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Study of Proteins Associated with Retinal Ganglion Cell Axon Misrouting in Ocular Albino Mouse Model

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A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Physiological Science

by

Yisha He

2016
ABSTRACT OF THE THESIS

Study of Proteins Associated with Retinal Ganglion Cell Axon Misrouting in Ocular Albino Mouse Model

by

Yisha He

Master of Science in Physiological Science

University of California, Los Angeles, 2016

Professor Professor Gordon L. Fain, Co-Chair

Professor Debora B. Farber, Co-Chair

Ocular Albinism (OA) is a disease characterized by hypopigmentation of the eyes and misrouting of the optic nerves. It is caused by mutations in the OA1 gene, which encodes a G-protein coupled receptor localized in the retinal pigment epithelium. Previously, microarrays carried out in the Farber lab comparing mRNAs from E15 control and Oa1\textsuperscript{-/-} mouse eyes showed that doublecortin and Creb-binding protein (CBP) were down-regulated in Oa1\textsuperscript{-/-} mice. To further explore if these proteins are involved in the Oa1 signaling cascade, I conducted a series of immunohistochemistry experiments to determine the localization of doublecortin, phosphorylated Creb (pCreb) and CBP in control and Oa1\textsuperscript{-/-} retina/RPE. In addition to confirming their down-regulation in Oa1\textsuperscript{-/-} retinas, my results suggest that doublecortin, pCreb and CBP are indeed involved in the Oa1 signaling cascade and may be related to the misrouting of the optic nerves in Oa1\textsuperscript{-/-} mice and OA patients.
The thesis of Yisha He is approved.

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2016
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Table of Contents

Introduction ........................................................................................................................................ 1
  Retina ........................................................................................................................................... 1
  Retinal Pigment Epithelium ........................................................................................................ 2
  RPE Melanosomes ....................................................................................................................... 4
  Ocular Albinism Type 1 ................................................................................................................ 5
  OA1 knock-out Mouse .................................................................................................................. 6
  OA1 protein and G protein-coupled receptors (GPCRs) ........................................................... 7
  Proteins interacting with the OA1 GPCR ....................................................................................... 8
  RGC Differentiation and Axonal Genesis .................................................................................... 10
  Axon Guidance at the Optic Chiasm ............................................................................................. 11
  Doublecortin .............................................................................................................................. 12
  Does OA1 signal through the cAMP-CREB pathway? ................................................................. 14

Purpose of my study ....................................................................................................................... 16

Materials and Methods .................................................................................................................. 17
  Animals ........................................................................................................................................ 17
  Immunohistochemistry (IHC) ...................................................................................................... 17
  Retina Whole Mount ................................................................................................................... 19
  Cell Culture ................................................................................................................................ 19
  Immunocytochemistry ................................................................................................................ 20
  Microscopy ................................................................................................................................... 21
  Antibodies ................................................................................................................................... 21

Results ............................................................................................................................................ 22
  Oa1 protein expression in mouse retina ....................................................................................... 22
  Dcx protein expression in mouse retina ....................................................................................... 22
  CBP and pCreb expression in ARPE-19 and ARPE-19/Oa1+ cells ............................................. 23
  pCreb expression in mouse retina ............................................................................................... 24
  CBP expression in mouse retina ................................................................................................. 25

Discussion ...................................................................................................................................... 34

References ....................................................................................................................................... 37
Introduction

Retina

Eyes are sense organs that allow vision for organisms. Vertebrates usually have a pair of eyes, each with an almost spherical shape. The surface of the eyeball consists of three basic layers: 1) the sclera and cornea - the outer layer, 2) the middle layer, formed by the choroid, the ciliary body and the iris, and 3) the retina - the inner layer. Between the choroid and the retina, there is a monolayer of epithelial cells: the retinal pigment epithelium (RPE).

Vision is the ability of the eye to form the image of an object and send the image to the visual centers of the brain. Light rays from the object pass through the cornea, aqueous humor, the pupil, the lens and the vitreous humor and finally reach the retina. Here is where the light signal is transformed into an electrical signal that is transmitted to the brain (Figure 1).

There are five primary kinds of neurons in the retina: photoreceptors, horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells (RGCs). Each of these neurons functions differently but all cooperate to allow vision to occur. The retina is a beautifully layered tissue. All the cells are distributed in different retinal layers. From outside to inside, the layers are: the Retinal Pigment Epithelium (RPE); the Photoreceptor Layer formed by rods and cones, which includes the Outer/Inner Segment Layer and the Outer Nuclear Layer (ONL) containing all the nuclei of the photoreceptors; the Outer Plexiform Layer (OPL) formed by the photoreceptor terminals and their synapses with horizontal and bipolar cells; the Inner Nuclear Layer (INL) composed by the nuclei of horizontal, bipolar and amacrine cells; the Inner Plexiform Layer (IPL) formed by all the synaptic terminals of these cells; the Ganglion Cell Layer (GCL) and the Nerve Fiber Layer (Figure
1). When light comes into the eye, it has to reach the photosensitive photoreceptors in the outer part of the retina to trigger the process of phototransduction, which through biochemical reactions converts the light signals into electrical signals. These signals are then transmitted through bipolar cells, RGCs and along the optic nerve, formed by RGC axons, to the brain.

![Figures a and b showing eye anatomy and retinal layers](Picture)

**Figure 1.** (a) Eye anatomy (Picture from lhon.global). (b) Retinal layers (Picture from wikipedia).

**Retinal Pigment Epithelium**

The RPE is a monolayer of epithelial pigmented cells adjacent to the photoreceptors. RPE cells are not neurons but are essential for retinal function. They closely interact with photoreceptors maintaining their excitability. Most importantly, RPE cells transform all-trans-retinal to 11-cis-retinal, needed by photoreceptors to maintain the visual cycle of the retina. RPE cells also are involved in the phagocytosis of shed photoreceptor outer segments (POS), helping in the renewal of photo-damaged POS: they absorb light energy to decrease photo-damage of POS. Moreover,
RPE cells function as transporters, moving ions, water, and metabolic end products from the subretinal space to the blood and nutrients from blood to photoreceptors; therefore, they stabilize the ion composition of the subretinal space. In addition to these functions, RPE cells secrete a variety of growth factors to maintain integrity of both the choriocapillaris endothelium and the photoreceptors, and secrete immunosuppressive factors to establish the immune privilege of the eye (Strauss, 2005).

