Calcium Binding Proteins Immunohistochemistry and Identification of Neurons in the Mammalian Pineal Gland of the African Giant Rat: *Cricetomys gambianus*

E. Bastianelli¹, K. Moutairou², M. T. Akélé-Akpo³, R. Darboux³ and R. Pochet¹

1 Laboratoire d’Histologie, Faculté de Médecine, Université Libre de Bruxelles, Bruxelles, Belgique
2 Département de Biologie cellulaire, Faculté des Sciences et Techniques, Université Nationale du Bénin, Cotonou
3 Unité d’Anatomie Pathologique, Faculté des Sciences de la Santé, Université Nationale du Bénin, Cotonou

Abstract. The presence of true neurons in the rodent pineal gland is still a matter of controversy. In this work, by using immunohistochemistry with five antibodies against calcium-binding proteins (calbindin-D28k, calretinin, calmodulin, neurocalcin and S-100β) and *Cricetomys gambianus*, a rodent belonging to Muridae family living in Africa, we were able to illustrate the presence of neurons in the pineal gland. Anti-calbindin-D28k and anti-calretinin labelled neurons belonging to two neural ganglia. One ganglion was localized in the anterior part of the gland near the pineal stalk and the other one in the posterior portion of the organ. Immunoreactive neurons are medium in size (15–20 μm) and have long thick processes running towards the stalk. Calretinin and calbindin-D28k positive neurons stained with different intensities. Thin processes were detected by anti-calretinin whereas thick processes were preferentially calbindin-D28k positive. Neurocalcin labelled a few smaller neurons and many thin processes within the ganglion. Calmodulin could not be detected immunochemically. Within the ganglia many astrocytic processes were S-100β positive. The afferent and the efferent pathways of the pineal ganglia remain to be elucidated.

Key words: Calcium – Calbindin – Calmodulin – Calretinin – Pinealocytes – Neurons

Correspondence to Dr Roland Pochet, Laboratoire d’Histologie, Faculté de Médecine, Université Libre de Bruxelles, 808 route de Lennik, B-1070 Bruxelles, Belgique E-mail rpochet@ulb.ac.be
Introduction

In rat, the most intensively studied rodent, the pineal gland is composed of two main cell subclasses: the pinealocytes which account for 90% of the cell total and the interstitial cells which include mainly astrocytes, pericytes and mast cells (Arstila 1967). Immunohistochemical studies have discriminated among pinealocytes and categorized them in distinctive subsets. Specific retinal antigen, S-antigen (Korf et al. 1985), rhodopsin (Huang et al. 1992) and recoverin (Korf et al. 1992, Bastianelli and Pochet 1994) recognize different pinealocyte subpopulations. Some pinealocytes express these proteins whereas others do not. Synaptophysin (Redecker and Bargsten 1993) and neuron-specific enolase (Mockett and Lapwood 1994) also label different subsets of pinealocytes. Recently, using calbindin-D28k, we were able to discriminate, in the rat, between type I and type II pinealocytes, or intraparenchymatous and perivascular pinealocytes (Bastianelli et al. 1993, Pochet et al. 1994). Interstitial cells in the pineal of rodents do not show such a diversity, but subsets have been described using GFAP (Borregón et al. 1993), S-100 (Moller et al. 1978), vimentin (Chakravarty 1992), calbindin-D28k (Bastianelli and Pochet 1995) or calretinin (Redecker et al. 1996). The presence of neurons, a third class of pineal cells, has not, until recently, been clearly shown and is not "generally accepted" ["The pineal of the rat does not contain any neurons" as stated in the recent textbook The Rat Nervous System by Paxinos (1995), p. 399]. However, already in 1973 David et al. have described the presence in the ferret of an intrapineal ganglion and the presence of intrapineal neurons has been noted in the rabbit (Romijn 1975), the ground squirrel (Matsushima and Reiter 1978), bats (Bhattacharya et al. 1990) and the chipmunk (Karasek et al. 1982). The presence of true neurons in the pineal gland increases the polysynaptic pathway conveying photic information whose physiological importance could be through a regulatory role on the rhythmic secretion of melatonin. Recently, by using anti-calretinin immunohistochemistry, we and another group (Novier et al. 1996) provided morphological description strongly suggesting that neurons were indeed present in rat pineal gland (Pochet et al. 1994, Bastianelli et al. 1995). In this work we present evidence for the presence of neurons clustered in two ganglia in a rodent pineal.

