

University of Groningen

Visualization of cholinceptive neurons in the rat neocortex

van der Zee, E.A.; Streefland, C.; Strosberg, A.D.; Schröder, H.; Luiten, P.G.M.

Published in:
Molecular Brain Research

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1992

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van der Zee, E. A., Streefland, C., Strosberg, A. D., Schröder, H., & Luiten, P. G. M. (1992). Visualization of cholinceptive neurons in the rat neocortex: Colocalization of muscarinic and nicotinic acetylcholine receptors. *Molecular Brain Research*, 14(4), 326-336.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Visualization of cholinceptive neurons in the rat neocortex: colocalization of muscarinic and nicotinic acetylcholine receptors

E.A. van der Zee^{a,*}, C. Streefland^a, A.D. Strosberg^b, H. Schröder^c and P.G.M. Luiten^a

^aDepartment of Animal Physiology, University of Groningen, Haren (The Netherlands), ^bLaboratoire d'Immunopharmacologie Moléculaire, Institut Cochin de Génétique Moléculaire, Paris (France) and ^cAnatomical Institute, University of Cologne, Cologne (FRG)

(Accepted 10 March 1992)

Key words: Acetylcholine; Muscarinic acetylcholine receptor; Nicotinic acetylcholine receptor; Neocortex; Rat

The present investigation analyzes the cellular distribution of muscarinic and nicotinic acetylcholine receptors in rat neocortex, by use of monoclonal antibodies raised against purified receptor proteins. The degree of colocalization of both types of receptors was determined by way of immunofluorescent double-labeling techniques. For both classes of receptors, pyramidal and nonpyramidal cells were found immunostained and an identical laminar distribution pattern of immunopositive neurons in the rat neocortex became apparent. A striking similarity in distribution of the two cholinergic receptor types was found in the frontal/motor and parietal cortex. Accordingly, we observed a high degree of colocalization of muscarinic and nicotinic acetylcholine receptors within immunopositive cortical neurons. Approximately 90% of the cholinceptive neurons expressed both types of receptors. The current data demonstrate that (i) the distribution of muscarinic and nicotinic cholinceptive neurons in the neocortex is present in identical laminar patterns and represent the same type of cells, (ii) both classes of cholinergic receptors are highly colocalized within cholinceptive neurons, which points at individual neurons as a likely site of interaction between muscarinic and nicotinic acetylcholine receptor-mediated processes.

INTRODUCTION

The cholinergic innervation of the cortical mantle is intimately involved in higher cognitive processes. In the last decade the cholinergic system received increasing attention in relation to function and dysfunction of learning and memory mechanisms, with special focus on the cholinergic synapse in the cerebral cortex^{5,6,10}. The cerebral cortex receives its topographically organized cholinergic input from the neurons of the nucleus basalis magnocellularis (nbm) as has been accurately established in recent years by several investigators^{21,26,27,41}. Lesions of the nbm as well as cholinergic receptor blockade were shown to severely impair performance on a variety of memory tasks^{13,20,31,40}.

Acetylcholine (ACh) exerts its influence on cortical target cells via two types of cholinergic receptors: muscarinic (mAChRs) and nicotinic acetylcholine receptors (nAChRs). mAChRs are G-protein mediated receptors coupled to second messenger systems^{36,45}, whereas nAChRs form monovalent cation channels mediating an inward flux of sodium ions¹⁹. The summarized impact of ACh can be described as facilitatory and thereby increasing the magnitude of cortical neuronal activity^{18,29}. It may be assumed that in this way, ACh through its re-

ceptors modulates the processing of incoming information to the cortex related to learning and subsequent plasticity. There is ample experimental evidence that both types of cholinergic receptors are involved in learning and memory processes. Blockade of either mAChRs or nAChRs leads to cognitive impairments^{5,7}. Moreover, recent studies clearly indicated complex interactions of mAChRs and nAChRs contributing to the cholinergic component of cognitive functioning. The cholinergic influence on neocortical electric activity, for example, appeared to be established by a joint contribution of both types of cholinergic receptors³⁹. Likewise, mAChRs and nAChRs interact on the regulation of spatial and passive avoidance learning, whereas the impact of combined muscarinic and nicotinic blockade on radial-arm maze performance of rats appeared to be stronger than the cumulative effects of each receptor blockade alone^{22,23}. However, the underlying mechanism of the latter findings is still poorly understood²⁴.

Little attention has been paid, so far, to the intracellular relationship of mAChRs and nAChRs. Recently, receptor protein immunocytochemistry enabled us to visualize mAChRs and nAChRs in the rat brain at the cellular and subcellular level^{30,42–44,47,48,49,55}. Since the intracellular relationship of mAChRs and nAChRs may

be a vital part of the functional interaction between the two types of cholinergic receptors, we currently examined their degree of coexistence by way of fluorescent immunocytochemical double-labeling techniques in three areas of the rat neocortex. The determination of the anatomical characteristics of the cortical cholinergic system may further substantiate our understanding of the electrophysiological and pharmacological properties of the cortical cholinergic processes.

MATERIALS AND METHODS

Subjects

In this study 15 male Wistar rats (b.wt. 300 g) were used. All animals were investigated for single-labeling of mAChRs, whereas in 10 cases also single-labeling for nAChRs was carried out. On brain sections of 10 animals, fluorescent double-labeling for mAChRs and nAChRs was performed, 5 of which were further processed for quantification of dual-labeled cells. Two cortical regions were studied in greater detail: frontal/motor cortex and parietal cortex (Table I). Zilles' atlas⁵⁴ of the rat cerebral cortex was used as a standard anatomical reference.

Tissue preparation

First, the animals were deeply anesthetized with sodium pentobarbital. Fixation of the brain was carried out by transcardial perfusion with 300 ml fixative composed of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4) at a perfusion speed of 20 ml/min, which was preceded by a short prerinse of saline. Immediately after fixation the fixative was removed by a perfusion with 150 ml 10% sucrose in 0.1 M PB. The brains were cryoprotected by overnight storage at 4°C in 30% sucrose in 0.1 M PB and subsequently coronally sectioned on a cryostat microtome at a thickness of 20 µm.

Immunocytochemical procedure

Muscarinic and nicotinic receptor proteins were visualized by means of the monoclonal antibodies M35 and WF6, respectively. The WF6 monoclonal IgG antibody was raised in mice using nicotinic receptor protein purified from membrane fragments of *Tor-*

pedo marmorate electric organ cells. The M35 monoclonal IgM antibody was raised in mice against muscarinic receptor protein purified from bovine forebrain homogenates. Further extensive descriptions of production and characterization of WF6 and M35 have been reported previously, as well as their use in immunocytochemistry^{2,3,4,12,30,42,44,47,50,52}

For single-labeling, free-floating brain sections were incubated 24 h at 4°C with the primary antibody solution in phosphate-buffered saline (PBS) containing mouse anti-mAChR IgM (M35, 1:2000) or mouse anti-nAChR IgG (WF6, 1:10). After rinsing, the sections were exposed for 2 h at room temperature (RT) to biotinylated rabbit IgG anti-mouse-IgM (mu-chain directed, 1:200; Zymed) or biotinylated sheep anti-mouse IgG (1:200; Amersham), respectively, followed by incubation in Streptavidin-HRP (1:200; Zymed). Finally, the sections were reacted under visual guidance with diaminobenzidine (DAB, 30 mg/100 ml Tris buffer, pH 7.4) and 0.01% H₂O₂ and mounted.

