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Short communication

Ultrastructural localization of cholinergic muscarinic receptors in rat brain cortical capillaries

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Abstract

Cholinergic innervation of the cerebrovasculature is known to regulate vascular tone, perfusion rate and permeability of the microvascular wall. Notably the cholinergic innervation of cerebral capillaries is of interest since these capillaries form the blood–brain barrier. Although there is a general consensus as to the presence of nicotinic and muscarinic receptors in the domain of the capillary wall, their precise anatomical position is unknown. The subcellular localization of muscarinic receptors in rat cortical capillaries was approached by way of immunocytochemistry at the ultrastructural level using monoclonal antibody M35 against muscarinic receptor protein. Binding of this antibody in the microvascular domain was found in 5% of the capillaries studied and was exclusively present in perivascular astroglia, and never in endothelium or pericytes. Combined with reported data on presynaptic cholinergic innervation the results indicate a cholinergic innervation pattern of non-directed presynaptic terminal structures in apposition to cholinergic perivascular astroglia with muscarinic receptor positive endfeet embracing the capillary basement membrane. The possible functional significance of such a cholinergic vascular innervation pattern is discussed with respect to capillary dynamics and barrier function.

Keywords: Electron microscopy; Acetylcholine; Astroglia; Muscarinic receptor; Microvessel

Considerable evidence implicates acetylcholine in the regulation of cerebral vascular dynamics and permeability. However, in spite of a large number of studies on various aspects of cholinergic innervation of cerebral vessels, our understanding of the structural and functional features of cholinergic microvascular regulation is still far from complete. The growing interest for cerebral capillaries is partly based on the notion that they collectively constitute the large mass of the fine vascularization of the brain and form the structural basis of the blood–brain barrier. It is mainly the condition and integrity of the capillary wall and the regulation of its properties that determine the effectiveness of the blood–brain interface. Activation of the forebrain cholinergic innervation, which appears to be of either basal forebrain or intracortical origin [7,9,24,25], yields vasodilatory effects, increases cortical blood flow and influences permeability of the blood–brain barrier [1,7,11,31]. Pharmacological modulation of such responses implicate mus-

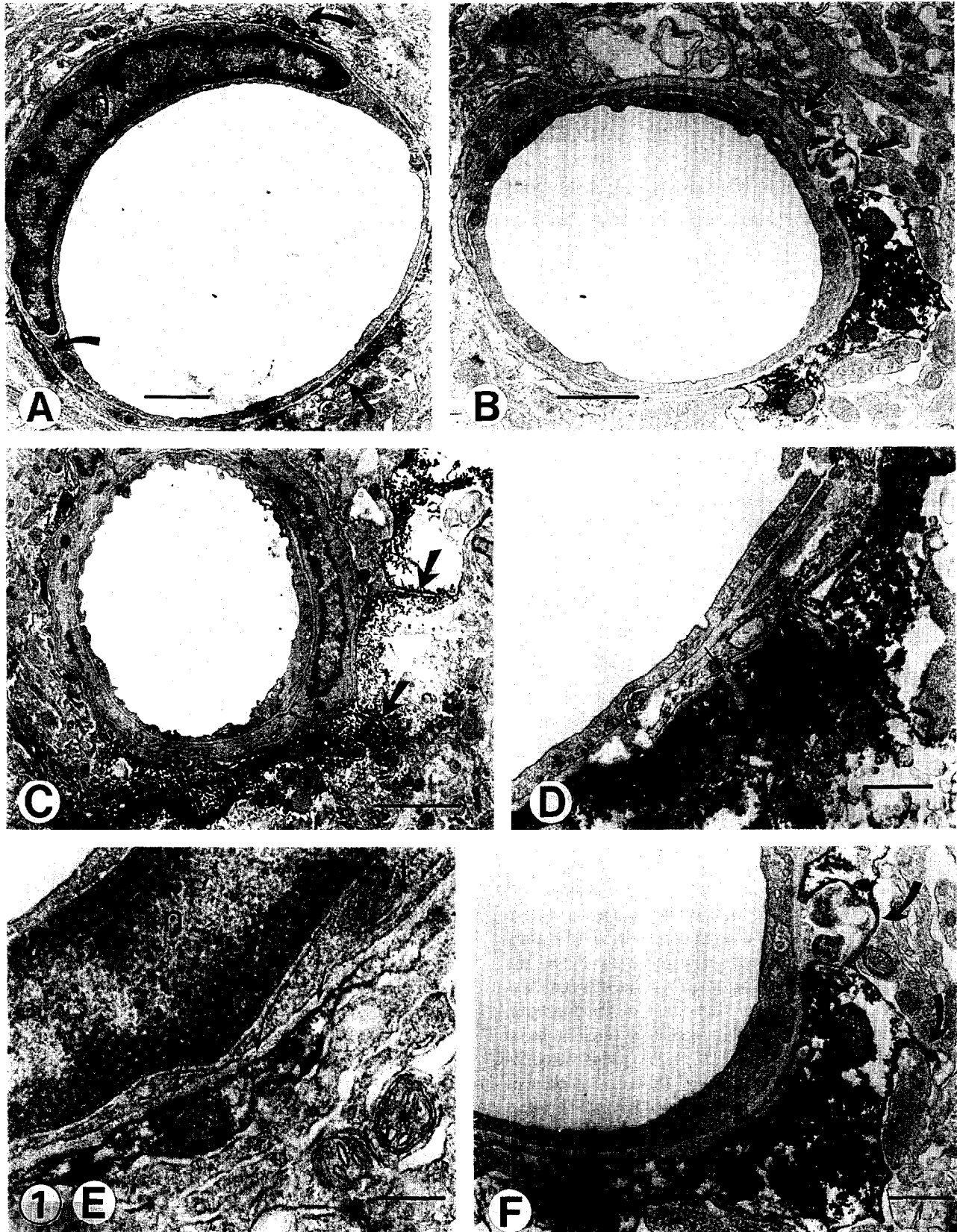
carinic receptors in the cerebrovascular environment [1,12,16,24,30].