Calretinin like parvalbumin and calbindin-D28k occurs in distinct subpopulations of neurons and both have been employed as neuroanatomical markers (Ceho 1990, Jacobowitz and Winsky 1991, Résibois and Rogers 1992, Andressen et al. 1993, Floris et al. 1994, Seto-Ohshima 1994). These proteins have an advantage over other neuronal markers in that, by virtue of their high solubility, they are usually present throughout the cytosol, even in the thin processes of neurons, therefore facilitating studies of neuronal shape and connectivity. The provide new and powerful tools to classify neurons, to reveal the mode of distribution of distinct neuronal populations, and to analyse cell class-specific morphological features at both the
light and electron microscopic level. This is best illustrated by the recent discovery and precise morphology, thanks to calretinin, of ultrastructural features of unipolar brush cells (UCB). The first precise description of this “novel” neuronal type was made after calretinin immunohistochemistry of the adult human cerebellar cortex (Braak and Braak 1993). Mugnaini and Floris (1994) by utilizing a variant of the classical Golgi/Del Rio-Hortega procedure which affords a much higher rate of impregnation have described the same new type of neuron they named unipolar brush cells and concomitantly used calretinin immunohistochemistry to analyse their distribution and ultrastructural features (Floris et al. 1994). Unipolar brush cells are the cortical neurons that are most densely immunostained with antisera to calretinin. They have rounded or ovoidal cell bodies (9–12 μm in diameter) that are intermediate in size between granule and Golgi cells, send out a thin axon, 0.3–0.5 μm in diameter, and commonly give rise to a single, short stubby dendrite, 2–3 μm in diameter, which usually divides only at the tip, where it forms a tightly packed group of branchlets covered by thin, spine-like appendages, resembling a paintbrush. The field occupied by the brush tip is rounded, ovoid, boxy or cap-shaped and its diameter is 10–30 μm in average, forming a neuropil island. The unipolar brush cells are highly concentrated in the flocculo-nodular lobe, the ventral ovula and the ventral paraflocculus. They are also in the cochlear nucleus and form a pathway between the floccullus and the cochlear nucleus. Electron microscopic analysis after calretinin immunostaining revealed that the soma, the dendritic stem and specially the dendrioles which terminate the brush-like tips send out short, non synaptic appendages. The dendrioles represent the main synaptic apparatus of the UCB and articulate tightly with a single mossy fiber rosette forming a glomerular array characterized by an extraordinarily extensive synaptic contact. Electrophysiological and electron microscopic observations (Mugnaini et al. 1997) indicate that the unusual synaptic ultrastructure may produce entrapment of neurotransmitter in the synaptic cleft. *Cricetomys gambianus* belongs to Muridae, is a wild animal, and is common throughout tropical Africa. These animals are omnivorous rodents whose physiological and morphological characteristics are poorly known.

*Cricetomys gambianus* lives in savannah and fallow areas. Adult animals weight between 1.2 and 2.0 kg. They generally do not drink water and eat fresh food (green fodder and palm nuts). In captivity, cannibalism and coprophagy are sometimes observed. Female cricetomes give birth four times a year and have two to four offsprings at each birth (Moutairou et al. 1996).

