MORPHOLOGY AND DISTRIBUTION PATTERNS OF GALANIN IMMUNOREACTIVE AXONS IN THE RAT BASAL FOREBRAIN COMPARISON WITH THE DISTRIBUTION PATTERNS OF GAD IMMUNOREACTIVE AXONS

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Comparing galanin and GAD immunoreactive axons in the rostral part of the basal forebrain complex, striking differences were observed regarding their distribution patterns and axonal morphology. In regions with very dense GABAergic innervation, galanin positive fibres were absent. The functional significance of these differences is not yet known. "Stalked" boutons were only formed by galanin containing axons, contrary to GABAergic axons which were endowed with boutons "en passage", only. In galanin immunoreactive sections counterstained with cresylviolet most of boutons were found to be distributed in the neuropil.

Galanin is supposed to be a neuropeptide influencing the release of acetylcholine from axon terminals or inhibiting the activity of cholinergic projection neurons by galanin containing interneurons (4, 6, 11). In the rat basal forebrain (BF), galanin was found to be colocalized in a subpopulation of cholinergic neurons located in the medial septal and diagonal band regions (12). The efferent projection of these double-labelled cholinergic neurons has been elucidated by use of fluorescent tracer injection in the hippocampal region, leading to their identification as galanin containing cholinergic septo-hippocampal projection neurons (129).

Contrary to the galanin containing neurons of the septal region, galanin reactive fibres were found to be immunolabelled also in rat brains which were untreated with colchicine. Their distribution pattern is not congruent with that of galanin immunostained cells but seems to be correlated with some cytoarchitectonically and im-

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munocytochemically defined subregions of the medial septal and diagonal band nuclei (9, 18) and differs remarkably from that of glutamic acid decarboxylase (GAD) or gamma-amino butyric acid (GABA) immunostained fibres. As GABA and galanin are known to influence the activity of contacted cholinergic neurons by inhibition or self-regulation (4, 6, 13), the comparison of their distribution patterns may give rise to some implications regarding the output regulation of the BF nuclear complex.

MATERIALS AND METHODS

Galanin and AChE Immunohistochemistry

Four adult male Wistar rats were anaesthetized with diethyl ether (two animals were given an injection of colchicine- 120 $\mu\text{g}/\text{rat}$ in 20 μl saline) by transcardial perfusion of isotonic saline (0.9%) followed by a fixative, containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After 15 min of perfusion, the brains were removed and the forebrains were dissected out. The tissue blocks were postfixed in the same solution at 4°C for 12 hr followed by another 12 hr in PB, containing 20% sucrose at 4°C. Frontal sections (50 μm) cut on a Vibratome (Oxford Lab., USA) were rinsed for 12 hr in PB (containing 0.2% Triton X-100) at 4°C. Alternate sections were stained using NADPH-diaphorase histochemistry according to Scherer-Singler *et al.* (15) and were counterstained using cresylviolet. The galanin immunohistochemistry with a polyclonal rabbit anti-galanin antiserum (Peninsula Labs., USA), the acetylcholinesterase (AChE) immunohistochemistry with a polyclonal rabbit anti-AChE antiserum (Dr. Grassi, Gif-sur-Yvette, France). The following sequential procedure (19) was carried out in: 1) normal goat serum (1 : 50, 60 min, room temperature); 2) antiserum to galanin (1 : 1000) or antiserum to AChE (1 : 1000) for 36 hr at 4°C; 3) goat anti-rabbit immunoglobulin (1 : 30, 1 hr., room temperature) and 4) rabbit peroxidase-antiperoxidase (PAP) complex (1 : 500, 2 hr, room temperature). After each incubation step, the sections were rinsed three times (10 min) in PB. All solutions with exception of diaminobenzidine (DAB) solution contained 0.2% Triton X-100. After DAB-H₂O₂ reaction, the tissue slices were mounted on glass slides, dehydrated and mounted in neutral balsam. In controls, the first antibodies were replaced by normal rabbit serum or omitted.

GAD Immunohistochemistry

Two adult male Wistar rats were perfused in diethyl ether anaesthesia with 50 ml 0.9% NaCl followed by a fixative containing 5% paraformaldehyde, 0.9% NaCl, 0.5% zinc salicylate in distilled water. The removed brains were postfixed for 2–3 hr in the same fixative at 4°C and rinsed overnight in 0.1 M Tris-buffered saline (TBS), pH 7.4. The forebrains were transversally cut with a Vibratome (50 μm). The sections were again rinsed in several changes of TBS. The GAD immunohistochemistry was performed with the polyclonal S3 GAD antiserum (14). The free-floating sections were stained by the following procedure: 1) 3% H₂O₂ in 10% methanol, 10 min; 0.5% Triton X-100, 10 min; 3) preincubation with 10% normal rabbit serum and 0.1 M D-L-lysine for 12 hr; 4) S3 GAD antiserum (from sheep), 1 : 2000 for 24 hr; 5) rabbit anti-sheep and goat immunoglobulins, 1 : 50 for 45 min; 6) peroxidase-antiperoxidase complex (PAP, from goat), 1 : 200 for 45 min. DAB was used as chromogen after intensification procedure according to Adams (1). All incubation steps were carried out at room temperature and the sections were rinsed in several

changes of TBS. Finally, the sections were mounted on glass slides with alcoholic gelatine. Specificity controls were carried out by omitting the primary antibody.