Structural aspects of the cholinergic innervation of cerebral vasculature have been characterized by a variety of anatomical, biochemical and ligand binding studies. This evidence may be summarized as the presence of the acetylcholine biosynthetic enzyme choline acetyltransferase (ChAT) in nerve endings that approach the capillary, however, without making direct contact to the endothelium [3,4,10,13]. To further complicate matters, ChAT was not only localized in nerve endings, but also in part of the microvascular endothelial cells [4,29]. The latter was, however, seriously questioned by others [10,22]. Furthermore, there is a general consensus as to the presence of capillary-associated cholinergic receptors, where both muscarinic and nicotinic receptors have been identified [14,16,21,34]. However, detailed knowledge on the nature of cholinergic structures, either vascular or perivascular, is lacking. Also postsynaptic specializations comparable to receptor-associated membrane densities were never found in the endothelial cells, pericytes and astrocytes that characteristically constitute the perivascular domain.

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In summary, the precise localization of acetylcholine receptors within the capillary bed is not known. Such knowledge, however, is of importance to understand the

anatomical organization of cerebrovascular cholinergic innervation and the potential mechanisms of its function, its role in cerebrovascular pathology, and effects of cholinergic



gic drug treatments. To answer this question, the anatomical localization of mAChR in rat cortical capillaries was studied employing the monoclonal antibody M35 against purified muscarinic receptor proteins [2,26,27,34].

