Topographic morphometry of the pineal gland of the rat. A 24-hours period, light-dark cycle and seasonal study

Francisco Martínez Soriano, Arantxa Blasco-Serra, Eva M. González-Soler, Salvador Hernández-Sánchez, Alfonso A. Valverde Navarro

Department of Human Anatomy and Embryology, Faculty of Medicine, University of Valencia (Spain)

SUMMARY

Classical studies pointed out to a possible division of rodents' pineal parenchyma in various regions and layers, also observing variations in nuclear sizes that could depend on luminosity cycles. The aim of this study is to analyze the morphological changes of nuclear sizes of pinealocytes that occur in the pineal gland of albino rats during different hours of the day, seasons and photoperiods, taking into account the different layers and regions. We studied differences on karyometric indices of pinealocytes of the peripheral (cortical) and central (medullary) layers of pineal gland in order to analyze the circadian and seasonal modifications, and establish whether these are indicative of functional differences between proximal, intermediate, and distal portions. Results showed that the total karyometric values of the distal area are clearly higher than those of the other two areas, and in turn those of the intermediate area are also significantly higher than those of the pars proximalis; and also, that there are significant differences between the peripheral and central karyometric indices of all the pineal regions analyzed. Moreover, there are significant evolutionary circadian, photophasic and seasonal differences between regions and the pineal layers analyzed.

Key words: Karyometric indices – Pineal gland – Circadian rhythms – Morphologic variations

INTRODUCTION

The pineal gland (named "pineal" by Galen because of its resemblance to pine nuts), also known as *conarium*, or *epiphysis cerebri*, is a small neuroendocrine organ present in the nervous system of vertebrates. It is located in the ceiling of the diencephalon, behind splenium corpus callosum, between habenularis and posterior commissures. Its main function is the rhythmic synthesis and release, during the dark hours of the day–night cycle, of melatonin. This control of melatonin production is known as an endogenous circadian timing system which is suppressed by light. This relationship between luminosity and the physiology of the pineal gland has been known for a long time (Wurtman and Axelrod, 1964, Axelrod et al., 1965, Merrit and Salkowski, 1959, Wurtman and

Corresponding author:
Francisco Martinez Soriano. Department of Human Anatomy and Embryology, Faculty of Medicine, University of Valencia, Av. Blasco Ibáñez 15, E-46010 Valencia, Spain. Phone: 34.96.386.48.08; Fax: 34.96.386.41.59. E-mail: martinfr@uv.es

Submitted: February 15, 2023. **Accepted:** April 6, 2023

https://doi.org/10.52083/XDNS9336

Ozaki, 1978). This circadian rhythm, or "clock", controls a number of behaviors such as the sleep–wake cycles, feeding, and cognition rhythms. Nocturnal secretion of melatonin is present in all species analyzed so far, but is interpreted differently depending on whether the animal is nocturnal or diurnal, and it guarantees a time-sensitive and ecologically well-adapted behavior of humans and animals (Macchi and Bruce, 2004; Sapède and Cau, 2014; Koch et al., 2015; Shoja et al., 2016). Moller and Baeres described that the main cell type in mammals' pineal gland are pinealocytes (95%), followed by glial cells (astrocytic and phagocytic subtypes). Pinealocytes are responsible for the synthesis and secretion of melatonin (Moller and Baeres, 2002; Aulinas et al., 2019).

The pineal gland has been studied from different morphological viewpoints in an attempt to establish links with the corresponding physiological rhythms parameters. Its size and anatomy vary significantly among vertebrates; but among them, it should be noted that the anatomy of the rodent pineal gland is considered, by diverse morphological characteristics, more complex (Quay and Renzoni, 1966; Becker and Vollrath, 1983; Matsushima et al., 1983; Cimas et al., 1992; Sakai et al.,1996; Borjigin et al., (2012).

In view of the variable length of the pineal gland in rodents, a classification in different types was proposed (Vollrath, 1979, 1981). The long, rod-like pineal organs that read the cerebellum and are closely related to the skull, belong to types A, AB, ABC, etc. The pineal gland of the rat is classified in this last type (Fig. 1).

In a similar line, various classical morphological and physiological animal studies suggest a possible division of the pineal gland parenchyma into an external ("cortex") and central ("medullar") layers (Quay and Renzoni 1966, Romijn, 1975, Matsushima el al.1983, Semm, 1983, Cimas et al. 1992, Hira, 1998), and revealed variations in nuclear size during different point-time.

Such size variations were also established between the peripherical (cortical) and central (medullary) gland regions. Although such cortico-medullary differences have not been confirmed by all authors of that time (Welsh et al., 1979, Heidbüchel and Vollrath,1983), they have been suggested by others, especially in relation to rodents (Milline et al.,1968, Blumfield and Tap, 1970, López-Iglesias et al., 1987; Martínez-Soriano et al., 2002). Some authors (Diehl et al, 1984) have reported cortico-medullary differences, although these were found to depend of the pineal region considered. In turn, Becker and Vollrath (1983) reported rhythmic differences in pinealocyte nuclear size within the peripheral but not in the central gland layer; besides, studies in different seasons (Popova et al, 1975) have shown the central and peripheral regions of the pineal gland differ in responsive capacity.

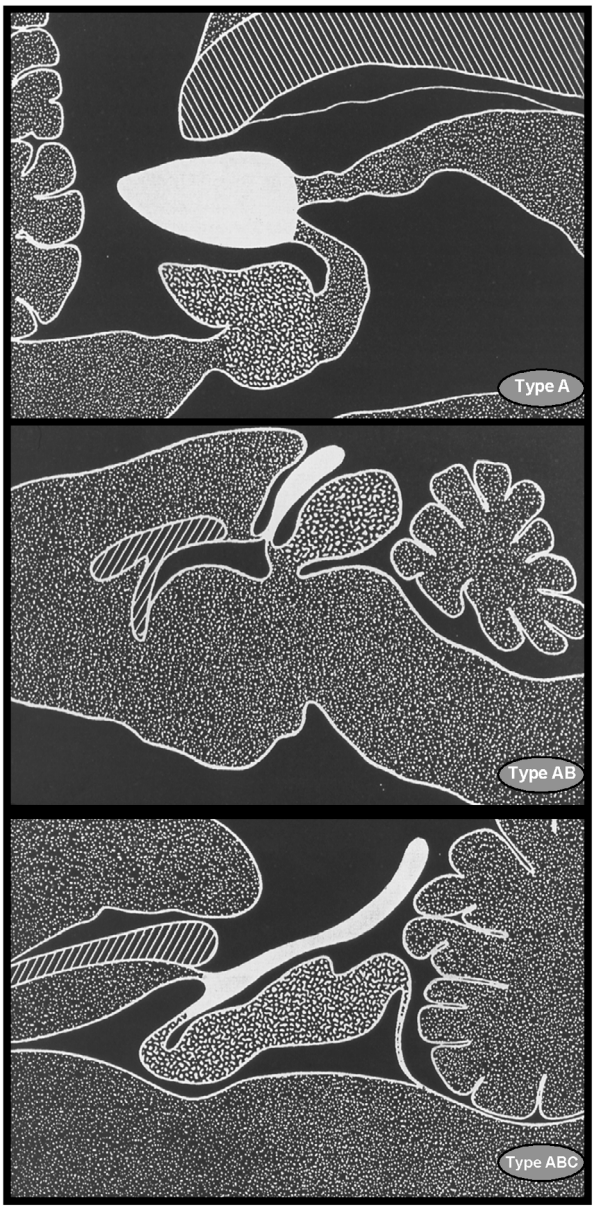

Fig. 1.- Different types of pineal gland in rodents. Based on the studies of Vollrath, 1979 and 1981.

