

Age-related morphometric changes in the pineal gland. A comparative study between C57BL/6J and CBA mice

R. Cernuda-Cernuda, J.J. Huerta, M. Muñoz Llamosas, M. Álvarez-Uría and J.M. García-Fernández

Department of Morphology and Cell Biology, University of Oviedo, Oviedo, Spain

Summary. Relatively little is known about the effects of melatonin on the aging of the pineal, the organ which is the main place for synthesis of this hormone. Using simple morphometric methods, some parameters of the pineal gland, such as total volume, number of pinealocytes and pinealocyte volume were estimated in two mice strains: normal CBA and melatonin-deficient C57BL/6J. Two age groups, 6 weeks and 10 months, were studied in order to evaluate possible differential age-related changes between both strains. Pineals of both strains have similar morphometric and morphological features at 6 weeks of age. This suggests that pineal development, which has already concluded at 6 weeks of age, is not affected by the absence of melatonin synthesis in the pinealocytes. Later on, CBA pineal showed an increase in size caused by cellular hypertrophy. In contrast, the C57BL/6J pineal volume decreased by loss of pinealocytes in the same period of time. Semithin sections analysed by light microscopy did not show that this cell death was evident in the C57BL/6J strain at any of the ages studied. Thus, a gradual loss of pinealocytes could be hypothesised in these pineals. These results suggest that pineal melatonin could have a role in the maintenance of pinealocyte viability and the increase of pineal size which takes place after development. The abnormal pattern observed in the C57BL/6J pineal should be taken into account in future studies on this gland.

Key words: Pineal, Mouse, CBA, C57BL/6J, Aging

Introduction

The secretion of the pineal hormone, melatonin, tends to attenuate throughout life (Reiter, 1994). Despite this, the mammalian pineal gland seems to be active in old subjects and few age-related morphological changes have been described in this organ. In fact, there are no

consistent signs for a significantly depressed pineal function in old age (Vollrath, 1981). An increase in weight was reported in the human pineal from birth onwards, reaching a maximum between 40 and 50 years of age, and there are no clear signs for a decrease beyond this age (Siuciak et al., 1990). Histological studies do not provide evidence for functional inactivity of the human pineal in old age. It still remains unclear whether melatonin and/or other factors synthesised in this gland are responsible for an attenuation of aging in other organs or in the pineal itself. The experiments by Pierpaoli and Regelson (1994) in pineal grafting from young to old mice seemed to demonstrate a role for pineal factors in the prolongation of lifespan. Since the mice strains they used -C57BL/6J and BALB/c- were melatonin-deficient in their pineals (Ebihara et al., 1987), the possible role of melatonin is dubious. Genetic analysis suggested that two mutant genes, which affect the activity of NAT and HIOMT, two enzymes required for the synthesis of melatonin, are responsible for this hormone deficiency at least in the C57BL/6J strain (Ebihara et al., 1986). Nevertheless, this is still a matter of debate, since Conti and Maestroni (1996) found a short-term peak of this hormone by HPLC determinations in the middle of the dark period in the pineal of these strains. Goto et al. (1989) reported that only 5 of a total of 36 inbred strains examined had pineal melatonin content. Among these, C3H and CBA mice showed a similar pattern of pineal melatonin rhythm and a similar peak level (150 pg/gland) for this hormone. The C3H strain normally supplied is retinally degenerate and little is known about the effect of retinal degeneration on pineal metabolism. Hence, we decided to compare two strains of mice, both with normal retinas. Thus, this paper deals with the influence of the two factors mentioned above: (i) presence of pineal melatonin production, and (ii) age, on some pineal morphometric features.

Materials and methods

In the present study, 20 male mice of two strains, distributed in groups of 5 individuals, were used for the

statistical analysis:

- C57BL/6J, melatonin-deficient, with normal retina (wild-type)

- CBA, with normal melatonin rhythm and normal retina

Two subgroups of age, 6 weeks and 10 months, were studied in each of the above two groups.

