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Invited Review

Architecture of the mammalian pituitary cholinergic system with observations on a putative blood acetylcholine sensor

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Summary. Acetylcholine (ACh) plays an important role in pituitary gland function. Little is known, however, about the source and trajectory of pituitary ACh, the location of pituitary cholinergic receptors, and the pathways along which the release of pituitary ACh is controlled. Therefore choline acetyltransferase (ChAT) immunoreactive profiles have been investigated in the rat median eminence and pituitary. Furthermore, both muscarinic- (mAChRp-L) and nicotinic receptor protein-like (nAChRp-L) immunoreactivity have been examined in the rat, rabbit, and cat pituitary. The results have demonstrated that the rat pituitary ChAT network is composed of neurons in the hypothalamic arcuate nucleus and a great number of terminals in the median eminence. In the pituitary, ChAT immunolabeled profiles were virtually absent. This suggests that much of the ACh acting on pituitary cells is released as a humoral factor from the median eminence. All the examined animals expressed mAChRp-L immunostained endocrine cells in the intermediate lobe. Apart from this, marked species differences in AChRp-L immunolabeled profiles have been found. In addition, strong mAChRp-L immunoreactive rod to cone-shaped bodies were detected associated with blood vessels of the anterior and intermediate lobes in the rat and rabbit, but not in the cat. The immunolabeling was present in particles on the body plasma membrane. These characteristics suggest that the function of these structures might be to sense pituitary blood ACh levels. Consequently the name *blood acetylcholine reading bodies* (BARBs) was adopted to indicate these structures. It is proposed that the BARBs may play a role in the feedback control of ACh release from the median eminence.

Key words: Hypophysis, Choline acetyltransferase, Cholinergic receptors, Blood acetylcholine reading body (BARB), Immunohistochemistry

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Histological classification of the pituitary

Traditionally the mammalian pituitary gland is divided into three parts, i.e., the posterior, intermediate, and anterior (including the pars tuberalis) lobes. This macro-architecture can be observed with the naked eye in aldehyde-fixed preparations, from larger animals, because of the different tissue texture of these parts. In each species the dimensions of each lobe may be different. For instance, in sections prepared in a mid-horizontal plane relative to the pituitary position *in situ*, the rat posterior lobe is relatively small, and situated in the center of the tissue slice. The intermediate lobe surrounds the posterior lobe as a thin sheath of cell islands. The anterior lobe occupies most of the pituitary gland as two large wings lateral to the intermediate lobe. In a similar section taken from the rabbit pituitary the intermediate lobe is a distinguishing feature. It penetrates deep into the anterior lobe, almost dividing this part of the pituitary into two halves. In the cat, on the other hand, the pituitary gland shows a relatively large posterior lobe, and two small wings of tissue on either side of the intermediate lobe form the anterior pituitary (for a review on the comparative anatomy of the pituitary gland see Harris and Donovan, 1966).

The modern understanding of pituitary histology is based on ultrastructural and (immuno)histochemical studies. The posterior lobe is primarily composed of afferent fibers and pituicytes, glial-like interstitial cells. Proteins synthesized by the hypothalamic magnocellular paraventricular (PVN) and supraoptic (SON) nuclei, e.g. vasopressin (AVP) and oxytocin (OXT), are transported along the axons to this part of the pituitary, and released at neurovascular endings and synaptoid contacts with pituicytes. The regulation of AVP and OXT release from the posterior lobe is mediated through a long-loop central nervous system (CNS) feedback circuit to the PVN and SON (e.g. Hatton and Walters, 1973), and locally via the pituicytes (see Boersma et al., 1993 and references therein). The exact nature of these

mechanisms, the local one in particular, are not well understood. The intermediate lobe is composed predominantly of endocrine and stellate cells. The former synthesize the precursor protein pro-opiomelanocortin, among other proteins, from which adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), ß-endorphin and other endogenous opiates are generated (e.g. Scott et al., 1976; Gianoulakis et al., 1979; Mains and Eipper, 1979). Apart from a long-loop CNS feedback circuit, hormone release is regulated locally by its own secretion (autoregulation) (e.g. Motta et al., 1965; Boscaro et al., 1988) via receptors which are present on the surface of the endocrine cells. The anterior lobe consists primarily of endocrine and folliculo-stellate (FS, a glial-like interstitial cell) cells. The endocrine cells synthesize, among others, ACTH, growth hormone (GH), thyroid-stimulating hormone (TSH), gonadotropin hormones (GnH), and prolactin (PRL). The FS cell is regarded as the intrinsic source of pituitary interleukin-6 (e.g. Vankelecom et al., 1993). Feedback control of anterior pituitary hormone secretion is established through the long-loop CNS circuit, autoregulation, and intercellular communication between different endocrine cells, and between endocrine and FS cells (e.g. Herbert, 1979; Denef and Andries, 1983; Allaerts et al., 1990).

Evidence for acetylcholine involvement in pituitary function

In the rat, the pituitary rank order of acetylcholine (ACh) concentration is: first the posterior lobe, followed by the intermediate lobe and, by a small margin, the anterior lobe (Egozi et al., 1988). It is not known if this rank order is a general phenomenon throughout mammalian species. In the rabbit posterior lobe, ACh is localized in nerve terminal vesicles (Lederis and Livingston, 1969, 1970) that may have issued from the hypothalamic arcuate nucleus (Tago et al., 1987). In the sheep posterior lobe a low density of muscarinic binding sites has been observed using autoradiography (Tolliver et al., 1981). Direct injection of ACh, or carbachol, into the rat posterior lobe *in vivo* or *in vitro* stimulates the release of AVP and OXT (Gosbee and Lederis, 1972; Gregg, 1985). ACh, choline acetyltransferase (ChAT), and acetylcholine esterase (AChE) activity has also been found in the intermediate lobe of the rat, rabbit, and domestic pig (Bridges et al., 1973; Gallardo et al., 1980; Barron and Hoover, 1983). In the frog intermediate lobe, muscarinic receptors (mAChRs) have been visualized by immunohistochemistry, whereas nicotinic receptors (nAChRs) have been detected by physiological methods (Lamacz et al., 1989). The latter type of receptor is present in the pig intermediate lobe as well (Zhang and Feltz, 1990). ACh application to frog intermediate lobe cells *in vitro* increases the secretion of α-MSH (Lamacz et al., 1989; Louiset et al., 1990). In the rat anterior lobe ACh receptors (AChRs), as well as ACh release, degradation and biosynthesis *in vitro*, have been reported (see Bridges et al., 1973; Carmeliet and Denef, 1988; 1989). This system, presumably acting via mAChRs, stimulates TSH and GH secretion and inhibits ACTH and PRL release (Rudnick and Dannies, 1981; Carmeliet and Denef, 1988).

Materials and methods

Twenty-two Wistar rats, twelve Dutch belted rabbits, three *specific pathogen free* (SPF) New Zealand White rabbits, and four domestic cats have been used. All animals were adult, kept in normal cyclic light, and had free access to food and water. They were treated in agreement with the ethical considerations included in the European Convention for the Protection of Vertebrate Animals. Light and electron microscopic analyses have been performed. For light microscopy the animals were anesthetized with veterinary Nembutal, prerinsed with saline, and perfused either with a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.05M cacodylate buffer, pH 7.4, or with 6% paraformaldehyde and 0.1% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4. The first fixative was used for rat tissue to be subjected to ChAT immunolabeling, and the second fixative for tissue to be subjected to cholinergic receptor protein (AChRp) immunostaining. After perfusion fixation the pituitary glands were removed and postfixed for two hours in the same fixative. From this material 60 µm vibratome or 40 µm cryostat sections were cut and collected in 0.05M Tris-buffered saline pH 7.6 (TBS).