Double-labeling experiments for the study of colocalization of both receptor types in the sequence of WF6/M35 were carried out with fluorescence techniques. For dual labeling, free-floating sections were sequentially exposed to each of the primary antibodies as for single-labeling. For WF6 the primary antibody step was followed by goat anti-mouse IgG Phycoerythrin-conjugated (1:50; Tago). After completion of the WF6 staining, the sections were incubated with M35 followed by biotinylated rabbit anti-mouse IgM (1:50; Zymed) and Fluorescein Isothiocyanate (FITC)-conjugated Streptavidin (1:50; Zymed). All incubations with fluorescent labels were performed in darkness. After antibody processing the sections were mounted and coverslipped in a 1:1 mixture of PBS and glycerin. The sections were studied and photographed with a Ploemopak Leitz fluorescence microscope with the appropriate filter blocks for FITC and Phycoerythrin labels, yielding a green and red fluorescence, respectively. Standard control experiments were performed by (a) omission of either or both primary antibodies in the incubation cycle, (b) primary antibodies incubated with the non-matching secondary antibodies (in the sequence of WF6 — biotinylated rabbit anti-mouse IgM — FITC-Streptavidin and M35 — sheep anti-mouse IgG phycoerythrin-conjugated) and (c) replacing the primary antibody by normal mouse serum. In all cases the controls yielded negative results, i.e. absence of any detectable labeling, excluding the appearance of possible crossreactivity of secondary antisera during the incubation cycle.

TABLE I

Numbers and percentages of single-M35, single-WF6 and double-labeled neurons per cortical layer in frontal/motor and parietal cortex

* The numbers of counted cells per cortical layer correspond to the numerical neuronal density in the middle portion of the various layers, see experimental procedures. **Layer 4 is based on area 4 of the frontal cortex as described by Lysakowski et al.²⁸. Sum = summation of cells in all layers.

Cortex area	Layer	Total no. of cells*	No. of single M35 (%)	No. of single WF6 (%)	No. of double labeled cells (%)
Frontal/motor	2-3	2108	56 (2.7)	141 (6.7)	1911 (90.7)
	4**	408	16 (3.9)	30 (7.4)	362 (88.7)
	5	3202	104 (3.2)	233 (7.3)	2865 (89.5)
	6	1403	37 (2.6)	109 (7.8)	1257 (89.6)
	Sum	7121	213 (3.0)	513 (7.2)	6395 (89.8)
Parietal	2-3	1287	44 (3.4)	80 (6.2)	1163 (90.4)
	4	305	8 (2.6)	28 (9.3)	269 (88.2)
	5	2108	53 (2.5)	124 (5.9)	1931 (91.6)
	6	926	17 (1.8)	61 (6.6)	848 (91.6)
	Sum	4626	122 (2.6)	293 (6.3)	4211 (91.0)

Degree of colocalization of M35 and WF6

The degree of colocalization was investigated in frontal/motor and parietal cortex (area 1) of 5 animals. Per animal, 12 radially oriented cortical strips of 125 μm (frontal/motor cortex) or 250 μm (parietal cortex) were analyzed through layer 1 to the border of layer 6b. The strips were taken at three coronal levels (Bregma 1.2, -1.3 and -3.3, according to Zilles' atlas⁵⁴), one strip per area per hemisphere. The data of all animals were pooled and presented in Table I.

Under the fluorescence microscope, a clear transition of the different cortical layers cannot unequivocally be established. Therefore, layers 2 and 3 were pooled in all areas studied. In the part of the 'motor cortex' (area 4 of the frontal cortex, according to Lysakowski et al.²⁸) of the frontal/motor area, a thin layer 4 could be distinguished. In each of the layers 2/3, 4, 5 and 6 only the middle part was analyzed in order to rule out mixing layers during quantification. Consequently, we aimed to determine the degree of colocalization per cortical area and layer, rather than the absolute numerical neuronal density per layer. In the quantified cortical profiles neurons were considered to be double-labeled if the cell body and/or dendritic processes revealed immunoreactivity for both types of receptors. Occasionally, single-labeled cell bodies but with double-labeled dendritic processes were encountered, or vice versa, probably caused by incomplete penetration of the respective antibodies. Nevertheless, as mentioned before, these cases were recorded as double-labeled neurons.

RESULTS

Distribution pattern for M35 and WF6

A consistent distribution pattern of muscarinic- and nicotinic-immunoreactive neurons was apparent for all animals studied throughout all neocortical areas. Only some minor differences were observed in laminar receptor distribution between the three cortical areas examined. In Figs. 1 and 2, an overview of the parietal cortex is given for both receptor types, which was generally identical for all cortical areas studied and resembled previous description of the muscarinic pattern^{30,47}. In brief, the cellular staining with M35 in the cortex revealed a clear laminar distribution (Figs. 1A, 2A). Predominantly layers 2–3 (Fig. 1C), and 5 (Fig. 1E) neurons showed strong immunoreactivity. In the superficial layers, immunopositive apical dendrites of layer 5 pyramidal neurons, traversing layer 4, branched heavily. Most of the M35-immunopositive astrocytes were present in the superficial layers and layer 6b, although some labeled astrocytes were found in other layers as well. Besides the cell bodies and apical dendrites of layer 5 pyramidal neurons, their basal dendritic complexes formed a plexus of immunoreactive processes, either cut longitudinally or

transversely (Fig. 1A,E). Layers 4 and 6 of the frontal/motor cortex and parietal cortex on the other hand showed considerably less immunostained neurons (Fig. 1A). However, somewhat higher numbers of positive neurons were observed in sublayer 6b of the parietal cortex, positioned adjacent to the white matter of the corpus callosum (Fig. 2A). In layer 6, both multipolar (Fig. 2C) and pyramidal-like (Fig. 2D) neurons were found M35-immunostained. Incidentally, M35-positive neurons were observed in the white matter.

