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Non-cellular autonomous neuroprotection by OTX2 in the retina

Bilal Mazhar

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Bilal Mazhar. Non-cellular autonomous neuroprotection by OTX2 in the retina. *Neurons and Cognition [q-bio.NC]*. Université Paris sciences et lettres, 2020. English. NNT : 2020UPSLE023 . tel-03339531

HAL Id: tel-03339531

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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

Préparée au Collège de France

**NON-CELL AUTONOMOUS ACTIVITY OF OTX2 IN THE
RETINA**

**NEUROPROTECTION AUTONOME NON CELLULAIRE
PAR OTX2 DANS LE RETINE**

Soutenue par

Bilal MAZHAR

Le 18 02 2020

Ecole doctorale n° ED 158

**Cerveau, Cognition,
Comportement (3C)**

Spécialité

Neuroscience



**COLLÈGE
DE FRANCE**
— 1530 —

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منہ آئی بات نہ رہندی اے
سچ کہو الیہ تے سرھانہڑ چھدا اے
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منہ آئی بات نہ رہندی اے

Bulleh Shah - بلھے شاہ

Many Thanks To...

...Alain for welcoming me into his laboratory. For strengthening my character. Thank you for teaching me various things, scientific and non-scientific things. I could not have worked at a more dynamic laboratory. I feel it has well prepared me for the future.

...Ken for welcoming me into the 'Pathologies Group.' You taught me many things from being better organized to thinking logically about experiments. You taught me essential skills for science and life in general. In addition, thank you for introducing me to culture and gastronomy.

...Olivia for too many things. Thank you for teaching me so many different experimental techniques and showing me the 'good way' to do things. And always knowing where things were in the lab. Without your daily kindness I would not have been able to function. Also thank you for dealing with the French administration for me.

...Raoul for showing me experimental work. Without your expertise I would not have complete my work. Thank you for showing me how to dissect, fix, and section the retina. And for all the cervical dislocations you did for me. Thank you for the wonderful conversations and your friendship.

...Yoko. Thank you for interesting conversations about life. We started working together and thank you for being supportive, helping me, and believing in me. Thank you for all the scientific conversations and advice.

...Jessica for always being there. Thank you for always being kind and available to help me with my experiments. And preparing anesthetic at the last minute. Also for countless cervical dislocation you did for my experiments.

...Javier for being a great colleague. Thank you for all the scientific conversations and a good sense of humor. Thank you for spending time at the Tennessee and giving me advice.

...Chantal thank you for lighting up the lab with good jazz music. And introducing me to fascinating jazz music.

...Ariel thank you for sharing your treasure trove of plasmids with me. And helping me manage the mice.

...Melanie thank you for being a good friend and always being there. Thank you for making so many phone calls for me.

...Camille, for all the wonderful times. It was nice to kick it off at the end of the day with someone who understood it all.

...Marion Thauvin thank you for your kindness. And always showing me the bright side of life and keep me positive.

...Julien Moulard, Charles-Felix Calvo, Pascal Ezan thank you for the wonderful conversation over lunch. It was great to eat and discuss with you. I needed your daily dose of positivism to keep me going.

...Alain Joliot and Edmond Dupont for a lot of support and help. Thank you for always sharing things from your lab and having the time to help me.

...Michel helping me troubleshoot molecular biology problems countless times.

...All the people from the laboratory who are not individually named. Thank you all for everything and all your support.

...All the staff of the gestion department...Nichole, Sophie, and Sandrine thank you for all your kindness and help over the years.

...to my family for supporting and understanding all the difficult moments of my life. I know it's been a long journey. But thank you for all your love, support and advice.

Abstract

Homeoproteins are transcription factors that are defined by their homeodomain and that play an essential role in establishing the body pattern. One such homeoprotein is OTX2. As other homeoproteins, in addition to cell autonomous activity, OTX2 can transfer between cells and exert non-cell autonomous activities.

Previous studies demonstrated that OTX2 has neuroprotective effect on adult retinal cells. OTX2 promotes the survival of adult retinal ganglion cells *in-vitro* and protects them against N-Methyl-d-aspartate (NMDA) toxicity *in vivo*, an adult model of glaucoma. Also, when *Otx2* is conditionally knocked out in retinal pigmented epithelium cells, this causes photoreceptor degeneration. And expression in retinal pigmented epithelium cells is sufficient for photoreceptors survival, showing that OTX2 in retinal pigmented epithelium cells is necessary for photoreceptor survival. However, it is not known if non-cell autonomous OTX2 transfer from retinal pigmented epithelium to photoreceptors has protective effect on photoreceptors.

The objective of this thesis is to characterize the non-cell autonomous activity of OTX2 in the retina in terms of visual function and photoreceptor survival. In the first part we blocked the transfer of OTX2 in the retina and assessed visual function using *PVCre::scFvOtx2* mice in which parvalbumin-expressing cells secrete an anti-OTX2 single chain antibody (OTX2scFv). The secreted OTX2scFv blocks OTX2 transfer within the retina with no effect on the retinal structure and anatomy. However, *PVCre::scFvOtx2* mice have reduced visual acuity and altered inner retinal physiology demonstrating a non-cell autonomous activity for OTX2 on inner retinal function.

In the second part we explored if OTX2 also has neuroprotective effect on photoreceptors. We established that OTX2 is present in the interphotoreceptor matrix of WT mice, where photoreceptor outer segments are located. We also found that exogenous OTX2 is able to bind glycosaminoglycans (GAGs) in the interphotoreceptor matrix on retinal fresh frozen sections. We also found that *in vivo* retinas, exogenous OTX2 is able to modulate mitochondrial reactive oxygen species pathway gene to protect retinal cells against oxidative stress.

This work demonstrated that non-cell autonomous OTX2 in the retina leads to an alteration in inner retinal cell function and causes the deficit in visual acuity. OTX2

binds to the OS and alters reactive oxygen species pathway genes in the retina. This may help to provide neuroprotection to photoreceptors cells.

Résumé

Les homéoprotéines sont des facteurs de transcription, elles régulent l'expression des gènes grâce à leur homéodomaine et jouent un rôle essentiel dans l'organisation du schéma corporel d'un organisme au cours du développement. OTX2 est une de ces homéoprotéines et, en plus d'une activité cellulaire autonome, OTX2 peut transférer entre les cellules et exercer un rôle non cellulaire autonome.

Des études antérieures ont démontré qu'OTX2 avait un effet neuroprotecteur sur les cellules rétiniennes adultes. OTX2 favorise la survie des cellules ganglionnaire rétinienne (CGR) adultes *in vitro* et dans un modèle adulte du glaucome *in vivo*. Dans ce modèle *in vivo*, OTX2 protège les cellules ganglionnaires rétiniennes contre la toxicité du NMDA. De plus, dans un modèle adulte, quand OTX2 est inactivé de manière conditionnelle dans les cellules de l'épithélium pigmentaire rétinien (EPR), cela provoque une dégénérescence des photorécepteurs (PRs). Son expression dans les cellules de l'épithélium pigmentaire rétinien est suffisante pour la survie des PRs, ce qui montre que l'expression d'OTX2 dans les cellules de l'EPR est importante pour la survie des PRs. Cependant, on ne sait pas si OTX2 provenant de l'EPR a un effet protecteur sur les PRs grâce à son action non cellulaire autonome.

L'objectif de cette thèse est de caractériser l'activité non cellulaire autonome d'OTX2 dans la rétine et son effet sur la fonction visuelle et de survie des PRs. Dans la première partie, grâce aux souris *PVCre::scFvOtx2* qui sécrètent un OTX2scFv, nous avons bloqué le transfert d'OTX2 dans la rétine et testé les fonctions visuelles. L'OTX2scFv sécrété bloque le transfert d'OTX2 dans la rétine et nous n'observons aucun effet sur la structure et l'anatomie de la rétine. Cependant, les souris *PVCre::scFvOtx2* ont une acuité visuelle réduite et une altération de la physiologie de la rétine interne. Ceci démontre une activité non cellulaire autonome d'OTX2 sur la fonction de la rétine interne.

Dans la deuxième partie, nous avons recherché si OTX2 a également un effet neuroprotecteur sur les PRs. Nous avons démontré qu'OTX2 est présent dans la matrice inter-photoréceptrice (MIP) de souris WT où se trouvent les segments externes des photorécepteurs. Nous avons mis en évidence des sites de fixations d'OTX2 dans la rétine. Nous avons constaté qu'OTX2 exogène est capable de se lier aux glycosaminoglycanes (GAGs) de la MIP sur des coupes de rétines congelées. Nous avons également observé que, dans des rétines *ex vivo*, OTX2 exogène est

capable de moduler un gène de la voie des espèces réactives de l'oxygène (ERO) mitochondriales afin de protéger les cellules de la rétine contre le stress oxydatif.

Ce travail démontre que l'effet non cellulaire autonome d'OTX2 dans la rétine provoque une altération des fonctions des cellules de la rétine interne ce qui cause une diminution de l'acuité visuelle. De plus, OTX2 se lie aux segments externes et altère l'expression des gènes impliqués dans la voie des ERO dans la rétine. Tout cela pourra contribuer à un effet neuroprotecteur des PRs.

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List of Acronyms

AAV	Adeno-associated Virus
ABAM	Antibiotic and antimycotic
AMD	Age related Macular Degeneration
AP-Nt-HD (AA)	Alkaline Phosphatase fused N-terminal of O with AA mutation
AP-Nt-HD (RK)	Alkaline Phosphatase fused N-terminal of O WT.
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
ChABC	Chondroitin ABC
CO ₂	Carbon dioxide
CRX	Cone-Rod Homeobox
DA	3,4-dihydroxyphenethylamine
DCFDA	2',7'-Dichlorodihydrofluorescein diacetate
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic
EN1	Engrailed 1
EN2	Engrailed 2
ERG	Electroretinogram
FBS	Fetal bovine serum
GAGs	Glycosaminoglycans
GPX1	Glutathione peroxidase 1
HCL	Hydrochloric acid
HP	Homeoprotein
HRP	Horseradish peroxidase
INL	Inner Nuclear Layer
IPM	Interphotoreceptor Matrix
LED	Light-emitting diode
Na ₂ HPO ₄	Disodium phosphate
NaCl	Sodium Chloride
NGF	Neural Growth Factor

NMDA	N-Methyl-D-aspartic Acid
NRL	Neural retina-specific leucine zipper proteir
ONL	Outer Nuclear Layer
OS	Outer Segment
OTX2	Orthodenticle Homeobox 2
OTX2scFv	Single chain antibody against Otx2
PAX6scFv	Single chain antibody against PAX6
PBS	Phosphate buffered saline
PEDF	Pigment epithelium-derived factor
PEN	Polyethylene naphthalate
PKCa	Protein kinase C
PNN	Perineuronal Net
PR(s)	Photoreceptor(s)
PV	Parvalbumin
PVCre	Paralbumin Cre Mice
PVDF	Polyvinylidene fluoride
RGC	Retinal Ganglion Cell
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPE	Retinal Pigmented Epithelium
RPE65	Retinal pigment epithelium-specific 65 kDa protein
RPM	Rotation per minute
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate-polyacrylamide
SOD1	Superoxide dismutase 1
TBS	Tris buffered saline
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VSX2	Visual System Homeobox
WB	Western Blot

Chapter 1. Introduction

Retina

A. Anatomy

The retina is a sensory organ at the back of the eye that transduces photons into neural signals. The neural signals are transmitted through layers of the retina and then to higher visual processing areas of the brain.

The outer nuclear (ONL) contains photoreceptor cells that transduce photons into neural signal. The outer plexiform layer (OPL) is where photoreceptors form synaptic contacts with bipolar cells. Bipolar cell bodies are located in the inner nuclear layer (INL).

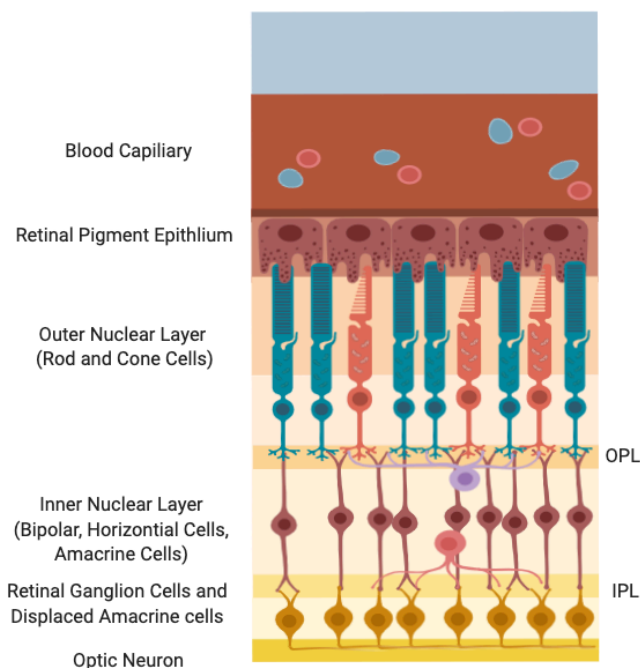


Fig 1. Mammalian retina

The retina is composed of four main cell layers. These are RPE, ONL, INL, and RGC layers. The photons of light are detected in the outer segments of the outer nuclear layer and the signal is transmitted to the brain via the optic nerve.

Photons enter the eye from the front through the lens. They pass through the retina and are absorbed by opsin molecules embedded in the outer segments (OS) of photoreceptors in the ONL. The ONL is composed of two main photoreceptor cell types. In nocturnal animals, such as mice, rods are the dominant type of

photoreceptors and contain rhodopsin in their outer segments. Rods are sensitive to photons and can detect a single photon (Okawa & Sampath 2007), allowing for animals to see in dim light. In trichromate animals, such as some primates, cone cells, which are sensitive to different wavelengths of light, are more abundant. Cone photoreceptors are active in daylight condition and the opsins embedded in cone cells are sensitive to long wavelength light from 420nm to 560nm. Cone cells respond to red, blue, and green color light that underlines color vision.

Photoreceptors form synapses with dendrites of bipolar cells in the outer plexiform layer (OPL). Bipolar cells are densely packed in the INL layer that also includes horizontal and amacrine cells. Bipolar cells can form synapses with both rods and cones. In the dark, photoreceptors release glutamate, which hyperpolarizes the ON bipolar cells and depolarizes the OFF bipolar cells (Purves & Cabeza, 2013). Under such conditions, photoreceptors have an abundance of cGMP that keeps the cGMP-gated Na⁺ channels open so that phosphodiesterase diminishes the supply of cGMP (Purves & Cabeza, 2013). This reduces the number of open Na⁺ channels causing hyperpolarization of the photoreceptor cell and less glutamate to be released (Strettoi et. al., 2010). This results in ON bipolar cells depolarization, while the OFF bipolar cells becomes hyperpolarized (Purves & Cabeza, 2013; Strettoi et. al., 2010).

Another cell type found in the INL is represented by the horizontal cells. Horizontal cells help to integrate and modulate photoreceptor output by providing them with an inhibitory feedback to photoreceptors. In the absence of light, glutamate from photoreceptor synapses is released, causing the horizontal cells to depolarize and this depolarization causes the hyperpolarization of nearby photoreceptors (Verweij et. al., 1996). In presence of light, photoreceptors release less glutamate, which causes hyperpolarization of the horizontal cell and depolarization of nearby photoreceptors (Verweij et. al., 1996; Verweij et. al., 2003). This negative feedback to photoreceptors allows for continuous generation and transduction of light signal into neurotransmitter signal (Masland 2012).

The ganglion cell layer, the most interior layer of the retina contains retinal ganglion cells (RGCs) and displaced amacrine cells. RGCs receive inputs from bipolar cells in the synaptic layer: the inner plexiform layer (IPL) (Masland 2012). Amacrine cells in the INL and GCL both modulate bipolar synaptic input on the RGC dendrites. The main RGC targets are the superior colliculus in the dorsal midbrain, thalamus, pretectum, and the hypothalamus. The latter structures project the visual information to the visual cortex. RGC axons form bundles that exit the retina at the

optic disk and enter the optic nerve. In rodents a majority of RGC axons cross at the optic chiasm. A fraction of RGCs contain a photopigment, melanopsin that allows for detecting light even in the absence of rods and cones. (Masland 2012). These intrinsically photosensitive RGCs project into the suprachiasmatic nucleus in the hypothalamus, and provide information for circadian rhythm (Masland 2012; Morgia et al., 2017).

The retinal pigmented epithelium (RPE), a tight junction pigmented monocellular layer of pigmented cells, provides essential functional support to the photoreceptors. RPE cells phagocytose the photoreceptor outer segments that contain toxic reactive oxidative species (ROS) that accumulate during phototransduction and photo-oxidative stress. This allows for photoreceptor to generate new outer segments. The RPE layer also forms the blood-retina barrier. It regulates the passage of nutrients, growth factors, and immune cells.

