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Differential expression and localization of brain-type and mitochondrial creatine kinase isoenzymes during development of the chicken retina: Mi-CK as a marker for differentiation of photoreceptor cells

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Abstract. The expression and the cellular- as well as subcellular-distribution of brain-type B-CK and mitochondrial Mi-CK during development of the chicken retina was studied by immunoblotting, immunofluorescence and immunogold methods. B-CK expression and accumulation in retina was high from early stages of embryonic development on, decreased slightly around hatching and remained high again during adulthood. At early stages of development (days 2–5), B-CK was more or less evenly distributed over the entire retina with the exception of ganglion cells, which were stained more strongly for B-CK than other retinal precursor cells. Then, at around day 10, the beginning of stratified immunostaining by anti-B-CK antibody was noted concomitant with progressing differentiation. Finally, a dramatic increase in staining of the differentiating photoreceptor cells was seen before hatching (day 18) with weaker staining of other cell types. At hatching, as in the adult state, most of the B-CK was localized within rods and cones. Thus, during retinal development marked changes in the immunostaining pattern for B-CK were evident. By contrast, Mi-CK expression was low during development in ovo and rose just before hatching with a predominant accumulation of this isoenzyme within the ellipsoid portion of the inner photoreceptor cell segments. Mi-CK accumulation in the retina coincided with functional maturation of photoreceptors and therefore represents a good marker for terminal differentiation of these cells. B-CK, present from early stages of retina development, seems to be relevant for the energetics of retinal cell proliferation, migration and differentiation, whereas the simultaneous expression of both B- and Mi-CK around the time of hatching indicates a coordinated function of the two CK isoforms as constituents of a PCr-circuit involved in the energetics of vision, which, in autophagous birds, has to be operational at this point in time.

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Introduction

In adult chicken retina two isoenzymes of creatine kinase (EC 2.7.3.2), brain-type B-CK and mitochondrial Mi-CK are expressed simultaneously at high concentrations. On the subcellular level the two CK isoenzymes have been shown to be spatially segregated in an isoenzyme-specific manner [44]. B-CK is present in soluble form in the inner segment of the photoreceptor cells, but is also associated with the plasma membrane of inner and outer segments [26]. By contrast, Mi-CK is strictly located within the ellipsoid portion of the inner segment of photoreceptor cells, where mitochondria are clustered [44].

Photoreceptor cells, like spermatozoa are structurally polar cellular systems. The places of energy production, glycolysis and oxidative phosphorylation, are almost exclusively located in the inner segment, whereas many energy-consuming reactions essential for vision take place in the outer segment. Like muscle (for review see [2, 40, 45]) and spermatozoa [37, 43], the energy metabolism of photoreceptor cells may also depend on a metabolic phosphocreatine circuit (PCr-circuit), shuttling PCr from mitochondria located within the ellipsoid portion of the inner segment to various subcellular sites of high energy requirement [15]. According to the PCr-circuit model, fractions of cytosolic B-CK-forming microcompartments with ATP-requiring systems in the inner as well as outer segments, and Mi-CK, exclusively located within mitochondria of the inner segment, are metabolically linked via phosphocreatine (PCr) representing a storage as well as a transport form of energy [45]. PCr, generated from glycolytic ATP via soluble B-CK or from mitochondrial ATP via Mi-CK, is thought to be the energy currency shuttling from the inner to the outer segment and back again in its dephosphorylated form (Cr). Thus, as far as CK is concerned, the topographic and enzymatic prerequisites for a PCr-circuit seem to be fulfilled also in retinal photoreceptor cells, where two CK isoforms are subcellularly compartmentalized and segregated in an isoenzyme-spe-

cific manner [15]. Recently, a cell-type specific distribution and developmental regulation of CK isoenzymes have been reported in the lens of the eye [11].

The objective of this work was to follow the maturation of the CK energy distribution system and to see whether expression of CK isoenzymes is temporally related to retinal function, which, in autophagous birds, should be fully operational at the time of hatching. Developmentally, photoreceptor cells are fully differentiated in chicken just a few days before hatching. Therefore, it was of interest to see at which point in time during retinal development an adult-like expression and distribution of CK isoenzymes was reached. Thus, we investigated the expression, accumulation and localization of both CK isoenzymes in whole retinas and specifically in photoreceptor cells of chickens during early embryonic development, around the time of hatching and during the post-hatching period, using biochemical as well as immuno-histochemical techniques on the light- and electron-microscopic levels.

Methods

Analytical procedures. Intact whole retinas were isolated by microdissection at various developmental stages from chickens. They were cleaned from adhering tissue. Retina extracts were prepared by homogenization of isolated retinas in 3–4 vol 5 m*M* hypotonic Na-phosphate buffer at pH 8.5 in a Polytron mixer. After incubation for 10 min on ice to swell the mitochondria within the tissue, the homogenate was made 50 m*M* in Na-phosphate at pH 8.5 and 0.2% in Triton X-100, homogenized again or sonified three times for 15 s, to extract Mi-CK from the mitochondria [34] and then centrifuged for 10 min at 50000 *g*. The supernatants were used for CK activity measurements.

For SDS-PAGE two aliquots of doubly concentrated sample buffer [18] were added to the total homogenate, warmed up to 90° C and centrifuged. Electrophoresis of such embryonic tissue extracts was performed according to [18] on 10% polyacrylamide sodium dodecyl sulfate (SDS)-gels, and the separated protein bands were blotted onto nitrocellulose as described [39, 43, 44]. For immunological identification of B- and Mi-CK subunits, affinity-purified rabbit anti-chicken B-CK and anti-Mi-CK antibodies, characterized extensively [34, 35, 43, 44], were used. The blots were first incubated for 2 h in phosphate-buffered saline (PBS) containing 10% horse serum and 5 mg/ml bovine serum albumin (BSA), followed by an overnight incubation with primary antibody diluted 1:500 in PBS containing 5 mg/ml BSA at 4° C. After three washes with PBS, the immunoreplicas were incubated with peroxidase-labeled goat anti-rabbit IgG diluted 1:5000 in PBS/BSA with 0.02% Nonidet-NP-40 for 2 h at room temperature, and then again washed three times with PBS. Subsequently, the staining colour was developed by adding 0.03% chloronaphtol plus 0.03% H_2O_2 in PBS. For semiquantitative analysis the developed immunoblots were subjected to densitometry using a computer-assisted Model 620 BioRad video densitometer.

