α-Internexin expression in photoreceptor-like cells of the developing chicken pineal gland

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Research article

Keywords: Pineal gland, visinin, α-internexin

Posted Date: February 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-200129/v1

Abstract

Background

It has been reported that some pinealocytes have similar functions to retina photoreceptors in many non-mammalian vertebrates. Two types of pinealocytes, rudimentary-receptor pinealocytes and secretory pinealocytes, have been identified in the pineal gland of juvenile gull, but whether they exist in the chicken pineal gland was unknown. Hence, the purpose of this study was to investigate chicken α-internexin (chkINA) expression patterns in photoreceptor-like cells of the developing chicken pineal gland.

Method

In this study, we observed distribution patterns of chkINA in pineal gland at post-hatching day (P) 1, P7, P14, P21, P35, and at young adulthood chicks by using Immunocytologic analysis, and Western blot analysis. The ultrastructure changes were also observed by electron microscope.

Results

Western blot and immunohistochemistry showed that chkINA expression was high in early developmental stages, but decreased across development. Visinin-immunoreactive cells were also detected during early development, reaching their greatest expression at post-hatching day 7, then gradually reducing over development. Further, confocal imaging revealed that chkINA and visinin were colocalized in photoreceptor-like cells of the chicken pineal gland. Ultrastructural observations revealed rudimentary-receptor pinealocyte morphology with cytoskeletal intermediate filaments in the embryonic chicken pineal gland. Therefore, we suggest that the existence of rudimentary-receptor pinealocytes might be highly relevant for photoreceptor-like cells.

Conclusions

We conclude that chkINA may be a useful photoreceptor-like cell marker for studying the chicken pineal gland. In particular, it could be a major cytoskeletal intermediate filament in rudimentary-receptor pinealocytes, which are homologous to photoreceptor cells in the chicken pineal gland. From an evolutionary viewpoint, we suggest that chkINA may be located not only in photoreceptor-like cells, but also in rudimentary-receptor pinealocytes.

Introduction

The pineal gland is an evagination from the roof of the diencephalon that is organized and shaped differently across species [1]. The pineal gland of fish and frogs is a vesicle connected to the roof of the diencephalon by a slender stalk with a cerebrospinal fluid-filled lumen that is opened to the third ventricle [2, 3]. In lizards and avian, the pineal gland shape becomes follicular. Vollrath (1981) described the pineal gland of mammals as formed in a gland shape and located in the epithalamus, near the center of the brain, between the two hemispheres [4, 5]. The avian pineal gland is composed of a very complex internal

structure with intricate functional organization, which may be due to its intermediate evolutionary place between lower vertebrates and mammals [6].

Three types of pineal glands can be distinguished based on the histological structure of avian pineal glands: saccular, tubule-follicular, and solid (Collin, Calas, & Juillard, 1976; Vollrath, 1981). Tubule-follicular pineal glands are present in pigeon (*Columba livia*), Japanese quail (*Coturnix japonica*), Muscovy duck (*Cairina moschata*), turkey (*Meleagris*), and young chicken (*Gallus Gallus*) [5, 7–9]. In chicken, the tubule-follicular-type pineal gland consists of many follicles and tubules with cavities. During development, the chicken pineal gland gradually loses its follicular features and takes on parenchymal structures, similar to those observed in most mammalian pineal glands [10](. The morphological change in the chicken pineal gland represents the dramatic transformation of its cell types [6].

The pineal gland produces melatonin, which helps to maintain circadian rhythm and regulate reproductive hormones [1]. In some of the lower vertebrates, the pineal gland has a sensory function to detect light. This kind of pineal gland is called the “parietal eye” because it has photoreceptors and is superficially situated in the brain [11]. The pineal gland is photoreceptive and endocrine in avian, but only endocrine in mammals [6, 12]. Although the pineal gland of postnatal mammals no longer has photoreceptors, there are other nerve conduction pathways that indirectly affect photosensory responses of the pineal gland [13–15].

The avian pineal gland contains three main types of cell populations: photoreceptor-like cells, pinealocytes, and supporting cells. Immunocytochemical characterization of the chicken and pigeon pineal glands has shown that pineal photoreceptor-like cells contain molecules that are very closely related to those expressed in retinal photoreceptors [16] indicating that they are modified or rudimentary photoreceptors. The photoreceptor-like cells organize radially around the lumen of each follicle and their outer segments are regressed and are less regular than the true photoreceptor cells [12]. The pinealocytes of the avian pineal gland are located among supporting cells near the luminal surface of follicles.

Based on the ultrastructure and the presence of outer segments, avian pinealocytes can be further characterized into three types: receptor pinealocytes, rudimentary-receptor pinealocytes, and secretory pinealocytes [5, 7]. In chickens, rudimentary-receptor pinealocytes are characterized by the presence of apical protrusions that lack membranous whorls [17]. Further, rudimentary-receptor pinealocytes are the predominate cells of the photoreceptor line, although receptor pinealocytes and secretory pinealocytes may also be present in young chicken [8, 17]. The supporting cells of the avian pineal gland mainly consist of ependymal- and astrocyte-like cells. Supporting cells occupy the pineal parenchyma and isolate the other cell types from the blood vessels surrounding the organ. Previous studies have shown that the supporting cells in domestic turkey contain numerous intermediate filaments (IFs) that fill the basal part of ependymal-like cells and most of the cytoplasm of astrocyte-like cells [18, 19].

Neuronal IFs are important cytoskeletal filaments expressed in neurons that can be characterized into five categories: low, middle, and high molecular mass neurofilament (NF) triplet proteins; α-internexin; and peripherin [20]. Neuronal IFs serve supporting and scaffolding roles in axon and dendrite outgrowth,

stabilization, and function [21]. The IF protein vimentin is also found in neurons, especially during early development and injury-induced axonal degeneration [22, 23]. The 66 kDa protein α-internexin was purified with IFs from the rat spinal cord and optic nerve [24]. α-Internexin is expressed in most neurons as they begin to differentiate, and its expression precedes NF triplet protein expression [25–27]. Unlike the NF triplet proteins, which are obligate heteropolymers, α-internexin can form homopolymers or assemble with NF proteins or vimentin [26–29]. Previous studies from our laboratory have molecularly cloned the mRNA sequence encoding the chicken α-internexin (chkINA) protein. chkINA expression was detected during the early stage of brain development and was the major IF protein in the parallel processes of the cerebellar granule neurons[30]. Moreover, chkINA was expressed in all neuronal lineages of the developing chicken retina, including photoreceptors [31].

Previous work has also shown that some cells may differentiate into neuron-like cells in the postnatal mouse pineal gland, and that these cells possess dual properties of neurons from the central and peripheral nervous systems (CNS and PNS, respectively). Further, the neuron-like cells may act as interneurons to convey signals to the pinealocytes [32]. Some pinealocytes in non-mammalian vertebrates also have similar functions to the retina photoreceptor in many non-mammalian vertebrates [33]. However, little is known about the molecular biology of pinealocytes and photoreceptor-like cells in non-mammalian vertebrates, leaving an evolutionary gap regarding the understanding of the pineal gland between mammals and non-mammalian vertebrates. The chicken is an essential model organism in developmental biology, but the role of chkINA in the developing and adult chicken pineal gland remains unknown. Hence, the purpose of this study was to analyze the spatial and temporal distribution patterns of chkINA in photoreceptor-like cells during chicken pineal gland development.