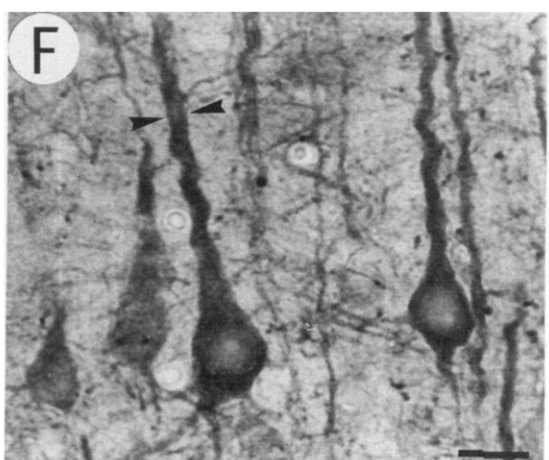
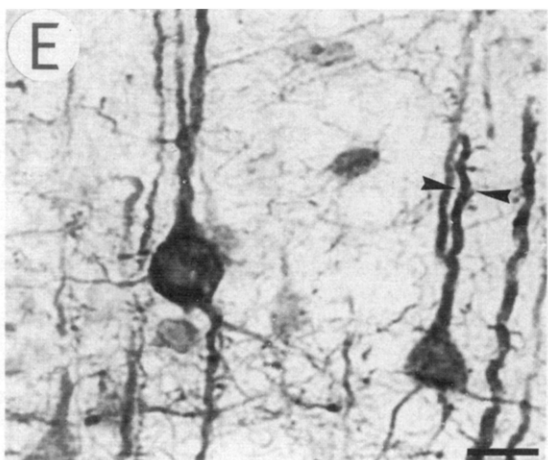
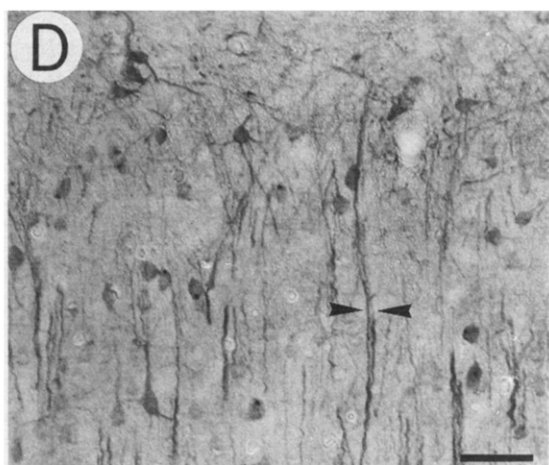
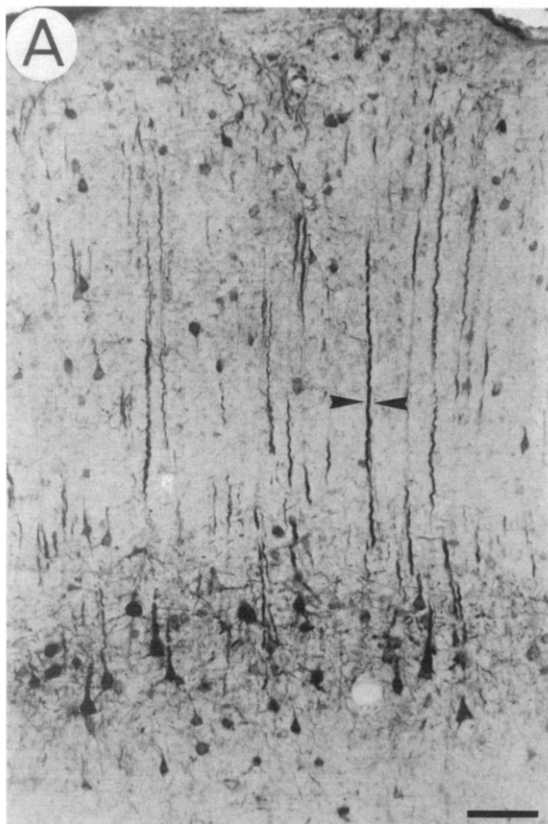
The distribution of WF6 immunoreactivity in the frontal/motor and parietal cortex resembled that of the occipital cortex as described by Schröder and coworkers⁴⁴. In short, WF6 predominantly visualized cell bodies in layers 2–3 and 5 (Fig. 1B). Layer 5 neurons were embedded in an immunoreactive plexus of basal dendrites (Fig. 1B,F), comparable to the neuritic network observed for M35. In layers 4 and 6, only limited numbers of immunoreactive fusiform and round cells were present with the exception of layer 6b, which contained several rows of WF6-immunopositive neurons located immediately above the white matter (Fig. 2B). Some labeled neurons were found embedded in the white matter as well (indicated by the arrow in Fig. 2B). Immunolabeled dendrites arising primarily from layer 5 pyramidal neurons, were found traversing the upper cortical layers perpendicular to the pial surface, and branched diagonally in the transition zone between layers 1 and 2 (Fig. 1D). Taken together, the above described pattern of WF6 immunoreactivity revealed a clear laminar distribution in all cortical areas similar to the M35 pattern.

Fluorescent double-labeling for M35 and WF6

The distribution patterns for muscarinic and nicotinic acetylcholine receptors showed considerable resemblance in laminar organization and cell typing, suggesting an extensive overlap for both types of cholinergic neuronal populations. In order to investigate the coexistence of muscarinic and nicotinic cholinergic receptors in more detail, fluorescent double-labeling experiments were performed. The degree of colocalization was studied in the frontal/motor and parietal cortex.

The results obtained with fluorescence were similar to the DAB results. WF6-phycoerythrin immunofluorescent precipitate was mainly of a fine granular appearance,

Fig. 1. Photomicrographs of M35 (left panel) and WF6 (right panel) in the parietal cortex. A,B: low power photomicrographs through layers 1–5 as indicated at the right of B, demonstrating identical laminar characteristics in distribution of muscarinic (A) and nicotinic (B) cholinergic neurons. Arrowheads point at immunopositive apical dendrites, traversing layer 4. C,D: numerous positive neurons of different cell type are present in layers 2–3. Apical dendrites arising from layer 3 and 5 pyramidal cells branch at the border of layer 1 and 2. E,F: high power photomicrographs of large neurons in layer 5 showing strong immunoreactivity for both receptor types. Besides massive apical dendrites (arrowheads), numerous basal dendrites are immunopositive. Bars in A, B = 85 μm , in C, D = 45 μm and in E, F = 20 μm .



while bright FITC immunofluorescence was detected for M35. In all layers of the cortical areas examined, a high proportion of double-labeled neurons was observed. The resemblance between M35 and WF6 immunolabeling is, for example, depicted in layer 2–3 and 5 of the frontal/motor cortex (Fig. 3). Both pyramidal and non-pyramidal neurons showed coexistence of M35 and WF6. Besides double-labeled cell bodies, numerous double-labeled dendritic processes could be observed, including both apical (arrowheads) and basal dendrites (arrows) (Fig. 3A,B). Immunoreactive astrocytes, most notably present in the superficial layers, were frequently observed as being single M35-positive. Only rarely throughout the cortical layers astrocytes were observed to be

double-labeled (Fig. 3C,D). This observation was done in only 2 of the 10 animals studied.

