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Analysis of calcium binding protein immunoreactivity in the claustrum and the endopiriform nucleus of the rabbit

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Abstract. The present paper describes parvalbumin, calbindin-D28k and calretinin immunoreactivity in the claustrum and endopiriform nucleus of adult rabbits. Studied neuronal populations are characterized by morphological heterogeneity. Four types were identified in each subpopulation of cells containing calcium binding proteins on the basis of the number of processes and their branching pattern. There were no spatial differences in the distribution of cells containing either parvalbumin or calbindin-D28k in the claustrum and endopiriform nucleus. Well documented presence of the various projective zones in the rabbit claustrum did not reflect the specific distribution of neurons containing calcium binding proteins, except those containing calretinin. Their localization may correspond with the limbic zone. We have found that the rabbit claustrum and endopiriform nucleus have different pattern of parvalbumin and calretinin immunoreactivity. The former was more intense in the claustrum and the distribution of cell types was significantly different from that in the endopiriform nucleus. Calretinin-positive cells were observed in the claustrum, while in the endopiriform nucleus they were scarce. The distribution of neither calbindin-D28k-ir neurons nor fibers allowed differentiation of claustrum and endopiriform nucleus. Significant differences between the claustrum and endopiriform nucleus observed in the rabbit might be related with ontogenetic as well as other (functional) factors.

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Key words: calbindin-D28k, calretinin, confocal laser scanning microscopy, morphology, parvalbumin

INTRODUCTION

The claustrum (Cl) and the endopiriform nucleus (EN) are telencephalic structures present in almost all mammals. Cl and EN are medially separated from putamen by the fibers of the external capsule. On the lateral side, claustrum and endopiriform nucleus neighbor, through the extreme capsule, insular perirhinal and piriform cortex (Kowianski et al. 1999, Woznicka and Kosmal 2003). Although some reports described this subcortical region of the mammalian brain as one entity, its structural diversity indicated by various approaches (development (Bayer and Altman 1991, Filimonoff 1966, Maciejewska et al. 1997, Puelles et al. 2000, Reblet et al. 2002), connections (Behan and Haberly 1999, Kowianski et al. 1998, LeVay and Sherk 1981, Majak et al. 2000, Pearson et al. 1982, Witter et al. 1988) and neurochemistry (Celio 1990, Druga et al. 1993, Gómez-Urquijo et al. 2000, Kowianski et al. 2001, Narkiewicz et al. 1988, Reynhout and Baizer 1999)) allows to distinguish two distinct structures.

Although there is a lot of data concerning morphology of claustrum and endopiriform nucleus, their functions are still not clear. Ettlinger and Wilson (1990) proposed the theory of involvement of the claustrum in sensory integration. According to this theory the claustrum is a relay area through which each sense (single sensory stimulus) can access other senses. It was supported by studies using different sensory combinations, e.g., audio-visual (Olson et al. 2002, Redoute et al. 2000), or tactile-visual (Banati et al. 2000, Hadjikhani and Roland 1998). The other possible explanation of this theory is the existence of numerous claustroneocortical projections organized in partially overlapping cortico-related zones (Kowianski et al. 1998, Sherk 1988).

Both Cl and EN are involved in spreading of epileptiform activity (Behan and Haberly 1999, Hoffman and Haberly 1996, Mohapel et al. 2000, 2001, O'Shea et al. 2000, Zhang et al. 2001). Most theories relate the epilepsy with a lack of balance between excitatory influences and inhibitory control over a network of connected neurons. Pathologies that selectively reduce the total number of inhibitory neurons would cause higher susceptibility to seizure activity (Roper et al. 1999).

Neuronal population of claustrum and endopiriform region comprises mostly projection neurons. Glutamate is their major neurotransmitter. According to Kowianski et al. (2001) some projection neurons of these regions contain nitric oxide synthase (NOS). In the claustrum the number of interneurons depends on the species studied and constitutes from 6 to 12% of the total number of neurons (Gómez-Urquijo et al. 2000, Gutiérrez--Ibarluzea et al. 1998, Spahn and Braak 1985). GABA is their neurotransmitter. Other substances present in the population of claustral interneurons are NADPH diaphorase (Hinova-Palova et al. 1997, Switka et al. 1994), NOS (Kowianski et al. 2001) and neuropeptides (Eiden et al. 1990, Kowianski et al. 2001).

In the endopiriform nucleus, the percentage of interneurons is similar to that in the claustrum (Gómez--Urquijo et al. 2000). Also some of their immunohistochemical properties are similar (Roberts et al. 1982, Sims et al. 1980). The above mentioned population of GABA-ergic interneurons in both structures seems to correspond with the populations of neurons containing calcium binding proteins (CaBPs): parvalbumin (PV), calbindin-D28k (CB) and calretinin (CR) (Druga et al. 1993, Real et al. 2003, Reynhout and Baizer 1999).

Neuronal population of the rabbit claustrum and endopiriform nucleus has not been characterized in detail yet in terms of transmitter content, location and synaptic relationships. Rabbit, among other species, due to its distinctness in morphology of this region (Kowianski et al. 1999) and in the pattern of distribution of calcium binding proteins in other structures (Gonzalez-Soriano et al. 2000) is the species of special interest. Differentiated neuronal population of studied structures may also reflect their distinct functional role; such data concerning electrophysiological properties of parvalbumin-ir and calbindin-ir interneurons of specific morphology were described in the rat striatum (Kawaguchi 1997).

The aim of this study was to assess qualitatively and quantitatively the distribution and relationships of CaBP-ir neuron populations in the rabbit claustrum and endopiriform nucleus, and to find whether this immunoreactivity reflects diversity of Cl and EN. Due to the role of calcium ion in epileptiform events (Hoffman and Haberly 1996), from the functional point of view, populations of neurons containing CaBPs and their relations with previously described different systems of connections (Kowianski et al. 1998, Majak et al. 2000) seem to be interesting.

METHODS

The material consisted of the five brains of adult New Zealand rabbits. The care and treatment of the animals

was in accordance with the guidelines for laboratory animals established by the National Institute of Health, as well as those by the local ethical committee. Animals were deeply anesthetized with Fentanyl (0.03 mg/kg i.p.) and Thiopental (80 mg/kg i.p.) and perfused transcardially with 250 ml of 0.9% saline containing 10 000 units of Heparin, followed by 1 000 ml of 4% solution of paraformaldehyde in phosphate buffer (pH 7.4 and 4°C). Immediately after perfusion, the brains were removed from the skulls and cryoprotected by floating in 30% sucrose phosphate buffer solution (pH 7.4 and 4°C) until sunk (1-3 days). Then, they were cut into 40-μm-thick coronal sections with a cryostat Jung 1800 (Leica, Germany). Eight adjacent sections were taken from every 1 200 μm, two – for cresyl violet staining (they were coverslipped with DPX (Fluka, Germany) and the remaining six – for PV, CB and CR immunohistochemistry. From each brain, a set of twenty sections was obtained for every staining.

