Immunocytochemical Localization of Melatonin in the Harderian Gland of Syrian Hamster

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ABSTRACT ***Background:*** **The Harderian gland (HG) is a tubulo-alveolar gland found within the ocular orbit of animals which present a nictitating membrane. The Harderian gland is regarded as an extrapineal melatonin producing organ and both, photoperiod and melatonin have been shown to exert an important role in the metabolism and morphological features of such a gland.**

Our results seem to support the presence of melatonin in the nuclei of the HG cells, although our studies have not definitively proved such presence.

Methods: **An Immunocytochemical anti-melatonin technique was done over free sections of Bouin fixed material obtained from Syrian hamsters. Some of the sections were embedded in an epoxy resin and studied under electron microscope.**

Results: **The presence of positive immunoreaction was observed at the level of the nuclear membranes and in close relation to chromatin. No differences were observed between males and females nor between pinealectomized animals and control ones.**

Conclusions: **Our results suggest the binding of melatonin to the cell nucleus in all cell types of the gland. These observations are in accord with the binding studies performed by Acuña-Castroviejo in purified cell nuclei of rat liver (Acuña-Castroviejo et al., 1994. J. Pineal Res., *16:*100–112) and the earlier one by Menéndez-Peláez et al. (1993a,b, J. Pineal Res., *15:*59–69; J. Cell Biochem., *53:*373–382) using the light microscope. Our results seem to support the idea of a nuclear action of melatonin and they agree with the observations of Carlberg and Wiesenberg (1995, J. Pineal Res., *18:*171–178) about the activation of some orphan receptors by melatonin.**

Key words: Harderian gland, Syrian hamster, Immunocytochemistry, Melatonin, Nuclear receptors

The Harderian gland is a tubulo-alveolar gland found within the orbit, on the posterior aspect of the eyeball in animals that possess a nictitating membrane. In addition, the Harderian glands of many rodent species contain several indolamines (Menéndez-Peláez, 1990) and large quantities of porphyrins (Margolis, 1971; Hoffman, 1971). For these reasons, such glands have been used as an alternative model to study indole and porphyrin production (Buzzell et al., 1990; Menéndez-Peláez et al., 1991).

It is well known that the Harderian gland of the Syrian hamster, but not that of the rat, exhibits marked sexual differences, both morphological (Hoffman, 1971) and biochemical (Marrufo et al., 1989; Pangerl et al., 1989; Menéndez-Peláez et al., 1991). Thus, the male gland exhibits two secretory cell types (type I and type II), while the female one shows a single type of secretory cell (type I) (Hoffman, 1971). Both photoperiod and melatonin have been shown to exert an important role in the metabolism and morphological characteristics of this gland (Menéndez-Peláez et al., 1988; Coto-Montes et al., 1994).

The studies related to the cellular localization of melatonin have been usually, conditioned by the available antibodies. However, specific antibodies, showing very little reactivity with other indoles have been developed (Kennaway et al., 1977; Arendt et al., 1977).

We know many facts, as the following ones: 1) the possible nuclear localization of melatonin in the retina and pineal gland (Mennenga et al., 1991), 2) the influence of melatonin in gene expression (Menéndez-Peláez et al., 1991), 3) the inhibition of DNA adducts production (induced by the administration of the chemical

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carcinogen safrole) by melatonin in the liver of rats (Tan et al., 1993), 4) the presence of melatonin binding sites in the nuclei of liver cells in rats (Acuña-Castroviejo et al., 1994), and 5) the demonstration of melatonin binding sites by means of binding techniques in nuclei of Harderian gland secretory cells of Syrian hamsters (data not shown).

All the above cited knowledge has prompted us to explore the possibility of a nuclear localization of melatonin. Thus, we have studied in this work, by electron microscopy, the subcellular distribution of melatonin in different cell types in the Harderian gland of Syrian hamster. We also consider the possible gender-associated differences.

MATERIAL AND METHODS

Animals

The animals used in this study were Syrian hamsters (*Mesocricetus auratus*) purchased from Charles River (Kingston, N.Y.). They were maintained in a windowless room providing a controlled temperature (20 ± 2°C) and lighting (14 h light/10 h darkness) environment. Animals were provided food and water "ad libitum." Eight female and eight male adult hamsters were used. Four male hamsters were pinealectomized eight days before sacrifice.

