was performed using the Analysis software (Soft Imaging System, GmbH, Germany) on a computer linked directly to CCD camera on the electron microscope. A mean of 55 varicosities at a final magnification of 20,500× was analysed. The analysis of the distribution of m2R was performed using the Analysis software (Soft Imaging System, GmbH, Germany) on a computer linked directly to CCD camera on the electron microscope. A mean of 55 varicosities at a final magnification of 20,500× was analysed. The immunoparticles were localized in association with two subcellular compartments: the plasma membrane and the cytoplasm. Firstly, the results were expressed as the proportion of immunoparticles for m2R associated with the different subcellular compartments in each neuron. For each neuron, the number of immunoparticles associated with each subcellular compartment was counted and the proportion in relation to the total number was calculated. Secondly, the results were also expressed in order to be able to compare the variation of immunolabeling for m2R in each subcellular compartment between young and aged rats. For each neuron, the number of immunoparticles associated with each compartment was counted in relation to membrane length (µm) for the plasma and nuclear membrane and to the surface of cytoplasm (µm²) for the endoplasmic reticulum, the endosome-like small vesicles and the unidentified compartment. For the Golgi apparatus, the values are expressed as the number of immunoparticles per Golgi apparatus. We also present a data called “total number of immunoparticles per surface of dendrite, soma or varicosity”, showing the total number of immunoparticles quantified in the profile on the micrograph. Our quantification demonstrate variations of the quantity of immunoparticles per neuronal profile, which we assume that it is proportional to the absolute number of receptors in these neuronal compartments. The values were compared using non-parametric Mann–Whitney test.

3. Results

3.1. Phenotypic analysis of the m2R distribution in cholinergic neurons of the NBM: light microscopic observations

3.1.1. Young rats

The double immunofluorescence experiments for m2R and ChAT demonstrated that there were at least three subpopulations of neurons in the NBM: neurons immunolabelled for both m2R and ChAT (m2R+/ChAT+) and

| Table 1 |
|-----------------|-----------------|
|                | m2R (A)         | ChAT (B)       |
| m2R + ChAT     | ChAT            |
| Young          | 87 ± 2%         | 13 ± 2%        |
| Aged           | 89 ± 1%         | 11 ± 1%        |
| m2R + ChAT     | m2R             |
| Young          | 90 ± 1%         | 10 ± 1%        |
| Aged           | 88 ± 2%         | 12 ± 2%        |

Quantification of neurons immunoreactive for m2R and/or ChAT at light microscopic level: analysis of double immunofluorescence experiments for m2R (CY3) and ChAT (FITC). (A) Proportion of ChAT immunoreactive neurons also labelled for m2R. For each animal, a mean of 49 ChAT immunoreactive neurons was analysed. The percentages are expressed as the mean ± S.E.M. of the number of neurons double labelled for m2R and ChAT or labelled only for ChAT, in relation to the total number of ChAT immunoreactive neurons. (B) Proportion of m2R immunoreactive neurons also labelled for ChAT. For each animal, a mean of 48 m2R immunoreactive neurons was analysed. The percentages are expressed as the mean ± S.E.M. of the number of neurons double labelled for m2R and ChAT or labelled only for ChAT, in relation to the total number of ChAT immunoreactive neurons. (C) A large-sized ChAT immunoreactive neuron (A′) is not immunoreactive for m2R (A). Scale bar: 10 µm.
neurons only labelled for m2R (m2R+/ChAT−) or for ChAT (m2R−/ChAT+) (Fig. 1). The analysis at LM level demonstrated immunoreactivity for m2R in almost all neurons immunolabelled for ChAT in the NBM (87% of ChAT labelled neurons) (Table 1). Few neurons of the NBM (10% of m2R labelled neurons) showing immunoreactivity for m2R but not for ChAT were also observed. A dense immunoreactivity for m2R was associated with the plasma membrane of perikarya and proximal dendrites (Fig. 1). A faint staining was observed in the cytoplasm.