The present study was carried out on the cerebral cortical front-limb motor area in the dorsofrontal parietal region [38] of six 3–4 month old male Wistar rats. The animals were deeply anaesthetized with sodium pentobarbital (60 mg/kg b.w., i.p.) and transcardially perfused with Zamboni fixative containing 2% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer. Vibratome sections at a thickness of 50 μm were thoroughly rinsed in phosphate buffered saline, the solvent for all following steps, and subsequently incubated with 0.1% H_2O_2 and 10% normal rabbit serum. This preincubation was followed by exposure to subsequently: monoclonal antibody M35 kindly donated by Prof. A.D. Strosberg, Paris (mouse anti-mAChR, IgM, 1:1000, overnight, 4°C), biotinylated rabbit anti-mouse IgM (Zymed, 1:200, 4 h, room temperature, RT) and HRP conjugated streptavidin (Zymed, 1:200, 2 h, RT). The HRP label was visualized by standard reaction with DAB and hydrogen peroxide in Tris buffer. The antibody M35 is a monoclonal IgM raised against purified muscarinic receptor protein from bovine forebrain homogenates. M35 was recently shown in transfected cell lines to be non-specific for muscarinic subtypes m1–m5 [8]. For further details see procedure descriptions in Van der Zee et al. [34,35]. Control sections were processed in a similar fashion but with omission of the primary antibody step or by replacement with a non-matching primary antibody. After immunocytochemical staining, selected pieces of cortical tissue, including control sections, were stained en bloc in 1% OsO_4 and uranylacetate, dehydrated and flat-embedded in epoxy resin and cut to ultrathin sections. The sections were collected on grids, contrasted with lead citrate and uranyl acetate and examined in a Philips 201 electron microscope.

In general, as extensively described in previous papers, immunoreactivity to M35 in the neocortex at light microscopic levels is present in various cell groups. These cell groups include major pyramidal cell populations in layers III and V, several non-pyramidal cell types in all layers except layer I, and in astroglia. Immunostaining in glial cells was present in all cortical layers, but was most prominent in the more superficial layers I–III. Astrocytic staining could easily be distinguished from neuronal pre-

cipitate by the characteristic irregular and darkly stained shape of the glial processes [6,34].

For unequivocal identification of capillary-related immunoreactivity of muscarinic receptor protein, the distribution and localization of the precipitates were studied in greater detail with electron microscopic resolution. This way a total of 800 capillaries were investigated in all cortical layers. In 43 vessels a positive immunoreaction was observed directly associated with structures of the capillary wall, which consists of a characteristic endothelial cell with its flattened nucleus, a thin basement membrane layer that commonly may be enclosing pericytes (Fig. 1A). Without exception immunostaining of M35 was exclusively localized in the perivascular astrocytes and not in endothelium, basement membrane or pericytes. In particular, the astrocytic endfeet in apposition to the basement membrane surrounding the endothelium were found to show immunoprecipitate for muscarinic receptor protein (Fig. 1D–F). The labelled vessel-contacting astrocytes were easily distinguished from neuronal labelling. The astrocytes revealed a characteristic irregular appearance and a conspicuous lack of typical neuronal specializations such as synaptic vesicles and synaptic membrane densities. In case of perivascular labelling, the immunoprecipitate was limited to individual glial cells and did not continue in adjacent astrocytic labelling thus yielding an often discontinuous staining pattern (Fig. 1B). Within the astrocytic structure there was an obvious lack of polarity of the immunolabel; the entire structure being marked with the immunoprecipitate. The labelled astrocytes were also devoid of any membranous densities that in neurons are associated with sites for receptor localizations in synaptic contacts as is generally also the case in dendritic postsynaptic labelling with M35 [26,27]. It may be concluded that labelling with the anti-mAChR antibody is confined to astrocytes and their processes in direct contact with the basement membrane in approximately 5% of the studied vessels.

A clear finding of the present study is that within the capillary complex binding of the mAChR protein antibody M35 is confined to the perivascular astrocytic endfeet, whereas no reactivity was present in the endothelial cells or pericytes, nor any non-cellular component of the capillary wall. In general the current results confirm several previous investigations demonstrating muscarinic receptors in microvascular preparations of the forebrain [15,21,30].

Fig. 1. A: survey of a cortical capillary with a smooth and thin layer of cytoplasm in which several mitochondria and a large flattened nucleus can be identified. The endothelium is surrounded by a thin basement membrane. Several immunolabelled astrocytic processes are present in apposition to the basement membrane (large arrows). B: astrocyte immunoreactive for muscarinic receptor protein. A large darkly labelled endfeet is connected by fine processes (large arrows) to extensions protruding into the neuropil. C: large astrocytic complex with separate mAChR immunostained compartments in direct contact with the perivascular basement membrane. The compartments are separated by fingershape extensions of the basement membrane (arrows). D: the basement membrane extensions as in C are indicated here at a higher magnification. Mitochondria are marked with an asterisk. E: higher magnification of an M35-positive astrocytic process juxtaposing the endothelium surrounding basement membrane (small arrows). F: further magnification of the same labelled astrocyte as in B illustrating the basement membrane contacting endfeet and fine immunoreactive processes (arrows). Scale bar in A and B = 1 μm , in C = 2.5 μm , in D and F = 0.5 μm and in E = 0.25 μm . Abbreviations: n. nucleus; cyt, cytoplasm.

