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The anatomical relationships between the serotonergic afferents and the neurons containing calcium-binding proteins in the rat claustrum

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Abstract. Claustrum is a telencephalic structure integrating information of various modalities. Proper functioning of this structure depends on the presence of a network of intrinsic connections. This includes GABA-ergic neuronal populations that also contain calcium-binding proteins (CaBPs). The goal of this study was to analyze qualitative and quantitative the 5-HT-containing fibers in the rat claustrum and to assess the relationships between these fibers and the populations of claustral neurons expressing CaBPs. We used the methods of immunocytochemistry and morphometry. The serotonergic fibers in the claustrum are heterogeneous, both with respect to their morphology and spatial distribution. Thin varicose fibers are more numerous and are homogeneously distributed within the claustrum. Remaining fibers were thicker and possessed larger varicosities. They were present mainly in the ventral part of the claustrum. Although the serotonergic fibers are found in the vicinity of claustral cells containing CaBPs, direct contacts between these fibers and cells are rare. Other mechanisms, including volume transmission, may possibly mediate serotonergic influences.

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Key words: serotonin, parvalbumin, calbindin D28k, calretinin, confocal microscopy, claustrum

INTRODUCTION

Serotonin (5-Hydroxytryptamine, 5-HT) is known to control numerous physiological and behavioral functions. Extensive evidence indicates that 5-HT modulates sleep, appetite, mood and emotions (e.g. linking fear and the neuroendocrine stress response) (Feldman et al. 1998, Graeff 2002, Halliday et al. 1995). Serotonin is also involved in learning and memory processes. This broad spectrum of activity is possible because of the diversity of influences of serotonin: it is acting through various receptors (Aghajanian and Sanders-Bush 2002, Barnes and Sharp 1999, van Hooft and Yakel 2003) that are differently expressed among neuronal populations (Freund et al. 1990, Gulyas et al. 1999, Paspalas and Papadopoulos 2001) and activate various transduction pathways (Hornung 2003, Leger et al. 2001).

In the claustrum, various subtypes of serotonergic receptors are expressed: 5-HT1A, 5-HT1C, 5-HT1E, 5-HT1F, 5- HT2A and 5-HT2C (Barnes and Sharp 1999, Hoffman and Mezey 1989, Hoyer et al. 2002, Rioux et al. 1999, Wright et al. 1995), indicating that this telencephalic structure that is present exclusively in mammals is an important target of the serotonergic action. Claustrum received relatively little attention of late, and its role is still poorly understood. Currently, claustrum is considered to be a structure belonging to the mesostriatal and mesolimbic dopamine pathways (Fuxe and Agnati 1985, Trinh et al. 2003) and to be involved in the cognitive processes (Sperner et al. 1996). According to fMRI and PET studies, claustrum plays an important role in the integration of information of various modalities (Banati et al. 2000, Calvert 2001, Hadjikhani and Roland 1998, Redoute et al. 2000).

Functional activity of the claustrum depends on the existence of massive bidirectional cortical connections and both intrinsic and extrinsic systems of control. Claustral projection neurons constitute the main part of the cortico-claustro-cortical loop involving motor, somatosensory, auditory, visual and limbic cortical areas (Dinopoulos et al. 1992, Gutierrez-Ibarluzea et al. 1999, Kowianski et al. 2000, LeVay and Sherk 1981, Li et al. 1986, Macchi et al. 1983, Morys and Sloniewski 1986, Sadowski et al. 1997). Contrary to other structures that are also involved in the integration of information (e.g. superior colliculus), claustral projection neurons are mainly unimodal. In the claustrum, polymodal neurons receiving information from more than one cortical area were observed rarely. The population of claustral interneurons consists of approximately 10% of the total number of neurons (Gomez-Urquijo et al. 2000, Kowianski et al. 1996, Spahn and Braak 1985). Interneurons are mainly inhibitory neurons containing GABA as the neurotransmitter. These neurons often contain other substances, including: calcium binding proteins (CaBPs) – parvalbumin (PV); calbindin D28k (CB) and calretinin (CR) (Druga et al. 1993, Real et al. 2003, Reynhout and Baizer 1999, Wojcik et al. 2004); somatostatin; cholecystokinin; VIP; and, NPY or nitric oxide synthase (Eiden et al. 1990, Guirado et al. 1989, Kowianski et al. 2001). Finally, claustrum is a target of the serotonergic, dopaminergic and cholinergic pathways and therefore is also influenced by other, more distant structures (Kitt et al. 1982, Guirado et al. 2003, Lipp and Nauta 1983).

