Calretinin-immunoreactive neurons in the claustrum of the guinea pig

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\section*{ABSTRACT}

\textbf{Background:} The claustrum is present in all mammalian species. Many aspects of its morphology and function remain subject to debate. It has been suggested that calcium-binding proteins influence neuronal activity in many structures.

\textbf{Objective:} The aim was to examine CR immunoreactivity in the claustrum of guinea pigs and compare it with that in other mammals.

\textbf{Design:} CR immunoreactivity in the claustrum was analyzed in five guinea pigs using immunohistochemical techniques. The distribution of CR-positive neurons in the dorsal claustrum and endopiriform nucleus (END), and their morphological characteristics were described.

\textbf{Results:} CR immunostaining in the dorsal claustrum and END of the guinea pig consisted of CR-positive neurons, fibers, and puncta of varying labeling density. The majority of these neurons displayed small- or medium-sized round, oval, and multipolar cell bodies with aspiny dendrites. CR-ir neurons prevailed in the periphery of both divisions. The dorsal claustrum was seen to have a weakly CR-ir central core surrounded by a rim of moderately positive neuropil.

\textbf{Conclusion:} CR immunoreactivity in the guinea pig claustrum was not as diffuse as in higher mammals, but non-uniformity was evident. CR-ir neurons are distributed in both the dorsal claustrum and END, mostly in the periphery.

\section*{Introduction}

The claustrum is a subcortical structure situated between the striatum and insular cortex in the mammalian brain. Owing to differences in cytoarchitecture, connectivity, and a relationship with the rhinal sulcus, the claustrum can be viewed as being comprised of two parts. The dorsal part adjoining the insular cortex is named the dorsal or insular claustrum (CLD), while the ventral part adjacent to the piriform cortex is called the endopiriform nucleus (END) (see 1, 2). In 2007, Paxinos and Watson (3) introduced an enhanced parcellation of the claustral complex in the rat. The CLD is further divided into dorsal (DCl) and ventral components (VCi). Similarly, the END is subdivided into three segments: dorsal (DEN), intermediate (IEN), and ventral (VEN) (3,4).

Studies on the expression of developmental regulatory genes specific to lateral and ventral pallial histogenesis indicate that the CLD is derivative of lateral pallium, while the END is considered derivative of ventral pallium (5,6). Both subdivisions also differ in their hodological characteristics, namely, cortical projections (1,2). The CLD and END consist of glutamatergic-projecting neurons and gamma-aminobutyric acidergic local interneurons coexpressing calcium-binding proteins (CBPs) and neuropeptides in various combinations (1,7). The large family of CBPs is classified as buffer proteins which may act as modulators of cytosolic calcium levels. Calretinin (CR) is a CBP with complex \( \text{Ca}^{2+} \)-binding kinetics. In addition, CR is involved in the processes of neuronal excitability and synaptic plasticity (8–10). Calretinin-immunoreactive (CR-ir) interneurons have been demonstrated to influence the activity of other interneurons (11).

The expression of CBPs has often been used as a marker for revealing and elucidating the functional subdivisions of the central nervous system. CR has been found in the claustrum of several mammalian species, with published data indicating striking interspecies differences in the distribution of CR-ir neurons and neuropil, as well as their somatodendritic characteristics. Specifically, CR-ir neurons have been reported in the claustrum of the rat (4), mouse (12,13), rabbit (14), cat (15), monkey (16), dolphin (17), and chinchilla (18). Pirone et al. (19) reported on CR-ir neurons in the human, chimpanzee, and crab-eating macaque claustrum. The aim of the current study is to examine the expression of CR immunoreactivity in another mammalian species, the guinea pig, with a particular focus on the somatodendritic morphology of CR-positive neurons in the CLD and END to use in the comparative

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analysis of differences between rodents and other mammalian species.

**Material and methods**

**Tissue preparation**

Experiments were carried out on five young adult, male, pigmented guinea pigs (350–400 g). Animals were maintained with ad libitum access to food and water, on a 12 h light/dark cycle. All animals were treated in accordance with the regulations and laws of the European Union (86/609/EEC). Animals were deeply and irreversibly anesthetized with an intramuscular injection of mixture of ketamine and xylazine, then transcardially perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4, 4ºC), immediately followed by 4% paraformaldehyde in phosphate buffer. The brains were removed, blocked, post-fixed for 6 h in 4% paraformaldehyde, and cryoprotected in a graded sucrose PBS run (10%, 20%, 30%). Afterwards, the blocks were cut into 40 µm coronal sections using a freezing microtome.