B. RPE and Photoreceptors

RPE cells are cuboidal cells situated at the back of the eye posterior to photoreceptor cells. RPE is essential for conversion and storage of retinoid, absorption of scattered light, secretion growth factors and phagocytosis of photoreceptor outer segment (Sparrow et al., 2010). Microvilli emanate from the apical surface of the RPE cells and interdigitate between photoreceptor outer segments. The microvilli greatly increase the surface area of the RPE and this allows for greater amount of OS to be engulfed (Sparrow et al., 2010).

These retinoids, including vitamin A, are transported to the RPE cells (Wongsiriroj et. a., 2008). Within RPE cells, RPE65 converts vitamin A into 11-cis retinol, which is the essential molecule involved in the visual cycle (Wongsiriroj et. a., 2008).

RPE prevents light scattering in the eye. The RPE cells have melanosomes that absorb excess light and prevent oxidative damage on the macula. The melanosomes prevents light scattering, thus improving the quality of the optical image. In addition, not absorbed scattered light causes oxidative damage to retinal cells.

Similarly to PEDF, VEGF is secreted by the RPE and promotes the survival of endothelial cells, but is specifically produced by RPE cells that do not receive enough oxygen (Holmes et. al., 2007). Retinal VEGF stimulates the formation of blood

vessels and is involved in eye vasculogenesis and angiogenesis. In age-related macular degeneration (AMD) patients, excessive VEGF results in proliferation of abnormal blood vessels (Ohno-Matsui et al., 2001). These abnormal blood vessels are fragile compared to typical blood vessels and, when damaged during aging or retinal diseases, blood and proteins leak into the retina. Bleeding and scarring lead to photoreceptor death and blindness.

Phagocytosis of outer segments of photoreceptors is another essential role of the RPE, which helps maintain healthy vision (Mazzoni, et al., 2014). The outer segments of the photoreceptors undergo constant shedding and renewal (Young 1967). On exposure to light, a tremendous amount of Reactive Oxidative Species (ROS) is produced in the OS and ROS are toxic to PRs. The engulfed outer segments contain high amounts of photo-oxidative products such as lipofuscin and N-retinylidene-N-retinylethanolamine (A2E) and other mitochondrial by-products (Kenvany & Palczewski 2010). By phagocytosing the outer segments, RPE cells neutralize these highly oxidative species (Azad et al., 2009; Datta et al., 2017).

Photoreceptor Degeneration Diseases

A. Retinitis Pigmentosa

Retinitis pigmentosa (RP) is one of the most common genetic disorder leading to blindness (Facts about Retinitis Pigmentosa, 2015; Sung et al., 1991; Zhu et al., 2018). The disease starts with the loss of photoreceptors, leading to night blindness and, with time, cone cells also start to die (Facts about Retinitis Pigmentosa, 2015; Bunker et al., 1984; Sung et al., 1991).

Inheritance patterns of retinitis pigmentosa have been identified as X-linked, maternal mitochondrial, autosomal dominant, and autosomal recessive (Bunker et al., 1984). So far, over fifty different mutations have been identified and associated to the disorder (Ferrari et al., 2011). A commonly mutated gene in the photoreceptors is *rhodopsin* (Hartong et al., 2006). Misfolding and missense mutations in the gene cause rhodopsin not to embed into the OS of the photoreceptors (Hartong et al., 2006; Berson et al., 1991). With age, the outer segments of the photoreceptors are eventually lost, resulting in retinal degeneration and blindness. A single rhodopsin gene mutation is responsible for twenty five percent of autosomal dominant forms of RP (Berson et al., 1991).

Another common inherited mutation is in retinoid isomero-hydrolase or RPE65 (Cai et. al., 2009). RPE65 is expressed in RPE cells and is important for phototransduction. During phototransduction, it converts all-trans-retinyl esters to 11-cis-retinol (Wolf 2005; Kiser et. al., 2015). Misfolding mutations and those that prevent an iron cofactor from binding to the enzymatic core induce RPE cell degeneration, causing that of photoreceptors and leading to blindness (Rivolta et. al., 2001; De Angelis et. al., 2002; Schorderet & Escher, 2009; Tang et. al., 2011; Borrego et. al., 1996; Dryja et. al., 1990).

Similarly, mutations in other RPE or photoreceptor genes such as *Crx*, *Nr2E3*, and *Lrat* also cause RP. It was demonstrated that mutations in *Crx* prevent terminal differentiation and survival of photoreceptor cells (Ibrahim et. al., 2018). CRX is a homeoprotein that binds to DNA of retinal genes (Ibrahim et. al., 2018; Stenson et. al., 2013). Frameshift or missense mutations in *Crx* prevents the protein from binding to its targets and photoreceptors are not terminally differentiated (Ibrahim et. al., 2018). Furthermore, a mutant form of CRX has been shown to partly interact with wild-type CRX and to disrupt its function, thus hindering retinal foveal development and leading to blindness (Stenson et. al., 2013).

B. Age-Related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) occurs at an older age, when the field of view slowly blurs until vision is lost in its center (Mehta, 2015). In rare cases, patients diagnosed with AMD also have been reported for hallucinations not due to mental illness (Abbott et. al., 2007). AMD pathogenesis is linked to oxidative stress, mitochondrial dysfunction and inflammatory responses in the eye (Thornton et. al., 2005). At early stages of the disease an accumulation of harmful products such as lipofuscin and drusen causes cellular homeostatic imbalance and degradation (Parekh et. al., 2009). These lipofuscin deposits occur in the ONL and the RPE layer, while the drusen deposits are localized between the Bruch membrane and the RPE layer (Pilgrim et. al., 2017). Early in the disease, these deposits cause thinning and depigmentation of the area resulting in central vision loss. Risk factors associated with AMD include dietary and lifestyle choices such as smoking, drinking, hypertension and fat consumption (Thornton et. al., 2005; Dasari et. al., 2011). Other factors include genetic mutations and mitochondrial disorders.

With regard to lifestyle risk factors, smokers are three times more likely to develop AMD in comparison to non-smokers (Thornton et. al., 2005). This is likely

due to cigarette smoke containing many toxic and carcinogenic compounds, which may initiate photoreceptor damage. Obesity, high cholesterol and high trans-fat-rich food increase chances to develop AMD. One of the features of AMD is the appearance of drusen bodies. These drusen bodies are accumulations of lipids and fat between the RPE and choroid. One proposed mechanism is that excessive amount of lipid in the eye blood circulation could impede blood flow. This would result in the disruption of blood capillaries and blood leakage into the eye eventually leading to photoreceptor death (Ratnayaka et. al., 2015).

Genetic causes can also lead to AMD-related photoreceptor death. Studies have shown that genes such as complement system proteins factor H (CFH), and factor B are strongly correlated with AMD (Fritsche et. al., 2010; Yates et. al., 2007; Haines et. al., 2005). CFH is a complement control protein that helps immune cells distinguish between the body's own cells and foreign entities. During an inflammatory response, this protein allows the immune cells to distinguish pathogen from body's own cells by inhibiting inflammation (Fritsche et. al., 2010). Mutations that do not allow CFH to distinguish between the body's own cells versus foreign entities cause an inflammatory response that damages the macula and the retina (Yates et. al., 2007). Other gene mutations include *arms2*, *htra1*, *serping1*, and *fibulin-5* (Ennis et. al., 2008). In hereditary AMD, reduction of translation of *arms2* by mRNA interference has been shown to reduce the symptoms (Fritsche et. al., 2008). In certain populations, a polymorphism in *htra1* promoter region is linked to greater chances of AMD (Cruz-Gonzalez et. al., 2013). A single nucleotide polymorphism G>A has been associated with increased HTR1 expression, resulting in AMD (Cruz-Gonzalez et. al., 2013).

There are two types of AMD. Dry AMD, also known as nonexudative AMD, is a form of AMD that does not involve leakage of fluids into the eye. Thus, photoreceptor degeneration is due to drusen deposits, oxidative stress, and mitochondrial dysfunction (Pilgrum et. al., 2017). Patients suffering from dry AMD exhibit minimal symptoms until vision loss occurs and this form accounts for roughly eighty percent of AMD patients (Gehrs et. al., 2006).

The second type of AMD is wet AMD or neovascular AMD. It results in abnormal blood vessel growth through Bruch's membranes. These newly proliferated blood capillaries are fragile and break easily (Rücker et. al., 2006). This results in leakage of blood and proteins in the macula. Continuous bleeding, leaking, and scarring of the blood vessels leads to irreversible retinal death and loss of vision.

Homeoproteins

A. Homeoprotein are Transcription Factors

Homeoproteins are transcription factors that are defined by their DNA-binding domain or homeodomain (Gehring 1992). During development homeoproteins regulate genetic networks that instruct the body plan. In 1967, Gehring's group identified the *Antennapedia* mutation in which the fly antenna is replaced, by a thoracic leg (Gehring 1967). The structural investigation into the Antennapedia gene led to identification of the homeobox. The homeobox encodes the homeodomain, a DNA binding domain of sixty amino acids, highly conserved among all homeoproteins and in all eukaryotes across eukaryotes, including fungi, metaphytes and metazoans (Carrasco et., 1984; McGinnis et al., 1984; Shepherd et al., 1984). The homeodomain encompasses a unstructured N-terminal arm and three well-defined alpha helices arranged in helix-loop-helix-turn-helix (Gehring et al., 1994). Helix 1 and 2 are arranged in an anti-parallel manner, while helix 3 is perpendicular to the axis created by the first two helices (Wolberger et al., 1991).

Homeoproteins function as transcriptional regulators of target genes (Krumlauf 1994). During development, homeoproteins are critical for embryonic polarity, cell migration, and differentiation (Prochiantz et. al., 2015; Mark et. al., 1997). They are critical for nervous system development by regulating morphogenetic events leading to stereotypic specification and patterning of the ectoderm (Mallamaci et. al., 1996). In the mouse, vertebrate orthologues of Drosophila homeoproteins are also involved in body patterning. The OTX family of homeoproteins (Otd fly orthologues) includes OTX1, OTX2, and OTX3 (Crx), all critical for the development of the murine nervous system, including the brain and the retina (Acampora et. al., 2005; Boncinelli et. al., 1993).

B. Unconventional Transfer of Homeoproteins

Traditionally, homeoproteins are described as cell autonomous acting DNA-binding transcriptional factors. However, in the early 1990s, Alain Prochiantz and colleagues discovered that many homeoproteins are able to translocate across plasma membranes. *In-vitro* experiments demonstrated that they were able to exit and enter neural cells without altering the plasma membrane (Roux et al., 1993).

Internalized homeoproteins were shown to exert a non-cell autonomous morphogenetic activity through DNA-binding (Joliot et al., 1991b). This cellular transfer supports direct non-cell autonomous homeoprotein signalling (Planques et al., 2018) and involves many if not all members of this family of transcription factors (Lee et al., 2019).

Homeoprotein intercellular transfer depends on two short peptide sequences present in the homeodomain (Figure 3). Internalization is entirely driven by the third helix (Derossi et al., 1994) within which a tryptophan in position 48 (TRP48) of the homeodomain is necessary for the translocation across the membrane (Derossi et al., 1996). The present model for internalization is that the third helix binds to extracellular membrane negative charges and that TRP48 digs into the lipid bilayers and provokes the formation of inverted micelles (Berlose et al., 1996; Derossi et al., 1996; Christiaens et al., 2002).

The transfer signals are in the homeodomain and thus highly conserved and present in most if not all homeoproteins. Even though the ability to transfer was demonstrated *in vitro* and *in vivo* for around 100 homeoproteins (Lee et al. 2019), actual physiological functions were only shown for a few of them including Engrailed (Brunet 2005; Wizenmann et al., 2009, Layalle et al., 2011), Pax6 (Di Lullo et al., 2012; Kaddour et al., 2019); Vax1 (Lee et al., 2019) and OTX2 (Sugiyama 2008). It is of note that in the three cases where the amount of extracellular homeoprotein has been calculated (Wizenmann et al., 2009, Di Lullo et al., 2012; Kaddour et al., 2019); it was found to be approximately 5% of the nuclear content.

The internalization signal in the homeodomain was reported on most if not all homeoproteins including EN1, EN2, OTX2, PAX6, and HOXB4 (Rekaik et al., 2015; Ohta et al., 2007; Chauhan et al., 2004; Apulei et al., 2019). Moreover, these homeoproteins have been shown to transfer between cells (Joliot et al., 1998; Prochiantz and Joliot, 2003; Volovitch et al., 1993). More recently, it has been shown that cellular transfer is a common feature of over hundred different homeoproteins (Lee et al., 2019). The transfer efficiency of homeoproteins differs, meaning that some homeoproteins transfer more than others. The protein binds to a negative charged area of the cell, which could be carbohydrates and/or lipids (Lensink et al., 2005). Then the plasma membrane is destabilized by the tryptophan at position forty-eight of the homeodomain of the lipid bilayer (Christiaens et al., 2002). This process

forms inverted micelles and allows homeoproteins to be internalized directly into the cell cytoplasm (Christiaens et al., 2002; Dupont et al., 2007).

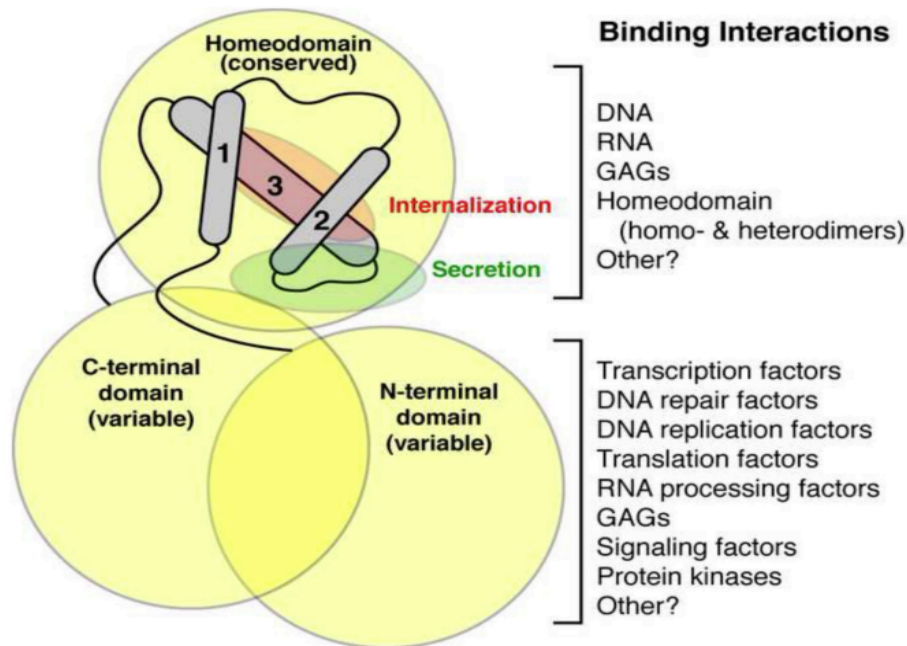


Fig 2. A scheme of homeoproteins showing the homeodomain

The sixty amino-acid-long motif allows homeoprotein secretion and internalization. In addition, the homeodomain is responsible for DNA, RNA, and GAG binding. Based on Prochiantz and Di Nardo, 2015.

If homeoproteins are able to transfer in this atypical manner then an interesting question is raised. How much of homeoprotein is transferred between cells? To answer this question Prochiantz’s lab has estimated that in vitro and in-vivo less than five percent of total homeoprotein can be found outside of the cell (Di Lullo et. al., 2011; Wizenmann et. al., 2009). This implies that only small amounts of homeoproteins are transferred between cells. Hence for our project this is essential because it is possible that a small amount of OTX2 from RPE is transferred to the OS of the PRs.

C. Roles of OTX1, OTX2, and OTX3 in Embryonic and Retinal Development

In the mouse, *Otx2* is expressed in the visceral endoderm and epiblast before gastrulation at embryonic day 5 (E5). At E7.5, *Otx2* is expressed throughout the

anterior part of the three germ layers, including the anterior neural plate that will give rise to the telencephalon, diencephalon, and mesencephalon (Acampora et al., 2001; 2009). During embryonic development, OTX1 and OTX2 are expressed in overlapping regions. By E10.5, expression of *Otx1* is maintained in the dorsal telencephalon whereas that of *Otx2* is reduced (Sakurai et al., 2010) and by embryonic and early postnatal stages *Otx2* expression is predominantly localized to the choroid plexuses, the retinal pigment epithelium, neural retina and regions of the diencephalon and mesencephalon (Nothias et al., 1998).