3.1.2. Aged rats

The quantitative analysis at LM level of double m2R and ChAT immunofluorescence experiments demonstrated that the percentage of neurons in each type of neuronal population (m2R+/ChAT+; m2R+/ChAT− and m2R−/ChAT+) was similar in young and old rats (Table 1). The proportion of neurons immunolabelled for ChAT and m2R was unchanged in aged rats compared to controls (89 and 87%, respectively) and similarly, the proportion of neurons immunolabelled for m2R but immunonegative for ChAT was also unchanged in aged rats compared to controls (12 and 10%, respectively).

3.2. Cellular and subcellular distribution of m2R in the cholinergic basolocortical neurons: electron microscopic observations

3.2.1. Young rats

3.2.1.1. Somato-dendritic compartment in the NBM of young rats. Cellular and subcellular localization of m2R

Perikarya: The ultrastructural studies of sections of NBM double labelled for m2R and ChAT confirmed that a large part of m2R immunoparticles were associated with the plasma membrane of cholinergic perikarya (43%) (Figs. 2 and 3). In the cytoplasm, m2R immunoreactivity was present in several compartments including endoplasmic reticulum (15%), nuclear membrane (2.4%), Golgi apparatus (11%) and small vesicles (1.3%). Twenty-seven percent of immunoparticles were not associated with a recognizable organelle (Fig. 3). Dendrites: Sixty-five percent of ChAT immunopositive dendrites in the NBM expressed m2R (data not shown). The quantitative analysis demonstrated that in cholinergic den-
Fig. 3. Quantitative analysis of the subcellular distribution of m2R in cholinergic perikarya of the NBM in young and aged rats using the pre-embedding immunogold method with silver intensification. (A) Proportion of immunoparticles for m2R associated with different subcellular neuronal compartments in young rats. For each neuron, the number of immunoparticles associated with each subcellular compartment was counted and the proportion in relation to the total number was calculated. Data are expressed as percentage ± S.E.M. A large part of immunoparticles is associated with the plasma membrane. In the cytoplasm, the immunoparticles are detected in association with the endoplasmic reticulum (er) and the Golgi apparatus (Golgi). A small proportion of immunoparticles are associated with small vesicles (small ves.) and the nuclear membrane (n. mb). A part of immunoparticles are not seen in association with any identified compartment (unident.). (B) Comparison of the localization of m2R immunoparticles in different subcellular compartments in cell bodies of cholinergic neurons in the NBM of young and aged rats. For each neuron, the number of immunoparticles associated with each compartment was counted in relation to the membrane length (µm) for the plasma membrane and the nuclear membrane and to the surface of cytoplasm (µm²) for small vesicles, the endoplasmic reticulum and unidentified compartment. Data are the result of counting four young and four aged rats in 10 perikarya per animal. The results are expressed as the mean ± S.E.M. in relation to an arbitrary unit (100) of the control values. In aged rats, the statistical analysis (non-parametric Mann–Whitney test) shows a decrease of the total number of immunoparticles associated with a decrease of the labelling detected both at the plasma membrane and in the cytoplasm. In the cytoplasm of aged rats, the analysis demonstrates a decrease of the number of immunoparticles associated with the endoplasmic reticulum and with unidentified compartments. *p<0.05; no symbol: not significant.

Fig. 4. Quantitative analysis of the subcellular distribution of m2R in cholinergic dendrites of the NBM in young and aged rats. (A) Proportion of immunoparticles for m2R associated with different subcellular neuronal compartments in young rats. For each cholinergic dendrite, the number of immunoparticles associated with each subcellular compartment was counted and the proportion in relation to the total number was calculated. Results are expressed as percentage ± S.E.M. The largest portion of immunoparticles is associated with the plasma membrane. In the cytoplasm, the immunoparticles are detected in association with small vesicles (small ves.) and with unidentified compartments (unident.). (B) Comparison of the localization of m2R immunoparticles in dendrites of cholinergic neurons in the NBM of young and aged rats. For each dendrite, the number of immunoparticles associated with each compartment was counted in relation to the membrane length (µm) for the plasma membrane and to the surface of cytoplasm (µm²) for small vesicles and unidentified compartment. Data are the result of counting four young and four aged rats in 40 dendritic profiles per animal. The results are expressed as the mean ± S.E.M. in relation to an arbitrary unit (100) of the control values. In aged rats, the statistical analysis (non-parametric Mann–Whitney test) shows a decrease of the total number of immunoparticles associated with a decrease of the labelling at the plasma membrane. *p<0.05; no symbol: not significant.

