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Original Article

Distribution of phosphorylated cyclic AMP response element binding protein (p-CREB-1) in rat retina

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Abstract: Purpose: The phosphorylation of cyclic AMP (cAMP) response element binding protein (CREB) is essential to the physiology of neuron development. In the present study, we sought to determine the expression profile and specific cell distribution of p-CREB-1 in the rat retina. Methods: Double immunofluorescence staining was employed to determine the developmental expression pattern and specific distribution of p-CREB-1 in adult rat retina. Results: Our data implicate a developmental expression pattern of both CREB-1 and p-CREB-1 in rat retina. The p-CREB-1 was prominently expressed in retinal neurons and rarely expressed in retinal glial cells, as evidenced by immunofluorescence assay. Conclusion: The developmental phosphorylation pattern and distinct expression localization of p-CREB-1 in rat retina suggest a physiological role for p-CREB-1 in the retinal development.

Keywords: p-CREB-1, retina neurocytes, retina development

Introduction

CREB-1, a pleiotropic leucine zipper transcription factor, mediates important mechanisms governing several physiological functions of the central nervous system ranging from development to plasticity and disease [1, 2]. The canonical pathway that leads to the activation of CREB-1 by phosphorylation of the Ser133 residue [3] has been implicated in the regulation of proliferation, differentiation, and neurogenesis in the developing neuron system [4, 5], and mediating synaptic plasticity, long-term memory formation and consolidation in the adult brain [6]. Its activity is triggered by increased intracellular levels of cAMP and Ca^{2+} influx in response to neurotransmitters, hormone stimulationand neuronal activity [7, 8]. Triggering this pathway subsequently initiates the transcription of multiple target genes [9]. During mammalian development neuronal activity, and possibly Ca^{2+} influx, is critical to the formation of synaptic connections, suggesting that phosphorylated CREB-1 (p-CREB-1) is involved in this physiological process [7, 10].

In embryonic cortical neurons, neuronal activity induces transcription of neuro-protective proteins such as Bcl-2 and brain-derived neurotrophic factor (BDNF) by a p-CREB-1-dependent mechanism [11]. In the adult hippocampus, CREB-1 phosphorylation occurs in response to a diverse array of stimuli and has been shown to enhance cell survival and neurogenesis [12]. Moreover, several lines of evidence suggest that p-CREB-1 integrates contextual information, regulates the repertoire of retinal precursor cells and acts as a neuronal survival moderatorduring retinal development [13, 14]. *In vivo* evidence indicates that decreased phosphorylation levels of CREB-1 are associated with retinal degeneration [15, 16]. Yet the physiological role of p-CREB-1 in the regulation of retina development remains unclear.

The vertebrate retina, a part of the central nervous system, contains seven major cell types and is still undergoing sustained development within a period of time after birth. During the early postnatal period, extracellular stimuli, such as neurotransmitters, trigger long-term phenotypic changes in multi-potent retinal progenitors, including cell differentiation and synaptic formation, to generate different retinal cell typesand form a functional retina [17].

Table 1. List of primary antibodies used in this study

Antigen	Host	Antibody source	Working concentration
CREB-1	Rabbit	CST 9197s	1:200
P-CREB-1	Mouse	Millpore 2361835	1:200
Protein kinase C, a isoform (PKC-α)	Rabbit	CST 2056	1:200
Microtubule-associated protein-2 (MAP-2)	Rabbit	Abcam ab96378	1:200
THY-1.1	Rabbit	Millpore MAB1406	1:200
Calretinin	Rabbit	Boster BA0681	1:100
Calbindin	Rabbit	Boster PB0017	1:100
Glutamine synthetase (GS)	Rabbit	Abcam ab49873	1:200
Glial fibrillary acidic Protein (GFAP)	Rabbit	Boster BA0056	1:100

Detailed information is given about the antibodies used, the host, the commercial source and catalog reference, as well as the working concentration at which each antibody was used depending on the application.

Several intracellular signaling cascades that phosphorylated nuclear transcriptional factors are involved in these sophisticated processes [7, 14]. Identifying in which cells the transcriptional factors have beenphosphorylated might facilitate the understanding of its biological roles. However, the distribution of these transcriptional factors such as p-CREB-1 has not been reported.

Given the importance of p-CREB-1 in neuron development, it is likely that this transcription factor is also involved in the regulation of retina development. Therefore, the expression profile and cell location of p-CREB-1 was immunochemically evaluated in rat retina under normal conditions by using double-staining assay. The new insight of this study might shed light on the potential involvement of p-CREB-1 inretinal development.