ChAT immunoreactive profiles in the rat pituitary system have been analysed using the primary antibody Ab17 (1:500, kindly provided by Prof. C. Cozzari, Rome, Italy). This antibody has a binding affinity (Kd) of 3.10^{11}M to rat ChAT (Cozzari, personal communication). The labeling of cholinergic cells and fibers in the rat CNS by this antibody was reported previously (e.g. Vetter et al., 1993; Umbriaco et al., 1994). In order to label muscarinic receptor protein (mAChRp) the antibody M35 (1:1000, Chemunex, France), raised against affinity purified calf forebrain mAChRp, was used. The production and properties of this antibody have been described earlier (André et al., 1984; Van Huizen et al., 1988; Van der Zee et al., 1989; Schröder et al., 1990). To detect nicotinic receptor protein (nAChRp) the antibody WF6 (1,4 E280; 05-04-91), (1:200, generously donated by Prof. A. Maelicke, Mainz, Germany) was used. This antibody was raised against nAChRp purified from membrane fragments of *Torpedo marmorate* electric organ. The production and properties of this antibody have been described in detail elsewhere (Maelicke et al., 1988, 1989; Conti-Tronconi et al., 1991; Van der Zee et al., 1992). For ChAT immunolabeling the antibodies were dissolved in TBS and 0.5% Triton X-100. For AChRp immunolabeling the antibodies were dissolved in TBS. Sections were incubated with the primary antibody solution for 2 to 4 days. Control staining was achieved by omitting the primary antibody from the solvent. In both cases tissue

was further processed by incubating the sections with the appropriate biotinylated secondary antibody and, thereafter, the avidin-biotin complex (ABC) to which peroxidase was conjugated. The peroxidase activity was detected using 3,3'-diaminobenzidine (DAB) as the chromogen. Subsequently the sections were rinsed in 0.1M phosphate buffer, mounted on glass slides, dehydrated in an ethanol series, cleared in xylene and coverslipped.

For electron microscopy the tissue was treated as described above except that a DAB-cobalt intensification was used instead of DAB. Hereafter the sections were rinsed in 0.1M phosphate buffer and inspected for the presence of labeling. The tissue parts of interest were dissected and put in 1.5% osmium tetroxide overnight. Subsequently the osmicated sections were dehydrated and embedded in plastic. Two to four semithin sections were made, using a diamond knife on a Reichert-Jung ultratome, to pre-evaluate the tissue. The rest of the plastic-embedded tissue was trimmed and cut into ultrathin sections of which some were contrasted with lead citrate and uranyl acetate. All ultrathin sections were viewed with a Philips CM 100 electron microscope.

Choline acetyltransferase profiles in the rat pituitary system

The antibody Ab17 always produced a clear staining of cholinergic profiles throughout the rat CNS. It did not

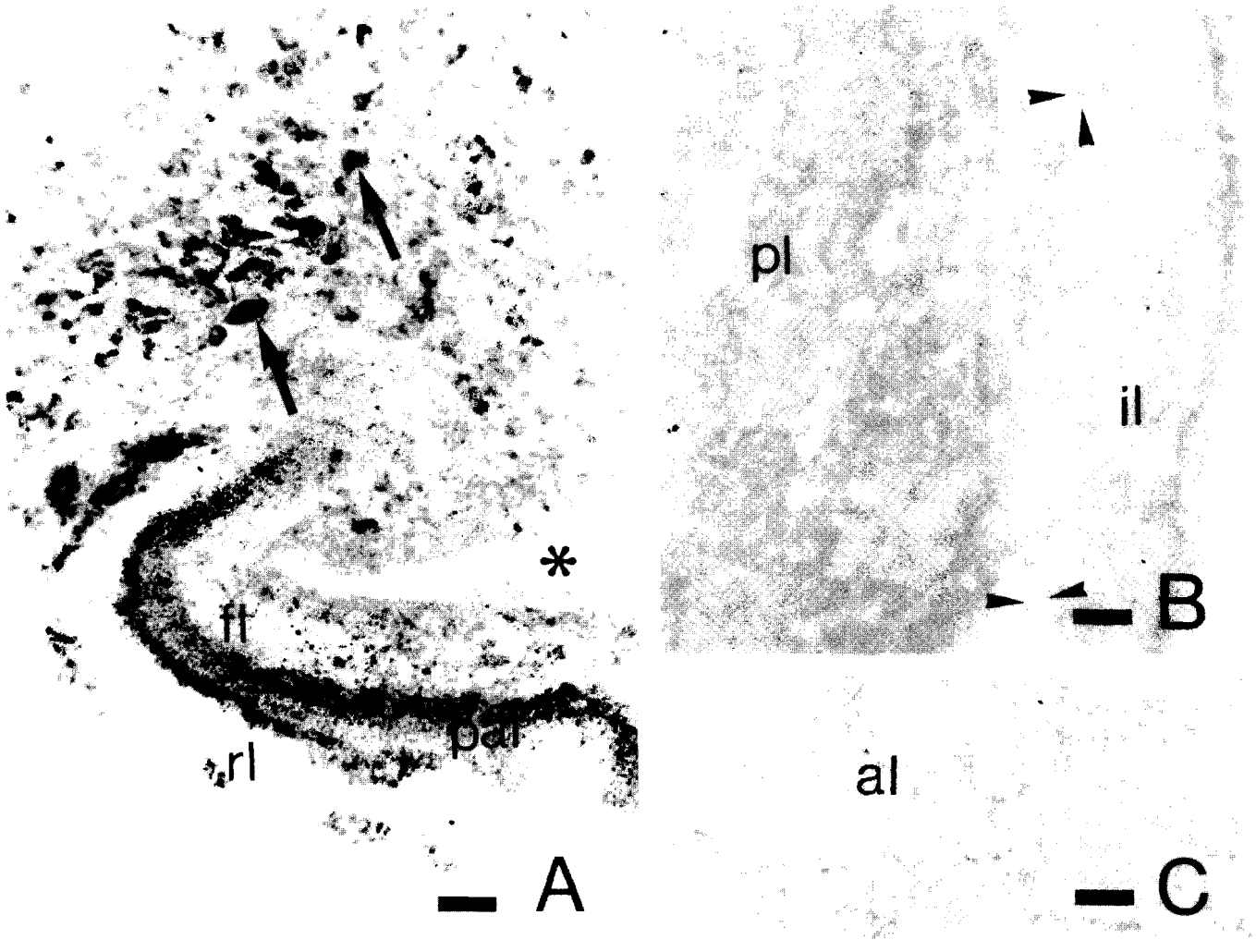

Fig. 1. A. Light photomicrograph (counterstained section) of ChAT-immunopositive cells (arrows) and terminals in the rat hypothalamic arcuate nucleus and the median eminence, respectively. Note that the ChAT immunoreactive terminals are aggregated in the palisade layer of the median eminence. The fibrous layer contains much fewer, but larger, terminals whereas the reticular layer is almost devoid of ChAT immunoreactive terminals. The asterisk indicates the third ventricle. Bar: 50 μm. **B, C.** Rat pituitary after simultaneous ChAT immunostaining with the brain section shown in panel A. Note the absence of cholinergic innervation in the rat pituitary. The arrowheads in panel B indicate unstained putative blood acetylcholine reading bodies (BARBs) associated with blood vessels in the rat intermediate lobe. Bar: 20 μm. fl: fibrous layer of the median eminence; pal: palisade layer of the median eminence; rl: reticular layer of the median eminence; al: anterior lobe; *il*: intermediate lobe; pl: posterior lobe.

stain rabbit tissue. Therefore, only the ChAT immunoreactive profiles of the rat pituitary system have been investigated. ChAT immunolabeled neurons and punctate varicosities were present in the rat hypothalamic arcuate nucleus and median eminence, respectively (Fig. 1A). The ChAT cell bodies in the hypothalamic arcuate nucleus, the presumed origin of the tubero-infundibular cholinergic system, have been recorded earlier by Tago et al. (1987). They measured 10-25 µm in diameter at their longest dimension, were largely ovoid in shape and frequently bipolar. Solid proof that the hypothalamic arcuate nucleus is the source of cholinergic fibers to the median eminence remains to be provided. The ependymal and subependymal layers of the median eminence (terminology after Kobayashi et al., 1970) contained few ChAT immunoreactive boutons. The fibrous layer, primarily composed of the neuroendocrine axons on their way to the posterior lobe, had a greater number of ChAT terminals. A distinguishing feature of these terminals was their size which was obviously greater than anywhere else in the median eminence. The palisade layer of the external median eminence displayed fine ChAT immunoreactive grains with such a high density that they formed a more or less continuous layer. The reticular layer (zona granulosa) of the median eminence lacked ChAT-labeled profiles except for a few fiber tracks that coursed towards the pial surface.

