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Time of origin of the rat pineal gland cells. A bromodeoxyuridine immunohistochemical study

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Summary. The immunohistochemical detection of bromodeoxyuridine (BrdU) was used to study the time of origin of the cells in the pineal gland of the rat. A study was made involving 17 groups of 4 rats each, administered with a single dose of bromodeoxyuridine (BrdU, 25 mg/kg) in 7 phases of the embryonic period (E15 to E21) and in 10 postnatal phases (between P0 and P30), followed by determination in each rat of the number of visible immune-labeled cells in the pineal gland 60 days after birth. The results show that approximately 60% of the pineal cells underwent the last division(s) prior to differentiation in the prenatal period between E18 and E21. The rest of the pineal cells originated after birth, particularly in the first 5 postnatal days.

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

Introduction

The mammalian pineal gland originates as an evagination of the roof of the diencephalon. In the rat, this evagination appears in 15.5-day-old embryos. Three types of processes transform this initial formation into the adult pineal gland: 1) cell proliferation; 2) morphogenetic changes; and 3) cell differentiation. The morphogenetic changes take place particularly in the embryonic period and consist of transformation of the pineal evagination into a tubular organ, the lumen of which (or pineal recess) ultimately disappears, giving rise to the solid appearance of the adult gland (Kappers, 1960; Clabough, 1973; Calvo and Boya 1981a,b; Fujieda et al., 1997). Cell differentiation in turn includes: a) appearance of the cells characteristic of the adult pineal gland (pinealocytes and interstitial or glial cells), and b) hypertrophy and development of the ultrastructural, biochemical and functional phenotype of these cell types. In the rat, the morphological differentiation of the pineal cell types takes place between the last days of embryonic development and the second postnatal week (Karasek, 1974; Steinberg et al., 1981; Calvo and Boya, 1981a, 1983). The growth of the pineal gland from the second postnatal week onwards is mainly due to both hypertrophy and morphological, biochemical and functional differentiation of the pinealocytes (Kappers, 1960; Blumfield and Tapp, 1970; Tapp and Blumfield, 1970; Calvo and Boya, 1983, 1984).

In all morphological studies of the development and postnatal evolution of the rat pineal gland, the presence of abundant mitoses during embryonic development and early postnatal phases is described (Kappers, 1960; Blumfield and Tapp, 1970; Tapp and Blumfield, 1970; Clabough, 1973; Calvo and Boya 1981a,b, 1983, 1984; Fujieda et al., 1997). Markers of DNA synthesis such as tritiated thymidine (H^3-thymidine) or bromodeoxyuridine (BrdU) have been used to quantify pineal proliferative activity during the embryonic (Calvo et al., 2000) and postnatal periods (Wallace et al., 1969; Carbajo et al., 1998; Calvo et al., 2000). In all these studies the quantification of cell proliferation has been based on the counting of labeled cells in samples harvested shortly after administration of the marker (normally one hour).

Labeling with BrdU or H^3-thymidine can also be used in application to long-term studies. Such studies are based on two principles: a) the marker (BrdU or H3-thymidine) is available for incorporation to DNA for only a short period of time (about one hour) after administration; and b) DNA is metabolically stable, as a result of which cell labeling intensity only decreases with successive cell division (being reduced by half with each division). In the course of organ development, if the timing of labeling coincides with the last cell divisions prior to differentiation, these cells will retain intense labeling when studied in the adult organ. In this way, by administering the marker in different phases of organ development, it is possible to determine the moment in which the cells present in the adult organ originate. Only one study of this kind has been published to date in relation to the pineal gland, though it is limited to very few postnatal phases (Wallace et al., 1969). Our present

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study uses BrdU labeling to determine the moment in which the pineal gland cells originate in the rat. To this effect we injected BrdU throughout pineal gland development, both in the embryonic stage (7 phases, from E15 to E21) and in the postnatal period (10 phases, from P0 to P30), followed by determination of the remaining number of labeled cells in the pineal gland 60 days after birth.

Materials and methods

A total of 68 Wistar rats 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) at the following gestational ages: E15, E16, E17, E18, E19, E20 and E21. After birth, the young animals were kept alive for 60 days, followed by sacrifice and sampling. In the postnatal group, BrdU was administered to the young rats at the same dose and using the same route as before, in the following phases: newborn (P0), P1, P3, P4, P5, P10, P15, P20, P25 and P30. All these rats were likewise sacrificed after 60 days. Each phase was represented by at least four rats from different litters.