Materials And Methods

Chicken (Gallus gallus**domesticus) sampling and maintenance**

Fertilized white leghorn chicken eggs were obtained from JD-SPF Biotech Company (Miaoli County, Taiwan) and incubated at 37.5℃ and 60% relative humidity. All animal experiments were conducted in accordance with the protocols approved by the Animal Care and Use Committee at the College of Medicine, National Taiwan University. Chicken embryo stages were determined according to Hamburger and Hamilton [34]. Chicken embryos were collected at embryonic day (E) 15 and chicks were collected at post-hatching day (P) 1, P7, P14, P21, P35, and at young adulthood (P90). For immunohistochemistry, the embryos were removed from the eggs after deep anesthesia induced by cooling on ice. Chicks were sacrificed by decapitation. The brains and eyes of the embryos and chicks were then removed and immersed in 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4) at 4℃ for 12–24 h, depending on the size. Specimens were embedded in optimal cutting temperature compound (Catalog #14020108926, Leica, Wetzlar, Germany), frozen in isobutene on dry ice, and stored at − 80℃ for cryostat sectioning, as described by Redies and Takeichi (1993) [35].

Protein extraction

Tissues were homogenized in protein radioimmunoprecipitation assay lysis buffer (Intron Biotechnology, Inc., Gyeonggi, Korea) using tissue grinders. Homogenized tissues were incubated on ice for 15 min, followed by sonication and centrifugation at 14,000 rpm at 4℃ for 15 min. Finally, supernatants were collected, and the protein concentration was measured using the Bradford protein assay (Bio-Rad, Hercules, CA). The collected protein extract was stored at − 80℃ until use.

SDS–PAGE and western blot analysis

Lysates were denatured with 1X sample buffer, boiled for 10 min, and chilled on ice. Proteins (50 µg per lane) were loaded onto 12% SDS–PAGE. Proteins were transferred to PVDF membranes (GE Healthcare Life Sciences, Canada) via wet electrophoretic transfer and blocked with 5% nonfat dry milk dissolved in 1X TBST (0.1% Tween-20 in 1X TBS) for 1 h at room temperature. The blot was incubated with appropriate primary antibodies diluted in 1X TBST in 5% nonfat dry milk at 4℃ overnight. The following primary antibodies were used in this study (please refer to Table 1 for a detailed list of all antibodies): rabbit anti-chkINA polyclonal antibody (RRID:AB_2827964, Liu and Chien, 2013), rabbit anti-tryptophan hydroxylase 1 (TPH1) polyclonal antibody (Antibodies-online, RRID:AB_2827965), mouse monoclonal anti-vimentin [Development Studies Hybridoma Bank (DSHB), Iowa City, IA, RRID:AB_528506], and mouse monoclonal anti-visinin (DSHB, RRID:AB_528510). Subsequently, the membranes were washed with 1X TBST and incubated with horseradish-peroxidase-conjugated secondary antibodies diluted in 1X TBST in 5% nonfat dry milk at room temperature for 1 h. The membranes were washed again and detected using enhanced chemiluminescence (GE Healthcare Life Sciences, Canada) and autoradiography.

Immunohistochemistry

Tissue sections were cut at a thickness of 16 µm by a cryostat (Leica CM3050S, Leica Microsystems, Nussloch, Germany), placed on microscope slides (Superfrost, Thermo Scientific, Waltham, MA), and processed for immunohistochemistry. Sections were fixed with ice-cold methanol for 15 min, then blocked with 3% fetal bovine serum (FBS) in PBS for 1 h. Subsequently, sections were incubated with primary antibodies in blocking solution (3% FBS in PBS) overnight at 4℃, rinsed in PBS, and incubated with secondary antibodies and Hoechst 33342 (1:1000, Molecular Probes, Invitrogen, Carlsbad, CA) diluted in PBS at room temperature for 1 h. Finally, sections were washed three times in PBST (0.1% Triton X 100 in PBS) and once in PBS for 5 min each wash, then mounted with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA). All images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Primary and secondary antibodies used in this study are detailed in Table 1.

Transmission electron microscopy

Chicken pineal glands were isolated and fixed in 2% glutaraldehyde and 2% PFA in 0.1 M PB overnight at 4℃. Following 1 h in 1% osmium tetroxide, tissue samples were dehydrated in a graded series of ethanol and embedded in epoxy resin (EMS, Hatfield, PA). Ultrathin sections (70 nm thickness) were collected on copper grids and stained with uranyl acetate and lead citrate. Transmission electron microscopy (TEM)

was evaluated using a Hitachi H-7100 electron microscope (Hitachi, Tokyo, Japan) equipped with a Gatan 832 digital camera (Gatan, Inc.).

Statistical analysis

Data was quantified using ImageJ 1.43 software (National Institutes of Health). Student t-test was performed and plotted by using GrapdPad Prism® 7.0 to compare the protein expression of two developmental stages. Significance was set at P-value < 0.05. All data are presented as mean values ± standard error.

Results

Spatial chkINA, visinin, and TPH1 protein expression in young adult chicken tissues

To determine spatial chkINA, visinin, and TPH1 protein expression, western blot was performed in several young adult chicken tissues (Fig. 1A). Immunoblotting using the anti-chkINA antibody yielded a band around 48 kDa. chkINA could be detected in the young adult telencephalon, optic lobe, cerebellum, retina, and pineal gland (Fig. 1B). These results revealed that chkINA was expressed mainly in the CNS. The calcium-binding protein visinin is expressed in cone photoreceptors of the chicken retina [36, 37]. Visinin was identified as a protein band around 24 kDa and was found in the retina and the pineal gland (Fig. 1C). The visinin results illustrated that some photoreceptor cells might exist in the pineal gland. TPH1, one of the three enzymes in the melatonin biosynthesis pathway, can be used as a pinealocyte marker [38]. The anti-TPH1 antibody was visualized as a dense protein band around 53 kDa in the young adult pineal gland (Fig. 1D). According to the TPH1 results, pinealocytes in the young adult pineal gland may participate in the melatonin biosynthesis pathway.

Temporal chkINA, visinin, TPH1, and vimentin protein expression in developing chicken pineal glands

To determine temporal chkINA, visinin, TPH1, and vimentin protein expression in developing chicken pineal glands, western blot was performed at E15, P1, P7, P14, P21, and young adulthood (Fig. 2A). We found that chkINA protein expression in the pineal gland was high at E15 and decreased over the following stages of development (Fig. 2B). This result demonstrates that chkINA expression was high in the embryonic stage and stably decreased after hatching, which shared a similar pattern as that found in the mammalian CNS [27]. Visinin expression in the pineal gland was the highest at P7, then gradually reduced from P7 to young adulthood (Fig. 2C). The visinin result suggests that the photoreceptor-like cell population may gradually increase, reach its highest level at P7, then decrease over the following developmental stages. TPH1 protein was expressed in a fluctuating pattern, with low levels on E15, P1, P14, and P21, and high levels on P7 and in young adulthood (Fig. 2D). This result suggests that pinealocytes may increase in number or activity during some developmental stages. Vimentin, a marker

for glial cells in the pineal gland [15], was detected at 57 kDa. It showed high protein expression on E15, which decreased slowly over the following developmental stages (Fig. 2E). This result suggests that the number of supporting cells might decrease during pineal gland development.

