An immunohistochemical study of vasoactive intestinal peptide and neuropeptide Y in the sheep pineal gland during prenatal development

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SUMMARY

In this immunohistochemical study we investigate the presence of vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) during embryonic development in the sheep pineal gland. We use ten ovine embryos at different stages of development (117 to 150 days of prenatal development). The VIP positive fibers were observed along the blood vessels, entering the pineal gland through the pineal capsule. We also observe isolated VIP positive nerve fibers between the pinealocytes. The presence of VIP-immunoreactive fibers in the pineal capsule supports a possible origin from a peripheral ganglion. The NPY fibers enter the gland through the pineal capsule occupying a perivascular localization. Some NPY nerve fibers leave the lobular septae and are localized intraparenchymally between the pinealocytes. Moreover, we believe that the majority of the NPY immunoreactive fibers originate, during the prenatal development of the sheep pineal gland, in the superior cervical ganglion; however, some fibers probably originate from the brain itself.

KEY-WORDS : neuropeptide Y - vasoactive intestinal peptide - pineal gland - sheep.

RÉSUMÉ

Étude immuno-histochimique du peptide intestinal vasoactif et du neuropeptide Y dans la glande pinéale ovine pendant le développement prénatal. Par S. REGODÓN, A. FRANCO, M’aT. REGODÓN, J. MASOT et E. REDONDO.

Dans le présent travail nous étudions par immunohistochémie la présence de peptide intestinal vasoactif (VIP) et de neuropeptide Y (NPY) pendant le développement embryonnaire de la glande pinéale ovine. Nous avons utilisé 10 embryons ovins à différentes étapes du développement (117-150 jours de développement prénatal). Les fibres VIP positives sont observées près des vaisseaux sanguins, pénétrant dans la glande pinéale au travers de la capsule pinéale. Nous avons également observé des fibres nerveuses VIP positives isolées entre les pinéalocytes. La présence de fibres VIP immunoreactives dans la capsule pinéale nous suggère une origine possible dans un ganglion périphérique. Les fibres NPY immunoréactives pénètrent dans la glande au travers de la capsule pinéale occupant une localisation pérvascu- laire. Certaines fibres nerveuses NPY abandonnent le septa lobulaire et se localisent dans le parenchyme entre les pinéalocytes. Finalement, nous pensons que la majorité des fibres immunoréactives au NPY naissent pendant le développement prénatal de la glande pinéale ovine, dans le ganglion cervical supérieur.

Introduction

The pineal gland, which develops in mammals from the diencephalic ependyma and is located during embryonic development between the anterior and the posterior commissure, has recently been [8, 22, 23] the object of considerable research.

It is precisely in seasonally polyestrous species such as sheep that the pineal gland assumes great importance since it is involved in photoperiod regulation through secretion of melatonin. When the nerve signal relating to the photoperiod information arrives at the pineal gland, the gland transforms it into a hormonal-type signal, through the secretion of melatonin. The melatonin is synthesized following the seroton in with the tryptophan as a precursor. Its synthesis occurs uniquely and exclusively during the period of darkness, in such a way that the duration of its secretion is the code that is going to translate the photoperiod information. Once secreted it is released into general circulation, displaying great variability in the plasmatic concentrations related to the size of the pineal gland and the number of pinealocytes of the gland. As for the catabolism, it is carried out at the hepatic level, being eliminated in the urine [2, 24]. The morphological differences between the prenatal and postnatal development of the pineal ovine gland have been previously reported by our work group [8, 22, 23]. Accordingly, the aim of this study was to acquire a better understanding of the ontogenesis of the sheep pineal gland.

In the central nervous system, vasoactive intestinal peptide (VIP) is predominantly present in neurons located in the cerebral cortex, the amygdala, and the hypothalamus [27]. VIPergic fibres have been shown to exert a potent vasodilatory effect. VIP is also a neuromodulator involved in the regulation of several neuroendocrine functions, the most well-established of which is regulation of the secretion of prolactin from the anterior pituitary [25]. The presence of vasoactive intestinal peptide (VIP) positive nerve fibers in the pineal gland of the sheep has been proven during postnatal development [5, 7], but never investigated, however, during prenatal development. The importance of a study of this nature lies in its originality and in the role that nerve fibers play in the regulation of melatonin secretion by the pineal gland. Previous studies point out the existence of these VIP fibers in numerous other species of mammals: the rat [13]; the mouse [14]; the cat [16, 30]; the dog [29] and the pig [30]. In all of these mammals, VIPergic fibres are located around the blood vessels, entering the pineal parenchyma from the pineal capsule VIPergic nerve fibers between the pinealocytes have also been detected. All these findings suggest the influence of VIP fibers in the functionality of the pinealocytes as well as of the blood vessels [9, 10].

