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CHOLINERGIC NEURONAL SYSTEM OF RAT AMYGDALA. AN IMMUNOCYTOCHEMICAL STUDY

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Abstract

The cholinergic neuronal elements of the amygdala (Am) in the rat were investigated by means of immunocytochemical technique for detection of the specific synthetic enzyme choline acetyltransferase (ChAT). Am contains a large number of ChAT-immunopositive axons and terminals but relatively few ChAT-immunoreactive neuronal perikarya. The region with higher density of cholinergic neurons is the amygdalostriatal transition area. These neurons are multipolar and exhibit intensive labelling of the perikarya and dendrites. They are similar (if not identical) to the large striatal cholinergic interneurons. Scattered ChAT-immunopositive neurons are present in the three subnuclei of the central nucleus of Am and in the two divisions of the anterior amygdaloid area. These cells are large, multipolar and the reaction product extends also in the dendrites. They are similar to the adjacent cholinergic neurons in the basal nucleus of Meynert (Ch4 group of Mesulam) but especially the neurons in the central nucleus are not so strongly labelled. Small immunolabelled perikarya are found in the nucleus of the lateral olfactory tract and in the basolateral nucleus. Practically all subdivisions of Am contain cholinergic axons and terminals but their density differs. The highest density of cholinergic axons is observed in the amygdalostriatal transition area, the basolateral nucleus and the nucleus of the lateral olfactory tract, and the lowest density is encountered in the lateral nucleus of Am. It appears that the great majority of cholinergic axons in Am represent afferent axons arising in the basal forebrain and in the mesopontine tegmentum, and only the amygdalostriatal transition area and regions containing small cholinergic neurons display an interneuronal axonal plexus.

Key words: acetylcholine, basal magnocellular nucleus of Meynert, bed nucleus of stria terminalis, extended amygdala, limbic system, neostriatum, parabigeminal nucleus

Introduction. The largest accumulation of cholinergic neurons in CNS is localized in the basal forebrain [1–3]. These neurons are concentrated in the medial septal

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nucleus, the nuclei of the diagonal band, and especially – in the basal magnocellular nucleus of Meynert in substantia innominata. These large cholinergic neurons are usually designated as Ch4 group, according to the nomenclature coined by MESULAM et al. [1]. Whilst the cholinergic innervation of the amygdala appears to be well established ([4–11] and many others), the data on the presence of cholinergic neuronal perikarya in Am are both scanty and contradictory [6, 12–15]. Here we present data on the cholinergic neuronal perikarya and axons in rat Am, based on an immunocytochemical detection of the acetylcholine biosynthetic enzyme, choline acetyltransferase (ChAT).

Material and methods. All procedures were carried out according to a standard protocol established by the Ethic Commission in the Medical University – Sofia. Young-adult Wistar Albino rats were used. For detection of ChAT immunoreactivity we followed the protocol of Prof. Michail S. Davidoff (personal communication to Prof. Andreas Wree). Under deep anaesthesia the animals were perfused transcardially with 150 ml phosphate buffered saline (PBS), followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The brains were postfixed for several hours at 4 °C in the same fixative. Brain blocks were dissected and transferred to 10% sucrose in 2% paraformaldehyde in 0.1 M PB to the next morning at 4 °C. The tissue blocks from the basal forebrain and the mesopontine tegmentum were soaked for 24 h in 20% sucrose in 0.1 M PB. Coronal and occasionally sagittal sections 30 µm thick were cut on Reichert Jung freezing microtome and were collected in PB. Following a brief rinse in PBS the sections were incubated in 0.3% Triton in PBS, followed by 10% normal rabbit serum (NRS) for 60 min. The sections were rinsed in PBS and transferred for 24 h in the primary antibody solution containing goat anti-ChAT, diluted 1 : 200 with PBS, containing also 0.2 Triton and 1% NRS. Following a thorough rinse in PBS, the sections were transferred to anti-Rat IgG-Biotin, diluted 1:250 with the same diluent (PBS-Triton-NRS). The sections were thoroughly rinsed with PBS and transferred for 60 min in the ABC complex, and rinsed again in PBS. The reaction product was visualized by the routine incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB), dissolved in Tris-HCl-buffer with 0.05% H_2O_2, with added 0.5% nickel ammonium sulphate. The reaction was stopped by a rinse in PBS. Since the ChAT immunostaining in Am was most often delicate, we preferred not to counterstain the sections but adjacent serial sections were stained with Cresyl violet for cytoarchitectonic orientation. The sections were mounted on chrome alum gelatin-coated slides and air dried. Finally, the sections were dehydrated in graded ethanols, cleared in xylene and coverslipped with Entellan. Also in rodents, the neuronal organization of Am is very complicated, and we followed the cytoarchitectonic delineation of Am nuclei as suggested by PAXINOS and WATSON [16].