Immunofluorescence-staining of paraffin-embedded embryonic chicken retinas. Whole chicken embryos (2–5 days of incubation), intact eyes (10 days in ovo) or eyecups (15 days in ovo and older) were embedded in paraffin as described [42]. The tissues were fixed with 3% paraformaldehyde in PBS for at least 2 h on ice, dehydrated through a graded series of ethanol, transferred to xylene and penetrated with paraffin (Paraplast tissue embedding medium, Lancer, Sherwood Materials, Ireland). Sections were cut, rehydrated by xylene and a reversed graded series of ethanol, and exposed to affinity-purified rabbit anti-chicken B-CK diluted 1:100 in PBS containing 10% horse serum and 1 mg/ml BSA. As controls preimmune sera or corresponding rabbit IgG were used at the same dilution. After exposure for 30 min at room temperature or overnight at 4° C, sections were washed three times with PBS and incubated with 1:50 diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG for 30 min at room temperature. After three washes with PBS, a drop of 50% glycerol in 0.1 *M* glycine-NaOH, pH 9.0 was placed onto the sections, which then were covered by a coverslip, examined with a Zeiss-Standard-18 epifluorescence microscope and photographed on Ilford HP-5 film.

Immunofluorescence staining of thin frozen sections of chicken retinas. After a circular incision had been made into the front of chicken eyes and the vitreous humor had been removed, eye cups were prefixed with 3% paraformaldehyde and 0.1% glutaraldehyde in PBS for 30 min on ice. Retinas were removed and fixed for an additional 2 h with the same fixative. Small pieces (2 × 3 mm) of retinas were immersed in 10% gelatin in PBS at 37° C; after solidification on ice, retina-containing gelatin blocks were cut (5 × 5 mm) and hardened with fixative for 30 min on ice. After the gelatin-stabilized retina blocks had been washed with PBS, immersed with 2.3 *M* sucrose in PBS overnight at 4° C [38] and frozen in liquid Freon 22, 0.5-μm sections were cut at −100° C with an Ultracut E ultramicrotome fitted with a cryokit FC4 (Reichert-Jung, Vienna, Austria). Sections were picked up with a drop of 2.3 *M* sucrose in PBS and mounted on coverslips. To wash out the sucrose, coverslips with sections, were placed on droplets of 0.1 *M* glycine in PBS. After blocking nonspecific binding sites with 0.2% gelatin, 0.5% BSA and 0.1 *M* glycine in PBS for 30 min at room temperature, the sections were incubated for at least 1 h with affinity-purified rabbit anti-B-CK or with specific anti-Mi-CK antibodies [34, 35] both diluted 1:100 in the blocking solution or with control IgG at the same dilution. After three washes with PBS, sections were incubated with FITC-conjugated goat anti-rabbit IgG diluted 1:100 for 30 min. The sections were washed in PBS and mounted in 50% glycerol/0.1 *M* glycine/NaOH in PBS at pH 9.0

Low-temperature embedding of embryonic chicken retina in Lowicryl K4M and immunogold staining of ultrathin plastic sections. Eye cups were prefixed with 3% paraformaldehyde/0.1% glutaraldehyde in PBS for 30 min at 0° C. Retinas were carefully removed and fixed for an additional 2 h in the same fixative. Dehydration and embedding in Lowicryl K4M were carried out according to [4] in a low-temperature embedding apparatus (LTE, Balzers, Liechtenstein) with a graded series of ethanol, progressively lowering the temperature to −35° C (PLT-method). Infiltration with liquid Lowicryl K4M resin [5] was performed at −35° C, and polymerization was achieved by indirect UV irradiation (360 nm) in a deep-freezer. Seventy-nanometer sections were cut with an Ultracut E ultramicrotome (Reichert) and processed for immunolabeling according to [31] and [32] using affinity-purified rabbit anti-chicken Mi-CK followed by goat anti-rabbit IgG coupled to 5-, 10- or 15-nm colloidal gold (Janssen Pharmaceutica, Beerse, Belgium). Sections were stained with 2% aqueous uranyl acetate for 5 min followed by lead citrate for 1 min and examined with a JEOL 100C electron microscope.

Results

Accumulation of CK isoenzymes during retina development: differential expression of B-CK and Mi-CK

In adult chicken retina, the presence of B-CK and Mi-CK has been demonstrated, and the two CK isoenzymes were shown to be localized mostly in the photoreceptor cell layer of the retina, where they were segregated on a subcellular level in an isoenzyme-specific fashion [44]. Here, an immunoblot analysis of the tissue extracts from