Neuropeptide Y (NPY), a naturally occurring neuropeptide originally isolated from the porcine brain [28], consists of 36 amino acid residues with a C-terminal tyrosyl-amide. It is widely distributed within the central and peripheral nervous system including the pineal gland [3]. The existence of neuropeptide Y positive fibers (NPY) has been proven in numerous species of mammals: the rat [12, 15, 31]; the hamster [19]; the cat [18]; the pig [21]; the cow [20] and the sheep [6]. However, its existence in the sheep pineal gland during prenatal development has not been reported. In these animals, a great quantity of NPY-positive fibers has been detected in the pineal capsule, in the perivascular cavities and in the pineal parenchyma between the pinealocytes. Immunoreactive fibers, although in lesser quantity, have also been observed in the posterior and habenular commissure, from where they enter towards the interior of the pineal gland, along the flow of the blood vessels. MOLLER et al., [19] demonstrate that the nature of these nerve fibers that arrive at the interior of the pineal parenchyma are sympathetic and, therefore, NPY-positive.

In this immunohistochemical study we investigate the existence of the VIP and NPY peptides during the embryonic development of the sheep pineal gland.

Material and methods

A) ANIMALS

Ten clinically healthy ovine embryos at different stages of development (117 to 150 days of prenatal development) were used for this study. To obtain embryos at different stages of development, a cesarean section was performed after synchronization of estrus, using hormonal techniques and uterine flushing. Fluorogesterone acetate was administered 14 days before introduction of males. Then 600 IU of pregnant mare serum globulin (PMSG) (Sigma Aldrich Quimica, Madrid, Spain) was inoculated. Cesarean section was performed from day 117 until day 150 following introduction of males. Animals were tranquilized with an intramuscular injection of 0.5 mg/100 kg body weight of propionyl phenothiazine and anesthesia was induced by intravenous injection of sodium thiopental (4 g in a 20 % aqueous solution). Once separated from maternal linking, embryos were euthanized by umbilical vein administration of 1 g sodium thiopental in a 20 % aqueous solution.

B) FIXATION OF TISSUES

The skull was opened by use of an oscillating surgical saw and the brain was carefully removed. The brain were immediately immersion fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 4 days. The sheep pineal gland and adjacent epithalamic regions were postfixed in the same fixative for about 24 h, and processed by paraffin-embedding methods. Sections 4 µm thick were cut and processed for immunohistochemistry.

C) IMMUNOHISTOCHEMICAL PROCEDURES

The immunohistochemical localization of VIP and NPY was performed by use of the avidin-biotin-peroxidase complex (ABC methods). First, the sections were deparaffinized, and rinsed for 15 min in PBS containing 1 % bovine serum albumin (BSA). They were then pretreated in 1 % H2O2 in PBS for 30 min to reduce endogenous peroxidase and follo-
wed by incubation in diluted (1:50) normal swine serum (Dako, Madrid, Spain) for 15 min. The sections were then incubated with a specific antiserum against VIP raised in rabbits (Sigma Aldrich Química, Madrid, Spain), diluted 1:600 in PBS and NPY raised in rabbits (Sigma Aldrich Química, Madrid, Spain) diluted 1:1000 in PBS for overnight at 20°C. After the incubation, the sections were washed for 3 x 10 min in PBS and then incubated with biotinylated swine anti-rabbit IgG (Dako, Madrid, Spain) diluted 1:1500 in PBS for 30 min at room temperature and washed in PBS for 3 x 10 min. They were then incubated with the ABC (Dako, Madrid, Spain) diluted 1:50 in PBS for 1 h. After diaminobenzidine reaction, a nuclear counterstaining with hematoxylin was applied.

The specificity of the staining reaction was determined in control experiments. They comprised prior absorption of the primary antibody (some sections were incubated for 24 h at 4°C with the specific antisera, which had been preabsorbed with the peptide fragments against which the antisera were raised -50 µg of the peptide fragment per 1 ml of the diluted antiserum-), substitution of the primary antibody by PBS or normal mouse serum 1:100, or omission of both primary and secondary antibodies.

Results

A) VASOACTIVE INTESTINAL PEPTIDE (VIP)

In all animals analyzed we found VIP immunoreactive fibers. Their distribution and localization was similar in all embryos of the experiment.