This study attempts to establish that these karyometric differences may be a consequence of varying conditions in natural luminosity. To do this, we have studied the variations that the karyometric index of the pinealocytes of the peripheral and central layers could experience during the established photoperiods in order to be able to analyze the 24-hour periods and seasonal modifications and establish if these are indicative of functional differences between the proximal (*pars proximalis)*, intermediate (*pars intermediate)*, and distal (*pars distalis)* areas of the rat's pineal gland.

MATERIALS AND METHODS

Animals

120 adult male Wistar rats that weighed 280 ± 20 g were used for this experiment. Only male rats have been used to avoid the complex interactions that melatonin and the estrous cycle of female rats may have had in the experiment (Chuffa et al., 2013).

Animals were housed in the Central Research Unit of the University of Valencia, with a controlled cycle of 12 hours light/12 hours darkness under natural circadian and seasonal luminosity (light/dark cycle) (i.e. 08:00-18:30 pm during the short photoperiod (Winter and Autumn) and 07:00-21:30 pm. for the long photoperiod (Spring and Summer), as established from Valencia Meteorological Centre information), and constant temperature (22 ± 2 °C) and humidity (55 ± 10%). All the animals came from litters born on similar dates. Animal experimentation was carried out in accordance with the European Community's Council Directive and was approved by the Ethics Committee of the University of Valencia. Animals were weighed weekly to determine any possible differences between groups. Rats were divided in four groups of 30 rats for each season. Water and food were given ad libitum.

Animals were sacrificed in groups of five, every four hours (06:00, 10:00, 14:00, 18:00, 22:00 and 02:00). This was carried out in Autumn (20/21 October), Winter (2/3 February), Spring (20/21 April) and Summer (1/2 July).

Perfusion

Animals were sacrificed after anesthesia with an intraperitoneal injection of sodium Nembutal (10%). Afterwards, they were perfused with 5% glutaraldehide following saline cleansing. Once removed, the pineal bodies were fixed and refixed in osmium tetroxide.

Electron microscopy study

Once the pineal glands were obtained, they were post-fixed in osmium tetroxide for 90 minutes and dehydrated with graded series of acetone, stained with 5% uranyl acetate and 1% phosphotungstic acid in 70% acetone, and finally embedded in Epon resin. Afterwards, tissue was cut transversely with an ultramicrotome into thin sections and (1 µm) stained with toluidine blue (Fig. 2).

Measurements of 100 peripheral (cortical) and 100 central (medullar) pinealocyte nuclei have been previously reported to be sufficiently representative for each animal (Cimas et al., 1992). These 100 nuclei were selected from four sections taken from the *pars distalis*, *intermediate* and *proximalis*. Nuclear size measurements were made in two layers of the gland (central and peripheral layers), which have different staining aspects (Romijn, 1975). Only clearly visible pinealocyte nuclei were considered. All four selected sections were at least 15 µm away from the preceding one to avoid including the same pinealocyte nucleus in more than one section.

Nuclear measurements were performed following Martínez-Salvador et al. (2018). Once the visual field of the preparation to be analyzed was acquired, the contour of the nuclei object of analysis was drawn using an electronic pencil, and then, all the content of the screen was eliminated, except that of the surface of the drawing. Then, VISILOG program estimated nuclear volume (V) using Jacobj's formula with the following karyometric indices: longer diameter (A), shorter diameter (B) and a constant (*k*). $V = \prod/6 \times A \times B2 \times k$. (Jacobj, 1935).

To obtain the value of *k*, we took a Neubauer camera and photographed a square of it, which we subjected to the same computer process as the pieces to be studied. This gave us figures 1720 times higher than expected. Since a 1000x mag-

