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Cellular proliferation in the rat pineal gland during postnatal development

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Summary. To establish a possible correlation between the rate of cellular proliferation and already documented functional and morphological characteristics of the rat pineal gland during postnatal development, the bromodeoxyuridine labelling method was used to evaluate the fraction of cells at the S phase of the cell cycle in paraffin sections from 1-, 7-, 14- and 28-day-old rats. Numerical density, taken as an indirect measure of cell hypertrophy, was also evaluated. During the first week after birth the percentage of S phase-cells in the rat pineal gland sharply decreased from around 9% to 1.3%. A smaller but also significant decrease was found from the 7th to the 14th postnatal day where S phase cells were less than 0.5% of all pineal cells. A very low percentage was also seen in samples from 28-day-old rats. Numerical density, namely, the total number of cells per surface unit of pineal section, decreased from birth to the end of the first month. This decrease was also steeper from birth to the 7th postnatal day than at any other period of the study. These results support the idea that a strong expansion of the cellular population of the rat pineal gland precedes morphological and functional maturation and opens the way to further exploration of the relationship between functional and proliferative responses of the pineal gland.

Key words: Pineal gland, Cellular proliferation, Development, Rat

Introduction

Biochemical and morphological studies have been made to characterize the ontogenic development of the rat pineal gland. The secretory biorhythm and the concentrations of serotonin and melatonin as well as the levels of the enzymes SNAT, HIOMT and tyrosine hydroxylase have been established by using biochemical techniques (Quay and Halevy, 1962; Quay, 1963; Quay and Renzoni, 1966; Zweig et al., 1966; Black and Mytilineou, 1976). Similarly, morphological studies (Blázquez et al., 1989) have clearly depicted the changes that take place during development of the pineal gland.

As well as for many other organs, several authors have tried to find out a positive correlation between functional activity and morphological features in the pineal gland (Quay and Renzoni, 1966; Diehl, 1981; Becker and Vollrath, 1983; Blázquez et al., 1989). It cannot be said that such a relationship is, so far, universally admitted. Efforts have also been made to correlate numbers of mitotic figures, as a sign of proliferative activity of pinealocytes, with functional activity. Varying results, sometimes contradictory, have come from these studies (Quay and Renzoni, 1966; Becker and Vollrath, 1983; Blázquez et al., 1989).

To define a possible correlation between morphological and functional features of the rat pineal gland with the proliferative activity of pinealocytes, the first task should be to avoid the many problems linked to the assessment of cellular proliferation based on the counting of mitoses (Carbajo and Carbajo Pérez, 1994), problems that have undoubtedly contributed to make the findings of previous studies obscure. The labelling of cells at the S phase of the cell cycle with bromodeoxyuridine (BrdU) to quantitate cellular proliferation might well be the technique of choice for this kind of study. Since the description of the BrdU-labelling (Gratzner, 1982), this technique has proved to be a simple and reliable method for the quantitation of cellular proliferation (Silvestrini et al., 1988; Böswald et al., 1990) and has been widely used to assess cellular proliferation in growing tissues (Carbajo-Pérez and Watanabe, 1990; Ikeda and Yoshimoto, 1991; Carbajo et al., 1992).

In an attempt to cast some light onto the hitherto poorly understood morphogenetic and growth and maturation phenomena that take place in the pineal gland during the first month of postnatal life, and in an attempt to correlate cellular proliferation with morphological and functional data clearly established for that critical period of pineal development, the BrdU-labelling was used to evaluate the proliferative activity in pineal tissue from 1-, 7-, 14- and 28-day-old rats. To explore the existence of possible sex differences in the pattern of cellular

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proliferation male and female glands were analyzed in each age group.

Materials and methods

Pregnant Sprague-Dawley rats were housed individually in plastic cages under a light-dark regimen of 12:12 h and were provided with food and water *ad libitum*. For breeding purposes, pregnant females were checked daily after the 14th day *postcoitum*. The day of birth of the pups was designated as day 0 of postnatal life. Animals were caged by litters and remained with their mothers from birth until the day of sacrifice. All experimental groups included at least 3 males and 3 females. Groups of 1-, 7-, 14- and 28-day-old rats received an intraperitoneal injection of 50 mg/kg body weight of BrdU (Sigma, St. Louis, USA) in saline (10 mg/ml) at midday (half-way through the light phase) and were killed one hour later by decapitation.