Immunohistochemical chkINA and visinin patterns in the developing chicken pineal gland

To confirm the temporal distribution of chkINA and visinin, we further examined the chkINA and visinin expression pattern in the chicken pineal gland using immunohistochemistry (Fig. 3.1). ChkINA immunoreactivity was abundant at E15 (Fig. 3.1A), decreased gradually from P1 to P21 (Fig. 3.1B-E), and remained low until young adulthood (Fig. 3.1F). The chkINA expression pattern was similar to the western blot result (Fig. 2B) and indicated that chkINA shows stronger expression during the embryonic stage compared to postnatal stages.

Visinin, the specific cone photoreceptor marker in chicken retina [36, 37], was used to identify photoreceptor-like cells in the

pineal gland. Visinin immunoreactivity was weakly detected at E15, P1, and in young adulthood, but was high at P7, P14, and P21 (Fig. 3.1G-L). The visinin immunohistochemical staining patterns during chicken development were in accordance with the western blot results (Fig. 2C). Together, these visinin results suggest that the number of photoreceptor-like cells in the chicken pineal gland may be the highest at P7, then gradually decrease.

chkINA was present in photoreceptor-like cells in the developing chicken pineal gland

According to our previous study, a significant number of visinin-expressing cells were also positive for chkINA in the developing chicken retina [31]. Therefore, we performed double-labeling immunostaining for anti-chkINA and anti-visinin antibodies in the developing pineal gland to investigate whether photoreceptor-like cells in the pineal gland also express chkINA. The stacked and single optical sections of the confocal images showed that chkINA colocalized with visinin in the photoreceptor-like cells of the developing chicken pineal gland (Fig. 3.2). This result suggests that the photoreceptor-like cells in the chicken pineal gland would express chkINA as one of their IFs and could be identified by using the anti-chkINA antibody.

Immunohistochemical TPH1 pattern in the developing chicken pineal gland

To investigate the pinealocytes in the developing chicken pineal gland, we performed immunofluorescence TPH1 staining at E15, P1, P7, P14, P21, and in young adulthood (Fig. 4.1). TPH1 immunoreactivity was detected at every developmental stage. TPH1 was weakly expressed at E15 and

P1, but strongly detected at P7 and in young adulthood. These results suggest that the amount of melatonin secreted by the pinealocytes increases across chicken development.

Visinin-positive photoreceptor-like cells appear to play a role in melatonin synthesis

Previous studies have shown that rudimentary-receptor pinealocytes are predominate in young chickens [8, 17]. Therefore, we observed whether rudimentary-receptor pinealocytes play a role in melatonin synthesis by performing double-labeling immunostaining for anti-visinin and anti-TPH1 antibodies (Fig. 4.2). Single optical sections of the confocal images (Fig. 4.2A'- D') showed that some visinin-positive photoreceptor-like cells, including rudimentary-receptor and receptor pinealocytes, expressed TPH1 at P7 and P14 in chicken pineal gland. Our results support that some photoreceptor-like cells play a role in synthesizing melatonin.

Ultrastructural study of pineal gland photoreceptor-like cells in the young adult chicken and pinealocytes in the embryonic chicken

Previous studies have demonstrated a regression of pineal photoreceptor structures during the phylogeny of bird [7, 39–42], but little was known about IF ultrastructure in the pineal gland. Therefore, we conducted TEM to observe IFs in the chicken pineal gland. We first found that some intra-luminal structures corresponded to the outer segments of photoreceptor-like cells in the young adult pineal gland (Fig. 5A-B). We also demonstrated that the outer segments of some photoreceptor-like cells showed evolutionary regression and their cytosolic component was lost, but the laminated membrane structure was preserved (Fig. 5C-D). However, no obvious IFs were identified in the young adult pineal gland. Considering the western blot and immunohistochemical results showing that chkINA was abundantly expressed at E15, we then checked the embryonic pineal gland for IFs. Cytoskeletal IFs were found in the embryonic chicken pineal gland (Fig. 5E-F). The ultrastructure, western blotting, and immunohistochemistry results suggest that IFs are present in pineal gland cell populations, and chkINA may be the major cytoskeletal IF in the chicken pineal gland.

Discussion

This study investigated the chkINA expression pattern in photoreceptor-like cells of the developing chicken pineal gland. Further, it identified photoreceptor-like cells, pinealocytes, and supporting cells by analyzing visinin, TPH1, and vimentin expression patterns in the pineal gland. Protein expression of chkINA, visinin, TPH1, and vimentin in the developing chicken pineal gland is summarized in Table 2. Further, we provide a schematic diagram showing the spatial and temporal distribution of chkINA, visinin, TPH1, and vimentin in the developing chicken pineal gland in Fig. 6.

chkINA expression in the chicken pineal gland

A previous study discovered that the neuronal IFs α-internexin and peripherin were present in the mouse pineal gland and that some cells that may postnatally differentiate into neuron-like cells possess dual CNS and PNS neuronal properties [32]. In addition, immunoreactivity for neuronal markers that could be attributed to pineal perikarya and nerve fibers has been found in the rat and guinea-pig [43]. It has previously been suggested that the pineal gland contains neurons or neuron-like cells, pinealocytes, and glial cells [44, 45]. Postnatal development and differentiation may lead to different expression of neuron-specific markers in different species [46]. In our study, chkINA protein levels were very similar to the immunohistochemical data and showed a gradual decrease in chkINA in the pineal gland as the chickens grew into maturity (Fig. 2B and 3.1A-F). It is well-known that the pineal gland originates from the central neural canal and that α-internexin is a marker for CNS neurons. Therefore, it is not surprising that chkINA was expressed in the pineal neuron-like cells throughout the life of the chicken.

The parenchymal cells of the pineal organ are derived from multipotent neural stem cells and gradually become more restricted in their developmental potential [47]. In other words, neural progenitors that give rise to a sequence of different photoreceptor cells and neurons would gradually be restricted to photoreceptor- or neuron-like cells. Further, α-internexin was expressed in most neurons as they began to differentiate, and its expression preceded NF triplet protein expression [25–27]. Therefore, our result that chkINA expression was high at E15 and gradually decreased over pineal gland development may be associated with neural progenitors becoming restricted to pineal photoreceptor- or neuron-like cells. Moreover, we suggest that chkINA may play a role at the embryonic stage during chicken pineal gland development.

chkINA is present in chicken retina photoreceptors during embryonic development and may be a useful marker for identifying neuronal lineages during retinogenesis [31]. Our results revealed that chkINA was identified not only in retinal photoreceptors, but also in pineal photoreceptor-like cells in the chicken. In a mouse model, α-internexin was also detected in the pineal gland, even though the immunopositive nerve fibers were not clearly defined [32]. Further, the zebrafish α-internexin inaa is distinctively expressed in the cone photoreceptors of the zebrafish retina and photoreceptor-like cells of the pineal gland [48, 49]. Taken together, these results suggest that α-internexin may be expressed in the pineal gland of many species.

The functional role of chkINA in the pineal gland photoreceptor-like cells needs to be further defined. Studies focusing on the expression pattern and possible functions affected by photoperiodic regulation should also be conducted in the chicken model. In addition, little is known regarding whether α-internexin in other vertebrate species functions in the cytoskeleton of pineal photoreceptor-like cells or pinealocytes. The expression pattern of α-internexin in the pineal gland of other vertebrates should be further investigated to understand the relationship between α-internexin and pineal photoreceptor-like cells from an evolutionary view.