In layer 5 of the parietal cortex, groups of large (22 μm on average) pyramidal neurons were found to contain both muscarinic and nicotinic cholinergic receptors (Fig. 4). Occasionally, small (8 μm on average) neurons localized amidst the large pyramidal cells were found to be single-labeled, either for WF6 or M35 (Fig. 4E,F; M35 single-labeled neurons). Large pyramidal neurons single-labeled for WF6 or M35 were only rarely detected. As is the case in all other cortical areas, dual immunolabeling was present both in cell bodies and in dendritic processes. Although layers 4 and 6 contained relatively few immunoreactive neurons, a high propor-

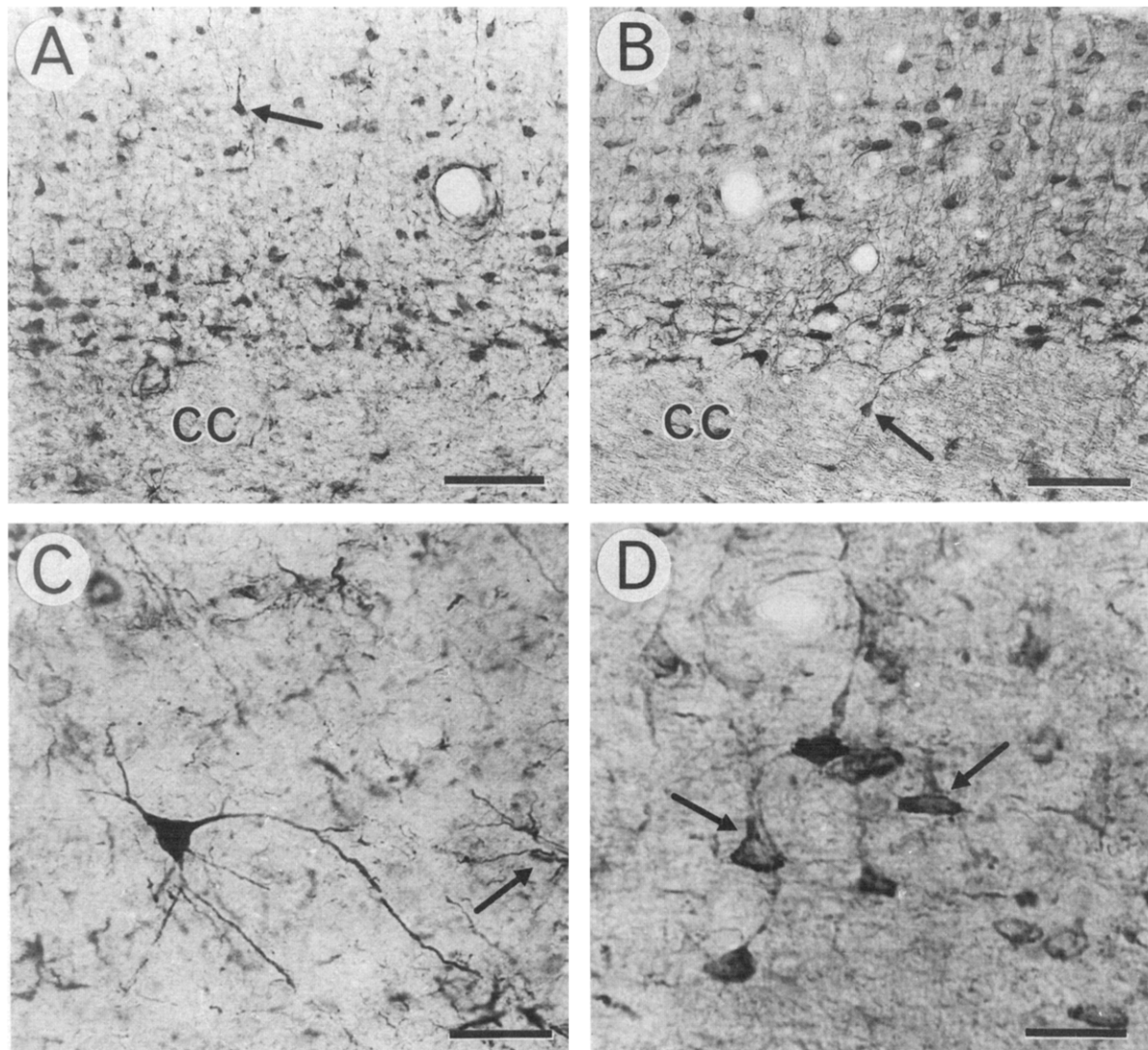


Fig. 2. Photomicrographs of M35 (A,C,D) and WF6 (B) in layer 6 of the parietal cortex. A,B: low power photomicrographs through layer 6, showing multipolar and pyramidal-like cells (arrow in A, for example) and the characteristic rows of muscarinic cholinergic (A) and nicotinic cholinergic (B) neurons just above the corpus callosum (cc). Occasionally, immunopositive neurons were observed embedded within the cc (arrow in B). C: a typical example of a strong M35-immunoreactive multipolar cell in deep layer 6. In this region, also numerous labeled astrocytes can be observed (arrow). D: high power photomicrograph of the pyramidal-like cells (arrows) found throughout the profile of layer 6. Bars in A, B = 125 μm , in C = 60 μm , and in D = 50 μm .