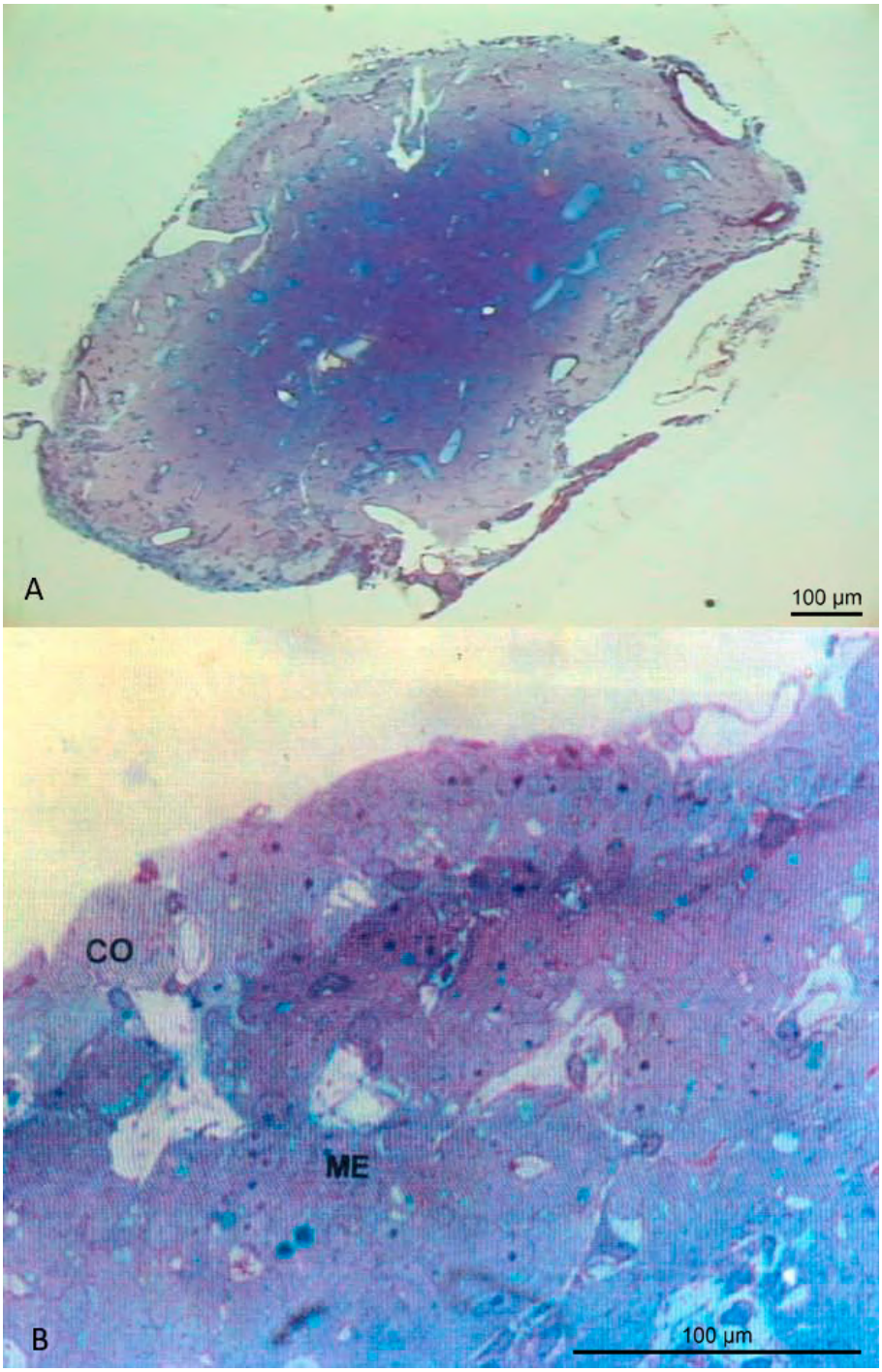

Fig. 2.- Immunohistochemical preparations (toulidine blue) of a pineal gland showing the difference between the cortical and medullary layers. CO: Cortical layer. ME: Medullary layer. Scale bars = 100 µm.

nification objective was used for the photographs, *k* would correspond to 1/1000, that is, to 1.72 the value of the constant that we need.

Statistical analysis

Statistics were done after a descriptive study. A comparative analysis of the variables was carried out by contrast and significance tests. Any P-values lower than 0.05 were considered statistically significant. An analysis of variance (ANOVA) or a Kruskall-Wallis test (depending on descriptive statistics) was used when comparing the means of more than two variables.

RESULTS

General Analyses

A general analysis of the mean volumes of the three regions shows that karyometric indices of

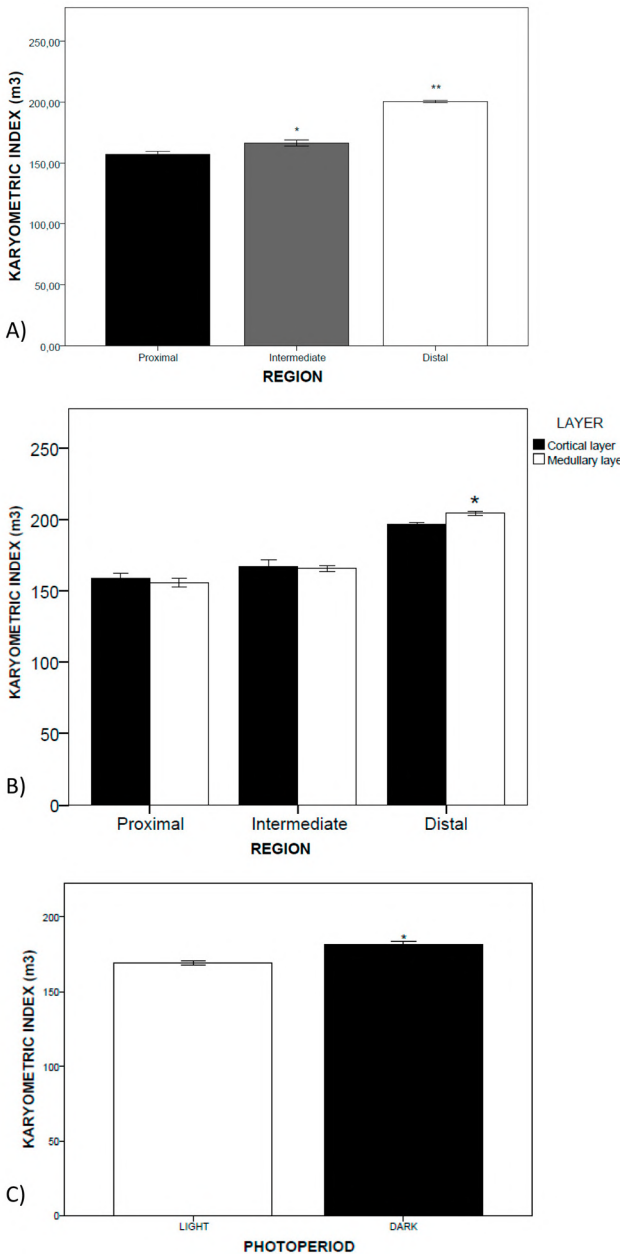

Fig. 3.- A) General comparison of karyometric indexes by regions. It can be seen that distal region has significant higher values than proximal and intermediate region. All data are presented as the mean +/- 2 SEM. *p<0,05 in respect to proximal region. **p<0,05 in respect to proximal and intermediate regions. **B**) General comparison of karyometric indexes by regions and layers. It can be seen that in the distal region, medullary layer values are significantly greater than the cortical ones, while cortical layer's karyometric indices in the intermediate and proximal tend to be higher. All data are presented as the mean +/- 2 SEM. *p<0,05 in respect to cortical layer of distal region. **C**) General comparison of karyometric indices depending on the photophase. There are statistically significant differences between the light and dark phases. All data are presented as the mean +/- 2 SEM. *p<0,05 in respect to light phase.

the distal region (or *pars distalis*) are significantly greater than those of the intermediate (or *pars intermediate*) and proximal (or pars *proximalis*) regions, being this last the region with the lowest karyometric indices (F=485,32, p<0,05) (Fig. 3A).

On the other hand, and once the sample has been segregated according to layers, a general analysis by layers shown that, in the p*ars distalis*, the medullary layer values are greater than the cortical ones (F = 76.140; p <0.01), while in the case of karyometric indices in the intermediate and proximal regions (or *pars*), cortical layer tend to be higher than the medullary, during dark phase, (F = 0.402; p> 0.05 and F = 1.517; p> 0.05; respectively). On the contrary, values of medullar layer are higher that cortical, but not in a significant manner (Fig. 3B).

General analyzes focused on karyometric indices depending on the photophase (light or dark), independently of other variables, show higher values in the dark photoperiod (t=-9,549; p<0,01) (Fig. 3C).

Proximal Region (*Pars proximalis*)

24-hour evolution

a. Cortical layer

Results show that evolution of karyometric indices throughout 24 hours is different, and constant alternating ascending and descending changes between the different time points of seasons can be observed. A substantial oscillation can be observed from 6 to 10 AM (Fig. 4).

b. Medullary layer
Circadian evolution of karyometric indices in the medullary layer happens to be different and alternating, but follows a more regular line compared to cortical layers (Fig. 4).