**Materials and Methods**

**Tissues**

Experiments were performed on 6 adult (aged 3 to 9 months) *Cricetomys gambianus*. The pineal gland was dissected and rapidly fixed in Helly’s fluid (68 mmol/l
potassium dichromate, 70 mmol/l sodium sulphate, 180 mmol/l HgCl₂ and 4% formaldehyde) Fixed tissues were routinely dehydrated, embedded in paraffin, and sections at 5μm or 7μm thick were prepared

**Antibodies**

Rabbit antiserum raised against chick duodenal calbindin-D28k was prepared as described (Spencer et al 1976) and routinely used at 1/6000 dilution. Its specificity was demonstrated previously (Pasteels et al 1990, Pochet et al 1991). Polyclonal antibody raised against S100β, was obtained from J Baudier (Grenoble, France) (Cocchia 1981, Baudier et al 1983) and used at 1/3000 dilution. Polyclonal antibodies raised against calretinin (Schwaller et al 1993) were obtained from SWant (Belinzona, Switzerland) and used at 1/1000 dilution. Three different rabbit polyclonal anti-calmodulin antibodies were used. They were prepared as described previously (Van Eldik and Watterson 1981, Van Eldik et al 1983) and termed 449, 465 and 860. Antibodies 449 and 465 were produced against performic acid-oxidized vertebrate calmodulin and 860 was made against a synthetic peptide of the COOH terminus (residues 134-148) of vertebrate calmodulin. Purified IgG fractions were used at 1/2000 dilution. Number 465 was routinely used as it gave higher signal versus background. Rabbit anti-recoverin was prepared as described (Polans et al 1993) and used at 1/1000 dilution. Polyclonal rabbit anti-serum raised against rat brain neurocalcin was prepared by Nakano et al (1992) and used at 1/1000 dilution. Anti-synaptophysin antibodies were obtained from Dako (Glostrup, Denmark) and used at 1/300 dilution. Endogenous peroxdydase staining was abolished by H₂O₂ (0.3%) pre-treatment (30 min at room temperature). Controls for specificity were performed using preincubated antibodies with an excess (2μg/ml) of pure antigen. No labelling occurred (not shown). S100β, calbindin and calretinin were purchased from SWant, calmodulin from Sigma (St Louis, USA), and neurocalcin was donated by Dr K Okasaki (Nagoya).

**Immunohistochemical staining**

Dewaxed and hydrated sections were processed for immunohistochemistry according to a peroxidase-antiperoxidase (PAP) procedure as described previously (Pasteels et al 1990, Pochet et al 1991). Serum dilutions were made up in Coons Veronal Buffered Saline (CVBS 10 mmol/l sodium veronal, pH 7.2, NaCl 0.9%) supplemented with 5% (v/v) normal sheep serum. The immunostaining sequence consisted of the following steps: 1) rinse in CVBS, 2) preincubation in 5%(v/v) normal sheep serum, 3) incubation with primary antibodies for 48 h at 4°C in a moist chamber, 4) incubation with sheep anti-rabbit immunoglobulin G (IgG) serum 1 100 dilution (Laboratoire d’Hormonologie et Immunologie, Marloie, Belgium), and 5) incubation with soluble rabbit PAP complex 1 300 dilution (DAKO, Glostrup, Denmark). Between each step, sections were thoroughly rinsed for 10
mn in CVBS. After the last rinse, staining was performed in a citrate phosphate buffer, pH 6.2, containing 0.5 mg/ml 3,3'-diaminobenzidine HCl (DAB; Sigma, St. Louis, USA) and 0.01% H₂O₂. All steps, except step 3, were performed at room temperature.

Results

General Description

By macroscopic examination, the *Cricetomys gambianus* pineal gland can be subdivided, as for other rodents, into a superficial and a deep portion. Medium length, measured in six animals, was 4 mm with a diameter of 1 mm. As in albino rats, a substantial portion of the parenchyma of the gland extends from the diencephalon to the vicinity of the cerebellum. Meningeal attachments were observed in the supero-posterior parts of the superficial gland which were connected by a thin pineal stalk to the cerebellum. This *Cricetomys gambianus* pineal gland can be described as a proximo-intermediate-distal gland corresponding to the αβC type (Reiter 1981; Vollrath 1981).

At the light microscope level, the pineal of adult animals was constituted to
2/3 of an anterior cellularized portion and the remaining 1/3 of a posterior more fibrous portion. The posterior portion was composed of a fibrous stroma, sometimes hyalized, containing rare fibrocytes. The anterior portion was characterized by small plain follicular structure surrounded by capillaries (Fig. 1).