Immunohistochemical staining procedure

Free floating sections were incubated in a solution composed of 3% normal goat serum (NGS) for PV and CB or 3% normal donkey serum (NDS) for CR, and 0.3% Triton X-100 in 0.01M PBS (pH 7.2) for 1 hour. The sections were then incubated at a temperature of 4°C with primary antibodies: mouse anti-PV (diluted 1:1 000, Sigma, USA) or mouse anti-CB (diluted 1:1 000, Sigma, USA) or goat anti-CR (diluted 1:1 000, Swant, Switzerland) in 0.01M PBS (pH 7.2) containing 3% solution of NGS or NDS and 0.1% Triton X-100. After 48 hours the sections were washed with PBS and incubated with the secondary antibody goat anti-mouse or donkey anti-goat coupled to Cy3 (diluted 1:800, Jackson, USA) for 2 hours. Finally, they were washed with PBS, mounted onto gelatin-coated slides, air-dried, and coverslipped with Keiser Gelatin (Merck, Germany). In control experiments, in which the primary antibody was omitted, no signal was detected.

Qualitative analysis

Immunohistochemically labeled slides were studied using a fluorescent microscope BX-51 (Olympus, Japan) and a confocal laser scanning microscopy (CLSM) – system MicroRadiance (Bio-Rad, UK), equipped with an Argon ion laser (American Laser Corporation, USA) and mounted on a light microscope Eclipse 600 (Nikon, Japan). The excitation filter 514 and emission long-pass filter E570LP were used to detect Cy3 fluorescence. CLSM images were obtained using 40× and 60× oil immersion objective lenses of NA = 1.3 and 1.4, respectively. The optimal iris was used for each magnification. In each case only the sections completely stained with fluorescence were taken into account. The images were recorded on the hard drive and analyzed using the software LaserSharp 2000 (Bio-Rad, UK).

Quantitative analysis

The amount of immunoreactive points or fibers in neuropil was expressed as high, moderate and low, whereas the cell number – quantitatively.

The following parameters of PV-, CB- and CR-ir cells morphology were examined using CLSM images: somatic area and somatic diameters (long and short) by means of Laser Pix 2.0 analyzer (BioRad, UK). More than 100 cells of given immunoreactivity were used for their estimation.

To compare immunoreactive neuronal subpopulations containing particular CaBPs, the number of cells per square millimeter was estimated in Cl and EN within the rostral two-fifth, mid two-fifth and caudal one-fifth of their length. The division was in accordance with previously published results (Wojcik et al. 2002). The borders of Cl and EN were marked as separate inclusion areas under low magnification (4× objective). To delineate the structures, the brain atlases (Girgis and Shih-Chang 1981, Shek et al. 1986), descriptions (Fleischhauer et al. 1980, Wojcik et al. 2002) and also the nearest cresyl violet section were used.

About 60% of these inclusion areas on sections were investigated. Neuronal profiles were counted with the aid of a 20× objective lens in systematic random test frames of the area equal to 13 000 μm^2 using C.A.S.T. Grid system (Computer Assisted Stereological Tool, Olympus, Denmark) working on a microscope BX-51 (Olympus, Japan). Along with the counting procedure, the cells were classified as belonging to one specified neuronal type and the percentage distribution of cells types was calculated for PV- and CB-ir cells. The CR-ir cells were excluded from the estimation of the cell density due to anisotropy in the claustrum and sporadic presence in the endopiriform nucleus.

The Chi-square test was used to estimate the differences in the distributions of neuronal types as well as of the size of cells. The significance level was 0.05.

RESULTS

Classification of cells

Four morphological cell types were identified in all subpopulations of cells containing CaBPs. The main criteria were the number of processes and their branching patterns. The presence of varicosities was not taken into account.

Type I contained multipolar cells usually with three to four thin processes of approximately equal thickness and small somata of round or oval shape (Fig. 1A).

Type II immunoreactive cells were multipolar with many (five or more) processes of variable thickness and medium- to large-sized somata. Among cells of this type polygonal, oval or round perikarya were observed. Cells immunoreactive for PV and CB were of differentiated shape, whereas those immunoreactive for CR were predominantly oval (Fig. 1B).

Type III consisted of bipolar cells of various sizes with two processes. In this type, cells immunoreactive for PV and CB were predominantly of fusiform shape, while among CR-ir ones round cells prevailed (Fig. 1C).

Cells with three rather thick processes and triangular, medium to large-sized somata belonged to type IV (Fig. 1D).

The set of estimates of morphometric parameters characterizing cells of all types is presented in Table I.