Results. The reaction product by the immunocytochemical demonstration of ChAT is dark brown by the "routine" reaction or intensively blue in experiments in which nickel ammonium sulphate was added to the DAB solution. The perikarya of the cholinergic neurons are filled with reaction product, except for the cell nucleus. Especially by the strongly immunostained neurons, the reaction product extends also in the dendrites, and they might be followed to distal arborizations. The cholinergic axons are also positive, so that the stem axons might be followed to the finest terminal arborizations.

All subdivisions of Am contain ChAT-immunopositive axons, and the density of the terminal arborizations strongly varies. Not all Am nuclei contain immunoreactive neuronal perikarya, and with few exceptions their number is low to moderate. In the central nucleus of Am (Ce) a moderate number of positive cells are seen: in the medial subnucleus (CeM) (Figs 1, 2), in the lateral subnucleus (CeL) (Fig. 3), and in the capsular subnucleus (CeC) (Fig. 4). The neurons are medium-sized, multipolar and less strongly stained than the adjacent prominent accumulation of the Ch4 group – the

basal magnocellular nucleus of Meynert (B). There is a moderately dense cholinergic axonal plexus, more prominent in CeC (Fig. 4). A significant accumulation of positive neurons are seen in both subdivisions of the anterior amygdaloid area (AAA): the dorsal (Fig. 5), and the ventral (Fig. 6). In the dorsal subdivision the labelled neurons are medium-sized, and in the ventral subdivision large positive cells are also seen. These neurons are very strongly immunostained and the reaction product extends for a long distance in their varicose dendrites (Fig. 6). In contrast to the fairly large number of positive perikarya, the cholinergic axonal plexus is moderate, and especially in the AAA ventral subdivision the stained axons are unevenly distributed. Extremely large number of immunostained axons is seen in the basolateral nucleus of Am (BL), so that at low magnification the borders of the elliptically shaped BL are sharply demarcated. Most of the cholinergic terminal axons display large varicosities connected with very thin intervaricose portions (Fig. 7). In BL there are also positive neurons. As a rule, these cells are small in size and round in shape, and their dendrites are very thin and weakly stained (Fig. 7). In the amygdalostriatal transitional area (AStr) a large number of evenly distributed, strongly positive multipolar neurons is present (Fig. 8). In the neuropil there is a dense cholinergic axonal plexus but the labelled fibres are extremely thin. In the medial nucleus of Am (Me) occasional immunolabelled neurons are seen. Somewhat more often such cells are seen in the anterodorsal subdivision of Me, as defined by Paxinos and Watson [16]. The labelled neurons are medium-sized and multipolar, with wavy dendrites that rapidly disappear from the plane of sectioning. In contrast to the paucity of positive perikarya, the neuropil of Me contains a dense cholinergic plexus, and the labelled axons display robust varicosities (Fig. 9). Two adjacent structures of the "olfactory" Am, the anterior cortical nucleus (ACo) and the nucleus of the lateral olfactory tract (LOT) display very characteristic labelling for ChAT (Fig. 10). In ACo the immunolabelled perikarya built a row in the deep portion of the nucleus, surrounded by a low number of cholinergic axons. Only towards the ventral brain surface there is a moderate cholinergic axonal plexus. In contrast, the superficial layer of LOT (LOT1) contains a very low number of cholinergic fibres. Their number increases in the intermediate layer (LOT2), and in the deep layer (LOT3) the density of labelled axons is very high, rivalling the amount of cholinergic axons in BL. Within this plexus positive neurons are scattered as well. They are small in size and most of them are only faintly immunostained.