Cellular and subcellular localization of SPR

The analysis at the LM level in the NBM demonstrated that all SPR positive neurons were also immunopositive for m2R. This observation allowed the study of the subcellular distribution of SPR as a control of the specificity of modifications of ultrastructural m2R localization. A dense immunoreactivity for m2R and SPR was associated with the plasma...
membrane of perikarya and proximal dendrites (Fig. 5), SPR immunoreactivity was present in 82% of m2R positives neurons (Table 2). Moreover, in accordance with previous study [7], our results demonstrated that all SPR immunoreactive neurons were also immunolabelled for ChAT (data not shown). Ultrastructural studies of sections of NBM labelled for SPR show that a large part of SPR immunoparticles were associated with the plasma membrane (47%) (Figs. 6 and 7A). In the cytoplasm, SPR immunoreactivity was present in several compartments including endoplasmic reticulum (17%), nuclear membrane (2%), Golgi apparatus (7%) and small vesicles (1%). Twenty-six percent of immunoparticles were not associated with a recognizable organelle (Fig. 7A).

3.2.1.2. Axonal compartment in the FC of young rats. The quantification of sections double labelled for m2R and VACHT at EM level demonstrated that 23% of cholinergic varicosities (VACHT immunopositive) expressed m2R immunoreactivity in young rats.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>m2R ± SPR</th>
<th>m2R</th>
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<tr>
<td>m2R immunoreactive neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>82 ± 2%</td>
<td>18 ± 2%</td>
</tr>
<tr>
<td>Aged</td>
<td>84 ± 1%</td>
<td>16 ± 1%</td>
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Quantification of neurons immunoreactive for m2R and/or SPR at light microscopic level: analysis of double immunofluorescence experiments for m2R (CY3) and SPR (FITC). For each animal, a mean of 47 m2R immunoreactive neurons was analysed. Percentages are expressed as the mean ± S.E.M. of the number of neurons double labelled for m2R and SPR or labelled only for m2R, in relation to the total number of m2R immunoreactive neurons. The statistical analysis (non-parametric Mann–Whitney test) shows no difference in the percentage of neurons expressing m2R also labelled for SPR, between young and aged rats.
nuclear membrane and to the surface of cytoplasm (in relation to the membrane length (number of immunoparticles associated with each compartment was counted and the proportion in relation to the total number was calculated. Data are expressed as percentage ± S.E.M. A part of immunoparticles are not seen in association with any identified compartment (unident.). (B) Comparison of the localization of SPR immunoparticles in different subcellular compartments demonstrated that the density of SPR located at the plasma membrane in old rats was significantly decreased (−51%) (Figs. 2 and 3B). In the cytoplasm of cholinergic neurons, a decrease of the number of immunoparticles for m2R associated with endoplasmic reticulum (−52%) and with the unidentified compartment (−44%) was also observed (Fig. 3B).

Dendrites: Our quantification data demonstrated a significant decrease of the number of ChAT positive dendrites also immunoreactive for m2R in old rats (−24%, data not shown). In cholinergic dendritic shafts, the ultrastructural analysis demonstrated a decrease (−37%) of the total number of immunoparticles per dendritic surface in old rats (Fig. 4B). Moreover, the analysis of the labelling associated with the different subcellular dendritic compartments demonstrated a significant decrease of the density of immunoparticles located at the plasma membrane in old rats (−30%) (Figs. 3B and 4B).