Seasonal Evolution

Focusing analytically on a seasonal analysis, results show that karyometric indices of medullary layer decrease progressively since the spring with respect to indices of the cortical layer, which slightly increase. This tendency becomes statistically significant in the summer (F=5,699; p<0,05) (Fig. 5).

Photophasic evolution

A more exhaustive analysis, taking into account the cell layers, results show a tendency of the karyometric indices to be higher in the medullary layer on the light photoperiod, with respect to the cortical layer (t=-3,396; p<0,01). Otherwise, karyometric indices of the medullary layer are smaller with respect to the cortical layer in the dark photoperiod (t=2,264; p<0,05) (Fig. 6).

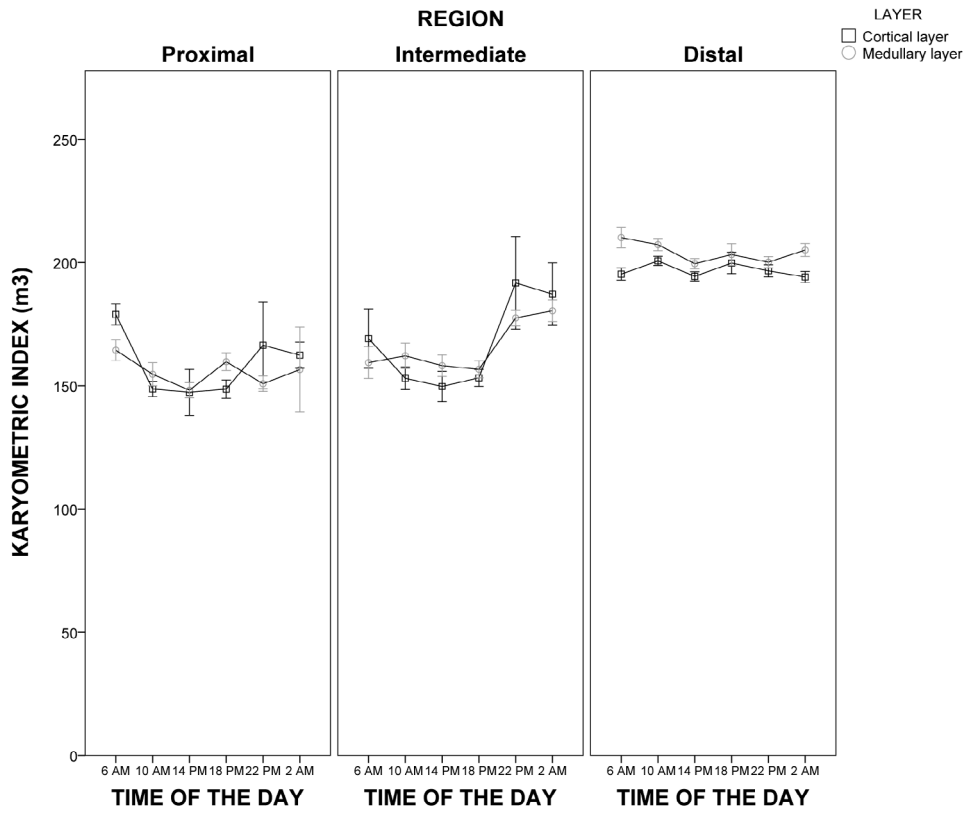

Fig. 4.- Evolution of karyometric indices in layers throughout 24 hours and divided by regions. All data are presented as the mean +/- 2 SEM.

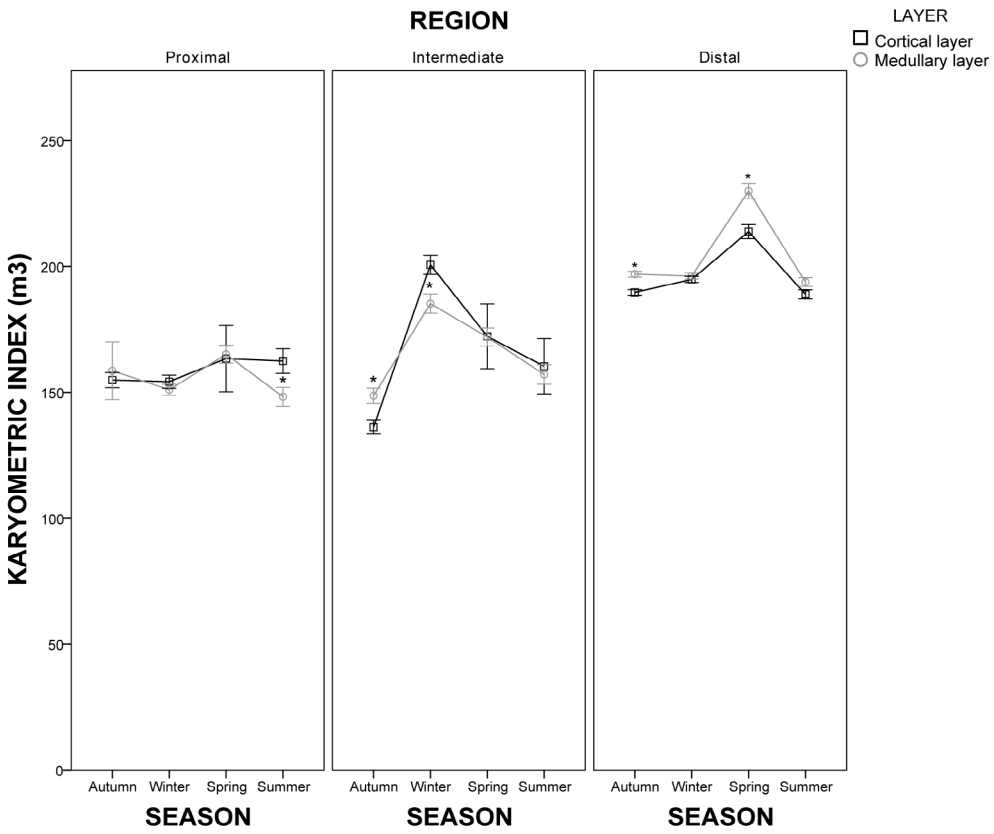

Fig. 5.- Seasonal evolution of karyometric indices in layers and divided by regions. A decrease of values can be seen on the medullary layers in respect to cortical layers on proximal region on summer and winter; on the other side, parallel curves can be appreciated on intermediate and distal regions, with some statistically significant points on the autumn-winter period (intermediate region) and in autumn and spring (distal region). All data are presented as the mean +/- 2 SEM. *p<0,05 in respect to cortical layer.

Intermediate Region (*Pars Intermediate*)

24-hour evolution

a. Cortical layer
Evolution of karyometric indices throughout 24 hours is different, observing a substantial oscillation from 18 to 22 PM (Fig. 4).

b. Medullary layer
Circadian evolution of karyometric indices in the medullary layer happens to be different and alternating, but, again, follows a more regular line compared to that of the cortical layer. Nevertheless, a similitude with changes in the cortical layer's karyometric indices can be seen on the 18-22 PM oscillation (Fig. 4).