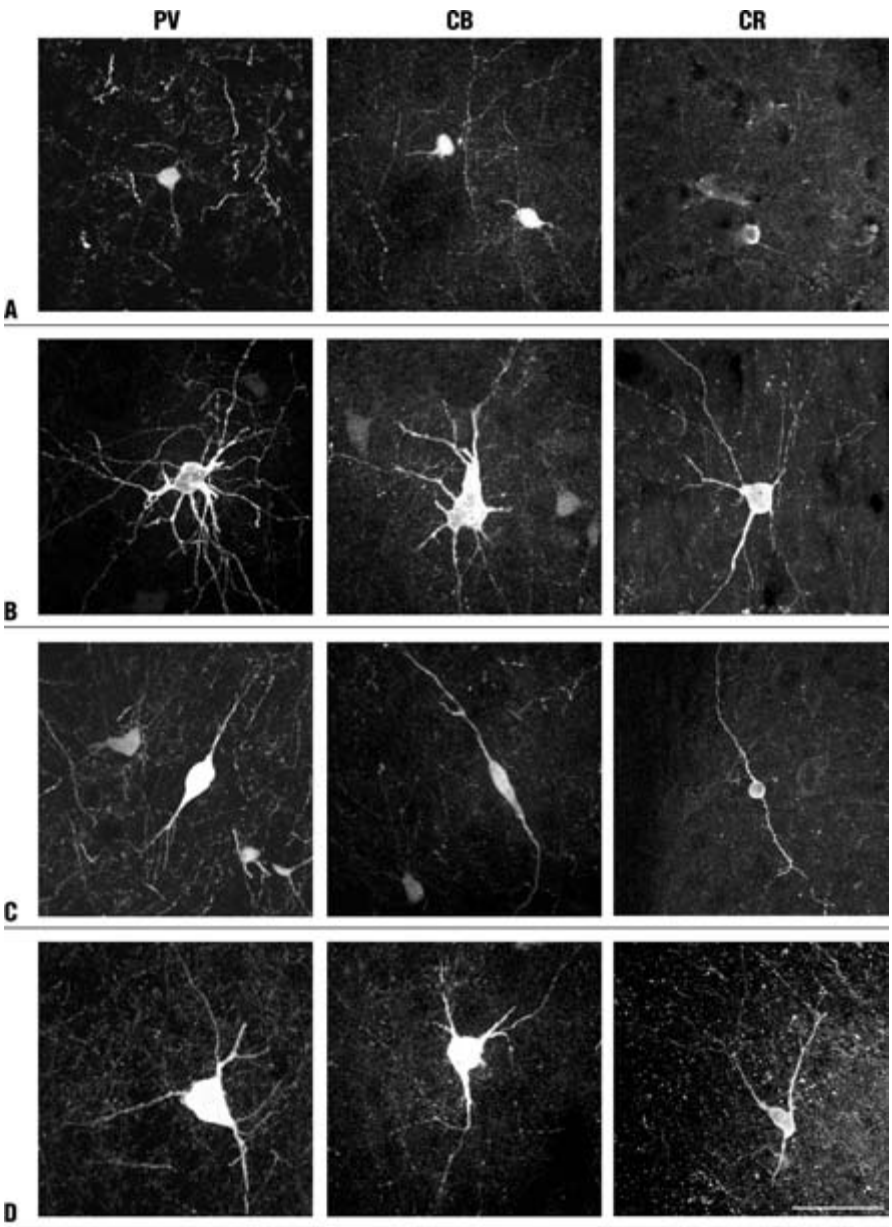


Fig. 1. Morphological types of cells containing calcium binding proteins in the claustrum and the endopiriform nucleus of the rabbit. (PV) Parvalbumin-ir (left column); (CB) calbindin-D28k-ir (central column); and (CR) calretinin-ir (right column). Stacks of confocal laser scanning microscope images. Scale bar = 0.05 mm. (A) Cells of type I – multipolar, with thin processes of approximately equal thickness and small somata of round or oval shape; (B) cells of type II – multipolar, with processes of variable thickness and medium- to large-sized polygonal, oval or round somata; (C) cells of type III – bipolar, with fusiform or round perikarya of various size; (D) cells of type IV – with three rather thick processes, and triangular medium- to large-sized somata.

The dendrites of cells of all neuronal types displayed an aspiny appearance. Dendritic branches were rarely observed only in the cells of type I, in the remaining types they could be easily distinguished.

For each CaBP all morphological types of cells are homogeneously distributed within distinguished parts of studied structures.

PV-immunoreactivity

Intense (high) PV-ir was found throughout the whole Cl and defined its area clearly, separated from surrounding structures by external and extreme capsules revealing an almost total lack of immunoreactivity (Fig. 2A). Dense meshwork of fibers oriented in all directions and numerous immunoreactive points were observed (Fig. 2B). They often formed basket-like plexuses surrounding unlabeled cells (Fig. 2D). Some fibers possessed varicosities.

The spatial distribution of PV-ir cells through Cl appeared homogeneous (Fig. 2F). The number of PV-ir profiles per unit area was moderate (58 ± 18 per mm^2). These strongly stained neurons belonged mainly (70%) to type II. Remaining population of cells was distributed almost equally among the other cell types (I, III and IV) (Table I).

PV-ir in EN was low (Fig. 2C). The lateral border of this structure was rather poorly distinguishable due to the similar pattern of immunoreactivity in relation to the neighboring deepest layer of the piriform cortex. In the neuropil of EN, only single PV-ir fibers with occasional varicosities were present. Very intense PV-ir points were sporadically observed on the surface of PV-ir cells (Fig. 2E).

The spatial distribution of PV-ir cells in EN (as in Cl) was characterized by homogeneity, but their number was very low (18 ± 9 per mm^2). These neurons belonged to type II and III (Table I). Differences were found in the

Table I

The set of morphometric parameters characterizing different morphological types of cells containing calcium binding proteins (PV, CB, CR) in the claustrum (Cl) and the endopiriform nucleus (EN)

		Type	Fraction (%)	Long diameter (mm)	Short diameter (mm)	Somatic area (μm^2)
CL	PV	I	11	13.4 ± 1.8	10.1 ± 1.6	94.2 ± 23.9
		II	70	23.1 ± 5.3	15.8 ± 3.3	237.7 ± 89.5
		III	10	28.5 ± 5.9	13.7 ± 3.2	222.3 ± 81.9
		IV	9	25.4 ± 5.8	16.3 ± 4.1	240.0 ± 87.9
	CB	I	17	14.5 ± 2.7	10.4 ± 1.3	100.8 ± 28.8
		II	51	22.4 ± 5.4	14.0 ± 3.1	194.4 ± 77.1
		III	23	24.6 ± 5.6	11.0 ± 2.4	174.0 ± 63.1
		IV	9	23.5 ± 5.7	16.2 ± 4.7	222.5 ± 97.7
	CR	I	4	9.1 ± 1.7	7.7 ± 1.6	78.9 ± 15.2
		II	42	19.4 ± 3.3	13.9 ± 2.3	152.9 ± 40.1
		III	19	22.2 ± 3.6	10.1 ± 1.1	130.8 ± 27.2
		IV	35	20.3 ± 4.1	11.7 ± 2.0	130.6 ± 40.8
EN	PV	I	9	15.1 ± 1.5	9.9 ± 1.2	106.8 ± 24.0
		II	47	22.8 ± 4.6	14.9 ± 2.5	220.0 ± 68.3
		III	35	25.9 ± 3.6	12.3 ± 3.2	195.7 ± 79.0
		IV	9	26.3 ± 2.8	16.7 ± 1.9	237.5 ± 40.1
	CB	I	17	14.3 ± 1.5	10.1 ± 1.7	93.5 ± 23.5
		II	49	21.2 ± 5.5	14.2 ± 3.3	183.7 ± 69.9
		III	25	24.0 ± 5.6	10.8 ± 2.7	149.7 ± 53.5
		IV	9	22.0 ± 4.4	14.2 ± 2.9	166.6 ± 45.1

Means and standard deviations (± SD) are given

distribution of cell types between Cl and EN – they were mainly related to the cells of type II (found more commonly in Cl) as well as of type III – more frequently observed in EN (Table I).

Comparison of PV-ir populations revealed that the distribution of somatic area of these cells showed a significant difference between studied structures – in Cl the amount of larger cells (between 200 μm^2 and 300 μm^2) was bigger than that in EN (χ^2= 8.5, df=3, P<0.04) (Fig. 3).

CB-immunoreactivity

Sections stained for CB showed low immunoreactivity in Cl and EN. The pattern of CB-ir was also similar in both studied structures (Fig. 4A-C). Moderate immunoreactivity of the fibers as well as of puncta in the neuropil was found. The fibers were thinner than PV-ir ones; the varicosities were often observed (Fig. 4C, D).

The spatial distribution of CB-ir cells in Cl and EN was homogeneous (Fig. 4E). Their number per unit area in Cl and EN did not differ significantly (53 ± 9 per mm^2 and 50 ± 22 per mm^2, respectively). In Cl, 51% of CB-ir cells belonged to type II, 23% to type III, 11% and 9% to types I and IV, respectively; in EN, the distribution of cell types was almost identical (Table I).

Comparison of CB-ir populations between Cl and EN revealed that the distribution of somatic area of these cells did not differ significantly (χ^2= 4.4, df=3, P>0.2) (Fig. 3).

CR-immunoreactivity

Due to the low CR-ir, Cl was clearly distinguishable throughout its whole extent from neighboring structures (Fig. 5A). In Cl, a moderate number of thin CR-ir fibers and immunoreactive puncta were present. Characteristically, strongly stained fibers arranged along the anteroposterior as well as dorsoventral direction marked the medial border of Cl (Fig. 5B, D). More medially, in the external capsule, an almost total lack of CR-ir was observed.

