Expression of calcium-binding proteins in the mouse claustrum

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Abstract

The present paper describes the distribution of three calcium-binding proteins (calbindin D28k, calretinin, and parvalbumin) in the mouse dorsal claustrum and endopiriform nucleus. The three calcium-binding proteins were distinctly expressed in structures of both the claustrum and the endopiriform nucleus. Calbindin was the calcium-binding protein showing the highest expression in the claustrum and the endopiriform nucleus. In contrast, calretinin-immunoreactive structures, particularly cell bodies, were very scarce in these regions. Both calbindin-immunoreactive and parvalbumin-immunoreactive neurons were more abundant in the claustrum than in the endopiriform nucleus, and more in rostral than in caudal levels. Nevertheless, calcium-binding protein immunoreactive neurons constitute a minority population of claustral neurons. The colocalization study of calbindin and parvalbumin immunoreactivities has demonstrated that both calcium-binding proteins are mostly expressed by separate claustral neurons in the mouse. On the other hand, our results on parvalbumin and calretinin immunoreactivity match a novel subdivision of the mouse claustrum mostly based on the pattern of cadherin expression [Neuroscience 106 (2001) 505]. In this sense, we propose that a specific zone of the dorsal claustrum with cell bodies that strongly express Rcad and cadherin-8 would be the selective target for parvalbumin-expressing fibers, and that they would be mostly avoided by calretinin-expressing axons.

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1. Introduction

The claustrum is a pallial subcortical region present in all mammals. Two parts of the claustrum can be distinguished: the dorsal part is usually called claustrum proper and it is located deep to the insular cortex (therefore it is also named dorsal or insular claustrum); the ventral part is called endopiriform nucleus and is located deep to the piriform cortex (Druga, 1966; Sherk, 1988; Dinopoulos et al., 1992). Krettek and Price (1977) considered a ventral division of the endopiriform nucleus located deep to the periamygdaloid cortex and adjoining ventral part of the middle region of piriform cortex. For the sake of clarity, from now on the dorsal claustrum will be referred to as claustrum whereas the ventral claustrum will be referred to as endopiriform nucleus.

The claustrum has extensive connections with the neocortex (Pearson et al., 1982; Macchi et al., 1983; Markowitsch et al., 1984; Li et al., 1986; Sloniowski et al., 1986; Sherk, 1988; Sadowski et al., 1997; Kowianski et al., 1998; Majak et al., 2000), whereas the endopiriform nucleus is hodologically related with the prepiriform and entorhinal cortices (Druga, 1971; Markowitsch et al., 1984; Witter et al., 1988).

Despite the evidence for hodologically different zones of the claustrum, previous cyto and chemoarchitectonic studies show an overall uniform structure (Sherk, 1988; Reynhout and Baizer, 1999). Recently, however, three subdivisions within the claustrum have been distinguished on the basis of specific cadherin expression patterns (Obst-Pernberg et al., 2001). It is not known whether these claustral subdivisions have different connections or play different functions.

In this context, the expression of calcium-binding proteins has probed to be very useful in revealing subdivisions in different regions of the central nervous system, notably the thalamus (Jones and Hendry, 1989; Dávila et al., 2000). Therefore, here we studied the…
distribution of three calcium-binding proteins (calbindin D28k, calretinin, and parvalbumin) in the mouse claustrum searching for specific distribution patterns putatively related either to hodologically-based subdivisions or cadherin expression patterns.

2. Material and methods

Fifteen adult OF1 mice (42–46 g body weight) were used in the present study. Throughout the experimental work animals were treated according to the European Communities Council directive (86/609/EEC) on treatment of experimental animals.

Mice were deeply anesthetized with sodium pentothal (65 mg/kg; Abbott Laboratories) and transcardially perfused with 0.1 M phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde, 0.1% glutaraldehyde and 0.2% picric acid in PBS at room temperature for 30 min. The brains were then removed and stored in 4% paraformaldehyde and 0.2% picric acid in PBS at 4 °C overnight; afterwards they were embedded in 4% agar and cut into 50-μm-thick frontal sections, using a vibratome. The sections were washed extensively in PBS prior to immunocytochemical staining with the peroxidase–antiperoxidase method.