The animals were killed by decapitation between 18:00 and 19:00 h. The Harderian glands were quickly dissected and immersed in Bouin fluid and were kept in this fixative mixture at room temperature overnight. Sections of 60 μm were obtained with a vibratome (Campden Instruments, Ltd., U.K.) and collected in TBS (Tris-buffered saline, pH 7.4, 0.01 M).

Immunocytochemical Procedure

The antimelatonin antiserum was purchased from Stockgrand, Ltd. The cross reactivity has been described in detail (Webley et al., 1985). For the present study, the immunohistochemical procedure was performed using an indirect method, with streptavidin-biotin-peroxidase complex (Hsu et al., 1981). Free sections were rinsed in TBS and incubated successively in the presence of either 1) normal sheep serum (20%) to reduce nonspecific binding (30 min in a moisture saturated chamber at 37°C); 2) antimelatonin goat antiserum at 1:500 (48 h in a moist chamber at 4°C). Control sections were covered with an equal quantity of tris buffer; 3) biotinylated anti-goat IgG at 1:200 (24 h in a moist chamber at 4°C); or 4) Streptavidin-peroxidase complex (Vectostasin) at 1:100 (24 h in a moist chamber at 4°C).

Sections were rinsed after each step in TBS (3 × 5 min). The streptavidin-biotin-peroxidase activity was revealed using 3'3'-diaminobenzidine tetrachloride (50 mg/100 ml) in the presence of 0.5% H_2O_2 in TBS.

Optic and Electron Microscopy Procedure

Some sections were postfixed in 1% OsO_4 for 2 h and the rest were covered with an equal quantity of tris-buffer. All sections were dehydrated and embedded in Spurr resin (Taab, Berkshire, U.K.). Semithin sections (1 μm) were obtained with an Ultracut E (Reichert-Jung) ultratome, stained with Toluidine blue, studied, and photographed with a Leitz Orthoplan microscope. Ultrathin sections were collected on copper grids, stained with uranyl acetate-lead citrate, and examined with a transmission electron microscope Zeiss EM-108 operating at 50 kV or a transmission electron microscope Jeol 2000 EXII at 120 kV depending on needs.

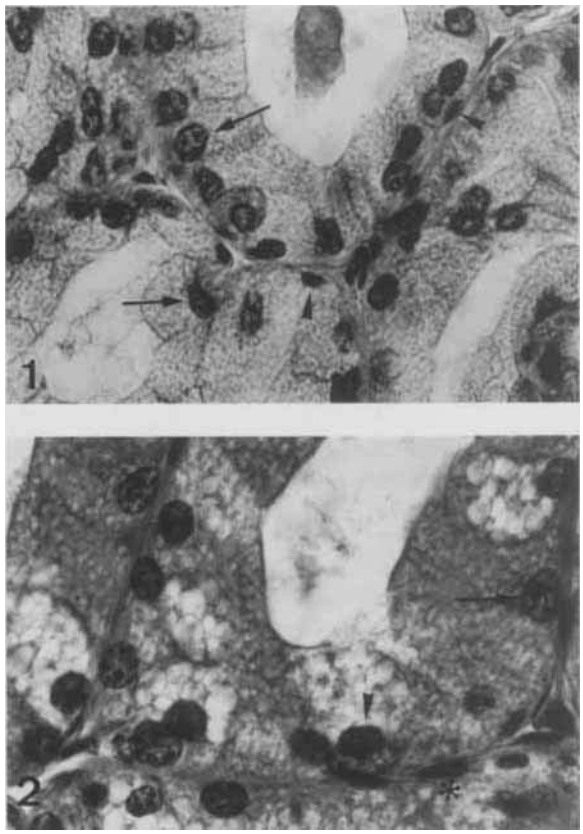


Fig. 1. Immunocytochemical demonstration of melatonin in the nucleus of type I cells (arrows) and in the nucleus of myoepithelial cells (arrowheads) of Harderian gland from female hamster. ×672.