In all three lobes of the rat pituitary, ChAT immunoreactivity was negligible to absent (Fig. 1B, C). Thus the rat pituitary cholinergic innervation is by no means equivalent in magnitude to the catecholaminergic (e.g. Saland et al., 1993) or serotonergic (e.g. Léránth et al., 1983) innervation. It must be noted that, although some researchers did measure ChAT activity (e.g. Barron and Hoover, 1983), or demonstrated AChE fibers (e.g. Whitaker and Labella, 1973) in the rat pituitary, actual ChAT immunoreactive structures have not yet been shown in pituitary sections. Carmeliet and Denef (1989), however, observed ChAT immunopositive staining displayed by cultured rat anterior lobe endocrine cells. It is known that one or more enzymes involved in ACh production, and ACh itself, may occur in cells that have been cultured in media (see Rama Sastry and Sadavongvivad, 1979). However, these enzymes do not necessarily function to synthesize ACh for neurotransmission or release purposes. Remote possibilities to reconcile this *in vivo-in vitro* controversy and some divergent biochemical results are: (1) the anterior lobe ChAT is of a different isoform than that from the brain, and (2) anterior lobe cells in culture can switch on *de novo* synthesis of (ACh) molecules. The latter phenomenon has been reported for neurons and neuroendocrine cells if exposed to changing or artificial environmental factors (e.g. Patterson and Chun, 1977; Djabali et al., 1993). The results of this study demonstrate that under normal circumstances the rat pituitary is neither innervated by a significant number of cholinergic fibers, nor is there an intrinsic ACh synthesis. This strongly designates the median eminence as a major source of rat pituitary ACh (see Egozi et al., 1988). Results from other studies suggest that this system occurs in other mammals as well (Tago et al., 1987).

At the ultrastructural level ChAT immunolabeling was present in large axon terminals (4-7 µm at the long axis) in the fibrous layer (Fig. 2A). These terminals had many mitochondria and some clear, and dense core vesicles. Part of these boutons established synaptic contact with other unlabeled profiles. In the electron photomicrograph presented in figure 2A, the ChAT immunolabeled profile seems to be the receptive part of an unlabeled axon terminal which establishes an asymmetric synapse. In the palisade layer many small axon varicosities were immunostained (Fig. 2B, C). These immunoreactive varicosities also contained a population of small clear vesicles, in addition to a few dense core vesicles, a typical feature of terminal varicosities of cholinergic nerves. Most of these small ChAT immunostained varicosities bordered at perivascular spaces without developing synaptic thickenings. These ultrastructural observations provide support for a release of ACh as a humoral factor from the median eminence.

Cholinergic receptors in the pituitary

The antibodies M35 and WF6 that were used to label mAChRp and nAChRp, respectively, have been described extensively in previous reports (e.g. André et al., 1987; Schröder et al., 1989; Van der Zee et al., 1992; Maelicke et al., 1988; Conti-Tronconi et al., 1991; Caffé 1994; Caffé et al., 1994). Briefly, these monoclonal antibodies were generated using protein isolated from tissue membrane fragments known to be enriched with the respective receptors. These antibodies, therefore, cannot claim to recognise a particular AChR of which several subtypes are currently known (see for instance Bonner et al., 1987, 1988; Swanson et al., 1987; Whiting et al., 1987; Hulme et al., 1990; Levey et al., 1991; Sargent, 1993). However, it is important to emphasize that both the M35 and WF6 antibodies have been subjected to extensive scrutiny. Competition studies and immunochemical analyses have unequivocally established their validity in detecting mAChRp and nAChRp, respectively (Leiber et al., 1984; Fels et al., 1986; André et al., 1987; Maelicke et al., 1989; Conti-Tronconi et al., 1991; McLane et al., 1992). The staining obtained using these antibodies in an immunohistochemical procedure will be referred to as mAChRp-, or nAChRp-like (nAChRp-L) immunoreactivity.

Rat

The rat pituitary displayed heterogeneous mAChRp-L immunoreactivity. At light microscopic level, the rank order of staining intensity was: first the intermediate lobe, followed by the anterior lobe, whereas the posterior

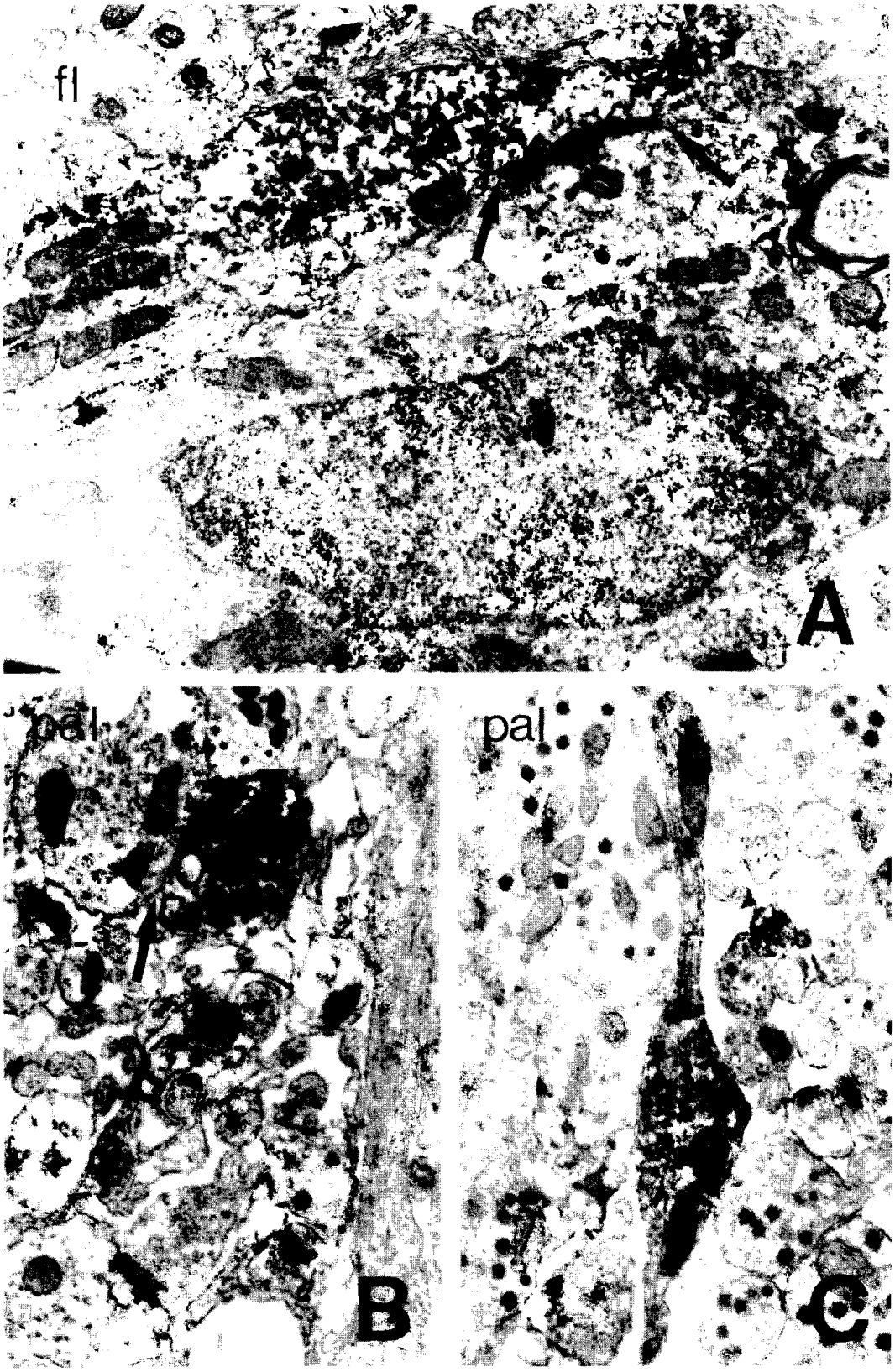

Fig. 2. Electron photomicrographs illustrating ChAT-immunolabeled terminals in the rat median eminence. **A.** Large terminal in the fibrous layer. Note the involvement of a synaptic specialisation (arrows). **B, C.** Small terminals in the palisade layer. Although a few synapse-like junctions have been observed (panel B, arrows) most ChAT terminals in this part of the median eminence bordered on the extravascular space without developing synaptic specialisations (panel C). x 28,000. Abbreviations as in figure 1.