Methods

Animals

All experiences involving animals strictly adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the procedure was approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center (Permit Number: SYXK (YUE) 2010-0058). The rats were housed in Ophthalmic Animal Laboratory of Zhongshan Ophthalmic Center, at Sun Yat-sen University, in an air-conditioned room with an ambient temperature of 16-26°C, a relative humidity of 40-70% and a 12-hour light-dark cycle. Food and water were available *ad libitum* and animal health was monitored by the animal care staff and veterinarian. The Sprague-Dawley (SD) rats were sacrificed by an intra-peritoneal injection of chloral hydrate (P3761, 60 mg/kg) (Sigma,St. Louis, MO) before we harvested the eyes.

Tissue preparation

The rats were anesthetized with chloral hydrate (5 mg/ml) and perfused with physiological saline and then 4% paraformaldehyde. Afterwards, the eyes were harvested and fixed in 4% paraformaldehyde overnight. Then, the eyes were dehydrated with graded sucrose solution and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, California, USA). Subsequently, the transverse sections of the rats' eyes were cut by using a microtome (Thermo Electron Corporation, Cheshire, UK), and mounted onto glass slide (CITOTEST, Jiangsu, CHINA), at a section thickness of 10 μm, and stored at -20°C until processed.

Immunofluorescence assay

Immunofluorescence was used to determine the specific location of p-CREB-1 in the mature retina. For immunofluorescence staining, the slides with tissue sections were washed with phosphate buffer saline (PBS) and permeabilized by 0.5% Triton X-100 (Sigma, St Louis, MO, USA) for 10 min, thenblocked with 10% normal goat serum (Boster, Wuhan, China) for 30 min. The sections were incubated with primary antibodies (**Table 1**) in a humidified chamber at 4°C overnight. After being washed with PBS for 15 min the secondary antibody (CST, Danvers, MA) was added to the sections at room tem-