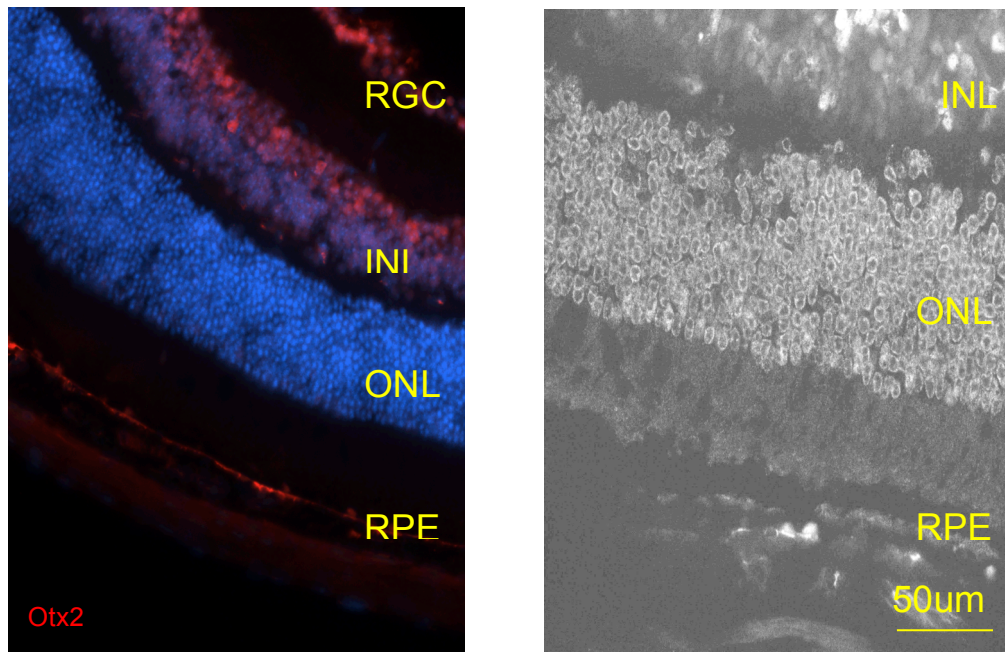


Fig 3. Localization of OTX2 in the Adult Retina of C57bl/6.

(A) OTX2 (red) is localized in all retinal cell layer, including the RPE, ONL, INL, and RGCs. Counterstaining with DAPI (blue). **(B)** Localization of *Otx2* (white) in ONL forming a ring like staining. ONL have inverted nuclear architecture where euchromatin lines the nuclear border as OTX2 staining reveals and heterochromatin localizes in the nuclear center. Scale bar is 50um for both images.

Previous work has demonstrated that during gastrulation, *Otx2* is expressed in cells from the anterior visceral endoderm producing signals that drive early specification and patterning of the neural plate (Ang et al., 1994; Simeone et al., 1993). *Otx2* null embryos lack rostral neuroectoderm and are embryonically lethal. These null mice also demonstrate major defects in body patterning and do not develop a head (Acampora et al., 1995; Matsuo et al., 1995). During embryonic development and postnatal development, neural ectoderm gives rise to retinal tissue. From E9 to P14, retinal progenitor cells start to differentiate into retinal cells such as

RGCs, bipolar cells, photoreceptors (rods and cones) and RPE cells. OTX2 orchestrates the genetic network in progenitor cells for them to mature into retinal cells. In addition to maturing progenitor cells, *Otx2* is expressed in the optic disk (Martinez-Morales et al., 2001; Sugiyama et al., 2008). This demonstrates the importance OTX2 homeoprotein during gastrulation, and regionalization of the rostral brain and retina.

In the mouse embryo complete deletion of *Otx2* is lethal. However, *Otx2* heterozygous mice are able to go through the process of gastrulation, development, grow, and reproduce (Bernard et al., 2014). But *Otx2*^{+/-} mice do exhibit retinal abnormalities. Mutations in *Otx2* causes ocular and retinal phenotypes such as microphthalmia and anophthalmia (Schilter et al., 2011). More precisely different mutant lines with increasing *Otx2* hypomorphism show morphological, physiological and behavioral defects with strengths and times of appearance correlated with the degree of hypomorphism (Bernard et al. 2014).

OTX2 plays a central role in photoreceptor cell maturation. In the postnatal retina, OTX2 expression (along with that of VSX2, BLIMP1 and other transcription factors) regulates photoreceptor maturation and homeostasis. It has been demonstrated that OTX2 is a direct regulator of *Vsx2*, while *Vsx2* is a strong regulator of *Blimp1* (Figure 4) (Brzezinski et al., 2010; Cepko., 2015). *Otx2* expression plays a role in the binary fate choice of rod versus bipolar cells. Initially, *Otx2* expression is higher compared to that of *Blimp1* (Cepko., 2015; Emerson & Cepko 2011). Then, as OTX2 initiates *Blimp1* expression, that of *Otx2* and *Blimp1* decreases and increases, respectively. This increased expression of *Blimp1* causes *Vsx2* to be expressed (Cepko., 2015; Brzezinski et al., 2010). This presents two possibilities for cell determination (Figure 4): if *Vsx2* and *Otx2* are highly expressed in a progenitor cell, it will differentiate into a bipolar cell, but if *Otx2* expression is low and *Vsx2* is absent it will differentiate into a rod photoreceptor (Kim et al. 2008, Brzezinski et al., 2013; Emerson & Cepko 2011). Therefore, the levels of expression of *Otx2* in the mouse embryo will have important consequences for photoreceptor maturation (Cepko., 2015).

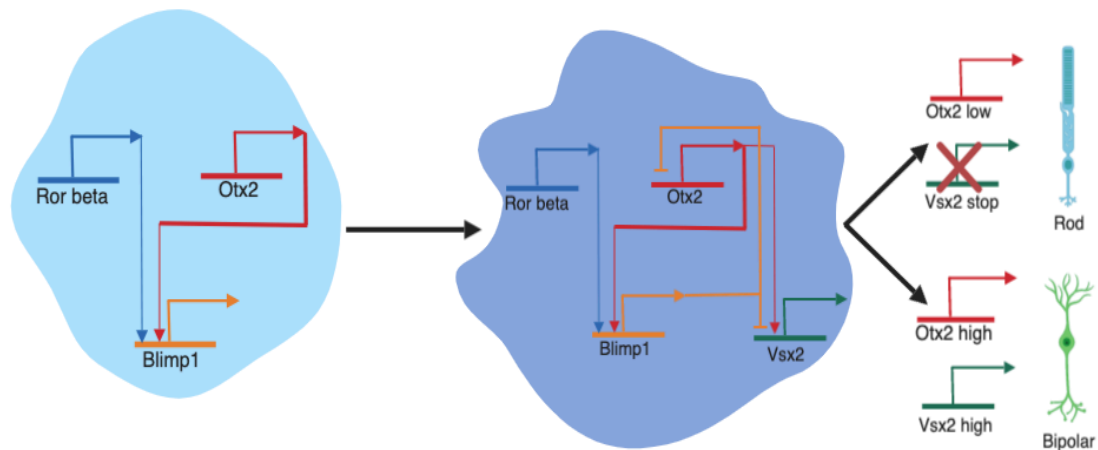


Fig 4. Regulatory genes involved in maturing photoreceptors.

At early stage of cell maturation *Otx2* and *RorBeta* are turned on. OTX2 and RorBeta bind to the promoter regions of *Blimp1*, and *Blimp1* turn binds to the *Vsx2*. The level of *Vsx2* and *Otx2* during maturation determine if the cell fate would be a photoreceptor or bipolar cell. If *Vsx2* is not available and *Otx2* expression is low the cell becomes a photoreceptor. However, high expression of *Otx2* and *Vsx2* leads to a bipolar cell. Adopted from Wang et. al., 2014.

Similar to OTX2, OTX3 (Cone Rod Homeobox protein or CRX) is vital for cell fate and homeostasis in mammalian retina. CRX is expressed in early post mitotic photoreceptor precursors and acts downstream of *Otx2*, though its expression is initiated by OTX2 (Nishida et al., 2003). CRX plays a role in expressing rhodopsin and S opsin gene synthesis and maintained in adult photoreceptors (Nishida et al., 2003). Moreover, CRX-deficient mice develop photoreceptors as WT mice, but they do not express photoreceptor genes such as *Pde6b* and *Sag*, nor have outer segments, eventually leading to retinal degeneration (Koike et. al., 2007). This highlights the critical role that OTX2 and CRX play in maintaining adult photoreceptors homeostasis. In humans, *Crx* is clinically interesting because mutations in *Crx* are known to cause retinal diseases such as Leber Congenital Amaurosis and Retinitis Pigmentosa (Swaroop et. al., 1999; Koike et al., 2007; Cepko., 2015).

D. Role of OTX2 in Rod Cells vs Cone Cells

Neural retina-specific leucine zipper (*Nrl*) transcription factor is expressed at E12 in rod photoreceptors cells (Austin & Cepko et al, 1995). *Nrl* determines rod cell differentiation by turning on multiple rod specific genes including Rhodopsin (Rehemtulla et al., 1996). A null mutation in *Nrl* causes the cells that were destined to

become rod photoreceptors to convert into cone cells, specifically into S cones (Mears et. al., 2001). This is because *Nrl*, in rod cells, directly or indirectly represses cone genes by activating a repressor *Nr2e3* (Oh et al., 2008; Corbo and Cepko, 2005). Hence, in *Nrl*-null models rod genes fail to be expressed and rod precursors experience a de-repression of cone genes, causing the rod to cone shift (Daniele et al., 2005).

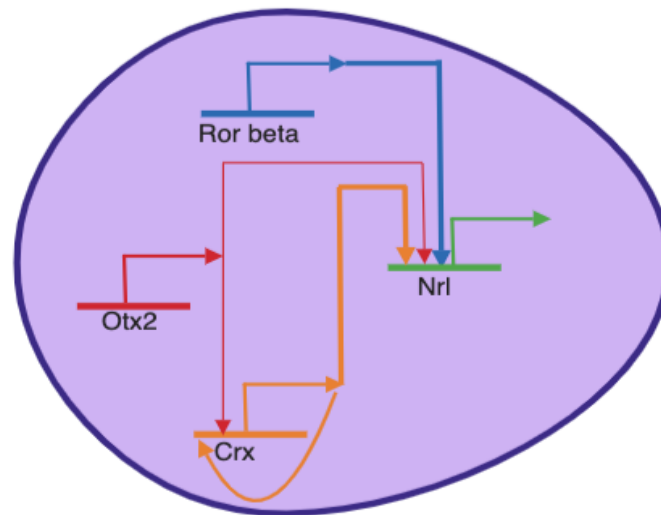


Fig 5. Regulator genes involved in maturation and maintaining of photoreceptors.

In terminally differentiated photoreceptor cells a constant expression of *Otx2* maintains expression of *Crx*, which turns on another homeoprotein, *NRL*. *NRL* causes the cells to differentiate into cone cells. *Ror beta* is also needed for *Nrl* expression. Based on Roger et. al. 2014.

As illustrated in Figure 6, recent studies have demonstrated a genetic interaction between *Otx2* and *Nrl*. *OTX2* and *CRX* directly regulate *Nrl* expression in developing mice by binding promoter sites close to its transcription start site (White et. al., 2016). In the genetic circuit *OTX2* and *CRX* play similar roles, even though both are needed for full photoreceptor development (Furukawa et al., 1999; Nishida et al., 2003). *OTX2* partially compensates for the loss of *CRX* (Samuel et. al., 2014) and it takes the loss of both *CRX* and *OTX2* in early postnatal retina to observe a dramatic loss in *Nrl* activity (Montana et al., 2011). In contrast, at later developmental stages and in the adult, *CRX* is able to compensate for *OTX2* loss to activate of *Nrl* transcription (Figure 6) (Montana et al., 2011). This suggests that while *OTX2* is

important in the retina to maintain photoreceptor adult phenotype, it can be substituted by CRX, after it has initiated *Crx* expression.

E. Role of OTX2 in Visual Phototransduction

In mice retina, there are seven million rods to fifty thousand RGCs (Jeon et. al., 1998). This makes a ratio of one RGCs to one hundred and forty rod cells (Jeon et. al., 1998). This implies that visual information generated by rod cells has to be passed to a lesser number of RGC, without a loss of information. Cells such as bipolar cells, horizontal cells, and amacrine cells modulate and pass along the information to the RGC (Hampson et. al. 1992; Weiler et. al., 1988).

Photons are captured by light sensitive G-protein coupled receptors (GPCRs) opsins. Rods predominantly have one type of opsin called rhodopsin. Cone cells in contrast have various opsins that respond to different wavelengths of light (Ebrey & Koutalos al., 2001). Within each opsin a 11-cis retinal molecule is covalently linked to form retinylidene protein (Kono et. al., 2008). When a photon is absorbed the 11-cis retinal undergoes photoisomerization into all-trans retinal, which changes the conformation of the opsin GPCR leading to signal transduction cascade in the outer segments of photoreceptors (Arshavsky et. al., 2002). This signal cascade causes cyclic GMP-gates cation channels to open and hyperpolarization of the photoreceptor (Molday & Molday, 1998).

One of the essential compounds for photosynthesis is vitamin A. Vitamin A is imported in the RPE cells as all-trans retinol. This all-trans retinol is converted into all-trans retinyl ester by an enzyme called Lecithin retinol acyltransferase (LRAT) (Herr et. al., 1992). The newly formed retinyl ester is stored in retinosomes in the RPE cells. The RPE cells convert all-trans retinyl ester into 11-cis retinol by the enzyme RPE65 (Masuda et al., 2014). This 11-cis retinol originating from the RPE cells, binds to Interphotoreceptor retinoid-binding protein (IRBP) and is transferred into the outer segments of the photoreceptors (Cideciyan et al., 2000; Fong et. al., 1999). IRBP are glycoproteins, which are found in the interphotoreceptor matrix (IPM) between the ONL and the RPE (Figure 6) (Parker & Crouch, 2009).

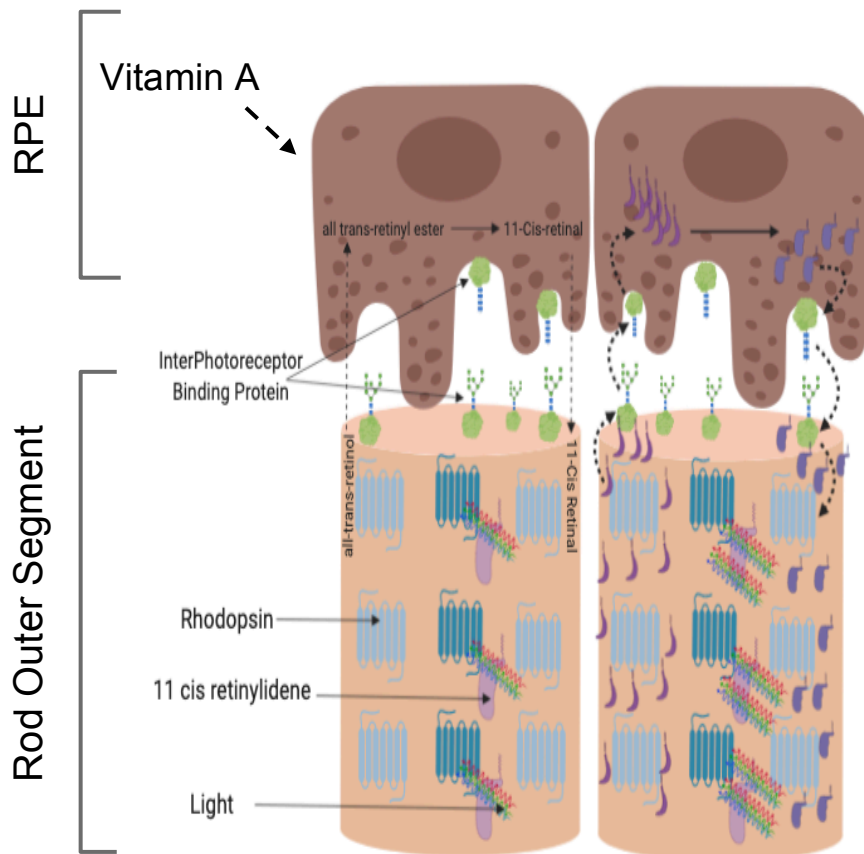


Fig 6. Phototransduction in the outer segments of photoreceptors

Phototransduction starts with entering of vitamin A to the RPE cell layer. Vitamin A is converted into 11-cis retinal and transported to the outer segment of the PRs. There it binds to opsins (GPCRs) and forms 11-cis retinylidene. When light hits 11-cis retinylidene, all-trans retinol is formed and a free electron is generated, which initiates the electron transport chain. Then all-trans retinol is converted into all-transretinyl ester in the RPE cell layer.

The 11-cis retinal is imported into the outer segments of the photoreceptors it covalently binds to opsin forming 11-cis retinylidene that can be photoactivated by a photon. The photon breaks the covalent bond between the opsin and its 11-cis retinylidene to produce all-trans retinylidene (Chen and et al., 2017). The all-trans retinylidene undergoes a hydration reaction to form all-trans retinal; itself converted to all-trans retinol by all-trans retinol dehydrogenase (atRDH) (Chen and et al., 2017). This all-trans retinol binds to IRBP proteins and is transferred into the RPE cells where it is converted into 11-cis retinol by LRAT and RPE65 enzymes and will be used again in the outer segments of the photoreceptor during phototransduction.