However, reports that mention mAChR specifically in endothelial cells could not be confirmed. This discrepancy may be related to the type of cerebral vessel studied [14,33]. We did not detect any M35 immunoreactivity in the endothelium of 800 capillaries, in which the endothelial cell plays an important role in the permeability properties of the blood–brain barrier. It should be noted, however, that also in the occasionally encountered arterioles, the endothelial cytoplasm was devoid of immunoprecipitate [32]. The possible technical pitfalls in our immunocytochemical study is minuted by the recent observation that the binding sensitivity of M35 is not different for the m1 (predominantly found in brain) and the m3 (endothelium) muscarinic receptor subtype [8]. Only the possibility that the amount of mAChRs in the endothelium is too low to detect cannot be excluded.

In case of microvascular ligand binding studies the endothelial localization may well be attributed to binding at glial perivascular structures [28,37]. The presence of functional AChRs in astrocytes was previously demonstrated in cultured astroglia by ligand binding, mAChR immunocytochemistry [17] and electrophysiological methods [18], and in brain tissue by use of specific muscarinic antibody binding [34]. Perivascular astrocytes, moreover, are endowed with protein kinase C [23] and respond with Ca^{2+} elevation after cholinergic stimulation [19], which supports a biochemical pathway to mediate muscarinic responses in the receptive astroglia. The localization of mAChR in perivascular astrocytes, as shown in the present study, matches particularly well with the detailed analysis of Hamel and coworkers of the microvascular innervation with the presynaptic cholinergic marker choline acetyltransferase [10,36]. Combined with their anatomical findings it appears obvious that cholinergic nerve terminals are in close contact with cholinceptive astrocytic endfeet, and that the cholinceptive astroglial cells may play an important yet undefined role in the neurogenic control of capillary function. This is further strengthened by a recent tracing study of Vaucher and Hamel who showed that generally all (cholinergic and non-cholinergic) nerve terminal from basal forebrain neurons apposing the capillary wall in the parietal cortex are separated from the basement membrane by a (sometimes very thin) glial leaflet [36]. In this respect it has long been known that the permeability condition of the microvascular endothelium of the brain capillaries is under influence of the surrounding astroglia [20]. Since no membrane specializations (e.g. synaptic contacts) are present between the cholinergic terminal [10] and adjacent muscarinic receptor-positive astrocyte (present study), the exact pathway by which release of ACh and activation of mAChR leads to changes in microvascular diameter or permeability remains unclear.

Alternatively, it may be considered that mAChR in the perivascular domain serve other functions than those related to regulation of vascular tone and permeability. Such alternatives are supported by various observations. Im-

munoreactivity to ChAT in endothelial cells has been reported [4], which is however not supported by others [10,22]. Therefore, the possibility of endothelium to potentially produce ACh is not likely. One can envisage, however, that besides a vascular-activating function of ACh, muscarinic receptors in perivascular astrocytes do serve as a scavenger for undesired ACh from circulation to the neuropil, or reversely from neuronal release to blood stream. In that sense astrocytes with mAChR may act as a barrier for excess ACh between circulation and neuropil, or vice versa, similar to its function in potassium or glutamate buffering [5]. In this line of reasoning it may well be that such a scavenger function of perivascular astrocytes is not limited to ACh but to a variety of neuroactive substances for which they may possess receptors. Second, we observed in light microscopic studies that muscarinic receptor immunoreactivity in astrocytes increases in aging rats [34] and even more after experimental damage of brain tissue [6,8]. It appears that this increased mAChR expression indicated by increased antibody binding reveals plastic changes that coincide with general glial activation reported for the above-mentioned conditions.

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