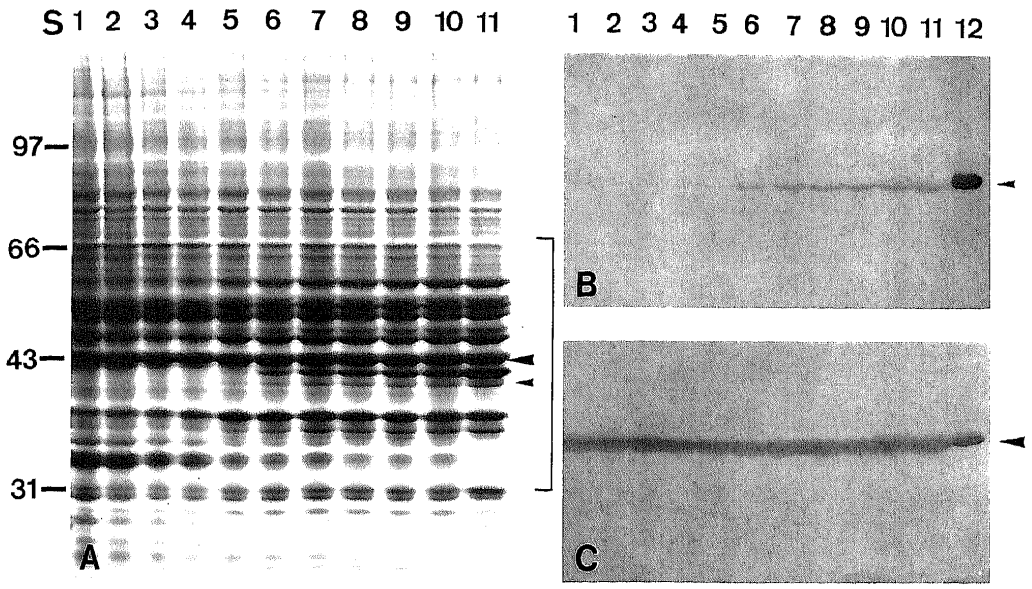

Fig. 1 A–C. Immunoblot analysis of retina extracts during chicken development. **A** Polyacrylamide (10%) SDS gel of retina extracts stained for protein with Coomassie Brilliant Blue. *Lane 1*, 8 days, *lane 2*, 10 days, *lane 3*, 12 days, *lane 4*, 15 days, *lane 5*, 18 days, *lane 6*, 20 days of embryonic development (in ovo); *lane 7*, at time of hatching; *lane 8*, 12 days, *lane 9*, 4 days, *lane 10*, 10 days, and *lane 11*, 8 weeks after hatching. Positions of B-CK and Mi-CK subunits migrating with an apparent Mr of 43000 and 42000 are indicated by a *large and small arrowhead*, respectively. The apparent electrophoretic mobility of the M_r standards (31-, 43-, 66-, 97-kDa, Biorad) are indicated in *lane S*. **B** Immunoblot of a parallel SDS-gel of the region *indicated* after staining with subunit specific Mi-CK antibodies. In *lane 12*, 3 μg purified Mi-CK was loaded. Note the relatively late appearance of Mi-CK signals only from day 18 on in ovo (*lane 5*). The immunostained band (Mi-CK) appears at a position corresponding to that indicated by the *small arrowhead* in panel A. **C** Immunoblot of another parallel SDS-gel, but stained with subunit-specific B-CK antibody for the presence of B-CK subunits. In *lane 12*, 1 μg purified B-CK was loaded. Note the significant expression of B-CK subunits throughout embryonic development to adulthood. The immunostained band (B-CK) appears at a position corresponding to that indicated by the *large arrowhead* in **A**

isolated whole retinas, from embryonic stages of day 8 in ovo (lane 1), through the time of hatching (lane 7) until adulthood (lane 11), with anti-Mi-CK (Fig. 1 B) and anti-B-CK antibodies (Fig. 1 C) revealed that B-CK was expressed and accumulated at high levels already at early embryonic stages of retinal development, whereas only traces of Mi-CK were detectable in retinas between days 8 and 15 in ovo (Fig. 1 B, lanes 1–4). However, after this lag period, Mi-CK rapidly accumulated from around day 18 in ovo onwards through hatching, and still increased to some extent from after hatching to adulthood (Fig. 1 B, lanes 5–11). Subsequent incubation of the immunoblots shown in Fig. 1 B, C with complementary antibodies led in either case to a doublet pattern corresponding to a superposition of the two separate panels (not shown). This, together with the fact that each antibody recognized only one single band with the correct apparent Mr (Fig. 1 A; large arrowhead, B-CK; small arrowhead, Mi-CK), is good evidence for the isoenzyme specificity of the anti-B and anti-Mi-CK used in this study. Semiquantitative analysis of the relative content of B- and Mi-CK of chicken retinas at different stages of development by densitometry of the immunoblots shown in Fig. 1 B, C (not shown) revealed that B-CK expression and accumulation was high from early stages of embryonic development, decreased slightly before and during hatching and then increased again to reach adult levels. By contrast, Mi-CK started to increase much later, that is just around the time of hatching. The different intensities of immunoblot staining for B-versus Mi-CK (Fig. 1 B, C) was not due to differential immunoreactivity of the two isoenzyme-specific antibodies, for the protein bands corresponding to the B- and Mi-CK subunit can be directly identified on the gel (see Fig. 1 A, large and small arrowheads). In addition, parallel experiments with electrophoresis of embryonic retinal extracts followed by CK-activity staining corroborated the prevalence of BB-CK and the relatively late appearance of Mi-CK (not shown).

The immunolocalization staining pattern for B-CK undergoes marked changes during retina development, and an adult-like pattern is reached at hatching

The neurogenesis of the vertebrate retina occurs in a succession of inseparable events: cell proliferation, cell migration and cell differentiation. These events have been studied by several groups who have described in detail the many physiological and structural changes taking place during retinal differentiation (see [12] for review).

Paraffin sections of retinas at different embryonic stages were stained by indirect immunofluorescence using B-CK antibodies followed by an FITC-conjugated second antibody (Fig. 2). On sections of 5-day-old embryonic retinas, more-or-less uniform staining of retina precursor cells was observed, with the exception of gan-

