Cell proliferation in the developing rat pineal gland. A bromodeoxyuridine immunohistochemical study

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Summary. The immunohistochemical detection of bromodeoxyuridine (BrdU) was used to study the cell proliferation in the developing rat pineal gland, from the appearance of pineal primordium in the embryonic day 15 (E15) until 30 days after birth. The results showed three different proliferative phases. From E15 to E21, the pineal gland shows a phase of rapid proliferation. The second phase corresponds to the first postnatal week, in which the number of labeled cells per surface unit decreases suddenly to values between 20% to 10% of those of embryonic period. From the second postnatal week onwards, the number of BrdU-positive cells progressively decreases.

Key words: Bromodeoxyuridine, BrdU, Pinealocytes, Pineal gland, Development, Proliferation

Introduction

The development of the pineal gland in the rat comprises three processes: 1) proliferation, i.e., mitosis of the immature pineal cells, to generate the cells present in the adult pineal gland; 2) morphogenesis, which includes changes in the form of the early pineal body, converting the initial evagination of the roof of the diencephalon into a tubular organ, followed by formation of the definitive solid gland; and 3) cell differentiation, which gives rise to the cell types present in the adult pineal gland.

The basic processes that occur during the embryonic development stage are proliferation and morphogenesis. Most of the studies made of the embryonic development of the rat pineal gland have focused on its morphogenesis - with only occasional comments on the abundance and location of the mitotic cells in the early pineal body (Kappers, 1960; Clabough, 1973; Calvo and Boya, 1981a,b; Fujieda et al., 1997). In a detailed study of the remodeling of the pineal epithelium during embryonic development, Fujieda et al. (1997) employed bromodeoxyuridine (BrdU) labeling to locate the

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proliferating cells, though they made no quantitative determinations of the labeled cells. In the postnatal period, both morphogenetic research of the changes in shape, size and location of the gland (Kappers, 1960; Blumfield and Tapp, 1970; Tapp and Blumfield, 1970; Calvo and Boya, 1984), and pinealocyte differentiation studies (Blumfield and Tapp, 1970; Calvo and Boya, 1983) have been made. Unlike in the prenatal period, some quantitative data on the proliferative activity of the pineal cells are available in the postnatal period (Quay and Levine, 1957; Wallace et al., 1969; Calvo and Boya, 1984); this information suggests that proliferation decreases rapidly in the first two weeks after birth.

The present study makes use of the immunohistochemical approach to detect bromodeoxyuridine (BrdU), with the aim of quantifying cell proliferation throughout the development period of the rat pineal gland, from the appearance of the early pineal primordium to 30 days after birth. As has been shown by many studies (Gratzner, 1982; DeFazio et al., 1987; Schutte et al., 1987; Miller and Nowakowski, 1988; Silvestrini et al., 1988; Magaud et al., 1989; Meyer et al., 1989; Böswald et al., 1990), this non-radioactive technique for the study of cell proliferation is simple, rapid, applicable to routinely processed material, and is as sensitive as tritiated thymidine based autoradiography.

Materials and methods

A total of 74 three-month-old adult female Wistar rats weighing 200-300 grams were used. The animals were housed in the laboratory with food and water *ad libitum*, and under controlled lighting conditions with a 12L:12D cycle (lights on: 8.00 am; lights off 20.00 pm).

Female rats were mated, and the day of the first appearance of spermatozoa in vaginal smears was denoted as embryonic day 0. The pregnant rats were injected intraperitoneally with BrdU (25 mg/kg dissolved in physiological saline) in the following gestational ages: E15, E16, E17, E18, E19, E20 and E21. One hour after injection, the fetuses were removed (at least two fetuses from four different pregnant rats in each stage). In the postnatal stage, BrdU was administered to the young rats at the same dose and

using the same route as before, in the following phases: newborn, P1, P2, P3, P4, P6, P7, P10, P15, P20, P25 and P30. Each phase was represented by at least four rats from different litters. The injected young rats were likewise sacrificed one hour after injection.