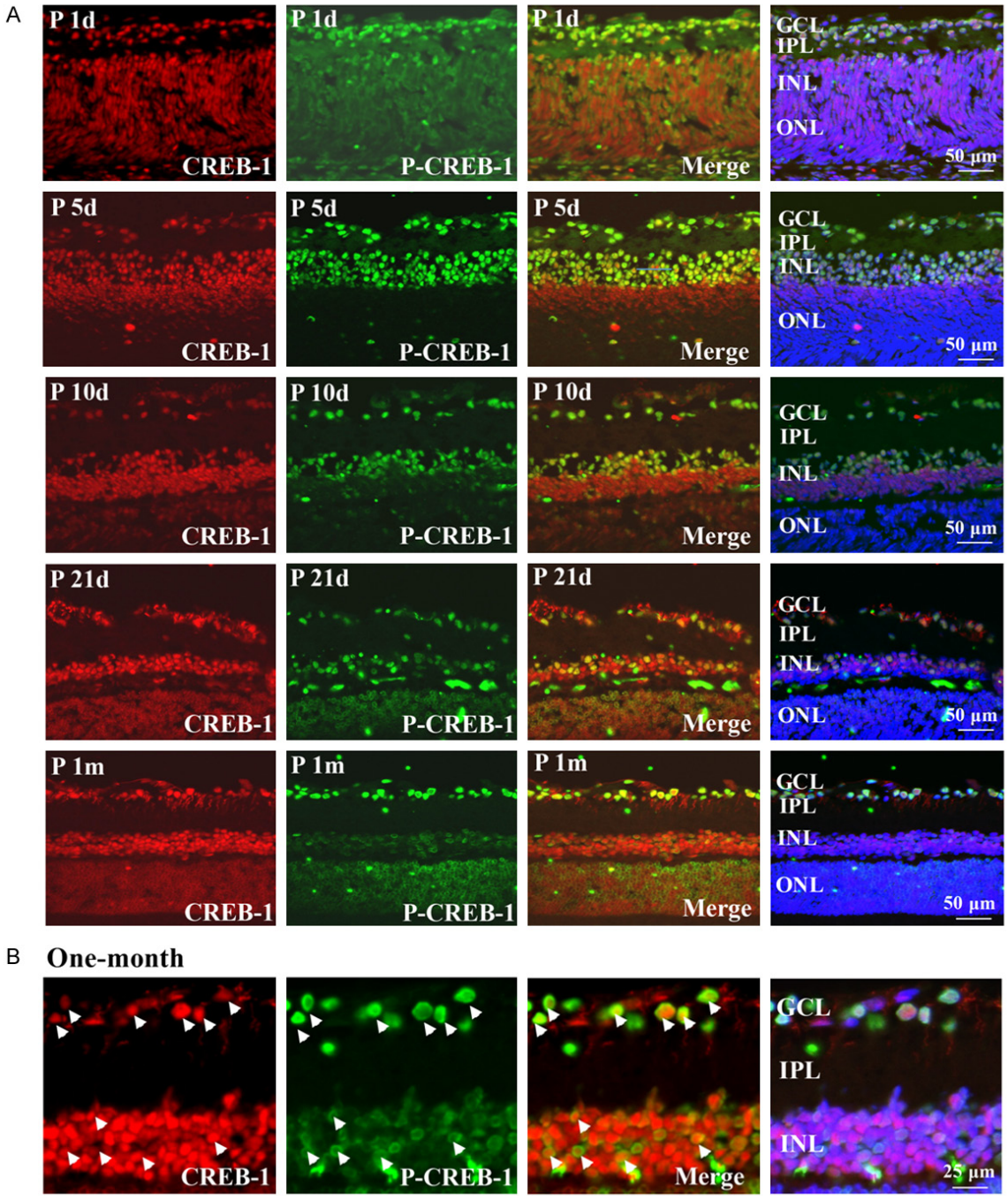

Figure 1. Developmental expression profile of CREB-1 and p-CREB-1 in rat retina. A. The expression of CREB-1 (red) and p-CREB-1 (green) in the rat retina were observed at postnatal day 1, day 5, day 10, day 21 and 1 month by using immunofluorescence staining. B. Confocal immunofluorescent images at higher-magnification of CREB-1 (red) and p-CREB-1 (green) staining in adult rat retina. Nuclei were stained with DAPI (blue). Arrows indicate examples of co-localization. Ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

perature as required, and the nuclei were stained with DAPI. Sections were rinsed with water and mounted with Fluoromount G (eBioscience, San Diego, USA). Photomicrographs were taken by using the LSM 5 Pascal confocal laser scanning microscope (Zeiss, Oberkochen, Germany). Images of the retinal sections were collected at 400× and 800× magnification.