The spatial distribution of CR-ir cells was anisotropic; they were localized in the medial border of Cl

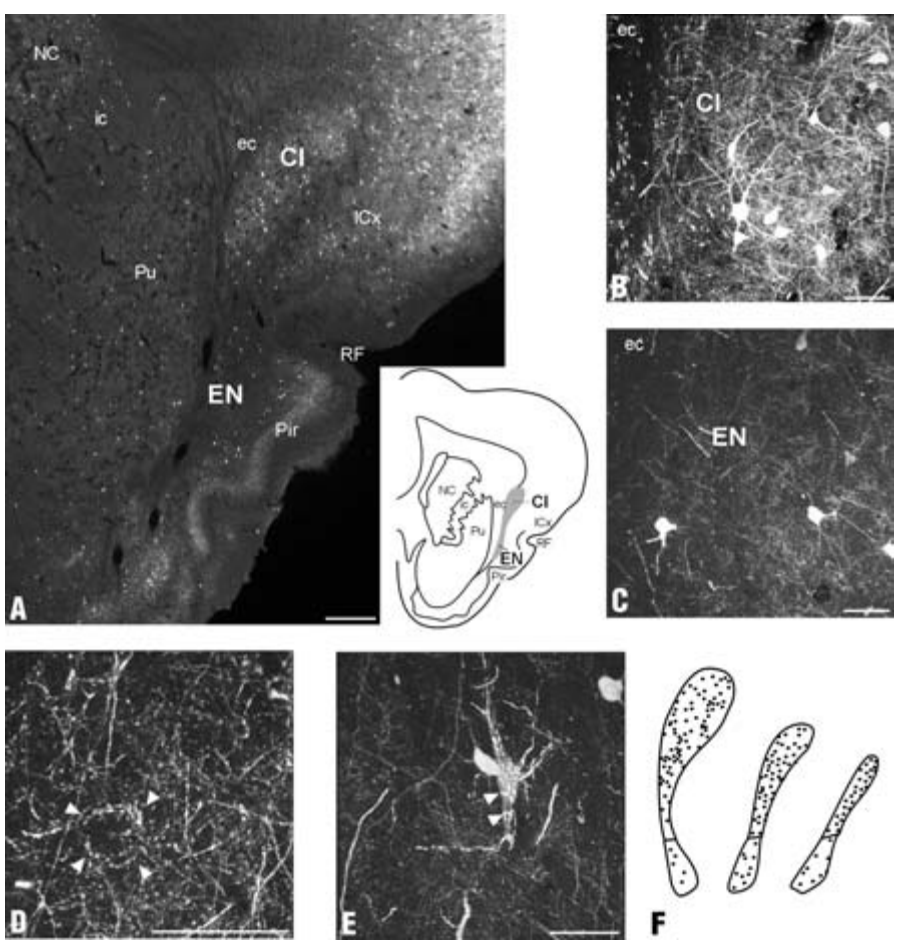
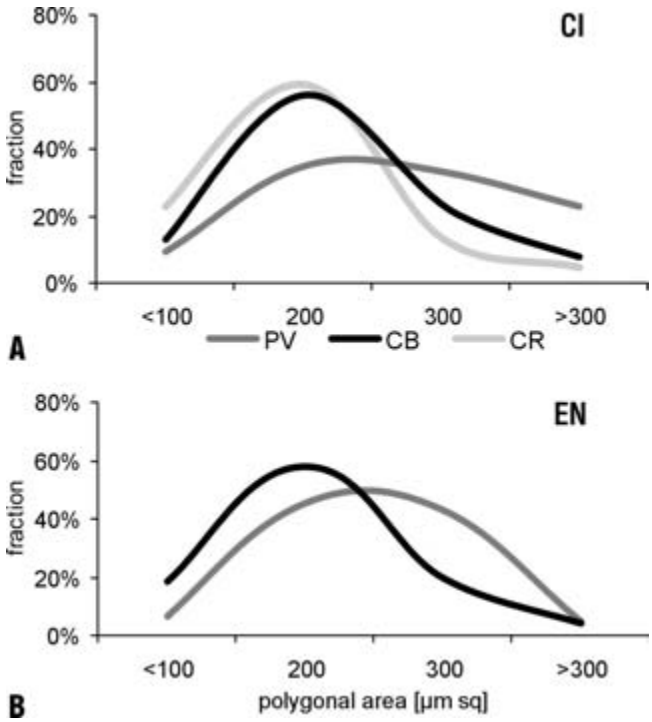


Fig. 2. Parvalbumin-immunoreactivity in the claustrum (Cl) and the endopiriform nucleus (EN). (A) Coronal section at bregma 3.5 mm; (B), (D) claustrum; (C), (E) endopiriform nucleus, (D) immunoreactive points on the surface of unstained cells (basket – arrowheads); (E) immunoreactive points on the surface of immunopositive cells; (F) spatial distribution of PV-immunoreactive cells on the coronal sections through the anterior, central and posterior part of the structures. One dot represents one labeled cell. Abbreviations: (ec) external capsule; (ICx) insular cortex; (ic) internal capsule; (NC) caudate nucleus; (Pir) piriform cortex; (Pu) putamen; (RF) rhinal fissure. (A) photomicrograph; (B)-(E) stack of confocal laser scanning microscope images. Scale bars: (A) 0.5 mm; (B)-(E) 0.05 mm.



(Fig. 5B, F). Although their number was small, they predominantly belonged to cells of type II (42%) or, interestingly, of type IV (35%).

The CR-ir in EN was low and immunoreactive somata were observed sporadically (a single cell per three or four coronal sections). Whereas the lateral border of EN was difficult to distinguish from the neighboring deepest layer of the piriform cortex, the medial border of EN was clearly defined due to the lack of immunoreactivity of the external capsule (Fig. 5C). In EN there were observed thin fibers arranged perpendicularly to the surface of the hemisphere, crossed by a considerably smaller number of strongly stained fibers (Fig. 5E).

Fig. 3. Distribution of somatic area of parvalbumin- (PV), calbindin-D28k- (CB) and calretinin- (CR) immunoreactive cells in the rabbit. (A) Claustrum; (B) endopiriform nucleus. Significant differences were observed between the distributions of size of PV- and CB-ir cells in both the endopiriform nucleus and the claustrum.