Pineal glands were dissected out and fixed overnight in Bouin's fluid. After dehydration in ethanol, the glands were embedded in paraffin wax and sectioned at a thickness of 4 μm. After deparaffinization, sections were incubated with a monoclonal antibody to BrdU plus DNase for 1 hour (Cell proliferation kit, Amersham, UK) and with peroxidase-conjugated rabbit anti-mouse IgG (1:50; Sigma) for 30 min. The reaction product was then visualized with 3-3'-diaminobenzidine. Sections were lightly counterstained with hematoxylin, dehydrated, cleared, and mounted with DPX (Fluka, Ulm, Germany).

The proportion of BrdU-labelled cells per 100 cells (labelling index; BrdU-LI) was calculated by counting labelled and unlabelled cells in 25 microscope fields (7497 μm^2 each field) selected at random from each of the animals of the different age groups. The numerical density (ND= number of cells/100μm^2) was calculated using the same microscopic fields. These parameters were obtained with an MIP image analyzer (Microm España, Spain), capturing the images with a x50 aberration-free objective lens (Carl Zeiss, Jena, Germany).

For statistical purposes, ANOVA was used at a level of significance of 95%. The Scheefe test was used for two group comparisons.

Results

BrdU-labelled nuclei were easily identifiable in pineal sections from all the groups of the study (Fig. 1). Direct examination under the light microscope revealed that in samples from younger animals BrdU-labelled nuclei clearly outnumbered the labelled nuclei found in older ones (Fig. 2). Very few labelled nuclei were found in pineal sections from 28-day-old rats (Fig 2d).

The numerical data concerning the proliferative activity of pinealocytes obtained following image processing and quantification are shown in Table 1. In both sexes, the LI was relatively high the day after birth and then decreased sharply up to the 14th postnatal day. The steepest decrease was found during the first week. After the 14th postnatal day the LI stabilized at very low levels. No sex difference was found.

The density of pinealocytes by surface unit, ND, (Table 2) decreased progressively and significantly from birth to the end of the first month. A statistically significant difference with respect to sex was only found at 14 days.

Discussion

Biochemical and physiological studies have demonstrated that the first four weeks of postnatal life are crucial for the functional maturation of pinealocytes. Thus, intrapineal 5-HT and melatonin levels reach values similar to those found in adult animals around the twelfth day (Quay and Halevy, 1962; Zweig et al., 1966). Simultaneously, HIOMT and SNAT reach values similar to those found in adult rats on the tenth and twentieth days, respectively (Ellison et al., 1972; Tamarkin et al., 1980) whereas tyrosine hydroxylase activity stabilises at around one month after birth (Black and Mytilineou, 1976). Also, towards the end of the third week of postnatal life a close correlation can be seen between SNAT activity, ß-adrenergic receptor activity, and the appearance of circadian rhythms in melatonin synthesis (Yuwilder et al., 1977; Cantor and Weiss, 1978; Weiss et al., 1979).

Several attempts have been made to set up a correlation between the functional activity of the pineal gland and certain morphological parameters. In this sense, Quay and Renzoni (1966), report cyclic variations in nuclear and nucleolar size and in the mitotic activity of rat pinealocytes suggestive of the existence of 24h rhythmicity in melatonin synthesis. This was later confirmed by Rudeen et al. (1975). In contrast, other authors (Diehl, 1981) found no significant modifications in pineal volume, whereas they did observe considerable

Table 1. Percentage of BrdU-Labelled cells (BrdU-LI).

AGE (days)	MALES (mean±SEM)	FEMALES (mean ±SEM)
1	9.73±0.63	9.39±0.43
7	1.32±0.07*	1.35±0.07*
14	0.35±0.01*	0.48±0.02*
28	0.15±0.01	0.24±0.02

*Significantly different ($p< 0.05$) with previous age from same sex.

Table 2. Number of pinealocytes per 100 μm^2 (ND).

AGE (days)	MALES (mean±SEM)	FEMALES (mean ±SEM)
1	3.09±0.08	3.17±0.03
7	2.17±0.02*	2.18±0.05*
14	1.73±0.02*	1.87±0.05*
28	1.35±0.02*	1.40±0.08*

*Significantly different ($p< 0.05$) with previous age from same sex.

