Calbindin-D28k and Calretinin in Chicken Inner Retina During Postnatal Development and Neuroplasticity by Dim Red Light

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ABSTRACT: **Members of the family of calcium binding proteins (CBPs) are involved in the buffering of calcium (Ca2+) by regulating how Ca2+ can operate within synapses or more globally in the entire cytoplasm and they are present in a particular arrangement in all types of retinal neurons. Calbindin D28k and calretinin belong to the family of CBPs and they are mainly co-expressed with other CBPs. Calbindin D28k is expressed in doubles cones, bipolar cells and in a subpopulation of amacrine and ganglion neurons. Calretinin is present in horizontal cells as well as in a subpopulation of amacrine and ganglion neurons. Both proteins fill the soma at the inner nuclear layer and the neuronal projections at the inner plexiform layer. Moreover, calbindin D28k and calretinin have been associated with neuronal plasticity in the central nervous system. During pre and early postnatal visual development, the visual system shows high responsiveness to environmental influences. In this work we observed modifications in the pattern of stratification of calbindin immunoreactive neurons, as well as in the total amount of calbindin through the early postnatal development. In order to test whether or not calbindin is involved in retinal plasticity we analyzed phosphorylated p38 MAPK expression, which showed a decrease in p-p38 MAPK, concomitant to the observed decrease of calbindin D28k. Results showed in this study suggest that calbindin is a molecule related with neuroplasticity, and we suggest that calbindin D28k has significant roles in neuroplastic changes in the retina, when retinas are stimulated with different light conditions.** © 2013 Wiley Periodicals, Inc. Develop Neurobiol 73: 530–542, 2013

Keywords: **chick; retina; neuroplasticity; calcium binding proteins; calbindin D28k; calretininl p38 MAPK; ERK**

INTRODUCTION

During pre and early postnatal visual development, the visual system shows high responsiveness to environmental influences. Retinal plasticity has been well-documented, and some authors described that the wiring of retinal circuits are modified in response to visual stimuli (Tian and Copenhagen, 2001; Fosser et al., 2005; Chan and Chiao, 2008; Lee et al., 2008; Di Marco et al., 2009). Specifically, we have analyzed plastic changes in serotoninergic networks in the chicken retina and we have argued that the serotoninergic retinal system is modified by light environment from postnatal day 9 (Fosser et al., 2005).

Additionally, it is well known that plastic changes involve modifications in calcium intracellular levels. After membrane depolarization, an increment in cytosolic calcium (Ca2+) occurs, closely related with the neuronal activity. The binding of Ca2+ to several cytoplasmic proteins initiate a cascade of events that

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led to neurotransmitter release or modifies the excitability of the cells or trigger gene expression (Berridge et al., 2003; Harzehim et al., 2010). Calcium triggers and/or modulates some cell behaviors through the regulation of kinases and phosphatases, which in turn are dependent of the action of the calcium binding proteins. The most common Ca2+ binding structural motif in proteins is the EF-hand, which fall into distinct families (Braunewell and Gundelfinger, 1999; Haeseleer et al., 2002; Babini et al., 2005). Calbindin D28k and calretinin are members of the EF-hand family, implicated in the regulation of intracellular free calcium in neurons and they are also considered as part of the calcium signaling toolkit (Berridge et al., 2003; Mojumder et al., 2008).

Some well known actions of CaBPs include: modifications in cytosolic calcium transients, cell excitability, promotion of neuronal bursting activity, protection of neurons against the deleterious effect of excessive Ca2+; short-term facilitation by amplifying [Ca2+] changes during repetitive activity and presynaptic induction of long-term potentiation (Baimbridge et al., 1992; Jackson and Redman, 2003; Westerink et al., 2006).

Through mixing and matching components from the "calcium signaling toolkit", cells acquire Ca2+ signals that fine-tune their physiological state (Harzehim et al., 2010). Signaling targets of the calcium influx are the CaBPs and Ca2+/CaBPs-dependent protein kinases. Some of CaBPs were thought to act as sensors by association with target proteins or a target-specific activation. Other members of the family only function as calcium buffers maintaining calcium at constant level in order to modulate different cellular processes (Gurden et al., 1998; Schwaller et al., 2002; Jouvenceau et al., 2002; Berridge et al., 2003; Roussel et al., 2006; Chaudhury et al., 2008; Schwaller, 2009). However, pure calcium buffers are under revision since calbindin-D28K, parvalbumin, and calretinin are involved in regulating synaptic strength (Schurmans et al., 1997; Edmonds et al., 2000; Jouvenceau et al., 2002; Blatow et al., 2003; Chaudhury et al., 2008; Schwaller, 2009).

Cytological and biochemical studies of CaBPs in the retina have been done with the purpose of explain the physiology of retinal circuitries, and several works have analyzed parvalbumin, calretinin, and calbindin-D28k expression in mammalian and non-mammalian retinas (Pasteels et al., 1990; Araki et al., 1997; Chiquet et al., 2005; Morona et al., 2008).

Although the existence of calbindin-D28K in the retina has been previously described, only a small number of studies were done showing how these neurons branch and organize their networks in the inner plexiform layer (IPL) (Araki et al., 1997; Haverkamp and Wässle, H., 2000). Calbindin-D28k belongs to the calmodulin family (Chard et al., 1995), being currently considered as a noncanonical sensor (Schmidt, 2012) and is expressed in several types of neurons (Ellis et al., 1991; Suzuki et al., 2004; Mojumder et al., 2008) but neither the characterization of each subtype of calbindin expressing neurons nor its role in retinal neuronal plasticity is still finished. Recently, in the visual system calbindin was associated with light processing and the circadian clock (Butler et al., 2011).

Similarly, calretinin was also considered as a buffer as well as having Ca^{2+} sensor functions (Schwaller, 2010). Some evidence was supported that calretinin contributes to the control of synaptic plasticity (Schurmans et al., 1997; Camp and Wijesinghe, Rajiv, 2009), neural excitability (Edmonds et al., 2000) and may sense calcium and respond to it and other signals (Palczewska et al., 2005). Calretinin is present in horizontal retinal neurons, as well as in a subpopulation of amacrine and ganglion neurons.