During embryogenesis, the generation of RPE and retina happen in concert. First, a layer of the prospective RPE and a layer of prospective neuronal retina are established. Then RPE cells start to mature with onset of melanogenesis and establishing their polarity. Afterwards, primordial photoreceptors differentiate by extending their outer segments, while RPE cells respond by elongating their apical microvilli into the subretinal space. At this stage, RPE cells develop both long microvilli that maximize the apical surface for epithelial transport, and short microvilli that form photoreceptor sheaths for phagocytosis of photoreceptor outer segments. They also differentiate in macular and peripheral retina areas. RPE cells around the macula are smaller in size but denser with melanosomes. With higher number of photoreceptors per RPE cells, they display higher turnover rate and higher enzyme activities. The development of RPE and retina depend on each other. RPE cells secrete factors that promote photoreceptor survival and differentiation. At the same time, RPE development depends on specific stages of photoreceptor development. Besides, the proper development of the retina is dependent on genes involved in the melanogenesis pathway of RPE cells (Strauss, 2005).
RPE Melanosomes

Melanosomes are organelles responsible for color and photo-protection in animal cells and tissues. Melanosomes contain black melanin and exist in the skin melanocytes, choroidal melanocytes and RPE cells. Melanosomes have been considered as lysosomal-related organelles, but also share features of secretory granules (Marks et al., 2001). Unlike in melanocytes, melanosomes in RPE are generated before birth and their pigment granules are retained throughout life. After generation, melanosomes are transported from the cell body to the apical processes in the RPE, in response to light onset (Wasmeier et al., 2008). Melanosomes in RPE are essential for RPE functions. They can protect the retina from the effects of light by absorbing the diffuse-light and they also act as electron acceptors. To help relieve oxidative stress, melanin acts as a detoxification agent quenching the peroxyradicals and reducing lipid peroxides. Besides, melanin helps regulating retinal metabolism by monitoring the zinc concentration in RPE through zinc binding, and protects the pigmented cells and adjacent tissues by adsorbing potentially harmful substances, which are then slowly released in non-toxic concentrations. Furthermore, melanosomes have also been demonstrated to participate in the phagocytosis of POS. Interestingly, deficit of RPE melanin pigment is associated with age-related macular degeneration, the leading cause of blindness (Schraermeyer et al., 1999).

Melanin plays a role in the development of the fovea and routing of optic nerves. As mentioned before, the proper development of the retina is dependent on proper melanogenesis in RPE cells. In albino animals where RPE melanogenesis appears abnormal, retina development is abnormal too. They also have an abnormality in the routing of optic nerves connecting the eyes and the brain. Moreover, the center retina is underdeveloped, and there is deficit in rod development (Glen, 1998).
Ocular Albinism Type 1

Albinism in humans is a congenital disorder characterized by the complete or partial absence of pigment in the skin, hair and eyes. There are two principal types of albinism: oculocutaneous albinism (OCA) and ocular albinism (OA). OCA, with an incidence of 1 in 20,000 people, affects the eyes, skin and hair. There are four types of OCA, all autosomal recessive diseases caused by mutations in genes that control the synthesis of melanin within the melanocytes. OCA1, OCA2, OCA3 and OCA4 are caused by mutations in the tyrosinase gene, P gene, the tyrosinase-related protein-1 (Tyrp1) gene and the membrane-associated transporter protein (MATP), respectively, which are all crucial genes in the synthesis of melanin.

OA is a less severe X-linked recessive disease affecting the eyes only. Ocular Albinism Type 1 (OA1), also called Nettleship-Falls syndrome, has an incidence of 1 in 50,000 people. OA1 is caused by mutations in the OA1 gene, which encodes the OA1 protein that is important in the formation of melanosomes. Instead of normal-sized melanosomes, OA1 patients have macromelanosomes (giant melanosomes) in their skin melanocytes and RPE cells, but a reduced amount of pigment in the RPE. Therefore, although their hair and skin have light color (they do not have white hair and skin as albinos), their eyes usually are very light blue and their visual acuity is seriously impaired. Also, because they have less pigmentation to protect the eyes from strong light, they show photophobia. In addition, they have an under-developed fovea, (foveal hypoplasia). Moreover, the significant refractive error of OA patients leads them to develop astigmatism, strabismus and nystagmus (involuntary movements of the eye). The macromelanosomes result from abnormal growth of single melanosomes. Hence, it is speculated that the OA1 protein may act as a stop signal for melanosome growing. (Shen et al., 2001)
In addition to macromelanosomes in the RPE, another important feature of OA1 patients is the misrouting of their optic nerves. In an albino eye, a higher proportion of optic nerve fibers cross to reach the opposite side of brain instead of going to the same side of brain, and this causes problems in their binocular vision. How OA1 mutation disrupts the growth of optic nerves remains not clear. In our lab, we have a group interested in this feature of OA and making efforts to study this problem.

