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The connections of the endopiriform nucleus with the insular claustrum in the rat and rabbit

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The connections between two parts of the claustrum in the rat and rabbit were studied using the highly fluorescent lipophilic carbocyanine dye (DiI). After the application of DiI crystal into the endopiriform nucleus, labeled fibers in the insular claustrum were observed in its part directly neighboring the insular cortex and capsula externa. Additionally, numerous projections into the piriform, insular and entorhinal cortices were present. The presence of connections between the endopiriform nucleus and insular claustrum suggests its role concerned with the processes taking part in the allocortical regions as well as in the limbic system.

key words: claustrum, endopiriform nucleus, connections, rat, rabbit, dil

INTRODUCTION

The claustrum is a relatively large telencephalic structure of uncertain ontogenetic origin and function. In all mammals two main parts: the dorsal (insular claustrum) and ventral (endopiriform nucleus – En or prepiriform claustrum) can be distinguished. These two parts of the structure reveal some important differences concerning the developmental pattern, connections, cellular structure and histochemical characteristics. Endopiriform neurons are generated in the palliostriatal ventricular angle, whereas claustral neurons develop in the neocortical neuroepithelium [1]. Both parts of the structure are generated in different periods of development, revealing the characteristic age gradient, according to which the endopiriform nucleus represents the older part of the structure.

There is strong anatomical evidence that both parts of the claustrum possess different patterns of connections. The dorsal part is mainly and reciprocally connected with the various regions of the isocortex [6,15,18,20,23], whereas the ventral one possesses connections with the entorhinal and piriform cortices [21,27–29], as well as with the olfactory and limbic structures [2,12].

The comparative anatomical studies performed on the representatives of selected species revealed that insular claustrum and endopiriform nucleus undergo some characteristic changes during the brain evolution [5,22]. Whereas in some representatives of Insectivora and Rodents the domination of the ventral part of the claustrum has been present, in the Carnivora and particularly in Primates it undergoes a substantial and relative reduction. This process may reflect general changes taking part in the evolution of the neo- and allocortex, which are the principal aims of the claustral projections. Both parts of the structure consist of neurons of differentiated sizes and shapes, as well as numerical density and total number [11]. There are also significant differences in the numbers of neurons containing parvalbumin and calbindin D-28k in these two parts of the rat claustrum [6].

In the light of these results, the physiological role of this structure still remains somewhat mysterious. The hypothetical cooperation of both parts of the structure in transferring information coming from the neocortical regions via claustrum into the allocortical (limbic) structures [12,27,29] is still not well documented because there is a lack of evidence of intraclaustral connections.

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The aim of this study is to characterize the presence and morphological pattern of connections between the ventral and dorsal parts of the claustrum in the rat and rabbit, by means of the lipophilic carbocyanine dye (1,1'dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate; DiI), applied on the fixed tissue.

MATERIAL AND METHODS

The fluorescent lipophilic carbocyanine tracer DiI, which is weakly fluorescent in water but highly fluorescent and quite photostable when incorporated into cell membranes, was used in this study. DiI exhibits distinct orange fluorescence and can be detected by using the standard rhodamine optical filter with excitation wavelength of 565 nm.

Eight adult Wistar rats (weight 250–300 g) and three New Zealand adult rabbits (weight 3.5–4 kg),of both sexes, were used. Care and treatment of the animals were in accordance with the guidelines for laboratory animals established by the National Institute of Health, as well as by the Local Ethical Committee of the Medical University of Gdańsk.

The animals were deeply anesthetized with diethyl ether — inhalation anesthesia — (rats) or fentanyl (0.03 mg/kg i.p.) and thiopental in a dose of 80 mg/kg i.p. (rabbits). Under deep anesthesia, the animals were perfused transcardially with 0.9% saline containing 10,000 units of heparin; followed by 4% solution of paraformaldehyde in phosphate buffer (pH 7.4 and 4°C) containing 0.1% EDTA (ethylenediamine tetraacetate disodium salt). Immediately after perfusion the brains were removed from the skulls and fixed in 4% solution of paraformaldehyde in phosphate buffer (pH 7.4 and 4°C) containing 0.1% EDTA for 1-2 days.

The EDTA was added in order to inhibit the diffusion of DiI out of membranes in preparations. This procedure improves the sharpness of labeled structures and eliminates the transneuronal labeling in the material [10].