The IPL of the retina is functionally stratified in an Off and On pathway wherein bipolar neurons synapse onto amacrine and ganglion neurons. A number of ganglion, amacrine, and bipolar cells, supporting the Off channel branch and interconnect at the outer part of the IPL, while the ON channel is connected at the inner half of the IPL (Nelson et al., 1978; Layer et al., 1997; Naito and Chen, 2004). Most of bipolar cells have their branches at the Off or the On sublamina, but recently it was describe that certain ON bipolar neurons can break the stratification rule by having synapses in both sublaminae (Hoshi et al., 2009). On the other hand, most of the amacrine neurons branch at the Off or at the On sublamina, and only a few, as the serotoninergic ones, ramify both at the On and Off sublaminae (Rios et al., 1997). In this context, we considered that there is a lack of *bona fide* information related to CaBPs at the IPL and inner nuclear layer (INL) of the retina, and related to the On or Off organization of retinal circuitries.

In this work we report different subtypes of calbindin and calretinin immunohistochemistry positive neurons in the inner retina from hatching to P12, taking into account the calbindin and calretinin networks and On-Off organization of the chicken retina. We also analyzed how calbindin D28k and calretinin are modified along postnatal development after rearing animals in dim red light conditions. Results show that only calbindin D28k undergoes modifications in our experimental days. To assess whether or not calbindin is involved in retinal neuronal plasticity we also analyzed phosphorylated p38 MAPK protein

expression, since this kinase was related with calbindin D28k function (Choi et al., 2001), as well as in the postnatal development of the visual system and plasticity processes (Oliveira et al., 2008).

MATERIALS AND METHODS

Animals

Fertilized eggs of White Leghorn strain were incubated in darkness at 38°C in a humidified incubator (56–58%) and rotated at regular intervals. Animals were distributed in two groups from hatching to P12. Control animals were reared in a white wall cage of about 50 × 50 cm^2 with cycles of 12 hs light and 12 hs darkness. The light in the cage proceeded from a unique bulb of 40 watts (Osram ASI 40, 390 lumen) placed 50 cm above the cage floor; light intensity in the center of the cage was about 300 lux. The other group, dim-light-reared animals, was raised from hatching to P12 in a similar cage whose walls were covered with black paper and illuminated with cycles of 12 hs darkness and 12 hs with a dim-red-light (Osram GO25 red, 25 watts, 20 lumen) placed 50 cm above the cage's floor yielding ~15 lux in the center of the cage. Chicken weights were monitored during the experimental period to avoid differences that could be attributed to corporal weight. The red light used for the dark-reared group allowed chickens to find their food; consequently they were neither light-deprived nor completely visually deprived.

The use of animals in these experiments was in accordance with the guidelines established by the NIH and the National Academy of Science, USA and approved by the Institutional Commitee for the care and use of laboratory animals (CICUAL), School of Medicine, University of Buenos Aires. Animals were housed in a room at about 24°C and received water ad libitum and Purina TM chick starter.

Tissue Processing

Chicks were anaesthetized with Pentobarbital and euthanized by decapitation. Retinas from 50 chickens from hatching to postnatal day 12 (P12) were isolated, and collected through selected experimental days. Experiments were performed at P6, P9, and P12. A central region of the retina, above the retinal pecten, was detached from the eye cup, fixed 3 h in a solution containing 4% paraformaldehyde, in 0.1*M* phosphate buffer, pH 7.4. Then, retinas were washed in phosphate buffer and cryoprotected by immersion in 10% (120 min) and 30% buffered sucrose (overnight) and then frozen at −20°C. Cryostat retinal sections (25 μm) were obtained using a slide microtome and collected in phosphate buffer saline (PBS) to perform floating sections immunohistochemistry. If retinas were not used immediately, they were stored at 4°C, in PBS, sodium azide 0.01% and used within the next 2 months.

Immunohistochemistry

Immunostaining was done with a modified procedure using biotin-avidin conjugates (Fosser et al., 2005). As primary antibody it was used an anti- calbindin-D28k and anti-calretinin antibody from SWANT (Swant Immunochemicals, Bellinzona, Switzerland). These antibodies were used in 1% normal goat serum, 0.3% Triton X-100, TS (Tris-HCl 50 m*M*, Na Cl 150 m*M*, pH: 7.4) for 24–48 h at 4°C. Negative controls were performed replacing primary antibody by goat serum. We followed our own immunocytochemical technique protocol in free floating slides (Fosser et al., 2005).

Monoclonal anti-calbindin D28k antibody is a mouse IgG1 produced by hybridization of mouse myeloma cells with spleen cells from mice immunized with calbindin D28k purified from chicken gut and was used 1:8000 and 1:5000. We decided to use 1:5000 since it gives us a better demarcation of calbindin D28k immunoreactive (CB+) networks (Supporting Information Fig. 1), as well as, a more suitable morphometric and densitometric analysis. At low dilutions of the antibody, only those neurons that have high amounts of calbindin or calretinin were seen, but it was very difficult to see and analyze their networks (Supporting Information Figs. 1 and 2). When retinas were overstained, a better morphological distinction of neurons in the INL and ganglion cell layer (GCL) could be seen, as well as a more accurate analysis of the pattern of distributions of calbindin and calretinin networks at the IPL (see Fig. 1 and Supporting Information Fig. 2).

In the chicken retina, antibodies to calbindin are known to label a few amacrine, bipolar, and ganglion cells, as well as cone photoreceptors including their axon terminals in the outermost stratum of the OPL (Pasteels et al., 1990).

The antiserum against calretinin is produced in rabbits by immunization with recombinant human calretinin containing a 6-his tag at the N-terminal and was used at 1:5000 (Swant Immunochemicals, Bellinzona, Switzerland). In chicken retinas, these antibodies are known to label two subtypes of horizontal cells and a few amacrine neurons (Fischer et al., 2007).