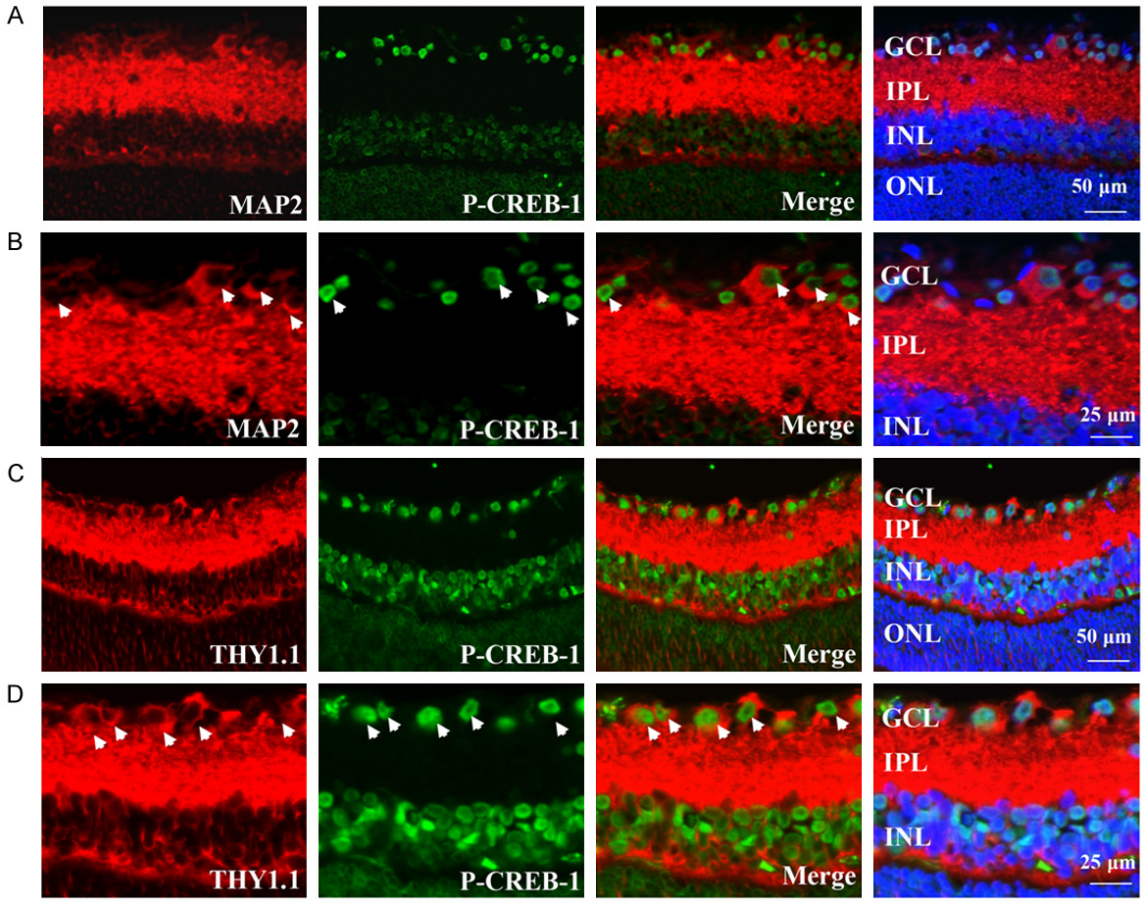

Figure 2. Immunolocalization of p-CREB-1 in the adult rat retina neurons. A. P-CREB-1 immunoreactivity in the rat retina co-localized with Map-2 positive cell. B. Confocal immunofluorescent images at higher-magnification of p-CREB-1 (green) and Map-2 (red) staining in adult rat retina. Nuclei were stained with DAPI (blue). Arrows indicate examples of co-localization. C. P-CREB-1 (green) immunoreactivity in the rat retina co-localized with THY1.1 (red) positive cell. D. Confocal immunofluorescent images at higher-magnification of p-CREB-1 (green) and THY1.1 (red) staining in adult rat retina. Nuclei were stained with DAPI (blue). Arrows indicate examples of co-localization. Ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

Results

The developmental expression profile of CREB-1 and P-CREB-1 in rat retina

In the central nervous system, almost every neuronal and glial cell appears to exhibit CREB-1 expression [18]. The activity of CREB-1 is triggered by signaling cascades that result in phosphorylation of CREB-1 in differentiated cells [19, 20]. Here, we analyzed the developmental expression profile of CREB-1 and p-CREB-1 in therat retinaat different time points by using a double-immunochemical assay. The p-CREB-1 antibody recognizes the peptide sequence containing the phosphorylated Ser133. Representative microscopic pictures demonstrated that both the CREB-1 and p-CREB-1 are expressed in the nuclei of each cell. No staining was observed in the sense controls (datanot shown).

Varying intensities of CREB-1 and p-CREB-1 staining were observedin the retina at different developmental stages (**Figure 1**). As shown in **Figure 1A**, at postnatal day 1, CREB-1 was constitutively distributed in the all layers of the retina; however, with ongoing retina developmentits expressionwas down-regulated in the outer nuclear layer (ONL) and gradually restricted to only the ganglion cell layer (GCL) and inner nuclear layer (INL). In contrast, p-CREB-1 was almost exclusively expressed inthe GCL of postnatal day 1 rats and profoundly up-regulated in the GCL and INL of the mature rat retina.