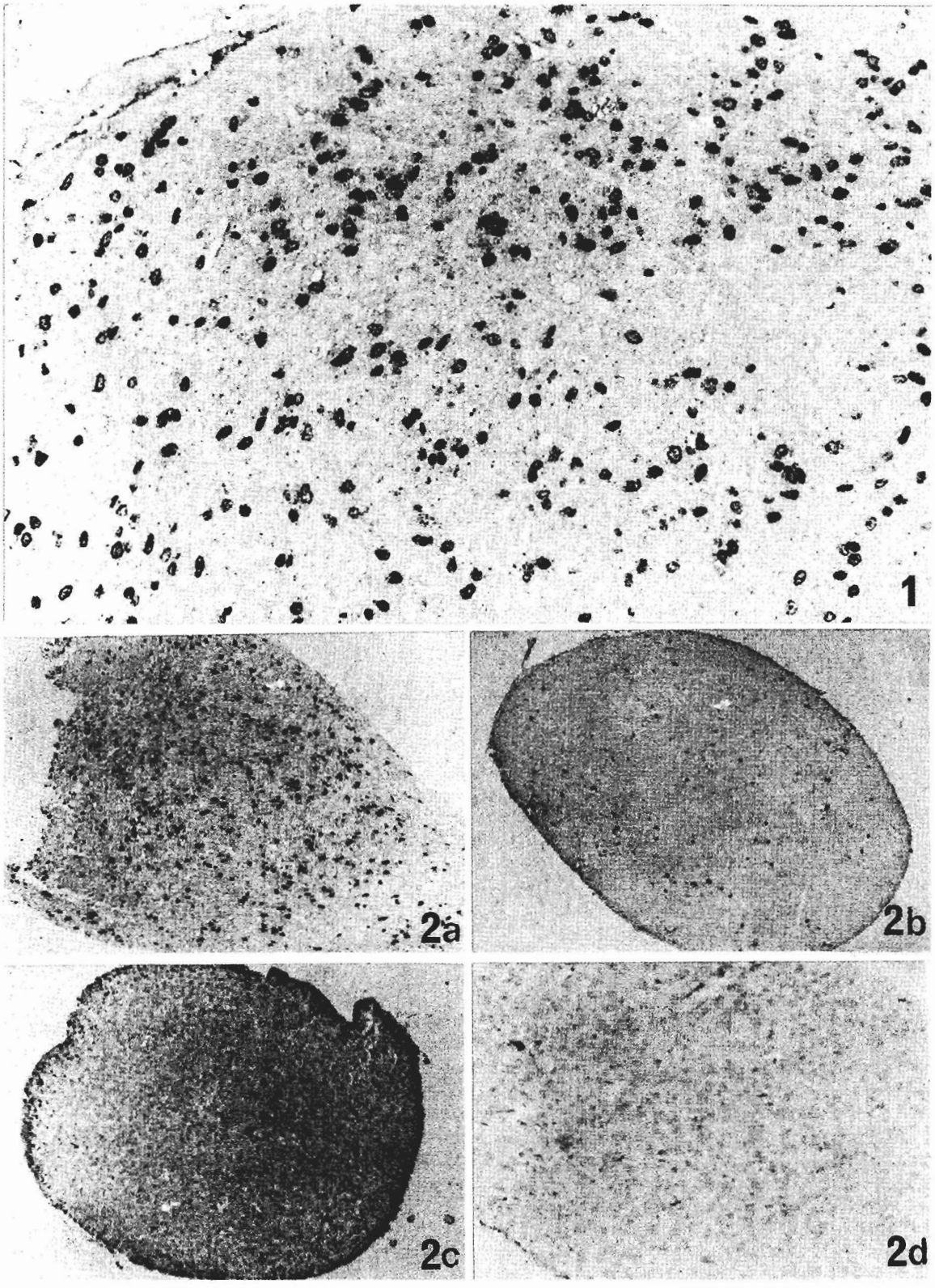

Fig. 1. BrdU-labelled nuclei clearly visible in a preparation corresponding to the pineal gland of a male rat at 1 day of age. x 338

Fig. 2. Pineal gland sections showing immunocyto-chemical detection of BrdU corresponding to male rats at 1 **(a)**, 7 **(b)**, 14 **(c)** and 28 **(d)** days of age. x 88

variation in the nuclear size of pinealocytes in different areas of the gland. Becker and Vollrath (1983) suggested that certain morphological parameters -such as pineal volume, nuclear size and mitotic activity- do not faithfully reflect circadian rhythmicity in melatonin synthesis by the pineal gland. However, Blázquez et al. (1989) report the existence of a certain correlation between melatonin concentrations and SNAT activity -on one hand- and the presence of dense bodies, lipid droplets, synaptic ribbons and mitotic bodies within the cytoplasm of pinealocytes and of granulated vesicles in pineal synaptic terminals, on the other.