For double-labeling secondary antibodies (Supplementary Fig. 2) we used a rabbit anti-mouse IgG conjugated to rhodamine-Red X 1:2000 and donkey anti-rabbit IgG conjugated to FITC 1:2000 (Jackson Immuno Research Laboratories).

Sections were incubated in a blocking solution for 2–4 h at 4°C and at that time, sections were transferred to the primary antibody solution for 18–24 h. After that, sections were washed in TS for 40 min and then incubated with the secondary antibody 1:2000 (sheep anti-rabbit biotin-SP, Chemicon, now Millipore) for 3 h, washed in TS for another 40 min and incubated with streptavidin-HRP conjugate, Chemicon 1:1000 for 2 h. After washing twice with TS, sections were washed with 0.1*M* acetate buffer pH 6 for 5 min. Development of peroxidase activity was carried out with 0.035% w/v 3,3′diaminobenzidine plus 2.5% w/v niquel ammonium sulfate and 0.01% H2O2.Sections were observed and photographed with a Zeiss Axiophot light microscope equipped with epifluorescence.

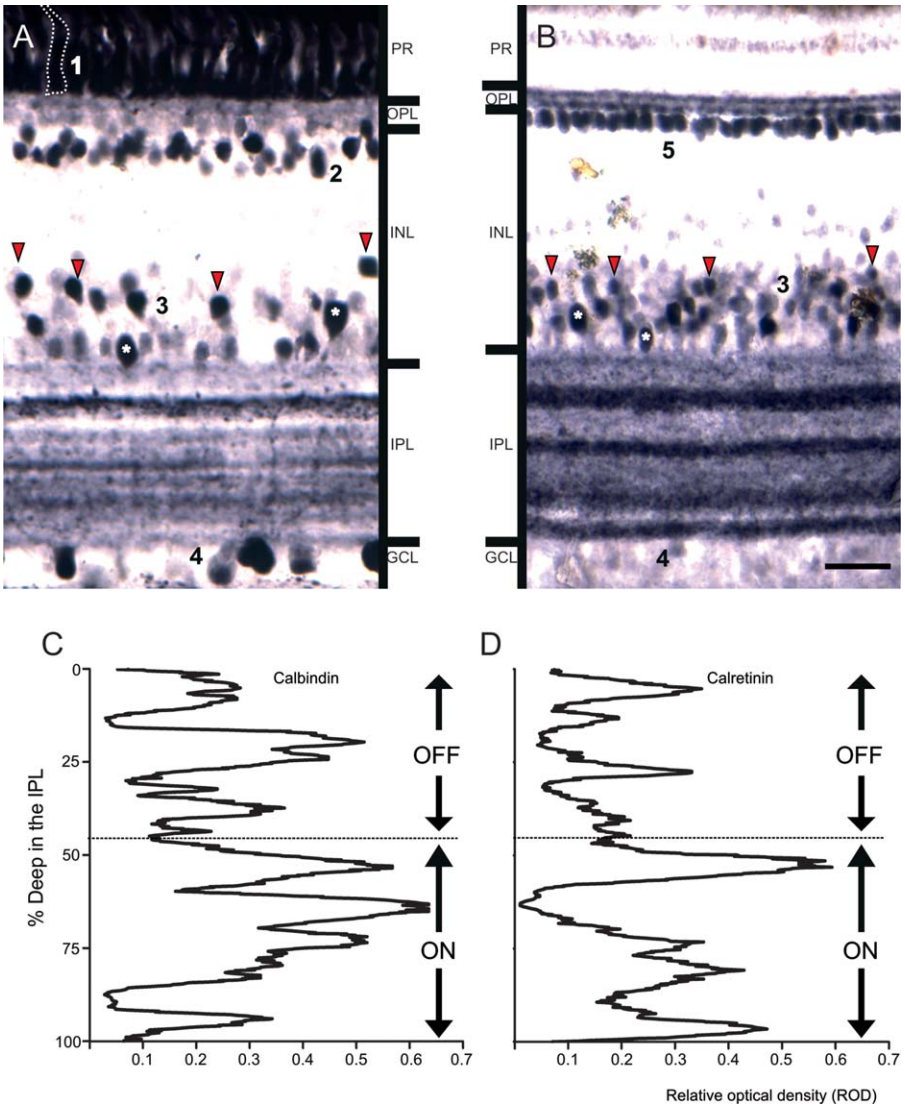

Figure 1 A,B**:** Calbindin and calretinin immunohistochemistry at hatching. A: Calbindin D28K immunoreactive neurons are stained at photoreceptor layer (1), the internal nuclear layer in which bipolar (2) and amacrine neurons (3) are labeled and at the ganglion cell layer (4). B. Calretinin is clearly seen at the INL staining horizontal (5), amacrine (3) neurons and also some ganglion cells (4) at the GCL. In A and B arrowheads are indicating the small populations of CB+ or CR+ neurons and asterisks the bigger ones. Like calbindin immunoreactive networks, calretinin networks are organized in several strata at the IPL. C,D: Charts showing the relative optical density profile through the IPL to show how CB+ or CR+ networks are organized. Stratification of calbindin (C) and calretinin (D) immunoreactive neurons are clearly observed at the IPL. At this layer, sets of ganglion, amacrine, and bipolar cell types, carrying the Off channel, arborize and interconnect in the outer part of the IPL, while the On channel is wired in the inner half of the IPL. PR, photoreceptor and outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Image Analysis

The morphometric analyses were performed using Image Pro 4, Media Cybernetics. The analysis were done only in the following conditions: (a) the section of retinas were perpendicular to the nerve layer, (b) sections belonged to central retina, and (c) the thickness of the inner plexiform layer (IPL) do not show significant differences between groups. IPL networks from chicken retina were analyzed from P6 to P12 for control and dark-reared conditions as

was previously used in our laboratory (Fosser et al., 2005). The relative area occupied by the calbindin and calretinin immunoreactive networks was analyzed and measured after performing segmentation between mark and background. The relative optical density (ROD) was measured to evaluate the intensity of calbindin and calretinin immunoreactivity. The measured area ROD was obtained according to the formula: ROD= log (256/mean gray). To integrate data from relative area and ROD, the integrated optic density (IOD) was used following the formula: IOD = area x average optical density (Image Pro, Media Cybernetics). IPL networks were measured separately and data were collected in an Excel (Microsoft Corp.) sheet.