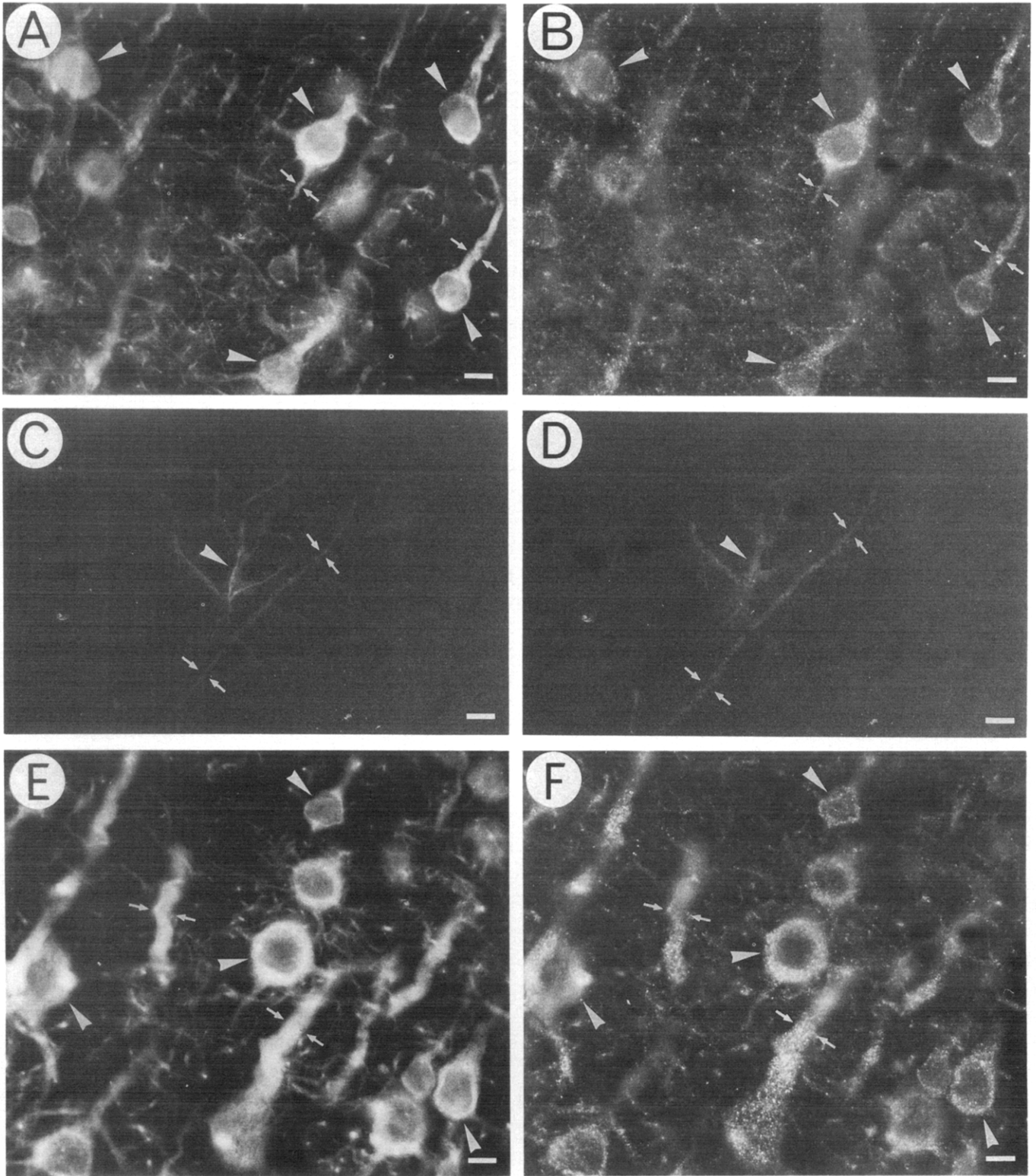


Fig. 3. Colocalization of muscarinic (left panel) and nicotinic (right panel) cholinergic receptors in different layers of the frontal/motor cortex. A,B: nearly all neurons in layer 3 show immunoreactivity for both antibodies. C, D: in layer 1-2, incidentally double-labeled astrocytes were observed in only 2 of 10 animals. In astrocytes, the distribution of nAChRs (D) revealed a more diffuse appearance than mAChRs (C). E,F: clear coexistence of mAChRs (E) and nAChRs (F) was apparent in layer 5. For all photomicrographs, arrowheads point at double-labeled cells. Small arrows point at double-labeled dendritic processes. Bars = 10 μ m.

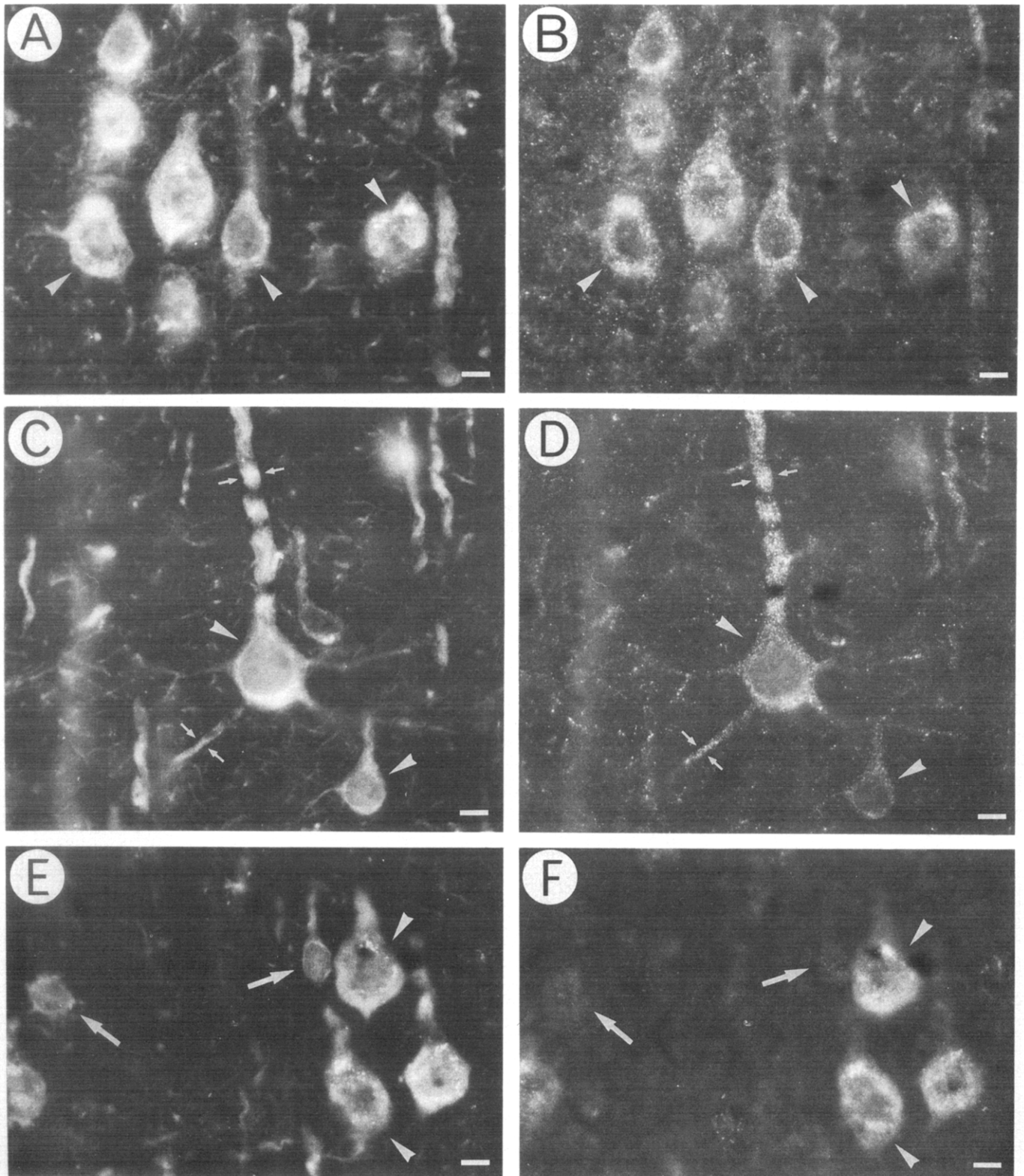


Fig. 4. Pairs of high power photomicrographs showing fluorescent double-labeling for muscarinic (left panel) and nicotinic (right panel) cholinergic receptors in layer 5 of the parietal cortex. Most neurons and dendrites are double-labeled (indicated by arrowheads and small arrows, respectively). Some single-labeled neurons are present as well (large arrows in E; F, single-labeled for mAChRs), most often observed in small ($8\ \mu\text{m}$ on average) neurons. Bars = $10\ \mu\text{m}$.

tion of these nerve cells proved to possess both types of cholinergic receptors.

Degree of colocalization of M35 and WF6

In Table I, the percentages of colocalization are presented for the two cortical areas studied. In these cortices, the middle portions of the cortical layers were quantified. Of the 5 animals used for quantification of the proportion of double-labeled cholinceptive neurons, no differences in degree of colocalization were observed between left and right hemispheres. Furthermore, the percentage of colocalization appeared to be constant for the three coronal levels studied, with only minor variation per cortical strip. The degree of colocalization was consistent for all cortical areas and for nearly all different layers examined. Therefore, the outcome per cortical strips of the individual animals were pooled per layer as well as presented in summation (Table I).