Then the brains were cut coronally into 3–5 mm-thick blocks. The crystal of DiI was inserted into the brain section using the tip of the micropipette held vertically to the frontal surface of the brain section. Then a small crystal of DiI was slightly pressed into the endopiriform nucleus under the stereomicroscope MZ8 (Leica; Germany). Subsequently, the brain sections were gently rinsed to remove loose DiI crystals and stored in fixative (4% solution of paraformaldehyde in phosphate buffer; pH 7.4 containing 0.1% EDTA) for three months in a dark place at room temperature.

Then the brains were embedded in 3% agar and cut in the transverse plane on the vibratome 1000 (Pelco; USA), to obtain 50–100-μm-thick sections, which were mounted on slides using 50% glycerol in 0.1 M phosphate buffer, coverslipped and studied in the fluorescent microscope Leica DMLB (Leica; Germany) equipped with the filter system, providing an excitation wavelength of 565 nm and microscope digital camera system DP10 (Olympus Optical Co., Japan). The histological sections were studied under the confocal scanning system MicroRadiance (Bio-Rad; UK) equipped with the Argon laser producing dichromatic light at 488 and 514 nm. The 514-nm line of this laser was applied to excite DiI, using an excitation filter 514 and an emission longpass filter E570LP. For 3D reconstruction, the image analysis program LaserSharp 2000 v. 1.0 (Bio-Rad; UK) was used.

Some of the sections were finally counterstained in 0.001% bisbenzimide (Boehringer Mannheim, Germany) in 0.1 M phosphate buffer. This staining revealed the outline of sections and identified the cortical cytoarchitecture.

RESULTS

The application of DiI into the endopiriform nucleus in the rat

DiI crystals were introduced into the rostral, medial and caudal parts of the endopiriform nucleus. In all cases the diffusion of the tracer around the place of DiI crystal application was observed (Fig. 1B). The claustrum in the rat is not well separated from the cortex (Fig. 1A), therefore the place of tracer application comprised also a small part of the surrounding piriform cortex in three animals.

In all cases we observed the labeled fibers in the insular claustrum (Fig. 1B, 1C, 1D), which were localized in the area directly neighboring the insular cortex and external capsule. Observed labeled fibers are randomly oriented: some of them pass almost parallel to the capsula externa while others run in various directions within the dorsal part of the claustrum (Fig. 1D). The dichotomic divisions of the neuronal fibers at acute angle are sporadically present in the dorsal part of the claustrum. There are multiple conspicuous varicosities along stained axonal fibers. No labeled neuronal somata are visible in the insular claustrum. Fibers running within the capsula externa and in the area corresponding to capsula extrema are very numerous. Some of them change their course penetrating to the adjacent structures: the insular cortex, striatum and more posteriorly to the amygdala.