In the phototransduction pathway, OTX2 is essential to maintain homeostasis and healthy functioning of the RPE and photoreceptors. OTX2 regulates the

expression of genes central to this biochemical process. Constant *Otx2* expression is needed in the RPE and the photoreceptor cell layers to bind promoter regions of *Lrat*, *RPE65*, *Irbp*, and *atRDH* and allow for their constitutive expression (Figure 8) (Masuda et al., 2014; Cideciyan et al., 2000; Bobola et al., 1999; Chen et al., 2017). If any of these proteins is not expressed phototransduction cannot occur because 11-cis retinal that would bind to opsin is not produced. And all-trans retinol from PRs would not be removed, which would have toxic effects on the cells. Insufficient OTX2 concentration in PRs results in reduced IRBP expression in the interphotoreceptor matrix, which in turn leads to the accumulation of lipofuscin precursor in the outer segments of the photoreceptors (Chen and et al., 2017). Under stressful conditions in the retina, the accumulated lipofuscin precursor undergoes an oxidative reaction to produce toxic lipid vesicles, which can cause PR death and blindness.

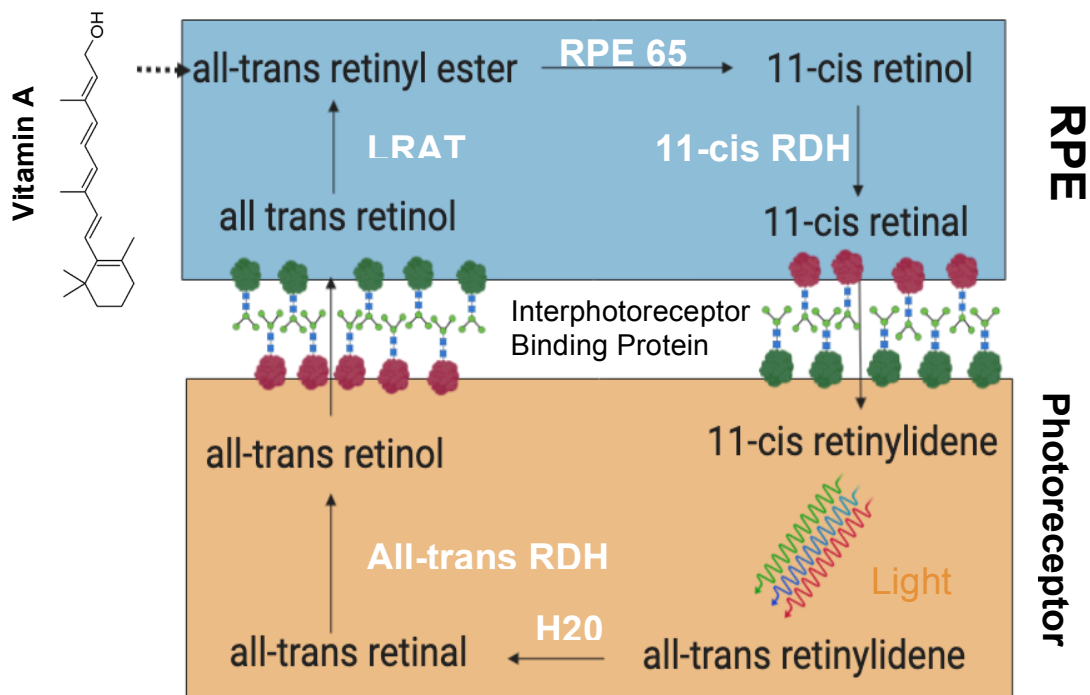


Fig 7. Phototransduction genes regulated by OTX2.

Genes (in white) involved in phototransduction pathway are regulated by OTX2. Vitamin A enters RPE cells and is converted into 11-cis retinol by OTX2 regulated RPE65. 11-cis retinol and all-trans retinol transporter genes are also regulated by OTX2.

F. OTX2 Survival of Adult Retinal Ganglion Cells

The axons of RGCs form the optic nerve, which is the sole link between the retina and the higher visual structure of the brain. A loss of adult RGCs is linked to diseases such as optic neuropathies and glaucoma that can lead to blindness (Osborne et al., 1999; Levin, 2007). *Otx2* is expressed in photoreceptor and bipolar cells, but not in the RGCs (Rath et al., 2007; Sugiyama et al., 2008). When exogenous OTX2 is injected in the eye it is taken up by the RGCs and trans-synaptically transferred to parvalbumin cells in the visual cortex (Sugiyama et al., 2008). In addition, it has been demonstrated *in-vitro* and *in vivo* that OTX2 protects RGCs from tissue disrupted and NMDA-induced death (Torero Ibad et. al., 2011). This neuroprotective effect of OTX2 on RGC is a non-cell autonomous effect (Torero Ibad et. al., 2011).

H. OTX2 Role in RPE Survival

Previous studies have shown that conditional knockout of *Otx2* in the RPE cell causes photoreceptor cells to degenerate. In the adult retina, OTX2 is produced in three retinal layers including RPE, ONL, and the INL (Figure 3). It has been shown that when *Otx2* is conditionally removed from adult RPE it causes degeneration of photoreceptors (Figure 8) (Housset et al., 2013). This suggests that OTX2 ensures long-term survivability of photoreceptors (Béby et al., 2010).

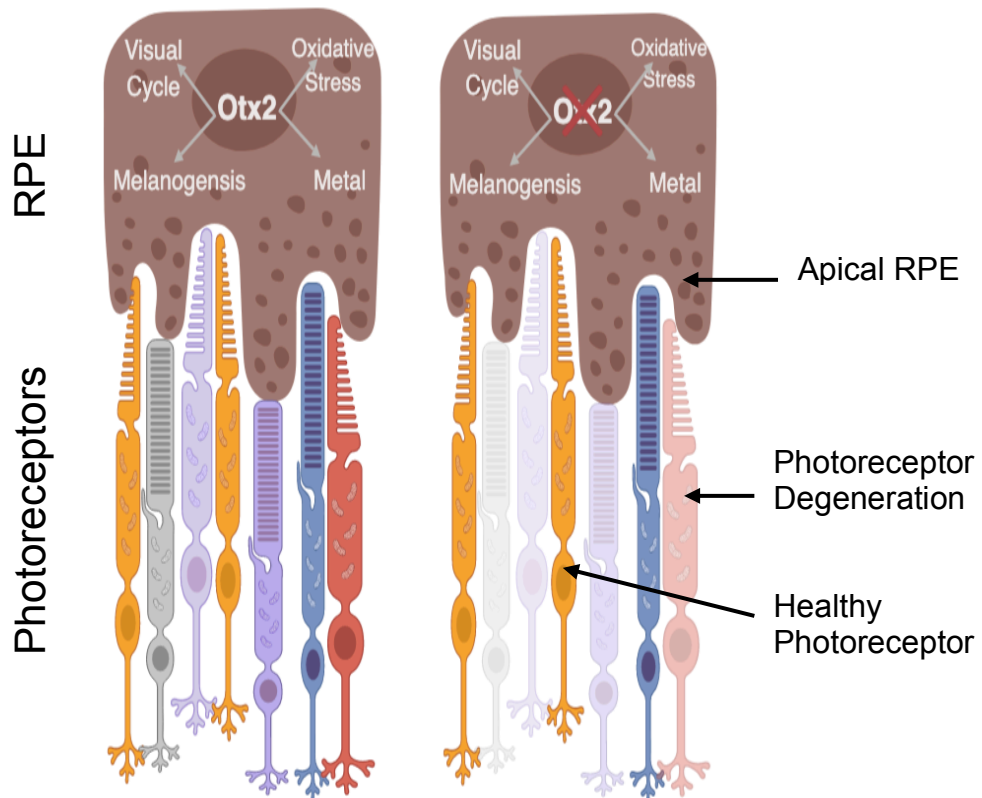


Fig 8. Potential role of Otx2 in healthy retina vs Conditional Knockout Otx2 in RPE.

When Otx2 is conditionally knocked out in the RPE layer it causes degeneration of photoreceptors. This could be because Otx2 deletion causes increased oxidative stress, melanogenesis, and effect the visual cycle. Adapted from Housset et. al., 2013.

Keeping in view the critical role of RPE in maintaining homeostasis of the adult photoreceptors we hypothesize that a homeoprotein, OTX2 could be secreted from the RPE and has a neuroprotective agent on adult photoreceptors.

OTX2 Activity

A. Cell Autonomous Activity

OTX2 functions both cell autonomously and non-cell autonomously in the retina. Studies have shown that OTX2 plays a role in terminally differentiating photoreceptors (mentioned above) and bipolar cells (Nishida et. al., 2003). Without OTX2 bipolar cells do not develop (Figure 4). In OTX2 conditional knocked out expression of protein kinase C, a marker of bipolar cell is down regulated (Koike et.

al., 2007). Electrograms revealed impaired electrophysiological effect on retinal bipolar cells due to reduced levels of *Otx2* (Koike et. al., 2007; Bernard et. al., 2014).

To study the cell autonomous effects of OTX2, our laboratory has developed *Otx2*^{+GFP}. In the *Otx2*^{+GFP} mice, one of the *Otx2* alleles is substituted with a GFP. This means that these mice have half or less dose of OTX2. Similarly another mouse line *Otx2*^{+AA} one allele of *Otx2* has AA substitution in it and this reduces the internalization specificity of OTX2. These mice are also viable, reproduce, and have normal head development (Bernard et al., 2014). Their retina develops normally and without anatomical defects. These two models of mice indicate that at a gross level, the severity of anatomical phenotype increases with decreasing OTX2 activity.

These two mice models have distinct differences between their photoreceptor activity and thickness of the ONL. Electroretinography on *Otx2*^{+AA} and *Otx2*^{+GFP} mice, in comparison to wild-type showed that photoreceptor cells of mutant were less active in comparison to wild-type cells at P30 and P60. In addition, the thickness of the neural retinal layers show demonstrable thinning. For example, a comparison of ONL of the *Otx2*^{+GFP} mice and wild type mice demonstrated that the neural retina of the transgenic mice was thinner. It was hypothesized that this could be due to lower OTX2 activity in the retina (Bernard et al., 2014).

B. Non-Cell Autonomous OTX2 Activity

Non-cell autonomous effects of OTX2 depends on its internalization by non OTX2 producing cells. Since the regions necessary for OTX2 internalization is in the DNA-binding homeodomain, altering this region will also affect OTX2's cell autonomous activity. We have developed a genetic approach for interfering with OTX2 non-cell autonomous activity by sequestering extra cellular OTX2 in the neural tissue (Bernard et. al., 2016). We engineered mice to express a secreted single chain antibody against OTX2. Constructs contain the heavy and light variable chains and despite the removal of the constant regions of the antibody, the single chain retains the antigen-binding capacity and specificity of the original antibody (Ahmad et al., 2012). Previously this method has been used to study other homeoproteins such as PAX6 in zebrafish and Engrailed in chick (Lesaffre et al., 2007; Wizenmann et al., 2009). In our study, scFv against *Otx2* is knocked into the *ROSA26* locus of C57BL6 in *Otx2scFv* mice. *Otx2scFv* expression is induced by Cre recombinase. When Cre is

expressed using a virus or Cre-TAT (cell internalization form of Cre) it recombines at LoxP sites, removing the stop cassette allowing the expression and secretion of the single chain antibody against OTX2 (Figure 9). *Otx2scFv* expression is detected by six MYC tag. The *Otx2scFv* plasmid construct has six Myc tags followed by a P2A skipping peptide. During translation, the skipping peptide between *Otx2scFv* and *GFP* undergoes a cleavage separating proline and glycine at C-terminal of the 2A peptide. The *Otx2scFv* peptide is separated from Green Fluorescent Protein (GFP).

These mice are referred to as heterozygous *Otx2scFv*^{tg/O}. To study non cell autonomous effects of OTX2 we crossed *Otx2scFv* with *PV-Cre* mice. Hence *Otx2scFv* occurs only in cells expressing PV.

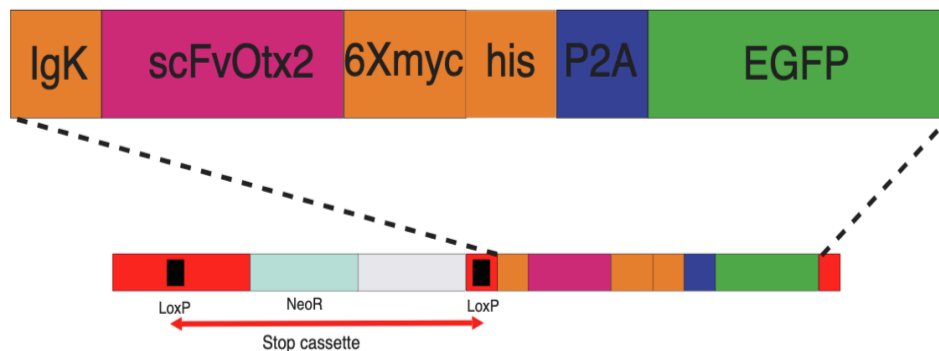


Fig 9. Gene construct of *Otx2scFv* mice.

When Cre is expressed, the stop cassette is removed, allowing the single chain antibody against *Otx2* and green fluorescent protein (as a reporter gene) to be produced. The scFvOtx2 bind to an extracellular *Otx2*. Based on Bernard et al., 2016.

To study the non-cell autonomous effects of *Otx2* and mutation in the RK doublet of *Otx2*, the *Otx2*^{+/AA} and *Otx2*^{AA/AA} mice were produced. Since *Otx2* deletion is embryonically lethal, it is impossible to develop knockout mice for *Otx2*. However, another approach to reduce transferred activity was to produce *Otx2*^{+/AA} mouse. In these mice one allele of *Otx2* has been mutated to prevent its internalization, but the mice are viable, fertile, and present no significant abnormalities (Martinez-Morales et al. 2001). However, depending on the genetic background of these mice some retinal abnormalities are present in these mice (Bernard et al., 2014). In *Otx2*^{+/AA} mice the RK doublet of the *Otx2* gene is mutated to be replaced with two alanine (A) residue. As a result the mice have reduced specificity of capture by PV-cells in the visual

cortex. It is important to note that the *Otx2* can still be transferred between cells but their transfer specificity is impaired (Beurdeley et al., 2012).

Previous studies on the brain has demonstrated the importance of non cell autonomous OTX2 activity. Non-cell autonomous transfer of OTX2 in juvenile mouse cerebral cortex was linked critical period and maturation timing. It has been shown that non-cell autonomous OTX2 controls the maturation of GABAergic fast-spiking parvalbumin interneurons and OTX2 is required for non-plastic state (Sugiyama et al. 2008; Beurdeley et al. 2012; Spatazza et al. 2013; Bernard et al. 2016). In the cortex choroid plexus release OTX2, which travels to and is internalized by parvalbumin cells. This results in maturation of the perineural nets (PNN). The interlocking role of PNN and OTX2 will be discussed in further details later on.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are composed of a repeating disaccharide unit (Pomin & Mulloy, 2018). GAGs are sulfated, polydisperse polysaccharides. Based on the difference of the repeated disaccharide unit they are classified in four main categories. These include heparin/heparan (HSPG), chondroitin sulfate/dermatan sulfate (CSGAG), keratan sulfate (KS), and hyaluronan (Pomin & Mulloy, 2018). In vertebrates, they act as joint lubricant, skeleton shock absorber, and are a major component of tissue extracellular matrix (Laurent et. al., 1996; Katta et. al., 2009).

Glycosamines such as heparin/heparan sulfate and chondroitin/dermatan sulfate are synthesised in the Golgi apparatus. Here the protein is posttranslationally modified by O-linked glycosylation (Pomin & Mulloy, 2018). Similarly the keratan sulfate are modified via N-linked glycosylation of the proteoglycan (Pomin & Mulloy, 2018; Hardingham & Fosang, 1992). However, the fourth form of GAG, hyaluronic acid or hyaluronan is not synthesised in the Golgi apparatus. Hence it is secreted immediately to the plasma membrane (Rilla et. al., 2005). Enzymes such as chondroitinase ABC (ChABC) are able to cleave GAGs.

A. Interaction of Glycosaminoglycans (GAGs) with Transcription Factors

Glycosaminoglycans can have tremendous variety and diversification. Disruption of GAG synthesis genes can cause physical defects and abnormalities such as macular corneal dystrophy (Pacella et. al., 2015). Modification in the position of primary sulfation can have a huge effect on the molecular and structural diversity of GAGs. For example, an octasaccharide can have structural combinations of over one million different sulfation patterns (Raman et. al., 2010; Sasisekharan & Venkataraman, 2000). This structural diversity is believed to play a role in promoting and interaction of bioactive molecules such as transcription factors, especially homeoproteins. This could suggest OTX2 could bind directly to GAGs or embedded proteins in the GAGs before internalization. And during the course of this thesis we investigated OTX2 binding to GAGs in the retina.