Cellular and subcellular localization of SPR
In accordance with our results obtained in young rats, the quantitative analysis at EM level of sections of NBM double labelled for m2R and ChAT demonstrated a decrease of the total number of immunoparticles per neuronal surface in old rats (−52%) (Fig. 3B). The analysis of the labelling associated with the different subcellular neuronal compartments demonstrated that the density of m2R decreased at the plasma membrane in old rats (−51%) (Figs. 2 and 3B). In the cytoplasm of cholinergic neurons, a decrease of the number of immunoparticles for m2R associated with endoplasmic reticulum (−52%) and with the unidentified compartment (−44%) was also observed (Fig. 3B).

3.2.2. Aged rats
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Cellular and subcellular localization of SPR
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Fig. 7. Quantitative analysis of the subcellular distribution of SPR in perikarya of the NBM in young and aged rats using the pre-embedding immunogold method with silver intensification. (A) Proportion of immunoparticles for SPR associated with different subcellular neuronal compartments in young rats. For each neuron, the number of immunoparticles associated with each subcellular compartment was counted and the proportion in relation to the total number was calculated. Data are expressed as percentage ± S.E.M. A large part of immunoparticles is associated with the plasma membrane. In the cytoplasm, the immunoparticles are detected in association with the endoplasmic reticulum (er) and the Golgi apparatus (Golgi). A small proportion of immunoparticles is associated with the nuclear membrane (n. mem) and small vesicles (small ves.). A part of immunoparticles are not seen in association with any identified compartment (unident.). (B) Comparison of the localization of SPR immunoparticles in different subcellular compartments in cell bodies of the NBM in young and aged rats. For each neuron, the number of immunoparticles associated with each compartment was counted and the proportion in relation to the total number was calculated. Data are expressed as percentage ± S.E.M. A large part of immunoparticles is associated with the plasma membrane. In the cytoplasm, the immunoparticles are detected in association with the endoplasmic reticulum (er) and the Golgi apparatus (Golgi). A small proportion of immunoparticles is associated with the nuclear membrane (n. mem) and small vesicles (small ves.). A part of immunoparticles are not seen in association with any identified compartment (unident.). (B) Comparison of the localization of SPR immunoparticles in different subcellular compartments demonstrated that the density of SPR located at the plasma membrane in old rats was significantly decreased (−51%) (Figs. 2 and 3B). In the cytoplasm of cholinergic neurons, a decrease of the number of immunoparticles for m2R associated with endoplasmic reticulum (−52%) and with the unidentified compartment (−44%) was also observed (Fig. 3B).

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Cellular and subcellular localization of SPR
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number of immunoparticles per terminal surface (immunoparticles) significantly decreases with aging. Quantitative analysis at the ultrastructural level, demonstrated a significant decrease of the density of cytoplasmic immunoparticles (~55%) leading to a decrease of the total number of immunoparticles per terminal surface (~20%) (Figs. 8 and 9B). However, no modification of the density of immunoparticles located at the plasma membrane was observed (Figs. 8 and 9B). The number of VACHT positive varicosities also immunoreactive for m2R was not modified in old rats (22%, data not shown).

4. Discussion

We report here the first description of the effect of aging on the subcellular localization of the m2 autoreceptor in all parts of the basalocortical cholinergic neurons. We have demonstrated that the subcellular distribution and membrane abundance of m2R is modified with aging and that these modifications vary with neuronal compartments: soma/dendrites in the NBM or terminals in the FC. (1) In control animals, in accordance with previous data obtained in mice [12], m2R is prominently located at the plasma membrane at postsynaptic sites in the NBM and at presynaptic sites in cortical varicosities. (2) In the soma-dendritic compartment in the NBM of aged rats, the percentage of cholinergic dendrites expressing m2R decreases. In parallel, a decrease of the total density of m2R immunoparticles associated with a depletion of cell surface receptors were observed in cholinergic cell bodies and dendrites of the NBM. This was accompanied by a decrease of cytoplasmic m2R immunoreactivity due to a decrease of the density of receptors associated with the endoplasmic reticulum and the unidentified compartments. In contrast, in aged rats, few changes were observed for SPR in neurons of the NBM. In the FC, the percentage of cholinergic varicosities expressing m2R was unchanged with aging and the density of plasma membrane m2R was not modified. However, the cytoplasmic number of receptors was decreased in cortical cholinergic varicosities of aged rats leading to a decrease of the total number of m2R immunoreactive sites.