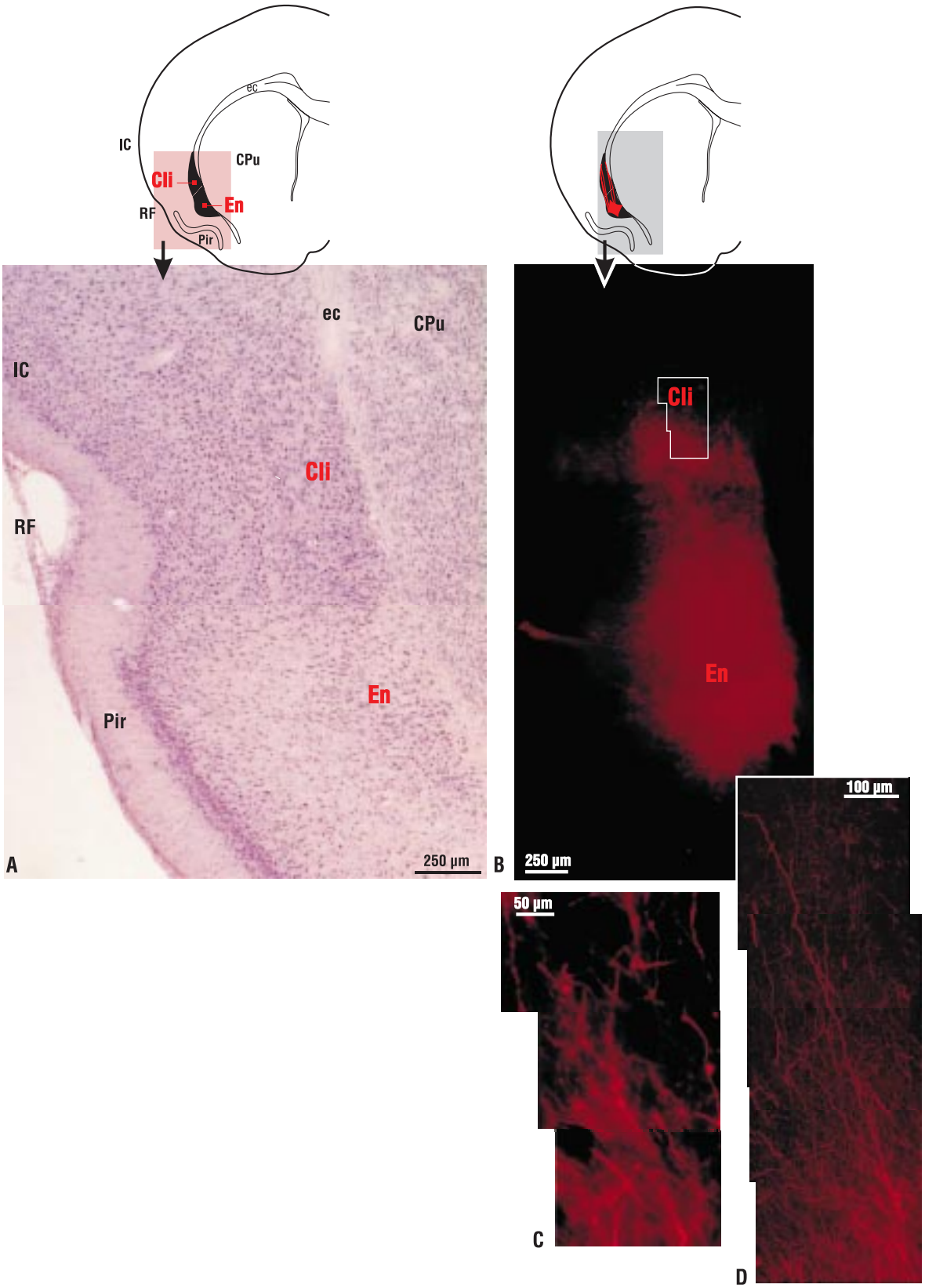

Figure 1. A) Localization of the insular claustrum and endopiriform nucleus on the coronal plane of the rat's brain on scheme and cresyl violet stained section. B) Injection of DiI into the endopiriform nucleus with bundles of labeled fibers passing to the insular claustrum as well as to the piriform and insular cortices. C) Higher magnification of the labeled fiber localized in the insular claustrum. D) 3-D confocal reconstruction of the bundle of fluorescent fiber going from the endopiriform nucleus to the central part of the insular claustrum. Abbreviations: Cli — insular claustrum, CPu — caudate-putamen, ec external capsule, En — endopiriform nucleus, IC — insular cortex, Pir — piriform cortex, RF — rhinal fissure.

In the endopiriform nucleus there are numerous labeled fibers which form a network denser than in the surrounding cortical areas. The highest number of neuronal fibers is present close to the crystal application site, but numerous ones, clearly distinguished, reach also the anterior or posterior end of the structure. In all cases labeled fibers going to the anterior and posterior piriform cortices and insular cortex are also present. The projections coming from the endopiriform nucleus to the piriform and insular cortices were more numerous than these terminating in the claustrum. Fibers projecting towards the piriform cortex have a more horizontal course - and towards the insular cortex – a steeper course. All projections from the endopiriform nucleus were observed ipsilaterally to the site of DiI crystal application.

The application of DiI into the endopiriform nucleus in the rabbit

The area of diffusion around the place of DiI crystal application was clearly visible (Fig. 2B). Contrary to the rat, in the rabbit it was totally located within the area of the endopiriform nucleus. Labeled fibers in the insular claustrum (Fig. 2B, 2C, 2D), similarly to the rat, were localized in the zone directly neighboring the insular cortex and external capsule.

In the rabbit, the external and extreme capsules were much better developed, and the claustrum was distinctly separated from the surrounding structures (Fig. 2A).

Projecting fibers, labeled after DiI insertion into the endopiriform nucleus, show a topographical pattern and morphology similar to the rat as described above. Nevertheless, in the rabbit some differences can be reported: 1) the presence of fibers running in cranio-caudal axis within the endopiriform nucleus and 2) the lack of arborization of the fibers going from the site of crystal insertion to the insular claustrum. In cases where the place of the crystal application includes capsules surrounding the claustrum, the pattern and morphology of the fibers were similar to the one already described in the rat.

The labeled fibers in the anterior and posterior parts of the piriform, entorhinal and insular cortices are found in the representatives of both species. Apart from cortical structures, there is also evidence of labeled projection to the striatum and amygdala.