**OA1 knock-out Mouse**

To obtain an animal model of ocular albinism, Oa1<sup>Δ/Δ</sup> mice were generated by introducing an Hprt cassette replacing the first exon of the Oa1 gene. First, the targeting vector, containing 3.5kb of sequence upstream of exon 1, 3kb of sequence 3’ of exon 1, the PGK-hprt expression cassette and an MC1-tk expression cassette was generated and inserted into embryonic stem (ES) cells through electroporation. Recombinant ES clones with the targeting vector recombined to the endogenous Oa1 locus were injected into C57BL/6 blastocysts, which were then implanted into the uterus of female mice. The chimeric mice obtained were crossed with C57BL/6 mice to produce either heterozygous carrier females or Oa1−/Y hemizygous males. At the appropriate time, these animals were crossed to generate Oa1<sup>Δ/Δ</sup> mice (Incerti et al., 2000). Electron microscope analysis has shown that Oa1<sup>Δ/Δ</sup> mice have hypopigmentation and macromelanosmes in RPE cells (Figure 2a), and horseradish peroxidase (HRP) labeling allowed to see the misrouting of their optic nerves. There is a reduction in the size of the uncrossed pathway and an increase in the size of the crossed pathway (Figure 2b). Electrophysiology measurements have indicated that retinal function is not affected by Oa1 disruption (Incerti et al., 2000). All these
features are very similar to those in OA1 patients. These Oa1−/− mice appear to be a suitable animal model for studying the OA1 disease.

Figure 2. (a) Electron micrographs show that macromelanosomes exist in Oa1−/− RPE, not in B6/NCrl RPE (Young et al., 2008). (b) Retrograde labeling results show that there are less uncrossed RGC cells in Oa1−/− mice than B6/NCrl (Young et al., 2008). (c) A schematic picture showing that in albino eyes, a higher proportion of optic nerve cross to reach the opposite side of brain, while a smaller proportion stay in the same side. (Neveu et al., 2007).

OA1 protein and G protein-coupled receptors (GPCRs)

GPCRs, also known as seven-transmembrane domain receptors, play an important role in cell signaling. GPCRs usually have an extracellular N-terminus, 7 transmembrane α-helices, and an intracellular C-terminus. When binding to a specific ligand, GPCRs go through conformational changes and act as a guanine nucleotide exchange factor (GEF) inside the cell, exchanging the
GDP bound to a G protein for GTP. This activates the G protein and its Gα subunit dissociates from the Gβγ subunits. Gα can then activate (or inhibit) the next protein in the GPCR signaling pathway. Thus the initial signal is expanded many times and finally it can control gene expression, cellular secretion, membrane permeability or have some other biological effects.

The OA1 protein has been shown to have the structure of a G-protein coupled receptor (GPCR). It has 7-transmembrane helices with 3 cytoplasmic loops and 3 extracellular loops. The OA1 protein is localized in late endosomal or lysosomal compartments as well as on melanosomal membranes (Samaraweera et al., 2001). It had been proposed that OA1 might act as a stop signal for melanosome growth (Shen et al., 2001). However, Young et al. (2008) demonstrated that OA1 interacts with Gαi3 and that in fact Gαi3 is the stop signal for melanosome growth.

There are more than 40 types of mutations reported in the OA1 protein, most of which occur in its N-terminus. The expression of OA1 protein starts early in embryo development. In fact, the transcription of OA1 in mouse eyes starts at embryonic (E) day 10.5, before RPE pigmentation, and is maintained through adulthood (Surace et al., 2000; Figure 5). Therefore, OA1 has a very important function in RPE pigmentation and retina development.

**Proteins interacting with the OA1 GPCR**

In order to explore the proteins interacting with OA1, we have previously compared mRNA expression in B6/NCrl and Oa1−/− by performing microarray experiments with E13 and E15 mouse eyes. There are just 4 proteins that are expressed differently at E13 (Figure 3a), whereas at E15, 51 genes are differentially expressed, 47 of them significantly down-regulated in Oa1−/− eyes (Figure 3b). Eleven of these genes were confirmed to be down-regulated by qRT-PCR, including
Gpr143 (another name of OA1), Erdr1, Doublecortin (Dcx), Rhox4b, Rbm5, Akap9, CREB-binding protein (CBP), Gnas, Tfap2b, Fubp1 and Zc3h11 (Figure 4). Among the encoded proteins, Dcx, as a microtubule associate protein (MAP), has drawn our attention for its function in axon guidance. Similarly, CBP has also been implicated in neurogenesis and axon guidance. So both Dcx and CBP might have a pivotal role in the Oa1-regulated RGC’s axonal routing.

Figure 3. (a) Microarray results show that in E13, only several genes are downregulated in Oa1−/− mouse eyes (Young et al., unpublished). (b) Microarray results show that in E15, 51 genes are downregulated in Oa1−/− mouse eyes (Guha et al., unpublished).
RGC Differentiation and Axonal Genesis

Mouse RGCs are born from E11 to postnatal (P) day 0, with a peak in their appearance occurring at E14.5 (Figure 5). Non-polar spherical RGCs are generated from multipotent retinal progenitor cells and they undergo RGC fate commitment during or just shortly after the terminal cell division adjacent to the RPE layer. These cells then extend a basal process toward the inner limiting membrane of the retina and their nuclei migrate through the basal processes to the inner surface of retina. Afterwards, RGCs extend their axons from the basal processes to the optic fiber layer. Meanwhile, the apical process that was attached to the outer limiting layer retracts, forming RGCs that are morphologically distinguished from other retinal cells. After generation, RGC axons grow further to the optic disk in the center of the retina, extend through the optic disc and then to the brain forming the optic nerve (Erskine et al., 2014; Zolessi et al., 2006).

Figure 4. qRT-PCR results confirm the downregulation of Gpr143 (Oa1), Dcx and CBP in Oa1−/− mouse eyes in E15 (Guha et al., unpublished).
Axon Guidance at the Optic Chiasm

In mammals, the optic nerve formed by axons of RGCs gather at the optic disc and project to image-forming nuclei such as the lateral geniculate nucleus (LGN), the visual part of the thalamus, and the superior colliculus (SC). Before making a decision to cross or not to cross the midline, optic nerves from both eyes first meet at the optic chiasm, which is located at the base of the hypothalamus. From there, RGC axons from the nasal side of the retina cross over to the opposite side of the brain (contralateral), while axons from the temporal retina turn back to the same side of the brain (ipsilateral). This enables the brain to incorporate signals from both eyes in order to achieve binocular vision. The species that have more ipsilateral axons attain better stereoscopic vision. In primates, the number of ipsilateral and contralateral axons is almost equal. In mouse,
whose eyes are further apart, only 3% to 5% of all RGC axons are ipsilateral. In animals that have albino eyes, the proportion of contralateral axons is higher than normal while that of ipsilateral axons is lower than normal, and this causes problems in their binocular vision.