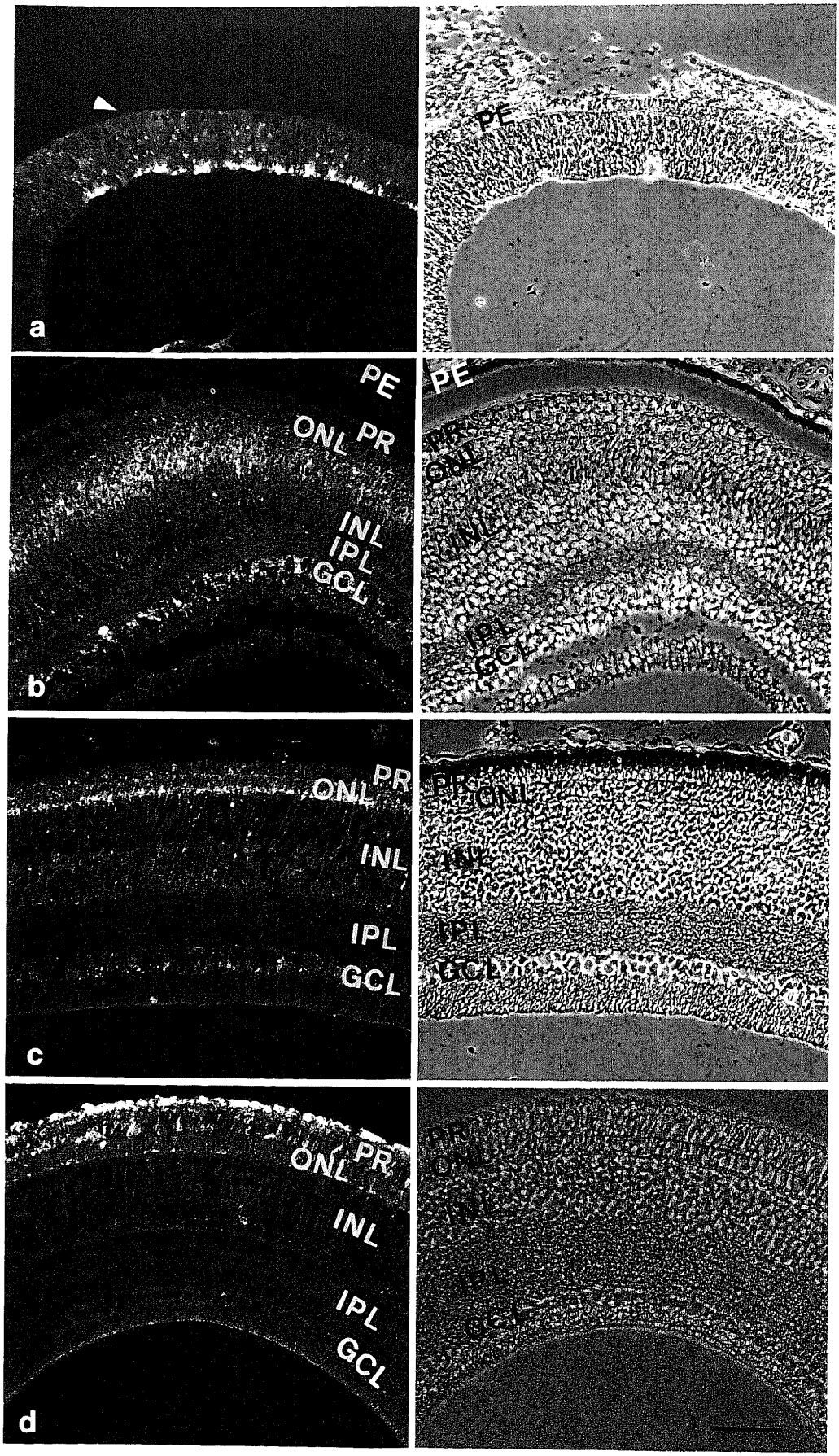

Fig. 2a–d. Changes in the immunofluorescence staining pattern for B-CK during development of the chicken retina. Indirect-immunofluorescence micrographs (*left-hand panels*) showing B-CK distribution in paraffin-sectioned chicken retinas of four different developmental stages. **a** Day-5 embryos. **b** Day-10 embryos. **c** Day-20 embryos at hatching. **d** Ten days after hatching. Corresponding phase-contrast pictures are *on the right*. All retinas are oriented with the pigmented-epithelium (*PE*) on top. *PR*, photoreceptor cell layer; *ONL*, outer nuclear layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. *Bar*, 50 μm. Note the relatively uniform distribution of B-CK in embryonic precursor cells with the exception of strongly stained ganglion cells at days 2–5 in ovo, the change to stratified staining during difffferentiation of retinal cells, and finally the accumulation of B-CK preferentially in the photoreceptor cell layer