Two conserved regions on the GAG thought to play a role in potential binding sites for GAG-protein interactions. These sites are XBBXB and XBBBXXB, where B is a basic residue of lysine or arginine and histidine, while X are hydrophilic residues (Cardin & Weintraub, 1989). Arginine residue binds tightly to GAGs than lysine residues. It has been suggested that the spacing of these residues determines protein-GAG interaction and binding specificity and protein folding may bring distal amino acids into play (Gandhi & Mancera, 2008). Under physiological conditions sulfate groups and carboxylic acid are deprotonated. This gives the GAG proteins a negative charge. These negatively charged groups form ionic clusters with positively charged basic amino acid on proteins. These ionic charges form a part of the GAG-protein interaction because other interactions such as van der Waal, hydrogen bonds, and hydrophobic interactions are also involved in forming GAG-protein interactions (Capila & Linhardt, 2002).

B. OTX2 interaction with GAG in Visual Cortex

Studies from our laboratory have demonstrated that OTX2 is captured by various neurons, including RGCs, DA neurons (DA, 3,4-dihydroxyphenethylamine), and PV-cells (Parvalbumin) (Torero Ibad et. al., 2011; Alvarez-Fischer et. al., 2011; Beurdeley et. al., 2012). OTX2 binds to cell surface binding sites of parvalbumin cell (PV-cells). During critical period of plasticity in the developing mouse visual cortex, PV-cells mature and are gradually wrapped by GAGs in a structure called PNNs. And

there is a strong molecular association between endogenous OTX2 and PNNs in the adult visual cortex (Beurdeley et. al., 2012). PNNs are extracellular matrix structures responsible for synaptic stabilization and can be found around neuron cell bodies and proximal neurites of the central nervous system (Flores & Mendez, 2014). PNNs play an important role in closure of the childhood critical period (Hensch 2005). They are negatively charged and composed of chondroitin sulfate proteoglycans (Balmer, 2016). In the cortex they are associated with inhibitory interneurons and maintain the excitatory/inhibitory balance (Hensch 2005).

Treating brain with ChABC hydrolysis disrupts the PNNs and reopens plasticity in the adult cortex and results in decreased concentration of endogenous OTX2 from cortex in PV-cells (Pizzorusso et al., 2002; Beurdeley et. al., 2012). These observations are consistent with the GAG-protein complex of PNNs participating in specific recognition of OTX2 prior to its internalization by PV-cells. OTX2 contains a short sequence at the beginning of the homeodomain that is responsible for GAG binding domain. The RK motif is sufficient and necessary for OTX2 to bind to PNNs (Beurdeley et. al., 2012). In the visual cortex WT OTX2 coupled to FTIC shows a high affinity for PNN (Beurdeley et. al., 2012). In addition, OTX2 containing the RK showed strong binding to disulfated chondroitin sulfates CS-D and CS-E (Beurdeley et. al., 2012). However, an AA mutation in the RK doublet motif of OTX2 is enough to prevent OTX2 internalization into PV-cells (Bernard et al., 2014). Hence, blocking endogenous OTX2 and its transfer into cells of the primary visual cortex. OTX2-AA has reduced capture by PV-cells. This means that OTX2-AA delays the critical period for brain when PNN of the PV-cells capture OTX2-AA. OTX2-AA localization to PV cells, reduces PV, and PNN expression, and reopens plasticity in adult mice (Beurdeley et. al., 2012). The source of the OTX2 can be from various parts of the brain, as OTX2 is present in choroid-plexus, dorsal lateral geniculate, and superior colliculus (Spatazza et. al., 2013). OTX2 from the retina can also reach the visual cortex (Sugiyama et. al., 2008). This makes OTX2 via GAG binding on PV cells a key regulator of PV-cell maturation and cerebral cortex plasticity during development and in adulthood (Spatazza et al., 2013a).

Mitochondria

A. Role of Mitochondria

Mitochondria are double membraned organelles found in all eukaryotic cells. Their primary role is to produce energy in the form of adenosine triphosphate (ATP). Mitochondria perform glycolysis followed by the Krebs cycle converting glucose into pyruvate and eventually into NADH and ATP. Mitochondria are essential for the survival of all cells and they are a number of neurodegenerative diseases such as Alzheimer, Parkinson, and Amyotrophic Lateral Sclerosis (ALS) associated with mitochondrial dysfunction. Unregulated production of reactive oxygen species (ROS) by the mitochondria can lead to excessive DNA, protein denaturation, and cross reacting with lipids in the brain (Verbon et. al., 2012; Son et. al., 2013;). Multiple studies have pointed out the role of superoxide anion and hydrogen peroxide in mediating neurodegeneration in Alzheimer disease (Drake et al., 2003; Butterfield & Lauderback, 2002).

B. ROS and Retinal Degeneration

Mitochondrial dysfunction is also described in retinal degenerative diseases such as AMD and retinitis pigmentosa. It is thought that ROS produced by the mitochondria and UV light in the photoreceptors cells is harmful to the host cell as well as to the mitochondria itself (Organisciak et. al., 2010; Tao et. al., 2019; Indo et. al., 2015). The natural mechanism to remove the ROS from the photoreceptors is for the OS to be shed and engulfed by the RPE cells. If this ROS is not removed from the PRs, it causes DNA damage, lipid accumulation resulting in drusen formation leading to retinal degeneration. For example, in AMD cumulative oxidative stress leads to damage to DNA, proteins, and lipids of the mitochondrial DNA. Moreover, deletions due to ROS causes damage to mitochondrial DNA and reduces DNA repair, leads to AMD (Barot et al., 2011; Ersoy et. al., 2014).

Mitochondria produce reactive oxygen species (ROS). Generation of ROS occurs during oxidative phosphorylation to synthesis ATP, in the inner mitochondrial membrane where electron transport chain takes place (Song et. al., 2018; Baccolo et. al., 2018; Pelley 2018). A partial leakage of electrons in the complex I and complex III of the electron transport chains can lead to partial reduction of oxygen to produce superoxide (Turrens, 2003; Murphy, 2009).

In the retina production and accumulation of ROS causes mtDNA damage. This process occurs in both normal and accelerated aging as well as with AMD. A mutation occurring in the *NADH dehydrogenase* was characterized by an increase in ROS production and interferes with visual cycle (Udar et. al., 2009; Kaamiranta et. al., 2019). In AMD patients a point mutation (mtA11812A>G) in NADH ubiquinone oxidoreductase gene is found to increase ROS stress. Patients with this mutation exhibit reduced protection against ROS (Sangiovanni et al., 2009). More recently mtDNA from AMD patient's retina was compared to retinal mtDNA from healthy patients. It was shown that in healthy people, aging was associated with mtDNA damage, but patients suffering from AMD had elevated levels of mtDNA damage (Karundharma et. al., 2010). Moreover, ROS damage to two nuclear genes was identified important element of AMD pathogenesis.

Many experiments and studies have demonstrated that ROS can also directly or indirectly cause damage to RPE (Masuda et. al., 2017; Rohowetz et. al., 2018). Hence, excessive amounts and improper removal of ROS in the neural retina can cause damage to the RPE cells leading to cause AMD (Zarbin et. al., 2004; Mao et. al., 2014; Lambros et. al., 2015). Previous studies show that inhibition of oxidative phosphorylation in postnatal mice resulted in mitochondrial degeneration in the RPE (Zhao et. al., 2011). In addition electron microscope imaging of RPE mitochondria has shown a decreased number or total loss of cristae and matrix density in aging and AMD patients in comparison to healthy subjects (Feher et. al., 2006).

In-vitro studies on RPE cells derived from AMD donors have revealed the effects of hydrogen peroxide (Kaarniranta et. al., 2019; Tao et. al., 2019). It has been shown that after forty-eight hours of incubation with hydrogen peroxide, RPE cells from AMD patients were more susceptible to oxidative stress induced cell death (Kaarniranta et. al., 2019). Moreover, mitochondria of RPE from AMD patients had reduced levels of ATP synthases and the cells were substituting ATP production by glycolysis (Golestaneh et. al., 2017). Taken together these results support the idea that mitochondria of RPE from AMD patients are dysfunctional and the RPE are in an energy crisis due to ROS damaged mitochondria leading to AMD pathogenesis.

In addition to ROS produced by the mitochondria, reactive oxidative species can also be produced by blue light entering the retina (Tao et. al., 2019). And these superoxides also contribute to mitochondrial damage. Mitochondria isolated from primary human RPE cells were exposed to blue light, which resulted in production of hydroxyl radicals, superoxides, and singlet oxygen (Godley et. al., 2005). The

excessive production of blue light induced ROS results in increased mtDNA damage in RPE cells. This suggests that blue light induced ROS is a risk factor for AMD by inducing mtDNA damage (Kaarniranta et. al., 2019).

Homeoproteins stimulate mitochondrial activity of ATP synthesis and release at least partially response for DA neuroprotection. Homeoprotein also protect against oxidative stress. OTX2 reduces DNA damage, so it may have neuroprotective effects in retinal cells such as PRs and/or RPE by mitigating mitochondrial dysfunction or reducing oxidative stress.

Conclusion

The objective of this thesis is to examine the cell autonomous and non-cell autonomous activity of OTX2 in the retina. OTX2 is a homeoprotein transcription factor expressed in the retina that can transfer between cells and that can act cell autonomously and non-cell autonomously. We used a genetic approach to block OTX2 transfer in the retina and found a degradation of visual function with no alteration in retinal structure or organization. Furthermore, the loss of visual acuity was associated with a change in inner retinal activity. These results provide evidence for OTX2 non-cell autonomous activity on retinal function.

In the second part we hypothesised that OTX2 from the RPE layer is transferred to the interphotoreceptor matrix (IPM) containing the outer segments of photoreceptors, and this transferred OTX2 plays a critical role in maintaining homeostasis of the photoreceptors or neuroprotection. We provide evidence that OTX2 is present in the interphotoreceptor matrix, but we were unable to determine if this OTX2 originated from RPE cells. We do show that endogenous OTX2 bind to interphotoreceptor matrix ex vivo and that this binding occurs with GAGs. Finally, preliminary results show that exogenous OTX2 reduces levels of harmful ROS in retina ex-vivo.

Résumé en Français

L'objectif de cette thèse est d'examiner l'activité cellulaire autonome et non cellulaire autonome d'OTX2 dans la rétine. OTX2 est un facteur de transcription de la classe des homéoprotéines. OTX2 est exprimé dans la rétine et peut transférer entre les cellules pouvant agir de manière autonome ou non. Nous avons utilisé une approche génétique pour bloquer le transfert d'OTX2 entre les cellules dans la rétine et avons constaté une dégradation de la fonction visuelle, sans altération de la structure ou de l'organisation de la rétine. De plus, la perte d'acuité visuelle a été associée à un changement de l'activité rétinienne interne. Ces résultats fournissent la preuve d'une activité d'OTX2 non cellulaire autonome sur la fonction rétinienne.

Dans la deuxième partie, nous avons émis l'hypothèse qu'OTX2 produit dans la couche EPR est transféré à la matrice interphotoréceptrice (MIP) contenant les segments externes des photorécepteurs et que, cet OTX2 transféré, joue un rôle essentiel dans le maintien de l'homéostasie des photorécepteurs ou de la neuroprotection. Les résultats de cette thèse fournissent des preuves qu'OTX2 est présent dans la MIP, mais nous n'avons pas pu déterminer si cet OTX2 provenait des cellules de l'EPR. Nous montrons qu'OTX2 endogène se lie à la MIP *ex vivo* et que cette liaison se produit grâce aux complexes carbohydrates extracellulaires. Enfin, les résultats préliminaires montrent qu'OTX2 exogène réduit les niveaux d'ERO nocifs dans la rétine *ex vivo*.

Chapter 2. Material and Methods

A. Viral Injection.

A. AAV5 hBEST-iCRE Virus Production.

An AAV5 containing hBEST1 promoter driving expression of enhanced *Cre* was produced by cloning hBEST promoter into AAV vector ITR genome plasmid. The viral cloning, production, and purification was done by Vector Biolabs. Vector Biolabs also performed sequence verification. The hBEST1 promoter was a kind gift from Dr. Donald J. Zack at University of John Hopkins Hospital.

All animal procedures are carried out in accordance to the guidelines set by the Ministry of Research and Ministry of Agriculture of France and institutional ethics committee.

a. Retro-orbital Injection.

C57BL/6J mice (purchased from Janvier France) at ages between P30-50 were anaesthetized with a mix of xylazine/ketamine (Imalgène 500 Virbac France, 80 mg/kg, Rompun 2% Bayer, 5 mg/kg) until pain reflexes were absent. Mice were placed on their side to provide access to the retro-orbital sinus. A 20G needle fixed to a 1ml syringe was inserted into the retro-orbital sinus and the virus was injected a final titer above 10^{10} virus particles in a volume of 100-200ul. The mice maintained alive in the A2 biosafety animal facility. After three weeks for viral expression, the mice were killed by cervical dislocation.

b. Subretinal Injection.

C57BL/6J mice were anaesthetized with a mix of xylazine/ketamine (Imalgène 500 Virbac France, 80 mg/kg, Rompun 2% Bayer, 5 mg/kg) until pain reflexes were absent. The eyes were dilated with atropine. Hydrogel was placed on eye and a small diameter coverslip was placed on top. Using a 20-22G needle a hole in the sclera was made 1 mm away from the limbus. Using a 10 μ l syringe (Hamilton), 2 μ l of virus was injected in the subretinal space of the eye. The injection was visually confirmed by the local detachment of the retina. Mice were maintained in the A2 animal safety facility for a minimum of three weeks and then killed by cervical dislocation and the eyes removed for analysis.

B. Plasmid and Proteins

A. Production of Alkaline Phosphatase Fusion Proteins.

For alkaline phosphatase (AP)-tagged fusion constructs, cDNA encoding for mouse *Otx2* was subcloned between the *Xho*I and *Xba*I sites of the pAPtag-5 vector (GenHunter). AP-tagged truncations were obtained by PCR, where AP-NtHD corresponds to OTX2 (1–98) and AP-HD (AP fusion with the OTX2 homeodomain alone) corresponds to OTX2 (38 –98). For production of AP-tagged fusion proteins, HeLa cells were plated on 6 well plastic plates in DMEM containing 10% fetal bovine serum (FBS) (Invitrogen). Cells were transfected using lipofectamine (Invitrogen) (Beurdeley et. al., 2012). Cells were incubated at 37°C, 5% CO₂ with 1:1 ratio of plasmid DNA and lipofectamine for five hours. After five hours the medium was changed to DMEM with 10% FBS. After three days, proteins in the cultured medium was collected and their concentration measured using western blot.

B. Otx2 Production Purification and Quantification.

Recombinant OTX2 protein was produced as described (Torero Ibad et al., 2011). Briefly, wild-type mouse OTX2 with a myc tag and a 6His tag in C-terminal position was cloned into pTrac plasmid (Invitrogen). Recombinant OTX2 protein was produced in bacteria (BL21 CodonPlus RP Stratagene) at 37°C overnight in auto-induced medium (MagicMedia, Invitrogen). Centrifugation at maximum speed for fifteen minutes was used to pellet the bacterial cells. The bacterial supernatant was discarded and after washing the bacterial mass with PBS, the mass was re-suspended in 20 mM phosphate, 0.5 M NaCl, and 5 mM imidazole and protein was extracted by lysis in 10 mM Tris, 100 mM Na₂HPO₄, 20 mM imidazole, 6 M guanidine HCl, pH 8. The lysate was centrifuged for 20 min at 20,000g and the supernatant passed through an affinity chromatography column charged with 100 mM NiSO₄ (HiTrap Chelating HP column, GE Healthcare). The recombinant protein was then eluted with 6 M guanidine HCl, 0.2 M acetic acid and immediately dialyzed against 20mM phosphate, 0.5 M NaCl. The protein solution was completed to 10% glycerol and frozen. OTX2 protein was solubilized in gel solubilization buffer (80 mM Tris, pH 6.8, 4 mM DTT, 1% glycerol, 1.2% SDS) and SDS-PAGE was carried out on a 12% gel. Serial dilutions of BSA were loaded in adjacent lanes. Proteins on the gel

were visualized with Bio-Safe Coomassie staining solution (Bio-Rad) and concentration was estimated by comparison with BSA (Torero Ibad et al., 2011).

D. Ex-vivo OTX2 Binding on Frozen Sections.

Cryosections (14 μm) from fresh frozen adult retina were mounted on glass slides and stored at $-80\text{ }^{\circ}\text{C}$. The sections were equilibrated at $-20\text{ }^{\circ}\text{C}$ for 20 minutes, then rinsed with 1X PBS buffer to wash away the Optimal Cutting Temperature Compound (OCT). The sections were immersed in methanol at $-20\text{ }^{\circ}\text{C}$ for 10 minutes and then washed three times with 1X PBS. Endogenous alkaline phosphatase was heat inactivated at $60\text{ }^{\circ}\text{C}$ for 25 minutes. The slides were washed and 10% FBS in 1XPBS was used as a blocking solution for minimum 1 hour at room temperature. Conditioned medium containing AP-tagged OTX2 protein and 1X protease inhibitor (Roche) was added and sections incubated at $4\text{ }^{\circ}\text{C}$ overnight. The slides were rinsed three times with 1X PBS, before AP development (NBT/BCIP kit; Vectorbio) for 2 hours to 3 hours until colored reaction product was apparent.