In general, approximately only 1 out of 10 cholinceptive neurons was found to be single-labeled, either for muscarinic receptors (3.0% and 2.6% in frontal/motor and parietal cortex, respectively) or nicotinic receptors (7.2% and 6.3% in frontal/motor and parietal cortex, respectively). The number of WF6 single-labeled neurons was approximately twice the number of M35 single-labeled neurons. The degree of colocalization did not strikingly differ for different cell types. For example, layer 5 of the frontal/motor cortex and parietal cortex contains both pyramidal and non-pyramidal neurons. In layer 5 of the frontal/motor cortex 3203 neurons were studied (Table I), of which 2850 were pyramidal neurons. From this group 2555 (89.6%) was double-labeled. Of the 352 studied non-pyramidal neurons, 310 (88.2%) were double-labeled. In layer 5 of the parietal cortex 2108 neurons were studied (Table I), of which 1729 were of the pyramidal cell type. A total of 1585 (91.7%) was found double-labeled, whereas of the 379 studied non-pyramidal cells, 346 (91.2%) appeared to be double-labeled. The single-labeled neurons were most frequently small-sized pyramidal neurons (8 μm on average) or small-sized non-pyramidal neurons (approximately 10 μm on average).

DISCUSSION

Distribution pattern for M35 and WF6

The distribution of muscarinic and nicotinic cholinceptive neurons in all cortical layers of the rat cerebral cortex in this investigation appeared to be well related to cerebral cholinergic innervation pattern, which is most dense in layers 2–3 and 5 of the frontal/motor cortex and parietal cortex^{11,16,28}. Furthermore, the distribution of muscarinic cholinceptive neurons in the

neocortex of the rat closely resembles that of the cat⁵⁰. In the visual cortex of both species, dense staining of pyramidal cells and dendritic processes was observed in layers 2–3 and 5. Like in cat and human brain material, M35-immunoreactive astrocytes in rat were found amidst cortical cell layers^{42,50}.

With respect to WF6-immunoreactivity, our results closely corroborated the labeling in the occipital cortex described by Schröder and coworkers⁴⁴. In the latter study, clear cortical lamination in WF6 immunoprecipitate and cell typing was described. In addition, the nAChR-localization found by Deutch and colleagues⁹, obtained with a monoclonal antibody produced in mice against affinity-purified nAChR from Torpedo marmorate electric organ membranes and applied to rat brain sections, revealed identical patterns of immunoreactivity. Immunoreactive neurons and associated dendrites were found throughout the cortical mantle in a laminar pattern, being most pronounced in layer 5 and in layers 2–3¹¹.

Like in the rat cerebral cortex, immunostained pyramidal and non-pyramidal perikarya accompanied by their dendritic processes were also observed in the human cerebral cortex for both M35 and WF6^{42,43,44}. The immunoreactivity predominated in pyramidal neurons in layers 5 and 3, although immunopositive non-pyramidal neurons in these layers were found as well. The neuropil displayed fibrillary and punctate structures, probably longitudinally and transversely cut dendritic ramifications^{30,42,47}. These characteristics proved to be very similar for mouse, golden hamster (unpublished data), rat, cat and human.

Fluorescent double-labeling for M35 and WF6 and degree of colocalization

The coexpression of mAChRs and nAChRs was apparent in all cortical regions. In the cortical areas studied, an overall percentage of double-labeled neurons of approximately 90% was observed. Moreover, this percentage appeared to be valid throughout the entire cortical mantle (data not shown). Of the single-labeled cells, slightly higher numbers of neurons were found to be exclusively nicotinic cholinceptive as compared to muscarinic cholinceptivity.

A near total overlap of muscarinic- and nicotinic immunoreactivity in certain parts of the cerebral cortex is not exceptional in itself. In a previous study, we described a near 100% codistribution of both cholinergic receptor markers in the suprachiasmatic nucleus and adjacent region of the rat hypothalamus⁴⁹. By contrast, in a study of cholinergic receptors in the embryonic chick retina, mAChRs and nAChRs occurred essentially on different classes of neurons, showing tissue-dependent

cholinergic receptor characteristics¹⁷. Electrophysiological studies on rat cerebral cortex do not reveal unambiguous data on coexistence of mAChRs and nAChRs within individual cholinergic neurons. Detecting both muscarinic and nicotinic responses after iontophoretically applied ACh is hampered by the experimental conditions needed for recording the nicotinic receptor-induced responses⁵¹. Therefore, the recent findings may be considered as valuable information for the understanding of cholinergic neurotransmission in the neocortex.

Nearly all astrocytes bearing cholinergic receptors appeared to be single-labeled for M35. Only in brain sections of two of the 10 animals studied, occasionally double-labeled astrocytes could be found. The presence of both mAChRs and nAChRs on astroglia, besides several other neurotransmitter receptors and second messenger systems, is well documented^{15,35}. Nevertheless, their role in these non-innervated cells is still a matter of speculation, but may well play a role in transmitter uptake from extracellular space.

WF6/M35 colocalization has recently been performed on human cortex material by Schröder and coworkers^{43,55}. In these studies, identical types of neurons as described in the present study were found to express both classes of cholinergic receptors. However, different percentages of colocalization as compared to the rat were obtained, implying differences between species. Schröder et al. described double-labeled cholinergic neurons comprising 30% of the cholinergic cell group, while 60% and 10% appeared to be single-labeled for M35 and WF6, respectively. In their investigation, however, the authors assumed that for technical reasons the amount of colocalization was slightly underestimated.

Binding sites recognized by M35 and WF6

Currently, the exact epitopes on the cholinergic receptors recognized by M35 and WF6 are unknown, but M35 most likely recognizes a conformational determinant only present on the active receptor^{3,4}, whereas the binding site of WF6 is on the same nicotinic cholinergic subsite as (but differs from) the α -bungarotoxin binding site^{8,52}. Nevertheless, the present results clearly show that most cholinergic cells, visualized under the physiological and biochemical conditions that result from mild tran-

scardial fixation of the receptor proteins, contain binding sites for both M35 and WF6 and should be considered as both muscarinic- and nicotinic cholinergic.

Functional consequences of cholinergic receptor colocalization

Our findings demonstrate a high incidence of colocalization of mAChRs and nAChRs within cholinergic cortical neurons. Muscarinic and nicotinic cholinergic receptors contribute in different ways to the excitability of the cholinergic neurons^{25,34,36,37,45}. However, different studies demonstrated that both receptor classes interact and contribute to learning and memory^{22,23,24,38,46}. For example, Messing and coworkers³² showed that both muscarinic and nicotinic agonists stimulate in an additive way rapid translocation of protein kinase C (PKC), a key molecule in learning and memory processes¹. Preliminary data on PKC/M35-colocalization in rat neocortex, as currently studied in our lab, revealed a high coincidence of mAChRs with PKC in several cortical cell types⁴⁸. By way of PKC stimulation, partly through the action of ACh on nAChRs and mAChRs in the cortex, the cholinergic neuronal cell group in the cortex may be an important substrate for memory processing and storage. A likely site of interaction is within individual cholinergic neurons, since both cholinergic receptor types are highly colocalized.