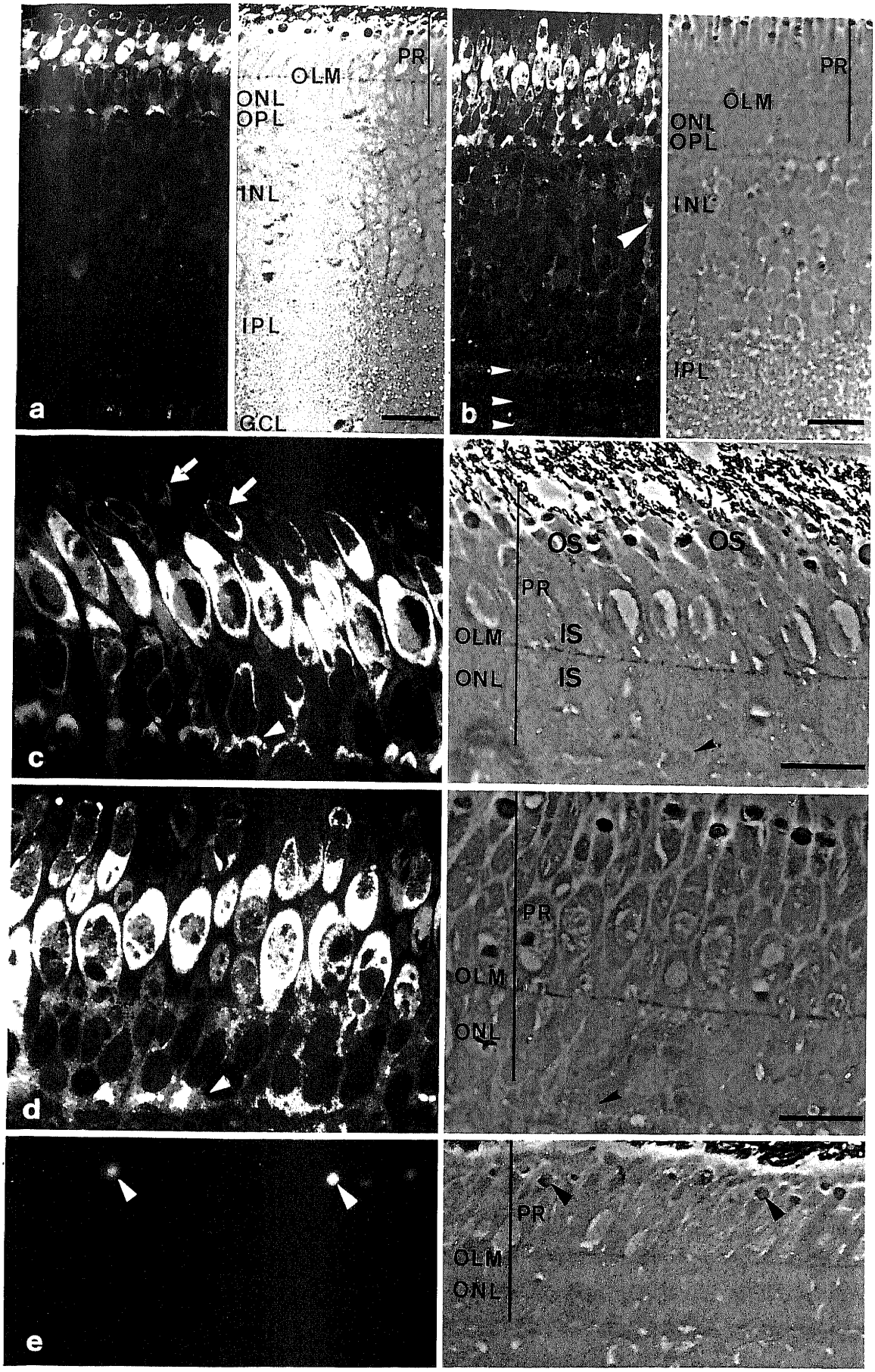

Fig. 3a–e. Comparison of B-CK distribution in retina at the time of hatching and at 10 days after hatching. Immunofluorescence staining of very thin cryosections of a retina from a chicken at hatching time (**a**, **c**); and of retina from a chicken 10 days after hatching (**b**, **d**). **a**, **b** Immunofluorescence micrographs with corresponding phase-contrast pictures, showing an overview of the different cell layers. **c**, **d** Higher magnification of the photoreceptor cell region at both developmental stages – at hatching (**c**) and 10 days after hatching (**d**). Note the strong labeling of photoreceptor inner segments, both cones and rods, and in part also of outer segment plasma membranes (*arrows* in **c**, but ring-like staining of rod outer segments is also visible in **a** and **d**) as well as staining of Müller cells (*large arrowhead*) in **b**) and stratified staining of the IPL (*smaller arrowheads* in **b**). In **c** and **d**, the strongly stained synaptic regions with horizontal cells are marked by *arrowheads*. Staining with preimmune control serum (**e**); *arrowheads* indicating autofluorescence of retinal oil droplets. *PR*, photoreceptor cell layers; *IS*, inner segments; *OM*, outer segments; *OLM*, outer limiting membrane; *IPL*, inner plexiform layer; *OPL*, outer plexiform layer; *other designations* as indicated in Fig. 2. *Bars*, 25 μm

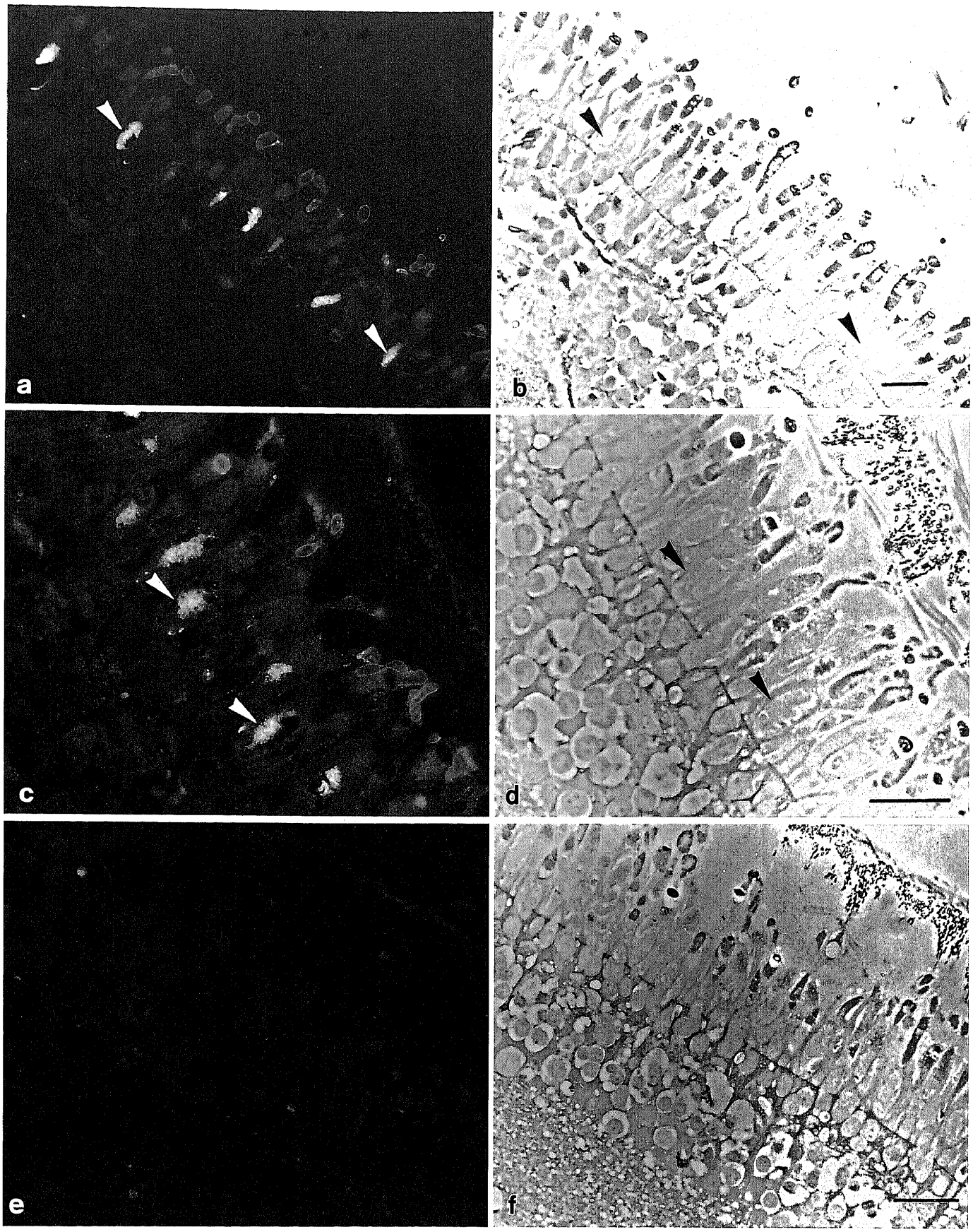

Fig. 4a–f. Localization of mitochondrial Mi-CK during development of the chicken retina. Immunofluorescence staining of a thin frozen section of retina from a chicken embryo 2 days before hatching (19 days in ovo (**a**, **b**); and from an adult chicken (**c**, **d**) for the presence of Mi-CK, using subunit-specific Mi-CK antibodies followed by an FITC-conjugated second antibody. Staining with the same concentrations of preimmune- and second antibody as a control (**e**, **f**). Note predominant staining of the ellipsoid portion of the inner segment of photoreceptor cells, especially of cones, containing clusters of mitochondria (**a**, **c**). No significant staining with Mi-CK antibody was obtained before day 18 in ovo. *Bars*, 25 μm