Fig. 2. Harderian gland from male hamster showing a nuclear localization of melatonin in the type I cells (arrows), type II cells (arrowheads) and myoepithelial cells (asteric). ×725.

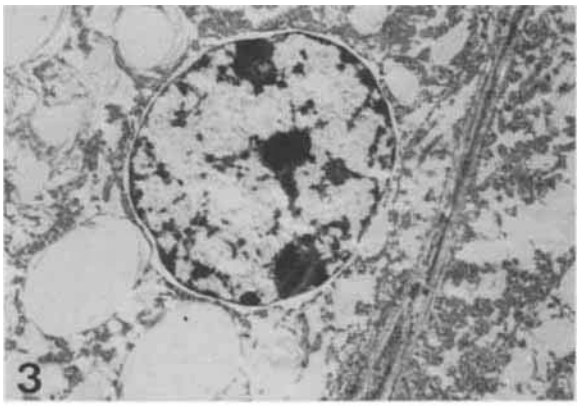
RESULTS

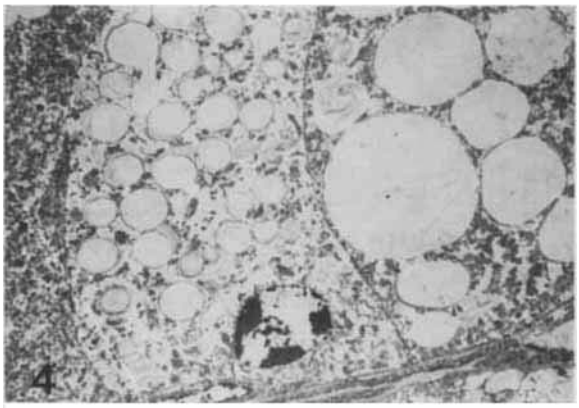
The processed material studied under O.M. showed a general positive response to the anti-melatonin treatment.

Such response was always circumscribed at the nuclear level, while cytoplasm showed no positive reaction.

The reaction was no restricted to any particular cell type. In this way, a positive reaction was observed at the level of secretory cells, myoepithelial cells and connective tissue cells. No differences were observed between males and females (Figs. 1, 2).

In a similar way, our observations under E.M. show a clear labeling at the level of the nuclear envelope and chromatin, but because of the inherent electrodensity of the chemicals normally used in the preparation of the samples for E.M. (OsO_4, $UO_2(CH_3.COO)_2.H2O$), it was very difficult to know whether the observed density was or not totally caused by such chemicals (Figs. 3, 4).





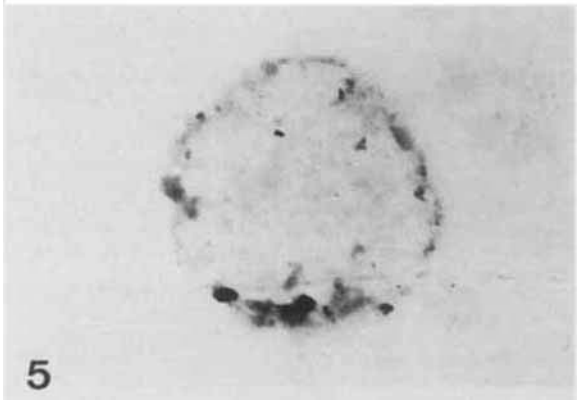


Fig. 3. Electron micrograph of a type II cell showing a positive reaction at the level of the nuclear envelope and chromatin. ×9,700.

Fig. 4. Electron micrograph of a type I cell showing the reaction to melatonin at nuclear level. ×4,100.

Fig. 5. Electron micrograph obtained from material not treated with osmium nor uranil acetate. A clearly positive circular area is observed. ×9,600.

To avoid this problem, we decided to study new samples avoiding the treatment by Os and U compounds, at the cost of a poorer structural image.

We have observed this material with a higher acceleration potential (120 kV) to diminish the intrinsic electrodensity of the tissues in relation to the truly positive areas.

Working in such conditions, we could observe the presence of clearly marked circular areas (Fig. 5). These areas were always related to the nuclear envelope and chromatin of the cellular nuclei. Because of the absence of external electrodensity sources, but the products of the performed immunoreaction, we can conclude that such labeling must be related to the presence of melatonin.