Only occasional labelled neurons are to be found in the basomedial nucleus of Am. No cholinergic perikarya are seen in the lateral nucleus of Am and in the intercalated cell masses. These three structures, however, display an appreciable number of cholinergic axons.

The most prominent axonal bundle that connects Am with other brain centres is stria terminalis (ST). Along its entire course ST contains a large number of strongly stained axons that enables the clear visualization of ST at a low magnification (Figs. 11, 12). Starting from Am, ST ascends in dorsocaudal direction, dorsal to the optic tract (OT) (lower part of Fig. 11). It curves caudally to the internal capsule (IC) and disappears from the plane of sectioning. Further, ST reaches the ventricular surface (upper part of Fig. 11) and runs in rostral direction dorsally to IC. The supracapsular part of ST contains interstitial neurons but only very rare they are immunostained (not shown). At prethalamic level ST descends abruptly towards the bed nucleus of ST (BST). By this descent the cholinergic axons in ST run medially to IC in which the most dorsal cholinergic neurons of the Ch4 group are seen. Within BST there is a profuse cholinergic axonal plexus. The BST lateral division, ventral part [16], located ventrally to the anterior commissure (CoA) contains also a significant number of immunopositive perikarya. Most of them are faintly stained but some perikarya display a strong reaction (Fig. 13).

As to be expected, all prominent cholinergic groups of the brain (Ch1-Ch8) are clearly identified in the present study. Portions of Ch4 are seen in Figs. 1 and 12. Its significant contribution to the extrinsic cholinergic axonal plexus in Am is well established (see Discussion section). As an additional source of cholinergic axons distributed within Am we suggest the parabigeminal nucleus (Ch8 group) located in the caudolateral part of the midbrain tegmentum (Fig. 14).

Discussion. It is a common belief that in contrast to the largest accumulation of cholinergic neurons in substantia innominata, the adjacent Am does not contain cholinergic neurons. In the chapter on the cholinergic systems in the most appreciated textbook on the human nervous system anatomy, SAPER [17] categorically stated: "There is no evidence for cholinergic neurons in the amygdala, but there is intense cholinergic innervation ...." Indeed, the lack of such neurons was declared inter alia by the team of M.-M. MESULAM et al. [1, 10 11] that started the systematic investigation of CNS cholinergic neuronal systems by means of ChAT immunocytochemistry. As it was mentioned in the Introduction, the data on the presence of cholinergic perikarya in Am are both scanty and contradictory. In a series of papers on age-related changes on the CNS, LOLOVA and DAVIDOFF [13] published data on severe reduction of cholinergic elements in the rat's Am, but they were less interested in the topographic distribution of ChAT-positive perikarya and axons. BUTCHER et al. [14] reviewed the organization of the central cholinergic neurons, and stated that they are only occasionally seen in Am. According to CARLSEN and HEIMER [6], ChAT-immunoreactive neurons are found in BL, NITECKA and FROTSCHER [12] found cholinergic neurons in AAA and Ce, and PHELPS et al. [15] declared that ChAT-positive cells are present in two structures of the "olfactory" Am: LOT and ACo.

The presently utilized modification of the ChAT immunocytochemistry proved to be very reliable and effective. There was no false positive labelling. For example, in the thalamus an abundant cholinergic axonal plexus was present, but the only region that contained immunopositive neurons was the medial habenular nucleus (the Ch7 group). The axonal bundles containing numerous cholinergic axons like ST sharply contrasted to the pale IC, ACo and OT. We confirm and extend the prior observations that reported cholinergic neurons in several different regions of Am. Interestingly, the cholinergic perikarya in AStr were not noticed in previous investigations. We regularly found a significant number of positive neurons in AStr. This transitional area pos-

Fig. 1. Low magnification photograph of the ventral portion of the basal forebrain and Am. Medial is to the right. Strongly positive, large neurons of the basal magnocellular nucleus (B) embedded in the ventral portion of the internal capsule (IC). The medial subnucleus of the central nucleus of Am (CeM) contains three cholinergic neurons (arrows) that are smaller and more weakly stained than the cells in B. × 100

