Influence of the light and dark phase of the cycle on the cellular proliferation in the pineal gland of the adult rat: A bromodeoxyuridine immunohistochemical study

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Abstract: The cellular proliferative activity in the adult rat pineal gland was studied using bromodeoxyuridine immunohistochemistry during the light and dark phases of an artificial 12L:12D photoperiod. The results showed statistically significant differences in the number of labeled cells between the light and dark phases, with the labeled cells being almost threefold more abundant in the light period. Minor changes were also found in the pineal gland volume between both periods. The decrease in the number of labeled cells in the dark phase of the cycle could be related to the well-documented antimitotic action of melatonin.

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Introduction

One of the more peculiar features of the pineal gland is its capability to show light–dark cycle changes. Numerous studies have described the existence of circadian rhythms in virtually every characteristic of the pineal gland, from the weight of the gland to its enzymatic activity levels [for review, see Vollrath, 1981; Klein, 1984].

In adult animals, pinealocytes are commonly described as nonproliferative cells. However, several studies during the fifties and the sixties showed that the mitotic activity of adult pinealocytes is not null, although the mitotic index values were described as extremely low [Quay and Levine, 1957a,b; Wallace et al., 1969]. Moreover, a circadian rhythm in the number of pinealocyte mitoses has been reported [Renzoni and Quay, 1964; Quay and Renzoni, 1966a]. Nevertheless, we still lack clear and confident data on the mitotic index of adult pinealocytes and its variations within the dark–light cycle. In fact, the investigations of Quay and Levine [1957a,b], Quay and Renzoni [1966], and Renzoni and Quay

[1964] were based upon a technique—metaphase counting with or without previous administration of colchicine—that is now considered a rather insensitive tool for the evaluation of mitotic activity. On the other hand, Wallace et al. [1969] used tritiated thymidine autoradiography, although their study focused on the postnatal development of the pineal gland. According to these authors, the percentage of marked pineal cells in 120-day-old rats appeared close to 0%, as shown in the graphics in their papers (no numerical data are included).

More recently, the immunohistochemical detection of bromodeoxyuridine (BrdU) has been introduced as a reliable method of studying cellular proliferation. This compound is incorporated in replicating DNA instead of thymidine and can then be detected by specific monoclonal antibodies. As extensive research has demonstrated [Gratzner, 1982; Schutte et al., 1987a,b; DeFazio et al., 1987; Miller and Nowakowski, 1988; Magaud et al., 1989; Meyer et al., 1989], this technique can be carried out in routine processed paraffin-embedded sections; it is

rapid, nonradioactive, and as sensible as tritiated-thymidine autoradiography.

In the present study, we report the presence of a light–dark rhythm in the proliferative cellular activity of the adult male rat.

Materials and methods

Twenty 3-month-old adult male Wistar rats, 200 to 300 g, were used in this study. Rats were kept in the laboratory with food and water ad libitum and under controlled lighting conditions with a 12D:12L cycle (lights on at 08:00; off at 20:00).

Ten rats (group LIGHT) received three intraperitoneal injections of BrdU (25 mg/Kg in saline) at 08:00, 12:00, and 16:00. Sacrifice was carried out at 19:00, one hour before the lights were turned off. The other ten animals (group DARK) received the same dose of BrdU at 20:00, 00:00, and 04:00 and were killed at 07:00, one hour before the lights were turned on. BrdU injections were administered under a very low intensity red light. Rats were killed by decapitation under deep ether anesthesia. A tissue brain block containing the pineal gland in situ was fixed by immersion in methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) for 12–16 hr at 4°C. After paraffin-embedding, serial sections 7 μm thick were mounted in chromagel-coated slides for immunolabeling.

Immunolabeling of BrdU incorporated in the replicating DNA was carried out using the immunoperoxidase indirect method [Taylor, 1986] with a monoclonal antibody. After deparaffinization of the sections, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min. For denaturation of DNA, the sections were incubated in 2N HCl for 20 min at 37°C, 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°C overnight, and in peroxidase-labeled rabbit anti-mouse secondary antibody (1:50) for 1 hr 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.

Negative controls consisted of immunostaining sections nondenatured with HCl and of rat pineal glands that were not treated with BrdU. In both cases, a lack of immunostaining was observed.

BrdU-marked cells were counted in one of four sections using a semiquantitative image analysis system (VIDS-IV) by a reader who was blind to the treatment groups. The volume of the gland was also estimated by measuring the surface area of the pineal gland in each of the sections. Statistical analysis (Student's t-test) was performed with a SPSS/PC+ statistical software package.

Results

The immunohistochemical detection of BrdU showed labeled nuclei in the pineal gland. These nuclei were widespread and located throughout the gland, showing no tendency to a specific topographical distribution. Immunostaining appeared mainly in interphase nuclei, the presence of positive mitosis or paired nuclei being extremely rare. Table 1 shows the counts and measurements.