The exact mechanism of the integration of information in the claustrum remains unclear. To better understand functions of this structure, we need more data on its internal connections. A previous study by Baizer (Baizer 2001) described the morphology of serotonergic afferents in the primate's claustrum and proposed that neurons containing CaBPs were a possible target of the 5-HT afferents. Therefore, the goals of this study were: (1) qualitative and quantitative analysis of the 5-HT fibers in the rat claustrum and (2) the description of the relationships between the 5-HT fibers and the subpopulations of claustral neurons that express the three calcium-binding proteins.

METHODS

Four adult rats (200–280 g) of the Wistar strain were used for these experiments. Care and treatment of animals were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the experiments were evaluated and approved by the Local Ethics Committee of the Medical University of Gdansk. The animals were deeply anaesthetized with lethal doses of Nembutal (80 mg/kg of body weight), then they were perfused transcardially with a 0.9% solution of NaCl with heparin, followed by a 4% paraformaldehyde solution in 0.1M phosphate buffer (pH 7.4). The brains were postfixed in 4% paraformaldehyde for 3–4 hours, and then cryoprotected in a 0.1 M phosphate buffer containing 10% sucrose (overnight at 4°C) and 30% sucrose (until sunk). The left hemisphere was

marked in each case by slight damage of the neocortex in the vicinity of the studied area. Coronal, 40-µm-thick, serial sections of the brain were cut on a JUNG 1800 cryostat (Leica, Germany). From each of the brain section sets, every sixth section was stained using the Cresyl Violet method (Clark et al. 1981), then each was air-dried and cover slipped with DPX (Fluka, Germany). A set of twelve sections from each brain (distance from Bregma +3.2 to –1.4, according to Paxinos and Watson [1997]) was stained for serotonin immunoreactivity. The same number of sections was double stained for serotonin- and parvalbumin- or calbindin D28k- or calretinin immunoreactivity. Initially, the free-floating sections were blocked with 3% normal goat serum (NGS) or 3% normal donkey serum (NDS) containing 0.3% Triton X-100 (TX) in 0.01M (pH= 7.2) phosphate buffered saline (PBS) for 1 hour and then they were incubated with the monoclonal rabbit anti-5HT antibody (1:3000; Sigma; USA) diluted in 3% NGS or 3% DGS for 48 hours in 4°C. After multiple rinses in PBS, the sections were incubated for 2 hours at room temperature together with the appropriate fluorophore conjugated secondary antibodies: Cy3- conjugated donkey anti-rabbit or Cy3-conjugated goat anti-rabbit (1:600; Jackson ImmunoResearch; USA). In the double staining method, the free floating sections were blocked with 3% NGS or 3% DGS containing 0.3% TX in 0.01M PBS (pH= 7.2) for 1 hour and then incubated together with a mixture of monoclonal rabbit anti-5HT antibody (1:3000; Sigma; USA) and either polyclonal goat anticalretinin (1:500; Chemicon; USA) or monoclonal mouse anti-parvalbumin (1:500; Sigma; USA), or monoclonal mouse anti-calbindin-D28k (1:1500; Sigma; USA) diluted in 3% NGS or 3% DGS for 48 hours in 4°C. After multiple rinses in PBS, sections were incubated for 2 hours at room temperature) with a mixture of the appropriate secondary antibodies: Alexa Fluor 488 goat anti-mouse or Alexa Fluor 488 donkey anti-goat (1:150; Symbios; Poland) or Cy3-conjugated donkey anti-rabbit or Cy3-conjugated goat anti-rabbit (1:600; Jackson ImmunoResearch; USA). Finally, all sections after immunohistochemical staining, were rinsed with PBS, mounted onto gelatin-coated slides, air-dried, and cover slipped with Keiser Gelatin (Merck, Germany). For the delineation of structures, the brain atlas of Paxinos and Watson (1997) and the nearest cresyl violet section were used. Only the left claustrum was analysed. For the initial analysis, immunohistochemically stained sections were examined with a BX-51 (Olympus, Japan) fluorescent microscope, and a confocal system Radiance 2100 (Bio-Rad, UK), equipped with a Krypton/Argon laser and mounted on a Eclipse 600 (Nikon, Japan) microscope, using the software LaserSharp 2000 v.4.0 (Bio-Rad, UK). The confocal laser scanning microscopy (CLSM) images were obtained using 40X and 60X oil immersion objective lenses of N.A. = 1.3 and 1.4, respectively. The Krypton/Argon laser produces monochromatic light at a wavelength between 488 nm and 568 nm. The 488-nm line was applied to excite the Alexa Fluor 488, while the 568-nm line was applied to excite Cy3. The optimal iris was used for each magnification. On the basis of a 3D reconstruction of the CLSM images, both, (1) the morphometric analysis of 5-HT fibers; and (2) the quantitative analysis of relationships between 5-HT fibers and claustral neurons containing calcium-binding proteins, was done. For the former analysis, images were collected with a resolution of 1024 × 1024 pixels under the 60X objective lens with the zoom set to 3.8. The pixel size equaled 0.05 µm. The thickness of each fiber, the area of the profiles of the varicosities and the distances between them were measured using the analysis program LaserPix v. 4.0 (Bio-Rad; UK). For the latter analysis, images were collected with a resolution of 512 × 512 pixels under the 60X objective lens with the zoom set to 3.3. The pixel size equaled 0.12 µm. Merge images were obtained by means of LaserSharp 2000 v.4.2 software (Bio-Rad, UK). Among neurons in direct contact with the 5-HT fibers, only those cells, which possessed 5-HT buttons on the surface of the bodies and/or proximal part of processes, (without a gap) were rated.