Visinin-positive photoreceptor-like cells in the chicken pineal gland

Visinin, a calcium-binding protein, was originally found to be expressed in photoreceptors of the retina [36, 37]. Our previous data also confirmed that visinin could be a good marker to identify retinal photoreceptors in the chicken [30]. However, the pineal glands of some non-mammalian vertebrates, including fish, frogs, turtles, and birds, are intensely visinin-immunoreactive [50]. A further study revealed that visinin in chicken pinealocytes was markedly increased after continuous light exposure, indicating pinealocyte photosensitivity and a possible role for visinin in photoreception [51]. In other words, environmental light could increase the population density of visinin-immunoreactive pinealocytes. The results of this study showed that visinin expression did not stably increase in the chicken pineal gland. Rather, it reached its peak at P7, and then gradually reduced to the level observed in young adulthood. It is suggested that visinin-immunoreactive pinealocytes in chicken may be differentially regulated by environmental light at different developmental stages.

Our single optical confocal section results (Fig. 4.2A'-D') demonstrate that TPH1 and visinin co-labeling was sparsely found in chicken pineal gland cells. TEM also indicated that some of the developing photoreceptor-like cells might undergo apoptosis or degeneration. A previous study suggested that pinealocyte phylogeny is homologous to retinal photoreceptors and that environmental lighting could influence the enzymatic activity and the circadian rhythm of pinealocytes [52].

Most avian pineal organs contain some pinealocytes that are modified neuroendocrine photoreceptor types. This type of pinealocyte has an inner segment-like structure with a cilium that may expand into irregular bulbous structures, a non-synaptic basal neurite, and an abundance of indoleamine-storing dense-core vesicles primarily located in the basal process [8, 40, 53]. In the chicken, this type of pinealocyte has irregular lamellar structures that resemble photoreceptor outer segments and basal neurites that contain synaptic ribbons adjacent to neuronal dendrites [8, 39, 42]. Therefore, we suggest that a few visinin-immunopositive photoreceptor-like cells in the chicken pineal gland may play a role in neuroendocrine function.

Melatonin synthesis is rhythmic and driven by an endogenous circadian clock, which is also regulated by environmental photic input. Hydroxyindole-O-methyltransferase (HIOMT), the last enzyme in the melatonin biosynthesis pathway, is present in modified photoreceptor-like cells, whereas pinealocyte-like cells are HIOMT positive only after hatching [54]. HIOMT mRNA localization in chicken and bovine pineal glands was also supported by *in situ* hybridization studies [55]. Considering the immunohistochemical pattern of TPH1 and visinin co-labeling in the developing chicken pineal gland (Fig. 4.2), we might deduce that TPH1-positive photoreceptor-like cells play a role in melatonin production. Nevertheless, the amount of melatonin synthesis in photoreceptor-like cells will need to be further studied.

Ultrastructure of photoreceptor-like cells in the chicken pineal gland

Reduced photosensory function and regressed lamellar outer segments have been reported in reptile and bird pineal glands [1, 7]. Interestingly, there were also some cells resembling the modified photoreceptors described in pike and lamprey pineal glands [56]. In the mammalian pineal gland, pinealocytes lack outer

segments and there are no photoreceptor-like cells [57, 58]. Evolutionary progress has produced a mammalian pineal gland that is no longer photosensitive, but only serves as a neuroendocrine organ [59]. It has been suggested that pineal outer segments are better developed in lower vertebrates than in mammals, in which several irregular photoreceptor membranes are present [57]. Indeed, our ultrastructural observations showed degenerating photoreceptor-like cells with regressed outer segments and lost cytosolic components in the young adult chicken pineal gland. Therefore, it might be suggested that photoreceptor-like cells in the young adult chicken pineal gland gradually lose their light sensory function. However, a previous study reported that even cells without outer segments may operate as photoreceptors when their plasma membrane is loaded with opsin [60]. It is unclear whether these degenerating photoreceptor-like cells with reduced outer segments can function as photoreceptors. Therefore, further study is needed to investigate the photosensory function of photoreceptor-like cells with degenerating outer segments in the young adult chicken pineal gland.

During rat pineal gland development, it is known that pinealocytes display some morphological features in common with developing photoreceptors, such as cilia with a 9 + 0 arrangement at 4 days after birth. However, these features disappear in rats older than 17 days [58]. Pinealocytes in many non-mammalian vertebrates strongly resemble the photoreceptor cells of the retina [33]. There also are two types of pinealocytes in the juvenile gull pineal gland: rudimentary-receptor pinealocytes and secretory pinealocytes. Several studies suggest that rudimentary-receptor pinealocytes are the predominate cells of the photoreceptor line, and that some pinealocytes with photoreceptor characteristics may also be present in the pineal gland of species like the pigeon, goose, and quail [7, 61]. In contrast, the pineal gland of the adult domestic fowl is solid lobular parenchymal and mainly formed by secretory pinealocytes [62]. According to these reports, the morphology of pinealocytes at early stage of pineal gland development might be in a “photoreceptor-like” period. Ultrastructural observations in our study found the same rudimentary pinealocyte morphology in embryonic chicken pineal glands as a previous study that found small-sized photoreceptor outer segments in chicken pinealocytes [63]. Our results may also correspond to modified photoreceptors, which may be precursors to chicken pinealocytes [53]. Transient photoreceptor-like elements found in developing pinealocytes and the ability to experimentally manipulate photoreceptor expression in pinealocytes *in vitro* also supports the close relationship between the pineal gland and retina [58, 64–66]. Therefore, it could be suggested that photoreceptor-like cells transform into rudimentary pinealocytes during chicken pineal gland development. Furthermore, ultrastructural observation revealed cytoskeletal IFs in the cell pedicle of pinealocytes in the embryonic chicken pineal gland. Our immunohistochemical data also demonstrated that chkINA, but not TPH1, was widely colocalized with visinin in the photoreceptor-like cells of the embryonic pineal gland. Taken together, these results suggest that chkINA might be a major cytoskeletal IF in rudimentary pinealocytes, which are homologous to photoreceptor-like cells, in the chicken pineal gland.

This study analyzed chkINA expression patterns throughout development of the chicken pineal gland. Our results showed that chkINA was abundantly expressed in the chicken pineal gland throughout development. These findings provide novel information regarding the use of neuron-specific IF protein markers that may be applied to neurobiological studies in chickens, especially for photoreceptor-like cells

of the chicken pineal gland. The chkINA expression and ultrastructure of photoreceptor-like cells of the pineal gland also suggest that chkINA could be a major cytoskeletal IF in rudimentary-receptor pinealocytes, and that this type of pinealocyte might be differentiated from photoreceptor-like cells in the chicken pineal gland.

Abbreviations

chkINA chicken α-internexin

CSF cerebrospinal fluid

Ifs intermediate filaments

NF neurofilament

E15 embryonic day 15

P1 post-hatching day 1

PB phosphate buffer

OCT optimal cutting temperature compound

RIPA radioimmunoprecipitation assay

FBS fetal bovine serum

TPH1 tryptophan hydroxylase 1

DSHB Development Studies Hybridoma Bank

TEM Transmission electron microscopy

OSs outer segments

Declarations

Acknowledgements

We thank the staff of the imaging core at the First Core Labs, National Taiwan University College of Medicine, for technical assistance.