During optic nerve development, there are three stages. From E12 to E13.5, early RGC axons from the dorsal-central (DC) retina enter the ventral diencephalon and form early crossed and uncrossed projections. After early projections are formed, from E14-E17, axons from temporal retina approach the chiasm midline and turn back to the ipsilateral optic tract while axons from nasal retina cross the chiasm and form the contralateral optic tract. In the late stage, from E17.5 to P0, newborn RGCs from all the retina project to the opposite side. (Petros et al., 2008; Figure 5)

The mechanism regulating cross or not cross decision is not completely clear yet, but progress has been made. On the one hand, in the ipsilateral development, EphrinB2 and EphB2 receptors function as a chemorepellent system, which is controlled by the transcription factor Zic2 expressed in the ventral-temporal (VT) retinal ganglion cell nucleus. On the other hand, NrCAM together with some other proteins seem to play a significant role in control of transcription factor Islet2 in the contralateral development (Petros et al., 2008).

**Doublecortin**

Doublecortin (Dcx) is a microtubule associated protein (MAP) that when mutated causes X-linked lissencephaly. It is expressed in post mitotic neurons during neuronal migration and neurite formation, so that Dcx is often used as a marker for neurogenesis. Doublecortin stabilizes microtubules and causes bundling. Male patients of X-linked lissencephaly have a smooth brain due to lack of migration of immature neurons, which normally promote folding of the brain surface.
Dcx protein has two “Doublecortin domains” near the N-terminal that are responsible for binding microtubules. Dcx mutation in these domains cause Dcx protein to lose its function. In human, Dcx mutation alone causes a serious problem in neuron migration and axon guidance, but in mouse, doublecortin-like kinase (Dclk) can compensate for Dcx when there is a Dcx mutation. Only if Dcx and Dclk are both mutated, mice show problems in axon guidance (Deuel et al., 2005). It has been shown that Dcx mutations not only affect microtubule and related proteins but also cause problems in F-actin distribution and actin-related proteins. Dcx mutated neurons have increased F-actin around the cell body and decreased F-actin in neurites and growth cones. The axons of these neurons are selectively deficient in axon guidance, but not elongation (Fu, et al., 2012).

Western blots from our lab show that Dcx has 2 molecular weight forms at E15 and P2. The lower band is no longer present in the adult retina (Figure 6). Comparison of B6/NCrl and Oa1−/− Dcx levels shows that these are much lower in the knockout mouse at E15 and P2, but that the difference is not as prominent in the adult retina. We hypothesize that Dcx may play an important role in axon guidance of optic nerves.
Does OA1 signal through the cAMP-CREB pathway?

There are two principal types of GPCR signal transduction pathways, the cAMP signal pathway and the phosphatidylinositol (IP3/DAG) signal pathway. In the cAMP signal pathway, after the G protein is activated or inhibited, the Gα subunit dissociates and binds to adenylyl cyclase (AC), which catalyzes the synthesis of cAMP from ATP. When cAMP is produced, it activates protein kinase A (PKA), which in turn regulates cell metabolism by phosphorylating corresponding
enzymes or the cAMP response element-binding protein (CREB). CREB is a transcription factor that can upregulate or downregulate the transcription of downstream genes. CREB is activated by phosphorylation, and various kinases, including PKA and Ca\(^{2+}\)/calmodulin-dependent protein kinases, are responsible for this reaction. When activated, CREB recruits other transcriptional coactivators to bind to the cAMP response element (CRE) in the promoter 5’ upstream region of genes. CREs contain a highly conserved nucleotide sequence, 5’-TGACGTCA-3’. After binding CREB, the transcription complex upregulates or downregulates gene expression. CBP is a protein that usually acts as a scaffold to stabilize additional protein interactions with the CREB transcription complex. Phosphorylated cAMP response element-binding protein (pCreb) is the activated form of CREB.

CBP was found to be downregulated in E15 Oa1\(^{-/-}\) mouse eyes by microarray and QPCR analyses. Thus it is easy to speculate that the GPCR Oa1 might signal through the cAMP-CREB pathway to control expression of genes such as Dcx. I have studied and will report, the expression of both pCreb and CBP in mouse retina.
Purpose of my study

In recent years, progress has been made in the understanding of how macromelanosomes are formed in OA1 patients or in Oa1−/− mice. However, the mechanism underlying the effect of OA1 mutations in the misrouting of the optic nerves remains rather mysterious, since the Oa1 protein is expressed in RPE cells, located in the outer retina, while the optic nerves are formed by the axons of RGC cells, located in the inner retina. We are very interested in this problem.

During embryo development, RGC cells are initially attached to the RPE cell layer. Thus it is possible that at that time, the mutated Oa1 protein in the RPE, through intercellular signaling, may cause the abnormal expression of some specific proteins in the RGCs and changing the normal axon guidance.

In order to explore the possible proteins encoded by genes responsible for optic nerve misrouting, mRNA expression in B6/NCrl and Oa1−/− mice were previously compared on microarrays of cDNAs using total RNA from E13 and E15 normal and Oa1−/− mouse eyes. Only 4 cDNAs were expressed differentially in E13, whereas in E15, 51 genes were differentially expressed in Oa1−/− eyes. Among these genes, that encoding Dcx draw our attention because of its function in axon guidance. QPCR experiments confirmed Dcx down-regulation. We then hypothesized that Dcx could be a pivotal protein in optic nerve routing. Another gene also shown to have lower level of transcription in Oa1−/− eyes at E15 is that coding for CBP. We think that Oa1 may regulate the transcription of Dcx through a signaling pathway that involves pCreb and CBP.