RESULTS

Galanin

Contrary to the cell bodies, the galanin containing axons were immunostained as well in colchicine treated as in untreated rat brains. Compared with the neighbouring nuclei of the lateral septum, the septal nucleus was poorly innervated by galanin containing fibres (Fig. 1a). They were absent inside the vertical (vDB) and horizontal (hDB) limb of the diagonal band, only the marginal and lateral border zones were innervated. A broad and very dense band of galanin containing fibres occupied the lateral border line of the hDB and its ventro-lateral extension, forming here an arrowhead-like figure in transversal sections (Fig. 1a). This band of fibres converged with another band of galanin positive fibres which were located medially to the nc. accumbens. This lateral fibre assembly could be followed up for caudal levels in serial sections. It was found to be distributed in the ventral pallidum (VP), but also in the substantia innominata-nc. basalis Meynert complex (SI-NB). In the latter one they showed their highest packing density in the ventral part beneath the zone surrounding the globus pallidus. Like in the hDB, the galanin containing fibres were absent in the nc. preopticus magnocellularis (NPM).

The stained axons represented terminal branchings rich in varicosities. These varicosities represented two types: one could be found in the course of the axon, the other one was situated at the tip of short stalks originating from the irregularly twisted axonal main shafts (Figs. 2a, b). These two types of varicosities occurred in the same axons. Most of the axons gave rise to short side-branches which likewise formed the "stalked" varicosities.

Two series were counterstained with cresylviolet to look for possible correlations between Nissl-stained cell bodies and the galanin immunoreactive fibres. These double stainings showed that the varicosities of galanin positive fibres seem to be randomly distributed in the neuropil. No preference for their location at the surface of neuronal somata could be registered (Fig. 1c).

One brain was stained for galanin and AChE immunohistochemistry in alternate transversal sections through the BF region. It could be seen that the most dense innervation with galanin positive fibres was found in regions of the BF nuclei where several AChE positive cell assemblies formed insular conglomerates. Such regions were the lateral border of the hDB, the ventral part of the bed nucleus of stria terminalis and the center of the ventral part of SI-NB.

GAD

Contrary to the distribution patterns of galanin containing axons, all BF regions were supplied with GAD containing fibres, but in different densities. The highest density of GAD positive axons in the BF was found in some regions where galanin

FIGS. 1a-c. a. Distribution patterns of galanin positive fibres in the septum and ventral pallidum.
b. Distribution patterns of GAD positive fibres in the septum and ventral pallidum.
c. Galanin immunoreactivity counterstained with Nissl technique. Cell somata are not surrounded by axonal varicosities.

FIGS. 2a-b. Galanin positive fibres, in Fig. b "stalked" varicosities are well visible.
c, d. GAD positive fibres, in d. varicosities of the "en passage" type can be seen.

reactive fibres were absent, i.e. the VP and the dorsal part of the SI-NB (Fig. 1b).

The morphology of GAD positive axons differed remarkably from that of galanin containing fibres. The axons appeared also poorly branched but the varicosities were larger and only of the "en passage" type. No "stalked" varicosities were present contrary to galanin positive fibres (Figs. 2c, d). In serial sections of colchicine treated brains, where galanin immunohistochemistry was combined with NADPH-diaphorase histochemistry, no double-labelling was obtained.

DISCUSSION

The fibres expressing galanin immunohistochemistry in the rat BF complex represent a type of axon which has been frequently found in telencephalic and diencephalic regions (5, 8). The characteristic feature of this morphologically determined type consists of very short side branches, known as stalked boutons, which are characteristic of cortical axons distributed in the dorsal lateral geniculate nucleus, for instance (3, 5). In this diencephalic region this morphological type of axon was found to contact the distal portions of dendrites (7).

In the rat BF complex a similar type, resembling the galanin positive fibres of this region, was also stained in Golgi-Kopsch series besides other axonal types (17). In our preparations, where the galanin immunoreactivity was combined with Nissl-staining, no signs of a preferential axo-somatic connectivity have been found, giving rise to the conclusion that they mainly contact the neuropil also in this region, i.e. dendrites.

The origin of the galanin immunostained axons in the rat BF is not yet known, as is not the possible content of other neuroactive substances which may come into question (10). This has to be further investigated by using immunohistochemistry. As numerous boutons, which apparently are peptidergic, were found at the electron microscopic level (2), it can be assumed that galanin containing terminals may form a part of them. The distribution patterns of galanin positive axons are of some interest, especially when compared with the distribution patterns of GAD immunoreactive axons (Brauer *et al.*, in preparation).

The high density of axons in regions where some assemblies of cholinergic cells occur, implies the assumption that special functional interrelations between these two neuronal components exist which have to be elucidated. One of these cholinergic subpopulations in the rostral BF is the outer assembly of cholinergic cells near the margin between hDB and VP. These cells have never shown coexpression of NADPH-diaphorase histochemistry (16), contrary to other cholinergic subpopulations where double-labelling occurs. It cannot be excluded that not yet known functional interrelations with regard to these findings and the specificity of galanin containing fibre innervation may exist. There is still a lack of information on possible functional differences between these subpopulations of cholinergic neurons. From our investigation, we know that the cholinergic neurons which contain NADPH-diaphorase do not appear identical with those coexpressing galanin immunoreactivity.

Comparing the distribution patterns of galanin and GAD immunoreactive fibres it seems to be obvious that there exist striking differences, which appear opposite in the septal and diagonal band region. Areas which are occupied by especially dense GAD innervation lack the supply of galanin immunoreactive axons. The functional consequences of this observation have to be elucidated. Whereas the action of

GABAergic axons—represented by GAD positive fibres—on neurons is known to be inhibitory, the function of the galanin positive terminals, as described here, is unknown partly because of the information lacking regarding a possible coexisting neuroactive substance, which seems not to be acetylcholine.

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