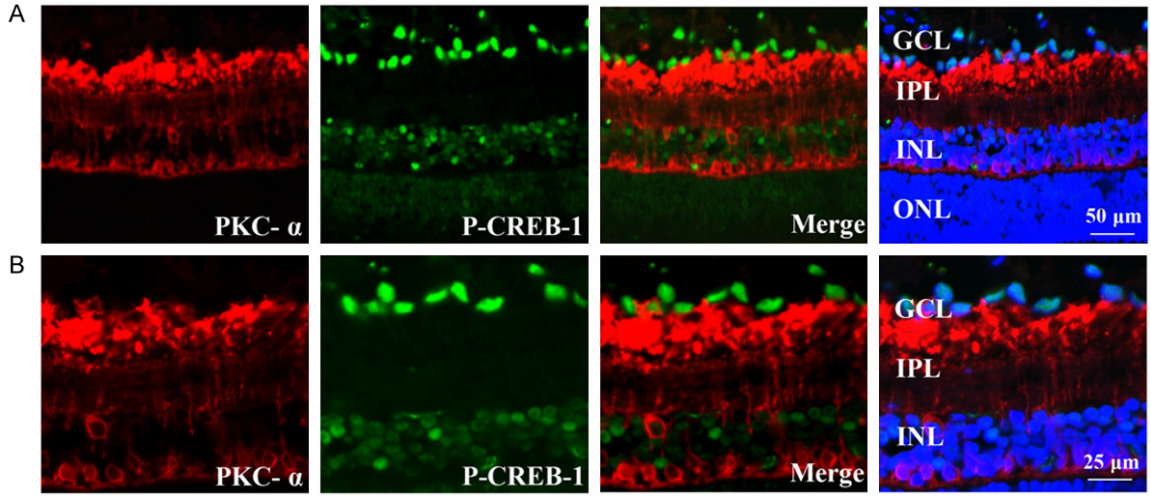

Figure 3. No clear co-localization was detected between PKC-α and p-CREB-1. A. Noimmunoreactivity of p-CREB (green) were observed in PKC-α (red) positive bipolar cells. B. Confocal immunofluorescent images at higher-magnification of p-CREB-1 (green) and PKC-α (red) staining in adult rat retina. Nuclei were stained with DAPI (blue). Ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

Interestingly, peak expression of p-CREB-1 in the INL was observed at postnatal day 5 and slightly decreased with age (**Figure 1A**). These temporal and spatial developmental expression patterns of CREB-1 and p-CREB-1 are in accordance with the proposed function as a regulator for retina development signals.

Moreover, higher-magnification images of adult rat retina demonstrated that (**Figure 1B**), about half of the retinal cells in the GCL and INL that expressed CREB-1 were immuno positive for p-CREB-1 regardless of its phosphorylation state. This expression pattern is similar to that observed in cortex [18]. Together, these lines of evidence indicate that the phosphorylation of CREB-1 might contribute to the differentiation of retina neurocytes.

Immunolocalization of p-CREB-1 in the adult rat retina neurons

The vertebrate retina consists of seven major neuron cell types. To determine the exact distribution of p-CREB-1 in retinal cells, double immunofluorescence staining was performed with specific markers of major neuronal phenotypes in the fully developed postnatal one-month rat retina. Given the lack of p-CREB-1 distribution in the ONL layer, the cellular analysis was restricted to the GCL and INL neurocytes.

The intensive staining of p-CREB-1 in the GCL suggested positive phosphorylation of CREB-1 in retinal ganglion cells (RGCs). The retinal neurons were labeled with microtubule-associated protein-2 (MAP-2) or THY1.1, the specific cellular makers for neurons and retinal ganglion cells, respectively. As shown in **Figure 2A** and **2B**, the MAP-2 stained neuronal axons and cell bodies were present in the GCL and IPL of retina. Thy1.1 expression was distributed specifically in ganglion cells (**Figure 2C** and **2D**), which were strongly expressed in the membrane and cytoplasm of the cells in the GCL and IPL. It was apparent that the cellular staining of p-CREB-1 co-localized with both MAP-2 and Thy1.1

Anti-protein kinase C-α (PKC-α) was used to label rod ON-bipolar cells and a subpopulation of amacrine cells [21]. As shown in **Figure 3A** and **3B**, the PKC-α staining was distributed in cytoplasm of the cells in the inner and outer parts of the INL, and the synaptic terminals of the IPL. No clear co-localization was detected between PKC-α and p-CREB-1. Horizontal cells localized in the outer parts of the INL, exhibiting dense plexus formed calbindin labelling, were not p-CREB-1 immunoreactive (**Figure 4A** and **4B**). However, calretinin-positive amacrine cells presented in the inner the INL and GCL, revealed only a few p-CREB-1 stained cells (**Figure 4C and 4D**). Taken together, these observations demonstrate that p-CREB-1 was promi-