Fig. 2. Detail from Fig. 1 (the delineated region). Three medium large, moderately immunostained neurons in CeM. × 200

Fig. 3. A solitary cholinergic neuron in CeL. × 200

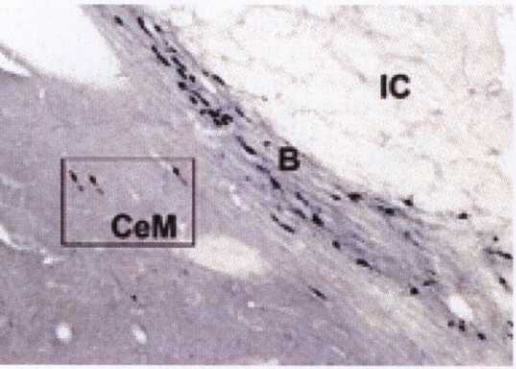
Fig. 4. Single immunostained neuron in CeC. × 200

Fig. 5. A group of cholinergic neurons in the dorsal division of AAA. The cells are medium-sized, elongated and strongly stained. × 400 →

Fig. 6. An accumulation of deeply stained cholinergic neurons in the ventral division of AAA. Some of the labelled neurons are with large dimensions. The strongly varicose dendrites are a characteristic feature of this area. × 400

Fig. 7. Extremely dense cholinergic axonal plexus in BL. There are also several small cholinergic neurons with rounded or oval perikarya and hardly visible very thin dendrites. × 200

Fig. 8 An accumulation of ChAT-positive medium-sized neurons in Astr. The neuropil contains numerous stained axons that, however, are considerably thinner than the axons in BL. × 200


IC
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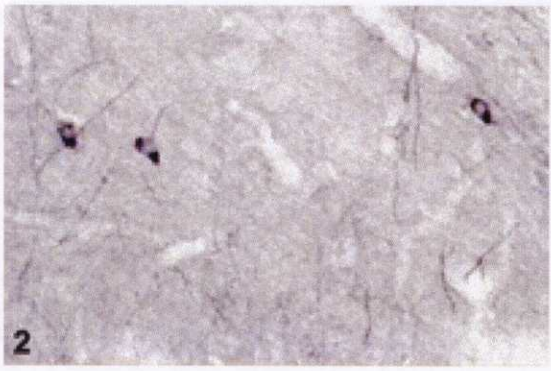
CeM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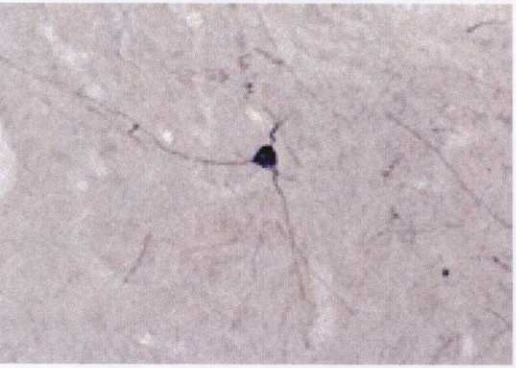

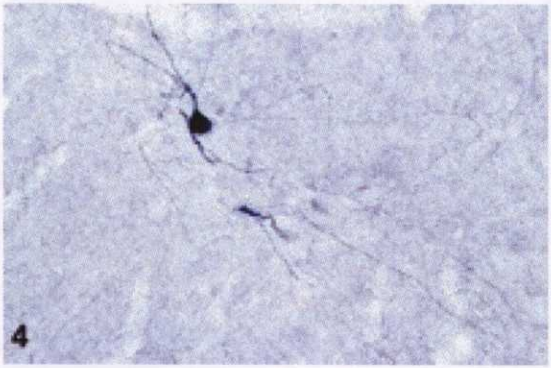
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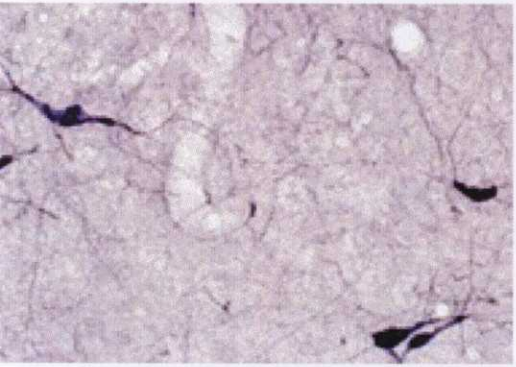