E. ChABC Treatment of Fresh Frozen Sections.

ChABC treatment was used to remove GAGs from fresh frozen cryosections of 14 μm thick sections of retina. Sections were washed with 1XPBS to remove OCT, followed by 10 minutes fixation with methanol at $-20\text{ }^{\circ}\text{C}$. The sections were incubated with ChABC (2-5U/ml Sigma) in 1X PBS and 1X protease inhibitor (Roche) at $37\text{ }^{\circ}\text{C}$ overnight. The next day the sections were heated to $60\text{ }^{\circ}\text{C}$ for 25 minutes to inactivate endogenous alkaline phosphatase followed by 10% FBS in PBS for 1 hour. Conditioned medium containing AP-tagged OTX2 protein (above) and 1X protease inhibitor (Roche) was added and sections incubated at $4\text{ }^{\circ}\text{C}$ overnight. The glass slides were rinsed three times with 1X PBS and the AP staining performed as above for 2 hours to 3 hours.

F. Immunohistochemistry.

Mice were killed by cervical dislocation, the eyes removed and placed in 1ml of 4% paraformaldehyde in 1X PBS in the dark for 2 hours at room temperature. After fixation the eyes were washed with 1X PBS three times and transferred to 20% sucrose solution at $4\text{ }^{\circ}\text{C}$ overnight or until the eyes sank to the bottom of the eppendorf tube. The eyes were embedded in OCT and frozen in isopentane at $-50\text{ }^{\circ}\text{C}$. Cryostat sections were cut at 14 μm thick and collected on glass slides.

Sections were washed with PBS for 5 minutes followed by permeabilization with 1% Triton-X in PBS for 30 minutes. The sections were rinsed with PBS three times and antigen retrieval was performed in 100mM glycine dissolved in PBS for 30 minutes at room temperature. After 30 minutes the sections were rinsed again with PBS three times. The sections were blocked with 10% normal goat serum and 1% tritonX-100 in PBS. Primary antibodies were diluted in fresh blocking solution and allowed to bind overnight at 4°C. The sections were washed with PBS and incubated with secondary antibodies (Thermo Fisher Scientific) for 1 hour. The sections were washed with PBS three times and mounted in DAPI containing mounting medium.

G. Laser Dissection of Photoreceptor Outer Segments.

Photoreceptor outer segments were dissected from fixed retina. The eyes were fixed in 4% PFA and 40-60um thick cryostat sections. Sections were mounted on steel framed slides with polyethylene naphthalate (PEN, Lecia). Sections were washed in PBS for 5 minutes and allowed to dry at room temperature. Using a Laser Microdissection System (Lecia LDM-7000) six to eight sections per retina containing the OS from the photoreceptors were recovered. The tissue was captured in a PCR tube. The tubes were centrifuged at maximum speed and stored at -20°C.

H. Western Blotting.

Proteins were extracted by the addition of 50 µl of RIPA buffer containing 1% Triton X-100, 140 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM Tris-Cl (pH 8), and 1 mM EDTA (RIPA buffer). The samples were incubated at 4°C for 1 hour and then DTT (Thermo Fisher Scientific) was added. The samples were centrifuged at 1000Xg for 5 minutes. The supernant were heated at 95°C for 10 minutes and proteins separated on NuPAGE 4%-12% Bis-Tris precast gels (Invitrogen) for 1 hour at 200 V and transferred onto a methanol-wetted PVDF membrane at 400 mA for 1 hour. The membrane was blocked in TBS, 0.2% Tween, 5% non-fat milk and then primary antibodies incubated in blocking buffer overnight at 4°C. The membrane was incubated with the corresponding HRP-coupled secondary antibodies for 1 hour at room temperature. Membranes were imaged using a LAS-4000 gel imager (Fujifilm).

Antibodies used are as following (Otx2 CD4 anti mouse 1/200), (Beta Actin anti mouse HRP 1/1000, Sigma), (Tubulin anti-mouse 1/500, Sigma), (GFP anti-Chicken 1/100, Abcam), (M2 anti Mouse, Sigma 1/1000), (Donkey anti mouse 548 1/800, Thermofisher Scientific), (Donkey anti Mouse HRP 1/1000, Thermofisher Scientific), (Goat anti chicken 1/800, Thermofisher Scientific).

I. RT-qPCR for *Otx2scFv* and Retinal Neuronal Markers.

RNA was extracted from fresh frozen retina of P30 mice with RNeasy lipid tissue mini kit (Qiagen). cDNA was generated with the Quantitect Reverse transcription kit (Qiagen). The RT-qPCR was performed on a LightCycler II 480 Instrument (Roche Life Science). Using the 2^{-DDCt} method, samples expression levels were normalized to *Hprt* and to PV-Cre mice levels. Primers for the following cell types were used: *Otx2scFv* (transgene expression), *Brn3a* (ganglion cells), *Chx10* (bipolar cells), *Syntaxin* (amacrine and horizontal cells), *Lim1* (horizontal cells), *Rhodopsin* (rods), *Red Opsin* (L/M cone cells).

Primer	Sequence
<i>Brn3a</i> (forward)	agg cct att ttg ccg tac aa
<i>Brn3a</i> (reverse)	cgt ctc aca ccc tcc tca gt
<i>Chx10</i> (forward)	aca cac agc cca tct tgg aag
<i>Chx10</i> (reverse)	tcg gtc act gga gga aac atc ttc ttc g
<i>Syntaxin</i> (forward)	ctc agt gag atc gag acc ag
<i>Syntaxin</i> (reverse)	gat cat gat ctt ctt cct gcg
<i>Lim1</i> (forward)	ttg gag ccg ggc gaa ctc at
<i>Lim1</i> (reverse)	aga cct cca agg ggc gtc cc
<i>Rhodopsin</i> (forward)	gga gca gca ccc gtt gga atg
<i>Rhodopsin</i> (reverse)	ccg taa gcc ggg aca ctt gtc a
<i>Red Opsin</i> (forward)	ggt ggt gat ggt ctt cgc ata c
<i>Red Opsin</i> (reverse)	ttg gag gtg ctg gaa agt tca g
<i>HPRT</i> (forward)	agc agg tgt tct agt cct gtg g
<i>HPRT</i> (reverse)	acg cag caa ctg aca ttt cta a

J. Electroretinogram.

Mice were anaesthetized with xylazine/ketamine (Imalgène 500 Virbac France, 100 mg/kg, Rompun 2% Bayer, 10 mg/kg). Tropicamide (Mydriaticum 0.5% Théa, France) was used to dilate the iris and the cornea was locally anaesthetized with oxybuprocaine hydrochloride. Gold electrodes were placed in contact with each eye, reference electrodes were placed subcutaneously in the submandibular area, and a ground electrode was placed subcutaneously on the back of the animal. ERG was performed with a mobile apparatus (SIEM Bio-Medicale, France) with LED lamps in a Ganzfeld chamber controlled by the VisioSystem software. ERG recordings were obtained from seven *PV-Cre* and nine *PV-Cre::Otx2-scFv* mice 30-31 days of age.

K. RNAscope.

In situ hybridization was performed on retinal sections using RNAscope assay. Eyes were removed from *PV-Cre* and *PV-Cre::Otx2-scFv* mice at P30. The eyes were fixed overnight at 4 °C in 4% paraformaldehyde. The next day the eyes were embedded in 20% sucrose at 4 °C overnight, followed by OCT. Sections of 40 µm thickness were mounted, allowed to dry, and stored at -20 °C. On the day of processing the sections were thawed to room temperature for 15 minutes before performing ISH according to manufacturer's protocol (Advanced Cell Diagnostics). In short, peroxidase was neutralized with hydrogen peroxide, followed by permeabilization with 1X TBST (0.1% Tween-20). ISH probes were hybridized for 2 h at 40 °C, signal was amplified in three steps and finally signal visualized either with Red or Far Red. We used probes *PV* (Mm-PV-C2 probe, 1:50 dilution) and *Myc* (Mm-MYC-C1 probe, 1:50 dilution).

L. Primary RPE Cultures.

Primary RPE cultures were established from P30 day old mice. They were isolated as described (Fernandez-Godino, et. al., 2016). Briefly, retinas were removed from the eye and warm 0.05% Trypsin-EDTA (Thermoscience) was added for 45 minutes at 37°C. Then the eye cups were shaken by hand to release the RPE cells in the trypsin. The trypsin solution was centrifuged at 340g for 10 minutes, trypsin discarded and the cells resuspended in Neurobasal medium containing

glutamine and ABAM (thermoscience). RPE cells plated on to coverslips and cultured at 37°C 5% CO₂.

M. Optomotor Test.

The optomotor test of visual acuity was used to screen for possible phenotype differences. P30 mice were used since it was thought that this age would allow sufficient time for the scFv to be expressed and secreted. The optomotor test was used as described in the literature and an optotype of 0.375 c/deg was used on the advice that this elicited robust responses (Torero Ibad et al., 2011; Bernard et al., 2014). Briefly, mice were placed on an elevated platform centered in a rotating drum. A square wave 100% contrast optotype of 0.375 c/deg was rotated at a speed of 2 rpm. Mice were filmed from above and scored in real time by two observers blind to genotype for head turns in the direction and speed of the moving grating. Any discrepancies between the two observers' real time score was resolved by analyzing the video. A Mann-Whitney U test was used to compare the genotypes due to the unequal variances.

Chapter 3. OTX2 Non-Cell Autonomous Activity Regulates Inner Retinal Function. (Article Submitted)

Raoul Torero-Ibad, Bilal Mazhar, Clémentine Vincent, Clémence Bernard, Julie Dégardin, Manuel Simonutti, Thomas Lamonerie, Ariel di Nardo, Alain Prochiantz and Kenneth L. Moya. BioRxiv: <https://doi.org/10.1101/2020.02.12.945584>. Submitted: eNeuro 13/02/2020.

Introduction

OTX2 is a homeoprotein transcription factor important for retinal development and maintenance. It is expressed early in the embryonic mouse optic vesicle and retinal pigmented epithelium (RPE) and is required for differentiation of photoreceptors by transactivation of CRX and NRL and for differentiation of bipolar cells (BPs) via regulation of PKCa (Martinez-Morales et al, 2001; Nishida et al, 2003; Koike, et al. 2007). In mice hypomorph for OTX2 we reported visual deficits, retinal physiological dysfunction and age- and OTX2-dependent degeneration of the retina (Bernard et al., 2014). In adult mice, knock down of OTX2 in RPE results in photoreceptor death demonstrating that continued expression of OTX2 in RPE is necessary for photoreceptor survival (Housset et al., 2013). Exogenous OTX2 protects adult RGCs against NMDA-induced excitotoxicity and preserves visual acuity (Torero-Ibad et al., 2011).

The capacity of homeoproteins (HPs) to transfer between cells allows different types of activities. HPs can act within the cells that produce them, thus in a cell autonomous fashion, but they can also exert their activity extracellularly or by transferring to cells that do or do not produce them, i.e., non-cell autonomously. Two separate sequences sufficient and necessary for homeoprotein cell exit and entry are in the DNA-binding homeodomain (for review, Di Nardo, 2018). A simple genetic approach cannot therefore be used to study their direct non-cell autonomous activity, as mutation of either sequence alters OTX2 DNA binding and thus also alters cell-autonomous activities. An alternative genetic approach was used to specifically target extracellular OTX2 in order to specifically study any direct non-cell autonomous activity. Mice expressing a neutralizing secreted anti-OTX2 single chain antibody (scFvOTX2^{tg/o} mice) were produced. The scFv sequence inserted into the ROSA locus is downstream from a stop cassette between two loxP sites. When Cre

recombinase is made available the stop cassette is excised allowing for the expression and secretion of the OTX2-scFv (Bernard 2016). In the retina parvalbumin (PV) is only expressed by RGCs and amacrine cells that do not express OTX2. Thus it is anticipated that in PV^{Cre}::scFvOTX2^{tg/o} mice, OTX2-scFv expressed and secreted from RGCs and amacrine cells sequesters extracellular OTX2 in the vicinity of the producing cells, thus blocking its non-cell autonomous activities. This strategy based on anti-HP scFv in vivo secretion has been used with success in several animal models to neutralize extracellular PAX6, ENGRAILED and OTX2 (Lesaffre et al., 2007; Wizenmann et al., 2009; Layalle et al., 2011; Bernard et al., 2016).

We show here that the sequestration of extracellular OTX2 by the OTX2-scFv secreted by RGCs and amacrine cells leads to a significant decrease in visual acuity. This decrease takes place in absence of any observable developmental defects, laminar abnormalities or changes in cell lineages. Electroretinogram (ERG) measurements show normal outer and inner nuclear function, but we measure a 2-fold increase in amplitude in the response to 20Hz flickers. Taken together, our results provide evidence for a direct non-cell autonomous activity of OTX2 homeoprotein for RGC function.

Results

In the retina, PV is expressed in RGCs and amacrine cells (Haverkamp and Wässle, 2000, Haverkamp et al., 2009). We were able to demonstrate expression of OTX2-scFv-Myc transgene in RGC layer (Fig 10). RT-qPCR shows significant expression of the OTX2-scFv transgene in the retina of PV::Cre;scFvOTX2^{tg/o} mice and no detectable expression in the retina of PV::Cre mice (raw ct PV::Cre = 38.2+1.7; PV::Cre;scFvOTX2^{tg/o} = 28.9+0.1) (Fig 11). Because the antibody is fused with GFP (Bernard et al., 2016), we immunoprecipitated the OTX2-scFv protein from PV^{Cre}::scFvOTX2^{tg/o} retina using an anti-GFP antibody followed by Western blot analysis. We detected a doublet at the expected molecular weight of the construct at about 78kD, that was not present in extracts from retina of PV^{Cre} control mice (arrow Fig 11B). This demonstrates the expression of the transgene and the presence of OTX2-scFv protein in the retina of PV^{Cre}::scFvOTX2^{tg/o} mice.

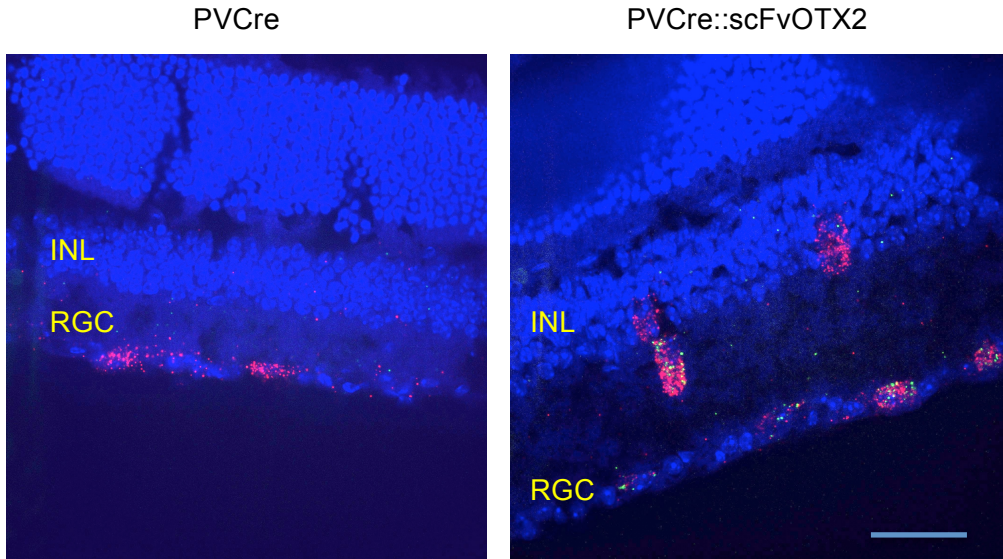


Fig 10. Co-localization of *scFvOtx2* in RGC cells and amacrine cells in retinal tissue.

In situ hybridization revealed co-localization of *Otx2scFv* (green) and parvalbumin (red) mRNA in retinal ganglion cells, amacrine cell, and INL of the *PVCre::scFvOTX2* mice. Scale bar 50 μ m.

