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Morphometric analysis of photoreceptive, neuronal and endocrinal cell differentiation of avian pineal cells: An *in vitro* immunohistochemical study on the developmental transition from neuronal to photo-endocrinal property

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ABSTRACT—Little is known about the developmental origin, determination and differentiation of different pineal immunoreactive cells in the avian group, and an experimental establishment is then required to explain the differentiation of cell types (i.e. photosensory, neural and secretory types). The present in vitro study suggests that the avian pineal organ is made up of multiple types of cells with different immunoreactivity at the ontogenic state (from embryonic day 9 to day 14), before it acquires the final photoendocrinal nature of the mature state. The morphometric analysis suggests that the developmental changes in the morphology of the quail pinealocytes appear to represent a condensed expression of the phylogenic development in the ontogeny. Several types of immunoreactive cells from a neuronal line were suppressed with maturation of developing pineal glands, while other cell types such as photoreceptive and endocrinal lines became more prominent. The melatonin level in the culture medium presented a high value up to 72 hr of culture, followed by a decrease as well as dampening of the level at the end of the culture possibly because the cultures were maintained in dark. The results of the present study, a combined analysis of morphometry and RIA, open a new line for research into the pineal development and cell differentiation.

Key words: pineal gland, quail, photoreceptor, neuron, melatonin

INTRODUCTION

The avian pineal organ exhibits remarkable differences in morphology and physiology compared to the mammalian pineal. The phylogenetic and ultrastructural peculiarities of the avian pineal gland have been well described in numerous studies (Collin, 1971; Oksche, 1971; Vollrath, 1981; Oksche *et al*., 1987). Further, the avian pineal organ is a favorite model for biologists to look into various functions of this enigmatic organ. Embryological studies have been performed using both morphological and biochemical techniques mainly with the chick pineal organ (Calvo and Boya, 1978; Oshima and Matsuo, 1988; Sato, 2001). The circadian oscillator and its mechanism of function including gene expression for melatonin have been established to some extent in the avian pineal gland (Nakahara *et al*., 1997). However, the avian pineal organ still needs elucidation in the broad sense of the ontogenic differentiation of various cell types (i.e. photosensory and secretory type). Further, little is known about the developmental origin of the organ and the molecular mechanisms involved in phenotypic determination and differentiation of pineal cells in the avian group.

Our understanding of the multipotentiality of pineal cells is based mainly on experiments using developing avian embryos (Araki, 2001). Avian pineal cells exhibit a wide spectrum of potentiality for phenotypic expression under cell culture conditions, including several types of photoreceptors, serotonin cells, HPC-1/Syntaxin positive cells and so on (Araki *et al*., 1992; 1993). We describe here a morphometric analysis of the developmental potency of different immunoreactive cells in the quail pineal cell culture in order to answer the following questions:

1. How does the developmental potency alter during onto-

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genic development of the avian pineal gland? To this end we cultured pineal cells from various embryonic stages, and performed immunostaining for iodopsin and pinopsin as photoreceptive phenotype, tyrosine hydroxylase and HPC-1 as neuronal phenotype, and serotonin and AVP as endocrinal phenotype.

2. How is cytodifferentiation, especially the development of sensory structures and hormone production of melatonin, linked in the developing quail pineal gland?

In view of melatonin's wide spread effect, the organs and cells producing melatonin and their fate during phylogeny are of considerable neurobiological interest. Aspects of photoreceptor pigments and phototransduction underline firm evolutionary and functional links between melatonin production and the generation of circadian rhythms. The capacity of the modified photoreceptor cells to produce melatonin has been corroborated by immunocytochemical demonstration of serotonin and HIOMT in these cells by various workers (Murakami *et al*., 1994; Nakahara *et al*., 1997). In the present study we recorded the melatonin content in the medium of alternate day culture using the RIA method described by Vakkuri *et al*. (1984). By comparing the results of melatonin secretion with those of morphometric analysis, it becomes possible to investigate the responses of biological systems to different stimuli and to study the effects of different agents which influence both secretory and intracellular molecules.