RESULTS

Serotonin immunoreactivity of the rat claustrum

According to the brain atlas by Paxinos and Watson (1997) claustrum extends from +3.0 to –1.4 from the Bregma on coronal sections. In sections stained with an anti 5-HT antibody the borders of the rat claustrum were not evident. Only the medial boundary of the claustrum was clearly distinguishable because of the low 5-HT immunoreactivity of the external capsule (Fig. 1), and only scattered 5-HT positive fibers were observed. The neighboring dorsal endopiriform nucleus was characterized by higher 5-HT immunoreactivity (Fig. 2). The pattern of the 5-HT immunore-

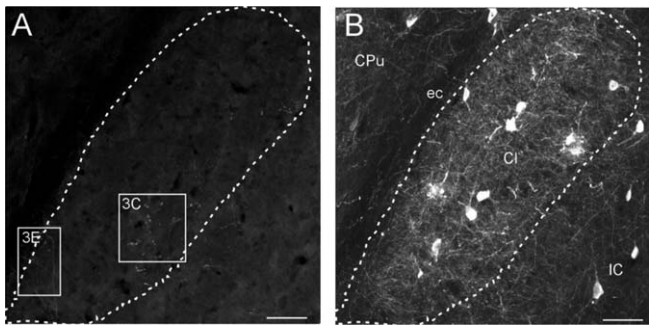

Fig. 1. (A) Photomicrographs of the rat claustrum showing 5-HT-immunoreactivity and relations of the claustrum with neighboring structures. The borders of claustrum (delineated) were hardly distinguishable. (B) Parvalbumin-immunoreactivity of the same region allowed clear differentiation of the structure. Abbreviations: (Cl) claustrum; (CPu) caudate-putamen; (ec) external capsule; (IC) insular cortex. Scale bar is 50 μm.

activity was investigated in coronal sections throughout the length of the claustrum (Fig. 2). A relatively weak network of fibers was revealed after 5-HT staining.

Three types of 5-HT fibers were distinguished on the basis of the morphological differences visible at higher magnification. Thin varicose fibers (0.5 ± 0.1 μm) were observed most frequently (Fig. 3A–B). Their varicosities were of small size (0.8 ± 0.4 μm^2), fusiform, and relatively densely packed (mean distance between varicosities equaled to 2.3 ± 0.9 μm). Those fibers were observed in the entire claustrum. In the anterodorsal portion of the claustrum relatively long fibers oriented mediolaterally prevailed, whereas in the posteroventral portion of the claustrum fibers were oriented mainly in the anteroposterior direction. The latter fibers were much shorter due to their perpendicular course. Thicker fibers (0.8 ± 0.2 μm) were observed much less frequently (Fig. 3C–D). They had spherical varicosities (diameter 1.9 ± 0.7 μm^2) that were loosely placed (mean distance - 3.8 ± 2.0 μm). This subpopulation of 5-HT fibers was observed in the ventral portion of the claustrum. The number of fibers on the coronal sections varied, but their highest amount was observed in the middle part of the claustrum. Some of the fibers of both types were arranged in the basket-like structures that encircled unstained cells (Fig. 3D). The rarest fibers (1 or 2 per section), were those without varicosities, straight and thick (1.3 ± 0.4 μm). They were observed almost exclusively near the medial boundary of the claustrum (Fig. 3E).