4.1. Effect of aging on the cellular and subcellular distribution of postsynaptic m2R in the NBM

In the NBM of aged rats, the decrease of the total number of m2R in cholinergic cell bodies and dendrites and the decrease of the number of cholinergic dendrites expressing m2R demonstrate that m2R is downregulated with aging. This downregulation is associated with a decrease of cell surface receptors in cholinergic neurons of the NBM. Very few data have focused on the status of m2R in the NBM of normal aged rats. Most studies have concerned the target brain areas of cholinergic neurons including cortex, hippocampus or amygdala where the cholinergic deficits correlates with cognitive decline in pathological aging, i.e. in Alzheimer’s disease [18,19,44]. However, our results are in accordance with the few existent data. Autoradiographic studies have reported a decrease of M2 muscarinic receptors in the cholinergic basal forebrain of aged rats [5,29]. This M2 pharmacological type correspond to m2R and m4R. It has been demonstrated that the m4R is not expressed or at very low level compared to...
m2R, in the cholinergic basal forebrain [23,47]. Thus, this decrease in M2 binding sites certainly reflects a decrease of m2R binding sites in these structures. Our study demonstrated that this decrease occurred specifically in cholinergic neurons.

Our results bring the first demonstration of the subcellular events that may contribute to downregulation of m2R: a decrease of the density of m2R at the plasma membrane and a decrease of the number of m2R associated with the endoplasmic reticulum. This decrease of the density of m2R associated with the endoplasmic reticulum may be explained by alterations of the m2R gene expression. In accordance with this hypothesis, a decrease of the level of m2R mRNA in old rats has been demonstrated in different brain structures including cholinergic basal forebrain nuclei [29]. Thus, a decrease of m2R synthesis may lead to a decrease of the total number of m2R in neurons, and notably to a decrease of cell surface receptors. Alterations in the targeting of m2R at the plasma membrane may also contribute to the decrease of the expression of m2R at the cholinergic cell surface in the NBM. However, the modifications of the subcellular distribution of m2R do not seem to be due to a general decrease of the activities of synthesis and membrane targeting of proteins, including other neurotransmitter receptors, in these neurons since the density of SPR in the endoplasmic reticulum and at the plasma membrane are unaltered in aged rats. This suggests that aging specifically affects the subcellular distribution of cholinergic m2 receptors.

Alterations in different processes regulating m2R synthesis, which could involve ACh or other messengers may also contribute to the decrease of the abundance of m2R at the plasma membrane. In this context, alterations of the supply in neurotrophic factors in aging, which is known to regulate gene expression [28], including muscarinic receptors [8,16,36] may contribute to the regulation of the subcellular distribution of m2R. Notably, exposure to NGF of telencephalic neurons modulates gene expression and abundance of binding sites of the different muscarinic receptors [16]. Trophic factors are also known to regulate muscarinic receptors gene expression in neurons of the superior cervical ganglia [24].

4.2. Effect of aging on the cellular and subcellular distribution of presynaptic m2R in the FC

We have demonstrated here that aging induces a decrease of the density of m2R in cholinergic varicosities of the FC, which is due to a decrease of cytoplasmic receptors with no modification of the abundance of m2R at the plasma membrane. This is the first demonstration that the compartmentalization of the m2R in cholinergic cortical terminals is modified with aging. An hypothesis to explain these results, may be that in normal conditions, a pool of intracytoplasmic receptors could be recruited for membrane targeting. With aging, this pool of receptors may be progressively downregulated favouring the targeting of receptors to the membrane. Alterations in axonal transport of m2R may also contribute to this decrease of m2R in cholinergic terminals. In this context, although not specifically shown for cholinergic neurons, alterations in cortical axonal transport have been demonstrated in aged people suffering from Alzheimer’s disease [9].