All the rats were sacrificed by decapitation under deep ether anesthesia. The cerebral block containing the pineal gland *in situ* was fixed in methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid) for 12-16 hours, at 4 ºC. Following embedding in paraffin, serial 7 μm sections were obtained and mounted on chromogelated slides.

Demonstration of BrdU taken up by the DNA was performed by the indirect immunoperoxidase method (Taylor, 1986), employing a monoclonal antibody. After deparaffinization of sections, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in metanol for 20 min. For denaturation of DNA, the sections were incubated in 2N ClH for 20 min at 37 ºC, followed by washing in 0.1M borate buffer and several PBS washes. The sections were sequentially incubated for 30 min in nonimmune rabbit serum (1:30) at room temperature, in mouse anti-BrdU monoclonal antibody (1:100) (Boehringer) at 4 ºC overnight, and in peroxidase-labeled rabbit anti-mouse secondary antibody (1:50) for 1 h at room temperature. The immunoreaction product was visualized with a freshly prepared solution of 3,3'-diaminobenzidine-tetrahydrocloride and the sections were finally counterstained with hematoxylin.

Application of the BrdU immunohistochemical technique to tissue sections not denaturalized by HCl, or to pineal gland sections from control rats not injected with BrdU yielded no immune staining.

The BrdU-labeled cells were counted in 8 zones measuring 26,643 μm^2 and selected on a random basis from each rat, using a semiautomatic image analyzing system (VIDS IV). The statistical analysis of the data was performed with the SPSS/PC+ statistical software package.

Results

The immunohistochemical technique for detecting BrdU showed the presence of labeled nuclei in all the phases studied. Labeling was always observed in interphase nuclei; in no case was mitotic or cytoplasmic labeling seen.

Figures 1-12 show the evolution in the number of BrdU-positive cells in a medial sagittal section of the pineal gland during 12 development phases, between E15 and P7. As is shown in Table 1, from P10 onwards, the number of labeled cells was very low; consequently, images of these phases are not shown.

In the early stages, the BrdU-positive cells were very abundant and were distributed homogeneously throughout the epithelium of the pineal primordium. Particularly in the first week after birth, the labeled cells tended to form irregularly shaped groups, while at the end of this week the labeled cells (now very scarce)

appeared isolated.

Table 1 and Fig. 13 show the labeling indices in the form of the number of BrdU-labeled cells per surface unit (26,643 μm^2) in all the phases studied. The number of labeled cells (mean ± standard deviation) is shown in Table 1. In all phases, the statistical analysis showed the data obtained to follow a normal distribution. The results of the global series were significant (p<0.001) according to analysis of variance (ANOVA).

Discussion

A first particularly apparent result during the embryonic period and first week after birth, was the high labeling index of the pineal gland compared with the neighboring structures (Figs. 1-12). Cell proliferation during pineal development is therefore a very intense and sustained phenomenon.

The labeled cell counts suggest the existence of three phases in cell proliferation in the course of pineal gland development. The first phase extends from the appearance of the pineal body in E15 to the end of the embryonic period. This is an intensely proliferative period, with values of about 230 labeled cells per 26,643 μm^2. The number of labeled cells increased rapidly up to E18, followed by an evident decrease - though high values persist in E21. To date, no quantitative data have been published on rat pineal gland cell proliferation during the embryonic period, though all the morphological studies conducted tend to comment on the abundance of mitoses during this period (Kappers, 1960; Clabough, 1973; Calvo and Boya, 1981a,b; Fujieda et al., 1997). As regards location, the labeled cells were homogeneously distributed throughout the pineal parenchyma during the embryonic period. This

Table 1. Number of BrdU-positive cells per surface unit during embryonic and postnatal pineal gland development

PHASE	NUMBER OF CELLS/26,643 μm^2
E15	168.5969±9.36343
E16	204.5756±3.5381
E17	256.7319±8.6776
E18	281.2195±7.1056
E19	256.7319±8.0994
E20	248.8269±10.6572
E21	216.9326±5.9307
P0	81.7407±3.2317
P1	54.9272±3.3650
P2	50.9440±3.2158
P3	48.4369±2.7841
P4	36.7167±2.6962
P6	22.0417±1.6119
P7	17.6056±1.2682
P10	5.3232±0.4129
P15	3.5728±0.3809
P20	1.0737±0.1012
P25	0.8392±0.1625
P30	0.3197±0.0879

Values are expressed as mean±standard deviation.

confirms the earlier results reported by Fujieda et al. (1997).