Free-floating sections were first incubated in 2% normal goat serum and 0.3% Triton X-100 in PBS at room temperature for 1 h, to block nonspecific binding of the antibodies and permeate the tissues, respectively, and then were transferred to the primary antibody. The three polyclonal antibodies, anti-parvalbumin, anti-calretinin, and anti-calbindin, were raised in rabbits (SWant, Bellinzona, Switzerland) and used at a dilution of 1:2000 for 18 h. After three washes in PBS for 45 min, the sections were incubated in goat anti-rabbit IgG (anti-CB: 1/1000; anti-PV: 1/500). To determine coexistence, we took into consideration only those cells clearly positive to calbindin or parvalbumin and displaying a wide nuclear profile.

Controls: as controls of the immunohistochemical method used in the present study, sections were processed according to an immunohistochemical procedure similar to that described above, except for that the primary antibodies were used at a higher concentration (anti-CB: 1/1000; anti-PV: 1/500). To determine coexistence, we took into consideration only those cells clearly positive to calbindin or parvalbumin and displaying a wide nuclear profile.
Fig. 1
Light microscopic images were photographed by using a Leica microscope equipped with a Nikon DXM1200 digital camera. Digital images were loaded into Adobe Photoshop software and converted to grayscale images. Brightness and contrast were adjusted for the final images. No additional filtering or manipulation of the images was performed. The final figures were composed and labeled with Adobe PageMaker software and printed with an Epson Photo 750 printer.

3. Results

The three calcium-binding proteins studied in this work were distinctly expressed in structures of both the claustrum and the endopiriform nucleus. We will next describe separately calbindin-immunoreactivity in the claustrum and in the endopiriform nucleus, and then calretinin- and parvalbumin-immunoreactivities, respectively.

3.1. Calbindin-immunoreactivity

3.1.1. Claustrum

Sections immunostained for calbindin showed an overall stronger staining of both neurons and neuropil than those stained for either of the other antibodies; accordingly numerous calbindin-immunoreactive (ir) neurons embedded in a moderately immunostained neuropil were found from rostral to caudal levels of the claustrum (Fig. 1A and B). Nevertheless, the number of immunostained cells decreased slightly at caudal levels. As in other parts of the telencephalon, two cell-staining patterns for calbindin were observed throughout the claustrum: darkly stained neurons with a Golgi-like appearance, and lightly stained cell bodies with barely visible processes (Fig. 1D and E). Cell morphologies varied from small, round or elongated neurons to medium-sized multipolar neurons (Fig. 1C and D). These distinct cell types appeared intermingled throughout the claustrum. Multipolar calbindin-ir neurons displayed typically three to five processes splitting into a few secondary aspiny dendrites. Sometimes, pairs of calbindin-ir multipolar neurons with apposed somata were observed within the claustrum (Fig. 1E).

The uniform background staining of the claustrum and adjacent cortical areas made it difficult to delineate the borders of the claustrum in calbindin immunostained sections (Fig. 1B). A moderately stained neuropil extended over the whole anterior–posterior extent of the claustrum. Neuronal staining consisted of varicose axons oriented in all directions and puncta surrounding numerous immunonegative cell profiles (Fig. 1C). Although many perisomatic calbindin-positive terminals were observed, they did not form pericellular baskets.

3.1.2. Endopiriform nucleus

As in the claustrum, calbindin-ir neurons in the endopiriform nucleus exhibited a variety of staining intensities, sizes, and morphologies. Small or medium-sized multipolar cells were the most common types, although pyramidal-shaped cell bodies were also found at intermediate levels of the dorsal endopiriform nucleus. In the dorsal endopiriform nucleus, the number of calbindin immunostained neurons was highest at rostral levels (Fig. 1A), decreasing at intermediate and caudal levels (Fig. 1F). On the other hand, the number of calbindin immunoreactive neurons in the ventral endopiriform nucleus was similar to that found in the caudal part of the dorsal endopiriform nucleus. Pairs of calbindin immunostained cells with apposed somata were also found in the endopiriform nucleus.

A uniform, moderately stained, neuropil extended throughout both the dorsal and ventral regions of the endopiriform nucleus (Fig. 1F). Calbindin-positive axons bearing varicosities were observed in this neuropil.

3.2. Calretinin-immunoreactivity

3.2.1. Claustrum

Calretinin immunostaining in the claustrum consisted of a few scattered positive cells against a background of stained fibers and boutons. Most calretinin-ir neurons were densely stained and exhibited small, round or elongated cell bodies (Fig. 2C), from which two or three aspiny dendrites arose. Some immunostained bipolar neurons located near the external capsule had their cell bodies and dendrites oriented parallel to it whereas other calretinin-ir neurons extended their dendrites, perpendicular to the external capsule and to the cortical surface.