Funding

This work was supported by a grant to C.-L. Chien (106-2312-B-002-003; 105-2320-B-002 -008 -MY3) from the Ministry of Science and Technology, Taiwan and a grant to W.-H. Peng (ISU 108-S-03) from I-Shou University.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request

Author contribution

CM Hao conceived of the study, carried out antibody sensitivity test, immunohistochemical staining, western blot, and statistical analysis and drafted the manuscript. ML Liao participated in the design of experiment and edited the manuscript. WH Peng carried out the ultrastructure observation and final approval of manuscript submission. CL Chien supervised the project.

Ethics declarations

Ethics approval and consent to participate

All animal work performed in this study was approved by and followed the guidelines of the Institutional Animal Care and Use Committee at the College of Medicine, National Taiwan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

Figures

Figure 1.

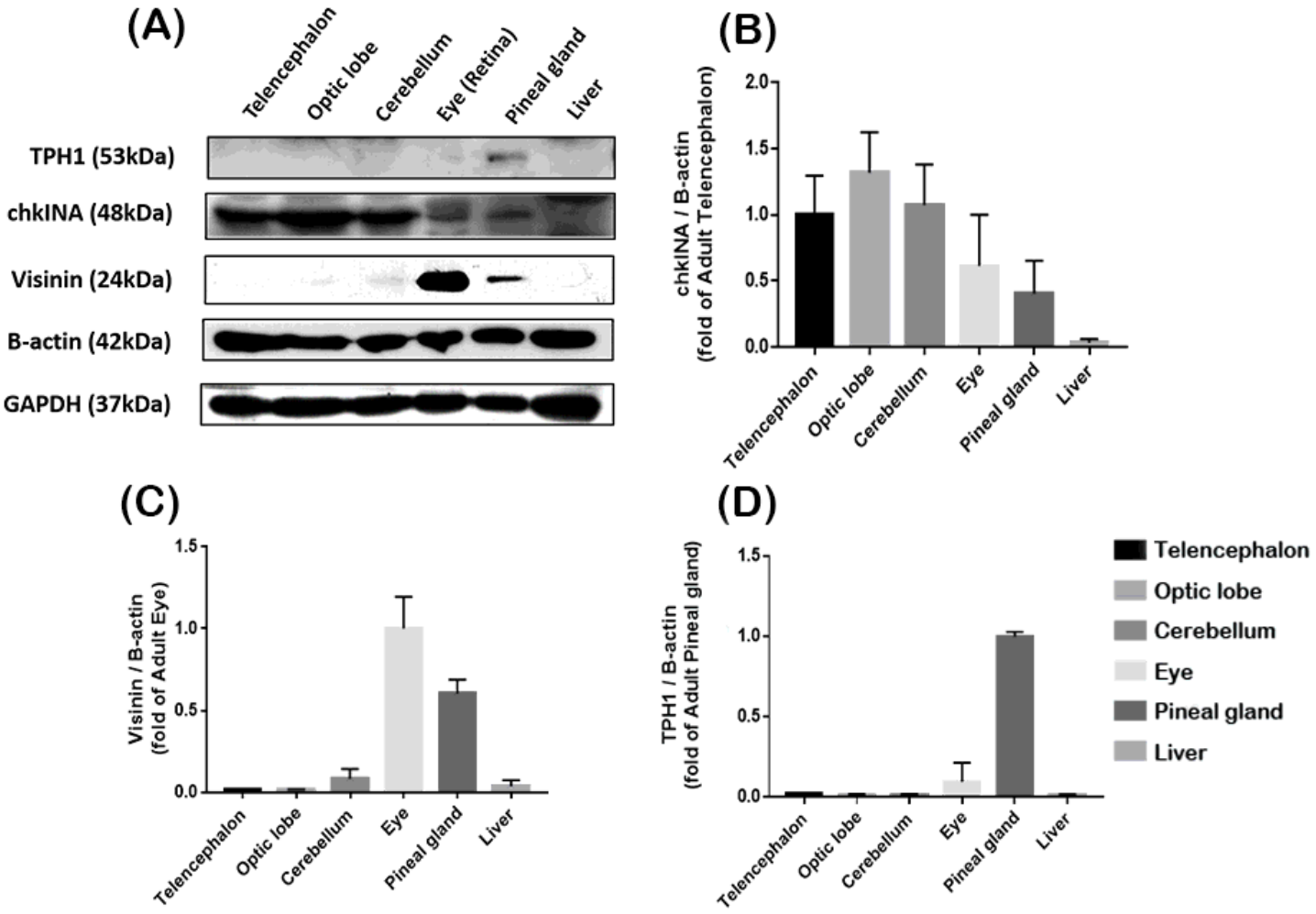

Figure 1

Spatial chkINA, visinin, and TPH1 protein expression in young adult chicken tissues. A: Protein extracts obtained from the young adult chicken telencephalon, optic lobe, cerebellum, retina, pineal gland, and liver were subjected to 12% SDS–PAGE followed by western blotting (n = 3). Specific antibodies against chkINA, visinin, and TPH1 were applied. Antibodies against β-actin and GAPDH were used as loading controls. B: chkINA protein was found mainly in the young adult chicken telencephalon, optic lobe, cerebellum, retina, and pineal gland. C: Visinin protein was found in the young adult chicken retina and the pineal gland. D: TPH1 protein was found in the young adult chicken pineal gland.