MATERIAL AND METHODS

Animals

Quail eggs were purchased from a local hatchery (Tokai-Yuki, Aichi, Japan) and were incubated in a dark humidified incubator at 38°C for the desired number of days. Principles of laboratory animal care were always followed during the present study.

Pineal disperse cell culture

Quail eggs were incubated in different batches. The disperse pineal cell culture was performed from pineal glands at embryonic 9th (E9), 11th (E11) and 14th (E14) days of incubation. Pineals were dissected out from embryos, and separated from extra tissues in Hanks' solution. After washing with phosphate buffered saline {PBS (–)}, pineals were treated with 0.25% trypsin (GIBCO BRL, Rockville, U.S.A.) in PBS(–) for 20 min at 37°C. Following further

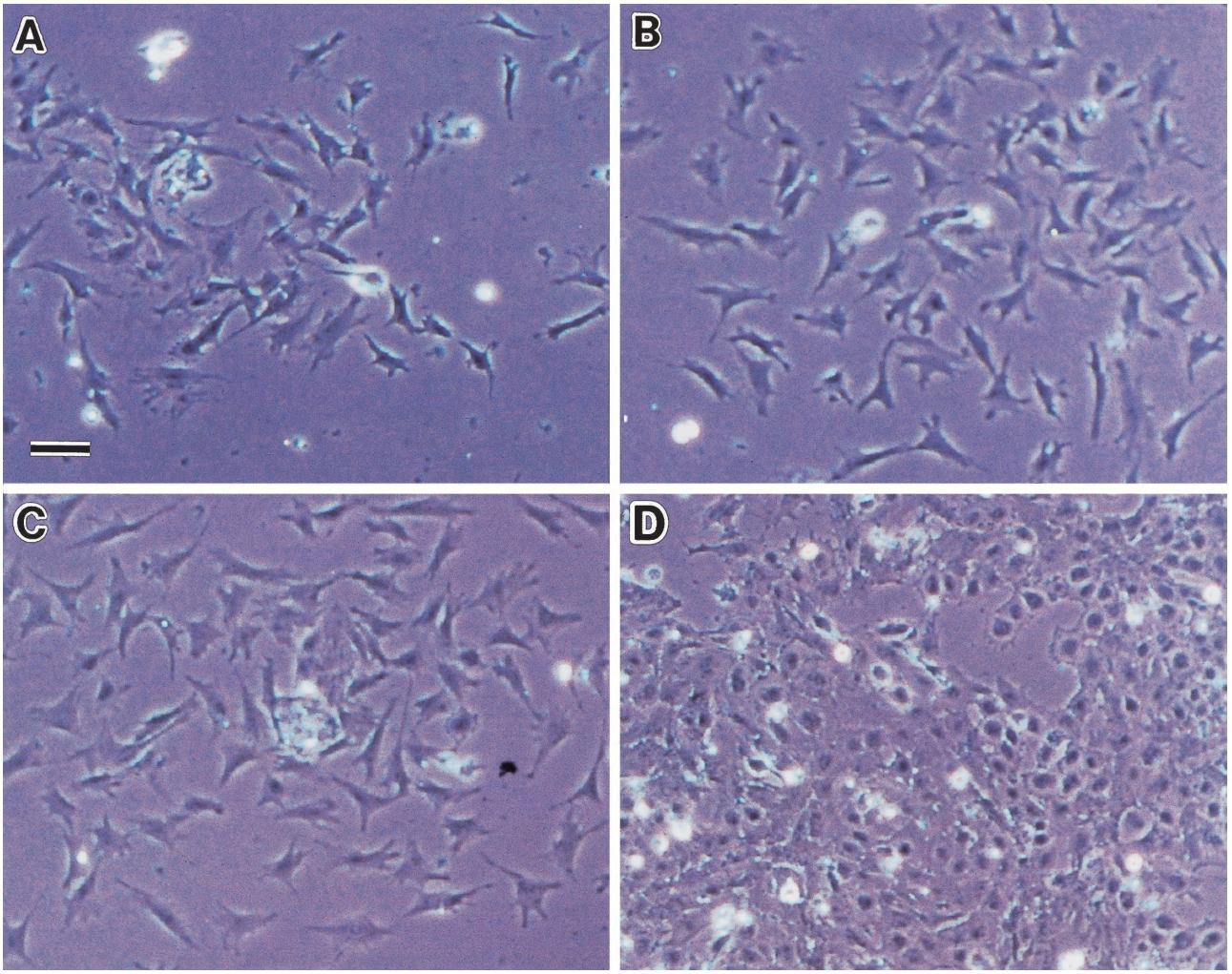

Fig. 1. Phase contrast microphotographs showing cultured pineal cells from (A) E9, (B) E11, (C) E14 embryos after 2 days of culture. (D) After 6 days of culture they showed a similar appearance to each other. Bar in (A) is 20 μm and applies to B, C and D.

washes in culture medium, they were dissociated by being gently pipetted through a Pasteur pipette. Dissociated cells derived from 2 pineals were seeded onto one plastic dish (35 mm, Iwaki Glass) pre-coated with collagen (Cell matrix, type-1-C, Nitta Gelatin, Osaka, Japan). The culture medium was Dulbecco's Modified Eagle MEM supplemented with Kanamycin sulfate (6 mg/dl), sodium pyruvate (5mg/dl), 0.6% glucose, and 6% FBS (fetal bovine serum). The medium was replaced every alternate day. The growth and vitality of culture were recorded by taking photographs under a phase-contrast microscope (Fig. 1).

After 6 days of culture, cultures were fixed for immunohistochemistry with glutaraldehyde (0.3%) for 10 minutes and then with paraformaldehyde (2%) in phosphate buffer (50 mM, pH 7.4) for 2–3 hr on ice. After several washes in PBS, cultures were processed for immunohistochemistry.

Immunohistochemistry