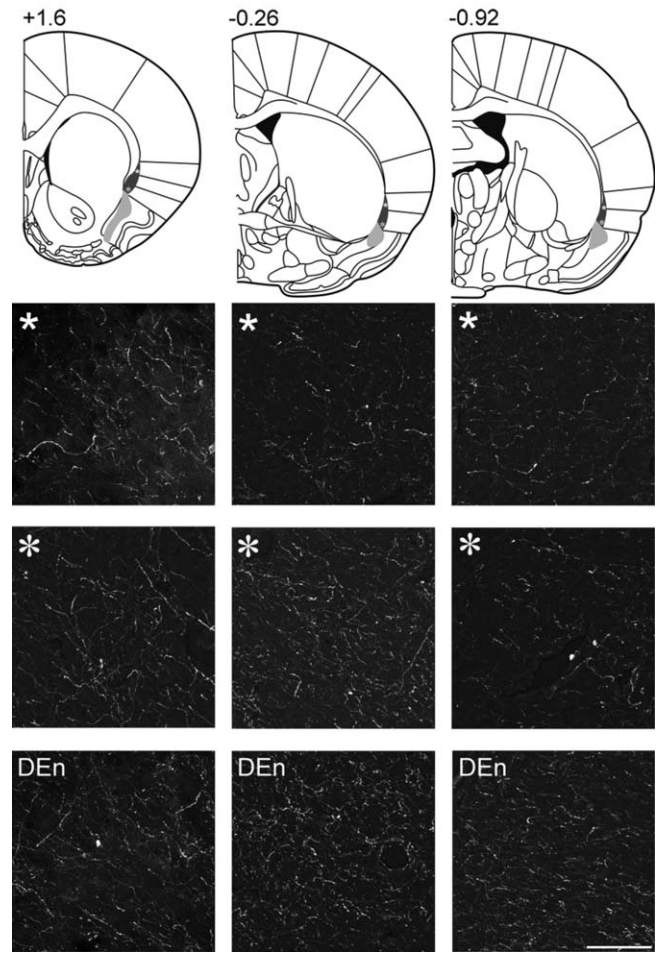

Fig. 2. Photomicrographs of the 5-HT immunoreactivity in the neuropil of the dorsal claustrum (*), ventral claustrum (*) and dorsal endopiriform nucleus (DEn). Specified levels correspond to sections from the atlas of Paxinos and Watson (1997). The dorsal endopiriform nucleus is colored gray on the corresponding drawings of the Paxinos and Watson atlas and the black-colored area dorsal to the gray area represent claustrum. Note higher 5-HT-ir in DEn, especially in the middle and posterior parts of the studied region. Coordinates are given in relation to Bregma. Abbreviations: (Cl) claustrum; (CPu) caudate-putamen; (Den) dorsal endopiriform nucleus; (ec) external capsule; (IC) insular cortex. Scale bar is 50 μm.

Relationships of serotonergic fibers with neurons containing calcium binding proteins

There was no co-expression of 5-HT and the studied calcium-binding proteins in the fibers (Fig. 4A–C). Sporadically, calbindin-D28k-immunoreactive fibers contacted 5-HT terminals (Fig. 4E), an arrangement corresponding to cartridges (axo-axonal terminals). 5-HT terminals were observed among some parvalbu-