Mice were maintained in the central animal care facilities under a constant photoperiod (12:12 h LD) and temperature conditions ($20 \pm 2^\circ\text{C}$), with standard food and tap water ad libitum. Animals anaesthetised with diethyl ether were sacrificed by decapitation at the same hour of the light-dark cycle to avoid possible circadian variations of the parameters to be measured, as some authors have described in other mammals (Becker and Vollrath, 1983). The whole pineal was extracted and fixed by immersion in a 0.1M pH 7.4 phosphate buffered 4% paraformaldehyde and 3% glutaraldehyde solution for three hours at room temperature. A postfixation in 1% osmium tetroxide for 2 hours at 4°C was also carried out. The samples were then dehydrated in acetone and embedded in araldite resin (Durcupan, Fluka). Semithin sections ($1\ \mu\text{m}$ width) were obtained with glass knives in a Reichert Jung 'Ultracut' ultramicrotome and stained with toluidine blue solution. In order to estimate the pineal volume, these sections were selected as follows: the first one was randomly chosen within the first $100\ \mu\text{m}$ of tissue; and the following sections were taken with a separation of $200\ \mu\text{m}$ until the entire pineal was sectioned. Profiles of the sections were drawn by means of a camera lucida in a Nikon light microscope. The area of the sections (S) was estimated calculating the area of the profiles drawn using the QWin Pro image digital processing system (Leica), and by dividing by the magnification. The total volume of the pineal was calculated by means of Cavalieri's direct estimator (Gundersen et al., 1988):

$$V_{\text{pineal}} = t \sum S_i; (t = 200\ \mu\text{m})$$

Morphometric analysis was carried out at the light microscope level to assess: (1) the volume density of pinealocyte nucleus and cytoplasm and space occupied by connective tissue, nerves and vessels; (2) the numerical density of pinealocytes; (3) the mean volume of pinealocytes, pinealocyte nuclei and cytoplasm

The point-counting method of Weibel (1979) was employed to calculate the relative volume of the gland components. Five resin semithin sections ($1\ \mu\text{m}$ width) separated by at least $200\ \mu\text{m}$ were randomly chosen from each gland. Six fields were randomly selected and photographed at a final magnification of $\times 1000$, $0.01\ \text{mm}^2$ being the area of gland tissue examined in each photograph. Thus, 180 random fields were morphometrically analysed per group. Measurements were made by placing a transparent screen with a square point lattice spaced by $1\ \text{cm}$ over each photograph and by counting the number of intersections on each component. The volume fraction of a particular component was calculated by dividing the total number of points over that component by the total number of points over the glandular tissue.

The numerical density of pinealocytes (N_V) (number of pinealocytes per unit volume of gland) was estimated according to the formula (López and Álvarez-Uría, 1994):

$$N_V = N_A / (T+D-2h), \text{ in which:}$$

N_A is the number of pinealocyte nuclei per unit area of section. 6 random fields of $2500\ \mu\text{m}^2$ were evaluated in each of the 5 sections mentioned above; thus, a total of 150 random fields per group.

T is the average thickness of the sections, which was estimated to be $1\ \mu\text{m}$.

D is the average nuclear diameter. 20 random pinealocyte nuclei per gland (100 nuclei per group) were measured to estimate nuclear diameter.

Finally, h is the height of the smallest recognisable cap section, which was arbitrarily estimated to be 10% of the nuclear diameter.

The total number of pinealocytes per gland was calculated by multiplying N_V by the volume of the gland. The average volume of a pinealocyte or of a pinealocyte fraction, such as nucleus and cytoplasm, was calculated by dividing the absolute volume occupied by each fraction by the total number of pinealocytes.

The data obtained were statistically compared using a one-way analysis of variance (ANOVA), and, when the F test was significant ($p < 0.05$), differences between specific means were tested by the Student-Newman-Keuls test.

The Scion Image program was used to obtain 3D-images of the whole pineals in every group. The profiles of the sections employed for the volume estimation were laid in an orderly way with a separation according to the same scale of magnification. The same inclination, 10° degrees from the vertical axis that provided a good sight of all the profiles, was chosen for all the representations.

In addition to this, observations at light microscope level were also made in semithin sections of CBA and C57BL/6J (5 individuals per group) of an intermediate age (5 months), in order to elucidate whether a loss of pinealocytes was or was not evident in the C57BL/6J strain.

Photographs of the semithin sections were taken with a Polaroid DMC1 digital camera.