The second phase corresponds to the first postnatal week. Coinciding with the time of birth, the presence of labeled cells decreased suddenly. This marked drop has not been previously described. In the following days, the decrease continued, though at a slower rate; as a result,

by the end of the first week, labeling decreased to about 7% of the index recorded during the embryonic period, and to 20% of the index observed at the time of birth. The decrease in pineal mitotic activity in the first week after birth has been previously described in autoradiographic (Wallace et al., 1969) and morphological (Calvo and Boya, 1984) studies. This decrease coincides with
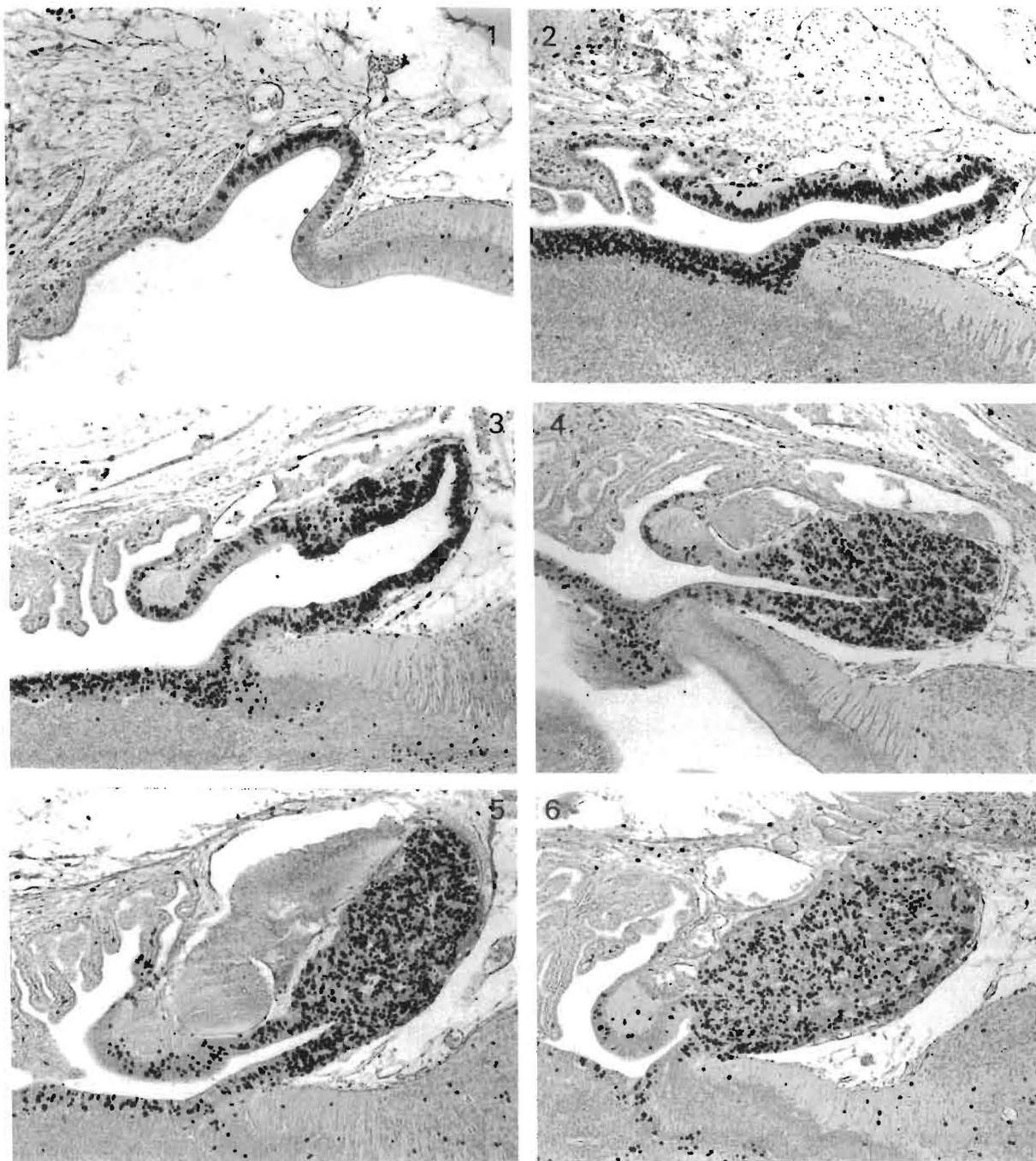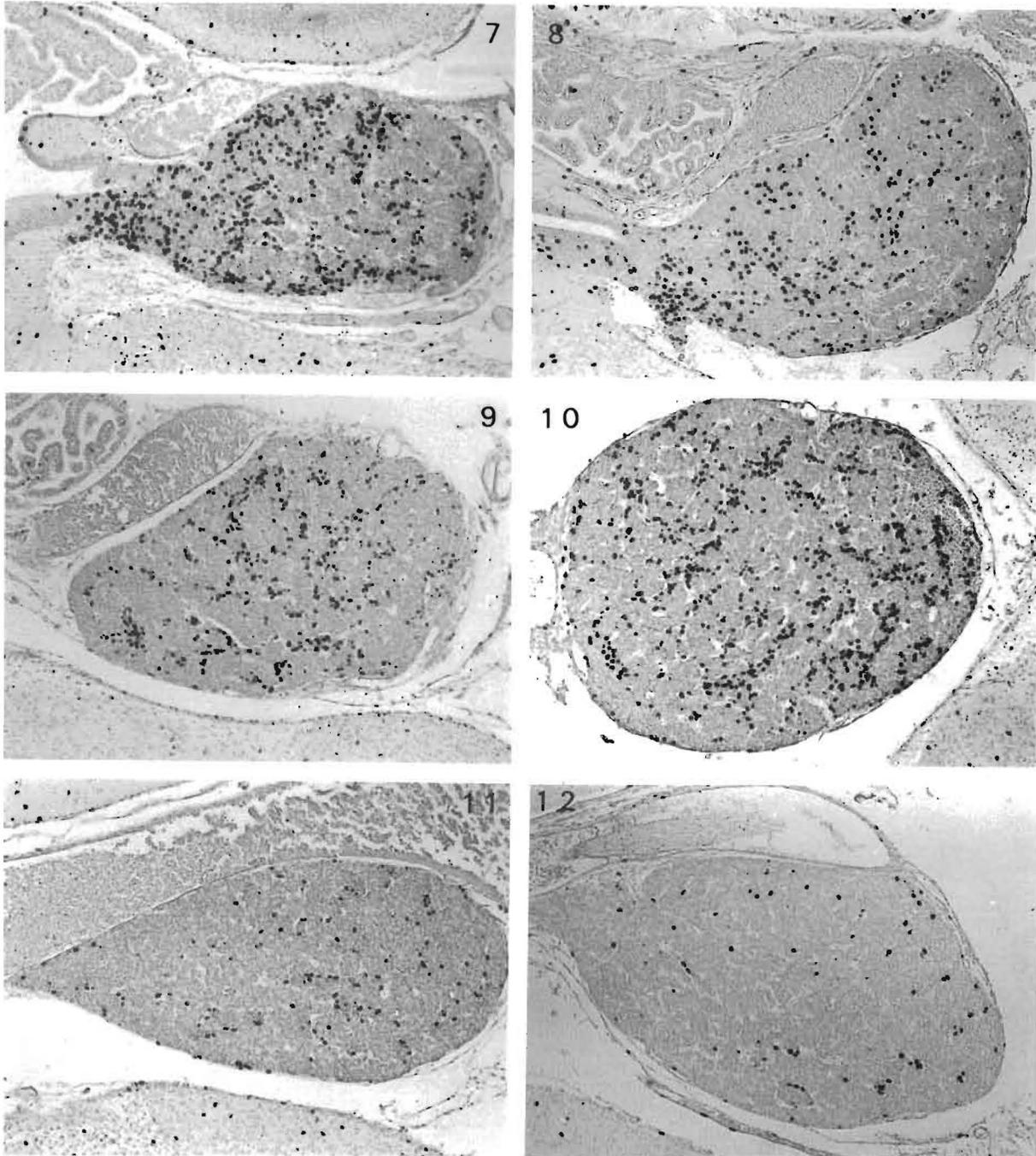