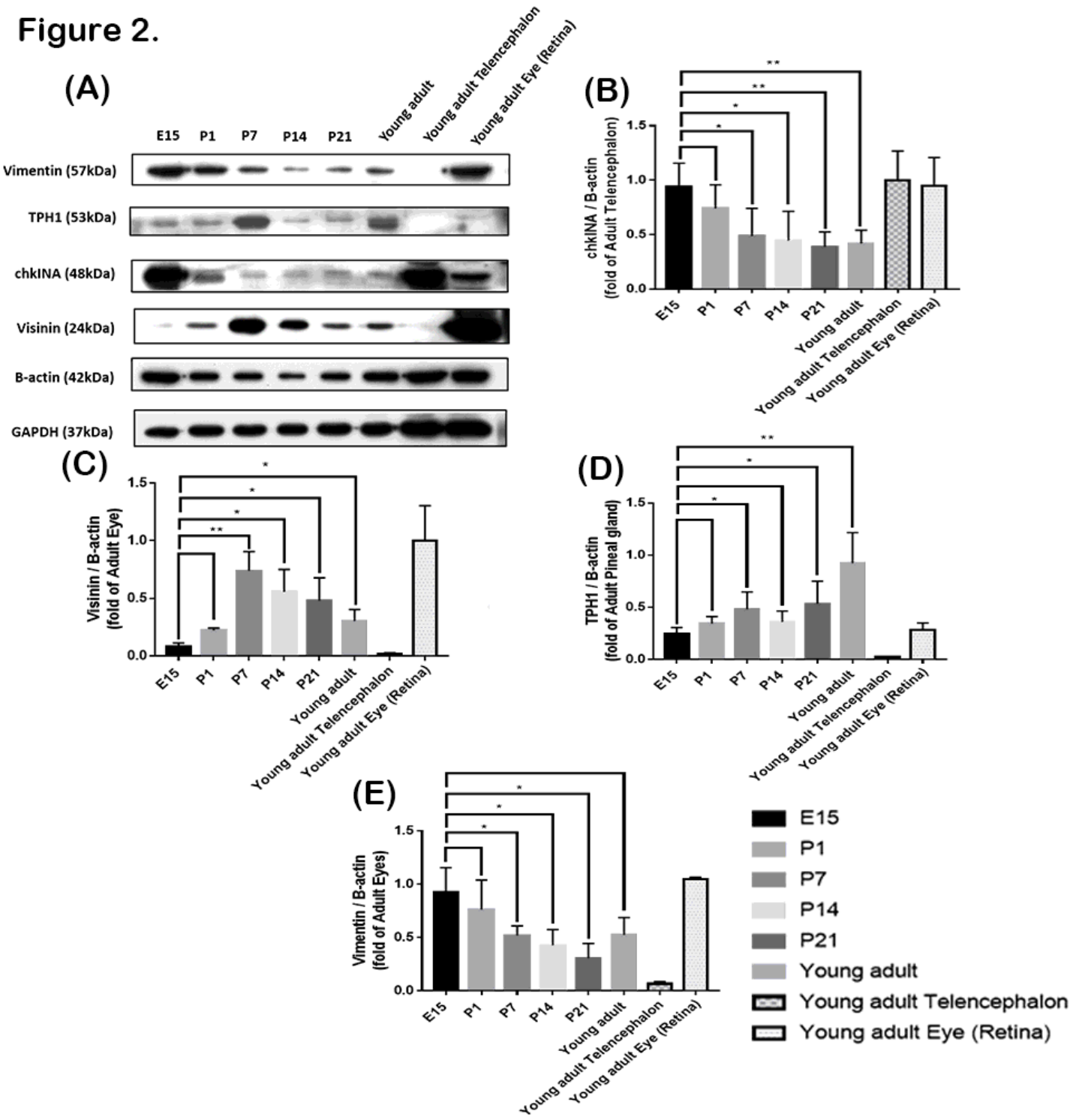

Figure 2

Temporal chkINA, visinin, TPH1, and vimentin protein expression in the developing chicken pineal gland. A: Protein extracts were obtained at different developmental stages (E15, P1, P7, P14, P21, and young adulthood) from the chicken pineal gland and young adult chicken telencephalon and retina. Proteins were subjected to 12% SDS-PAGE followed by western blotting (n = 5). Specific antibodies against chkINA, visinin, TPH1, and vimentin were applied. Antibodies against β-actin and GAPDH were used as loading controls. B: chkINA protein expression was high on E15, then decreased over pineal gland

development. C: Visinin protein expression was the highest at P7, then decreased from P7 to young adulthood. D: TPH1 protein expression was low at E15, P1, P14, and P21, but high at P7 and in young adulthood. E: Vimentin protein expression was high at E15 and decreased slowly across pineal gland development. *: P < 0.05; **: P < 0.01.

Figure 3.

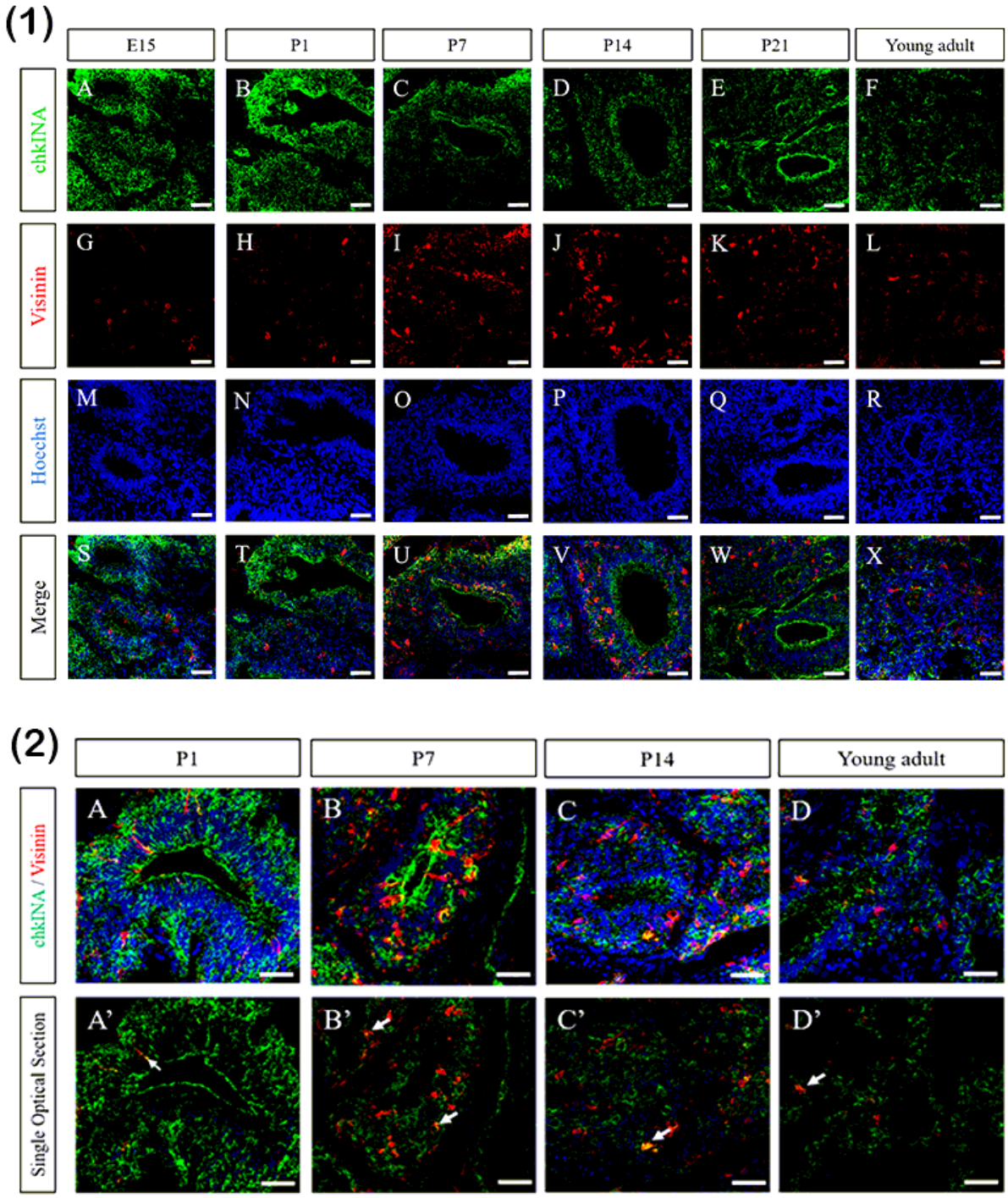

Figure 3

Distribution patterns of chkINA and visinin during chicken pineal gland development. 1: Chicken pineal tissues from six developmental stages (E15, P1, P7, P14, P21, and young adulthood) were sectioned. Immunohistochemical staining was conducted for the polyclonal anti-chkINA (green, A-F) and monoclonal anti-visinin (red, G-L) antibodies. All sections were counterstained with Hoechst 33342 (blue, M-R) to identify pineal cell nuclei. The merged images are shown in S-X. Immunoreactivity for chkINA in the pineal gland was robustly detected at E15 (A) and P1 (B), but decreased across pineal gland development (C-F). Immunoreactivity for visinin, which identified photoreceptor-like cells, was detected easily at P7 (I) and P14 (J), but decreased in young adulthood (L). Scale bars = 25 μm. 2: Pineal tissues from four developmental stages (P1, P7, P14, and young adulthood) were sectioned. The merged images (A-D) were stained for the polyclonal anti-chkINA (green) and monoclonal anti-visinin (red) antibodies. Single optical sections (A'-D') of the pineal gland were taken from dorsal to ventral with an 0.5 μm interval using a confocal laser scanning fluorescence microscope. chkINA was colocalized with visinin in the photoreceptor-like cells (arrows) of the developing chicken pineal gland. Scale bars = 25 μm.