In contrast to the piriform cortex and to the subjacent striatum, the claustrum is characterized by the presence of a dense calretinin-ir neuropil consisting of both stained fibers and puncta (Fig. 2A and B). Nevertheless, the neuropil immunostaining was unevenly distributed within the claustrum. A calretinin-negative neuropil zone, virtually devoid of immunoreactive fibers or puncta, appeared as an oval region in the core of the claustrum (Fig. 2B and C). This oval region was also largely devoid of calretinin-ir neurons, which, if present, were found at the periphery of the region (Fig. 2C). The calretinin-negative region was clearly visible from intermediate to caudal levels of the claustrum since it was surrounded by immunopositive structures, including a thin deep layer of neuropil that separates it from the fibers of the external capsule (Fig. 2C), and a superficial layer of neuropil continuous with the deep layers of the neighboring insular cortex.
3.2.2. Endopiriform nucleus

The overall calretinin immunostaining in the endopiriform nucleus was similar to that of the claustrum and the insular cortex. Few scattered neurons embedded in a moderate-to-dense immunoreactive neuropil were found in the endopiriform nucleus. The most abundant calretinin-ir cell type consisted of small bipolar cells with the elongated cell body and processes oriented parallel to the external capsule. Bipolar neurons were observed in both the dorsal and ventral endopiriform nuclei.

Neuropil staining was highest at intermediate levels in the region of the dorsal endopiriform nucleus adjacent to the claustrum (Fig. 2B). For the rest of the dorsal endopiriform nucleus and the whole ventral endopiri-
form nucleus, the neuropil was moderately immunostained for calretinin (Fig. 2D). Calretinin immunoreactive axons were fine and displayed small varicosities.

3.3. Parvalbumin-immunoreactivity

3.3.1. Claustrum

Parvalbumin immunostaining in the claustrum consisted of a number of immunoreactive neurons surrounded by a dense meshwork of stained fibers and puncta (Fig. 3C and D). Both darkly and lightly stained neurons were found throughout the claustrum but they were less abundant at the caudal part of the claustrum. Most parvalbumin-ir cells were medium in size and had multipolar cell bodies (Fig. 3C) with several thin, beaded dendrites that extended for long distances.

Parvalbumin-ir varicose axons were oriented in all directions within the claustrum (Fig. 3C and D). However, it was unclear whether these axons formed perisomatic contacts.

In sections immunostained for parvalbumin, the claustrum was characterized by the presence of a sharply defined, moderately stained patch of neuropil particularly evident at intermediate and caudal levels of the claustrum (Fig. 3B). The deep layers of the adjacent agranular insular cortex presented also a patch of moderately stained neuropil, and between these two neuropil patches there was an intervening immunonegative cell-poor zone that was most apparent at intermediate levels of the claustrum (Fig. 3B).

The patch of parvalbumin immunopositive neuropil in the claustrum roughly corresponds to the calretinin-negative oval area described above.

3.3.2. Endopiriform nucleus

A number of parvalbumin-ir neurons embedded in a plexus of immunostained varicose axons and processes were found at rostral levels of the dorsal endopiriform nucleus (Fig. 3A). At intermediate and caudal levels of the dorsal endopiriform nucleus, the number of positive neurons and axons decreased dramatically: very few immunoreactive neurons were observed, and the neuropil was almost negative at these levels (Fig. 3E). The parvalbumin immunoreactivity pattern in the ventral endopiriform nucleus was similar to that of the caudal part of the dorsal endopiriform nucleus.

Most parvalbumin stained neurons located in the rostral part of the dorsal endopiriform nucleus were medium in size and exhibited multipolar morphologies, without a specific orientation of their cell processes. In the ventral endopiriform nucleus, some multipolar immunoreactive neurons with the cell body and main dendrites oriented parallel to the pial surface were observed (Fig. 3F).

3.4. Colocalization study

We carried out a colocalization study for the two major calcium-binding proteins present in neurons of the mouse claustrum, i.e. calbindin and parvalbumin, using post-embedding immunocytochemistry on adjacent semithin sections. The analysis was made in the dorsal claustrum, since it was the claustral region with more positive neurons for either calcium-binding protein.