In the work that I have carried out, we studied the expression pattern of Oa1, Dcx, pCreb and CBP in normal and Oa1−/− retinas in an effort to figure out the mechanism of optic nerve misrouting.
Materials and Methods

Animals

C57BL/6NCrl (B6/NCrl) and congenic Oa1 knockout mice (Oa1<sup>−/−</sup>) were obtained from The Charles River Laboratories in the United States and Italy, respectively, and were bred at the vivarium of the University of California, Los Angeles (UCLA). The Oa1<sup>−/−</sup> mice were confirmed to be congenic at the Murine Genetic Analysis Laboratory of the University of California, Davis, by examination of 1448 SNPs throughout the genome, with the exception of a region surrounding the Oa1 gene on the X chromosome, where 129s1/SvImj alleles associated with the targeting vector were detected between 139 and 159 Mb.

Immunohistochemistry (IHC)

Eyes of E14.5, E15.5, P2 and Adult B6/NCrl and Oa1<sup>−/−</sup> mice were used in this study. Adult B6/NCrl and Oa1<sup>−/−</sup> mice were anesthetized with isoflurane and then sacrificed with cervical dislocation. Eyes were enucleated and fixed in 4% paraformaldehyde/0.1M phosphate buffer on ice for 1 hr. A small hole was then made on the cornea of each eye and the eyes were put back into 4% paraformaldehyde and rotated for 1 hr at 4°C. The cornea and lens were then removed and the eyecups were fixed in 4% paraformaldehyde and rotated at 4°C overnight. P2 mice were anesthetized with isoflurane until they were dead and the corneas and lenses were not removed from their eyecups.

To get E14.5 and E15.5 mouse embryos, female mice of pup-bearing age were placed in cages where a reproductive male mouse had stayed for 2 days to get used to the male’s smell. The specific male was then put back into that cage for them to mate overnight. The next morning, females were
separated from the male and their vaginal plugs were checked. If a female had a plug, that day would be counted as E0.5. On the 14th or 15th day, pregnant females were anesthetized with isoflurane and sacrificed with cervical dislocation. Their uteri were dissected and embryos were taken out. Their heads were cut, fixed in 4% paraformaldehyde and rotated at 4°C overnight.

On the second day, the mouse eyecups or embryo heads were rinsed 3 times with PBS (10 mins each time) and then rotated in 10% sucrose for approximately 3 hrs, at 4°C. The tissues were then placed in 30% sucrose and rotated in 4°C overnight, followed by 4 hrs rotation in half 30% sucrose and half OCT. The tissues were then placed in molds with several drops of OCT inside. The orientation of the eyecups and heads was adjusted and molds were filled with OCT and placed on dry ice for 40 mins. Molds were stored at -80°C until they were sectioned with a cryostat-microtome. 10 um cross-sections of eyes and heads were mounted on glass slides.

Sections were blocked with one drop of serum [10% goat or donkey serum (depending on the primary antibody used) from Sigma-Aldrich Inc. in IHC wash buffer] on each for 1 hr, washed with IHC wash buffer [PBS/TBS (TBS for phosphorylated proteins) + 0.5% Triton 100] once for 5 mins and incubated with primary antibody (in IHC wash buffer) at 4°C overnight. The next morning, sections were washed with IHC wash buffer 3 times, 5 mins each time, incubated in secondary fluorescent antibody (in IHC wash buffer) for 1 hr, again washed with IHC wash buffer and finally washed with PBS or TBS once for 5 mins. They were then incubated with 1:10,000 DAPI in PBS or TBS for 7 mins and washed with PBS or TBS two times, 5 mins each time. After air dried for 2 hrs, the slides were mounted with mounting solution and covered with coverslips. Slides were sealed with nail polish at the edges and preserved at room temperature.
Retina Whole Mount

Adult B6/NCrl and Oa1+ mice were anesthetized with isoflurane and sacrificed with cervical dislocation. Eyes were enucleated, cleaned and a small hole was made on each before they were fixed in 4% paraformaldehyde, 4°C overnight. After removing the anterior segment by cutting a circular path along the ora serrata, the lens and vitreous humor were gently squeezed out from the eyecups. The optic nerves were then cut and the retinas were dissected from the eyecups. Retinas were then cut into 4 petals each and was placed in a well of a 24-well plate for blocking in serum at 4°C overnight. Next morning, they were washed with IHC wash buffer (3 times, 20 mins each) and incubated with primary antibody (in IHC wash buffer) for 3 to 4 days. Following this, retinas were washed with IHC wash buffer (3 times, 20 mins each), incubated with secondary fluorescent antibody (in IHC wash buffer) for 2 days, washed with PBS (3 times, 20 mins each) and incubated in DAPI for 1 hr. Then the retinas were mounted with mounting medium on glass slides and covered with coverslips. The slides were sealed with nail polish.

Cell Culture

ARPE-19 is a spontaneously immortalized cell line of human RPE cells. This cell line was obtained from American Type Culture Collection (ATCC). ARPE-19 cells showing stable overexpression of OA1 protein (ARPE-19/OA1+) were previously generated in our laboratory by transfection of OA1 in ARPE-19 cells. These two cell lines were used in this study.

Cells were grown to confluence in 75 cm² culture flasks in 1:1 mixture of Dulbecco’s Modified Eagle Medium and Hams F12 medium. A brief protocol of cell culture is described as follows. First, culture medium was removed and the cells were washed with PBS. Then the cells were
incubated with 1.0 to 2.0 ml of Trypsin-EDTA solution for 5 to 15 mins until they were observed dispersed under the microscope. 6.0 to 8.0 ml of culture medium was added into the flask, and the cells were further dispersed by gentle pipetting up and down. Finally, the cell suspension was divided into 3 aliquots and each was transferred to a new flask with enough culture medium and incubated in 95% air/5% CO₂ at 37°C. The culture media was renewed every 2 days. The cells were cryopreserved in liquid nitrogen with DMEM/F12 plus 5% DMSO as freeze medium.