Cultures of E9, E11 and E14 pineal cells were tested for immunoreactivity for serotonin, arginine vasopressin (AVP), iodopsin, pinopsin, tyrosine hydroxylase (TH) and HPC-1/Syntaxin1A following the ABC method. The immunohistochemical method used has already been fully described in the previous papers (Araki *et al*., 1990; 1992). Briefly, cultures were treated with 2% FBS in PBS containing 0.2% Triton X-100 (PBS-TX) for 30 min at room temperature. They were incubated with one of the primary antibodies overnight at 4°C, followed by additional incubation for 1 hr at room temperature. After washing in PBS-TX, they were incubated with biotinylated antibody solution for 40 min at room temperature and then with the avidin-biotin peroxidase solution (VectoStain ABC kit; Vector Laboratories, Burlingame, California). The immunoreactive sites were visualized by using diaminobenzidine. The primary antibodies used were anti-serotonin, anti-AVP (supplied by Gumma University, Japan), anti-iodopsin, anti-pinopsin, anti-tyrosine hydroxylase (Sigma, Saint Louis, U.S.A.) and anti-HPC-1/syntaxin1A (Sigma). They were diluted to at least 1:1000 with PBS-TX containing 2% FBS. Their specificity has been fully described in previous papers (Araki *et al*.,1992; 1993; Inoue *et al*., 1992; Okano *et al*., 1997).

Determination of immunoreactive cell number

The number of immunoreactive cells was counted under a microscope at a high magnification (using an objective lens of x20) in at least 20 randomly chosen fields for each culture dish. Three separate experiments were performed for each embryonic stage and subjected to the cell number analysis, and the mean values and standard deviations were calculated.

RIA of Melatonin

The RIA of melatonin was performed by using I-125 melatonin following the method of Vakkuri *et al* (1984). Rabbit anti-melatonin antibody (HAC-AA92-03RBP86) was supplied by Gunma University (Japan) and used at a dilution of 1:250000. Radio iodinated melatonin (specific activity: 2200 Ci/mmol) was used at 4000 dpm per tube. The lower and upper detection limits were 0.25 and 25 pg/culture medium containing tube respectively. Inter and intra-assay coefficients of variation were 18.5% and 8% at the 5 pg reference, respectively. Statistical significance was analyzed with the Student's t-test.