Seasonal Evolution

In this area the values of both layers are very similar throughout the long photoperiod seasons, and results show an evolution curve overlapping in the spring-summer period, and those of the peripheral layer become greater in the winter. Results show significant differences between layers in the autumn (t= -3,52, p<0,05) and winter (t=4,03, p<0,05), the short photoperiods (Fig. 5).

Photophasic evolution

General results for the region show statistically significant differences between the light and dark photoperiods. Taking into account the cell layers, results show high medullary layer karyometric indices with respect to the cortical layer during the light period (t=-2,066; p<0,05); and on counterpoint, karyometric indices of the medullary layer are smaller with respect to the cortical layer in the dark photoperiod (t=2,105; p<0,05) (Fig.6).

Distal Region (*Pars Distalis*)

24-hour evolution

a. Cortical layer
Results show that karyometric indices' curves are very similar independently of the time of the day (Fig. 4).

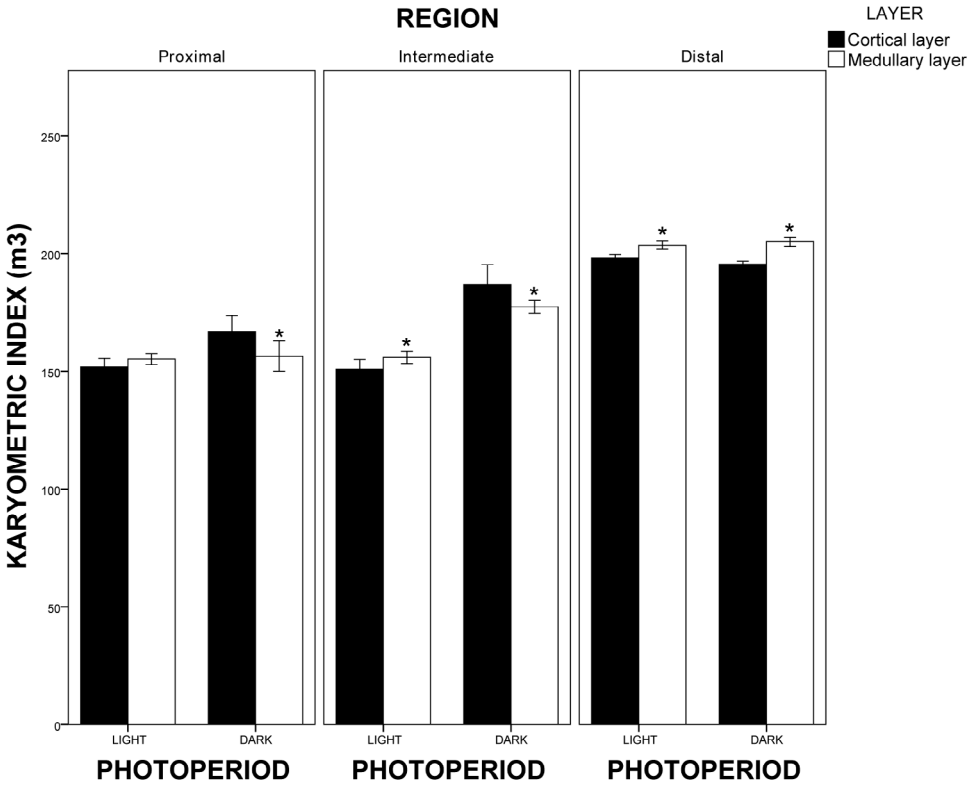

Fig. 6.- Photophasic evolution of karyometric indices in layers and divided by regions. In the proximal region, it can be seen that there is an increasing tendency of these indexes on the cortical layer in darkness in respect to the light photoperiod. This is the opposite in the medullary layer. Also, indexes are higher in the medullary layer on the light photoperiod, in respect to cortical layer; on the contrary, they are higher in the cortical layer on the dark photoperiod. In the intermediate region, values are significantly higher in both layers in the dark photoperiod. In the distal region, results show that indexes are similar in both light and dark photoperiods. Nevertheless, there's a significant decrease of values on the cortical layer in respect to medullary layer in the dark photoperiod. All data are presented as the mean +/- 2 SEM. *p<0,05 in respect to cortical layer.

b. Medullary layer
   In the medullary layer, it can be observed that the general evolution of the circadian curves is very similar to those of the cortical layer (Fig. 4). Interestingly, even though the curves are quite regular and have no large oscillations, karyometric indices are higher during all day than those seen in the other regions.

Seasonal Evolution

Results show that the karyometric indices' curves are similar, practically parallel. Nevertheless, results also show that the medullary layer has higher values; these differences are statistically significant on autumn and spring (Fig. 5).

Photophasic evolution

General results for the region did not show statistically significant differences between the light and dark photoperiods. Nevertheless, there are significant differences: in both photoperiods (light and dark), the medullary layer's karyometric indices are higher than those of the cortical layer (t=-4,489; p>0,05; t=-8,138; p<0,01; respectively) (Fig. 6).

DISCUSSION

Starting by analyzing the general aspects of the results, we can observe that the total karyometric values of the distal region are clearly higher than those of the other two regions, and in turn those of the intermediate region are also slightly (but significantly) higher, over those of the pars proximalis (Fig. 3). Within each of these three regions there are also differences, since while in the distal region the medullary layer values are higher than the cortical ones, in the intermediate and proximal portion the cortical values tend to be higher, but no statistically significant differences are found in between them (Fig. 3).

If we go on to analyze the variations that occur in the cortical and medullary layers of each of the pineal regions throughout the four seasons, (Fig. 5), two interesting circumstances are observed: the first is that, while in the distal and intermediate portions the evolution of both layers maintains a certain parallelism throughout all the seasons, in the proximal part the behavior during spring and summer is different (and opposite), and it becomes parallel in the autumn and winter seasons, which would suggest an influence of the short photoperiod seasons on the functioning of both layers in this pineal segment. Another aspect to highlight along the same lines is that, while in the distal region the values of maximum cortical and medullary activity occur in the spring season, in the intermediate season they occur in the winter season; and in the proximal region, it is maximum in the spring for the medullary layer and in the summer for the cortical layer, which indicates that the seasonal influence is not produced in a uniform way over the entire gland. Likewise, these variations are another piece of data that points to the difference in functional behavior between all the factors analyzed in this work, variations that undoubtedly seem to be determined by the photophysical characteristics (geomagnetic variations, wavelengths of solar radiation...) of seasonal evolution, which influence each one of the pineal regions differently (Gerasimov et al., 2014).