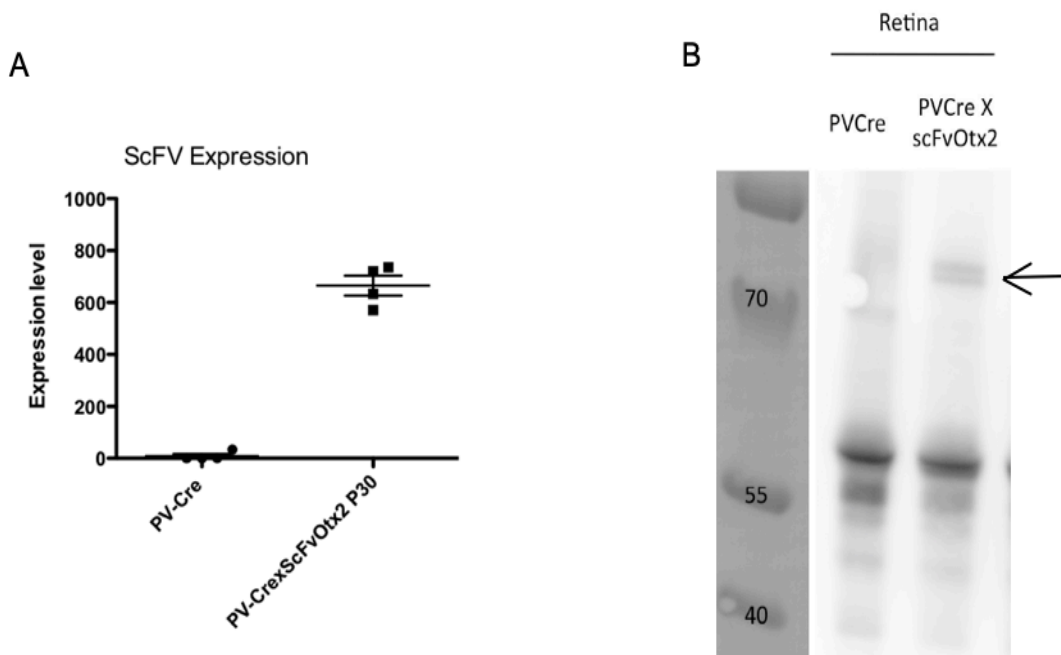


Fig 11. Expression *scFvOtx2* in mouse retinal tissue.

(A) RTqPCR for *OTX2scFv* mRNA was carried out on extracts from *PVCre* and *PVCre::scFvOTX2tg/o* mice and raw ct values normalized and converted to arbitrary units (a.u.). (B) Retinal lysates from *PVCre* and *PVCre::scFvOTX2tg/o* mice were immunoprecipitated for GFP followed by Western blotting with anti-MYC. Molecular weight markers are in the left lane in kDa.

One-month-old mice were tested for visual acuity by examining their optokinetic response to a 100% contrast optotype square wave grating of 0.375 c/deg spatial frequency. Bernard et al (2014) reported that this spatial frequency stimulates an optimal response and the number of head movements in response to this optotype is sensitive to changes in visual acuity due to loss of RGCs (Torero-Ibad et al., 2011). A significant reduction in visual performance in the $PV^{Cre}::scFvOTX2^{tg/o}$ mice was observed compared to PV^{Cre} littermates (Fig 12). As an additional control, we also evaluated $PV^{Cre}::scFvPAX6^{tg/o}$ mice. PAX6 homeoprotein is reported to also have non-cell autonomous activities (Di Lullo et al., 2011), but is not expressed in the mature retina and these mice had no visual acuity loss. These results show that interfering with extracellular OTX2 activity in the retina leads to decreased visual acuity.

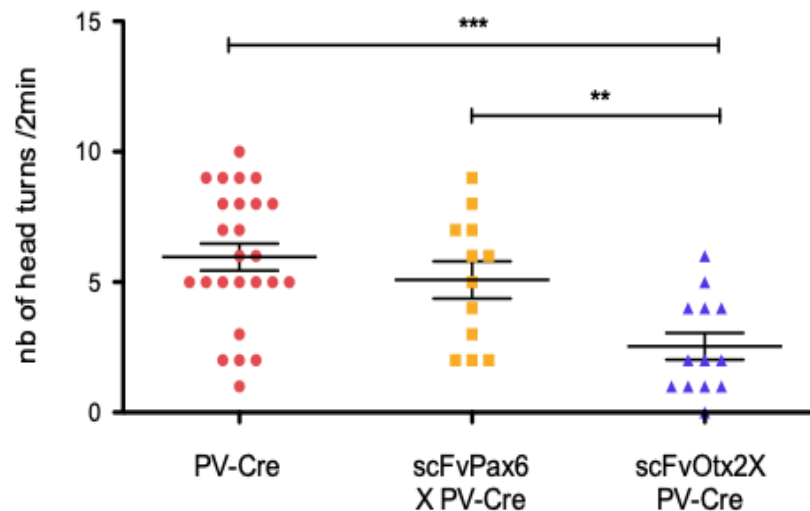


Fig 12. Visual Acuity Test for PV^{Cre} and $Otx2scFv$ mice.

Thirty-day-old mice were subjected to the optomotor test using an 100% contrast optotype of 0.375c/deg. PV^{Cre} and $PV^{Cre}::Pax6scFvtg/o$ mice made an average of about five head turns during the 2 min test period. $PV^{Cre}::Otx2scFvtg/o$ mice made significantly fewer head turns revealing reduced visual acuity. ** $p < 0.01$ or *** $p < 0.001$, Mann-Whitney U test, two tailed.

In search of a cellular correlate, we analyzed retinal structure by hematoxylin and eosin staining of sections from 1 month old mice. There was no difference in thickness of the outer nuclear layer (ONL, photoreceptors) nor the inner nuclear layer (INL; horizontal, bipolar, amacrine and Müller glial cells) between $PV^{Cre}::scFvOTX2^{tg/o}$ and PV^{Cre} littermates (Fig 13). Nor could we find a difference in the number of cells in the ganglion cell layer (GCL) containing RGCs and displaced amacrine cells. These results show no gross structural abnormalities in the retina of

PV^{Cre}::scFvOTX2^{tg/o} mice nor obvious differences in the number of cells in the ONL, INL or GCL.

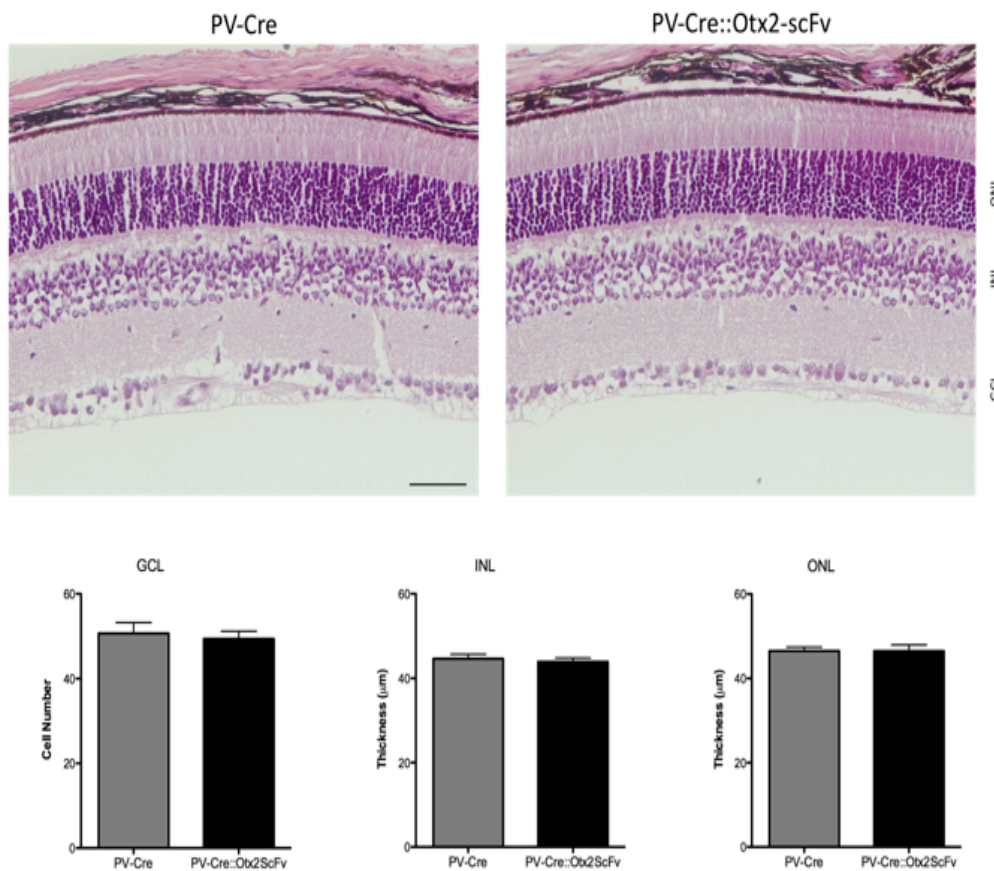


Fig 13. scFvOtx2 does not Affect the Thickness of Retinal Cell Layer.

Expression of *scFvOtx2* does not alter retinal organization. In retina from mice expressing *scFvOtx2*, all retinal layers and their organization and their approximate size are similar to their PVCre littermates. Also the number of cells in the GCL, the INL thickness and the ONL thickness are similar between the to genotypes. Scale bar: 50µM. Abbreviations: GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

qPCR was reported to be useful to estimate retinal cell number (Torero-Ibad et al., 2011). We observed no differences between P30 PV^{Cre}::scFvOTX2^{tg/o} and PV^{Cre} littermates in terms of mRNA for BRN3A (RGCs), syntaxin (amacrine and horizontal cells), CHX10 (bipolar cells), rhodopsin (rod photoreceptors) and red/green opsin (long/medium wavelength cone photoreceptors) (Fig. 14). A small increase in LIM1 expression by horizontal cells was observed which might reflect a non-cell autonomous repressor activity of OTX2 on LIM1 expression (Nakano et al., 2000). Thus, based on these markers, histological studies and cell counting, it can be concluded that all cell types involved in retinal visual processing are present in normal numbers.

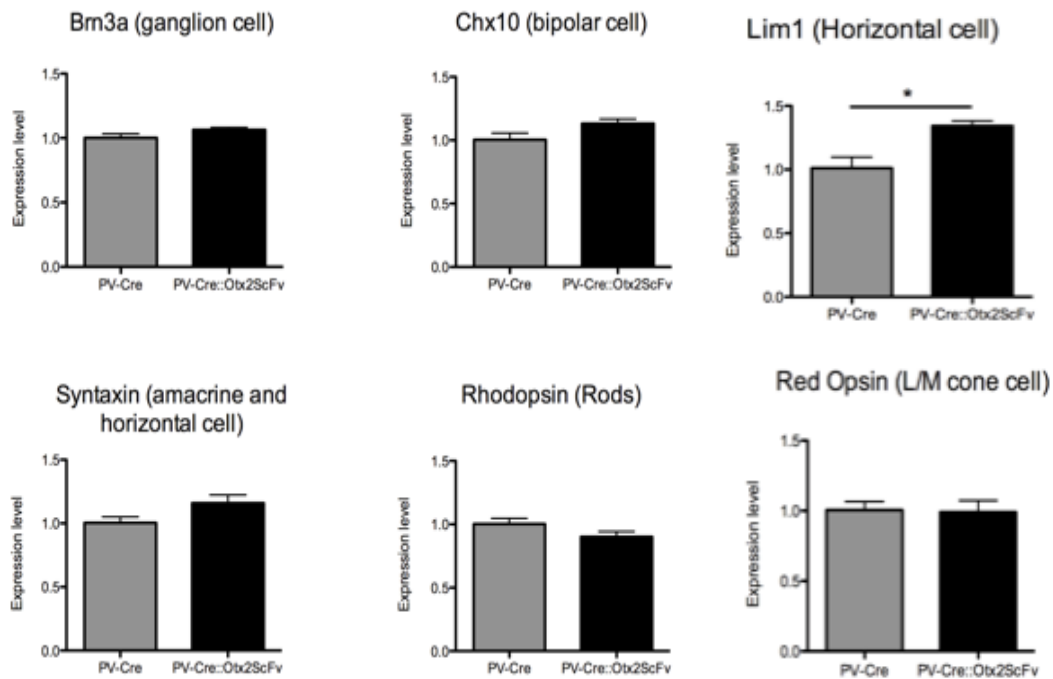


Fig 14. Measurement of retinal cell types-specific by RT-qPCR.

Genes expressed in RGCs, bipolar cells, amacrine cells, rods and cones are similar in the two genotypes. *LIM1* mRNA found in horizontal cells is significantly increased in the OTX2-scFv expressing mice. Expression levels normalized to PV-Cre P30. $P < 0.05$, Mann-Whitney U-test, two -tailed.

To determine if there was a functional defect in photoreceptors and/or bipolar cells we turned to ERG. The negative a-wave reflects photoreceptor hyperpolarization in response to light. The amplitude is related to photoreceptor number and the implicit time to photoreceptor physiology (Lamb and Pugh, 2004; Hood and Birch, 1992; see Weymouth and Vingrys, 2007 for review). There was no difference in a-wave amplitude or implicit time in the $PV^{Cre}::scFvOTX2^{tg/o}$ mice compared to PV^{Cre} littermates indicating normal number and function of photoreceptors (Fig 15 and 16).

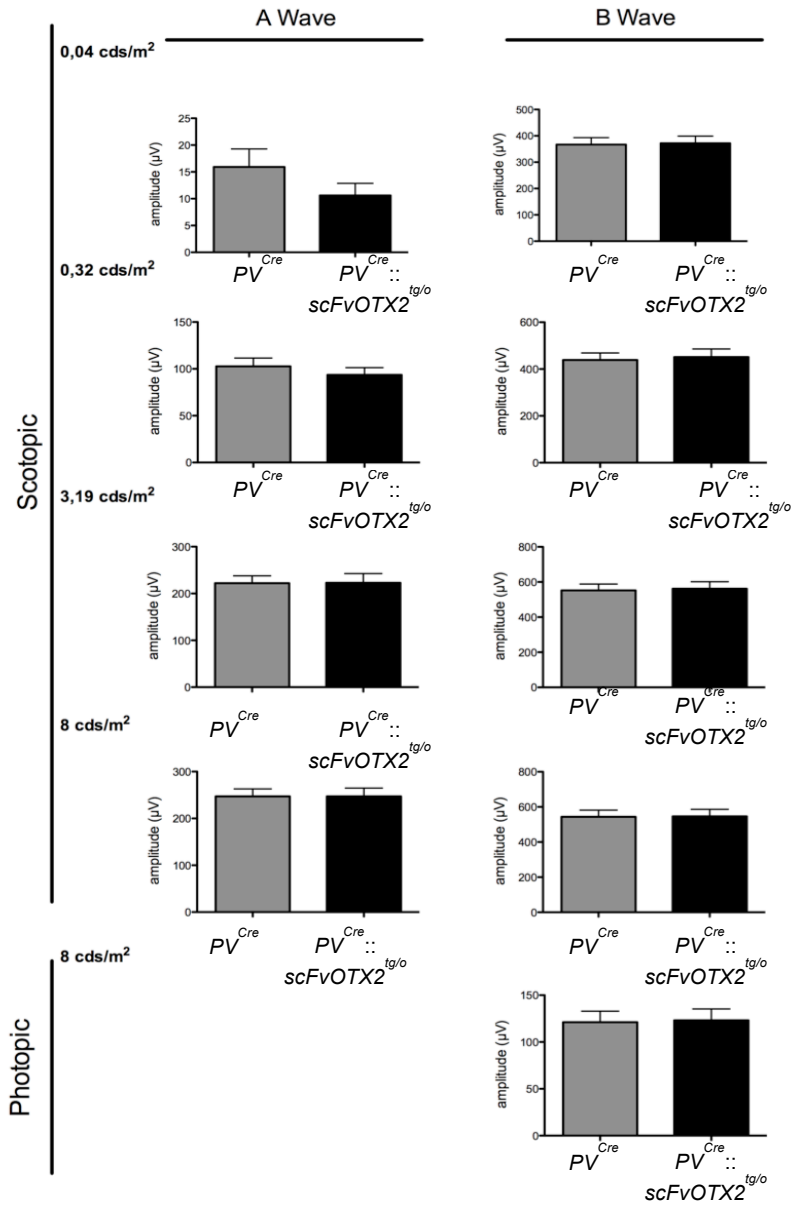


Fig 15. A- and B- waves under Scotopic and photopic Conditions.

A- and b-wave amplitude in scotopic and photopic lighting conditions and at different light intensities is similar in the two genotypes of one-month-old mice.