**Immunocytochemistry**

Autoclaved coverslips were coated with 0.01% Poly-D-lysine solution for 1 hr at room temperature and rinsed with water 3 times, 5 mins each. Coated coverslips were dried and exposed to UV overnight. RPE cells were seeded and cultured on coated coverslips for several days. Then, coverslips were washed with PBS for 5 mins and fixed with 4% paraformaldehyde for 30 mins in 24-well plates. Cells were thereafter washed in ice cold PBS, two times, 5 mins each, and incubated in 170 ul of blocking buffer [3% of goat or donkey serum (depending on the primary antibody to be used) in PBS or TBS (TBS for phosphorylated proteins) with 0.25% Triton X-100] at room temperature for 30 mins. The cells were incubated with primary antibody (in PBS or TBS 0.1% Tween 20, PBST or TBST), at 4°C, overnight, washed three times with PBST, 5 mins each, followed by incubation with secondary antibody (in the blocking buffer) for 30 mins in room temperature and washed again 3 times in PBST, 5 mins each. The coverslips were then mounted on slides with a small drop of prolong gold DAPI mount, with the cells facing down, and sealed with nail polish.
Microscopy

Pictures were taken with an Olympus FluoView FV1000 confocal microscope and processed using Olympus FluoView software (Olympus America Inc., Lake Success, NY).

Antibodies

Primary antibodies

<table>
<thead>
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<th>Name</th>
<th>Information</th>
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</thead>
<tbody>
<tr>
<td>OA1</td>
<td>OA1 (C6), sc398602, mouse monoclonal IgG, Santa Cruz Biotechnology (Dallas, TX)</td>
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<tr>
<td>Doublecortin</td>
<td>Doublecortin (C18), sc8066, goat polyclonal IgG, Santa Cruz Biotechnology (Dallas, TX)</td>
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<td>CBP</td>
<td>CBP (C20), sc-583X, rabbit polyclonal IgG, Santa Cruz Biotechnology (Dallas, TX)</td>
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<tr>
<td>pCreb</td>
<td>pCreb(Ser133), 87G3, Rabbit monoclonal IgG, Cell Signaling Technology (Danvers, MA)</td>
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<tr>
<td>Rbpms</td>
<td>A kind gift from Brecha Lab, UCLA</td>
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<tr>
<td>Calbindin</td>
<td>Calbindin D28K (H-50), sc-28285, rabbit polyclonal IgG, Santa Cruz Biotechnology (Dallas, TX)</td>
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Secondary antibodies

<table>
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<tr>
<th>Antigen</th>
<th>Description</th>
</tr>
</thead>
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<td>Alexa Fluor® 568 goat anti-mouse IgG (H+L), Abcam PLC (San Francisco, CA)</td>
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<tr>
<td>Alexa Fluor® 594 goat anti-mouse IgG (H+L), Abcam PLC (San Francisco, CA)</td>
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<tr>
<td>Alexa Fluor® 488 goat anti-rabbit IgG (H+L), Abcam PLC (San Francisco, CA)</td>
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<td>Alexa Fluor® 568 donkey anti-goat IgG (H+L), Abcam PLC (San Francisco, CA)</td>
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<tr>
<td>Alexa Fluor® 594 donkey anti-mouse IgG (H+L), Abcam PLC (San Francisco, CA)</td>
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Results

**Oa1 protein expression in mouse retina**

Oa1 protein has been generally acknowledged to be expressed in RPE cells since early embryo development through adulthood. However, no study has shown whether it is expressed in the rest of the retina. I have found using IHC experiments that Oa1 is expressed also in the inner retina. At E14.5 and E15.5, Oa1 protein is expressed in the normal mouse embryo at the very inner part of retina, just in the neurites of presumed ganglion cells (Figure 7a). At P2, Oa1 expression has increased in the normal retina and is seen in a whole layer of migrating cells that will constitute the GCL and possibly, some amacrine cells. Neurites are still primarily labeled (Figure 7b). In the normal adult retina, Oa1 is expressed in ganglion cells and their projections (Figure 7c). Oa1 is not expressed in the Oa1⁻⁻ retina, neither at P2 nor in adult Oa1⁻⁻ mice (Figure 7b, 7c).

**Dcx protein expression in mouse retina**

In the embryonic retina, Dcx can already be seen at E14.5 in the inner border of the retina, at E15.5 Dcx expression in this area has increased. It is mainly visible in a large proportion of cells of the inner retina (Figure 8a). By P2, Dcx is observed in a well-defined ganglion cell layer, also expressed in the projections of a large number of cells (Figure 8b). However, in the adult control retina, Dcx is mainly expressed in the ganglion cells. Moreover, at the outer border of the INL the Dcx expression probably correspond to localization in the horizontal cells (Figure 8c).

As a marker of neurogenesis, Dcx co-localizes with Rbpms and Calbindin in the retinal GCL of B6/NCrl control mice (Figure 9a, 9b). Rbpms is a RGC marker expressed in the cell body and Calbindin is also a RGC marker expressed in their projections. The co-localizations of Dcx with
Rbpms and Calbindin make it clear that Dcx is present in RGC cell bodies and projections. Moreover, the co-localizations of Dcx and Oa1 in the GCL of wild-type mouse retina suggest that Oa1 may influence RGC Dcx expression not only by intercellular signaling starting in the RPE but also by intracellular signaling in the GCL (Figure 9c). A retina whole mount picture illustrates more clearly that in the GCL, Dcx is expressed in RGCs and in the smaller amacrine cells. (Figure 9d).