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 °C. 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 2 N ClH for 20min at 37°C, followed by washing in 0.1 M 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 °C 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-tetrahydrochloride 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 and non-labeled nuclei 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 used to detect BrdU revealed the presence of labeled nuclei in all the phases studied, from E17 onwards. Labeling was always seen in interphase nuclei – no labeled mitotic figures or cytoplasmic labeling being observed at any time.

Figs. 1-12 show the evolution of the number of BrdU-positive cells in a middle sagittal section of the pineal gland in 10 phases between E17 and P30. Table 1 shows the total number of labeled cells per unit surface area (26,643 μm^2) (mean ± standard deviation) in all the

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
E17	8.5555±1.4307
E18	43.6667±3.3994
E19	64.8333±4.5549
E20	94.4375±6.033
E21	79.0000±4.579
P0	51.7619±3.1583
P1	44.5000±2.6695
P3	40.0000±2.831
P4	32.0556±2.8256
P5	30.3333±3.0553
P10	1.8170±0.1225
P15	1.1722±0.0841
P20	0.4103±0.0313
P25	0.3170±0.0247
P30	0.1545±0.0173

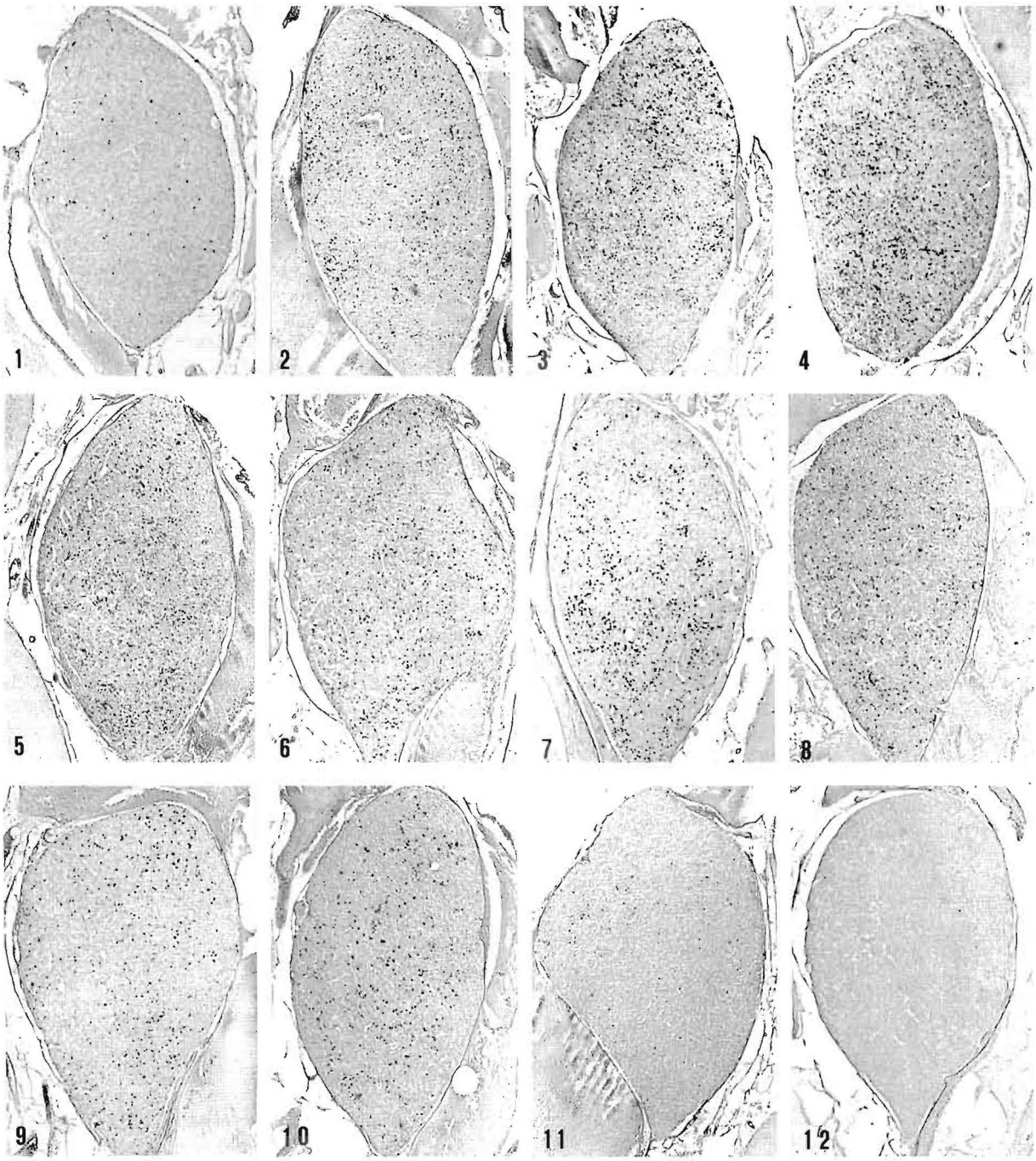
Values are expressed as mean ± standard deviation

Table 2. Labelling index expressed as number of BrdU+ cells per 100 cells.