The VIP positive fibers were few and were located mainly in the proximal part of the pineal gland. Isolated VIP positive fibers could be observed in the distal zone of the pineal gland. We found these VIP positive fibers localized along the blood vessels (Fig. 1), entering the pineal gland through the pineal capsule (Fig. 2) and also inside the capsule. We observed isolated nerve fibers leaving the capsule and situating themselves between the pinealocytes (Fig. 3). Finally, VIP positive nerve fibers were detected in the connective tissue septae (Fig. 4).

**FIGURE 1.** — Embryo, 120 days. VIP-immunoreactive fibers located close to blood vessels x 180.
**FIGURE 2.** — Embryo, 150 days. Fibers VIP positive enter the gland from the capsule x 180.
**FIGURE 3.** — Embryo, 150 days. Fibers VIP positive dispersed between pinealocytes x 220.
**FIGURE 4.** — Embryo, 130 days. Presence of VIP-immunoreactive nerve fibers in the connective tissue septae x 220.
B) NEUROPEPTIDE Y (NPY)

In all embryos analyzed NPY immunoreactive fibers were found. The NPY positive fibers were abundant in all of the surface area of the pineal gland. These fibers enter the pineal gland through the pineal capsule, passing to the connective tissue of the glandular septae, occupying a perivascular location (Fig. 5). Many fibers leave the lobular septae and are arranged intraparenchymally, between the pinealocytes (Fig. 6). These NPY positive nerve fibers, although still abundant in all of the glandular surface area, were less numerous in the cortical glandular zone (Fig. 7) than in the medullar zone of the pineal gland (Fig. 8).

Discussion

The presence of VIP positive nerve fibers has been proven in the sheep pineal gland during prenatal development at least from day 117 of pregnancy.

The origin of the VIP positive fibers has been the subject of numerous controversies, without general unanimity. The majority of the studies, in adult mammals [1, 2, 4-7,11-22, 31], point to these fibers originating from the parasympathetic pterygopalatine ganglion. This can be shown in the work of SHIOTANI et al. [26] in the gerbil and COZZI et al. [5] in the sheep. In the sheep, these authors point out that the VIP positive fibers present in the pineal capsule could originate in the peripheral ganglion of the head. Nevertheless, this sole origin contradicts what has been pointed out by UEMURA et al. [29] in the dog, who show that the superior cervical ganglion could be a source of VIP immunoreactive nerve fibers. In fact, the removal of this ganglion considerably diminishes the number of VIP immunoreactivity in the vascular structures of the brain. Due to this, it is possible to consider multiple origins for the VIP fibers in the sheep, coinciding with what has been described by COZZI et al. [5].

The localization was fundamentally perivascular, coinciding with the observations in adult mammals in general [17] and in the sheep in particular [5]. Some fibers are situated...
intraparenchymally. COZZI et al. [5] attributed this finding to the fact that the large lobes of the sheep pineal gland force the perivascular-situated fibers to cover great distances for the release of peptides.

In our observations, the VIP fibers were situated in all of the pineal surface area, being more numerous in the cortical zone than in the medullar zone, coinciding with that described by COZZI et al. [5]. However, SHIOTANI et al. [26] in the gerbil and MIKKELSEN et al. [14] in the rat did not detect VIP fibers in the deep pineal gland.

The presence of specific receptors for VIP on the pinealocytes, shown by means of auto-radiography, in the rat and the gerbil [1, 11], show a direct effect of VIP on the functionality of the pinealocytes. COZZI et al. [4] describe the influence of VIP fibers on the release of melatonin by the effect on the blood vessels.

In the same way as for VIP fibers, the presence of NPY fibers has been proven in sheep pineal glands during prenatal development.

The localization of the NPY nerve fibers, coinciding with that described in other species of adult mammals [17], was mainly perivascular, although intraparenchymal localization was also observed. The distribution of these fibers in the pineal parenchyma was not homogeneous; [12, 31] in the rat pineal gland and [20] in the bovine pineal gland reported a high density of NPY positive fibers in all of the glandular surface area. In the hamster, MOLLER et al. [18] describe a greater presence of NPY positive fibers in the medullar zone than in the cortical glandular zone, similarly shown by MOLLER et al. [18] in the cat and PRZYBYSKA-GORNOWICZ et al. [21] in the pineal of the pig.

With respect to its procedence, MOLLER et al. [19] in the pineal gland of the hamster, describe the presence of NPY fibers following the removal of the superior cervical ganglion, from which it is possible to talk of a central innervation originating in parasympathetic extra-cerebral ganglia. Contradictory opinions are those MOLLER et al. [18], in the pineal gland of the cat, argue that the origin of some NPY fibers from parasympathetic gangliaons must be excluded due to the scarcity of immunoreactive fibers in ganglionectomized animals. The origin of these extra-parasympathetic fibers is probably the brain.

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References