The pinealectomized animals showed the same labeling as that of intact animals.

The tissue conservation for electron microscopy study was not perfect because Bouin was not the best fixative mixture, but fixatives containing picric acid and paraformaldehyde were necessary for obtaining a good reaction when the antibody was used at low concentration (Menéndez-Peláez and Reiter, 1993a).

DISCUSSION

The results of the present study suggest the binding of melatonin to nuclei of all cell types in the Syrian hamster Harderian gland. Anyway, it results hard to believe that all cell types in the gland produce melatonin. It is much more likely only some cells produce the hormone, while other cells get melatonin from outside.

As the locations of positive reaction are not apparently changed after pinealectomy, it could be possible that some cells in the gland produce melatonin and it gets into adjacent cells by a paracrine mechanism.

Our studies, at E.M. level, corroborated the earlier one, performed by Menéndez-Peláez et al. (1993b) in O.M., which showed an accumulation of melatonin in the nuclear fraction of cells from Pineal gland, Harderian gland, and other tissues.

In the present study, melatonin labeling is not apparent in cytosol and plasmalemma, while it is clearly observed at nuclear level. Menéndez-Peláez et al. (1993b) by radioimmunoassay studies revealed that melatonin was also found in the cytosol, although at immunocytochemical undetectable levels. The meaning of such observations may be that cytosolic melatonin, unlike the nuclear one, is not tightly bound to cell components and therefore is lost during the immunocytochemical procedure. In this sense, it is possible that nuclear melatonin is bounded tightly to some kind of proteins.

The possible localization of melatonin at the level of chromatin has been recently proposed (Mennenga et al., 1991; Menéndez-Peláez et al., 1993b), but our results, working without contrast chemicals, show that melatonin is found much frequently at nuclear membrane than in close relation to the chromatin. These observations corroborate the binding studies performed by Acuña-Castroviejo et al. (unpublished) which have revealed the existence of more binding site density in the nuclear membrane than in chromatin.

Clearly the Pineal gland is an important source of melatonin about its accumulation in other organs. However, some melatonin is not pineal in origin. So, Harderian gland synthesizes melatonin (Menéndez-Peláez et al., 1987) and the fact that pinealectomized animals show detectable levels of this indole in the nuclear fraction of some tissues seem to point in the sense that pinealectomy does not totally abolishes melatonin in mammals.

Our observations support the idea of a nuclear action

of melatonin. Till now, it was generally felt that melatonin acts almost exclusively via specific membrane binding sites situated at discrete locations throughout the organism (Krause and Dubocovich, 1991). However, the importance of nuclear effects of melatonin is increasing.

The evidence of a melatonin binding site in purified cell nuclei from rat liver (Acuña-Castroviejo et al., 1994) and in cell nuclei from hamster Harderian glands (Coto-Montes et al., unpublished), the genomic effects of melatonin proposed in such different tissues as the adrenal and the Harderian glands (Menéndez-Peláez et al., 1991; Persengiev et al., 1991), and the melatonin potent action as a chromatin protector (Tan et al., 1993) strongly supports this hypothesis.

Moreover, it has been recently reported by Carlberg and Wiesenberg (1995) that some receptors (RZRα, RORα1, and RZRβ), belonging to the orphan nuclear receptor superfamily, are activated to a different extent by melatonin, with binding specificities in the low nanomolar range (Becker-André et al., 1994; Wiesenberg et al., 1995).

These authors have also identified a functional analogue of melatonin, the thiazolidine dione CGP 52608, which can bind to the high affinity nuclear receptor but not to the membrane receptor. This is a very important finding, because it is possible that such specific ligand could help to differentiate between nuclear and membrane signaling of melatonin.

In addition, the identification of a natural RZRα responding gen (5-lipoxigenase) in human B lymphocytes (Steinhilber et al., 1995) is one of the most conclusive probes, until now, about the presence of nuclear receptors for melatonin.

The results described herein provide a clear appointment for this suggestion, since melatonin appears to have specific nuclear binding sites, mainly in the nuclear membrane but also associated to chromatin.

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