Results

In both strains, the volume of the whole pineal was similar at 6 weeks of age. Despite the fact that C57BL/6J pineal is slightly smaller than CBA pineal, no statistical differences were found at this age. The total number of pinealocytes and also the average pinealocyte volume were similar at this age in both strains (Table 1, Fig. 1). As can be observed in the computer representation of the section profiles, C57BL/6J pineal is more flattened than CBA pineal (Fig. 2) in both ages studied. On the other hand, pineals of both strains showed a variable enlargement of the superficial part, and poorly developed intermediate and deep portions. Micrographs obtained of the semithin sections showed a similar appearance in

Age-related changes in mouse pineal

Table 1. Comparison of pineal gland morphometric data between 6-week-old and 10-month-old mice of the CBA and C57BL/6J strains.

	CBA		C57BL/6J	
	6 weeks	10 months	6 weeks	10 months
Pineal volume ($\times 10^6 \mu\text{m}^3$)	105.07 \pm 7.83	135.58 \pm 8.32*	97.44 \pm 1.01	64.28 \pm 7.42*
Total number of pinealocytes per gland	58048 \pm 6086	60608 \pm 4092	61510 \pm 4742	36640 \pm 2769**
Vv (%) of nerves, vessels and connective tissue	14.67 \pm 0.27	13.71 \pm 1.73	11.25 \pm 0.56	11.45 \pm 1.03
Average volume per pinealocyte (μm^3)	1566 \pm 63	1934 \pm 38***	1396 \pm 47	1566 \pm 149
Nuclear fraction (μm^3)	353 \pm 14	329 \pm 18	344 \pm 10	375 \pm 17
Cytoplasmic fraction (μm^3)	1213 \pm 45	1604 \pm 35***	1050 \pm 54	1191 \pm 144

Each value is the mean \pm S.E.M. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (p values refer to comparison with 6-week-old mice). Data of volume density (Vv) of pinealocytes, which are complementary to Vv of space occupied by nerves, vessels and connective tissue, are not represented. Vv of pinealocytes, pinealocyte fractions (nucleus and cytoplasm) and their absolute volumes per gland are also omitted, since they were used to estimate the respective values per pinealocyte.