Culture media were collected from the culture plates and frozen at –20°C till RIA assay. The melatonin content of an appropriate sample (50 ml) was determined by RIA. Radioiodinated melatonin and melatonin used as standards were purchased from PerkinElmer (Boston, U.S.A.) and Sigma, respectively. The assay was validated for culture medium without sample extraction. No significant interference was noted for the culture medium, which was detectable in the standard assay. The lower and upper detection limits of the assay were 7.5 and 1000 pg/ml. Melatonin release in the medium was noted under constant darkness with a 15 minute light pulse during the change of the culture medium. Each point representing the mean±SE of 4 replicated culture media is presented in Fig 4.

RESULTS AND DISCUSSION

1. Morphometric analysis of different immunoreactive cells in culture

During embryonic development from E9 to E14 the histological appearances of pineal glands remained mostly constant although there appeared a slight increase in the number of follicles from E9 to E11. In most follicles of E14 pineal glands, a parafollicular layer was seen surrounding the follicular cells, dividing the follicular cells into two different layers (Araki *et al*., 1992). Cells in the parafollicular layer were round in shape and smaller than the follicular cells as previously described by Calvo and Boya (1978).

Phase contrast microscopic observations of disperse cell cultures of E9 pineals on every alternate day initially presented slower growth rates than cultures from the other two stages. At the end of the experiment the patterns of growth and cellular texture of E9, E11 and E14 were very similar to each other (Fig.1). No significant differences were noticed in the total cell number per culture dish among cultures from three different stages. These observations are fundamentally the same as those described previously by Araki *et al* (1992).

In all stages tested immunoreactive cells were found in the culture reacting to the above-mentioned antibodies (Fig. 2). A morphometric analysis (measurement of cell number) of all the immunoreactive cells is given in Fig. 3. The cell number of each cell type varied considerably; serotonin (5-HT), arginine vasopressin (AVP) and syntaxin1A/HPC-1 immunoreactive cells were predominant in population, and others like iodopsin (I), pinopsin (PI) and tyrosine hydroxylase (TH) were much less predominant (Fig. 3). We will describe the results by dividing these immunohistochemically detectable substances into three categories according to their properties.

Endocrinal substances (serotonin and AVP): The numbers of serotonin (5-HT)-immunoreactive cells in cultures from E9 and E11 appeared stable (Fig. 2E, 3), but the number of 5-HT immunoreactive cells increased significantly in E14 culture, suggesting that as the hatching day nears, the synthetic activity of the pineal increases for melatonin and that serotonin acts as the substrate, for this. During embryonic development and the post-hatching period, it has been reported that the 5-HT immunoreactivity reaches a peak in one-day-old quails and then slowly decreases with increasing age (Oshima and Matsuo, 1988). Our previous study also showed that no pineal cells are positively stained for 5-HT in E11 pineal glands and that numerous immunoreactive cells are observed in the E14 pineal but are still stained only faintly (Araki *et al*., 1992).