The labeled fibers were present exclusively ipsilaterally to the tracer application site.

DISCUSSION

Technical considerations of the DiI labeling

In the present study, a tracer from the group of fluorescent carbocyanine dyes-DiI [19] was used. We decided to choose this tracer because of the possibility of applying it in the fixed tissue. This method, although time-consuming, enables the insertion of the crystal of the DiI tracer more precisely and without contamination of the surrounding structures than performing pressure injections of the tracer solution during stereotactic procedure. This seems to have a very important meaning for the results, especially in relationship with the structures of our interest. Once applied to the tissue, the DiI diffuse laterally within the plasma membrane, resulting in staining of the entire cell and producing detailed labeling of fine neuronal projections. The dye usually does not transfer from the labeled to the unlabeled cells, unless the membrane of a labeled cell is disrupted, and apparently does not transfer through gap junctions. The transfer of these probes between intact membranes is usually negligible. The combination of the confocal microscopy with the application of a lipophilic membrane dye led to the conclusion that it offers an excellent opportunity for the investigation of structural features as well as their connections [19].

Morphological features of the En projecting fibers

After application of the DiI crystal into the endopiriform nucleus, the labeled fibers in the dorsal part of the claustrum were detected. They were localized directly neighboring the insular cortex and capsula externa. In the literature only limited information concerning the anterogradely labeled fibers, connecting the endopiriform nucleus and insular claustrum is available [2]. This projection consists of sparse axons, except for the immediate vicinity of injection site. However, the exact significance of the interclaustral connections is still not explained satisfactorily, there are some concepts which stress the possible role of both parts of the claustrum in transferring (and probably integrating) the information coming from the neocortex into the limbic system structures, in particular entorhinal cortex and hippocampus [17,27,29]. In some pathological conditions, the demonstrated interclaustral connections may constitute a possible way of spreading epileptic seizures