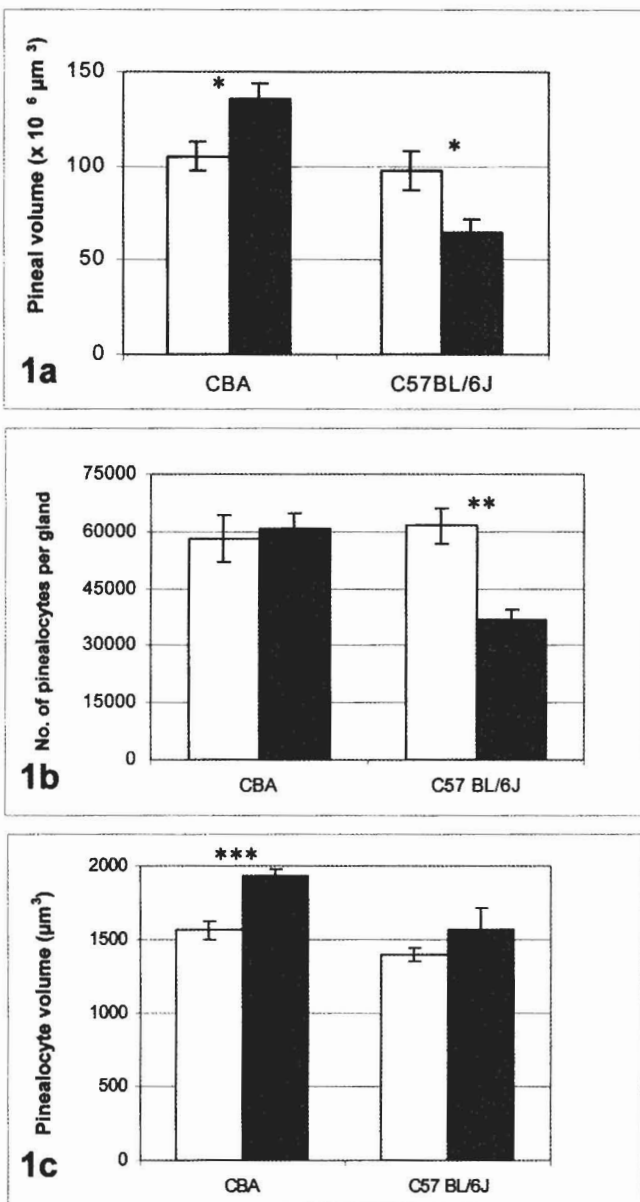


Fig. 1. The effects of age on the most representative morphometric data of the strains analysed are represented in these three histograms. **a.** The pineal volume of CBA increases significantly with age; in contrast, the pineal of C57BL/6J shows a significant parallel decrease in the same period of time. **b.** The total number of pinealocytes per gland does not differ in the CBA strain between both groups of age, but does decrease significantly in the C57BL/6J 10-month-old group. **c.** The pinealocyte volume increases in CBA mice, but does not show statistical difference between both ages in the C57BL/6J strain. Groups of 6 weeks of age are represented in white colour and groups of 10 months of age in grey colour. Values represent the mean, and error bars, the SEM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

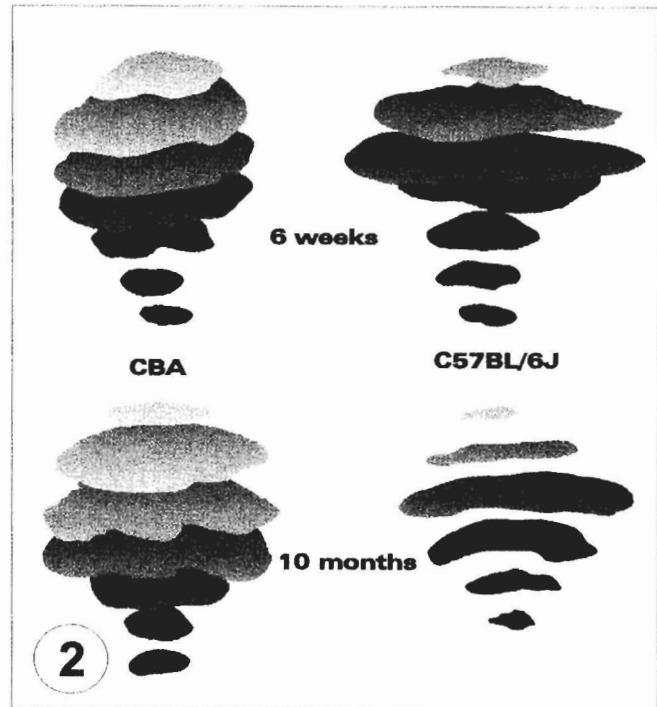


Fig. 2. Computer drawings of representative glands of the four groups studied. These images, which are obtained with the profiles of the sections employed for volume estimation, provide a good idea of pineal volume and morphology. C57BL/6J pineals, which are more flattened than those of CBA mice, show a decreased volume at 10 months of age, whereas the latter increase their size in the same period of time. x 35

Age-related changes in mouse pineal

both strains at 6 weeks of age (Figs. 3a,b).

CBA pineal size increased significantly with age ($p < 0.05$), whereas the C57BL/6J strain showed a parallel decrease in their pineal volume (Table 1, Fig. 1a). In the CBA strain, no statistical differences were found in the total number of pinealocytes per gland between the two

ages analysed (Table 1, Fig. 1b). Since the size of the nuclei estimated was also similar in both age groups, the increase in the pineal volume appeared to be caused by an increase of the pinealocyte cytoplasmic volume. In fact, the mean volume of pinealocytes increased significantly with age in the CBA strain ($p < 0.001$; Table