**Immunohistochemistry**

Initially, free-floating sections were incubated in 2% normal goat serum and 0.3% Triton X-100, then transferred to the primary antibody (rabbit polyclonal anti-calretinin, 1:2,000 for 24 h; Chemicon/Merck, Billerica, MA). This was followed by incubation in biotinylated goat anti-rabbit immunoglobulin G (1:200 for 3 h; Vector Laboratories, Burlingame, CA). Lastly, a standard avidin–biotin complex (ABC) kit was used (Vector Laboratories, Burlingame, CA). The peroxidase reaction was developed using 3,3′-diaminobenzidine (DAB) as the chromogen (Sigma-Aldrich/Merck, St. Louis, MO). All steps were carried out at room temperature with gentle agitation. Alternate sections were stained with cresyl violet to delineate cytoarchitecture and borders. To test for reaction specificity, incubation with primary antibody was omitted from the control sections, which resulted in the absence of specific immunostaining.

**Quantitative study**

Light-microscopic images were acquired with an Olympus BX 51 microscope equipped with an Olympus DP 70 digital camera. Digital images were imported in Photoshop (Adobe) and used to acquire data and plot the distribution of CR-ir cells. Measurements of neuronal size (longest diameter and cross-sectional area) were taken under a 40 × objective by means of imaging software (Cell¹, Olympus). The mean and standard deviation (± SD) of the longest diameters and cross-sectional areas of cell bodies were calculated. In total, we measured 235 neurons in the CLD and 405 neurons in the END. Selected CR-ir neurons with distinct dendritic trees were drawn using a camera lucida attachment.

**Results**

CR immunoreactivity was observed in the neurons, fibers, and neuropil of both the CLD and END subdivisions of the guinea pig claustrum (Fig. 1). Specific to the CLD, we noted a core of weakly CR-ir ovoid or triangular area of neuropil bordered by a rim of moderately CR-ir neuropil which was characterized by the presence of moderate-to-strongly positive short segments of fibers oriented in various directions, as well as a moderate number of puncta (Figs. 2 and 4). In contrast, the CR-ir core contained a lower density of positive fibers and a small number of puncta. Both subdivisions of the CLD (core and rim) contained CR-ir neurons slightly prevailing in the periphery of the CLD (Figs. 2 and 3). We were able to discern thin, weakly positive dorsoventrally oriented fibers and similarly oriented elongated neurons in the external capsule.

**Figure 1.** Low-power photomicrograph illustrating calretinin immunoreactivity in the claustrum (CLD) and endopiriform nucleus (END) of the guinea pig. Coronal section at AP 9.0 mm according to Luparello (23). The areas outlined by the dashed lines (A, B, C) are seen in high magnification in Fig. 4. INS = insular cortex; LA = lateral amygdalar nucleus; PIR = piriform cortex; STR = striatum. Scale bar = 2 mm.
With respect to the END, moderately labeled neuropil contained in its medial half a ventrodorsally oriented area of weaker CR immunoreactivity (Fig. 2). This weaker area was evident only in the rostral and intermediate third of the DEN. Although CR-ir neurons were found throughout the CLD and DEN, they were most abundant in the periphery of these nuclei. In the rostral half of the CLD, the majority of CR-ir neurons were located in its lateral aspect (Fig. 3).

Based on our observations and analysis, it is estimated that approximately 90% of CR-labeled CLD and END neurons exhibited moderate-to-strongly positive immunoreactivity, as seen in the cytoplasm, nucleus, and proximal dendritic segments (Fig. 4).

In strongly positive perikarya, label filled the cell body to the point of obscuring the nucleus. Dendrites on both the CLD and END were noted to branch into secondary and (less frequently) tertiary segments, the majority of which could be traced for distances of up to 80 µm from the cell body (Fig. 5). Dendrites of all of the neuronal types were smooth and aspiny, but beaded and undulated proximal dendritic segments were frequently discernible.

With respect to the CLD, the most prevalent neuronal shape was oval-to-round (78%), followed by multipolar (10%) or triangular (8%) perikarya. As for the END, oval-to-round perikarya comprised 45% of our sample, while multipolar and triangular neurons were seen less frequently (35% and 11%, respectively; Fig. 5). The multipolar and triangular neurons

Figure 2. Higher power photomicrograph showing calretinin immunoreactivity in the claustrum (CLD) and endopiriform nucleus (END). Asterisks mark areas of weaker immunoreactivity in the CLD and END. The large arrow indicates the level of the rhinal sulcus. Abbreviations as in Fig. 1. Scale bar = 200 µm.