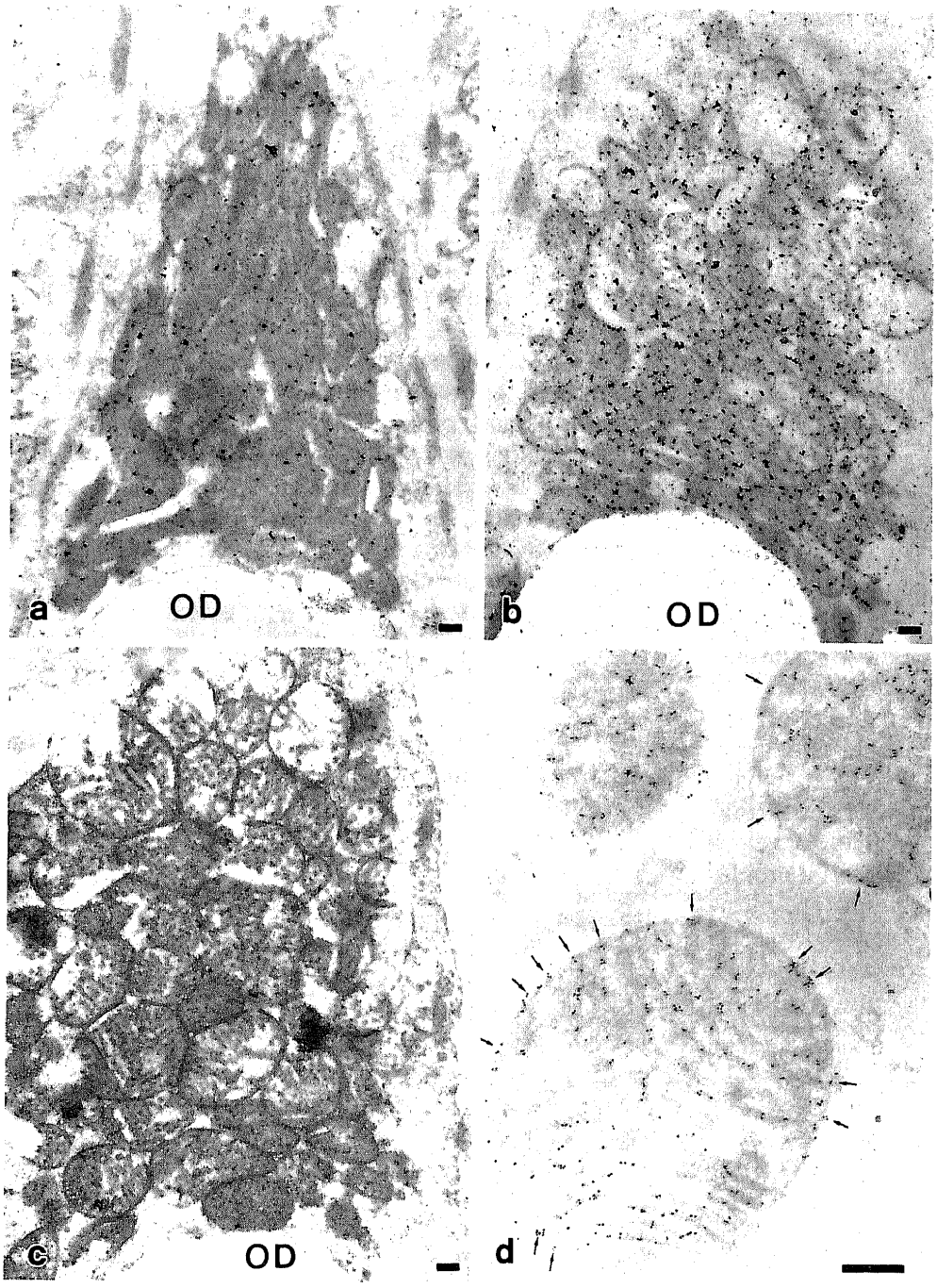

Fig. 5a–d. Immunogold localization of mitochondrial Mi-CK in developing chicken retina. Electron micrographs of longitudinal sections through embryonic chicken retinas 15 days (**a**) and 19 days (**b**) in ovo, after embedding in Lowicryl K4M and immunogold staining with Mi-CK antibodies followed by goat anti-rabbit IgG coupled with gold particles, showing a view of the ellipsoid region of cone inner segments with the characteristic accumulation of mitochondria and with retinal oil droplets (OD). Note faint and strong immunogold labeling of *mitochondrial clusters* in day 15 and day 19 embryonic retinas, respectively. At high magnification the specific staining by Mi-CK antibodies can be assigned to the cristae membranes as well as to those sites where inner and outer mitochondrial membranes are in close contact (**d**, *arrowheads*), e.g., at the so-called mitochondrial contact sites [1]. No significant staining is seen with control antibody (**c**). *Bars*, 250 nm