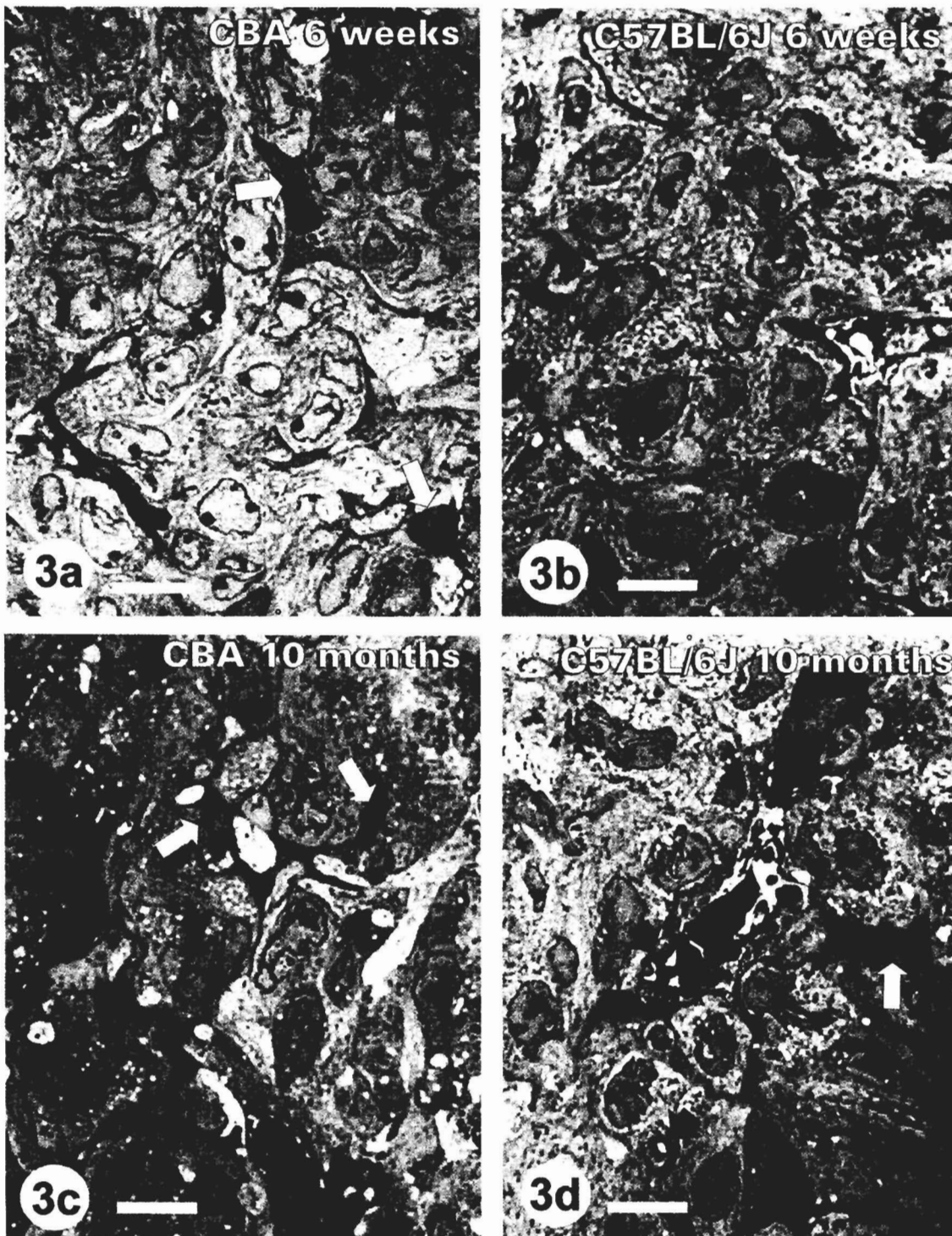


Fig. 3. Representative images of semithin sections of the groups of mice used for the morphometric study. The pinealocyte nuclei density of 6-week-old CBA mice (3a) is apparently similar to that observed in C57BL/6J mice of the same age (3b). In contrast, 10-month-old CBA mice (3c) showed slightly more dispersed pinealocyte nuclei than those in C57BL/6J pineals of the same age (3d). The density of pinealocyte nuclei of the latter is quite similar to that of the images obtained from the 6-week-old groups. Black arrows point to myelinated nerve fibres, white arrows indicate some interstitial cells. $\times 400$, Bar: $10 \mu\text{m}$.

1, Fig. 1c), and the cytoplasmic fraction was obviously responsible for this increment ($p < 0.001$; Table 1). The changes in pineal volume were also evident in the three-dimensional computer images (Fig. 2). A diminution of pinealocyte nuclei density, which corresponded to an increase in the cytoplasmic fraction could also be seen in some micrographs obtained of 10-month-old CBA mice (Fig. 3c). In contrast, the C57BL/6J pineal, which was melatonin-deficient, showed a significant decrease in size in the same period of time ($p < 0.05$; Table 1, Fig. 1a). The decrease in size of the pineal in this strain seemed to be caused by a diminution of the number of pinealocytes ($p < 0.01$; Table 1, Fig. 1b), since the average pinealocyte volume, either nucleus or cytoplasmic fractions, remained statistically unaltered (Table 1, Figs. 1c, 3b,d). On the other hand, both strains showed that the percentage of space occupied by nerves, vessels and connective tissue did not change significantly between the two age groups studied.