Differences between the medullary and cortical layers also exist in each of the regions according to photoperiods, since while in the distal part the karyometric indices of the medullary layer are always higher in both photophases, in the intermediate and proximal region the medullary layer's values are higher during the light photoperiod, but the cortical layer's values are higher during the dark period (Fig. 4). All these data point to the global existence of differences in the karyometric indices between the three pineal portions analyzed, and also to differences between the peripheral and central layers of each of the pineal regions analyzed, differences that seem to be mediated by the photophase.

Analyzing more specifically the 24-hour period evolution of the cortical and medullary layers (Fig. 6), we observe that the cortical layer of the proximal and intermediate regions tends to show the higheest oscillations, and has a different evolution than the medullary layer. Otherwise, both the scortical and medullary layer of the distal region follow a similar evolution, being the medullary layer's karyometric indices higher than those of the cortical layer; and these indices of the distal region are higher than those expressed by the other regions.

In view of all these circumstances, it seems evident that the behavior of the cortical and medullary layers in each of the pineal segments is different, and that seasonal, circadian and photophasic factors are not the only ones that must be intervening in this difference. In any case, these results support and reaffirm the importance of the effect of the season, the photophase and the 24-hour variations according to the topographic location of the pinealocyte within the gland. This would explain the discordant results of the different experts in the field regarding the variation of the karyometric indices both in the peripheral (cortical) or central (medullary) layer, since, they may be different depending on the time, the season and the region in which the measurement is made: thus, , while in the proximal and intermediate parts the cortical values seem to be higher, in the distal region the higher values are the medullary ones.

Our results coincide in supporting the existence of two zones/layers: peripheral (cortical) and central (medullary); and three regions (pars): proximal, intermediate and distal, which would be functionally different in the pineal gland of the rat, as pointed out by the team of Hira (Hira et al., 1988); this functionality would be determined by a combined influence of the geomagnetic and photophasic aspects of the 24-hour rhythms, seasons and light-dark photophases, as suggested in turn by the Cimas and Martínez-Soriano teams (Cimas et al., 1992; Martínez-Soriano et al., 2002). In this sense, Matsushima's team (Matsushima et al., 1993) showed that the volumes of pinealocytes in the pineal glands of rats subjected to a magnetic field during the months of April and October experienced volumetric differences between both layers and regions, in a proximal-distal direction and even between day and night, but these differ-

ences were vaguely significant in the intermediate zone. Photophasic differences were quite evident in the month of April and disappeared during the month of October. Authors concluded by suggesting that the influence of a magnetic field could exert a control mechanism of the day/night rhythms of the pinealocytes of the rat's pineal gland.

Some other published data would support this hypothesis: it is known that 90% of pineal cellularity is made up of pinealocytes, and within this population two subtypes have been described, α and β: while α constitutes 5% of pinealocyte population, β constitutes the remaining 85 (Pévet, 1977; Moller and Baeler, 2002). Receptors for adrenergic (Adrb1, Adra1b, Drd4) and cholinergic (Chrna3, Chrnb4) agonists have been found in them, and both express high levels of up to 49 different transcriptionists, which are found in the pineal gland and in the retina (Coon et al., 2019). Pinealocytes α have the specialized role of the methylation of N-acetyl-serotonin-methyl-transferase (ASMT), which is produced and released by pinealocytes β, so both constitute a fundamental set in the elaboration of melatonin, with transcriptional changes that occur between night and day, especially in β-type pinealocytes (Mays et al., 2018).

It is also known that melatonin receptors are widely expressed not only in the Central Nervous System (CNS), but in numerous peripheral tissues, which determines that the circadian rhythm of circulating pineal-derived melatonin can have important effects on the temporary functional organization of almost all peripheral organs, without this influence being necessarily involved in the feedback through the Suprachiasmatic Nucleus (Hardeland, 2013).

Pituitary adenylate-cyclase activating polypeptide (PACAP) is a neuropeptide that was isolated in the hypothalamus and localized in the central and peripheral nervous systems. Specific receptors for it have been found in the pinealocyte membrane and in nerve fibers that access the pineal gland. This neuropeptid stimulates the secretion of melatonin (Liu and Moller, 2000; Moller and Baeres, 2003).

On the other hand, it is also known that the distal pineal region receives a large number of vegetative afferent terminals from the superior cervical ganglion, via *nervus pinealis*. As these terminals descend towards the proximal region of the pineal gland, they decrease in number and practically almost disappear. On the contrary, numerous fibers of central origin are present in the proximal region, which attach to the pineal through the habenular and posterior commissures. Furthermore, the evident differences in the structure of both regions already suggest that both of them may be functionally different, the intermediate region being a transition zone between the other two.

Our results support the data provided in previously cited works and reaffirm the importance of the effect of season, photophase and 24-hour periods on the topographic location of the pinealocyte within the gland. Furthermore, this is thefirst study carried out in rats in a systematic way (together with Hira et al., 1998, and to the best of the authorss knowledge) which, taking into account not only the photophasic, daily or seasonal factors, but also the layers), can establish that differences in the karyometric indices, found according to the most proximal or distal, could be a reason that explains the discordant results of the different authors regarding the greater or lesser volume of the karyometric indices of the peripheral or central zone, since, as already expressed above, these may be different depending on the time, season and the region in which the measurement is carried out: thus, it can be clearly observed that, while in the proximal and intermediate parts the cortical values seem to be higher, in the distal, on the contrary, the higher values are the medullary ones.

This morpho-functional topographic distribution is suggestive of the possible existence of several different layers or "organs" in the pineal parenchyma, and would justify the application and use of the term "pineal complex" to the whole, (as suggested by Vollrath, 1985) since it could be an organ with sub-organs of functional characteristics and different cyclic elements in its interior that respond to geomagnetic and photophasic influences. Electrophysiological data existing in the literature could support this opinion. Indeed, several classic authors pointed to the existence

of pineal zones with different registers, and even pinealocytes with different electrical activity at rest or activity, day/night or under the stimulation of different hormones and chemical substances (Dafny, 1975; Semm and Vollrath, 1979, 1980; Reuss and Vollrath, 1984; Reuss et al., 1984). The existence of circadian, ultradian and infradian rhythms (Vollrath, 1981) in melatonin secretion also points in this direction.

On the other hand, it is also interesting to note that, according to existing data in the literature (Quay and Renzoni, 1966; De la Guardia et al., 1988; Giménez-González et al., 1991; Guillot-Valls et al., 1995), the medullary layer of the pineal gland of the rat is more susceptible to changes such as magnetic field influences or luminosity of different natures, than the cortical layer; in this same way, Martínez-Soriano et al. argued that the combined, seasonal, photophasic and lunar synodic influences are more specific on the medullary zone than on the peripheral one (Martínez-Soriano et al., 2002). So, it can be deduced that latitude andthe variation of light radiation and geomagnetic action are factors that could influence pineal functioning, and therefore its nuclear dynamics (Quay,1963; Cuello and Tramezzani, 1969).