Figure 2. Immunohistochemistry of the anterior part of *Cricetomys gambianus* hematoxylin counterstained and using anti-calretinin (A), anti-calbindin (B), anti-synaptophysin (C) and anti-S-100β (without counterstaining) (D). Only pinealocytes are immunoreactive for calretinin, calbindin and synaptophysin. Near the stalk some interstitial cells are S-100β-Ir. Scale bar = 10 μm
**Immunohistochemistry**

Further characterization was achieved by using immunohistochemistry and antibodies against 7 proteins: calbindin-D28k, calmodulin, calretinin, neurocalcin, recoverin, S-100β and synaptophysin. Calmodulin and recoverin could not be detected in any cell type (not shown). Calbindin and calretinin-Ir were detected in many pinealocytes (Fig. 2A and B) but also in two neuronal ganglia (a superior and an inferior) in which both fibers and perikarya could be visualized (Fig. 3A and B). These two ganglia were located between the anterior and posterior portions of the superficial pineal body. Synaptophysin-Ir was also detected in pinealocytes (Fig 2C) whereas neurocalcin and S-100β labelled many processes within the ganglia (Fig. 4A and B). Immunohistochemical data allowed to distinguish three cell types: pinealocytes, interstitial cell and neurons.

**Pinealocytes**

Hematoxylin-eosin staining revealed that pinealocytes were distributed in small

![Figure 3. Immunohistochemistry showing calretinin (a) and calbindin (b) positive neurons clustered into the ganglia. Neurons were stained with different intensity. Fibre arrays were strongly positive. Scale bar = 15 μm](image)
compact follicles. They had large pale cytoplasm with a secretory polarity. Their nuclei were basally located and showed dark mottled chromatin with two prominent nucleoli (Fig. 1). Pinealocytes were easily detected using anti-calbindin-D-28k and anti-calretinin antibodies (Fig. 2A and B). Approximately half of pinealocytes per follicle were immunoreactive, but presenting different staining intensities. Although overlapping immunoreactivity could not be excluded the majority of pinealocytes were either calbindin-D28k-Ir or calretinin-Ir. Calbindin-D28k allowed a detailed morphological analysis such as short running processes inside follicles (Fig. 2B). Most nuclei were also labelled. Synaptophysin was labelling pinealocytes without specific localisation, about one third displayed immunostaining with medium to strong intensity (Fig. 2C). Calmodulin and recoverin were not detected in *Cricetomys gambianus* pineal gland.

**Interstitial cells**

One main type of interstitial cells was detected using hematoxylin-eosin staining
They were distributed in crown at the periphery of the pineal follicles, in the vicinity of capillaries (Fig. 1). They exhibited a small fusiform shape with a centrally located dark nucleus. The cytoplasm appeared uniformly stained and no chromatin or nucleolus were visible. These cells were calbindin-D28k negative, and poorly S-100/3 positive. The calretinin and calbindin-D28k positive ones were found homogeneously throughout the superficial part of the pineal gland. The S-100/3 positive cells were scarce (Fig. 2D) and not directly observed in contact with the follicles except in the ganglia where they formed a dense network (Fig. 4B).

**Neurons**

Two distinct neuronal nuclei were detected in the pineal gland, one localized in the deepest part near the pineal stalk and the other one in the most superficial parts of the gland. Immunoreactive neurons were medium sized (15–20µm) (Fig. 3A and B) and had long thick processes running towards the stalk. Calretinin and calbindin-D28k positive neurons were staining with different intensities. Thin processes were detected by anti-calretinin whereas thick processes were prefentially calbindin-D28K positive. Neurocalcin labelled few smaller neurons and many thin processes within the whole ganglia (Fig. 4A). Within the ganglia many astrocytic processes were S-100/3 positive (Fig. 4B).