glion cells, which were stained strongly from the beginning (Fig. 2a). The cuboidal cells of the pigmented epithelium were not stained (Fig. 2a, arrowhead). At around day 9–10, when all retinal layers can be identified unambiguously [21], B-CK staining started to get a stratified appearance with ganglion cells (in the ganglion cell layer, GCL), as well as the layer where bipolar cells of the inner nuclear layer accumulate, being stained specifically (Fig. 2b). In addition, there was some diffuse staining of developing photoreceptors and of those layers where later during development horizontal cells and amacrine cells become prominent (Fig. 2b). In the inner nuclear layer two additional regions, which were composed mainly of outer and inner bipolar cells, were stained. At day 20 in ovo, when outer and inner segments of photoreceptor cells are assuming their mature shape [24] and the synaptic connections in the two plexiform layers have been formed [48], B-CK distribution changed markedly. Most of the fluorescence was now detected in the photoreceptor inner segments and in the synaptic region of these cells (Fig. 2c). In addition, staining of ganglion cells and the long processes of bipolar cells was also observed. Ten days after hatching the labeling of photoreceptor cells was even stronger, and most of the other cell types, with the exception of horizontal cells just below the outer nuclear layer (ONL), and possibly also of Müller cells within the inner nuclear layer (INL), were stained only rather weakly by the B-CK antibody (Fig. 2d). The specific staining by anti-B-CK of the latter two cell types is seen more clearly on cryosections (see Fig. 3a–c). Although the total amount of B-CK did not change very much during retinal devel-

opment and differentiation (see Fig. 1), the regional and cellular distribution of this CK isoenzyme changed markedly during this process.

B-CK distribution during posthatching development

Cryosections from a chicken retina at hatching and from a retina at 10 days posthatching were prepared by the gelatin support technique [44] and stained by indirect immunofluorescence using anti-B-CK followed by an FITC-conjugated second antibody. This method yielded an adult-like pattern of B-CK distribution even at hatching (Fig. 3a) and even more so at 10 days posthatching (Fig. 3b), that is: very strong fluorescence in the photoreceptor cells, both in rods and cones, strong staining of some Müller cells within the inner nuclear layer (Fig. 3b large arrowhead), weak, stratified staining of synaptic connections within the inner plexiform layer (Fig. 3b, small arrowheads) and staining of ganglion cells. At higher magnification of the photoreceptor cell region of retinas from hatched chickens, B-CK fluorescence was localized mainly in the inner segments and in the synaptic region. Weaker, often ring-like staining was found in outer segments (Fig. 3c). The staining at hatching (Fig. 3c) looked very similar to that at 10 days after hatching (Fig. 3d), indicating that B-CK distribution remained rather constant during postnatal development of the retina. Staining with preimmune IgG under the same conditions was negligible (Fig. 3e). The intracellular resolution obtained by this cryomethod was superior to that obtained with paraffin sections. However, cryosectioning of adult chicken retina, and even more so of embryonic retina, with good structural preservation, especially of the photoreceptor cell layer, is very difficult and not suited for routine work.

Immunocytochemical localization of Mi-CK in embryonic and posthatching chicken retina

On thin cryosections of retinas, specific staining by anti-Mi-CK of the ellipsoid portion of inner segments, mainly of cones, where mitochondria are clustered, was observed starting at a time point just before hatching (Fig. 4a, c). At the same time very little staining by anti-Mi-CK in other layers of the retina could be seen by this method, and staining with preimmune serum was negligible (Fig. 4e). The earliest embryonic stages at which appreciable fluorescence staining with anti-Mi-CK was detectable were at day 18 in ovo (not shown) and at later stages, which is in agreement with the results of the immunoblot analysis (Fig. 1).

Ultrastructural localization of Mi-CK in mitochondria of photoreceptors by immunogold electronmicroscopy

A view of Lowicryl K4M-embedded and immunolabelled inner segments of chicken photoreceptor cones (day 15 and 19 in ovo, corresponding to Fig. 5a, and b, respectively) with the characteristic accumulation of mitochondria within the ellipsoid portion and with oil droplets (OL) behind these clusters of mitochondria, is depicted in Fig. 5. While the immunogold labeling of mitochondria by anti-Mi-CK was rather weak at day 15 in ovo, strong labeling of mitochondria with gold particles was visible with very low background staining. At high magnification (Fig. 5d), the gold-labeling nicely showed the specific association of Mi-CK antigen with the cristae membranes of mitochondria, confirming that Mi-CK is located at the outer face of the inner mitochondrial membrane [34]. In addition, clustered immunogold staining was also seen at the periphery of mitochondria where inner membranes were in close proximity with the outer membrane (Fig. 5d, arrowheads), which corresponds to biochemical results [1] that demonstrated the presence of Mi-CK at mitochondrial contact sites. Staining with control IgC was negligible (Fig. 5c). At these stages, mitochondria of other cell types including rod cells were generally stained much more weakly by this method (not shown).

Discussion

Presence of CK isoenzymes in retina and early events of retinal development

The localization studies of B-CK clearly showed that B-CK was expressed in different retinal cell types depending on the stages of retinal development. Around day 5, when the retina is in a rapid growth phase, B-CK was found to be present in most retinal cells, presumably representing precursor cells of both glial and neuronal cells. At this stage premature ganglion cells, amacrine cells, horizontal cells and receptor cells have already entered their last cell cycle, which is completed by day 5. Overall mitotic activity decreases by day 9, and on day 10 or 11, the major retinal layers can be identified unambiguously [21]. At this latter stage, B-CK started to appear in stratified layers and was present in the ganglion cell layer as well as in the inner nuclear layer where presumably outer bipolar cells [48] were stained. Thus, the accumulation of B-CK in these different retinal cell types is correlated temporally with their differentiation and spatial stratification (for review see [12]).

In the embryonic retina, Mi-CK was not detectable by immunofluorescence or by immunogold methods in any other cells (not shown), except for photoreceptor cells, where this CK isoenzyme started to accumulate in rather large quantities at around 4–5 days before hatching (Figs. 4, 5). However, in the adult stage, most or all of these neurally derived cells, which expressed B-CK, were also coexpressing some Mi-CK (unpublished observation), although at considerably lower levels than in differentiated photoreceptor cells (shown in Fig. 5). Although no direct amino acid sequence data are available yet, native gel electrophoresis of retina extracts followed by staining for CK activity (not shown here) indicates that the mitochondrial CK isoform expressed in retina is the ubiquituous brain-Mi-CK isoform [35, 47] and not the sarcomeric-type cardiac Mi-CK isoenzyme [13, 14, 16, 34].