As the BrdU-labelling of cells at the S-phase of the cell cycle has proved to be a reliable technique to evaluate cellular proliferation in growing tissues (Carbajo-Pérez and Watanabe, 1990; Ikeda and Yoshimoto, 1991; Carbajo et al., 1992) this technique was used here to depict as clearly as possible the pattern of cellular proliferation of the pineal gland during the postnatal period. By using this technique the problems linked to the evaluation of cellular proliferation based on the counting of mitoses were avoided.

As already pointed by Quay and Renzoni (1969) and Becker and Wollrath (1983) cellular proliferation in the pineal gland, as in many other endocrine organs (McNicol et al., 1989, Carbajo-Pérez et al. 1991) may follow a circadian rhythm. Thus, when designing our study protocol it was ensured that all animals would receive the labelling dose of BrdU at the same time of day. From a methodological point of view any fixed time round the clock might have served to guarantee an unbiased study, as far as circadian modifications of cellular proliferation are concerned. Based on the following reasoning the middle of the light period was decided as the time for the BrdU injection: there are data supporting the notion that the highest number of mitotic bodies appear in the pineal gland during the dark period (Becker and Vollrath 1983); if together with this we take into account that the wave of S phase precedes in about 6-9 hours the wave of mitotic cells as shown in different tissues (Barbason et al., 1987; Carbajo-Pérez et al. 1991) the peak of S phase cells in the pineal gland, if any, should be found toward the middle of the light period as already described in other endocrine organs (Carbajo-Pérez et al. 1991). Despite the above reasoning concerning methodological questions, at present it is not known whether or not the fraction of S phase cells of the rat pineal gland follows a circadian rhythm, and neither is it known if such a rhythm would already be present at early stages of postnatal development (studies are in progress to clarify these questions).

This study shows that the proliferative activity of pinealocytes decreases from birth up to the end of the seccond week, thereafter persisting at very low values until the end of the first month. Noteworthy is that the decrease in the proportion of S phase-calls was sharp during the first week after birth and then very gentle up to the end of the second week. This represents a slight but clear difference between the postnatal evolution of cellular proliferation in the pineal gland and that described in other endocrine organs such as the pituitary gland. In the pituitary gland either in the anterior (Carbajo-Pérez and Watanabe, 1990) or in the intermediate lobe (Carbajo et al. 1992) the steepest decrease in the proliferative activity is seen between the 7th and 14th postnatal days, while in the pineal gland, as reported here, on the 7th postnatal day the percentage of S phase cells is already relatively small. It is tempting to suggest that there is a link between the timing of the modifications of cellular proliferation and the evolution of morphology and function in the pineal gland. In this sense, shortly after the end of the first week, when cellular proliferation sharply decreases, the intrapineal levels of 5-HT and melatonin reach similar levels to those found in adult rats (Quay and Halevy, 1962; Zweig et al., 1966) and this is followed by the activation of SNAT and HIOMT enzymes (Klein and Lines 1969; Rudeen et al. 1975) and by the appearance in pinealocytes of the organelles related to the biosynthetic activity of the gland (Blázquez et al., 1989). Simultaneously to this sequence of events the ND of the pineal gland progressively decreases, this being interpreted chiefly as cell hypertrophy (Carbajo-Pérez and Watanabe 1990) and being closely linked to cellular maturation.

The results reported here support the idea that a strong expansion of the cellular components of the rat pineal gland takes place during the first week after birth and that concomitant with a severe reduction of the potential for growth the gland progressively achieves morphological and functional maturation. Further to this, this report opens the way for the study of old problems such as the correlation between circadian rhythms of melatonin secretion and rhythms of cellular proliferation and opens the way for further exploration of the relationship between functional and proliferative responses of the pineal gland.

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