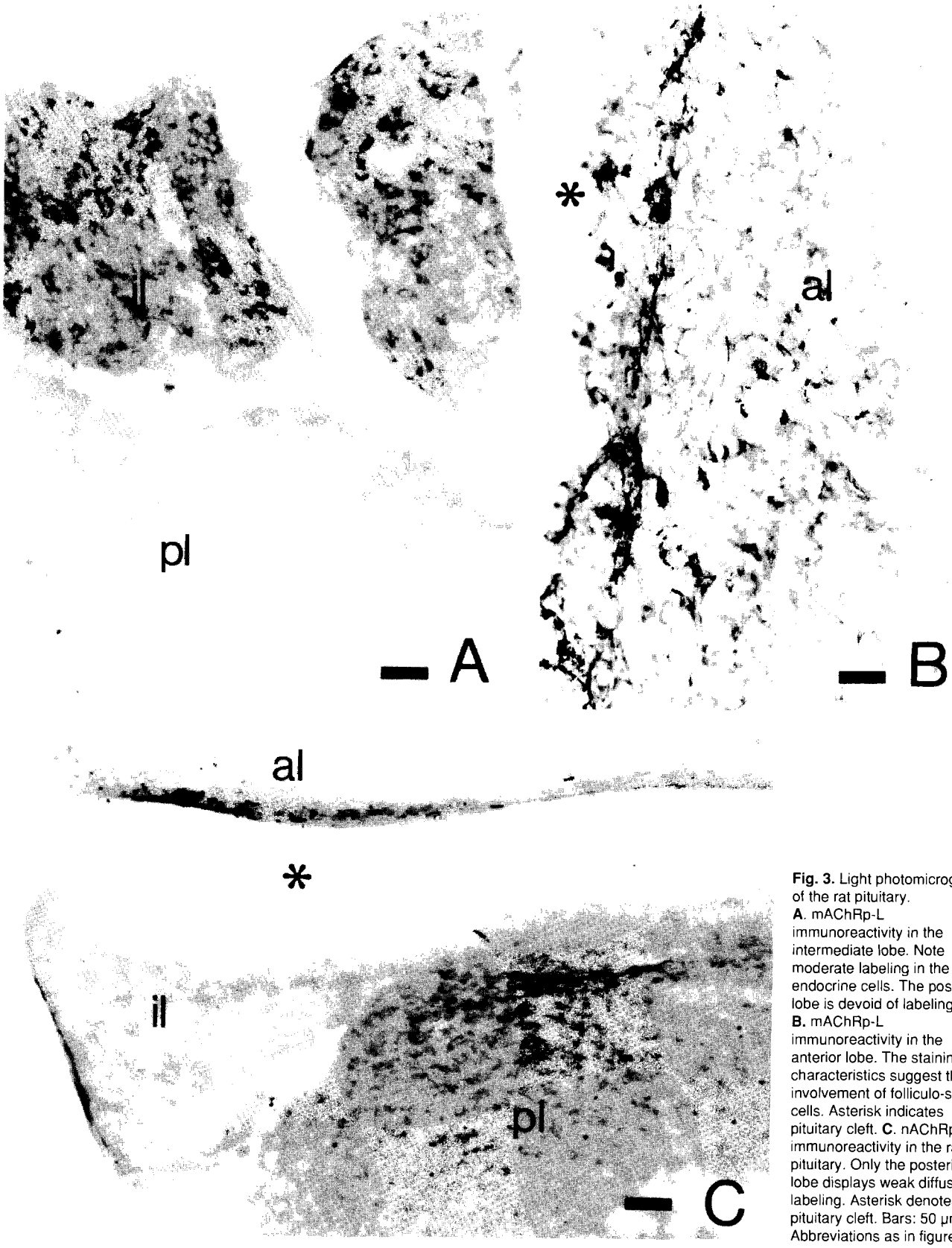

Fig. 3. Light photomicrographs of the rat pituitary. **A**. mAChRp-L immunoreactivity in the intermediate lobe. Note moderate labeling in the endocrine cells. The posterior lobe is devoid of labeling. **B.** mAChRp-L immunoreactivity in the anterior lobe. The staining characteristics suggest the involvement of folliculo-stellate cells. Asterisk indicates pituitary cleft. **C**. nAChRp-L immunoreactivity in the rat pituitary. Only the posterior lobe displays weak diffuse labeling. Asterisk denotes the pituitary cleft. Bars: 50 µm. Abbreviations as in figure 1.

lobe remained largely unlabeled. Within the intermediate lobe most, if not all, endocrine cells expressed moderate cytosolic mAChRp-L immunoreactivity. No interstitial cells or fibers were labeled either in the intermediate or the posterior lobe of the rat pituitary (Fig. 3A). In the anterior lobe, mAChRp-L immunostaining was expressed in an area bordering on the intermediate lobe, next to the pituitary cleft, presumably by some FS cells. Endocrine cells, vascular tissue, and most of the interstitial cells did not show mAChRp-L immunopositive staining (Fig. 3B). This distribution of mAChRp-L immunolabeling is similar to that observed in the frog as reported by Lamacz et al. (1989). It also agrees with the distribution of binding sites of [^{3}H]-quinuclidinyl benzilate (QNB), a non-selective high-affinity mAChR antagonist, as measured in tissue homogenates. When the latter method is employed the anterior lobe displays 2-3 times more signal than the posterior lobe (e.g. Hoover and Hancock, 1983). In addition, using tissue sections, some studies have found the highest QNB binding sites in the region of the rat pituitary cleft, followed by moderate levels in the anterior lobe and low levels in the posterior lobe (e.g. Hoover and Hancock, 1983). However, not all available data are in agreement. Michels et al. (1986), for example, have found the highest QNB binding sites in the posterior lobe, followed by the anterior lobe. It can be concluded from the current immunolabeling pattern that the mAChRs near the pituitary cleft are not related to the vasculature, as was assumed previously, but