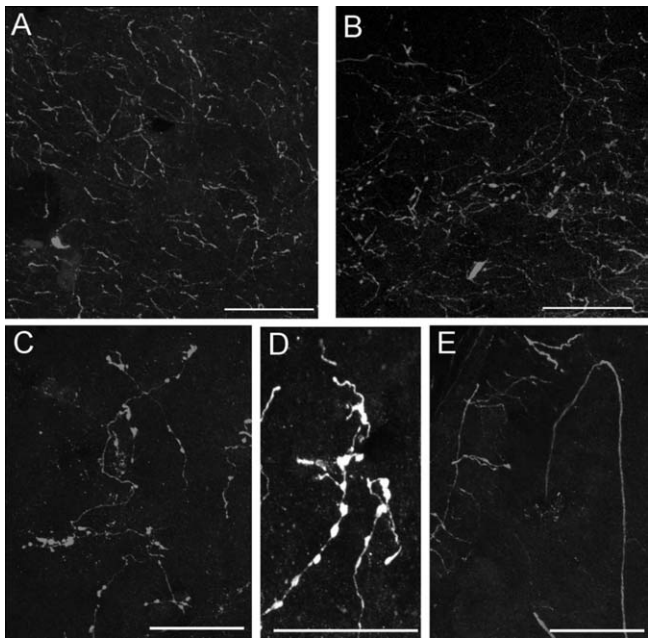

Fig. 3. Photomicrographs of the 5-HT-immunoreactive fibers of different morphology in the rat claustrum: thin varicose fibers from the dorsal part of claustrum (A, B), thick fibers with larger varicosities from the ventral part of the claustrum (C, D), and thick fibers without varicosities from the medial (E) part of the claustrum. Scale bar is 25 μm.

min immunoreactive fibers and terminals clustered around unstained cells (basket-like plexuses, Fig. 4D). Some 5-HT varicosities were found in a close vicinity of cell bodies containing calcium-binding proteins (Fig. 4F–H), but direct contacts appeared rarely. The CLSM analysis revealed the existence of very few

Table I

Percentages of cells containing studied calcium binding proteins that possessed direct contact with 5-HT-ir fibers and terminals

	PV		CB		CR	
Case	n	%	n	%	n	%
Ra 03-001	5/109	4.6%	7/106	6.6%	18/101	17.8%
Ra 03-002	4/66	6.1%	16/149	10.7%	10/68	14.7%
Ra 03-003	2/53	3.8%	21/136	15.4%	9/40	22.5%
Ra 03-004	2/66	3.0%	5/54	9.3%	2/21	9.5%
TOTAL	13/294		49/445		39/230	
Mean		4.4%		10.5%		16.1%
SD		1.3%		3.7%		5.5%

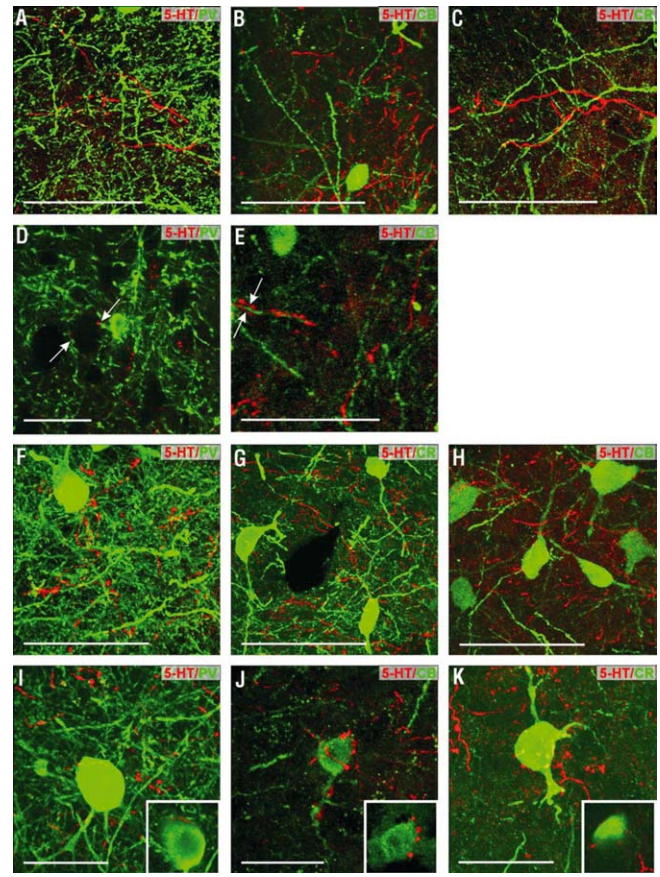

Fig. 4. Photomicrographs of the immunoreactivity for calcium binding proteins and 5-HT. Left column – parvalbumin, middle column – calbindin, right column – calretinin. There was no colocalization of 5-HT with the calcium binding proteins within fibers (A–C). 5-HT- and PV-ir terminals on the surface of the non-labeled cell (basket, D, arrows). 5-HT-ir terminals on CB-ir fibers (arrows, E). 5-HT fibers in the close vicinity of cell bodies containing the studied calcium binding proteins (F–H). Direct contacts of the 5-HT-immunoreactive fibers (I) and terminals (J–K) with cells containing CaBPs. Stacks of confocal images. Lower-left corner photomicrograph shows one 0.05 μm layer. Scale bar equals to 50 μm (A–H) and 25 μm (I, K).