Since on the whole-retina level B-CK is expressed much earlier and at significantly higher concentrations than Mi-CK, it is suggested that B-CK, in early embryos, may have a function that is independent of Mi-CK expression, e.g., it might be involved in energy buffering as well as in maintaining high local ATP/ADP ratios within proliferating, rapidly growing and differentiating cells. Recent studies in our laboratory showed that in chicken retina and brain there are different B-CK species [27] with distinct N-termini [28], which arise by alternative splicing [46]. Additional subspecies of B-CK arise by phosphorylation [20, 29] and differ kinetically from the nonphosphorylated forms [29]. Since phosphorylation of tubulin and tubulin-associated proteins, for example, have been shown to be correlated with their subcellular sequestration into supramolecular structures within the brain [6], it will be very interesting to study the subcellular distribution of these different B-CK-subspecies. Some of the B-CK present in isolated rod outer segments seems to be specifically associated with the plasma membrane [15].

The expression of high levels of B-CK in *Müller cells* of differentiated retina (Fig. 2c, d; and Fig. 3b) was unexpected, since these cells, like other glia cells, have been considered to play exclusively a structural role, e.g., providing a scaffold for the orderly array of retinal cells and guiding the migration of neuronal cells. However, more-recent research indicates that glia cells are quite active, e.g., in spatial buffering and uptake of K^+ released from neurons as well as in neurotransmitter metabolism, both consuming large amounts of energy [22]. In this context it is interesting to mention that, for example, *Bergmann* glia cells of the cerebellum also contain surprisingly high levels of CK, both B-CK and Mi-CK [33]. In chicken, in contrast to what has been reported for human and rat brain tissue [14], no significant amounts of MM-CK were found either in retina or brain (not shown).

Mi-CK appearance and photoreceptor cell differentiation

On ultrathin plastic sections of retinas from chicken embryos at 15 and 19 days in ovo, Mi-CK was labeled with immunogold almost exclusively in mitochondria of photoreceptor cells, especially of cones. Neither mitochondria from neurons nor from Müller glia cells were stained significantly at this stage of development. Therefore, we conclude that the Mi-CK signals on the immunoblot (Fig. 1B) are mainly attributed to Mi-CK expression within the photoreceptor cells. Already around day 9, when the overall mitotic activity of chicken retina decreases, cells at the ventricular surface of the retina are discernible as receptor cells by their structural organization [21]. Although neither inner- nor outer segments have been formed at this stage, the characteristic accumulation of mitochondria apical to the nucleus is already present, and the photoreceptor cells show a bipolar organization [21], but do not yet contain Mi-CK.

During the following days the ellipsoid and the paraboloid are formed, and the inner segments start to protrude through the outer limiting membrane at around day 15. Now, traces of Mi-CK were detectable by immunoblotting and immuno-EM. In parallel, at this time outer segment formation occurs by regular production of infoldings of the plasma membrane. At day 18, outer segments grow and the disks lose contact with the plasma membrane. At day 20, photoreceptor cells assume their mature shape (for review of photoreceptor differentiation see [12, 21]) and in parallel also accumulate significant amounts of Mi-CK.

Our data show that Mi-CK accumulation in embryonic retina temporally coincides with photoreceptor maturation and is correlated with cell differentiation rather than with cell proliferation, which is completed by day 10–12 [21]. GTP-kinase activity in chicken retina follows an accumulation pattern very similar to that of Mi-CK, and it has been shown that the major rise of GTP-kinase activity is between day 13 and day 21 in ovo [10] indicating that the expression of this enzyme is also correlated temporally with photoreceptor elongation and maturation. The appearance and accumulation of Mi-CK during retinal development seems to represent a good index for the terminal differentiation of photoreceptor cells, and antibodies against Mi-CK may be of possible use to follow the differentiation of retinal cells or retinal explants cultured in vitro [19].

B-CK and Mi-CK are coexpressed in terminally differentiating photoreceptor cells: Involvement of CK isoenzymes in the energy homeostasis of vision

Both CK isoforms, B-CK and Mi-CK, were shown by immunohistochemistry to be segregated within the photoreceptor cells. The coexpression and compartmented localization of the two CK isoenzymes in these cells, at a time in development (hatching) when the photoreceptors have to be operational in autophagous birds, strongly point to an important role of CK-isoenzymes in the energy metabolism of visual function, a highly energy-dependent process [17, 23]. Thus, the function of CK isoenzymes in photoreceptor cells may be similar to that of other cells and tissues with large fluctuating energy demands, e.g., muscle [2, 30, 40, 45] and spermatozoa [37, 43], in which a phosphocreatine circuit (PCr-circuit) involved in the replenishment and transport of energy as well as in the regulation of local subcellular ATP/ADP ratios has been postulated [41, 45].

It was shown that mitochondrial activity depends directly upon the amount of light absorbed by the visual pigment in the outer segments [8]. It was concluded that the mitochondria in the ellipsoid portion of the inner segment must somehow be connected to the transduction and signalling mechanisms of the outer segment. In this respect the spatial segregation of CK isoenzymes, allowing for a PCr-circuit from the inner to the outer segment, as well as the structure and function of Mi-CK as octameric "energy channeling" molecules with a central cavity [34, 36], allowing for efficient transphosphorylation of mitochondrial ATP to PCr, would be the basis for such as PCr-circuit [41, 45] working in photoreceptor

cells. Similar to the situation in spermatozoa [37, 43], this would facilitate in the highly polarized photoreceptor cells the "transport" of energy from sites of ATP production, that is from mitochondria and glycolysis (both in the inner segment) to sites of energy consumption, e.g., the light-sensitive, ATP-dependent reactions in the outer segment.

It will be interesting to study the expression of CK isoenzymes in retina from insessorial birds, in which the expression of Mi-CK is expected to be delayed until well after hatching. Such late expression of Mi-CK would be in accordance with the fact that, for example in mammalian brain and cardiac muscle, Mi-CK accumulation starts postnatally [3, 7] when the metabolic maturation of brain and heart functions are being acquired respectively. It was shown recently by ^{31}P-NMR experiments that the flux of energy through the CK reaction increased concomitantly with the postnatal expression of Mi-CK in rabbit heart [25]. Therefore, as in mammalian heart and brain, the accumulation of Mi-CK in chicken retina at a time of morphological and physiological maturation argues for an involvement of Mi-CK, together with B-CK, in the energetics of vision.

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