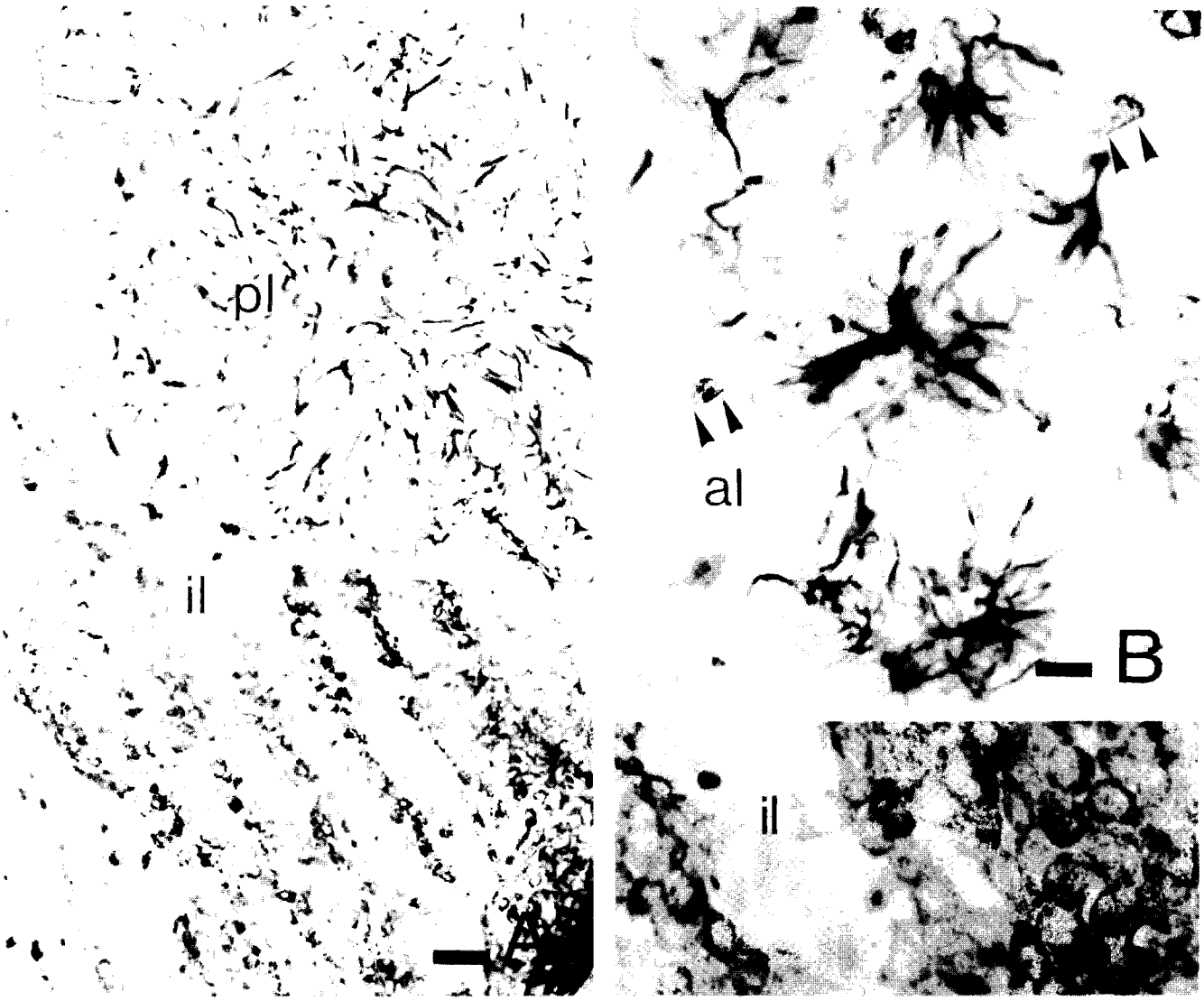

Fig. 4. Light photomicrographs of the rabbit pituitary. **A.** mAChRp-L immunoreactive fibers and endocrine cells in the posterior and intermediate lobes, respectively. Bar: 50 µm. **B.** mAChRp-L immunoreactive folliculo-stellate cells in the anterior lobe. The arrowheads indicate mAChRp-L immunostained putative blood acetylcholine reading bodies (BARBs). Bar: 20µm. **C.** nAChRp-L immunoreactive endocrine cells in the intermediate lobe. Bar: 20 µm. Abbreviations as in figure 1.

located on FS cells.

In the rat the most intense nAChRp-L immunoreactivity was seen in the posterior lobe. However, this staining was too diffuse for reliable allocation to specific structures. The endocrine cells of the intermediate lobe or profiles of the anterior lobe never displayed any nAChRp-L immunolabeling (Fig. 3C). This staining pattern agrees well with the observation by Michels et al. (1986) who found very low density [^{125}I]α-bungarotoxin (α-BTX), a putative nAChR probe, binding sites over the rat pituitary.

The current observations collectively indicate that

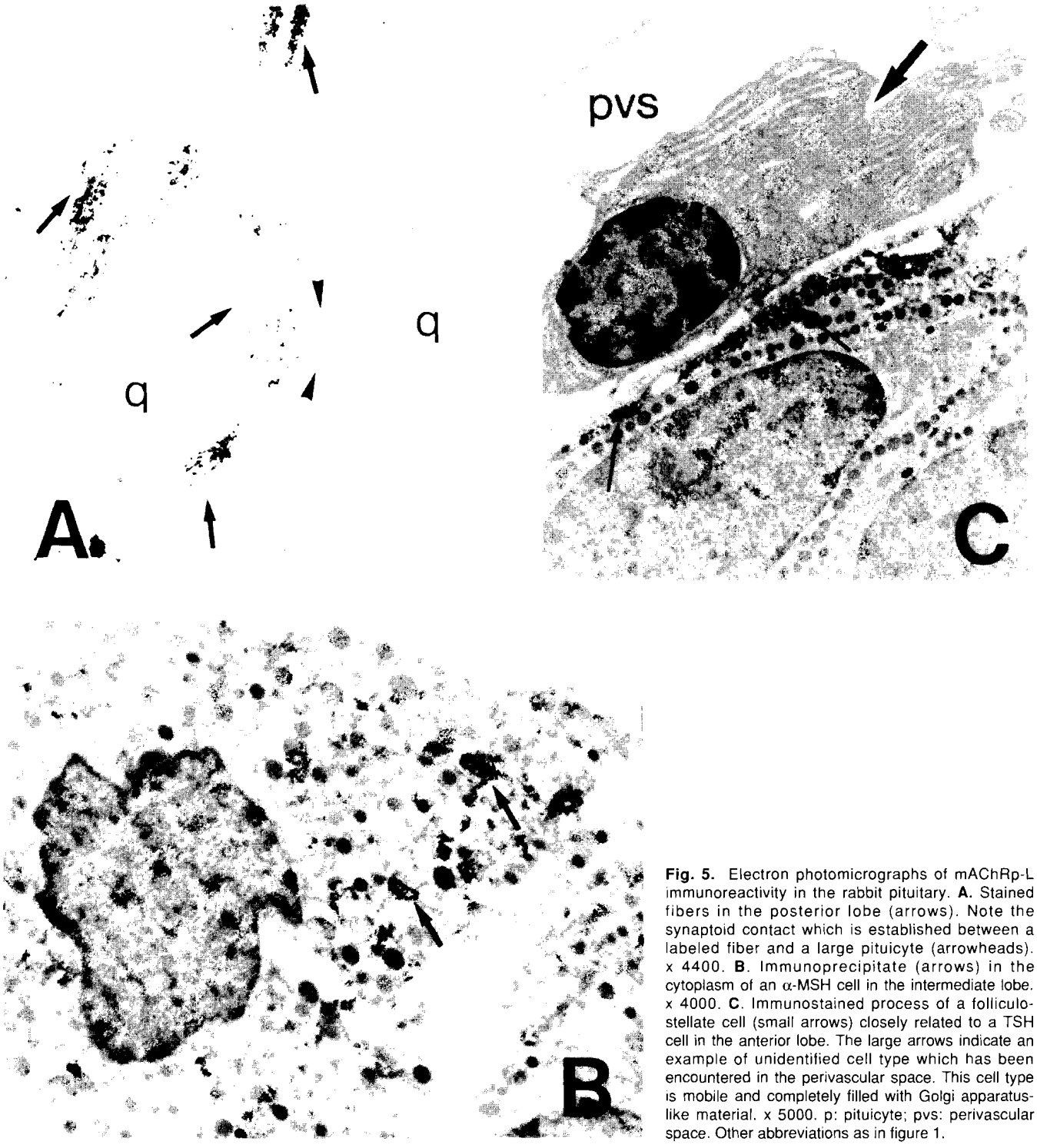

Fig. 5. Electron photomicrographs of mAChRp-L immunoreactivity in the rabbit pituitary. **A.** Stained fibers in the posterior lobe (arrows). Note the synaptoid contact which is established between a labeled fiber and a large pituicyte (arrowheads). x 4400. **B.** Immunoprecipitate (arrows) in the cytoplasm of an α-MSH cell in the intermediate lobe. x 4000. **C.** Immunostained process of a folliculostellate cell (small arrows) closely related to a TSH cell in the anterior lobe. The large arrows indicate an example of unidentified cell type which has been encountered in the perivascular space. This cell type is mobile and completely filled with Golgi apparatus-like material. x 5000. p: pituicyte; pvs: perivascular space. Other abbreviations as in figure 1.

mAChRs are expressed by intermediate lobe endocrine cells and, to a lesser degree, on FS cells in the anterior lobe. Within the posterior lobe low levels of nAChRs may be present. AChRs in the rat pituitary were not examined at the ultrastructural level.