Figure 4.

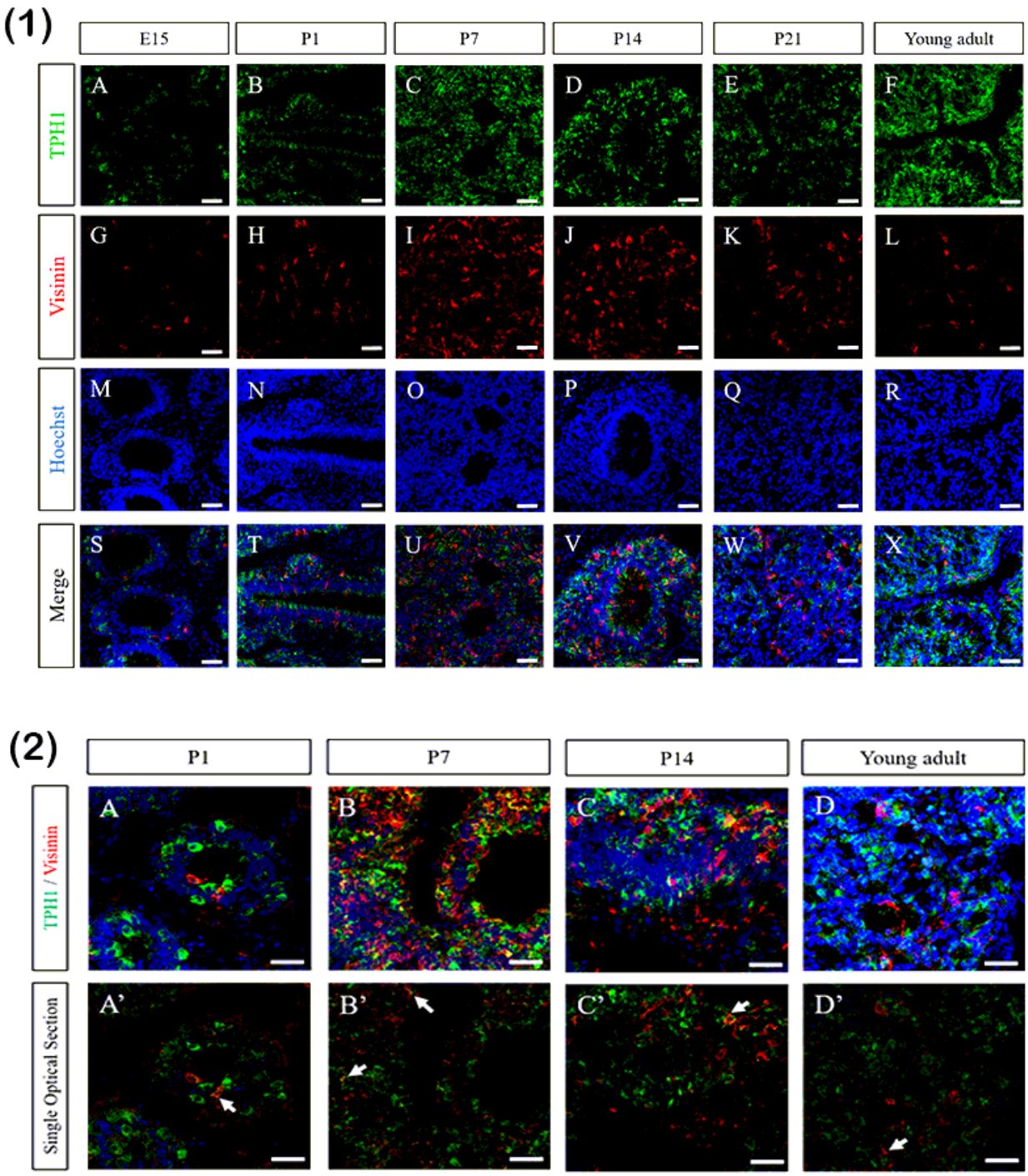

Figure 4

TPH1 and visinin distribution patterns during chicken pineal gland development. 1: Pineal sections were collected at six developmental stages (E15, P1, P7, P14, P21, and young adulthood) and sectioned. Immunohistochemical staining for the polyclonal anti-TPH1 (green, A-F) and monoclonal anti-visinin (red, G-L) antibodies was conducted. All sections were counterstained with Hoechst 33342 (blue, M-R) to identify pineal cell nuclei. The merged images are shown in S-X. TPH1 immunoreactivity was easily

detected at P7 (C) and increased across pineal gland development (D-E). Strong TPH1 immunoreactivity was detected in the young adult pineal gland (F). Visinin immunoreactivity was easily detected at P7 (I), P14 (J), but decreased in young adulthood (L) in the pineal gland photoreceptor-like cells. Scale bars = 25 μm. 2: Visinin-positive photoreceptor-like cells appear to play a role in melatonin synthesis. Pineal sections were collected from four developmental stages (P1, P7, P14, and young adulthood) and sectioned. Immunohistochemical staining for the polyclonal anti-TPH1 antibody (A-D, green) and monoclonal anti-visinin (A-D, red) was conducted. Pinealocytes of the chicken pineal gland were immuno-labeled by the TPH1 antibody, whereas visinin-immunopositive photoreceptor-like cells were weakly labeled by TPH1. Single optical sections (A'-D') of the pineal gland were taken from dorsal to ventral with an 0.5 μm interval using a confocal laser scanning fluorescence microscope. Limited co-labeling of TPH1 and visinin was found in the chicken pineal gland. Scale bars = 25 μm.

Figure 5.