Arginine vasopressin (AVP) antibody also reacted

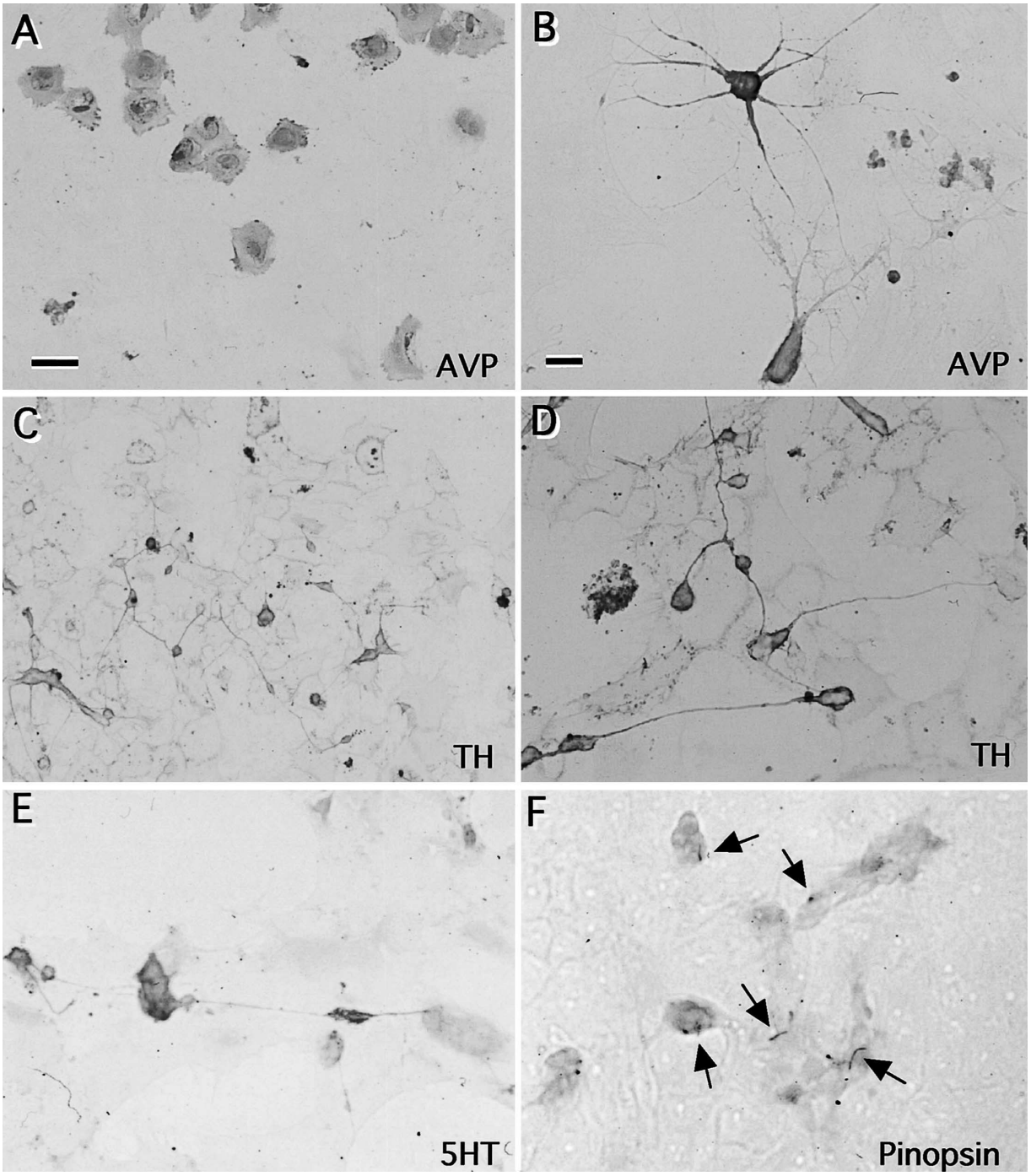

Fig. 2. Photomicrographs of various immunoreactive cells. (A,B) Arginine vasopression-positive cells. Flat epithelial cells are the dominant cell type, but occasionally neuronal cells with numerous processes are also observed. (C,D) Tyrosine hydroxylase-positive cells. Positive cells usually possess several long neuritic processes. (E) Serotonin-positive cells. Some cells are flat and some show a neuronal appearance. (F) pinopsin-positive cells. Immunoreactive products are usually observed on short thick processes (arrows). Bar in (A) is 50 μm and applies to (C) and (D). Bar in (B) is 20 μm and applies to (D) and (F).

intensely with high number of cells at the E9 stage (Fig. 2A). The positive cell numbers increased gradually as pineals for culture materials were removed from more developed embryos, and the AVP immunoreactive cell number was highest in E14 cultures (Fig. 3). The AVP immunoreactivity was localized mostly in flat epithelial cells, and occasionally it was found in the perikariya and neurite-like processes of some neuronal cells (Fig. 2A, B). Although no data are currently available on the immunohistochemical localization of AVP in developing avian pineal glands, these results indicate that endocrinal properties are acquired by pineal cells only in the late stage of development.