Rabbit

All the three lobes of the rabbit pituitary displayed mAChRp-L immunolabeled profiles with more or less uniform intensity. It is important to note that, when processed simultaneously, the mAChRp-L immunostaining intensity in the rabbit pituitary exceeded that of the rat (compare Figs. 3 and 4). It is not clear if this phenomenon is in any way related to the marked interspecies differences in pituitary ACh content (Bridges et al., 1973). The rabbit posterior lobe showed a dense plexus of randomly orientated, smooth mAChRp-L immunoreactive processes. Many, if not all, of the intermediate lobe endocrine cells showed intense cytosolic staining (Fig. 4A). In the anterior lobe the most obvious mAChRp-L immunoreactivity involved large FS cells (greater than 50 µm in diameter), occupying a hexagonal space typical of the microfollicle arrangement (Fig. 4B). The immunolabeled FS cells were absent from the center of the anterior lobe (see Fig. 7B), thus establishing some regional preference of these cells. Apart from the FS immunostaining, faint labeling was also observed on some endocrine cells (see Fig. 7B). The rabbit posterior and anterior lobes were devoid of nAChRp-L immunoreactivity. However, the endocrine cells of the rabbit intermediate lobe showed high

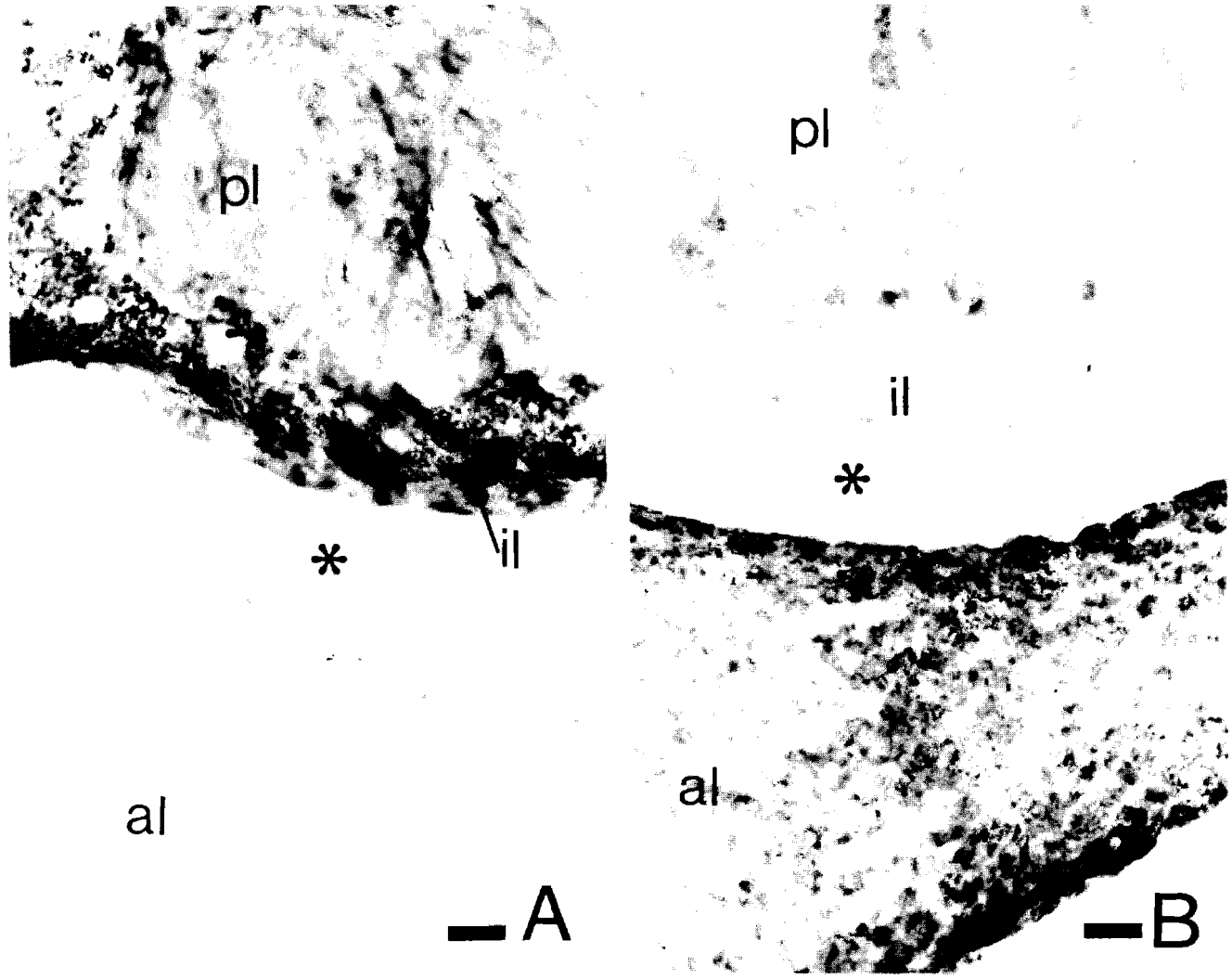

Fig. 6. Light photomicrographs of the cat pituitary. **A.** mAChRp-L immunoreactivity. The posterior lobe displays diffuse labeling of fibers whereas the endocrine cells in the intermediate lobe are clearly and intensely stained. In contrast, the anterior lobe is devoid of any immunolabeling. **B.** nAChRp-L immunoreactivity. Note the reverse order of immunostaining in the intermediate and anterior lobes with respect to the mAChRp-L immunoreactivity in the same location. Asterisks in A and B indicate pituitary cleft. Bar: 100 µm. Abbreviations as in figure 1.

intensity cytosolic staining (Fig. 4C). To date there are no reports describing the distribution of QNB and α-BTX binding sites in the rabbit pituitary.

The intense mAChRp-L immunoreactivity in the rabbit pituitary was further analysed at the ultrastructural level. In the posterior lobe the immunostaining was present in terminals containing small vesicles and making axosomatic synaptoid contacts with major (type I) pituicytes (Fig. 5A). Connections with type IV fibers (terminology after Takey and Pearl, 1984) were also observed. Neurovascular endings showing mAChRp-L immunolabeling were not found. The intermediate lobe endocrine cells displayed DAB precipitate over the cytoplasm (Fig. 5B), whereas in the anterior lobe, extensions of mAChRp-L immunolabeled FS cells were seen in close association with the endocrine cells (Fig. 5C). No clear synaptic contacts have been observed between these FS cells and the endocrine cells. Figure 5 also illustrates a frequently encountered unidentified mobile cell type. This cell is completely filled with Golgi apparatus-like material and situated in the perivascular space.

Cat

In the cat a plexus of mAChRp-L immunolabeled smooth fibers was found in the median eminence and infundibulum stalk. The internal zone of the median eminence contained thick, randomly orientated fibers. From this plexus thinner fibers ran, parallel to each other, in a ventral direction through the external zone of the median eminence. These fibers terminated at the beginning of the reticular layer where the primary portal system is located. No mAChRp-L immunostained fibers were present in the reticular layer. Apart from this network, stained smooth fibers coursed through the infundibular stalk, parallel to its long axis, to penetrate the posterior lobe (not shown). Within the cat pituitary mAChRp-L immunoreactive fibers spread out in the posterior lobe where they displayed the same diffuse image as has been previously observed for nAChRp-L immunopositive fibers in the rat posterior lobe. Many, if not all, of the endocrine cells of the intermediate lobe, including a group of cells facing the residual cavity of Rathke's pouch, showed clear cytoplasmic mAChRp-L immunolabeling. In contrast to the rat and rabbit, the cat anterior lobe (including pars tuberalis) was entirely devoid of mAChRp-L immunoreactivity (Fig. 6A).

The cat median eminence and pituitary stalk also contained nAChRp-L positive fibers, as has been described above for the mAChRp counterpart. In the cat pituitary nAChRp-L immunoreactivity was, apart from the diffuse staining in the posterior lobe, quite different from that of the rat and rabbit. Contrary to the rabbit, the endocrine cells of the cat intermediate lobe were not nAChRp-L immunolabeled. Contrary to the rat and rabbit, the cat anterior lobe endocrine cells consistently showed intense cytoplasmic nAChRp-L immunolabeling (Fig. 6B). AChRp expression in the cat pituitary was not examined at the ultrastructural level.

Observations on a putative pituitary blood acetylcholine sensor

In the rat (Fig. 7A) and rabbit (Fig. 7B-D), strong mAChRp-L immunostained bodies, of unknown identity, were observed associated with blood vessels in the intermediate and anterior lobes. In the rat just a few bodies were observed predominantly in the intermediate lobe, whereas in the rabbit many such profiles were observed predominantly in the anterior lobe. These structures showed neither nAChRp-L immunoreactivity, nor were they distinctly visible after Nissl, or control immunohistochemical staining procedures. In figure 1B the arrowheads indicate two structures which presumably represent these bodies in their unstained state.