All measurements were done after true color images (RGB 24bits) were converted to gray scale, 8 bit-pixel. Data were expressed as mean ± standard deviation (SD) of a minimum of eight animals per group and experimental age. Results were statistically analyzed using two-tailed Student's *t*-test.

Western Blot Analysis

Western blot analyses were performed on retinal extracts that had been homogenized in a Potter-Elvehjem homogenizer, in 3 vol of TS containing 1 m*M* EDTA, 0.5% Triton X-100, 0.1% sodium dodesylsulfate (SDS),1 m*M* PMSF and protease inhibition cocktail (Sigma-Aldrich). The homogenate was centrifuged at 900*g* for 10 min in order to discard pigmented epithelium. The supernatant was used in the western blot. Protein concentration in each sample was assayed by the BCA Protein Assay Kit (Thermo Scientific Pierce), by triplicate and the results averaged. Aliquots of tissue samples corresponding to 30 μg of total protein in sample buffer containing 4% SDS and 10% mercaptoethanol were loaded onto SDS-12% polyacrylamide gels. The proteins were electrotransferred to a PVDF membrane in Tris-glycine-methanol buffer.

The membrane was blocked for 1 h at room temperature in a blocking solution containing 5% normal goat serum, 0.1%, Tween-20, and PBS (pH 7.4). The membrane was then incubated overnight at 4°C with primary antibodies in the blocking solution. The following primary antibodies were used in the western blot experiments; mouse monoclonal anti-calbindin D28k (Swant Immunochemicals, Bellinzona, 1:5000), rabbit polyclonal anti-CR (Swant Immunochemicals, Bellinzona, 1:5000), mouse monoclonal anti p-p38 (Santa Cruz BT, sc-7973, 1:200), kindly gifted by Dra. S. Fiszer de Plazas and mouse monoclonal anti p-erk (Santa Cruz BT, sc-7383, 1:750) kindly gifted by G. Cremaschi and mouse anti-GAPDH (Santa Cruz BT; 1:5000) for a loading control. The membrane was rinsed three times with 0.05% Tween-20 in PBS for 10 min each, followed by incubation for 1 h at room temperature with HRP donkey anti-mouse IgG or anti-rabbit IgG, 1:2000 (Millipore). The blot was washed three times for 20 min each and then processed for DAB/Ni immunochemistry. Development of peroxidase activity was carried out with 0.04% w/v 3,3′diaminobenzidine (Sigma-Aldrich) plus 2.5% w/v nickel ammonium sulphate (Baker) and 0.01% H_2O_2. The reaction was stopped by adding tap water and then with distilled water.

RESULTS

Retinal Pattern of Expression of Calbindin and Calretinin: Subtype of Neurons and Plexus

Calbindin D28k and calretinin are normally expressed in different retinal neurons and only few neurons co-express both proteins (see Supporting Information Fig. 2). There are at least 2 subtypes of CB+ amacrine neurons. One type of CB+ amacrine cells has a round big soma which is located just at the boundary between IPL and INL [asterisk in Fig. 1(A) and Supporting Information Fig. 2(D)]. These neurons are homogenously distributed along the INL, and there are 5 or 6 somas between them, including either immunoreactive small calbindin neurons or immunonegative ones. The other subpopulation of CB+ neurons are smaller, they are localized 2 or 3 somas above the boundary between INL and IPL [arrowhead in Fig. 1(A) and Supporting Information Fig. 2(D)]. They look pale when a diluted solution of the antibody was used. Sometimes it can be observed that these neurons have a single primary dendrite which then gives some branches into the IPL forming in this way the calbindin sublayers.

Photoreceptors are also immunostained with anti-calbindin antibody. However, not all the cones are stained, just only those whose terminals are in the most external sublayer (double-cones). Cones are labeled from the inner segment to the synaptic ending [outlined as 1 in Fig. 1(A)], but in this work we did not analyze double-cone neuroplasticity. Besides, bipolar and ganglion neurons are also CB+. A row of bipolar neurons are labeled just below horizontal neurons [Fig. 1(A)]. Ganglion cells are occasionally seen, and future analysis must be done to discard if they were amacrine displaced neurons or not.

Calretinin immunoreactive (CR+) neurons are mainly detected at the INL. In Figure 1(B) photoreceptors are negative and some horizontal neurons are positive, about 60% of horizontal neurons are immunoreactive for calretinin, as was reported by other authors (Fischer et al., 2007). In a similar manner that CB+ amacrine neurons, two different populations of CR+ cells can be described. One CR+ amacrine type of neurons has a round soma which is located near the internal edge of IPL [asterisk in Fig. 1(B) and Supporting Information Fig. 2(E)]. The

other CR+ neurons are smaller, more abundant than the smallest CB+ and they are also localized 3 somas above the internal edge of INL [arrowhead in Fig. 1(B) and Supporting Information Fig. 2(E)].

Calbindin and Calretinin Sublaminar Pattern at IPL in Normostimulated Retinas

Immunostained retinas with anti-calbindin D28k and anti-calretinin antibodies showed a multiple sublaminar pattern at the IPL [Fig. 1(A,B), Supporting Information Fig. 1], which is easily distinguished through a densitometric analysis. In Figure 1(C,D) a densitometric characterization of immunoreactive networks of CB+ and CR+ at the IPL is shown. Furthermore, and considering amacrine CB+ neurons position, at least five rows of amacrine cells are observed into the INL. These neurons are quite different not only by their retinal position but also for their size, morphology, and calbindin immunostaining (by densitometric analysis).