An interesting issue to point out as a limitation of the present study is the exclusive use of male animals. As already mentioned, the use of females has been avoided due to the well-known influence and interaction of melatonin and reproductive hormones in mammalian species (Ozaki et al., 1978; Tamura et al., 199; Chuffa et al., 2013; Takahashi et al., 2021). In fact, there are studies that, after surgical removal of the pineal gland in females, found interesting changes in cycle hormones, but in turn evidenced that there is melatonin synthesis in sites other than the pineal gland (Dardes et al., 2000). Given all these complicated but interesting interactions, and considering that their study was not part of this project, it was decided to dispense with females. Still, it would be interesting that these types of studies begin to be carried out also in females as a future line of research, and, in turn, compare these results with those already existing in males.

ACKNOWLEDGEMENTS

The authors thank Prof. Francisco Montes-Suay for his help in statistical analysis.

FUNDING

This work was supported by FIS (health research fund - Fondo de investigación en salud) grants, designed by Carlos III Health Institute (ISCIII), to the project “Chronobiology of the pineal gland. Morphometric and embryological analysis”, [Ref. P1031081].

REFERENCES

AULINAS A (2019) Physiology of the Pineal Gland and Melatonin. In: KR Feingold (ed.). Endotext. MDText.com, Inc.

AXELROD J, WURTMAN RJ, SNYDER SH (1965) Control of hydroxyindole-O-methytransferase activity in the rat pineal gland by environmental lighting. *J Biol Chem,* 240: 949-954.

BECKER UG, VOLLRATH L (1983) 24-hour variation of pineal gland volume: Pinealocyte nuclear volume and mitotic activity in male Sprague-Dawley rats. *J Neural Transm,* 56(2-3): 211-221.

BORSIGIN J, ZHANG L, CALINESCU AA (2012) Circadian regulation of pineal gland Rhytmicity. *Mol Cell Endocrinol*, 349: 13-19.

BLUMFIELD MG, TAPP E (1970) Measurements of pineal parenchymal cells and their nuclei in the albino rat at different ages. *Acta Morphol Neerl Scand,* 8: 1-8.

COON SL, CONG F, HARTLEY SW, HOLTZCLAW L, MAYS JC, KELLY M, KELLEY MW, MULLIKIN JC, RATH MF, SAVASTANO LE, KLEIN DC (2019) Single cell sequencing of the pineal gland: the next chapter. *Front Endocrinol,* 20(10): 590.

CHUFFA LG, SEIVA FR, FÁVARO WJ, AMORIN JP, TEIXEIRA GR, MENDES LO, FIORUCCI-FONTANELLI BA, PINHEIRO PF, MARTÍNEZ M, MARTÍNEZ FE (2013) Melatonin and ethanol intake exert opposite effects of circulating estradiol and progesterone and differentially regulate sex steroid receptors in the ovaries, oviducts and uteri of adult rats. *Reprod Toxicol,* 39: 40-49.

CIMAS C, MARTÍNEZ-SORIANO F, RUIZ A (1992) Circadian and seasonal corticomedullary variations in pinealocyte nuclear size: a comparative and statistical analysis. *Histol Histopathol,* 7: 679- 687.

CUELLO AC, TRAMEZZANI JH (1969) The epiphysis cerebri of the Weddell seal: its remarkable size and glandular pattern. *Gen Comp Endocrinol*, 12(1): 154-164.

DAFNY N, MCCLUNG R, STRADA SJ (1975) Neurophysiological properties of the pineal body. 1. Field potentials. *Life Sci,* 16: 611-620.

DARDES RC, BARACAT EC, SIMÕES MJ (2000) Modulation of estrous cycle and LH, FSH and melatonin levels by pinealectomy and sham-pinealectomy in female rats. *Prog Neuropsychopharmacol Biol Psychiatry*, 24(3): 441-453.

DE LA GUARDIA F, MARTINEZ SORIANO F, RUIZ TORNER A, OLCINA P (1988) Pinealocyte karyometric modifications in the albino rat following the application of magnetic fields. *Z Zellforch Microsk Anat Histochem,* 102(4): 609-618.

DIEHL BJM, HEIDBÜCHEL M, WELKER HA, VOLLRATH L (1984) Day/night changes of pineal gland volumes and pinealocyte nuclear size. Assesses over 10 consecutive days. *J Neural Transm,* 60: 19-29.

GERASIMOV AV, KOSTYUCHENKO AS, SOLOVIEVA AS, OLOVNIKOV AM (2014) Pineal gland as an endocrine gravitational lunasensor: manifestation of moon-phase dependent morphological changes in mice. *Biochemistry,* 79(109): 1316-1323.

GIMÉNEZ-GONZÁLEZ M, MARTÍNEZ-SORIANO F, ARMAÑANZAS E, RUIZ-TORNER A (1991) Morphometric and structural study of the pineal gland of the Wistar rat subjected to the pulse action of a 52 Gauss, (50 Hz) magnetic field. Evolutive analysis over 21 days. *J Hirnforsch,* 32(6): 779-786.

GUILLOT-VALLS MD, HERNÁNDEZ-GIL-DE-TEJADA T, MARTÍNEZ-SORIANO F (1995) A morphometric and statistical study of the effects of soft laser (He-Ne) irradiation on the pineal gland. *Histol Histopathol,* 10(2): 351-358.

HARDELAND R (2013) Chronobiology of melatonin beyond the feedback to the suprachiasmatic nucleus-consequences to melatonin dysfunction. *Int J Mol Sci,* 14(3): 5817-5841.

HEIDBÜCHEL V, VOLLRATH L (1983) Morphological findings relating to the problem of cortex and medulla in the pinal gland of rats and hamsters. *J Anat,* 136: 723-734.

HIRA Y, SAKAI Y, MATSUSHIMA S (1998) Quantitative light microscopic study on the heterogeneity in the superficial pineal gland of the rat. *Anat Rec,* 250(1): 80-94.

JACOBJ JL (1935) Die Zellkerngrösse beim Menschen. Ein Beitrag zur quantitativem Zytologie. *Z Zellforch Microsk Anat Histochem,* 38: 161-240.

KOCH M, FERREIRÓS N, GEISSLINGER G, DEHGHANI F, KORF HW (2015) Rhythmic control of endocannabinoids in the rat pineal gland. *Chronobiol Int,* 32(6): 869-874.

LIU W, MOLLER M (2000) Innervation of the rat pineal gland by PACAP-immunoreactive nerve fibers originating in the trigeminal ganglion: a degeneration study. *Cell Tissue Res,* 301(3): 369-373.

LÓPEZ-IGLESIAS C, ARIAS JC, ALVAREZ-URÍA M (1987) The rat pinealocyte during the strous cycle. A morphometric study. *Arch Anat Microsc Morphol Exp,* 75: 19-27.

MACCHI MM, BRUCE JN (2004) Human pineal physiology and functional significance of melatonin. *Front Neuroendocrinol,* 25(3-4): 177-195.