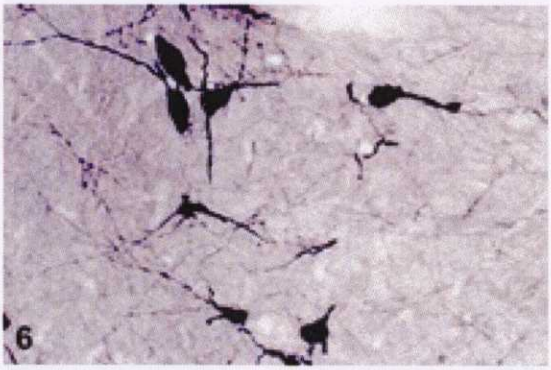
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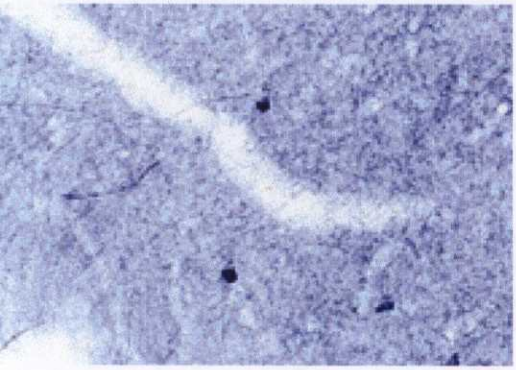

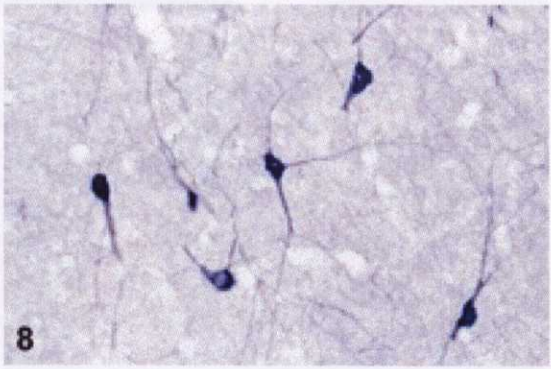
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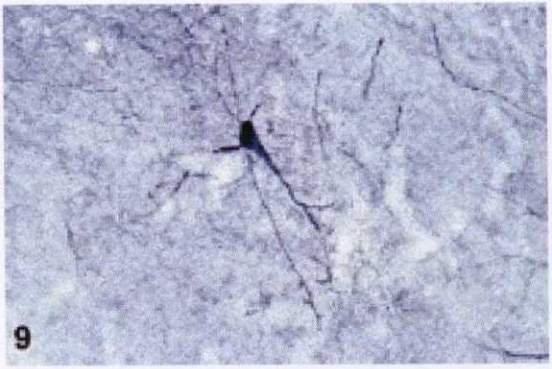
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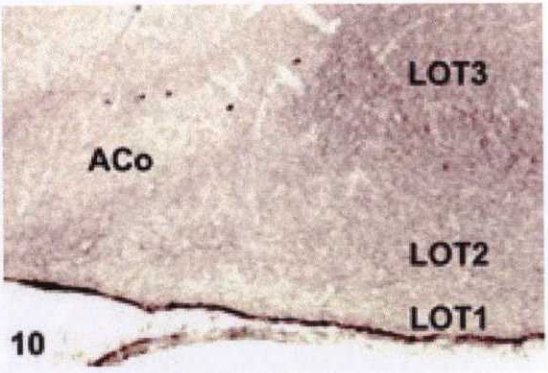
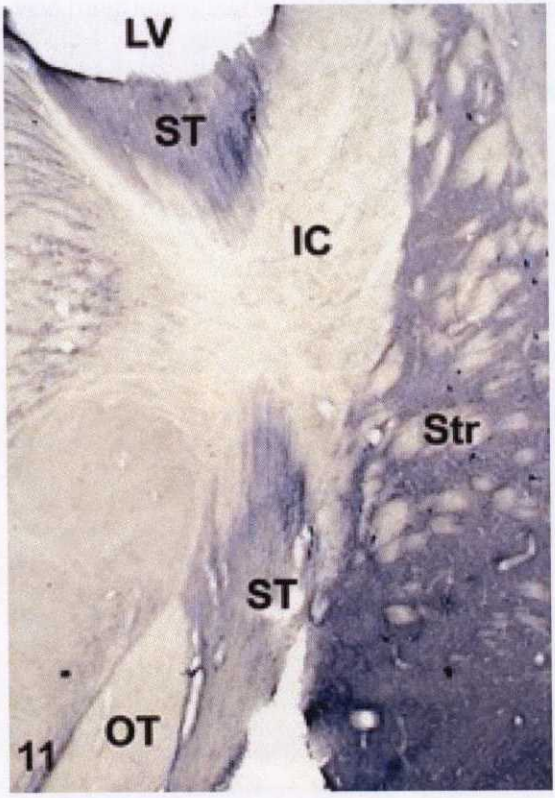