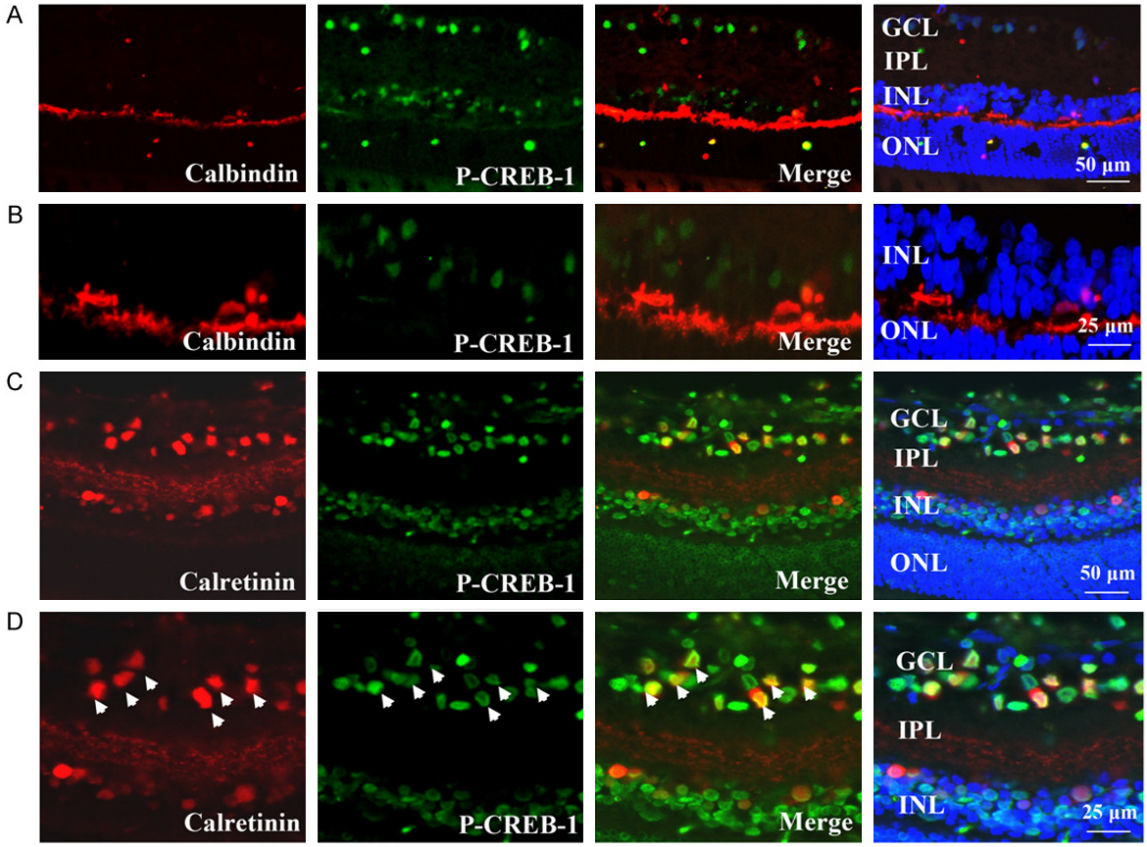

Figure 4. P-CREB-1 immunoreactivity were shown in few amacrine cells but nothorizonal cells. A. Horizontal cells stained with calbindin (red), were not p-CREB-1 immunoreactive (green). B. Confocal immunofluorescent images at higher-magnification of p-CREB-1 (green) and calbindin (red) staining in adult rat retina. Nuclei were stained with DAPI (blue). C. Partially calretinin-positive (red) amacrine cells stained with p-CREB-1 (green). D. Confocal immunofluorescent images at higher-magnification of p-CREB-1 (green) and calretinin (red) staining in adult rat retina. Nuclei were stained with DAPI (blue). Arrows indicate examples of colocalisation. Ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

nently expressed in the retinal neurons, but not in all neuron cell types of the retina.

Immunolocalization of p-CREB-1 in the adult rat retina glial cells

To assess the expression of p-CREB-1 in retinal glial cells, double labelling experiments were performed for p-CREB-1 with glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS), markers for astrocytes and Muller cells, respectively. In one-month old rat retina, GFAP was expressed in the outer parts of the GCL, and no co-localization between GFAP and p-CREB-1 were observed (**Figure 5A** and **5B**). Additionally, the Muller cells, which were labelled using an antibody against GS that span the entire neuro-retina, were not p-CREB-1 immunoreactive (**Figure 5C** and **5D**). This evidence indicates that p-CREB-1 was rarely expressed in the rat retinal glial cells.

Discussion

In the present study, we first demonstrated the developmental phosphorylation of CREB-1 in rat retina; this suggests a regulator role for p-CREB-1 in retinal developmentand cell classes. Moreover, in adult rat retina, p-CREB-1 was prominently expressed in the retinal neurons while rarely expressed in the retinal glial cells.