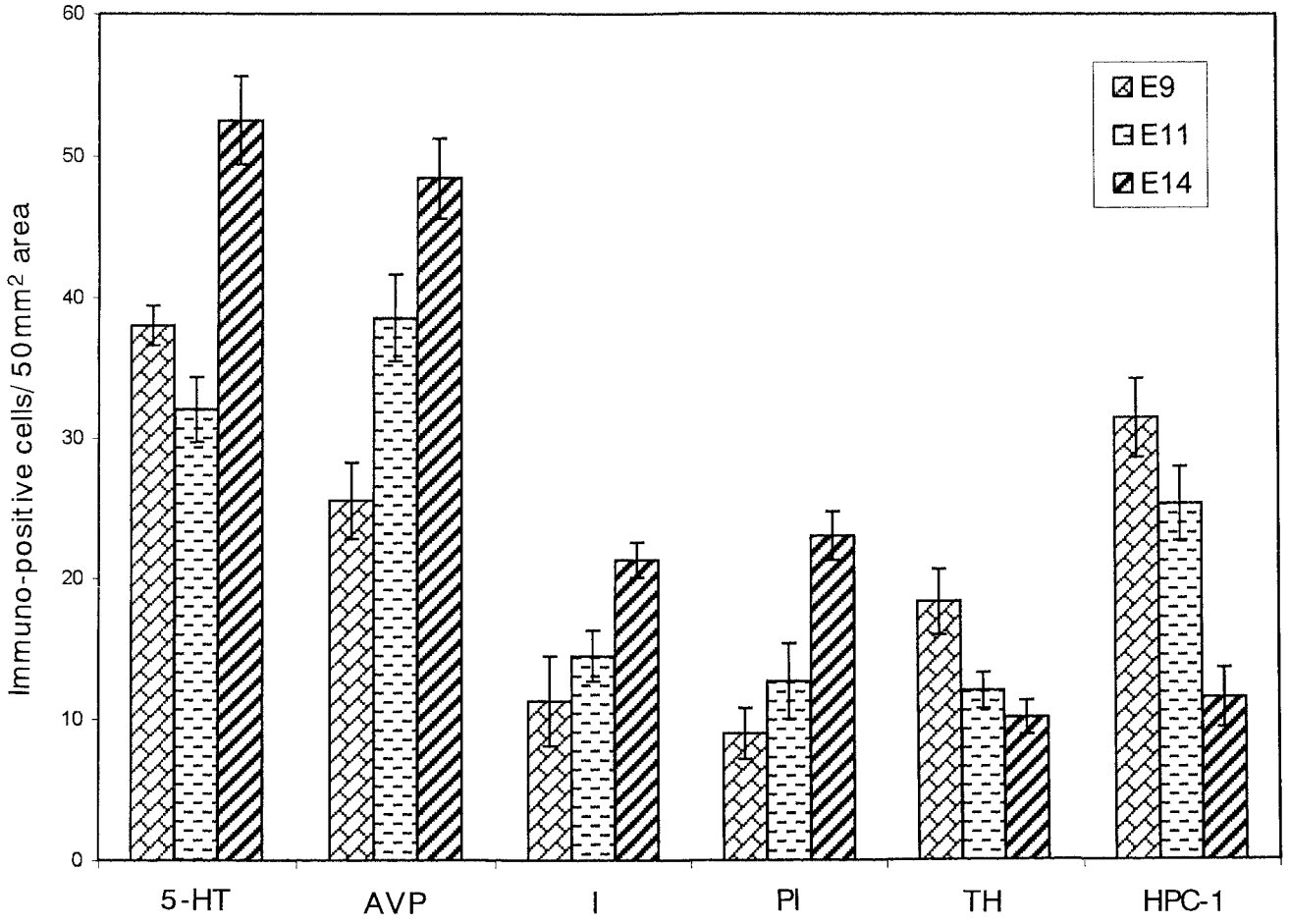

Fig. 3. Morphometric analysis of immunoreactive cells in pineal disperse cell culture of quail embryos from age E9 to E14. Pineal cells from E9, E11 and E14 embryos were cultured for 6 days. They were fixed and stained for one of 6 substances, 5-HT (serotonin), AVP (arginine vasopressin), I (iodopsin), PI (pinopsin), TH (tyrosine hydroxylase) and HPC-1/Syntaxin1A.