The pattern of CB+ and CR+ networks varies when both antibodies are compared (Fig. 1 and Supporting Information Fig. 2), but with both antibodies, two main zones could be analyzed considering the simplified functional version of retinal On/Off channels proposed by Famiglietti and Kolb (Famiglietti and Kolb, H., 1976). In this study we used for analysis the outer 40% of the IPL as the Off channel, where cells are hyperpolarized upon an increase in illumination, and the rest of the IPL as the On channel, whereas cells are depolarized by light [Fig. 1(C,D)], since it was proved to be useful in other nonmammalian species (Djamgoz and Wagner, 1987; Layer et al., 1997; Djamgoz et al., 2001; Naito and Chen, 2004).

Effect on Calbindin Responsiveness in Dim-Light-Reared Chicken Retinas

In the chicken inner retina, calbindin positive cells constitute a heterogeneous group of neurons that includes different types of amacrine cells, a subtype of bipolar neurons and ganglion cells. In this study, the dim red light did not affect the global pattern of the CB+ sublayers of the IPL through postnatal development [Fig. 2(A–F)]. Western blot and densitometric analysis (Fig. 2(G,H)] between normal and dim-light-reared group of animals have shown a decrease at P9 and P12 in calbindin D28k, as well as in the densitometric immunohistochemistry study (IOD, Fig. 2(I)]. Morphometric analyses not only confirmed the western blotting results but also allowed us to study in particular the calbindin networks at IPL. On the other hand, since IOD allow us to perform a detailed analysis of calbindin strata at IPL, a new analysis was done and plotted as a bar chart in which the Off and On layers of the IPL were analyzed, but these results did not show significant differences between the Off and On channels of the IPL [Fig. 2(J)].

Since the IPL is composed of axons and dendrites of ganglion, bipolar and amacrine cells and in order to rule out that the observed changes in the IPL were related to changes in the number of neurons, we analyzed the density of CB+ neurons observed along 100 μm of the naso-temporal axis. No significant difference was found in the number of CB+ neurons in the INL, neither in the GCL (Fig. 2 and data is summarized in Table 1), nor in the stratification profile through the IPL (Supporting Information Fig. 1), between C-r and D-r retinas. These results ratify that the plastic modifications observed on calbindin D28k immunoreactivity is at the synaptic level due to early postnatal hypostimulation.

Effect on the Calretinin Responsiveness in the Inner Retina in Dark-Reared Animals

Large populations of horizontal and amacrine cell are immunoreactive to calretinin but only a few neurons at the GCL (Figs. 1, 3, and Supporting Information Fig. 2). Although the CR+ lamination in the IPL is less defined than CB+ sublayers, seven CR+ strata could also be described at early postnatal stages and similarly as calbindin the outer region of the IPL was considered as Off sublayer, but we could not observe differences between them [Fig. 3(J)]. In chickens reared with dim red light a tendency to diminish the IOD was observed, and data in D-r animals is about half of control ones at P12. However, due to the high variability between animals we could not see any statistical differences [Fig. 3(H)].

The effect of the red dim-light on the CR+ pattern in the IPL, IOD statistical analyses and western blotting analyses did not show significant differences for all the stages analyzed [Fig. 3(A–F) and (G–H)]. The density of CR+ neurons, both in the INL and GCL was also analyzed, but no significant difference were found in the total number of cells neither at the INL nor in the GCL (Table 1).

Effect on Mitogen-Activated Protein Kinases (MAPKs) in the Retina in Dim Red Light-Reared Animals

There are four well-characterized subfamilies of mitogen-activated protein kinases (MAPKs):