Figs. 1-6. Changes in the number of BrdU-labeled cells in a medial sagittal section of the pineal gland during the embryonic period, in the following phases: Fig. 1) E15; Fig. 2) E17; Fig. 3) E18; Fig. 4) E19; Fig. 5) E20; and Fig. 6) E21. Immunohistochemical technique for the detection of BrdU. x 65

the onset of morphological differentiation of the different pineal cell types (Karasek, 1974; Steinberg et al., 1981; Calvo and Boya, 1983) and biochemical differentiation of the gland - including not only the expression of specific enzymes but also their rhythmic activity (Håkanson et al. 1967; Ellison et al., 1972; Klein et al., 1981; Ribelaiga et al., 1998).

In the first postnatal week, the BrdU-labeled cells tend to form irregular groups. These groups correspond probably to immature cells or pinealoblasts previously described in light (Tapp and Blumfield, 1970; Calvo and Boya, 1984) and electron microscopic (Calvo and Boya, 1983) studies.

Finally, the third phase began in the second week

Figs 7-12. Changes in the number of BrdU-labeled cells in a medial sagittal section of the pineal gland in the first week after birth. The following phases are shown: Fig. 7) P0; Fig. 8) P1; Fig. 9) P3; Fig. 10) P4; Fig. 11) P6; and Fig. 12) P7. Immunohistochemical technique for the detection of BrdU. x 65
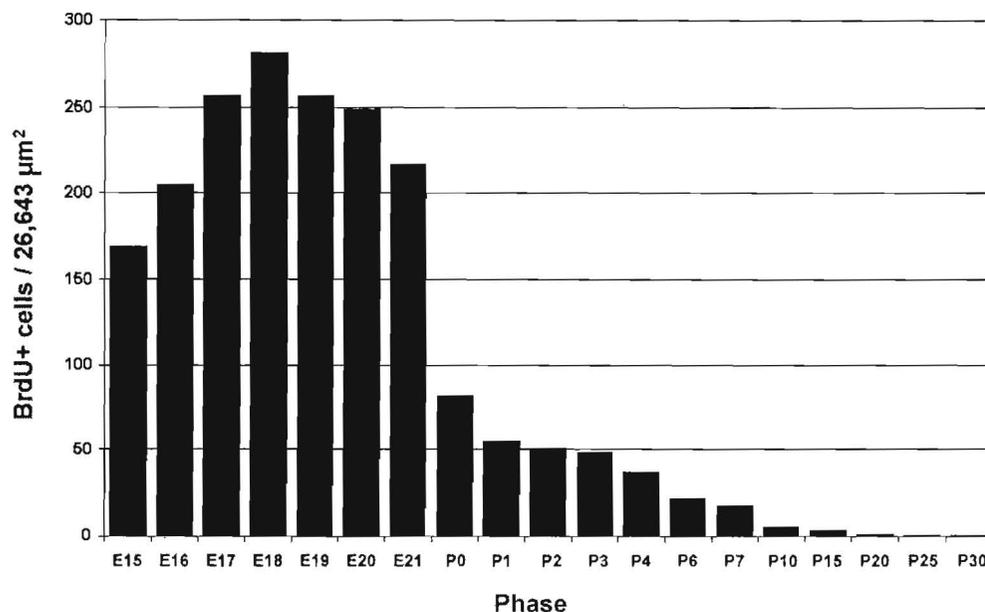
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Fig. 13. Variations in the number of BrdU-labeled cells per surface unit (26,643 μm^2) during rat pineal gland development.

after birth. Between P7 and P10 a sudden decrease in labeling (almost one third) was again recorded. From this point onwards, the decrease became slow and gradual, until very low indices were recorded at the end of the first month.

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