LOT3
ACo
LOT2
LOT1
10


LV
ST
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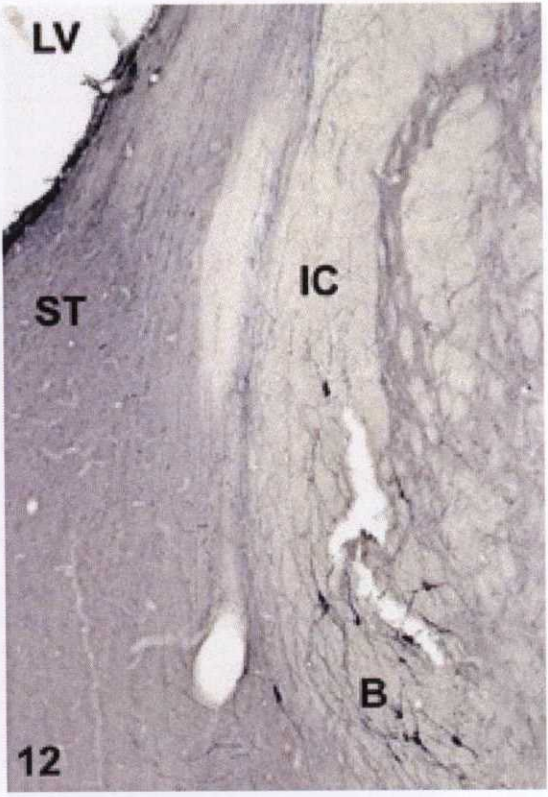
Str
ST
OT
11


LV
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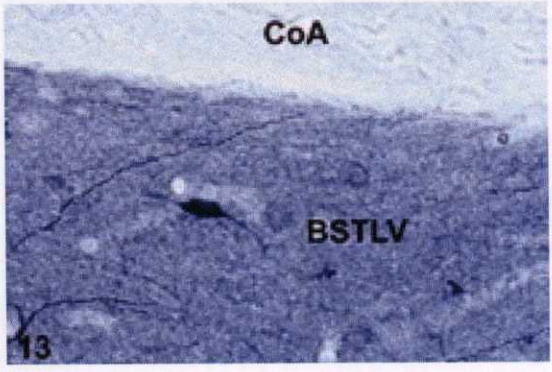
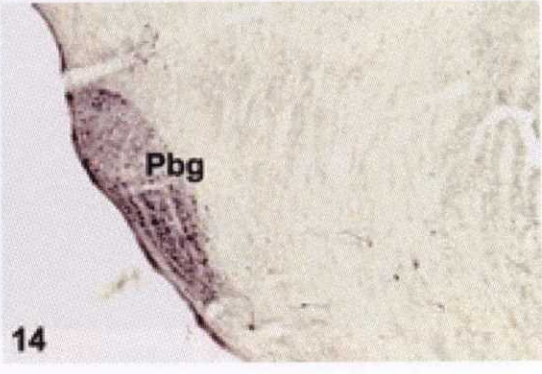