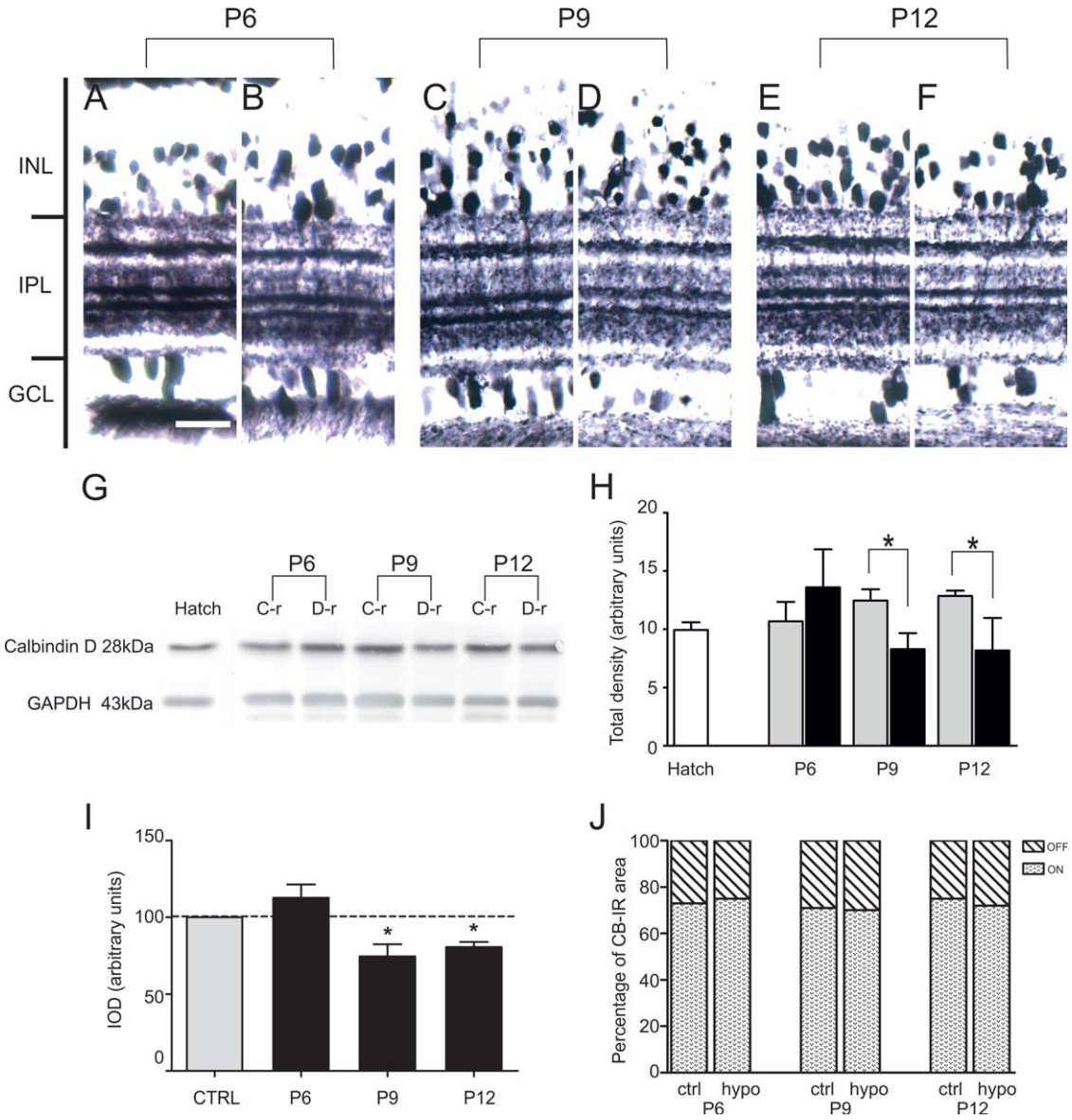

Figure 2 A–F: Microphotographs showing calbindin expression pattern in postnatal chicken retinas from P6 to P12 demonstrating their networks at several strata into the IPL. The most external layers are related to the Off channels of the retina, nearly 40% of this layer, while the rest of the IPL belongs to the On channel. A, C, and E: Control-reared chicken retinas (C-r) illustrating the immunoreactive protein patterning at the IPL. B, D, and F: Dim-light-reared chicken retinas (D-r). Note that these networks have a similar pattern than those observed in the control-reared chicken retinas (A, C, and E). G–H: Calbindin D28k western blot (G) and densitometric analysis (H) showing significant differences between normal vs. dim red light conditions. Results were expressed as a bar chart (H) and confirm significant difference at P9 and P12 (asterisk=$p < 0.05$; Student's *t*-test). I, integrated optical density of networks strata, expressed as a percentage respect to normostimulated retinas (Ctrl). A low amount of calbindin D28k was detected in P9 and P12 D-r retinas (asterisk= $p < 0.05$; Student's *t*-test). J: Bar chart analysis of On and Off layers at the IPL in control and dim-light-reared chickens; not significant differences were seen between them. INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; GAPDH, Glyceraldehyde 3 phosphate dehydrogenase. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1 Density of CB+ and CR+ Neurons in the Inner Retina

	Calbindin D28k		Calretinin	
Stage/condition	INL	GCL	INL	GCL
P9 / C-r	30.66 ± 6.02	9.33 ± 1.15	106.33 ± 13.31	5.33 ± 0.57
P9 / D-r	37.0 ± 1.73	10.00 ± 1.0	117.0 ± 10.44	7.0 ± 1.0
P12 / C-r	35.0 ± 2.58	10.75 ± 1.89	108.0 ± 7.02	4.33 ± 0.57
P12 / D-r	33.66 ± 3.21	10.66 ± 3.21	101.66 ±3.05	5.0 ± 1.0

Values represent the mean of the number of neurons ± standard deviation observed along 100 μm of the naso-temporal axis, in control (C-r) and dim-light-reared (D-r) chicken retinas. Values are the average of at least three paired separate experiments run for each experimental day. Individual experiments were composed of three to five tissue sections of each animal. The number of CB+ or CR+ neurons between control and dim-light-reared groups not showed significant differences during our experimental days (Student's *t*-test).

extracellular signal-regulated kinase (ERK) 1/2, ERK5, c-Jun N-terminal kinase (JNKs) and p38 MAPKs (Cuenda and Rousseau, S., 2007). To test whether or not calbindin is involved in retinal neuronal plasticity we also analyzed phosphorylated p38 MAPK (p-p38 MAPK) and phosphorylated ERK (p-ERK) protein expression. Western blot analyses of these kinases showed an increase in p-p38 both in C-r and D-r chickens retina during postnatal development (Fig. 4). Moreover, at P9 and P12 a decrease in the phosphorylation of p38 MAPK was observed between C-r and D-r. Phosphorylated ERK showed a statistically non-significant trend to decrease after rearing chickens in dim red light (Fig. 4).

DISCUSSION

In this work we have analyzed the calbindin and calretinin profiles by immunohistochemistry and western blot, in control and dim red light-reared chickens. It was already described that calbindin D28k is expressed in several subtypes of retinal neurons, sometimes co-expressed with other CaBPs (Bennis et al., 2005; Ichikawa et al., 2005) and here we focus on amacrine neurons networks. As was described, calbindin and calretinin are fast buffers related with neuronal plasticity (Berridge et al., 2003; Jackson and Redman, 2003; Roussel et al., 2006). Our results related to calbindin D28k, a member of the calmodulin superfamily of calcium-binding proteins, point out in this direction.

It has been suggested that calbindin D28k can also act as an activity-dependent sensor that targets membrane cytoskeleton-bound myo-inositol monophosphatase in central neurons, being this interaction important since it may affect calcium buffering by calbindin (Schmidt et al., 2005). The relationship between calbindin and membrane/cytoskeleton phosphatases has been recently studied in cerebellar Purkinje neurons (Schmidt et al., 2005) and the role of calbindin containing synapses in paired pulse facilitation has also been established as a novel presynaptic mechanism for activity-dependent control of synaptic gain in the mouse (Blatow et al., 2003). Interestingly, a study related with our work demonstrated that in MN9D dopaminergic neuronal cells the expression of calbindin-D28K induces neurite outgrowth in a p38 activity-dependent manner (Choi et al., 2001).

Our results show that changes in calbindin immunoreactive networks are closely correlated with environmental cues—dim red light rearing—and allow us to hypothesize that calbindin could act as fast calcium buffer, therefore working as a neuronal activity-dependent sensor, as was previously suggested in other areas of the CNS (Blatow et al., 2003; Roussel et al., 2006). More interestingly, when p38 MAPK was analyzed, we observed differences in this kinase, which suggests a relationship between calbindin and retinal neural plasticity.