Neuronal substances (Tyrosine hydroxylase and Syntaxin/ HPC-1): Tyrosine hydroxylase (TH)-immunoreactive cells followed a reverse pattern from the above mentioned endocrinal substances (Fig. 3). TH-immunoreactive cells often showed long branching neurite-like processes, as previously mentioned (Fig. 2C, D) (Araki *et al*., 1994). Intensely TH-immunoreactive cells were noted in E9 cell cultures with a gradual decrease in number from E11 to E14 cultures. This appears to be consistent with the previous observations that TH-immunoreactive cells are found only transiently during embryonic development (E9-E11) and disappear in the matured pineal organ of the quail (Araki *et al*., 1993). Other studies also suggest that neural cells degenerate during the late embryonic stage and after hatching (see a review by Sato, 2001).

HPC-1/Syntaxin1A immunoreactive cells were highest in number in the E9 cultures with a gradual decrease from E11 to E14 cultures (Fig. 3). The immunoreactive cells mostly showed a neuronal morphology with long branching neurite-like processes. Thus, the cell numbers of both TH- and HPC-1-immunoreactive cells decreased as the culture materials were removed from more developed embryos. These results indicate that the potency for neuronal differentiation is gradually lost during embryonic development, and are consistent with the previous results of *in vivo* developing pineals (Araki *et al*., 1992; 1993).

Visual pigments (pinopsin and iodopsin): Several retinal opsins have already been described in different pineal photoreceptor populations (Araki *et al*., 1992; Yamao *et al*., 1999). Pinopsin is a blue to green light sensitive visual pigment specifically found in pineal photoreceptors (Okano *et al*., 1997). This photoreceptive molecule is known to mediate the photic entrainment of the endogenous circadian clock, which derives rhythmic production of melatonin and hence is the circadian pacemaker in the avian pineal gland (Okano and Fukada, 2001). Anti-pinopsin antibody reveals intensely positive images for most of the membrane structures that make contact with the lumen of the follicles in the intact pineal follicles (Hirunagi *et al*., 1997; Yamao *et al*., 1999). In the present study the antibody stained only the string and bulb shaped structures of the pineal cells (Fig. 2F), the morphology of which resembles those of retinal photoreceptor cell. The number of pinopsin immunoreactive cells increased with increasing age of the cultured embry-

onic pineals. High number of pinopsin-positive cells was noted in E14 culture cells. These results are consistent with those reported by Yamao *et al* (1999).

Iodopsin immunoreactivity was observed on the whole plasma membrane covering from cell to fine neurite-like processes of cells both under culture condition and in the pineal organ of the adult quail (Masuda *et al*., 1994). The number of iodopsin immunoreactive cells was significantly higher in cultured cells from E14 pineals than in those from E9 and E11 (Fig. 3). Thus, it is indicated that the developmental potency for photoreceptors becomes more enhanced as embryonic development proceeds. The two types of visual pigments showed a very similar pattern both in the cell number and rate of increase as shown in Fig. 3. Several fundamental differences, however, were found in the *in vivo* expression patterns of these two visual pigments; during embryonic development visual pigments such as iodopsin and rhodopsin are found later on E14, while pinopsin immunoreactivity was first observed as early as E9 (Araki *et al*., 1992; Yamao *et al*., 1999), while in E11 pineals, most of cytoplasmic protrusions in the pineal luminal cavity have already been positively stained for pinopsin (Yamao *et al*., 1999). The present morphometric results of pinopsin and iodopsin suggest that the present culture conditions are less favorable for the production of pinopsin than that of iodopsin, particularly in cultured pineal cells from earlier embryos. This will be partly due to the fact that differentiation of pinopsin-immunoreactive cells is susceptible to dissociation procedure with trypsin (Yamao *et al*., 1999).

2. Hormonal analysis of melatonin

To determine the onset of melatonin secretion and to monitor it along with the developmental changes of the pineal organ, the media of one-day cultures from E 9 to E14 cultures were collected to note any conspicuously different amounts of melatonin. In pilot studies it was adjusted in such a way that the melatonin content of the samples showing a range of measurement (15–640 pg/ml) of the RIA by serial dilution. Undiluted samples of medium extracts from one-day cultures revealed values just at the lower range of detection after 72 hr of culture, although a high amount of melatonin was detected in culture medium after 24 hr. Samples of medium extracts from one-day cultures of pineal cells from E9 to E14 embryos were assessable at 0.5, and 0.1 dilutions, respectively. To eliminate inter assay variations, all measurements of this study were ultimately performed with a single assay. The results from RIA determinations of 24 hr melatonin discharge into the culture medium are shown in Fig. 4. The culture was performed in the dark with exposure to a 15 min light pulse during the time of the medium change every day. We recorded a single point assay, which already has been reported by others suggesting a moderate value of melatonin, perhaps with no persisting rhythm under the above conditions (Murakami *et al*, 1994). The melatonin in the medium by the disperse pineal cells was maintained at a moderately high level under