Figure 3. Spatial distribution of calretinin-immunoreactive (CR-ir) neurons in the claustrum and endopiriform nucleus. Numerals indicate stereotaxic planes according to Luparello (23). Each dot represents a single CR-ir neuron. M = medial direction; L = lateral direction; 13.4 = rostral section; 8.2 = caudal section. Arrows indicate the level of the rhinal sulcus.
displayed radially oriented dendrites, while oval and round cells typically exhibited bipolar dendrites. In the majority of CR-positive neurons in the CLD and END, the dendrites were noted as being sparsely branched. When compared with our measurements of CLD-ir neurons, the DEN-ir neurons had a larger diameter and cross-sectional area of CR-ir neurons. Specifically, CLD-ir cells averaged 14.4 ± 2.4 µm in diameter and 105.5 ± 31.6 µm² in area. END-ir neurons averaged 16.98 ± 3.27 µm in diameter (15% greater than CLD) and 155.9 ± 53.6 µm² in area (32% greater than CLD) (Table 1).

**Figure 4.** High-power photomicrographs of calretinin-immunoreactive neurons and neuropil in areas outlined by the dashed lines in Fig. 1. (A, B) dorsal claustrum; (C) endopiriform nucleus. Scale bar = 100 µm.
Discussion

Collectively, calcium-binding protein-ir neurons comprise a minority (12.3%) of cells in the human claustrum (20). These proteins are primarily expressed in segregated and disparate populations. CR-ir neurons have been found in the claustrum of numerous mammalian species. With the exception of rodents (e.g. mouse, rat, and guinea pig) (4,13,21), CR-ir neurons are broadly distributed throughout the claustrum (e.g. in rabbit, cat, dolphin, monkey, and human) without any specific relation to functional territories or parcellation (14–17,19). Pirone et al. (19) reported that CR-ir neurons in human claustrum are sometimes arranged in clusters.

Although Paxinos et al. (22) initially reported that the rat claustrum can be demarcated by the absence of CR labeling, subsequent studies reported CR-positive labeling in the CLD and DEN. Real et al. (13) described weak CR immunoreactivity in the ventral part of the mouse dorsal claustrum (VCI), with moderate-to-strongly immunoreactive neuropil in the dorsal part of the dorsal claustrum (DCI) and throughout the subdivisions of the END (i.e. DEN and VEN). Similar patterns of CR immunoreactivity were described in the rat dorsal claustrum (CLD) (4). The ventral part of the rat dorsal claustrum (VCI) is seen as having characteristically weak CR positivity, mostly expressed as a small number of isolated thin fibers and the absence of puncta, with a paucity of CR-positive neurons in the lateral aspects. With regard to CR immunoreactivity in the END of the rat (4), its three subdivisions (i.e. DEN, IEN, and VEN) are noted to display weak to moderate labeling, as also seen in the mouse (13). A complementary pattern of immunoreactivity is observed in the neuropil of guinea pig CLD, but the difference between labeled neurons in the central core of the DCI compared with its periphery was less distinct (21, present data). In yet another species of rodent, the chinchilla, differences in the degree of immunostaining between the central core and periphery of DCI were not reported (18). The dissimilar core/periphery labeling seen in the guinea pig DCI has not been reported in other mammals (e.g. rabbit, cat, dolphin, monkey, and human claustrum) (14–17,19). In the rabbit, Wójcik et al. (14) reported extremely sparse numbers of CR-ir neurons in the END, while in the CLD their numbers were moderate.

Multipolar, triangular, and fusiform-shaped neurons can be found in all subdivisions of the CLD and END in the mouse, rat, chinchilla, and guinea pig (present data); however, their proportions and type are seen to vary by subdivision [rat (4); guinea pig (present study)]. CR-ir neurons in the claustrum of the cat exhibited two distinct types. The first type had elongated cell bodies with two major dendrites emerging from the poles. The second type had irregularly shaped somas with dendrites emerging from several points and extending in all directions. The size of both types of neurons was in the range 20–30 µm (15). However, the dendrites of CR-positive claustrum neurons in rodents are almost exclusively aspiny in nature. Comparable somatodendritic morphologies have been reported in the claustrum of other mammals (e.g. rabbit, cat, monkey, and human), the one exception being the dolphin, where a population of small
(10 μm) unipolar, bipolar round, fusiform CR-ir neurons has been found (17).

Lastly, from a functional perspective, it is of interest to note that CR-ir rat claustrum neurons have dendritic processes which extend into adjacent structures (i.e. striatum and insula), and are seen to be reciprocal in nature (4). This was not evident in the present study, or in the claustrum of other representative mammalian species (i.e. mouse, rabbit, cat, dolphin, monkey, and human) (13–19).

Disclosure statement

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