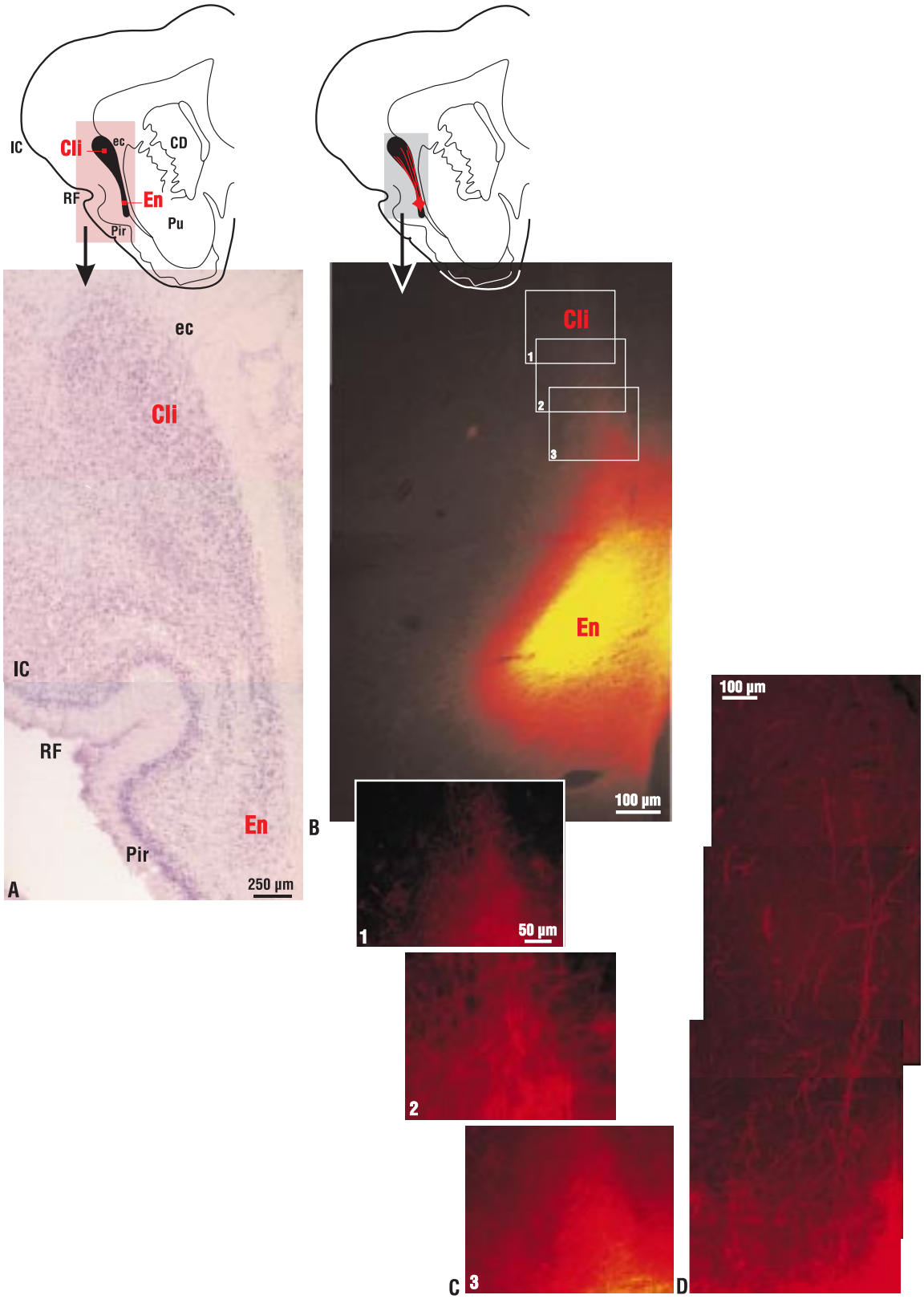

Figure 2. A) Localization of the insular claustrum and endopiriform nucleus on the coronal plane of the rabbit's brain on scheme and cresyl violet stained section. B) Injection of DiI into the endopiriform nucleus with bundles of labeled fibers passing to the insular claustrum as well as to the piriform and insular cortices. C) 1–3. Higher magnification of the labeled fiber localized in the insular claustrum. D) 3-D confocal reconstruction of the bundle of fluorescent fiber going from the endopiriform nucleus to the central part of the insular claustrum. Abbreviations: Cli — insular claustrum, CD — caudate nucleus, ec — external capsule, En-endopiriform nucleus, IC — insular cortex, Pir — piriform cortex, Pu — putamen, RF — rhinal fissure.

from the primary epileptogenic sites (e.g. piriform cortex, "area tempestas") throughout the endopiriform nucleus and the insular claustrum [9,16,24]. The endopiriform nucleus may generate long duration population bursts, which according to some authors [25] may be responsible for the initiation of seizure activity in some pathological conditions.

After the application of DiI into the rostral, middle and caudal parts of the endopiriform nucleus, in the rat and rabbit, numerous labeled fibers were present within the nucleus. The concentration of the fibers is the highest close to the crystal application site. However, the labeled fibers extending towards both the rostral and caudal limits of the structure were observed. The concentration of the neuronal fibers within the endopiriform nucleus outnumbers those in the surrounding regions of the piriform and insular cortices. These results remain in agreement with the observations of other authors, performed on the basis of experiments with anterograde tracing [2,12]. In the light of some published results there are significant differences in the concentration of labeled fibers in the antero-posterior direction, independently from the tracer application site [2].

Our results showed substantial and widespread projections from the endopiriform nucleus to the anterior and posterior divisions of the piriform cortex. The presence of these projections has been demonstrated by Golgi staining [26] and axonal transport methods [2,7,12,13].

Also physiological results indicate the presence of close connections between the endopiriform nucleus and the piriform cortex [8,9]. These reciprocal and numerous connections between the endopiriform nucleus and the piriform cortex may result in its modulatory function in relationship to this area. We discussed the significance of these connections in the normal and pathological conditions separately [12].

Apart from the above-mentioned connections, fibers projecting to the insular cortex are detected in all studied cases. These connections, documented by our observations, may be confirmed by the results of electrophysiological studies according to which the paroxysmal epileptiform activity can develop in layer VI of the agranular insular cortex, in synchrony with that in the endopiriform nucleus [4].

Our observations of numerous labeled axons ending in the entorhinal cortex correlate with some other reports [2,3,13]. Similarly, the projections to the perirhinal cortex [2], as well as to the amygdaloid complex [2,12,14] were described by means of other methods.

Present results suggest that the ascending connections from the ventral to the dorsal parts of the claustrum exist, and that those pathways enable the exchange of information between them. The number of the projecting fibers remains unexpectedly small, being less numerous than the projection reaching the piriform or insular cortices. This observation can suggest the important role of the endopiriform nucleus in the processes of the fast transmission of the information from the limbic region to the neocortex throughout the insular claustrum, which can be important in physiology for learning and memory, as well as in pathological conditions in the development of epileptic seizures.

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