In addition, by comparing immunostained semithin sections with the adjacent Nissl-stained semithin section, we obtained quantitative data about the relative density of calbindin- and parvalbumin-immunoreactive populations.

Calbindin- and parvalbumin-immunoreactive neurons constituted a minority of the claustral neurons, accounting for only 5.4% (n = 122) and 7.9% (n = 178) of the total number of counted cells (n = 2260), respectively. Neurons expressing either of the two proteins represent 12.3% of the claustral cells. In addition, calbindin- and parvalbumin-immunoreactive cells are mostly segregated populations (Fig. 4): more than 87% of parvalbumin-ir cells do not express calbindin (156 out of 178) and, conversely, 82% of calbindin-ir cells do not express parvalbumin (100 out of 122), whereas only 22 out of 2260 cells coexpress both proteins.
Fig. 3
4. Discussion

Although little is known about the exact functions of the calcium-binding proteins parvalbumin, calbindin and calretinin, they have provided useful markers of specific neuronal subpopulations in studies of the neuronal circuitry of the cerebral cortex and other brain regions, and they reveal chemoarchitectonic subdivisions in different regions of the central nervous system. Results of the present study indicate the existence of distinct immunoreactivity patterns for each of the three calcium-binding proteins in both the claustrum and the endopiriform nucleus as it has been reported in other mammals, and also reveal details not described previously. We will next discuss our results in comparison with other mammals and then analyze the calcium-binding protein expression patterns in the light of recently described subdivisions within the mouse claustrum.

Calbindin and parvalbumin immunoreactivities have been reported in the claustrum and endopiriform nucleus in other mammals but inter-specific differences exist regarding the expression pattern of each protein. In the rat, parvalbumin and calbindin exhibit a largely complementary distribution pattern: parvalbumin-immunoreactivity is concentrated in the claustrum, whereas calbindin-immunoreactivity prevails in the endopiriform nucleus (Celio, 1990; Druga et al., 1993). From our results in mouse, we cannot demonstrate this complementary distribution pattern between calbindin and parvalbumin; instead, we showed that for either of these calcium-binding proteins, more immunostained neurons were observed in the claustrum than in the endopiriform nucleus, and more in rostral than in caudal levels.

Our colocalization analysis has demonstrated that, in spite of a similar distribution pattern, parvalbumin and calbindin are mostly expressed by separate claustral neurons in the mouse. In this context, it is to be noted that in the rat cortex parvalbumin- and calbindin-ir cells represent two classes of GABAergic interneurons displaying morphological and neurochemical specificity (Celio, 1986; Hendry et al., 1989; Kosaka and Heizmann, 1989), and indirect evidence suggests that calcium-binding protein immunoreactive neurons may also represent, at least in part, inhibitory local circuit neurons in the rat claustrum (Druga et al., 1993).

With regards to calretinin, it has been claimed that the rat claustrum is demarcated by the absence of calretinin staining (Paxinos et al., 1998). However, calretinin immunoreactive structures have been reported in the monkey claustrum (Reynhout and Baizer, 1999) and in the endopiriform nucleus of the guinea pig (Frassoni et al., 1998). Our results in the mouse showed that calretinin staining consisted of a few positive cell bodies embedded in a moderate neuropil both in the claustrum and the endopiriform nucleus. Calretinin is present in the claustral regions of monkeys, guinea pigs, and mice. Thus, the reported absence of calretinin in the rat claustrum could be due to inter-specific differences or to a differential sensitivity of the immunocytochemical method.

Calcium-binding protein immunoreactive neurons constitute together a small subset (12.3%) of the claustral neurons in the mouse, as demonstrated by our study on semithin sections. This low incidence of calcium-binding protein immunoreactive neurons in the claustrum was to be expected if they represent different subpopulations of GABAergic interneurons, as suggested above, since GABAergic neurons in the rabbit claustrum accounted only for 12% of all neurons in the same claustral samples (Gómez-Urquijo et al., 2000).

These data on the low incidence of inhibitory claustral neurons are also in agreement with the presence in the rat claustrum of numerous latexin-immunoreactive neurons (Arimatsu et al., 1992), most of which are glutamate positive (and GABA negative), suggesting that they are excitatory projection neurons (Arimatsu et al., 1999).