Discussion

According to Vollrath's classification (1981), the pineal shape of both strains could correspond to type $\alpha\beta C$, since a relative enlargement of the distal region was always present in all the cases studied.

On the other hand, an epiphyseal growth had already been reported in mice from birth and 40 days of age, when a cellular hypertrophy occurs, and between 60 and 90 days (Ito and Matsushima, 1967). Our results in normal CBA mice could be somehow compared to what other authors have previously reported in the pineal of humans and in other mammals (Vollrath, 1981). Brednow and Korf (1998) recently reported that in adult animals the pineal is more than 2 times bigger in normal C3H mice than in C57BL mice. This is in accordance with our findings in animals of 10 months. These authors also observed a less pronounced, although evident, difference in the pineal size in the young subjects. However, we did not find any statistical difference in volume between pineals of either strain studied at 6 weeks of age. As can be seen in our results, normal CBA pineal growth is caused by a cellular hypertrophy that takes place within the period studied. In contrast, the volume of C57BL/6J pinealocytes did not vary significantly. This represents an important difference between both strains. A decrease in the pineal volume with age in the C57BL/6J strain could imply a decrease in its global synthetic activity. This might explain the success of pineal grafting experiments (Pierpaoli and Regelson, 1994). Since C57BL/6J pineals are melatonin-deficient, some other factors, different from melatonin, should be responsible for lifespan elongation observed in old mice which were recipients for the pineal of young donors in such experiments. As the age interval selected for this study, 6 weeks to 10 months, is restricted to a period of the adult life of mice, further studies are necessary to analyse possible changes in the mouse pineal in more advanced stages of life. The results

presented in this paper suggest that pineal melatonin might play an important role not only in the increase of pinealocyte activity in normal mice within this period of age, but also in the maintenance of pinealocyte viability. Some authors (Wurtman et al., 1964) reported that 3H -melatonin is uptaken mainly by the pineal when administered intravenously in the cat. The role of the melatonin within the pineal itself is still unknown and, to our knowledge, no melatonin receptors were found in this organ (Weaver et al., 1989; Siuciak et al., 1990). Nevertheless, melatonin was found to be a potent free radical scavenger and antioxidant (Reiter et al., 1997). The free radicals, which are a natural result of aerobic metabolism, represent one of the major potential causes of age-related destruction of neuronal tissue (Reiter, 1995 a). Melatonin has also proved to prevent apoptosis in some cell types such as thymocytes (Sainz et al., 1995), neuronal cells (Mayo et al., 1998), etc. If the C57BL/6J pineal definitely lacks melatonin synthesis, this could imply that the uptake of melatonin synthesised in other organs such as the Harderian gland, retina, etc., would not be sufficient for a satisfactory growth of the pineal. Such organs might be responsible for the short duration peak observed in this strain by Conti and Maestroni (1996). Self-produced melatonin or a nocturnal elevation of longer term could be necessary for a normal activity of pinealocytes. In fact, one of the theories which involves this hormone in the mechanism of aging, assumes that the duration of nocturnally elevated melatonin is important in these processes (Reiter, 1995b).

A diminution of pineal activity by a decrease in size does not imply a lack of normal circadian rhythmicity in the C57BL/6J strain. The melatonin synthesised outside the pineal, or other important factors might also be involved in the circadian activity of these mice and of other mammals. Could the absence of normal production of this hormone be inducing a premature aging process in this gland? Further studies in other mice strains are necessary in order to elucidate these questions.

In conclusion, since the C57BL/6J pineal does not seem to exhibit a regular pattern of growth in the adult life of mice, the CBA strain seems to be more appropriate as a model for pineal studies.

Acknowledgements. We wish to thank José Manuel López García and Ana Domínguez Sanjurjo for their assistance in the statistical analysis of the data; and Esther Alcorta Azcue for her help and suggestions with the Scion Image program. The present study was supported by a grant from the Spanish DGICYT (PB94-1326) and BIOMED 2 (PL96-2327) to JMG-F.