The phosphorylation of CREB-1 plays a vital role at a central converging point of pathways mediating the developmental regulated processes in neurons, including cell differentiation, synaptic connections and neurogenesis [1, 2, 22]. Here, we demonstrated that CREB-1 was markedly expressed and extensively dis-

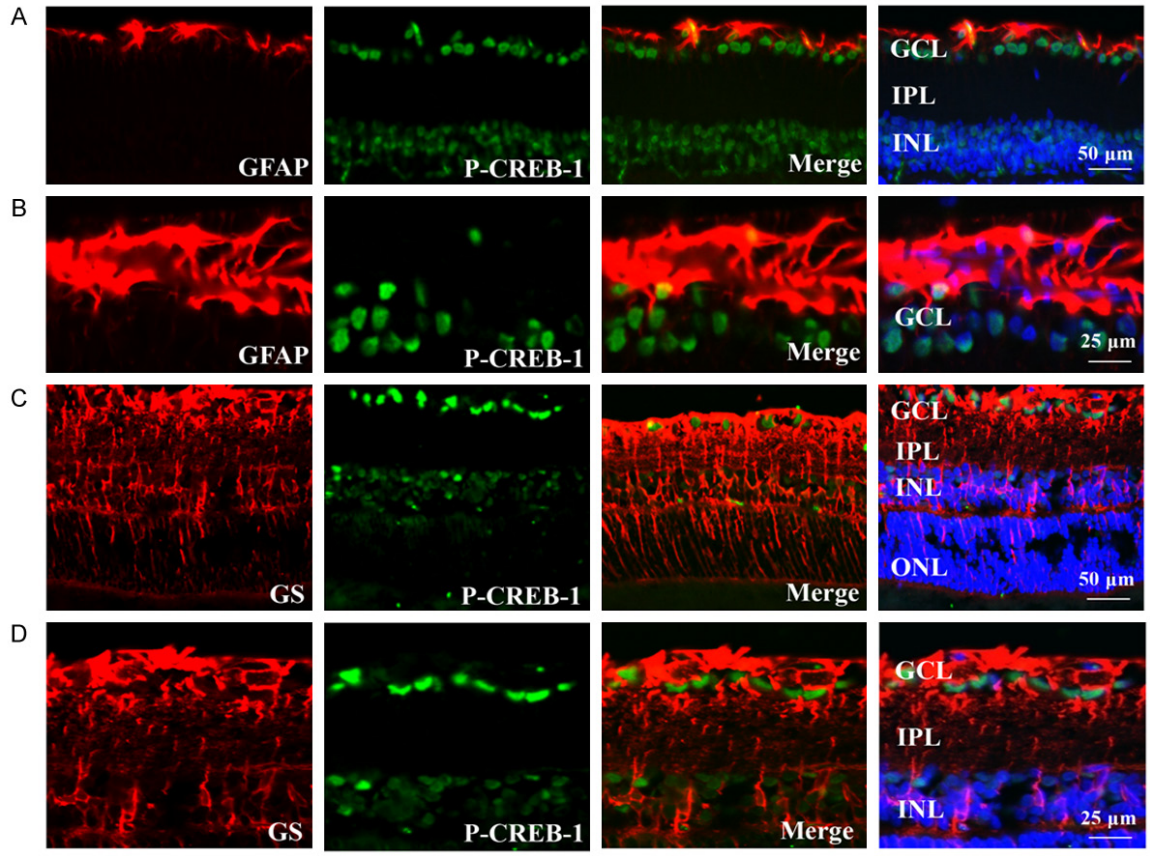

Figure 5. Immunolocalization of p-CREB-1 in the adult rat retina glial cells. A. No co-localization between p-CREB-1 (green) and GFAP (red) were observed in rat retina. B. Confocal immunofluorescent images at higher-magnification of p-CREB-1 (green) and GFAP (red) staining in adult rat retina. Nuclei were stained with DAPI (blue). C. No co-localization between p-CREB-1 (green) and GS (red) were observed in rat retina. D. Confocal immunofluorescent images at higher-magnification of p-CREB-1 (green) and GS (red) staining in adult rat retina. Nuclei were stained with DAPI (blue). Ganglion cell layer (GCL), inner nuclear layer (INL), inner plexiform layer (IPL), outer plexiform layer (OPL), and outer nuclear layer (ONL).