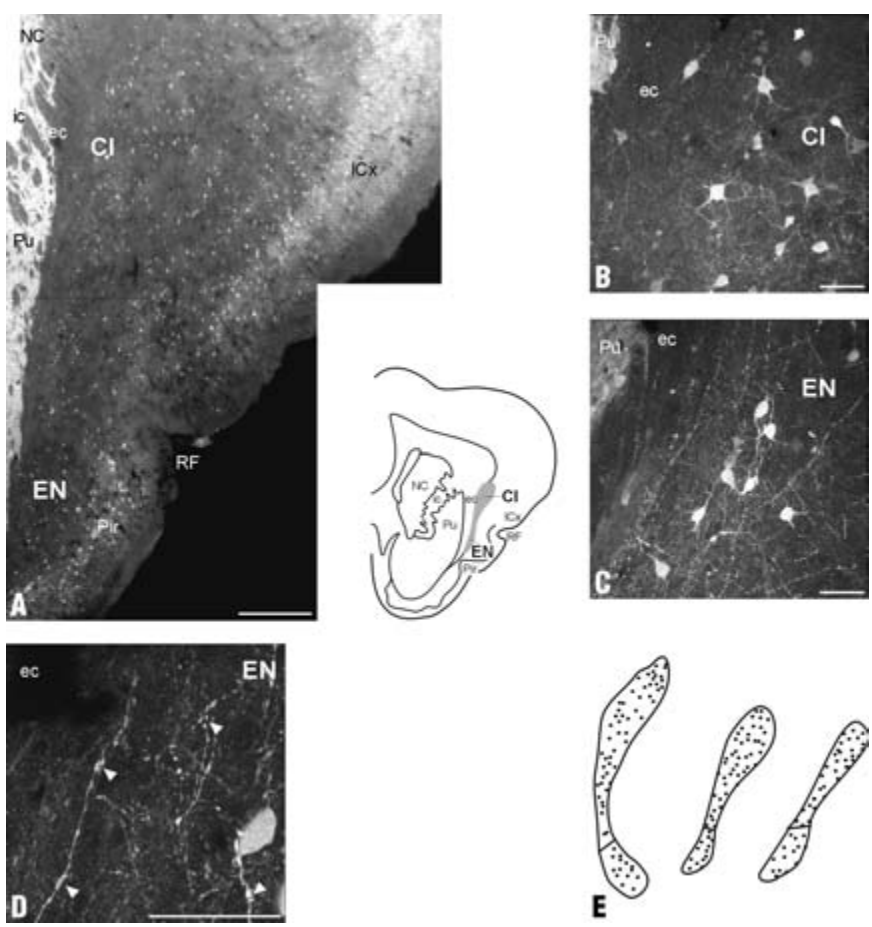


Fig. 4. Calbindin-D28k-immunoreactivity in the claustrum (Cl) and the endopiriform nucleus (EN). (A) Coronal section at bregma 3.0 mm; (B) claustrum; (C), (D) endopiriform nucleus; (D) immunoreactive fibers within the endopiriform nucleus with varicosities (arrowheads); (E) spatial distribution of CB-immunoreactive cells on the coronal sections through the anterior, central and posterior part of the structures. One dot represents one labeled cell. Abbreviations: (ec) external capsule, (ICx) insular cortex; (ic) internal capsule; (NC) caudate nucleus; (Pir) piriform cortex; (Pu) putamen; (RF) rhinal fissure. (A) Photomicrograph; (B)-(D) stack of confocal laser scanning microscope images. Scale bars: (A) 0.5 mm; (B)-(D) 0.05 mm.

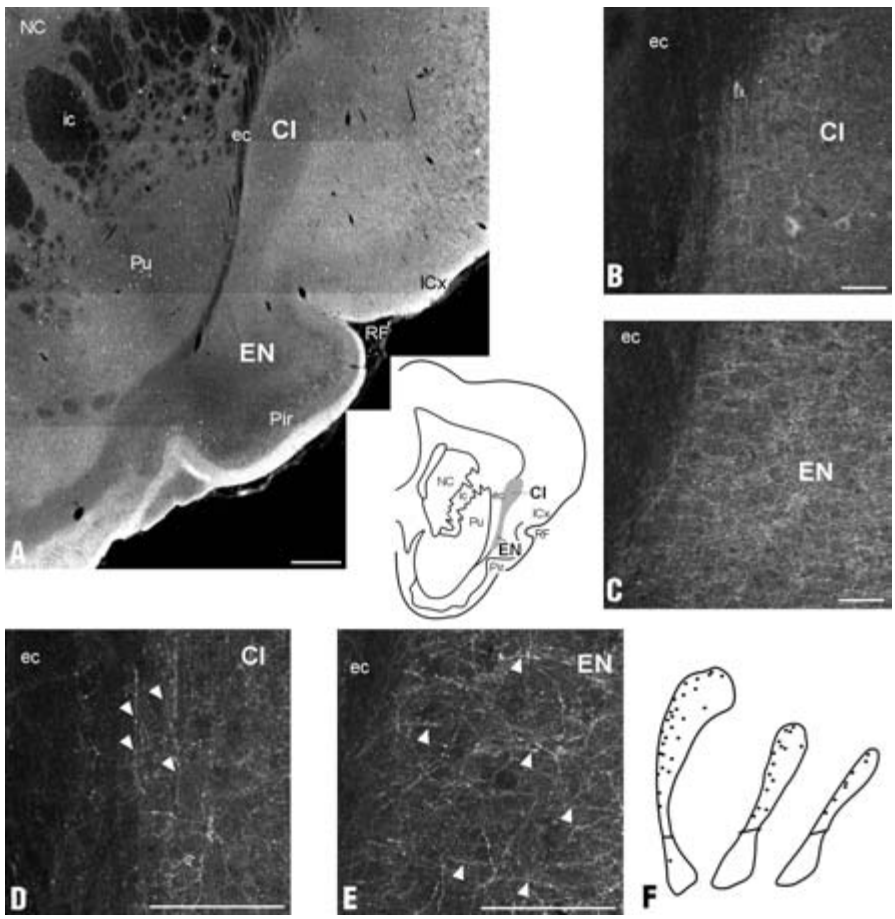


Fig. 5. Calretinin-immunoreactivity in the claustrum (Cl) and the endopiriform nucleus (EN). (A) Coronal section at bregma 2.5 mm; (B), (D) claustrum; (C), (E) endopiriform nucleus (D) immunoreactive fibers parallel to the external capsule (arrowheads); (E) immunoreactive fibers perpendicular to the external capsule (arrowheads); (F) spatial distribution of CR-immunoreactive cells on the coronal sections through the anterior, central and posterior part of the structures; one dot represents one labeled cell. Abbreviations: (ec) external capsule; (ICx) insular cortex; (ic) internal capsule; (NC) caudate nucleus; (Pir) piriform cortex; (Pu) putamen; (RF) rhinal fissure. (A) Photomicrograph; (B)-(E) stacks of confocal laser scanning microscope images. Scale bars: (A) 0.5 mm; (B)-(E) 0.05 mm.

DISCUSSION

The present study provides the first qualitative and quantitative data on the distribution and relationships of CaBP-ir neuron populations in the rabbit claustrum and endopiriform nucleus.

The data concerning morphology of PV-ir cells indicate variability of their shapes in different species (rat (Druga et al. 1993), mouse (Real et al. 2003) and monkey (Reynhout and Baizer 1999)). In the rat Cl and EN, the population of PV-ir cells consists of round and oval neurons (Druga et al. 1993), in the rabbit, fusiform, triangular and polygonal neurons have been also observed by us. In the mouse, polygonal cells prevail (Real et al. 2003). In the monkey, besides cells of circular, oval or elongated somata, some cells with bodies resembling cortical pyramidal neurons were observed (Reynhout and Baizer 1999). The latter can possibly correspond to cells of type IV observed by us in the rabbit, but not found in the rat (Druga et al. 1993).