References

- Becker U.G. and 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, 211-221.
- Brednow K. and Korf H. (1998). Morphological and immunocytochemical

Age-related changes in mouse pineal

- features of the pineal organ of C3H and C57BL mice at different stages of postnatal development. *Cell Tissue Res.* 292, 521-530.
- Conti A. and Maestroni G.J.M. (1996). HPLC validation of a circadian melatonin rhythm in the pineal gland of inbred mice. *J. Pineal Res.* 20, 138-144.
- Ebihara S., Hudson D.J., Marks T. and Menaker M. (1987). Pineal indole metabolism in the mouse. *Brain Res.* 416, 136-140.
- Ebihara S., Marks T., Hudson D.J. and Menaker M. (1986). Genetic control of melatonin synthesis in the pineal gland of the mouse. *Science* 231, 491-493.
- Goto M., Oshima I., Tomita T. and Ebihara S. (1989). Melatonin content of the pineal gland in different mouse strains. *J. Pineal Res.* 7, 195-204.
- Gundersen H.J.G., Bendtsen T.F., Korbo L., Marcussen N., Moller A., Nielsen K., Nyengaard J.R., Pakkenberg B., Sorensen F.B., Vesterby A. and West M.J. (1988). Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. *APMIS* 90, 379-394.
- Ito T. and Matsushima S. (1967). A quantitative morphological study of the postnatal development of the pineal body of the mouse. *Anat. Rec.* 159, 447-452.
- López J.M. and Álvarez-Uría M. (1994). Effects of ovariectomy and ageing on the structure and ultrastructure of the female Syrian hamster Harderian gland: a stereological analysis. *Anat. Embryol.* 189, 409-419.
- Mayo J.C., Sainz R.M., Uría H., Antolín I., Esteban M.M. and Rodríguez C. (1998). Melatonin prevents apoptosis induced by 6-hydroxydopamine in neuronal cells: implications for Parkinson's disease. *J. Pineal Res.* 24, 179-192.
- Pierpaoli W. and Regelson W. (1994). Pineal control of aging: effect of melatonin and pineal grafting on aging mice. *Proc. Natl. Acad. Sci. USA* 91, 787-791.
- Reiter R.J. (1994). Pineal function during aging: attenuation of the melatonin rhythm and its neurological consequences. *Acta. Neurobiol. Exp.* 54 Suppl, 31-39.
- Reiter R.J. (1995a). Oxidative processes and antioxidative defense mechanisms in the aging brain. *FASEB J.* 9, 526-533.
- Reiter R.J. (1995b). The pineal gland and melatonin in relation to aging: a summary of the theories and of the data. *Exp. Gerontol.* 30, 199-212.
- Reiter R.J., Carneiro R. C. and Oh C.S. (1997). Melatonin in relation to cellular antioxidative defense mechanisms. *Horm. Metab. Res.* 29, 363-372.
- Sainz R.M., Mayo J.C., Uría H., Kotler M., Antolín I., Rodríguez C. and Menéndez-Peláez A. (1995). The pineal hormone melatonin prevents in vivo and in vitro apoptosis in thymocytes. *J. Pineal Res.* 19, 178-188.
- Siuciak J.A., Fang J. M. and Dubocovich M.L. (1990). Autoradiographic localization of 2-[¹²⁵I]iodomelatonin binding sites in the brains of C3H/HeN and C57BL/6J strains of mice. *Eur. J. Pharmacol.* 180, 387-390.
- Vollrath L. (1981). The pineal organ. In: *Handbuch der mikroskopischen Anatomie des Menschen*. Vol. VI/7. Oksche A. and Vollrath L. (eds). Springer Verlag. Berlin. pp 1-665.
- Weaver D.R., Rivkees S.A. and Reppert S.M. (1989). Localization and characterization of melatonin receptors in rodent brain by in vitro autoradiography. *J. Neurosci.* 9, 2581-2590.
- Weibel E.R. (1979). Practical methods for biological morphometry. In: *Stereological methods*. Vol 1. Weibel E.R. (ed). Academic Press. London. pp 1-145.
- Wurtman R.J., Axelrod J. and Potter L.T. (1964). The uptake of ³H-melatonin in endocrine and nervous tissues and the effects of constant light exposure. *J. Pharmacol. Exp. Ther.* 143, 314-318.

Accepted June 5, 2000