At first this observation met with scepticism, and was suspected of being an artifact. Two categories of artifact are common when immunohistochemical procedures are employed; those due to procedural factors and those due to pathological processes. Procedural artifacts may, for instance, result from contamination by ingredients in reagents (e.g. iron in water), debris from other tissues (e.g. when multiple tissues are processed simultaneously), airborne particles (e.g. dust, elements released by the investigator), non-immunological labeling (pseudo-peroxidase staining), etc. The following strategies were adopted in order to prevent these artifacts: (1) Pyrogen-free (bottled) water, and reagents from different sources were used. (2) Equipment was cleaned or sterilized before tissue sectioning. (3) The staining procedure was performed in a dust-free environment, i.e. a laminar flow cabinet, used for antibiotic-free tissue culture (see Caffé et al., 1989). In addition, none of the following primary antibodies directed against AVP, OXT, Zebrin-II, D2 (dopamine) receptor, photoreceptor rod (AO) and cone visual pigments (COS-1 and COS-2) resulted in labeling of these bodies. Although these measures cannot completely exclude the event of a false positive observation, in practice they strongly indicate that the immunohistochemical staining of these bodies was not an artifact due to procedural factors. Infections of laboratory animals constitute another source of artifacts. The image of the observed mAChRp-L immunostained bodies was remotely reminiscent of haemoflagellate parasites, in particular of the genus *Trypanosoma*. The possible manifestation of such a parasitic infection in Europe, across animals that are asymptomatic, is very small but not impossible. It is known that in host blood, haemoflagellates are polymorphic during their life cycle, showing elongated and stumpy forms. To test for this possibility three *specific pathogen free* (SPF) New Zealand White Rabbits, claimed to be without any parasitic infection, were analysed. Without exception the mAChRp-L immunoreactive bodies could be observed in the pituitary of these SPF animals. In addition,

examination of, (1) stained blood films, (2) the pituitary tissue by the Giemsa procedure, and (3) the ultrastructure of the units, to be described below, strongly indicated that these bodies were not members of a known haemoflagellate parasite. Thus, the occurrence of these bodies was then recognized as an authentic

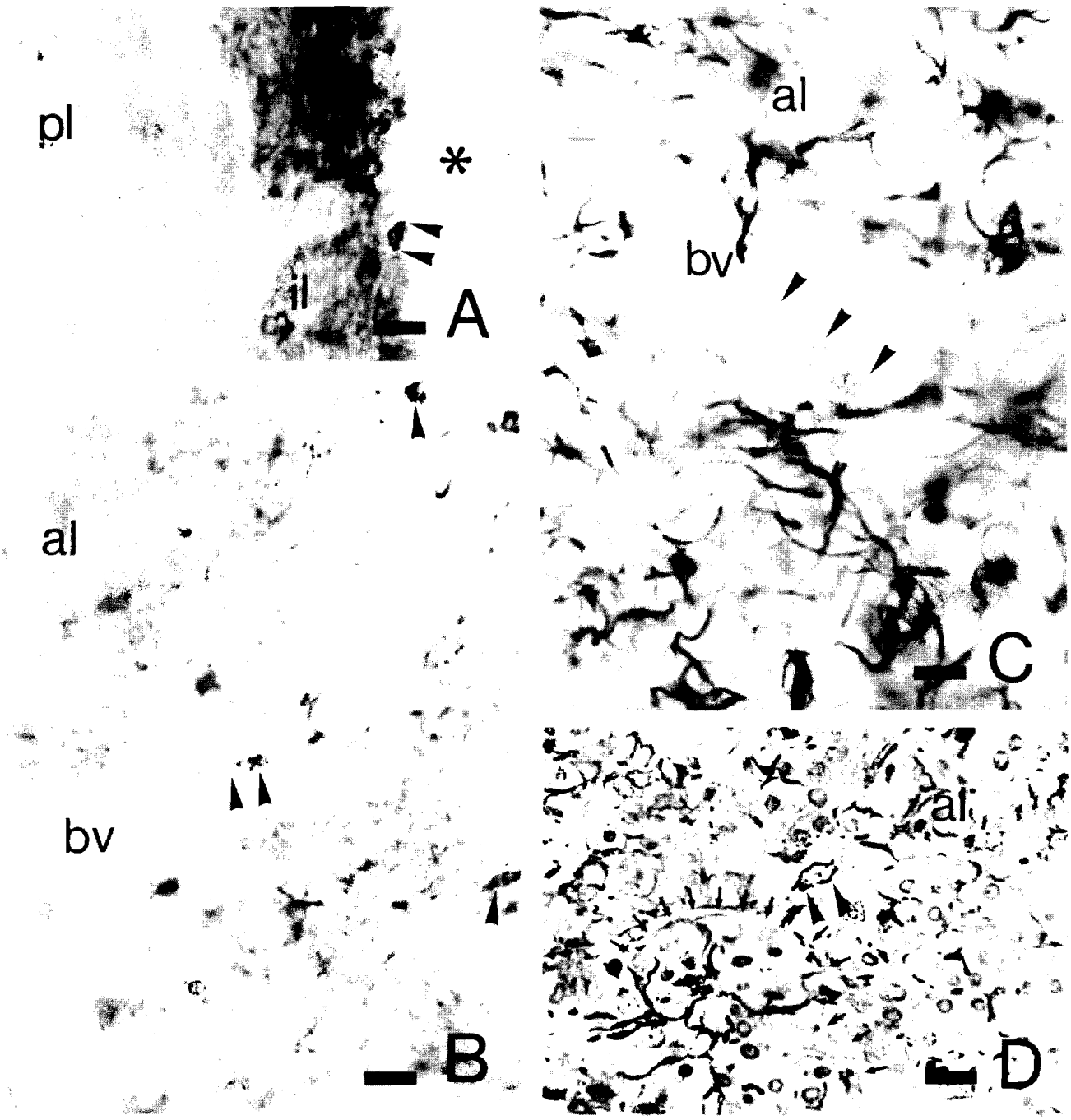

Fig. 7. Light photomicrographs of the rat intermediate lobe **(A)** and rabbit anterior lobe **(B - D)** illustrating mAChRp-L immunostained blood acetylcholine reading bodies (BARBs) closely related to blood vessels. In the rat only a few BARBs are present, whereas many can be observed in the rabbit tissue (arrowheads). Note the existence of a head and tail part (B, D), and rod (C) or cone-like (D) forms. In panel C immunostained particles can be clearly seen on the body outer surface. In panel D (counterstained semithin section) this outer surface staining of the BARB (arrowheads) can be better appreciated as a black limiting membrane. Note the configuration of a mAChRp-L immunostained folliculo-stellate cell within a typical follicle arrangement (small arrows in panel D). Asterisk indicates pituitary cleft. Bar in A and B: 50 µm. Bar in C and D: 20 µm. bv: blood vessel. Other abbreviations as in figure 1.

phenomenon.

The gross morphology of these bodies was composed of a head part and a connecting stem. The head was rod to cone-shaped, i.e. elongated or stumpy, in outline measuring 20 µm to greater than 40 µm in the long axis and 10-30 µm in the short axis. The stem was tubular and, on occasion, could be traced for as long as 100 µm within the capillary lumen (Fig. 7A-D). Some instances of branching were observed as well (not shown). The bodies were not evenly dispersed over the anterior lobe but concentrated in the center of the tissue. At their highest density, in 60 µm thick sections, more than ten of these bodies were counted within one mm^2 area. At light microscopic level the AChRp-L immunolabeling was clearly expressed as granules over the body surface suggesting that receptor protein molecules were directly exposed to the blood (Fig. 7C, D).

The fine structure of the body (Fig. 8A, B) was characterized by the presence of a surface membrane that was frequently damaged during processing, probably due to the delicate nature of this membrane and the mild fixation. At the outer surface of the body intensely mAChRp-L immunoreactive particles were located. These receptor protein particles were circular,