Calbindin Neuronal Subtypes

Calbindin-D28k expression was previously described in chicken retina but the authors did not attempt to systematize these neurons from a morphological point of view (Pasteels et al., 1990; Hamano et al., 1990). In this study we could describe at least five rows of amacrine cells into the INL, which are different considering their retinal position, size, morphology, and densitometric properties as a relative parameter of calbindin D28k amount. In addition, double cone photoreceptors are also immunoreactive as well as a few bipolar and ganglion neurons [Fig. 1(A) and Supporting Information Fig. 1(A)]. When high dilutions of the antibody are used, only one type of the amacrine cells can be clearly seen, which are the big ones and few smaller amacrine CB+ cells. This data point out about the heterogeneity of CB+ neurons.

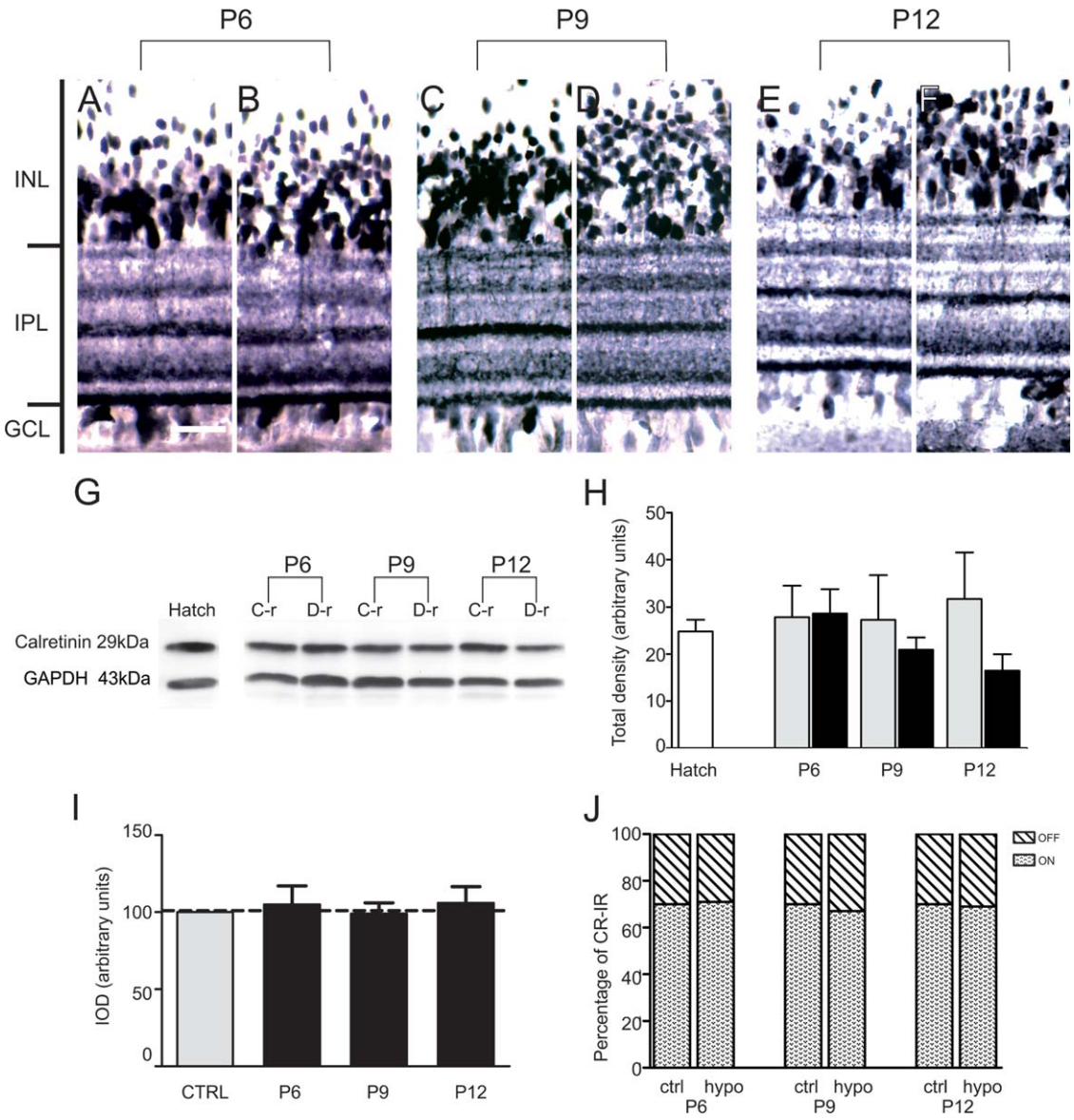

Figure 3 A–F: Immunohistochemical expression pattern of calretinin in chicken retinas from P6 to P12. Similar considerations as in Figure 2 can be done about the On and Off layers of the retina and calretinin networks can be observed as strata into the IPL. A, C, and E: Control-reared chicken retinas (C-r). B, D, and F: Dim-light-reared (D-r) chicken retinas. Again, there are neither new strata nor differences in the number of amacrine or ganglion between our experimental animals. G–H: Western blot (G) and densitometric analysis (H) of calretinin did not show differences between chicks reared in control vs. dim-light conditions. I–J: Morphometric analysis of calretinin neurons and their networks. Note that no significant differences were observed between the On and Off sublayers at IPL (Student's *t*-test). PE, retinal pigment epithelium; PR, photoreceptors; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; ROD, relative optical density. Scale bar = 20 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Neuroplasticity of Calbindin Immunoreactive Networks

The IPL was analyzed taking into account the distribution of dendrites and axons, in retinal Off-On channels, as was described above (Results). However, other authors have suggested that the dendritic tree of the IPL could be described in several sublayers. The application of a histochemical procedure