Contrary to PV-ir cells, morphology of CB-ir cells in both Cl and EN among species is, to a large extent, similar. Almost the same three types of cells are observed in a mouse, rat, rabbit and monkey: the small multipolar cells, the large multipolar cells and bipolar ones (Druga et al. 1993, Real et al. 2003, Reynhout and Baizer 1999). Cells of triangular shape observed by us in the rabbit, and by Druga et al. in the rat, are not observed in the monkey.

Data on the morphological differences on CR-ir across species are only fragmentary. Among various types of CR-ir cells, those of bipolar shape were the most numerous in the monkey (Reynhout and Baizer 1999) and mouse (Real et al. 2003). This observation does not agree with our data indicating the prevalence of cells of type II and IV. However, the detailed analysis of 3D-reconstruction of some bipolar cells (especially those with more than two processes) can classify them as cells of type I or II.

Present study has shown that populations of CaBP-ir neurons are not homogeneous but comprise several subpopulations based on morphology of neuronal process, perikaryal shape and size. Druga and coauthors (1993) and Reynhout and Baizer (1999), on the basis of data concerning CaBP-ir neuronal populations of the

studied region in the rat and monkey, suggested almost exclusive (except some of PV-ir neurons in monkey) confinement of these subpopulations to interneurons. According to previous studies from various species, based on Golgi staining (Braak and Braak 1982, Brand 1981, Dinopoulos et al. 1992, LeVay and Sherk 1981, Mamos et al. 1986, Rowniak et al. 1994, Spahn and Braak 1985) Cl and EN neurons without spines on dendrites are probably interneurons, while those with spines are projective ones. In the rabbit Cl and EN (Wasilewska and Najdzion 2001), all types of neurons, in Golgi staining, possessed spines; only among multipolar neurons – aspiny ones were sporadically observed. It indicates that population of interneurons in rabbit Cl and EN is rather small. It was confirmed by other authors (Gómez-Urquijo et al. 2000, Kowianski et al. 1996); according to their data only 6-12% of neurons in Cl and 12% in EN were neurons of a local network.

Although immunohistochemical labeling for CaBPs cannot directly indicate the presence of spines, "spine-like" structures were described (Pitkänen and Amaral 1993). In the rabbit, such structures were not observed, so at least part of the subpopulation of cells containing CaBPs in Cl and EN may be treated as interneurons. Moreover, morphological properties of CaBP-ir neurons of type I–III correspond to those of GABA-ergic cells described in the rabbit claustrum by Gomez-Urquijo and coauthors (2000).

Another observation that can speak in favor of hypothesis of the critical role of CaBP in the local neuronal network is the fact that in the rabbit claustrum, PV-ir basket-like plexuses very often surround unlabeled cells. Such plexuses were also observed in the amygdaloid nuclei (Kemppainen and Pitkänen 2000, McDonald and Betette 2001, Pitkänen and Kemppainen 2002, Sorvari et al. 1995) and neocortex (DeFelipe 1997). The presence of plexuses indicates that at least part of the population of PV-ir cells observed by us in the rabbit consists of GABA-ergic basket cells and can be treated as interneurons (Hendry et al. 1989). Also the similarity of morphology between cortical interneurons containing CaBPs (DeFelipe 1997, Frassoni et al. 1998, Kubota and Jones 1993) and CaBPs neurons of Cl and EN can also confirm this hypothesis, as according to morphological and physiological data (Shibuya and Yamamoto 1998), the populations of claustral and neocortical neurons are similar. The ontogenetic relations of the claustrum, endopiriform nucleus and cortex should be also taken into account (Puelles et al. 2000, Swanson and Petrovich 1998).

The presence of CaBPs in projective neurons was described for various structures (DeFelipe 1997, Kawaguchi 1997, Lewis et al. 2001), also in the rabbit (Gonzalez-Soriano et al. 2000). The possibility that in this species, the part of the neuronal population containing CaBPs in Cl and EN are projective cells cannot be excluded. The morphological similarity of Cl and EN cells of type IV in the rabbit to GABA-negative amygdaloid neurons (especially those CB- and CR-ir, (Kemppainen and Pitkänen 2000)) as well as cortical pyramidal neurons (DeFelipe 1997) could sustain this hypothesis. Moreover, varicosities observed by us on immunoreactive fibers in Cl and EN may correspond to previously described fibers connecting Cl and EN (Lipowska et al. 2000), as well as connecting Cl with cortical areas (Zhang et al. 2001). PV-ir fibers, being putative axon terminals of neurons originating from the mediodorsal thalamic nucleus, were also observed in the prefrontal cortex (Lewis et al. 2001).

In spite of fact that CaBPs are not confined to any specific neuronal subpopulation in claustrum and endopiriform nucleus, CaBP-ir allows to differentiate these structures. According to our results PV- and CR-immunoreactivity can show it most clearly. Even taking into account both qualitative and quantitative data (spatial distribution of cells, the number of cells per unit area, distribution of neuronal types, distribution of somatic size, immunoreactivity of neuropil) CB-ir is similar in both studied structures. In the rat, CaBP-ir also allows clear differentiation of Cl and EN (Druga et al. 1993). Rat endopiriform nucleus shows more intense CB-ir as compared with the claustrum, while the latter is characterized by larger number of PV-ir cells, larger amount of PV-ir processes and points.

Significant differences between Cl and EN observed in rat and rabbit might be related with ontogenesis. Previous study (Bayer and Altman 1991) showed that neurons of Cl were generated in the neocortical neuroepithelium, whereas neurons of EN – in neuroepithelium of the lateral ventricular angle. According to the other report (Puelles et al. 2000), neurons of Cl and EN derive from the lateral and ventral pallium, respectively. The latest studies on rabbit embryos (Reblet et al. 2002) indicate that neurons of Cl originate from two distinct populations of cells from both lateral and medial walls of the lateral ventricular angle, whereas neurons of EN come only from medial one.

On the contrary, in the monkey no significant differences in the CaBPs immunoreactivity between Cl and EN

are present (Reynhout and Baizer 1999). It indicates that other than developmental factor takes part in forming of final neurochemical properties of these structures.

While spatial homogeneity of cells containing PV or CB in the rabbit as well as in other species (Druga et al. 1993, Reynhout and Baizer 1999) does not fit the well documented presence of the various projective zones in the claustrum of different species (Kowianski et al. 1998, Pearson et al. 1982, Sadowski et al. 1997), distribution of CR-ir neurons in the rabbit may correspond to the limbic zone in this species (Majak et al. 2000).

Claustrum is placed among other structures as a convergence zone where crossmodal processing takes place (Banati et al. 2000, Calvert 2001, Hadjikhani and Roland 1998), although axons of claustral cells projecting to specified cortical areas and also sending collaterals to other cortical areas are very rarely (if at all) observed (Kowianski et al. 1998, Macchi et al. 1983, Majak et al. 2000). Thus, the possibility that neurons containing calcium binding proteins (present in all projection zones) are engaged in crossmodal processing cannot be excluded.