In Alzheimer's disease (AD), memory impairment has been related to a loss and/or shrinkage of cholinergic neurons in the basal forebrain, depleting the cerebral cortex from its cholinergic input^{14,33,53}. As a consequence to the presynaptic depletion, the cholinergic neurotransmission upon cholinergic neurons will be diminished. The widely accepted pharmacological model of Scopolamine-induced cognitive impairment as a model for AD limits itself to mAChRs. However, ACh as the endogenous transmitter will act on both receptor types. Since this study demonstrates that the muscarinic and nicotinic cholinergic receptors are present in the same cholinergic target cells, a more complete restoring of the cortical cholinergic cell function in learning and memory processes will require drug administration for both receptor subclasses.

REFERENCES

- 1 Akers, R.F., Lovinger, D.M., Colley, P.A., Linden, D.J. and Routtenberg A.. Translocation of protein kinase C activity may mediate hippocampal long-term potentiation, *Science*, 231 (1986) 587-589.
- 2 André, C., De Backer, J.P., Guillet, J.C., Vanderheyden, P., Vauquelin, G. and Strosberg A.D., Purification of muscarinic acetylcholine receptors by affinity chromatography, *EMBO J.*, 2 (1983) 499-504.
- 3 André, C., Guillet, J.G., De Backer, J.P., Vanderheyden, P., Hoebeke, J. and Strosberg A.D., Monoclonal antibodies against the native or denatured forms of muscarinic acetylcholine receptors, *EMBO J.*, 3 (1984) 17-21.
- 4 André, C., Marullo, S., Guillet, J.G., Convents, A., Lauwereys, M., Kaveri, S., Hoebeke, J. and Strosberg A.D., Immunohistochemical studies of the muscarinic acetylcholine receptor, *J. Recept. Res.*, 7 (1987) 89-103.

- 5 Bartus, R.T., Dean, R.L., Beer, B. and Lippa, A.S., The cholinergic hypothesis of geriatric memory dysfunction, *Science*, 217 (1982) 408–417.
- 6 Bartus, R.T., Dean, R.L. and Flicker, C., Cholinergic psychopharmacology: an integration of human and animal research on memory. In H.Y. Meltzer (Ed.), *Psychopharmacology: The third Generation of Progress*, Raven, New York, 1987, pp. 219–232.
- 7 Blozovoski, D., Deficits in passive avoidance learning in young rats following mecamlamine injections in the hippocampal-entorhinal area, *Exp. Brain Res.*, 50 (1983) 442–448.
- 8 Conti-Tronconi, B.M., Diethelm, B.M., Wu, X., Tang, F. and Bertazzon, T., α -Bungarotoxin and the competing antibody WF6 interact with different amino acids within the same cholinergic subsite, *Biochemistry*, 30 (1991) 2575–2584.
- 9 Deutch, A.Y., Holliday, J., Roth, R.H., Chun, L.L.Y. and Hawrot, E., Immunohistochemical localization of a neuronal nicotinic acetylcholine receptor in mammalian brain, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 8697–8701.
- 10 Durkin, T., Central cholinergic pathways and learning and memory processes: presynaptic aspects, *Comp. Biochem. Physiol.*, 93A (1989) 273–280.
- 11 Eckenstein, F.P., Baughman, R.W. and Quinn, J., An anatomical study of cholinergic innervation in rat cerebral cortex, *Neuroscience*, 25 (1988) 457–474.
- 12 Fels, G., Plümer-Wilk, R., Schreiber, M. and Maelicke, A., A monoclonal antibody interfering with binding and response of the acetylcholine receptor, *J. Biol. Chem.*, 261 (1986) 15746–15754.
- 13 Flicker, C., Dean, R.L., Watkins, D.L., Fisher, S.K. and Bartus, R.T., Behavioral and neurochemical effects following neurotoxic lesions of a major cholinergic input to the cerebral cortex in the rat, *Pharmacol. Biochem. Behav.*, 18 (1985) 973–981.
- 14 Gaykema, R.P.A., Nyakas, C., Horvath, E., Hersh, L.B., Matjenyi, C. and Luiten, P.G.M., Cholinergic fiber aberrations in nucleus basalis lesioned rats and Alzheimer's disease, *Neurobiol. Aging*, in press.
- 15 Hösl, L., Hösl, E., Della Briotta, G., Quadri, L. and Heuss, L., Action of acetylcholine, muscarine, nicotine and antagonists on the membrane potential of astrocytes in cultured rat brainstem and spinal cord, *Neurosci. Lett.*, 92 (1988) 165–170.
- 16 Houser, C.R., Crawford, G.D., Salvaterra, P.M. and Vaughn, J.E., Immunocytochemical localization of choline acetyltransferase in rat cerebral cortex: a study of cholinergic neurons and synapses, *J. Comp. Neurol.*, 234 (1985) 17–34.
- 17 James, W.M. and Klein, W.L., Localization of acetylcholine receptors on isolated CNS neurons: cellular and subcellular differentiations, *J. Neurosci.*, 8 (1988) 4225–4238.
- 18 Juliano, S.L., Ma, W., Bear, M.F. and Eslin, D., Cholinergic manipulation alters stimulus-evoked metabolic activity in cat somatosensory cortex, *J. Comp. Neurol.*, 297 (1990) 106–120.
- 19 Kelly, J.S. and Rogawski, M.A., Acetylcholine. In M.A. Rogawski and J.L. Barker (Eds.), *Neurotransmitter Actions in the Vertebrate Nervous System*, Plenum, New York, 1985, pp. 143–197.
- 20 Kopelman, M.D. and Corn, T.H., Cholinergic 'blockade' as a model for cholinergic depletion, *Brain*, 111 (1988) 1079–1110.
- 21 Kristt, D.A., McGowan, Jr. R.A., Martin-MacKinnon, N. and Solomon, J., Basal forebrain innervation of rodent neocortex: studies using acetylcholinesterase histochemistry, Golgi and lesion strategies, *Brain Res.*, 337 (1985) 19–39.
- 22 Levin, E.D., McGurk, S.R., South, D. and Butcher, L.L., Effects of combined muscarinic and nicotinic blockade on choice accuracy in the radial-arm maze, *Behav. Neural Biol.*, 51 (1989) 270–277.
- 23 Levin, E.D., Rose, J.E., McGurk, S.R. and Butcher, L.L., Characterization of the cognitive effects of combined muscarinic and nicotinic blockade, *Behav. Neural Biol.*, 53 (1990) 103–112.
- 24 Levin, E.D. and Rose, J.E., Nicotinic and muscarinic interactions and choice accuracy in the radial-arm maze, *Brain Res. Bull.*, 27 (1991) 125–128.
- 25 Luetje, C.W., Patrick, J. and Séguéla, P., Nicotine receptors in the mammalian brain, *FASEB J.*, 4 (1990) 2753–2760.
- 26 Luiten, P.G.M., Gaykema, R.P.A., Traber, J. and Spencer, Jr. D.G., Cortical projection patterns of the magnocellular basal nucleus subdivisions as revealed by anterogradely transported Phaseolus vulgaris leucoagglutinin, *Brain Res.*, 413 (1987) 229–250.
- 27 Luiten, P.G.M., Spencer, Jr. D.G., Traber, J. and Gaykema, R.P.A., The pattern of cortical projections from the intermediate parts of the magnocellular nucleus basalis in the rat demonstrated by tracing with *Phaseolus vulgaris* leucoagglutinin, *Neurosci. Lett.*, 57 (1985) 137–142.
- 28 Lysakowski, A., Wainer, B.H., Bruce, G. and Hersh, L.B., An atlas of the regional and laminar distribution of choline acetyltransferase immunoreactivity in rat cerebral cortex, *Neuroscience*, 28 (1989) 291–336.
- 29 Ma, W., Höhmann, C.F., Coyle, J.T. and Juliano, S.L., Lesions of the basal forebrain alter stimulus-evoked metabolic activity in mouse somatosensory cortex, *J. Comp. Neurol.*, 288 (1989) 414–427.
- 30 Matsuyama, T., Luiten, P.G.M., Spencer, Jr D.G. and Strosberg, A.D., Ultrastructural localization of immunoreactive sites for muscarinic acetylcholine receptor proteins in the rat cerebral cortex, *Neurosci. Res. Commun.*, 2 (1988) 69–76.
- 31 Mayo, W., Kharouby, M., Le Moal, M. and Simon, H., Memory disturbances following ibotenic acid injections in the nucleus basalis magnocellularis of the rat, *Brain Res.*, 455 (1988) 213–222.
- 32 Messing, R.O., Stevens, A.M., Kiyasu, E. and Sneade, A.B., Nicotinic and muscarinic agonists stimulate rapid protein kinase C translocation in PC12 cells, *J. neurosci.*, 9 (1989) 507–512.
- 33 Mesulam, M.M., Mufson, E.J. and Rogers, J., Age-related shrinkage of cortically projecting cholinergic neurons: a selective effect, *Ann. Neurol.*, 22 (1987) 31–36.
- 34 Metherate, R., Tremblay, N. and Dykes, R.W., Acetylcholine permits long-term responsiveness in cat primary somatosensory cortex, *Neuroscience*, 22 (1987) 73–81.
- 35 Murphy, S. and Pearce, B., Functional receptors for neurotransmitters on astroglial cells, *Neuroscience*, 22 (1987) 381–394.
- 36 Nathanson, N.M., Molecular properties of the muscarinic acetylcholine receptor, *Annu. Rev. Neurosci.*, 10 (1987) 195–236.
- 37 Rasmusson, D.D. and Dykes, R.W., Long-term enhancement of evoked potentials in cat somatosensory cortex produced by co-activation of the basal forebrain and cutaneous receptors, *Exp. Brain Res.*, 70 (1988) 276–286.
- 38 Riekkinen, Jr. P., Sirviö, J., Aaltonen, M. and Riekkinen, P., Effects of concurrent manipulations of nicotinic and muscarinic receptors on spatial and passive avoidance learning, *Pharmacol. Biochem. Behav.*, 37 (1990) 405–410.
- 39 Riekkinen, Jr. P., Koivisto, E., Sirviö, J. and Riekkinen, P., Joint modulation of neocortical electrical activity by nicotinic and muscarinic receptors, *Brain Res. Bull.*, 27 (1991) 137–139.
- 40 Santucci, A.C. and Haroutunian, V., Nucleus basalis lesions impair memory in rats trained on nonspatial and spatial discrimination tasks, *Physiol. Behav.*, 45 (1989) 1025–1031.
- 41 Saper, C.B., Organization of cerebral cortical afferent systems in the rat. II. Magnocellular basal nucleus, *J. Comp. Neurol.*, 222 (1984) 313–342.
- 42 Schröder, H., Zilles, K., Luiten, P.G.M. and Strosberg, A.D., Immunocytochemical visualization of muscarinic cholinergic receptors in the human cerebral cortex, *Brain Res.*, 514 (1990) 249–258.
- 43 Schröder, H., Zilles, K., Luiten, P.G.M., Strosberg, A.D. and Aghchi, A., Human cortical neurons contain both nicotinic and muscarinic acetylcholine receptors: an immunocytochemical double-labeling study, *Synapse*, 4 (1989) 319–326.
- 44 Schröder, H., Zilles, K., Maelicke, A. and Hajós, F., Immunohisto- and cytochemical localization of cortical nicotinic cholinergic receptors