CoA
BSTLV
13


Pbg
14

sesses morphological features of both Am and Str. Thus, it appears plausible that the presently reported cholinergic neurons in AStr are similar (if not identical) to the large cholinergic interneurons in the striatum [18]. NITECKA and FROTSCHER [12] proposed that the cholinergic neurons in AAA and Ce constitute the intraamygdaloid extension of the basal magnocellular nucleus. This speculation was apparently influenced by the just appearing concept of the "extended amygdala" coined by ALHEID and HEIMER [19]. We partially agree with the suggestion of Nitecka and Frotscher [12]. The cholinergic neurons in AAA are not a direct continuum of the Ch4 group but the neuronal features, especially of the larger neurons in the ventral subdivision are practically the same with the prominent Ch4 neurons. On the other hand, the cholinergic neurons in Ce are immediately adjacent to the ventral band of the Ch4 group (see Fig. 1) but the Am neurons are smaller and clearly less intensely stained than the robust neurons in B. The dense cholinergic axonal plexus in BL was reported repeatedly by the Mesulam's group [10,11], but only CARLSEN and HEIMER [6] encountered cholinergic perikarya in BL. We confirm their finding, and propose that the BL cholinergic neurons are interneurons contributing to the dense axonal plexus in BL that has mainly an extrinsic origin [8, 10, 11]. In the "olfactory Am", we extend the observation of PHELPS et al. [15], providing data on the differential topical distribution of cholinergic cells and fibres in ACo and LOT.

The amount of cholinergic axons in Am is very large. According to EMRE et al. [10], the density of these axons reached levels that were higher than in any other part of the forebrain except for the striatum. Thus, it is clear that the abundant cholinergic axons in ST are afferent to Am and arise in the basal forebrain. There is a general agreement that it appears as a sole source of the cholinergic innervation. We would like to point several other sources of afferent cholinergic axons to Am. We recently described projections to the amygdala from the pedunculopontine tegmental nucleus, the Ch5 group [1] and from the laterodorsal tegmental nucleus, the Ch6 group [1] but this projection is probably moderate [20]. On the other hand, we described also a prominent projection from the parabigeminal nucleus [20]. The cholinergic neurons in Pbg (shown in Fig. 14) were first described as the Ch8 group by MUFSON et al. [21].

In conclusion, Am contains a moderate cholinergic neuronal population, characteristically distributed in several nuclei, and the cholinergic population is not uniform. The density of cholinergic axons in Am is also variable, with largest concentrations in

Fig. 9. A single positive medium-sized neuron in Me surrounded by numerous cholinergic axons. × 200

Fig. 10. Low magnification photograph of the basal sector of Am. The deeper part of ACo contains a row of positive neurons. The three layers of the LOT contain cholinergic axons that are extremely numerous in the deep layer (LOT3). × 100

Fig. 11. Low magnification photograph demonstrating the strongly stained cholinergic axons in ST that are in contrast with OT and IC. Medial is to the left. Note the strong staining of Str that contains also numerous cholinergic neurons. × 100

← Fig. 12. Low magnification photograph demonstrating the abrupt prethalamic descent of ST towards BST. Medial is to the left. Lateral to the ST is the unstained IC. It contains the most dorsal cholinergic neurons of the Ch4 group (B) that are large and deeply stained. × 100

Fig. 13. The pale upper part of the figure is the CoA that contains no cholinergic axons. Ventral to CoA in the BSTLV are observed numerous cholinergic axons and terminals, one deeply stained neuron and several faintly stained perikarya. × 200

Fig. 14. Low magnification photograph of the caudolateral mesencephalic tegmentum. Medial is to the right. The small but sharply demarcated region containing densely arranged small cholinergic neurons is the Ch8 group – the parabigeminal nucleus (Pbg). Medial to it several scattered cholinergic neurons belong to pars dissipata of the pedunculopontine nucleus (Ch5 group). × 100

BL and LOT. Although part of the cholinergic axons in Am are derived from local circuit neurons, the great majority are emitted by the basal forebrain, and from the cholinergic groups in the mesopontine tegmentum [1, 20, 21].

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