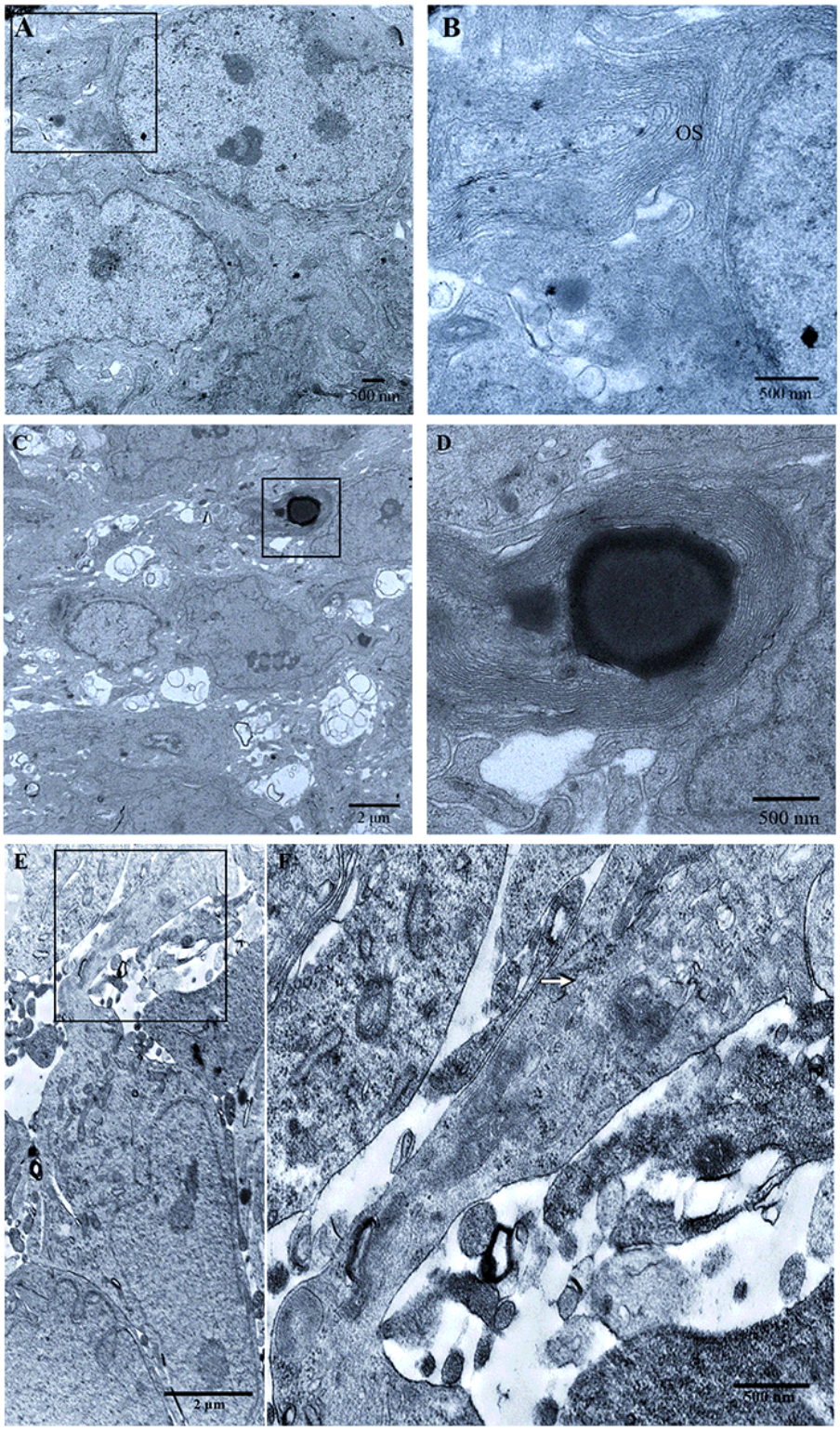

Figure 5

Ultrastructural images of pineal gland photoreceptor-like cells in the young adult chicken and pinealocytes in the embryonic chicken. A: Photoreceptor-like cell arrangement in the young adult chicken pineal gland was demonstrated using low magnification TEM images. B: The outer segments of photoreceptor-like cells could be identified using laminated membrane structures. C: A degenerating outer segment of a photoreceptor-like cell was shown in a low magnification TEM image. D: The enlarged

image of the square in C. The outer segment was regressed and the cytosolic component was lost, but the laminated membrane structure was preserved. E: Pinealocytes in the embryonic chicken pineal gland were revealed using low magnification TEM images. F: The enlarged image of the square in E. IFs (arrow) were present in the pinealocyte pedicle in the embryonic chicken pineal gland. Scale bars are indicated on the figures.

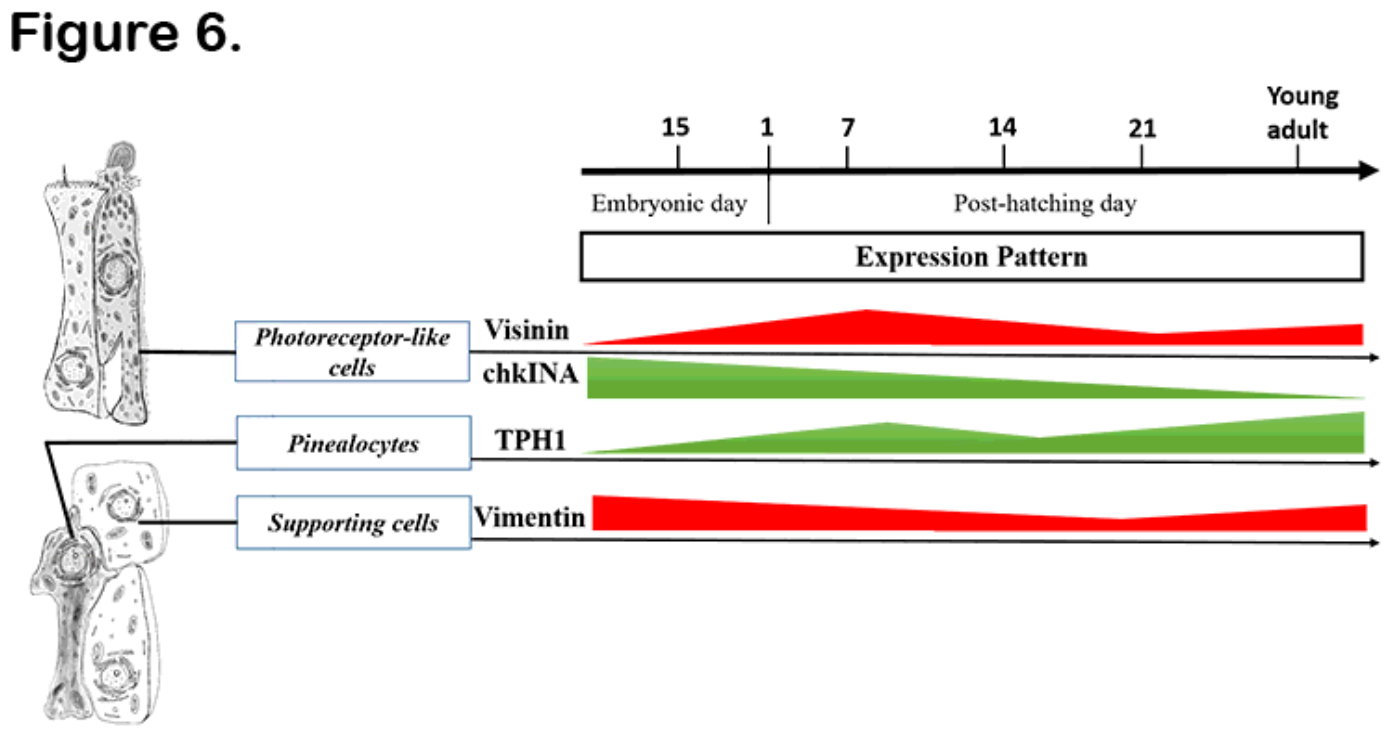

Figure 6

Schematic diagram summarizing the distribution of chkINA, visinin, TPH1 and vimentin in the developing chicken pineal gland. In summary, chkINA expression was high in early developmental stages, but decreased across development. Visinin expression was high at P7 and P14, and gradually decreased during the following developmental stages of pineal gland. Pinealocytes marker TPH1 was detected strongly at P7 and in young adulthood. The intermediate filament protein vimentin was high at E15, and decreased slowly over the following developmental stages.

Supplementary Files

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