MARTÍNEZ-SALVADOR J, RUIZ-TORNER A, BLASCO-SERRA A, MARTÍNEZ-SORIANO F, VALVERDE-NAVARRO AA (2018) Morphologic variations in the pineal gland of the albino rat after a chronic alcoholisation process. *Tissue Cell*, 51: 24-31.

MARTÍNEZ-SORIANO F, ARMAÑANZAS E, RUIZ-TORNER A, VALVERDE-NAVARRO AA (2002) Influence of light/dark, seasonal and lunar cycles on the nuclear size of the pinealocytes of the rat. *Histol Histopathol,* 17: 205.212.

MATSUSHIMA S, MORISAWA Y, AIDA Y, ABE K (1983) Circadian variations in pinealocytes of the chinese Hamster, Cricetulus griseus. A quantitative electron microscopic study. *Cell Tissue Res,* 228: 231-244.

MATSHUSIMA S, SAKAI Y, HIRA Y (1989) Twenty-four changes in pinealocytes, capillary endothelial cells and pericapillary and intercellular spaces in the pineal gland of the mouse. *Cell Tissue Res,* (255): 323-332.

MATSUHIMA S, SAKAY Y, HIRA Y, KATO M, SHIGEMITSU T, SHIGA Y (1993) Effect of magnetic field on pineal gland volume and pinealocyte size. *J Pineal Res,* 14(3): 145-157.

MAYS J, KELLY MC, COON SL, HOLTZCLAW L, RATH ME, KELLEY MW, KLEIN DC (2018) Single-cell RNA sequencing of the mammalian pineal gland identifies two pinealocytes subtypes and cell type-specific daily patterns of gene expression. *PLoS One*, 22: 13(10): e0205883.

MERRIT JH, SULKOWSKI TS (1969) Alterations of pineal gland biorhythms by N-methyl-3-piperediyl benzilate. *J Pharmacol Exp Ther,* 166: 119-124.

MILLINE R, KRSTIC R, DEVECERSKI V (1968) Sur le comportement de la glande pinéale dans des conditions de stress. *Acta Anat,* 71: 352-402.

MOLLER M, BAERES FM (2002) The anatomy and innervation of the mammalian pineal gland. *Cell Tissue Res,* 309(1): 139-150.

MOLLER M, BAERES FM (2003) PACAP-containing intrapineal nerve fibers originate predominantly in the trigeminal ganglion: a combined retrograde tracing- and immunohistochemical study of the rat. *Brain Res,* 984(1-2): 160-169.

OZAKI Y, WURTMAN RJ, ALONSO R, LYNCH HJ (1978) Melatonin secretion decreases during the proestrous stage of the rat estrous cycle. *Proc Natl Acad Sci USA*, 75(1): 531-534.

PÉVET P (1977) On the presence of different populations of pinealocytes in the mammalian pineal gland. *J Neural Transm,* 40(4): 289-304.

POPOVA NK, KOLAEVA SG, DIANOVA I (1975) State of the pineal gland during hibernation. *Bull Exp Biol Med,* 79: 467-468.

QUAY W, RENZONI A (1966) Twenty-four hour rhythms in the pineal activity activity and nuclear and nucleolar dimensions. *Growth,* 30: 315-324.

RENZONI A, QUAY WB (1964) Daily karyometric and rnitotic rhythm of pineal parenchymal cells in the rat. *Am Zool,* 4: 416-417.

REUSS ST, VOLLRATH L (1984) Electrophysiological properties of rat pinealocytes: Evidence for Circadian and ultradian rhythms. *Exp Brain Res*, 55(3): 455-461.

REUSS ST, SEMM P, VOLLRATH L (1984) Electrophysiological investigations on the central innervation of the rat and guinea-pig pineal gland. *J Neural Transm*, 60(1): 41-43.

SAKAI Y, HIRA Y, MATSUSHIMA S (1996) Regional differences in the pineal gland of the cotton rat, Sigmodon hispidus: light microscopic, electron microscopic, and immunohistochemical observations. *J Pineal Res,* 20(3): 125-137.

SAPÈDE D, CAU E (2013) The pineal gland from development to function. *Curr Top Dev Biol,* 106: 171-215.

SEMM P, VOLLRATH L (1979) Electrophysiology of the guinea-pig pineal organ: Sympathetically influenced cells responding differently to light and darkness. *Neurosci Lett*, 12: 93-96.

SEMM P, VOLLRATH L (1980) Electrophysiological evidence for circadian rhytmicity in a mamalian pineal organ. *J Neural Transm,* 47: 181-190.

SEMM P (1983) Neurobiological investigations of the pineal gland and its hormone melatonin. In: Axelrod J, Fraschini F, Velo GP (eds). *The Pineal Gland and its Endocrine Role. NATO Advanced Science Institutes Series* (Series A: Life Sci), vol 65. Springer, Boston, MA.

SHOJA MM, HOEPFNER LD, AGUTTER PS, SINGH R, TUBBS RS (2016) History of the pineal gland. *Childs Nerv Syst,* 32(4): 583-586.

TAKAHASHI T, OGIWARA K (2021) Roles of melatonin in the teleost ovary: A review of the current status. *Comp Biochem Physiol A Mol Integr Physiol*, 254: 110907.

TAMURA H, NAKAMURA Y, TAKIGUCHI S, KASHIDA S, YAMAGATA Y, SUGINO N, KATO H (1998) Melatonin directly suppresses steroid production by preovulatory follicles in the cyclic hamster. *J Pineal Res*, 25(3): 135-141.

VOLLRATH L (1979) Comparative morphology of the vertebrate complex. In: Ariëns Kappers J, Pévet P (eds). *The pineal of vertebrates including Man*. Prog Brain Res, 52: 25-38. Elsevier, Amsterdam.

VOLLRATH L (1981) The Pineal Organ. Handbuch der Mikroskopischen Anatomie des Menschen VI/7. *J Anat,* 135(2): 440-442.

VOLLRATH L (1985) The pineal gland of Mammals. An organ or a complex? In: Mess B, Rúzsas Cs, Tima L, Pévet P (eds). *The pineal gland. Current State of Pineal Research*. ISBN: 0-444-80629-6. Elsevier Science Publisher, Amsterdam, Netherlands, pp 27-33.

WELSH MG, CAMERON IL, REITER RJ (1979) The pineal gland of the Gerbil Meriones unguiculatus. I. Morphometric analysis over 24- hour period. *Cell Tissue Res,* 294: 95-109.

WURTMAN RJ, AXELDOD J (1964) Light and melatonin synthesis in the pineal. *Fed Proc,* 23: 206.

WURTMAN RJ, OZAKI Y (1978) Physiological control of melatonin synthesis and secretion: Mechanism generating rhythms in melatonin, methoxytryptophol and arginine,vasotocin levels and effects on the pineal of endogenous catecholamines, the estrous cycle, and environmental lighting. *J Neural Transm*, 13: 59-70.