PHASE	BrdU+ CELLS/100 CELLS
E17	2.43
E18	13.80
E19	21.96
E20	36.81
E21	28.11
P0	16.79
P1	14.10
P3	12.50
P4	9.77
P5	9.20
P10	0.51
P15	0.33
P20	0.11
P25	0.09
P30	0.04

phases studied. These means are graphically reflected in Figure 13.

The light microscopic study of the labeled cells revealed variations in labeling intensity from one cell to another, with the distinction of two different types of cell: strongly labeled cells, with intense immune-labeling of the entire nucleus, and weakly labeled cells containing only a few immunopositive granules with no labeling elsewhere in the nucleus. In Figure 14, the total BrdU-positive cells in phases between E18 and P5 are



Figs. 1-12. Mid-sagittal section of the rat pineal gland showing the BrdU-positive cells at postnatal day 60 when BrdU administration is carried out in phases E17 **(Fig. 1)**, E18 **(Fig. 2)**, E19 **(Fig. 3)**, E20 **(Fig. 4)**; E21 **(Fig. 5)**, P0 **(Fig. 6)**, P1 **(Fig. 7)**, P3 **(Fig. 8)**, P4 **(Fig. 9)**, P5 **(Fig. 10)**, P10 **(Fig. 11)**, and P30 **(Fig. 12).**

differentiated into each of these two cell types. Data from P10 onwards are not included because of the small number of labeled cells present in these later phases.

Table 2 shows the corresponding labeling indices, i.e., the proportion of labeled cells per 100 pineal cells, in the phases studied.

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

Our results show cell generation in the pineal gland to take place over a prolonged period, mainly between phases E17 and P5. The only previous study of this kind published in the literature (Wallace et al., 1969) reported most of cell proliferation in the rat pineal gland taking place in the postnatal period. However, this earlier study did not include prenatal phases, as a result of which the authors were unable to directly assess prenatal participation. In contrast, our data show approximately 60% of the rat pineal gland cells to be originated before birth.

On summing up the percentages of labeled cells from all the phases studied, the result exceeds 100% (reaching 166.5%). This is probably because the pineal cells maintain their proliferative activity for a number of days, and thus a given cell will be marked in more than one phase, for as long as its proliferative activity persists. Our results relating to the proportion of intensely and weakly labeled cells suggest that cell proliferation in the pineal gland is quite limited in time, and that cells labeled in a given phase undergo few divisions before differentiating. If this interpretation is correct, then cell proliferation during rat pineal development could be maintained by a pool of immature cells from which the pineal cells differentiate (thereby leaving the mitotic cycle) – particularly in phases E18 and P5. These immature cells have been described as being numerous in the embryonic period and, in the form of cell groups or nests, also in the early postnatal period (Calvo and Boya, 1983; Calvo et al., 2000).

On contrasting our findings with those reported by Wallace et al. (1969), the number of the labeled cells of the latter study (deduced from graphic representations, since no numerical data were supplied) are clearly far superior to our results. According to Wallace et al. (1969), on injecting H^3-thymidine in 6-hour-old rats, up to 60% of the adult pineal gland cells appear labeled. If injection is performed at two days of life, the corresponding percentage is reportedly 50%. These percentages seem very high, particularly when considering that, according to our data, postnatal proliferation generates less than half of the cells found in the adult pineal gland. Examination of the graphic representations provided by Wallace et al. (1969), shows that the immense majority of the labeled cells (over 95%) in the adult phase correspond to what the authors refer to as "lightly labeled cells", with a labeling intensity only slightly greater than background. In contrast, in our study, the proportion of weakly labeled cells throughout the postnatal period was around 50%. An explanation for this could be the lower immunohistochemical sensitivity of BrdU in detecting weak labeling. Nevertheless, according many authors this technique lacks background staining, and moreover has been shown to be as sensitive as autoradiography with H^3-thymidine (Gratzner, 1982; Schutte et al., 1987; DeFazio et al., 1987; Miller and Nowakowski, 1988; Silvestrini et al., 1988; Magaud et al., 1989; Meyer et al., 1989; Böswald et al., 1990). Alternatively, it is also possible that many of these weakly labeled cells described by Wallace et al. (1969), might actually be attributable to background labeling rather than to true cell labeling. On the other hand, according to the data presented by Wallace et al. (1969), the pineal cells appear to maintain a constant and notable proliferative