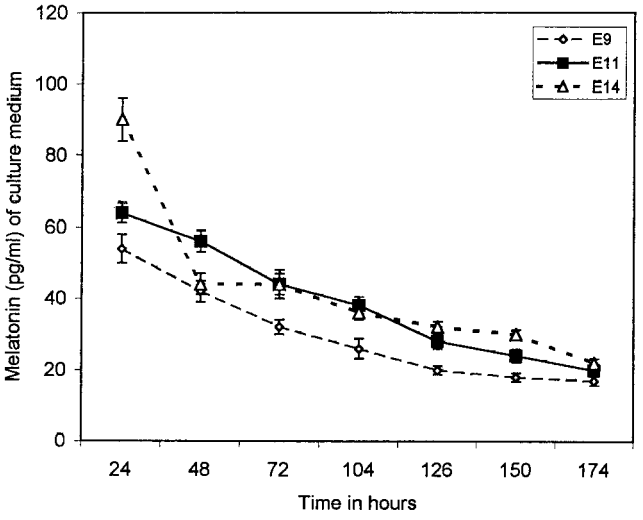

Fig. 4. Results of 24 hr melatonin release in the culture medium by cultured pineal cells from E9, E11 and E14 quail embryos.

culture condition for 72 hr, after which a decrease and dampening were noted. Hence, we may suggest that the mechanisms regulating melatonin synthesis in the avian pineal gland are established during embryonic life (E9) and that the quail pineal gland is not a dominant pacemaker in the animal's circadian system.

The cell culture technique appears to be an excellent experimental approach to elucidate the developmental potency of embryonic pineal cells. Our cell culture condition for embryonic avian pineals affords a good system to reveal the control mechanism of the phenotypic expression of particular cell types. Such studies are of considerable importance for the understanding of how this organ develops into its unique function only at a restricted region in the developing diencephalon. The so-called retino-potency of pineal cells has been described in culture with immunohistochemical methods, and photoreceptor-specific visual pigments and neuron specific markers were localized in cultured avian pineal cells (Araki *et al*, 1993; Araki, 2001; Mano *et al*, 1999).

The temporal sequence of the development of chick pineal cells with the distinction of cytogenesis (until day 9 of incubation) and cytodifferentiation (embryonic day 9–14) has partially been explained by Ohshima and Matsuo (1988). According to them, cytogenesis and cytodifferentiation take place at the same time during the second half of egg incubation but probably at different sites in the pineal organ.

Diurnal changes in the serotonin immunoreactivity were noted in the culture cells of the chick (Oshima and Matsuo, 1991). We showed that quite a few serotonin immunoreactive cells were differentiated in the disperse culture condition and that the number increased as the embryonic stage of culture material advanced. In addition, the relatively high content of melatonin released in the culture medium indicates that our culture condition provided a good experimen-

tal system to investigate how the pineal organ develops the circadian oscillator system during embryonic development. Pinopsin is an important visual pigment for the lighting regulation of the pineal oscillator (Okano *et al*., 1997). A recent study suggested a possibility that cells must be in contact with each other for 2–3 days after the onset of pinopsin synthesis (Yamao *et al*., 1999). This idea is consistent with the present observations that pineal cells from E9 are mostly negative for pinopsin when cultured, but that those from E14 become clearly positive for pinopsin. Light is considered another factor affecting pinopsin expression, since experiments using cultured chicken pineal glands show a light-dependent increase in pinopsin mRNA level (Takanaka *et al*., 1998). However, looking into the differentiation pattern of various cell types in the present culture, the light does not seem to have been a regulatory factor for cell type switching during the ontogenic development of avian pineals.

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