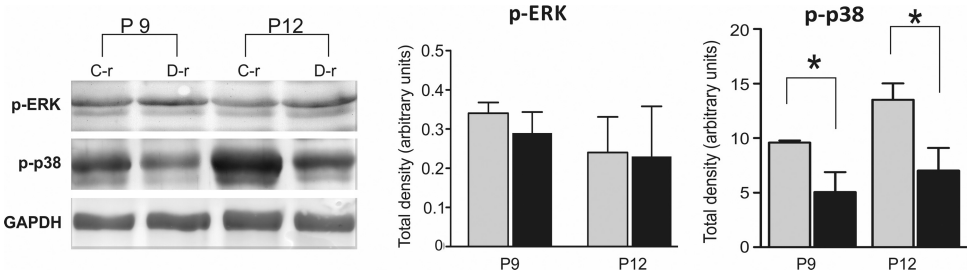

Figure 4 Immunoblot analysis of the phosphorylated forms of MAPKs in chicken retinas at P9 and P12. The total optical density was plotted for p-ERK and p-p38. The p-p38 graph shows a decrease in the phosphorylation of p38 MAPK in dim red light conditions. No changes were observed in the phosphorylation of ERK in the same experimental conditions. C-r, control; D-r, dim-light-reared; GAPDH, Glyceraldehyde 3 phosphate dehydrogenase. Results were expressed as bar charts (asterisk = $p < 0.05$; Student's *t*-test).

allowed Shen et al. to describe five sublayers in chicken retina (Shen et al., 1956). Lucifer yellow and DiI label of ganglion cells allowed to describe eight sublayers into the IPL chicken retina (Naito and Chen, 2004). We performed a densitometric analysis through the IPL [Fig. 1(C)] and observed eight CB+ layers, but we preferred to use the Off-On dendritic/axon organization proposed by Famiglieti et al. (1976) since this organization fits better with our analysis.

Plasticity of calbindin in chicken retina can be described considering our immunohistochemistry and western blot results. Western blot allow us to analyze the whole protein pool of calbindin in retina, but poor information about Off-On response of calbindinergic or calretinergic networks. On the other hand, an immunohistochemistry analysis of CB+ and CR+ can give us an accurate measure about neurons that synthesize calbindin or calretinin, the On-Off networks, as well as plastic changes in CB+ and CR+ neurons.

Dim light-reared animals showed a decrease in the optical density in their CB+ networks from P9 [Fig. 2(A–F,I–J)] analyzed by immunohistochemistry. These changes, seems not to be related with a decrease in the number of neurons in the INL or in the GCL because neuronal population density remained unchanged in both control and dim-light-reared chickens. Immunohistochemistry analysis have shown modifications in IOD value which could be related to changes in the area occupied by dendrites and axons from CB+ neurons that branch into the IPL. It could be possible that the changes shown in the IPL are the consequence of modifications in the stratification pattern of the neurons and subsequently in the quantity of CB. This point is supported since our western blot analysis showed that the amount of calbindin D28k as well as the morphometric data was reduced in the retina of dim-light-reared chickens. To our knowledge this is the first report providing evidence that calbindin D28k may act in retinal neural plasticity in close relationship with light. Recently, it was demonstrated in mice that calbindin modulates the photosensitivity of different components of the nonimage-forming visual system, playing an important role in transducing photic intensity information (Butler et al., 2011).

The relationship between calbindin and retinal neural plasticity was studied by p-p38 MAPK and p-ERK immunoreactivity, since both kinases are related to calcium signaling (Lee et al., 2000; Takeda and Ichijo, 2002; Katz et al., 2006). Furthermore, it was established that calbindin is involved in neuronal differentiation and in the process of neurite extension through p38 MAPK. Overexpression of calbindin D28K induces neurite outgrowth in dopaminergic neurons via activation of p38 MAPK, but not other MAPKs (Choi et al., 2001).

Our results show that p-p38 MAPK but not p-ERK is concurrent with calbindin modifications. In the central nervous system was demonstrated that p38 MAPK is involved in long- and short-term depression (Bolshakov, 2000; Guan et al., 2003; Xiong et al., 2006) and that ERK1/2 is involved in long-term potentiation (English and Sweatt, 1996; Liu et al., 2011). Calcium entering at the plasma membrane can induce a number of downstream signaling pathways, among them, the activation of MAPKs (Berridge, 1998; Berridge et al., 2003; Berridge 2012). Furthermore, an involvement for p-p38 MAPK in plasticity processes was suggested in the rat developing visual system (Oliveira et al., 2008). Our results are consistent with studies in which p38 MAPK is involved in the regulation of synaptic plasticity (Thomas, 2004; Correa and Eales, 2012). All together, these data suggest us that also in the chicken retina calbindin could exerts its actions through p38 MAPK, supporting our hypothesis that calbindin D28k is implicated in neuroplastic changes in the retina.

Calretinin Immunostained Networks

Calretinin was suggested to have a role in the maintenance of calcium dynamics and neuronal excitability (Edmonds et al., 2000; Gall et al., 2003; Camp and Wijesinghe, 2009). Although there are significant data associating calretinin with the mechanisms of neuroplasticity (Schurmans et al., 1997; Gurden et al., 1998; Cheron et al., 2008), here in this work it could not be found substantial differences in the CR+ networks in the IPL of dim-light-reared animals. Furthermore, no statistically differences were seen in calretinin western-blotting analyses, although a slight tendency at P9 and P12 was seen in the retinas of dim-light-reared chickens. As could be noted in Figure 3 there are differences between control and dim-light-reared groups, although due to the high variability of the results, no significant differences were registered. It is possible that our densitometric and morphometric analyses were not sharp enough to detect small changes in CR+ neurons. However, since P9 and P12 retinas showed a tendency to change the expression pattern of the protein CR under the different rearing conditions used in this study. Therefore, our results do not support a role for calretinin in neural plasticity.

CONCLUSIONS

Dim-red-light-reared animals showed a decrease of CB+ amount at the IPL networks from P9. Epigenetic environmental influences are crucial in the wiring of the nervous system and our results show that calbindin is an important player in visual adaptation, which should be considered as an important part of the biology machinery in retinal neuroplasticity.

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