tributed in all layers of the retinathat are still undergoing development in postnatal day one rats, and developmentally down-regulated in the ONL as the rats aged. Conversely, p-CREB-1 expression was predominated in the GCL in the postnatal dayone rat retina, while strong immunore activeactivity of p-CREB-1 was observed in the GCL and INL of the one-month old rat retina. This observation is consistent with previously reported patterns of p-CREB-1 staining in adult retina of mouse, dog and cat [23, 24]. As in other parts of the nervous system, various physiological stimuli or cellular stress, such as peptide hormones, growth factors and light exposure [25-27], could induce the phosphorylation of CREB-1 in retinal neurons. The phosphorylation of CREB-1 mediates the transcription of neurotrophic factors and triggers intracellular signaling pathways, there by modulating the proliferation and dictating the differentiation of neurocytes. The developmental phosphorylation of CREB-1 in the retina observed in the present study indicates that CREB-1 might-be involved in the physiological process of the development of retinal cell classes and architecture as well as maintaining neuronal properties.

To identify the differentiation processes of cells that p-CREB-1 has participated in, we analyzed the expression distribution of p-CREB-1 in the adult rat retina. Based on the staining pattern with p-CREB-1 antibody and retinal cell markers antibody, our data indicates that, although every neuronal and glial cell throughout all retinal layers appears to exhibit CREB-1 immunoreactivity, p-CREB-1 was prominently expressed in the retinal neurons but rarely expressed in

the retinal glial cells. These observations are consistent with previous reports demonstrating that p-CREB-1 is predominantly expressed in post-mitotic cells [18-20, 28]. Persistent and strong activation of CREB-1 phosphorylation is associated with neuronal survivalsinceit has been suggested to provide neuroprotective signals in times of cellular stress [29]. A close relationship between CREB-1 phosphorylation and neuronal survival has been reported [30, 31]. Drug-induced phosphorylation of CREB-1 promotes proliferation and morphological maturation of neurons [32, 33]. Application of lithium, a well-known neuroprotective agent, induces marked enhancement of CREB-1 phosphorylation in the cortex [33]. In line with these studies, our results suggest strong involvement of p-CREB-1 in the development of retinal neurocytes. Moreover, in adult rat retina, phosphorylated-CREB-1 expression in CREB-1 immunopostive cells was observed. Such moderate activation of CREB-1 may be necessary for the basal maintenance of signal transduction to ensure neuronal integrity [2, 4]. Additionally, given that there is strong phosphorylation of CREB-1 in the retinal neurons, not glial cells, in the GCL and INL during development; it is likely that CREB-1 controls the neurogenic transcriptional programs that regulate the repertoire of retinal precursor cells. Neuron cell proliferation and differentiation are sophisticated processes, coordinated by several extrinsic factors and neurotrophins. Although CREB-1 phosphorylation mediates the transcription signaling of neurotrophin factors, considerable evidence suggests that in developing neurocytes, neurotrophin factors could enhance the phosphorylation of CREB-1 [26]. Thus, populations of retina cells expressing p-CREB-1 could be either positively or negatively selected in retinal development. However, the mechanism underlying the involvement of p-CREB-1 mediated differentiation in retina, and whether the phosphorylation level of CREB-1 is associated with cell directional differentiation, requires further investigation.

Zuo et al. suggested that restoring CREB-1 phosphorylation in the retina might be a potential therapeutic strategy to prevent excitotoxic retinal degeneration [33]. Moreover, under the normal circumstances, p-CREB-1 is primarily expressed in the GCL and INL of the adult rat retina, while weakly expressed in the ONL where it is mostly constituted of photoreceptors. However, strong activation of CREB-1 phosphorylation in photoreceptor cells in response to injury stress was observed in previous studies [23, 34]. In photoreceptor degeneration animals and ocular penetrating trauma animal models, p-CREB-1 immunostaining is prominent in the ONL [34, 35]. Mónicaet al. reported that Müller-derived progenitors respond to NMDA receptor activation through CREB-1 phosphorylation [36]. Although p-CREB-1 is weakly expressed in the ONL of rat retina, these lines of evidence suggest that p-CREB-1 might be associated with a neuroprotective response in retina cells in the ONL.

In conclusion, the present study reveals the distinct expression localization of p-CREB-1 in rat retina, demonstrating that p-CREB-1 might be involved in the physiological development processes of retinal cell classes and architecture. Clearly, further investigation is needed to explore the underlying mechanisms by which p-CREB-1 mediates the development processes of the retina. Collectively, this study not only gives a new insight into the regulation of p-CREB-1 in retinal development, but it might also offer a therapeutic target to currently untreatable retinal diseases.

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Disclosure of conflict of interest

None.

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