direct contacts between structures containing 5-HT and neurons containing parvalbumin. Using the light microscopic analysis, we could not exclude the existence of a direct contact in only 5% of all PV immunoreactive cells (Table I). In these cases the thin 5-HT fibers were clearly located on the surface of PV positive cells (Fig. 4I). Those cells were of a large size and multipolar shape. In the case of neurons containing calbindin D28k or calretinin, direct contacts with 5-HT structures were observed more frequently and these

were not excluded in the case of about 10% and 16% of all immunoreactive cells respectively (Table I). These cells were also large-sized and multipolar or triangular. In contrast to the PV-immunoreactive neurons, 5-HT terminals were observed frequently at the surface of CB-ir and CR-ir cells (Fig. 4J–K).

DISCUSSION

This study provides new morphological data concerning serotonergic afferents in the rat claustrum and completes previous investigations of serotonergic innervations of this structure in the rat (Vertes 1991, Vertes and Martin 1988), hamster (Morin and Meyer-Bernstein 1999), cat (Leger et al. 2001), and primates (Baizer 2001). We have observed heterogeneous subpopulations of the 5-HT fibers in the rat claustrum both with respect to their morphology, spatial distribution and density.

The diversity of serotonergic fibers in the rat claustrum is not unique for this structure. This has been reported earlier in the cerebral cortex of lizards (Guirado et al. 1989), domestic chick (Metzger et al. 2002), rat (Papadopoulos et al. 1987), rabbit (Bjarkam et al. 2003), cat (Leger et al. 2001, Mulligan and Tork 1988, 1989, 1993), monkey (DeFelipe and Jones 1988, Hoyer et al. 2002, Smiley and Goldman-Rakic 1996, Takeuchi and Sano 1984) and human (DeFelipe et al. 2001, Hornung 2003, Trottier et al. 1996). This morphological heterogeneity of serotonergic fibers has been explained by their different origins. There are two main sources of the 5-HT afferents of the forebrain: the median raphe nuclei (MnR) and the dorsal raphe nuclei (DR). Both nuclear groups extend from the pons to the level of the midbrain tegmentum. Fibers arising in these nuclei are characterized by different morphology of varicosities and different vulnerability to some neurotoxic agents (Mamounas and Molliver 1988). A study using the anterograde tracer PH-L (Kosofsky and Molliver 1987) showed that fibers arising from neurons of DR are very thin and typically have small, pleomorphic varicosities that are granular or fusiform in shape (type D axons). In contrast, fibers of the MnR neurons are characterized by large, spherical varicosities (type M axons) and by variations in axonal diameter.

The subpopulations of 5-HT fibers distinguished in the present study suggest that the rat claustrum receives axons from neurons of both raphe nuclei. This finding may be important for understanding the role of claustrum in the limbic system. Interestingly, MnR efferents concentrate in certain areas of the limbic cortex (e.g. dentate gyrus, posterior cingulate and entorhinal areas) and parietal cortex, whereas DR axons innervate the entire cortex. Previously, there was no evidence in the literature that MnR is a source of 5-HT innervation for the claustrum.

According to our observations, thin fibers with fusiform and densely packed small varicosities predominate in the rat claustrum. In the light of previous findings, these seem to be axon terminals of DR neurons. Our study with retrograde transport (FluoroGold tracer) (unpublished data) and previous studies (Vertes 1991, Vertes and Martin 1988) with anterograde transport (PHAL and [3H] Leucine) indicated that DR is the source of the 5-HT innervation of the rat claustrum. Morin and Meyer-Bernstein (1999) obtained similar results in the hamster.

Claustrum is the area with the highest density of 5-HT2A receptors in the forebrain (Leysen 2004, Rioux et al. 1999) and, according to Blue and colleagues (Blue et al. 1988), there is a close spatial relationship between the 5-HT2 receptors and thin 5-HT axons in the forebrain areas, especially in the neocortex and striatum. This suggests that this type of receptors may be selectively linked to a particular type of 5-HT axon terminals, namely those that belong to axons sent by neurons of the DR nucleus. Alternatively, the heterogeneity of serotonergic fibers observed by us may be related to the function of a specific neuronal subpopulation they supply. This is supported by a previous study by Baizer (2001) and the theory of Morin and Meyer-Bernstein (1999). Morin and Meyer-Bernstein (1999) postulated that the morphology of serotonergic fibers does not depend on their origin but rather on their target.