CONCLUSION

In the rabbit claustrum and endopiriform nucleus, populations of parvalbumin, calretinin and calbindin D28k containing neurons are not homogenous. Four morphological types can be distinguished in each population. The morphology of most calcium binding proteins containing neurons indicates that they are elements of local neuronal network. Significant differences between Cl and EN in the distribution of cells containing CaBPs were observed in the rabbit. They could be possibly related with ontogenesis.

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REFERENCES

Banati RB, Goeres GW, Tjoa C, Aggleton JP, Grasby P (2000) The functional anatomy of visual-tactile integration in man: a study using positron emission tomography. Neuropsychologia 38: 115-124.

Bayer SA, Altman J (1991) Development of the endopiriform nucleus and the claustrum in the rat brain. Neuroscience 45: 391-412.

Behan M, Haberly LB (1999) Intrinsic and efferent connections of the endopiriform nucleus in rat. J Comp Neurol 408: 532-548.

Braak H, Braak E (1982) Neuronal types in the claustrum of man. Anat Embryol (Berl) 163: 447-460.

Brand S (1981) A serial section Golgi analysis of the primate claustrum. Anat Embryol (Berl) 162: 475-488.

Calvert GA (2001) Crossmodal processing in the human brain: insights from functional neuroimaging studies. Cereb Cortex 11: 1110-1123.

Celio MR (1990) Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35: 375-475.

DeFelipe J (1997) Types of neurons, synaptic connections and chemical characteristics of cells immunoreactive for calbindin-D28K, parvalbumin and calretinin in the neocortex. J Chem Neuroanat 14: 1-19.

Dinopoulos A, Papadopoulos GC, Michaloudi H, Parnavelas JG, Uylings HBM, Karamanlidis AN (1992) Claustrum in the Hedgehog (*Erinaceus europaeus*) brain: Cytoarchitecture and connections with cortical and subcortical structures. J Comp Neurol 316: 187-205.

Druga R, Chen S, Bentivoglio M (1993) Parvalbumin and calbindin in the rat claustrum: an immunocytochemical study combined with retrograde tracing from frontoparietal cortex. J Chem Neuroanat 6: 399-406.

Eiden LE, Mezey E, Eskay RL, Beinfeld MC, Palkovits M (1990) Neuropeptide content and connectivity of the rat claustrum. Brain Res 523: 245-250.

Ettlinger G, Wilson WA (1990) Cross-modal performance: behavioural processes, phylogenetic considerations and neural mechanisms. Behav Brain Res 40: 169-192.

Filimonoff IN (1966) The claustrum, its origin and development. J Hirnforsch 8: 503-528.

Fleischhauer K, Zilles K, Schleichher A (1980) A revised cytoarchitectonic map of the neocortex of the rabbit (*Oryctolagus cuniculus*). Anat Embryol (Berl) 161: 121-143.

Frassoni C, Radici C, Spreafico R, De Curtis M (1998) Calcium-binding protein immunoreactivity in the piriform cortex of the guinea-pig: selective staining of subsets of non-GABAergic neurons by calretinin. Neuroscience 83: 229-237.

Girgis M, Shih-Chang W (1981) Stereotaxic Atlas of the Rabbit Brain. Warren H. Green, Inc., St. Louis, 70 p.

Gómez-Urquijo SM, Gutiérrez-Ibarluzea I, Bueno-López JL, Reblet C (2000) Percentage incidence of gamma--aminobutyric acid neurons in the claustrum of the rabbit and comparison with the cortex and putamen. Neurosci Lett 282: 177-180.

Gonzalez-Soriano J, Gonzalez-Flores ML, Contreras-Rodriguez J, Rodriguez-Veiga E, Martinez-Sainz P (2000) Calbindin D28k and parvalbumin immunoreactivity in the rabbit superior colliculus: An anatomical study. Anat Rec 259: 334-346.

Gutiérrez-Ibarluzea I, Gomez-Urquijo SM, Arana-Arri E, Bueno-López JL, Reblet C (1998) Quantitative distribution of GABAergic neurons in the rabbit claustrum. Eur J Neurosci 10: 187.

Hadjikhani N, Roland PE (1998) Cross-modal transfer of information between the tactile and the visual representations in the human brain: A positron emission tomographic study. J Neurosci 18: 1072-1084.

Hendry SHC, Jones EG, Emson PC, Lawson DEM (1989) Two classes of cortical GABA neurons defined by differential calcium binding proteins immunoreactivity. Exp Brain Res 76: 467-472.

Hinova-Palova DV, Paloff A, Christova T, Ovtscharoff W (1997) Topographical distribution of NADPH-diaphorase-positive neurons in the cat's claustrum. Eur J Morphol 35: 105-116.

Hoffman WH, Haberly LB (1996) Kindling-induced epileptiform potentials in piriform cortex slices originate in the underlying endopiriform nucleus. J Neurophysiol 76: 1430-1438.

Kawaguchi Y (1997) Neostriatal cell subtypes and their functional roles. Neurosci Res 27: 1-8.

Kemppainen S, Pitkänen A (2000) Distribution of parvalbumin, calretinin, and calbindin-D_{28k} immunoreactivity in the rat amygdaloid complex and colocalization with gamma-aminobutyric acid. J Comp Neurol 426: 441-467.

Kowianski P, Dziewiatkowski J, Kowianska J, Morys J (1999) Comparative anatomy of the claustrum in selected species: A morphometric analysis. Brain Behav Evol 53: 44-54.

Kowianski P, Morys J, Dziewiatkowski J, Karwacki Z, Bobek-Billewicz B, Narkiewicz O (1996) Neurons of the claustrum projecting to the motor and somatosensory cortex in the rabbit – assessment with the method of retrograde transport of fluorescent tracers. Folia Morphol 55: 350-352.

Kowianski P, Morys J, Karwacki Z, Dziewiatkowski J, Narkiewicz O (1998) The cortico-related zones of the rabbit claustrum – study of the claustrocortical connections based on the retrograde axonal transport of fluorescent tracers. Brain Res 784: 199-209.

Kowianski P, Timmermans JP, Morys J (2001) Differentiation in the immunocytochemical features of intrinsic and cortically projecting neurons in the rat claustrum – combined immunocytochemical and axonal transport study. Brain Res 905: 63-71.

Kubota Y, Jones EG (1993) Colocalization of two CaBps in GABA cells of rat piriform cortex. Brain Res 600: 339-344.

LeVay S, Sherk H (1981) The visual claustrum of the cat. I. Structure and connections. J Neurosci 1: 956-980.

Lewis DA, Cruz DA, Melchitzky DS, Pierri JN (2001) Lamina-specific deficits in parvalbumin-immunoreactive varicosities in the prefrontal cortex of subjects with schizophrenia: Evidence for fewer projections from the thalamus. Am J Psychiatry 158: 1411-1422.

Lipowska M, Kowianski P, Majak K, Jagalska-Majewska H, Morys J (2000) The connections of the endopiriform nucleus with the insular claustrum in the rat and rabbit. Folia Morphol 59: 77-83.