Our work bring for the first time specific information about the effects of aging on the m2R as a presynaptic autoreceptor. In fact, most autoradiographic studies have been devoted to the status of M2 binding sites in the cortex of aged rats and thus of m2R since m4R is not expressed or is expressed at very low levels in the rat cortex [23,48]. However, autoradiography takes in account pre- and postsynaptic receptors and it is now well established that some noncholinergic neurons expressed m2R as a postsynaptic receptor in the cortex of the rat. In addition, this technique does not discriminate between auto- and hetero-receptors since m2R is also expressed by non-cholinergic terminals in the frontal cortex [23,26,37]. In this context, our system of analysis appeared the most suitable to specifically study the status of m2R in cholinergic terminals and thus to bring information concerning m2R as the presynaptic autoreceptor in the FC. Moreover, autoradiographic studies of muscarinic receptors in aged rats, have lead to conflicting results since M2 binding has been reported both increased [35] or decreased [1,5,29] in the cortex of aged rats. These discrepancies could be explained by differences in the specificity of the ligands or in binding methods. In aged humans, a decrease of the binding of M2 receptors in cortical synaptosomes, that are located at presynaptic sites, has been reported [32]. Alternatively, an increase of the binding of M2 in aging has been reported in humans in vivo [34]. This discrepancy in the modifications of M2 binding may be due to species differences. It may also be attributed to postsynaptic receptors since m2R has been demonstrated to be expressed by some cortical neurons. The pre- and postsynaptic m2R may be submitted to opposite regulations, as we demonstrate for m2R in the present work. Since the ligand used by Podruchny et al. (2003) is specific for m2R [20] and since we show here a decrease of m2R immunoreactivity in cholinergic terminals, the increase of M2 binding may be due to an increase of postsynaptic m2R. Moreover, the decrease of ACh during aging may lead to a decrease of the number of receptors occupied by the native ligand and thus an increase of free sites for the radioactive ligand without change in the abundance of m2R at the plasma membrane as we describe in this study. Alternatively, the affinity of receptors but not the total number of receptors may have been modified. Recent studies, using knock-out mice, have indicated that auto-inhibition of ACh release is mediated by m2R in the cortex [50]. Cortical potassium-induced ACh release is decreased in aged rats. However, blockade or activation of muscarinic receptors by atropine [27] or oxotremorine [6], increases or decreases, respectively, ACh release in the same extend in aged and young rats. This suggests that muscarinic presynaptic regulation of ACh is unaltered with aging. In this context, our results showing no modification of the membrane abundance of m2R in aged rats, together with these previous data, suggest that m2R...
functions as presynaptic autoreceptor are unaltered with aging.

A decrease of the number of cholinergic terminals associated with a shrinkage of persistent ones have been reported in the cortex of aged rats [46]. However, even if the number of cholinergic varicosities is decreased, we have demonstrated that the proportion of these terminals expressing m2R is unaltered with aging. Thus, aging might specifically affect cholinergic terminals that do not express m2R which could explain the difference of results concerning ACh release when different stimuli are used (local depolarisation or blockade of muscarinic presynaptic receptors).

5. Conclusion

Our results suggest that cholinergic dysfunctions observed with aging are associated with change in expression and localization of m2R. The decrease of the density of m2R at the plasma membrane in the NBM may alter neuronal activity triggered by the stimulation of these receptors. Thus, modifications of the cholinergic neuronal reactivity induced by the decrease of the number of membrane receptors may contribute to the general decrease of the cholinergic basalocortical transmission with aging.

We have observed different effects of aging on the density of m2R at the plasma membrane in somato-dendritic and axonal compartments. This suggests that the mechanisms regulating the membrane targeting of m2R differs according to its localization at the plasma membrane in the different parts of the neuron and that these mechanisms are differentially altered with aging.

Our anatomical results, showing that the availability of m2R at the membrane of cholinergic terminals in the frontal cortex are not altered with aging, are consistent with the previous biochemical findings demonstrating that the inhibitory effect of m2R on ACh release is still present in the cortex of aged rats [6,27].

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