- noceptors in rat and man, *Brain Res.*, 502 (1989) 287–295.
- 45 Strosberg, A.D., Structure function relationship of proteins belonging to the family of receptors coupled to GTP binding proteins, *Eur. J. Biochem.*, 196 (1991) 1–10.
- 46 Tilson, H.A., McLamb, R.L., Shaw, S., Rogers, B.C., Peditakis, P. and Cook, L., Radial-arm maze deficits produced by colchicine administered into the area of the nucleus basalis are ameliorated by cholinergic agents, *Brain Res.*, 438 (1988) 83–94.
- 47 Van der Zee, E.A., Matsuyama, T., Strosberg, A.D., Traber, J. and Luiten, P.G.M., Demonstration of muscarinic acetylcholine receptor-like immunoreactivity in the rat forebrain and upper brainstem, *Histochemistry*, 92 (1989) 475–485.
- 48 Van der Zee, E.A., Cazaubon, S. and Luiten, P.G.M., Colocalization of muscarinic cholinergic receptors with protein kinase C isozymes in the rat neocortex, Abstr. XIth Int. Congr. Pharmacol., *Eur. J. Pharmacol.*, 183 (1990) 751.
- 49 Van der Zee, E.A., Streefland, C., Strosberg, A.D., Schröder, H. and Luiten, P.G.M., Colocalization of muscarinic and nicotinic receptors in cholinceptive neurons of the suprachiasmatic region in young and aged rats, *Brain Res.*, 542 (1991) 348–352.
- 50 Van Huizen, F., Strosberg, A.D. and Cynader, M.S., Cellular and subcellular localisation of muscarinic acetylcholine receptors during postnatal development of cat visual cortex using immunocytochemical procedures, *Dev. Brain Res.*, 44 (1988) 296–301.
- 51 Vidal, C. and Changeux, J.-P., Pharmacological profile of nicotinic acetylcholine receptors in the rat prefrontal cortex: an electrophysiological study in a slice preparation, *Neuroscience*, 29 (1989) 261–270.
- 52 Watters, D. and Maelicke, A., Organization of ligand binding sites at the acetylcholine receptor: a study with monoclonal antibodies, *Biochem.*, 22 (1983) 1811–1819.
- 53 Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T. and DeLong, M.R., Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain, *Science*, 215 (1983) 1237–1239.
- 54 Zilles, K., *The Cortex of the Rat. A Stereotaxic Atlas*, Springer, Berlin, 1983.
- 55 Zilles, K., Schröder, H., Schröder, U., Horvath, E., Werner, L., Luiten, P.G.M., Maelicke, A. and Strosberg, A.D., Distribution of cholinergic receptors in the rat and human neocortex. In M. Frotscher and U. Misgeld (Eds.), *Central Cholinergic Synaptic Transmission*, Birkhäuser, Berlin, 1989, pp. 212–228.