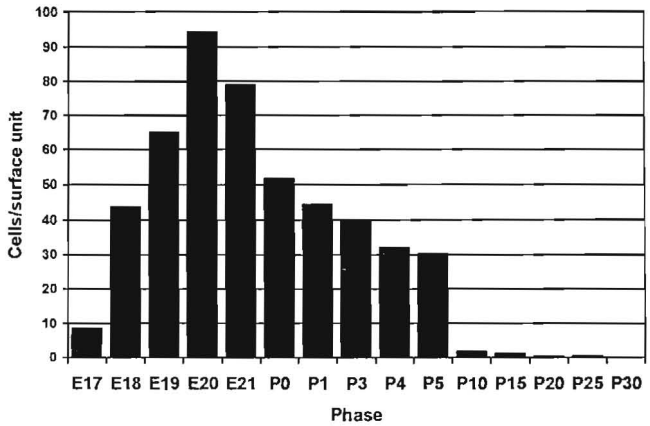


Fig. 13. Variations in the number of BrdU-labeled cells per surface unit (26,643 mm^2) during rat pineal gland development.

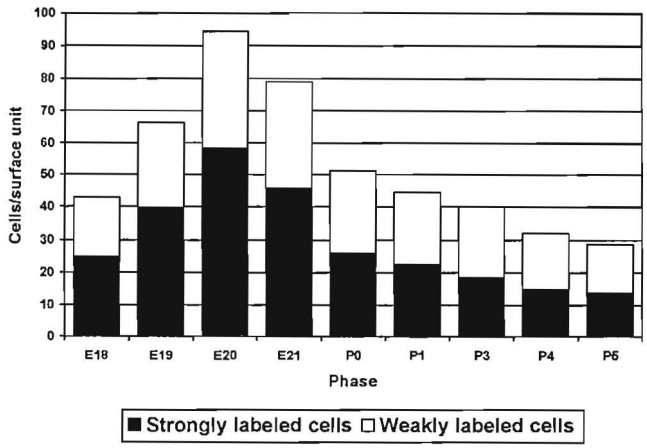


Fig. 14. Proportions of strongly and weakly labeled cells between E18 and P5.

activity throughout postnatal life. In effect, according to these authors, as time elapses after the injection of H^3-thymidine, the percentage of intensely labeled cells decreases, with a correlative increase in the proportion of "lightly labeled cells". This indicates that the intensely labeled cells are dividing and thereby diluting the marker. The problem is that, according to Wallace et al. (1969), this decrease continues in the adult pineal gland. Thus, rats injected in the neonatal period and studied at 60 days of age, show labeling of 50% of the cells (6% strongly labeled and 44% lightly labeled), while after 120 days, these same rats show labeling of 60% of the cells (2% strongly labeled and 58% lightly labeled). However, the studies of postnatal development of the pineal gland coincide that pineal proliferative activity after 60 days of age is practically absent (for review, see Vollrath, 1981; Calvo et al., 1997, 2000). Lastly, there are also other discrepancies between the results published by Wallace et al. (1969) and those of posterior reports. In effect, various studies conducted with different methods agree that a sudden drop in rat pineal gland cell proliferation takes place at around 7-10 days after birth (see review in Vollrath, 1981). This drop causes proliferation in the pineal gland after 15 days to be less than 1/20 of that present in one-day-old rats (Carbajo et al. 1998, Calvo et. al. 2000). However, according to Wallace et al. (1969), the percentage of labeled cells one hour after injection decreases only from 15% in 6-hour-old rats to 10% in 13-day-old animals.

The present results regarding the timing of the origin of the adult pineal gland cells, together with our earlier work demonstrating cell proliferation in the developing gland (Calvo et al., 2000), provide a comprehensive view of cell proliferation and differentiation during rat pineal gland development. Furthermore, the present study provides data not previously reported regarding cell differentiation in the prenatal period of rat pineal development. Likewise, our results suggest that changes are required in our conception of how cells are generated in the rat pineal gland – based up to this point on the single autoradiographic study on the subject published by Wallace et al. (1969). In contrast to the hypothesis of these authors, it seems that most pineal cells are generated before birth. Future experimental studies of the modifications in pineal gland cellularity should take these new findings into account.

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