Macchi G, Bentivoglio M, Minciacchi D, Molinari M (1983) Claustroneocortical projections studied in the cat by means of multiple retrograde fluorescent tracing. J Comp Neurol 215: 121-134.

Maciejewska B, Morys J, Berdel B, Dziewiatkowski J, Narkiewicz O (1997) The development of the rat claustrum – a study using morphometric and *in situ* DNA end labelling techniques. Eur J Anat 1: 137-143.

Majak K, Kowianski P, Morys J, Spodnik J, Karwacki Z, Wisniewski HM (2000) The limbic zone of the rabbit and rat claustrum: A study of the claustrocingulate connections based on the retrograde axonal transport of fluorescent tracers. Anat Embryol (Berl) 201: 15-25.

Mamos L, Narkiewicz O, Morys J (1986) Neurons of the claustrum in the cat; a Golgi study. Acta Neurobiol Exp (Wars) 46: 171-178.

McDonald AJ, Betette RL (2001) Parvalbumin-containing neurons in the rat basolateral amygdala: Morphology and co-localization of calbindin-D28k. Neuroscience 102: 413-425.

Mohapel P, Hannesson DK, Armitage LL, Gillespie GW, Corcoran ME (2000) Claustral lesions delay amygdaloid kindling in the rat. Epilepsia 41: 1095-1101.

Mohapel P, Zhang X, Gillespie GW, Chlan-Fourney J, Hannesson DK, Corley SM, Li XM, Corcoran ME (2001) Kindling of claustrum and insular cortex: comparison to perirhinal cortex in the rat. Eur J Neurosci 13: 1501-1519.

Narkiewicz O, Nitecka L, Mamos L, Morys J (1988) The pattern of the GABA-like immunoreactivity in the claustrum. Folia Morphol 47: 21-30.

O'Shea MS, Rosen JB, Post RM, Weiss SRB (2000) Specific amygdaloid nuclei are involved in suppression or propagation of epileptiform activity during transition stage between oral automatisms and generalized clonic seizures. Brain Res 873: 1-17.

Olson IR, Gatenby JC, Gore JC (2002) A comparison of bound and unbound audio-visual information processing in the human cerebral cortex. Cognit Brain Res 14: 129-138.

Pearson RCA, Brodal P, Gatter KC, Powell TP (1982) The organization of the connections between the cortex and the claustrum in the monkey. Brain Res 234: 435-441.

Pitkänen A, Amaral DG (1993) Distribution of calbindin-D(28K) immunoreactivity in the monkey tem-

poral lobe: The amygdaloid complex. J Comp Neurol 331: 199-224.

Pitkänen A, Kemppainen S (2002) Comparison of the distribution of calcium-binding proteins and intrinsic connectivity in the lateral nucleus of the rat, monkey, and human amygdala. Pharmacol Biochem Behav 71: 369-397.

Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JLR (2000) Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J Comp Neurol 424: 409-438.

Real MA, Dávila JC, Guirado S (2003) Expression of calcium-binding proteins in the mouse claustrum. J Chem Neuroanat 25: 151-160.

Reblet C, Alejo A, Blanco-Santiago I, Mendizabal-Zubiaga JL, Fuentes M, Bueno-López JL (2002) Neuroepithelial origin of the insular and endopiriform parts of the claustrum. Brain Res Bull 57: 495-497.

Redoute J, Stoleru S, Gregoire M-C, Costes N, Cinotti L, Lavenne F, Le Bars D, Forest MG, Pujol JF (2000) Brain processing of visual sexual stimuli in human males. Hum Brain Mapping 11: 162-177.

Reynhout K, Baizer JS (1999) Immunoreactivity for calcium-binding proteins in the claustrum of the monkey. Anat Embryol 199: 75-83.

Roberts GW, Woodhams PL, Polak JM, Crow TJ (1982) Distribution of neuropeptides in the limbic system of the rat: The amygdaloid complex. Neuroscience 7: 99-131.

Roper SN, Eisenschenk S, King MA (1999) Reduced density of parvalbumin- and calbindin D28k-immunoreactive neurons in experimental cortical dysplasia. Epilepsy Res 37: 63-71.

Rowniak M, Szteyn S, Robak A, Klawon M (1994) The types of neurons in the claustrum of bison bonasus: Nissl and Golgi study. Folia Morphol 53: 231-237.

Sadowski M, Morys J, Jakubowska-Sadowska K, Narkiewicz O (1997) Rat's claustrum shows two main cortico-related zones. Brain Res 756: 147-152.

Shek JW, Wen GY, Wisniewski HM (1986) Atlas of the Rabbit Brain and Spinal Cord. Karger, Basel, 139 p.

Sherk H (1988) The claustrum and the cerebral cortex. In: Sensory-Motor Areas and Aspects of Cortical Connectivity (Eds. E.G. Jones and A. Peters). Cerebral Cortex, Vol. 5. Plenum Press, New York, London, p. 467-499.

Shibuya H, Yamamoto T (1998) Electrophysiological and morphological features of rat claustral neurons: An intracellular staining study. Neuroscience 85: 1037-1049.

Sims KB, Hoffman DL, Said SI, Zimmerman EZ (1980) Vasoactive intestinal polypeptide (VIP) in mouse and rat brain: An immunocytochemical study. Brain Res 186: 165-183.

Sorvari H, Soininen H, Paljärvi L, Karkola K, Pitkänen A (1995) Distribution of parvalbumin-immunoreactive cells and fibers in the human amygdaloid complex. J Comp Neurol 360: 185-212.

Spahn B, Braak H (1985) Percentage of projection neurons and various types of interneurons in the human claustrum. Acta Anat 122: 245-248.

Swanson LW, Petrovich GD (1998) What is the amygdala? Trends Neurosci 21: 323-331.

Switka A, Scheuermann DW, Adiaensen D, Timmermans J-P, Narkiewicz O (1994) NADPH-diaphorase enzyme activity, serotonin and tyrosine hydroxylase immunocytochemistry in the claustrum of the domestic cat. In: Verhandlungen der Anatomischen Gesellschaft (Ed. W. Kühnel). Gustav Fischer Verlag Jena, Stuttgart, p. 182.

Wasilewska B, Najdzion J (2001) Types of neurons of the claustrum in the rabbit – Nissl, Klüver-Barrera and Golgi studies. Folia Morphol 60: 41-46.

Witter MP, Room P, Groenewegen HJ, Lohman AH (1988) Reciprocal connections of the insular and piriform claustrum with limbic cortex: an anatomical study in the cat. Neuroscience 24: 519-539.

Wojcik S, Dziewiatkowski J, Kowianski P, Ludkiewicz B, Jagalska-Majewska H, Morys J (2002) Qualitative and quantitative study of the postnatal development of the rabbit insular claustrum. Int J Dev Neurosci 20: 113-123.

Woznicka A, Kosmal A (2003) Cytoarchitecture of the canine perirhanal and postrhinal cortex. Acta Neurobiol Exp (Wars) 63: 197-209.

Zhang X, Hannesson DK, Saucier DM, Wallace AE, Howland J, Corcoran ME (2001) Susceptibility to kindling and neuronal connections of the anterior claustrum. J Neurosci 21: 3674-3687.

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