4.1. Different zones within the dorsal claustrum

Recently, three novel subdivisions within the mouse claustrum based on the pattern of cadherin expression and cytoarchitecture have been distinguished (Obst-Pernberg et al., 2001): a superior, an intermediate and an inferior part. The superior and intermediate parts are located deep to the insular cortex whereas the inferior part is located deep to the dorsal part of the piriform cortex. Cytoarchitectonically the intermediate part is characterized by the presence of cell aggregates. The superior and intermediate parts contain densely packed cell bodies that strongly express R-cadherin (Rcad; Obst-Pernberg et al., 2001) and cadherin-8 mRNA (Korematas and Redies, 1997), whereas the inferior part shows weak expression of Rcad, and only contain scattered cell bodies expressing cadherin-8 mRNA. In addition, the superior part shows a very strongly immunoreactive neuropil for Rcad and moderately labeled for cadherin-N (Ncad); the intermediate part is strongly immunoreactive for Rcad and moderately to strongly labeled for Ncad; and the inferior part is weakly labeled for Rcad and moderately labeled for Ncad (Obst-Pernberg et al., 2001).

Our results on parvalbumin and calretinin immunoreactivity match these novel subdivisions of the mouse claustrum. Thus, in parvalbumin-immunostained sections a patch of moderately immunoreactive neuropil occupies a position just deep to the ventral part of the agranular insular cortex. This parvalbumin positive zone is bordered dorsal and ventrally by claustral zones virtually devoid of immunostained neuropil. The dorsal
negative zone lies deep to the dorsal part of the agranular insular cortex and to the dysgranular cortex, whereas the ventral zone negative for parvalbumin is continuous with the endopiriform nucleus. In calretinin immunostained sections, on the other hand, a complementary pattern was observed: the intermediate region was virtually devoid of calretinin immunoreactive fibers, and dorsal and ventral calretinin-positive zones bordered it. It is tempting to relate these three zones of the dorsal claustrum showing a distinct calcium-binding protein expression pattern to the three subdivisions described by Obst-Pernberg and coworkers on the basis of cadherin expression patterns (Obst-Pernberg et al., 2001). These authors suggest that the selective adhesion of neural structures that express the same types of cadherin contribute to the formation of gray matter areas, neural circuits and functional connections in the postnatal forebrain of the mouse. In this sense, we propose that the cell aggregates of the intermediate zone of the dorsal claustrum with cell bodies that strongly express Rcad and cadherin-8 would be the selective target for parvalbumin-expressing fibers, and that they would be mostly avoided by calretinin-expressing axons. We cannot conclude whether these fibers have an intrinsic or extrinsic origin.

While these novel subdivisions of the mouse dorsal claustrum appear to be well documented from a cyto and neurochemical point of view, it is difficult to relate them to the different functional zones that have been described in the mammalian claustrum on the basis of its connections. In mammals with a well developed claustrum the claustro-neocortical connections are topographically organized, with an anterior part of the claustrum linked mainly with motor and prefrontal cortices, a central part linked with somatosensory cortex, a posterior claustrum related to visual cortex, and a ventral zone connected with auditory cortex (Pearson et al., 1982; Macchi et al., 1983; Sherk, 1988; Morys et al., 1996). In the rat claustrum, however, two main cortico-related zones have been described, an anterodorsal sensorimotor and a posteroverentral visuoauditory zones (Sadowski et al., 1997).

Our results indicate that the incidence of calcium-binding protein-expressing neurons is higher in rostral than caudal levels of the mouse dorsal claustrum. Since all hodological studies confirm at least two different functional zones in the claustrum, an anterior and a posterior region, it is likely that calcium-binding protein-expressing neurons, putatively GABAergic cells, are more represented in claustral intrinsic circuits related with the limbic and motor cortices rather than with the visuoauditory regions. As suggested for somatostatin-, neuropeptide Y-, and vasoactive intestinal peptide-ir neurons in the rat claustrum (Kowianski et al., 2001), calcium-binding protein-expressing neurons do not appear to play a significant role in the claustro-cortical projection but are most probably involved in modulation and information transfer in the claustrum.

It is to be noted that the complementary expression of calretinin and parvalbumin in patches of neuropil in the mouse claustrum is best seen at intermediate/posterior levels in the rostrocaudal axis of the claustrum. It
cannot be determined, however, whether these claustral zones correspond with claustral regions with specific cortical connections. In this sense, it would be of interest to perform combined immunohistochemical-fiber tracing experiments in order to study the specific connections of the chemoarchitectonic subdivisions of the dorsal claustrum.

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References