**Discussion**

The presence of neurons in the rat pineal is not yet commonly recognized (see page 399 of Paxinos 1995). Only recently a few data provided morphological evidence strongly suggesting that neurons were indeed present in the rat pineal gland (Pochet et al. 1994; Bastianelli et al. 1995; Novier et al. 1996). The present work provides further evidence for the existence of true neurons in the rodent pineal. Cricetomys gambianus, an African rodent rarely studied but presenting many anatomical similarities with the albino rat (Codjia et al. 1990), allowed us to detect true neurons in its pineal. This was made possible because first, in contrast to rat pineal, Cricetomys gambianus pineal gland contains neural ganglia and second, most of the neurons and processes clustered in the ganglia could be seen upon immunostaining using anti-calbindin-D28K and anti-calretinin. Furthermore, the use of immunostaining using neurocalcin, another intracellular calcium-binding protein (Hidaka and Okazaki 1993), present in different neuronal subpopulations (Bastianelli and Pochet 1993; Bastianelli et al. 1995) allowed the labelling of many processes and a few small neurons in the ganglia, and gave a complementary aspect reinforcing data obtained with the two previous antibodies. Finally, S-100/3, an astroglial cell marker (Celio 1996) gave an immunohistochemical picture which perfectly fits the ganglia architecture as detected with the 3 other neuronal antigens. Electron microscopy can be expected to provide further evidence of the neuronal structure of those gan-
glion cells. At this stage, we may consider what could be the possible physiological significance of neurons in the mammalian pineal gland. The vertebrate pineal organ rhythmically synthesizes and secretes melatonin during nighttime and forms an essential component of the photoneuroendocrine system which allows humans and animals to measure and keep time. In mammals, the pineal organ has lost both the direct light sensitivity and the capacity to generate circadian rhythms, and melatonin biosynthesis is regulated by retinal photoreceptors and a circadian oscillator located in the suprachiasmatic nucleus of the hypothalamus. Due to this spatial separation, it is thus understandable that the photoneuroendocrine system of mammals comprises neuronal and neuroendocrine pathways which interconnect its components. Recent data (Larsen et al. 1998) provide further evidence on the synaptology of the neural circuitry controlling the rhythmic secretion of melatonin by the rat pineal whose neurons seemed to be connected to each other through gap junctions (Condorelli et al. 1998). Although this study does not provide data on the connections between these ganglia and the brain, preliminary data indicated that neurons from these two ganglia seem to project through the pineal stalk toward the cerebellum. If this is true, it could be an entirely new situation as previous studies have shown that the pineal stalk connects the pineal gland to the diencephalon in many species.

Acknowledgements. This research was supported by grant from the FRSM and the C U D (Coopération Universitaire au Développement)

References

Arstila A U (1967) Electron microscopic studies on the structure and histochemistry of the pineal gland of the rat Neuroendocrinology 2 (Suppl.), 1—101
Bastianelli E, Pochet R (1993) Sexual dimorphism among calbindin-D28k immunoactive cells in the rat pineal body Histochemistry 100, 449—455
Bastianelli E, Pochet R (1994) Calbindin-D28k, calretinin and recoverin immunoreactivities in developing chick pineal gland J Pineal Res 17, 103—111
Bhattacharya A, Frampton J, Stephan H (1990) The megachiropteran pineal organ A comparative morphological and volumetric investigation with special emphasis on the remarkably large pineal of Dobsonia praedatrix J Anat 168, 143—166


Celso M R (1990) Calbindin D-28k and parvalbumin in the rat nervous system Neuroscience 35, 375—475

Celso M R (1996) Guidebook to the Calcium-binding Proteins Sambrook & Tooze, Oxford

Chakravarty N (1992) The roles of calmodulin and protein kinase C in histamine secretion from mast cells Agents Actions 36, 183—191


Codina J T C, Heymans J C, Adjallala H F (1990) Description du comportement de coprophagie chez les cricétoèmes (Cricetomys gambianus, Waterhouse), rongeurs africains Cah Ethol Appl 10 (2), 143—150


Floris A, Difio M, Jacobowitz D M, Mugnaini E (1994) The unipolar brush cells of the rat cerebellar cortex and cochlear nucleus are calretinin-positive A study by light and electron microscopic immunocytochemistry Anat Embryol (Berlin) 189, 495—520


Final version accepted February 27, 1999