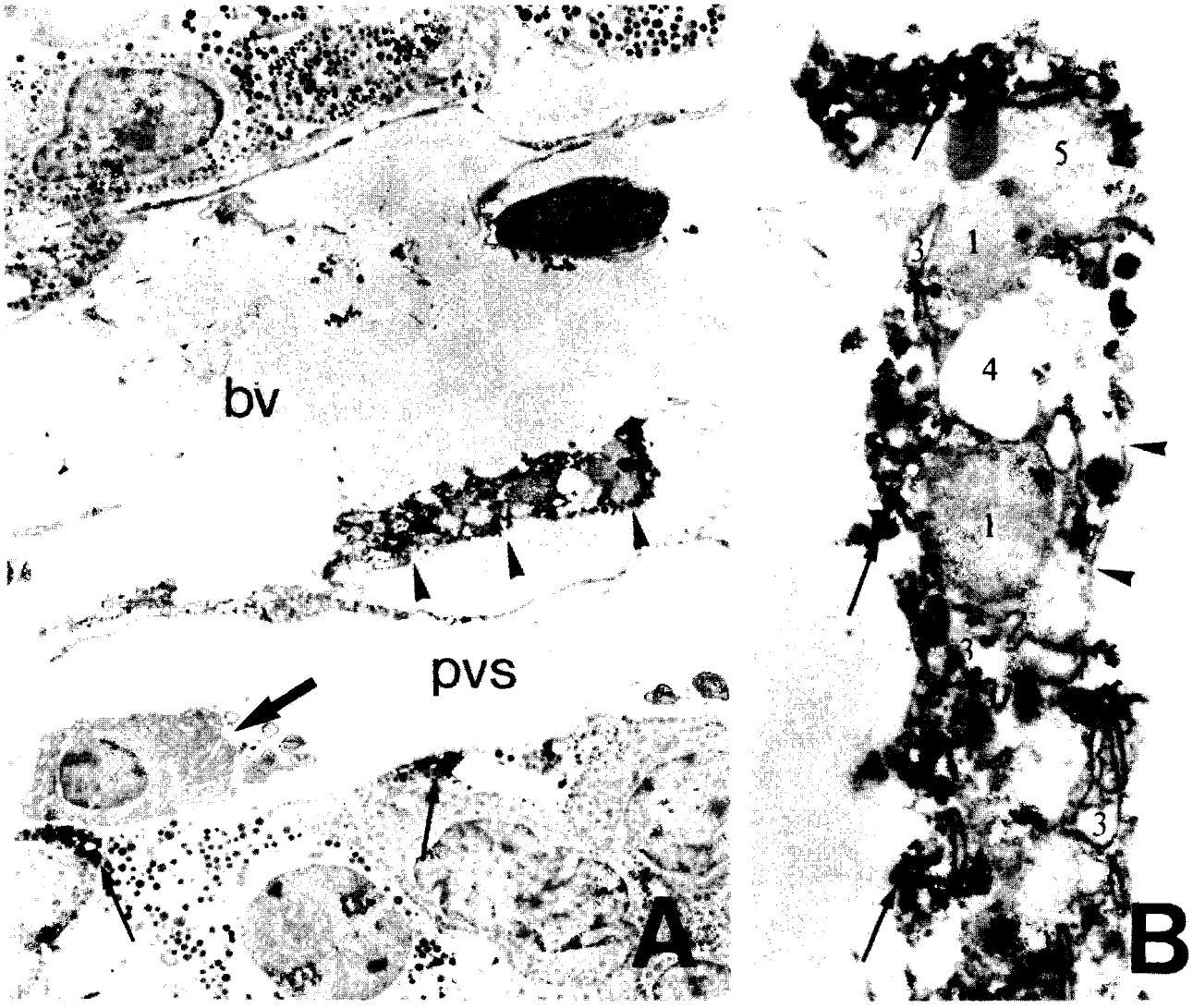

Fig. 8. Electron photomicrographs illustrating the ultrastructural features of a blood acetylcholine reading body (BARB). **A.** Low magnification showing the BARB lying within a blood vessel (bv) with the tail presumably attached to the blood vessel wall. Note the great difference in morphology between the BARB and endothelial cells (endo), endocrine cells, and an unidentified cell type (thick arrow) completely filled with Golgi apparatus-like material in the perivascular space (pvs). Small arrows point to processes of immunostained folliculo-stellate cells. x 4000. **B.** Higher magnification of the BARB. The head part is characterized by a plasma membrane (arrowheads) which is frequently ruptured during processing. Note the intensely mAChRp-L immunostained particles arranged at the body outer surface. These receptor protein particles have a thick stained membrane enclosing an unstained core. Futhermore, the body has a unique composition of cellular organelles. Some of these organelles are numbered and currently designated as: (1) nucleus-like organelle, (2) lipid droplet-like organelle, (3) Golgi apparatus, (4) vacuole, (5) amorphous material. x 23,000.

reminiscent of a vesicle, with the outer membrane darkly immunoreactive and the core less stained. The receptor protein particles were supported by amorphous material, reminiscent of the gelatinous membrane that supports the otolithic crystals on the maculae in the inner ear. The fine internal organization of the head part further displayed a, presumably random, arrangement of various organelles. Typical observations included: (a) one or two nucleus-like profiles (it is important to note that no clear nucleolus was observed in these nucleus-like organelles nor did the nuclear matrix show deeply stained chromatin patches similar to the nuclei of the endocrine or endothelial cells in the same section), (b) a near absence of mitochondria, (c) an extensive Golgi apparatus, (d) lipid-like droplets, and (e) amorphous material. This ultrastructure and collection of organelles does not comply to any known parasite or pituitary profile. Consequently these findings further support the authenticity of the bodies as well as the fact that they expose mAChRp particles to the blood.

What could be the relevance of this observation? It has been reported that ACh is present in whole blood (Kawashima et al., 1993). It might originate from mononuclear leukocytes, vascular endothelial cells, or the limited cholinergic innervation of blood vessels (Parnavelas et al., 1985; Kawashima et al., 1990). Here histological evidence has been presented suggesting that much of the pituitary ACh is released from the median eminence into the portal blood as a humoral factor. The pituitary gland is the primary target for these humoral factors and their release is strictly adjusted in response to the demands of the organism (e.g. Egozi et al., 1982; Simpson et al., 1985). The pathways, via which this feedback control is established, are well-known for some hypothalamic peptidergic releasing factors. It is conceivable that such feedback circuits should also exist for the non-peptidergic humoral factors, e.g. ACh and epinephrine (e.g. Gibbs, 1985). Because, unlike epinephrine, (blood) ACh is rapidly hydrolyzed by (circulating) esterases any ACh sensor must be localized in the vicinity of the releasing site. The location of the observed mAChRp-L immunoreactive bodies within the portal vessels is thus appropriate for functioning as a pituitary ACh monitor. However, if these bodies were to be part of a feedback circuit then they must be connected to the CNS. If this is the case it is unknown, but attachments between the body tail part and its environment have indeed been observed (see Fig. 8A). In addition, it must be remembered that the animals which were used in this study were perfused extensively. Most, if not all, blood cells were removed from the pituitary circulation. The fact that these mAChRp-L immunoreactive bodies are still present in the blood vessels indicates a secure attachment to the pituitary tissue. Thus it would appear logical to postulate the working hypothesis that these bodies compose a system of receptors for blood ACh. Each body was called a *blood acetylcholine reading body* (BARB). The receptors are likely to be part of the feedback pathway controlling ACh release. The observation that this system might not be present in the cat interestingly coincides with the absence of mAChRp-L immunoreactive structures in the anterior lobe of this species.

General conclusions

The presence of ChAT cell bodies in the hypothalamic arcuate nucleus have either been denied (Rodriguez-Sierra and Morley, 1985) or claimed (Tago et al., 1987). During this investigation ChAT cell bodies in the hypothalamic arcuate nucleus and a plexus of terminals in the median eminence have been observed in the rat. The fine structure of the axon terminals and varicosities in the median eminence indicate that ACh is released locally as a humoral factor. Collectively the current features of this cholinergic network strongly imply that much pituitary ACh comes via the portal system, and not via direct innervation or by intrinsic pituitary ACh synthesis (see Egozi et al., 1988). Results from other studies suggest that this system is present in other mammals as well.

With regard to the distribution of pituitary AChRps, the picture appears to be less straightforward. Two of the three examined species expressed mAChRp-L immunoreactivity in fibers of the posterior lobe, and all animals showed staining in the cytoplasm of the intermediate lobe endocrine cells. Beyond this, marked species differences were found in the distribution of immunolabeled profiles. Species differences in distribution and density of pituitary AChR ligand binding sites were also recorded. Therefore it appears that pluralism in expression of pituitary AChRs is genuine and prominent. The situation in each species should be known and taken into account when pituitary cholinergic mechanisms are considered.

The release of ACh as a humoral factor serving the pituitary receptors is likely to be controlled by some feedback circuit close to the median eminence. A system has been observed which, based upon its location, organisation, and staining properties presents itself as a suitable candidate for the receptive part of this feedback circuit. Each unit of this system was called a blood acetylcholine reading body (BARB). However, this matter is far from settled; it remains possible that these BARBs represent a hitherto unknown histopathological phenomenon. Further research is needed before a more definite conclusion can be reached.

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