Discussion

Our results demonstrate that there is cellular proliferative activity in the adult rat pineal gland and that this activity varies depending on the light–dark cycle.

Assuming that the mean duration of the S phase is about eight hours, the dosing schedule in this experiment (three doses of BrdU, one every four hours for a period of 12 hours) would permit us to stain all the cells that initiate DNA replication in each period of the cycle. Several other technical considerations should be pointed out to support the validity of the data. First, we chose an artificial 12L:12D cycle to avoid any potential effect of the different duration of the natural light–dark periods on the population of BrdU-positive cells. Moreover, BrdU administration was done the same day to both groups in order to avoid hypothetical seasonal or day-to-day differences. Second, given the well-known influence of the estrous cycle on the pineal gland activity, we restricted our analysis to male rats. On the other hand, the counting of BrdU immunopositive cells was performed in the whole gland, serially cut to prevent section sample bias. Finally, counting was carried out by an investigator blind to the study group distribution, in one of four 7 μm-thick sections, with the aim of not repeating the count of the same nuclei in consecutive sections. Given this methodological approach, we

Table 1. Influence of light and dark phases of the cycle on pineal gland volume and BrdU-positive cells

	Group LIGHT	Group DARK	P value
Gland volume (mm^3)	0.445 ± 0.019	0.37993 ± 0.02	<0.05
BrdU-positive cells/gland	1,908 ± 229	675.3 ± 84.3	<0.001

Values are expressed as mean ± standard deviation.

believe that our findings should represent the real proliferative activity of the adult pineal gland in this species.

BrdU immunostaining was mainly found in interphase nuclei, positive mitotic figures and paired nuclei being almost negligible. Therefore, the vast majority of the cells that incorporated BrdU in their DNA had not yet started the mitotic process at the moment of sacrifice.

According to previous reports, cellular proliferation in the adult rat pineal gland is very scarce. Quay and Levine [1957a,b], using a colchicine technique, described a decrease of the mitotic index from the first postnatal month to trace values thereafter. Quay and Renzoni [1966], with the same method, reported that at the time of peak proliferative activity in the adult rat, no more than nine mitotic figures per gland were found. However, the low sensitivity of the colchicine method for evaluating cell proliferation probably explains the low proliferation figures that these investigators reported.

According to the graphics of Wallace et al. [1969], in which a more accurate method of evaluating cell proliferation was used (tritiated thymidine autoradiography), there is almost negligible proliferative activity in the 120-day-old rat (no numerical data were shown). However, the design of the experiment was not highly reliable given the small number of animals (n = 2) and that the counts were done in a single middle section of the gland.

The results of our study show that the cellular proliferative activity of the adult rat pineal gland varies depending on the phase of the light–dark cycle, with the highest proliferative activity during the light phase and the lowest during darkness. Proliferative activity during the light phase is almost threefold higher than in the dark phase. This trend has been previously reported by Renzoni and Quay [1964] and Quay and Renzoni [1966]. On the other hand, Nir et al. [1971] have also shown an increase in the pineal DNA content during the light phase of the cycle, although the difference was not statistically significant. Our results confirm these observations and provide the approximate number of cells that begin DNA replication in each phase of the cycle.

The explanation for this difference in the proliferative activity of the gland depending on the light–dark cycle could be related to the well-documented antimitotic action of melatonin [Banerjee and Margulis, 1973; Lapin and Ebels, 1981; Blask, 1984]. Several studies have demonstrated that melatonin decreases the mitotic activity of many cell types and it has even been claimed that this hormone may play a role as an antineoplastic agent. Our results can be interpreted as indirect support of the antimitotic action of melatonin, given the concurrent nocturnal increase of melatonin levels and the lower cellular proliferative activity during the dark phase of the cycle.

Our study has also shown that pineal volume exhibits some minor changes, although statistically significant, depending on the phase of the light–dark cycle, with the maximum volume reached during the light phase. However, it would be hard to assume that these differences are due to changes in the parenchyma of the gland, given the rapid oscillations that appear. It seems more reasonable that the changes in the volume of the gland are secondary to variations in the connective tissue stroma (dilatation of the connective spaces, blood supply increase) and/or of the intercellular spaces [Krstic, 1975]. Thus, as Quay [1974] reported, pineal canaliculi show a circadian rhythm, being broad during the day and narrow during the night.

The methodology that we used in the study does not allow us to identify accurately the proliferating pineal cell type. Some of the immunostained cells are located close to blood vessels and could potentially correspond to connective cells or perhaps to wandering cells. Many other cells are unequivocally located within the parenchymal cell cords, but the specific parenchymal cellular type is even more difficult to identify. Many of these marked parenchymal cells display elongated nuclei without evident nucleoli, and this suggests that they may be glial pineal cells. Moreover, we have previously shown that the population of this particular cell type increases in adult and very old rats [Boya and Calvo, 1984; López-Muñoz et al., 1991; Borregón et al., 1993]. Further studies are needed to identify the exact nature of the proliferating cell types in the adult rat pineal gland.

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