Interestingly, at P2 in the Oa1−/− retina, Dcx is expressed in the GCL, in a very similar way to that in wild-type P2 retina. However, Dcx expression in the adult Oa1−/− retina is reduced considerably from that in the adult control retina, both in the RGCs and the horizontal cells (Figure 8b, 8c).

**CBP and pCreb expression in ARPE-19 and ARPE-19/Oa1+ cells**

Figure 10a shows ARPE-19 cells expressing pCreb (green), Oa1 (red) and the emerged image of both and Figure 10b shows the ARPE-19 cells that had been transfected with Oa1 (ARPE-19/Oa1+), also expressing pCreb (green), Oa1 (red) and the merged image of both. As can be seen, there is a tremendous increase in both pCreb and Oa1 expression in the OA1-transfected cells. Similar results were obtained for CBP (Figure 10c, 10d). Both pCreb and CBP are expressed in the nuclei of ARPE-19 and ARPE-19/Oa1+ cells. The increased expression of pCreb and CBP with Oa1 expression illustrates that both pCreb and CBP are downstream proteins in the Oa1 signaling pathway and are positively influenced by Oa1.
**pCreb expression in mouse retina**

Figure 11a shows that pCreb is expressed extensively in E14.5 wild-type eyes. It can be observed in RPE, retina and the epithelial cells of the lens, but not in the optic nerve (Figure 11b). At this stage, pCreb is densely expressed in the RPE, and sparsely throughout the whole retina, most concentrated at its inner border (Figure 11c). At the same time, Dcx expression is also mainly at the inner border of the retina, but there is no obvious co-localization of Dcx and pCreb (Figure 11d). By E15.5, pCreb expression in wild-type retina becomes much lower. At this age, it is present not only in the retina and the epithelial cells of the lens (Figure 11e), but also in the optic nerve (Figure 11f). pCreb expression pattern and level in the embryonic retina changes a lot from E14.5 to E15.5, from the RPE cells and throughout the whole retina to the inner retina and the optic nerve (Figure 11g). At E15.5, Dcx is also observed in the inner border of retina. However, pCreb is expressed in nuclei while Dcx is expressed in projections of the same cells (Figure 11h).

At P2, in control mice, pCreb is expressed in the nuclei of cells of the inner retina and of the innermost rows of the retinoblastic layer (Figure 12a, left), whereas in adult wild-type animals, pCreb is in the nuclei of many but not all cells of the GCL and the INL (Figure 12b, left).

There is an obvious difference in pCreb expression between P2 wild-type and Oa1<sup>−/−</sup> retina. In P2 Oa1<sup>−/−</sup>, pCreb is no longer present in retinoblasts and is only in two rows of nuclei of the inner retina (Figure 12a). However, in adult mice, this difference becomes not observable: pCreb is similarly expressed in the GCL and the INL of both wild-type and Oa1<sup>−/−</sup> retinas (Figure 12b).
**CBP expression in mouse retina**

At E14.5, CBP is expressed extensively in the eyes of wild-type embryos. It can be observed in RPE, retina, the vitreous humor and the lens and its epithelium (Figure 13a), as well as in the optic nerve (Figure 13b). Figure 13c gives a clearer image of CBP expression in retina at E14.5. It is present in RPE cells, most retinoblasts and the nerve fiber layer. From E14.5 to E15.5, CBP expression level and pattern change tremendously in wild-type retina: it is present in much lower amount only in the inner retina, the lens epithelium (Figure 13d), the optic nerve (Figure 13e) and the nerve fiber layer (Figure 13f).

At P2, in control mice, CBP is expressed in cells of the inner retina and of the innermost rows of the retinoblastic layer (Figure 14a, left) while in adult control animals, CBP is expressed in most of the cells in GCL and INL (Figure 14b left). Similar to pCreb, at P2, CBP is present only in the inner retina of Oa1<sup>−/−</sup> mice (Figure 14a). However, CBP is expressed in both the INL and the GCL in both wild-type and Oa1<sup>−/−</sup> retinas (Figure 14b).
Figure 7. (a) Oa1 expression in E14.5 and E15.5 control mouse retina. (b) Oa1 expression in P2 control and Oa1<sup>−/−</sup> mouse retina. (c) Oa1 expression in adult control and Oa1<sup>−/−</sup> mouse retina. (DAPI stains nuclei blue and expression of Oa1 is in red.)
Figure 8. (a) Dcx expression in E14.5 and E15.5 control mouse retina. (b) Dcx expression in P2 control and Oa1<sup>−/−</sup> mouse retina. (c) Dcx expression in adult control and Oa1<sup>−/−</sup> mouse retina. (DAPI stains nuclei blue and expression of Dcx is in red.)
Figure 9. (a) Dcx and Rbpms expression in control mouse retina. (b) Dcx and Calbindin expression in control mouse retina. (c) Dcx and Oa1 expression in control mouse retina. (d) Dcx and Rbpms expression in adult wild-type mouse retina GCL. [DAPI stains nuclei blue, expression of Oa1 is in red and expression of Rbpms and Calbindin is in green, expression of Dcx is in red in (a), (b) and (d), green in (c).]
Figure 10. (a) pCreb and Oa1 expression in ARPE-19 cells. (b) pCreb and Oa1 expression in ARPE-19/Oa1+ cells. (c) CBP and Oa1 expression in ARPE-19 cells. (d) pCreb and Oa1 expression in ARPE-19/Oa1+ cells. (DAPI stains nuclei blue, expression of Oa1 is in red and expression of pCreb and CBP is in green.)
Figure 11. (a) pCreb expression in E14.5 wild-type mouse retina (10x, whole eye). (b) pCreb expression in E14.5 wild-type mouse retina (40x, optic nerve). (c) pCreb expression in E14.5 wild-type mouse retina (40x). (d) pCreb and Dcx expression in E14.5 control mouse retina. (e) pCreb expression in E15.5 wild-type mouse retina (10x, whole eye). (f) pCreb expression in E14.5 wild-type mouse retina (40x, optic nerve). (g) pCreb expression in E14.5 wild-type mouse retina (40x). (h) pCreb and Dcx expression in E15.5 control mouse retina. (DAPI stains nuclei blue, expression of pCreb is in green and expression of Dcx is red)
Figure 12. (a) pCREB expression in P2 control and Oa1−/− mouse retina. (b) pCREB expression in adult control and Oa1−/− mouse retina. (DAPI stains nuclei blue, expression of pCREB is in green)
Figure 13. (a) CBP expression in E14.5 wild-type mouse retina (10x, whole eye). (b) CBP expression in E14.5 wild-type mouse retina (40x, optic nerve). (c) CBP expression E14.5 wild-type mouse retina (40x). (d) CBP expression in E15.5 wild-type mouse retina (10x, whole eye). (e) CBP expression in E15.5 wild-type mouse retina (40x, optic nerve). (f) CBP expression in E15.5 wild-type mouse retina (40x). (DAPI stains nuclei blue, expression of CBP is in green.)
Figure 14. (a) CBP expression in P2 control and Oa1<sup>−/−</sup> mouse retina. (b) CBP expression in adult control and Oa1<sup>−/−</sup> mouse retina. (DAPI stains nuclei blue, expression of DBP is in green.)
Discussion