The rat claustrum is very similar to the primate claustrum (Baizer 2001) with regard to the pattern of spatial distribution of serotonergic fibers. We found the 5-HT fibers in all areas that are the known projection zones in the claustrum (motor, somatosensory, auditory, visual and limbic). With the help of three-dimensional CLSM analysis, we showed that in the posteroventral portion of the rat claustrum, the 5-HT fibers were oriented in the antero-posterior direction. This may explain the presence of stained puncta (cross-sections of fibers on the coronal sections) as described in the primate claustrum. In both species,

differences in spatial distribution of the 5-HT fibers might be related and thus specify the posteroventral portion of the claustrum, known as the visual claustrum.

While gamma-aminobutyric acid and glutamate have an established synaptic function in CNS, there are suggestions that the 5-HT neurotransmission is predominantly paracrine (Bunin and Wightman 1999). One of the forms of paracrine neurotransmission is volume transmission – a process mediated *via* short distance diffusion in the extracellular space. The majority of the 5-HT receptors in the claustrum belong to the subtype 5-HT2A (Barnes and Sharp 1999, Rioux et al. 1999) and most likely, as in the forebrain (Jansson et al. 2001), are involved in the volume transmission. In the rat claustrum the 5-HT fibers often possessed various varicosities, but basket-like structures were observed relatively rarely. In the cortical areas, the synaptic or non-synaptic signaling nature of the 5-HT varicosities is still controversial. Various studies have shown that conventional synapses are formed by only 20–46% of the 5-HT positive varicosities (DeFelipe and Jones 1988, Gulyas et al. 1999, Seguela et al. 1989, Smiley and Goldman-Rakic 1996). This discrepancy between a small number of direct contacts of the 5-HT immunoreactive structures with the claustral cells and a large number of serotonergic receptors (Barnes and Sharp 1999, Hoffman and Mezey 1989, Hoyer et al. 2002, Rioux et al. 1999, Wright et al. 1995) supports the theory that volume transmission is the most important factor for 5-HT neurotransmission in the claustrum.

There is also some morphological evidence, although scanty, indicating direct connections between serotonergic fibers and neurons in the rat claustrum. Baizer (2001) proposed that the 5-HT fibers in the primate claustrum contact preferentially the larger projection neurons that contain calbindin D28k or parvalbumin. Taking into account connections and developmental similarities between the claustrum and cortex, proposition of Baizer seems plausible. In various cortical areas serotonergic afferents preferentially innervate distinct subclasses of interneurons (Hornung et al. 1990, Mulligan and Tork 1987, Paspalas and Papadopoulos 2001). Among them, the CaBPs containing neurons are often targeted. Hornung and Celio (1992) found selective innervation of the CB- but not PV-containing interneurons by 5-HT axons in all areas of the neocortex and in the hippocampal formation of the marmoset. Similar results were obtained in the rat hippocampus (Freund et al. 1990). Smiley and Goldman-Rakic (1996) showed that dendritic shafts of interneurons are the major targets of the 5-HT synapses in the monkey prefrontal cortex. There are also reports describing serotonergic innervation of cortical projection neurons (Papadopoulos et al. 1987, Takeushi and Sano 1984).

CONCLUSIONS

We used confocal laser microscopy analysis of immunohistochemically double stained sections to verify the proposition of Baizer (2001). This method is valid for evaluating the relationships between fibers and neurons (Aznar et al. 2004). Our colocalization analysis has demonstrated that direct contacts between the CaBPs containing neurons and 5-HT fibers are rare in the rat claustrum. Approximately 95% of PV immunoreactive cell bodies have no direct contact with 5-HT fibers, whereas among CB- and CR-containing neurons this fraction is 90% and 84%, respectively. Moreover, the real fraction of cells that form a synaptic connection with 5-HT afferents is probably considerably lower, considering the scarcity of synaptic contacts on the 5-HT varicosities that was mentioned above. Therefore, our data suggest that neurons that are submitted to the influence of claustral PV cells may be the target of serotonergic fibers.

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