The most commonly studied abnormality in Oa1⁻/⁻ animals is the misrouting of the optic axons from the temporal retina across the optic chiasm (Incerti et al., 2000; Lauronen et al., 2005). Exactly how Oa1 exerts its effects on the RGCs to influence the decussation of the optic axons remains unsolved. One possibility is that the G-protein coupled receptor, Oa1, influences the migration of the RGC axons through a signaling cascade that affects the expression of molecules responsible for directing axonal guidance.

Our analyses here demonstrated down-regulation in the expression of a MAP, Dcx, along with a transcription factor, pCREB, and its co-factor CBP, in the eyes of Oa1⁻/⁻ mice specifically in the critical phases of embryonic and postembryonic development that could be linked to the misrouting of the RGC axons and the aberrant development of the binocular visual pathway, characteristic of these animals.

Control B6/NCrl mice showed strong Dcx immuno-reactivity in immature cells and cell processes in the E14.5 and E15.5 retina, a stage when retinal cells are not yet organized into different layers. In adults the protein was expressed by a population of RGCs positive for Rbpms, a marker of RGCs. Dcx was also found in subsets of adult amacrine cells positive for calbindin. Our results here on Dcx expression in the ganglion cell layer of retina are compatible with previous studies of its role in cell migration within the immature retina, and in dynamic neuronal plasticity in the mature retina (Fu, et al., 2012). Compared with the control B6/NCrl, the Oa1⁻/⁻ mouse retina showed a reduced expression of Dcx in P2 and in adult mice. The down-regulation of Dcx in developing and adult Oa1⁻/⁻ mice retina here hints to its possible role in the misrouting of the RGC axons in these animals. We also provided evidence of Dcx expression by discrete cells in the GCL
which are also positive for Oa1. This suggests that the Oa1 protein could direct the navigation of RGC axons through the expression of Dcx in wild type retina.

Immuno-histochemical staining for pCreb, CBP and Oa1 in RPE-retinas from control B6/NCrl vs Oa1−/− mice helped us to understand that Oa1, the transcription factor Creb and its cofactor CBP are functionally linked as well as co-players in the pathway directing optic axon navigation. In post embryonic P2 retina, pCreb and CBP were down-regulated in the inner neuroblastic layer in the knockout animals where there is no Oa1 protein expression, a fact that was never reported before. The change in the spatiotemporal expression of pCreb and CBP in wild-type mice, from the RPE towards the RGCs from E14.5 to E15.5 further suggests that the melanosome specific GPCR, Oa1 might be signaling downstream through pCreb-CBP. Additionally, the up-regulation of pCreb as well as CBP in the human RPE cell line, ARPE-19, over-expressing Oa1 protein, confirms that Oa1 might be regulating the Creb expression in RPE cells.

These results indicate that Oa1 might influence pCreb expression in the RPE cells and the nascent RGCs while they are attaching to RPE cells, before E14.5. Then, these RGCs migrate to the inner retina, and from this point on, Oa1-activated pCreb might control Dcx transcription allowing the expression of Dcx protein in RGC cell bodies and projections of the normal, control retina. We hypothesize that the reduced levels of Dcx in the absence of Oa1 (in Oa1−/− mice) may affect the routing of RGC axons. This might be similar in OA1 patients, where OA1 mutations may decrease pCreb activity, resulting in abnormal levels of Dcx and misrouting of the optic nerve fibers.
Difficulties that I experienced during my studies

In this study, we met a lot of difficulties and there are many things that we are not satisfied with. The main problem that we had was that there is no good antibody for Oa1. We tried almost all the polyclonal Oa1 antibodies in the market. None of them was reliable. We finally used a monoclonal antibody that was recently produced to produce all the Oa1 IHC results in this study. However, we think that Oa1 expression in RGCs still needs to be further confirmed with more experiments. We also tried Co-immunoprecipitation (Co-IP) experiments to explore proteins that are in the Oa1 signaling pathway. Here again, these efforts were not successful because the quality of Co-IP is largely dependent on antibody quality. In order to better study the OA1 disease, a more stable and reliable antibody needs to be generated.

We were not able to get E14.5 and E15.5 Oa1−/− animals. We are having a difficult time maintaining the Oa1−/− mouse colony, for these mice don’t produce well. After giving birth, the mothers either don’t feed or eat the babies. I was not able to get Oa1−/− embryos or P2 throughout this last quarter. IHC is a good way of showing the location of protein expression, however not accurate in quantifying the amount of expression. Experiments have to be repeated for several times to make sure the expression level differences are true.
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