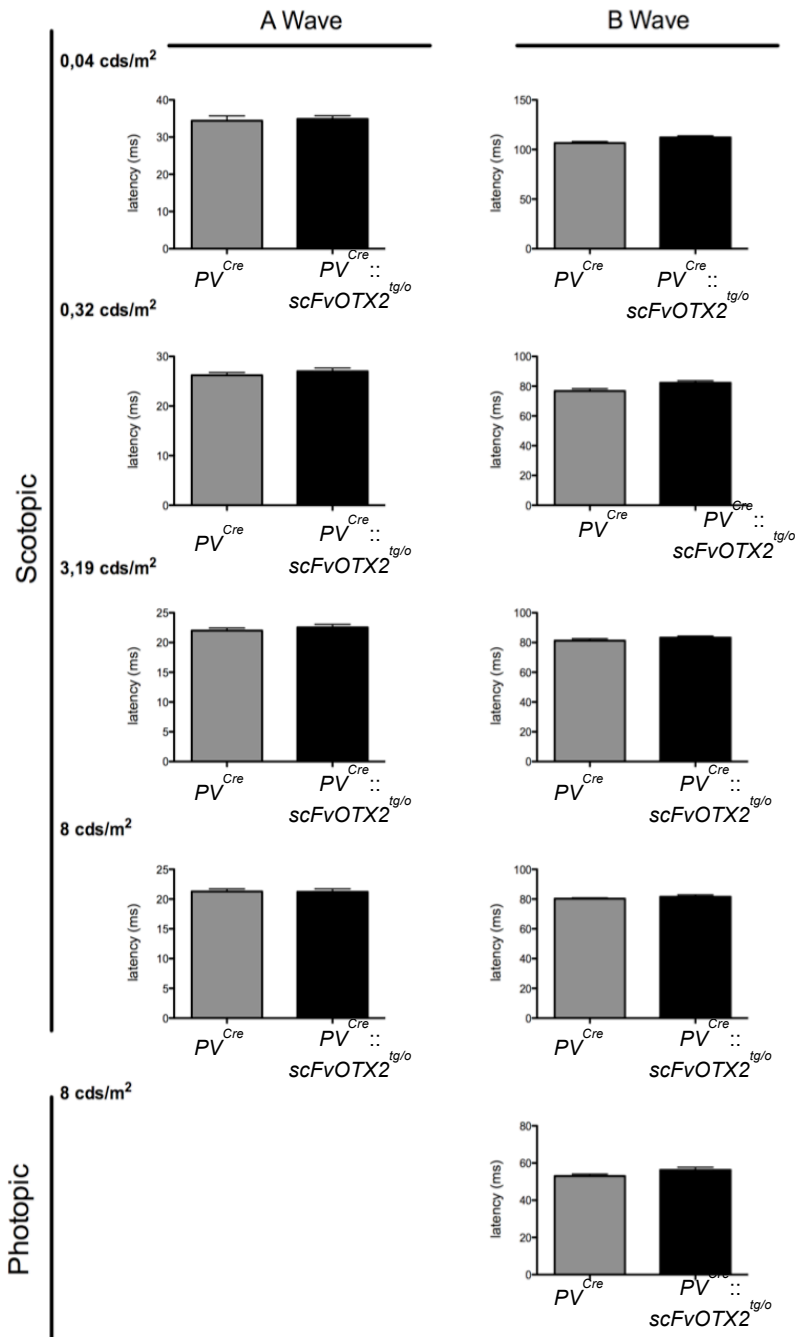


Fig 16. A- and B-waves implicit times.

ERG a- and b-wave implicit times. There were no differences in scotopic or photopic implicit time pooled for left and right eye between the two genotypes.

The b-wave is mainly driven by bipolar activity and the amplitude reflects number and the implicit time bipolar function. Both were indistinguishable between $PV^{Cre}::scFvOTX2^{tg/o}$ mice and PV^{Cre} littermates indicating normal number and response of bipolar cells. Interestingly, the $PV^{Cre}::scFvOTX2^{tg/o}$ mice showed a significant 2-fold increase in amplitude in the response to 20Hz flickers, suggesting inner retinal dysfunction (Fig 17).

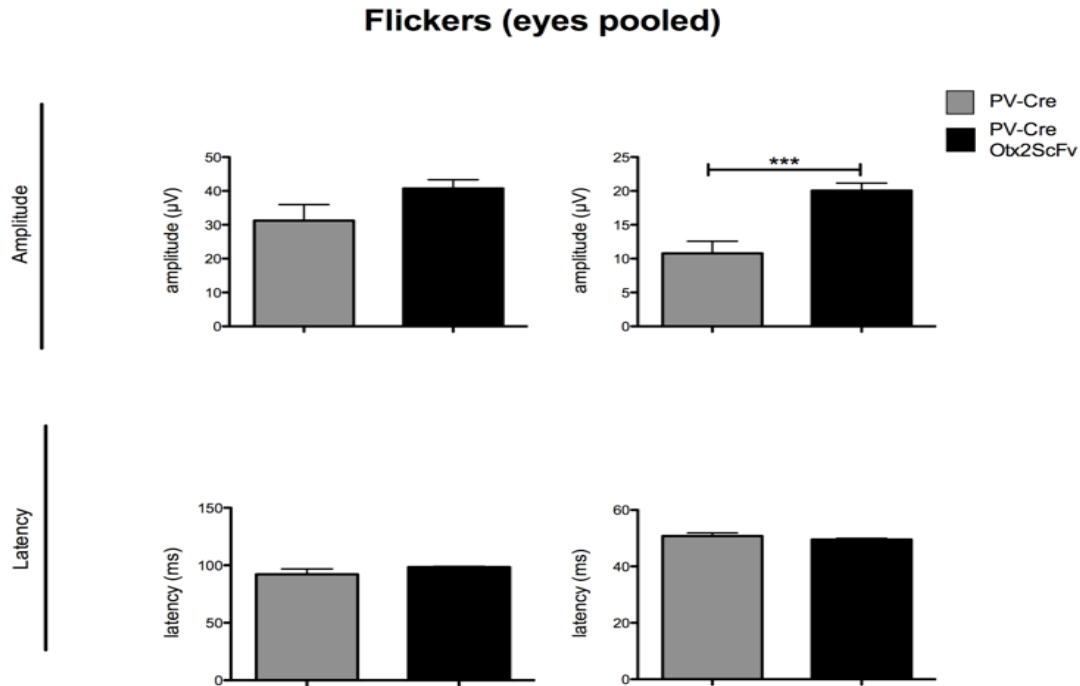


Fig 17. Measurement of Photoreceptor Functionality.

Upper: The amplitude of the flicker response to 10Hz stimulation is slightly increased and at 20Hz the response is significantly doubled in amplitude. Lower: There was no difference in the implicit time of the flicker response between the mice expressing OTX2scFv and their PV^{Cre} littermates. $P < 0.005$, Mann-Whitney U-test, two-tailed.

Discussion

OTX2 homeoprotein is part of the family of transcription factors that transfer between cells and have neuroprotective effects in the brain (Sugiyama et al., 2008; Torero-Ibad et al., 2011; Rekaik et al., 2015; see Di Nardo et al, 2018 for review). We were interested in assessing the importance of the direct non-cell autonomous activity of OTX2 in the retina. Since the amino acid sequences for OTX2 to exit and enter cells lie in the DNA binding homeodomain, mutations in these sequences might alter DNA binding and thus cell autonomous activity. Bernard et al., (2016) used a genetic approach to neutralize extracellular OTX2 by expressing a secreted anti-OTX2 single chain antibody. Here, we used this approach to drive expression of the OTX2-scFv in cells, which do not themselves produce OTX2, thus eliminating a putative cell autonomous effect of the antibody. PV is reported to be in all amacrine cells in rats, cats, bats and rabbits (Lee et al., 2004). In the mouse, direct immunofluorescence showed low PV expression in amacrine and displaced amacrine and strong PV expression in RGCs (Haverkamp and Wässle, 2000). A more sensitive transgenic approach using the PV promoter to drive expression of

enhanced GFP (Haverkamp et al., 2009) confirmed this expression pattern and showed that PV was expressed in some amacrine cells in the INL with notably strong expression in RGCs (Haverkamp et al., 2009). Kim and Jeon (2006) and later Yi et al., (2012) using single cell iontophoresis and retrograde tracing followed by PV localization showed that at least eight different RGC types express PV.

Parvalbumin starts to be expressed in RGCs around P11, after the birth of all retinal cell types (Cepko et al., 1996; Young, 1985). Thus, it is to be expected that interference with OTX2 non-cell autonomous activity starting at the earliest at P11 would have no effect in establishing the normal layers and structure of the retina, as we observe. It was reported that in OTX2 hypomorphic mice, in which both cell autonomous and non-cell autonomous phenotypes can be expected, there were fewer photoreceptors at P30, but those that were present had normal a-wave implicit time (Bernard et al., 2014). In PV^{Cre}::scFvOTX2^{tg/o} mice, the presence of normal numbers of OTX2-expressing photoreceptors and bipolar cells and their normal ERG a-wave and b-wave activity, respectively, confirms that OTX2 cell autonomous activity in these cells is not affected by expression of the secreted OTX2-scFv. It further suggests that OTX2 non-cell autonomous activity on these cells does not contribute measurably to their survival and function, at least at one month of age.

Nevertheless, in spite of normal outer and inner layer structure and function, the PV^{Cre}::scFvOTX2^{tg/o} mice showed diminished visual acuity. This was not simply due to the expression of an scFv since PV^{Cre}::scFvPAX6^{tg/o} mice did not show any loss of visual acuity compared to PV-Cre mice. RGC loss leads to a decrease in visual acuity (Torero-Ibad et al., 2011) but here, we observed no change in RGC number in PV^{Cre}::scFvOTX2^{tg/o} mice. In light of normal ERG a- and b-wave activities and normal RGC number, we can propose that the loss of OTX2 signaling induces inner retinal physiological dysfunction as suggested by the altered flickers response. The flickers response in macaques has been shown to be largely driven by spiking inner retinal neurons since it is increased at some frequencies by TTX and NMDA (Viswanathan et al., 2002). The same direction of change is observed in PV^{Cre}::scFvOTX2^{tg/o} mice, indicating that the decrease in OTX2 signaling alters the physiology of inner retinal neurons, among which are the RGCs which import OTX2 from other retinal cell types (Sugiyama et al., 2008). The flickers response at 20 Hz has been suggested to reflect function of the cone OFF-pathway (Tanimoto et al., 2015). Single OFF RGC recordings would be required to directly demonstrate such a change.

PV is expressed in other regions of nervous system, and interfering with OTX2 non-cell autonomous activity in these regions might contribute to the reduction of visual acuity. Indeed, OTX2 reaching PV cells in primary visual cortex is essential for visual plasticity (Sugiyama et al., 2018) and blocking it using the OTX2-scFv approach interferes with the critical period for visual plasticity (Bernard et al, 2016). However, the optokinetic response does not depend on visual cortex since large lesions of posterior cortex do not diminish visual acuity, indicating that the brain circuits for this response are subcortical (Douglas et al., 2005). The basic circuit for the optokinetic response is retinal input to the nucleus of the optic tract that sends input to the reticular tegmental nucleus of the pons with output going to the vestibular nucleus driving the oculomotor nucleus (Wada et al., 2014). None of these structures are known to express OTX2 and only the vestibular nucleus has PV cells. Thus, the only structure in the basic circuit that has both PV and OTX2 expression is the retina. Although the mechanisms are not yet fully understood, the flicker response is similar to what is observed with TTX and NMDA, suggesting a defect in signaling to inner retinal neurons, in particular to RGCs that receive OTX2 from outer sources. We conclude that interfering in vivo with OTX2 non-cell autonomous activity in the mature retina leads to an alteration in inner retinal cell function and causes the observed deficit in visual acuity.

Résumé en Français

L'homéoprotéine OTX2 fait partie de la famille des facteurs de transcription qui transfèrent entre les cellules et ont des effets neuroprotecteurs dans le cerveau (Sugiyama et al., 2008 ; Torero-Ibad et al., 2011 ; Rekaik et al., 2015 ; voir Di Nardo et al., 2018 pour un résumé). Nous nous sommes intéressés à l'évaluation de l'importance de l'activité non cellulaire autonome directe d'OTX2 dans la rétine. Les séquences d'acides aminés permettant à l'OTX2 de sortir et d'entrer dans les cellules se trouvant dans l'homéodomaine qui contient le site de liaison à l'ADN, des mutations dans ces séquences pourraient modifier la liaison à l'ADN et donc l'activité autonome des cellules. C. Bernard et ses collaborateurs (2016) ont utilisé une approche génétique pour neutraliser l'OTX2 extracellulaire en exprimant un anticorps anti-OTX2 simple chaîne sécrété (OTX2-svFv). Ici, nous avons utilisé cette approche pour stimuler l'expression de l'OTX2-scFv dans les cellules, qui ne produisent pas elles-mêmes OTX2, éliminant ainsi un effet cellulaire autonome supposé de l'anticorps.

La parvalbumine est connue pour être exprimé dans toutes les cellules amacrines et CGRs chez le rongeur (Lee et al., 2004; Haverkamp et Wässle, 2000). La parvalbumine commence à être exprimée dans les CGRs autour de P11, après la naissance de tous les types de cellules rétinienne (Cepko et al., 1996 ; Young, 1985). On peut donc s'attendre à ce que l'activité non cellulaire autonome d'OTX2 qui commence au plus tôt à P11 n'ait aucun effet sur l'établissement normal des couches et de la structure de la rétine, comme nous l'observons. Il a été signalé que chez les souris hypomorphes pour OTX2, chez lesquelles on peut s'attendre à des phénotypes à la fois autonomes et non autonomes, il y avait moins de photorécepteurs à P30, mais ceux qui étaient présents avaient un temps de réponse implicite normal (Bernard et al., 2014). Chez les souris PVCre::scFvOTX2tg/o, le nombre de PRs et de cellules bipolaires est normal et les ondes a et b de l'ERG étaient normales, ce qui confirme que l'activité autonome d'OTX2 dans ces cellules n'est pas affectée par l'expression de l'OTX2-scFv sécrété. Ceci suggère en outre que l'activité autonome non cellulaire d'OTX2 sur ces cellules ne contribue pas de manière mesurable à leur survie et à leur fonction, au moins à l'âge d'un mois.

Néanmoins, malgré une structure et une fonction normales des couches externe et interne, les souris PVCre::scFvOTX2tg/o ont montré une acuité visuelle diminuée. Cela n'était pas simplement dû à l'expression du scFv puisque les souris

PVCre::scFvPAX6tg/o n'ont pas montré de perte d'acuité visuelle par rapport aux souris PV-Cre. La perte de CGR entraîne une diminution de l'acuité visuelle (Torero-lbad et al., 2011) mais ici, nous n'avons observé aucun changement du nombre de CGR chez les souris PVCre::scFvOTX2tg/o. Le fait que les ondes a et b de l'ERG soient normale et que le nombre de CGR soit normal nous permet de proposer qu'OTX2 induit un dysfonctionnement physiologique de la rétine interne comme le suggère la modification de la réponse à la lumière scintillantes. Il a été démontré que la réponse aux scintillements chez les macaques est largement déterminée par la décharge des neurones rétiniens internes, puisqu'elle est augmentée à certaines fréquences par le TTX et le NMDA (Viswanathan et al., 2002). Le même type de modification est observée chez les souris PVCre::scFvOTX2tg/o, ce qui indique que la diminution de la signalisation non cellulaire autonome d'OTX2 modifie la physiologie des neurones rétiniens internes, qui internalisent OTX2 venant d'autres types de cellules rétiniennes parmi lesquels se trouvent les CGRs (Sugiyama et al., 2008). Il a été suggéré que la réponse aux scintillements à 20 Hz reflète la fonction de la voie OFF du cône (Tanimoto et al., 2015). Des enregistrements de CGRs OFF unique seraient nécessaires pour démontrer directement un tel changement.

Chapter 4. Non cell autonomous OTX2 activity on PRs

Introduction

In adult retina *Otx2* is expressed in the INL, ONL, and the RPE layer, but the protein is also found in RGCs. Previous work has shown that conditional knockout (cKO) of OTX2 from all retinal *Otx2* expressing cells leads to gradual degeneration of photoreceptors (Béby et. al. 2010). A follow up study showed that cKO of *Otx2* specifically in the RPE caused gradual photoreceptors degeneration (Housset et. al., 2013). Furthermore the re-expression of OTX2 in RPE of Mice in which OTX2 was conditionally KO in the retina was sufficient for PR survival (Housset et. al., 2013). These studies show the importance of *Otx2* expression in RPE cells for PR survival and highlight the role of RPE OTX2 even in the absence of OTX2 in neural retina including the PRs.

We hypothesised that RPE OTX2 can be secreted and acts directly on PRs to promote cell survival. Alternatively non-cell autonomous activity of OTX2 on PR survival could arise from cell autonomous OTX2 regulating PR survival factors expressed in RPE cells. This RPE cell autonomous OTX2 could affect expression of other non-cell autonomous factors that promote survival of PRs.

To begin to examine the first possibility we first determined if OTX2 was present in the IPM between the RPE and ONL and if then there were extracellular binding sites in the IPM. Since, previous work in the brain showed the importance of GAGs in OTX2 binding, we examined if exogenous OTX2 bound to retinal GAGs. We then tried to demonstrate secretion of OTX2 from RPE cells *in vitro* and *in vivo*. Finally, we reduced endogenous OTX2 in the retina and expressed OTX2 and OTX2 YL specifically in the RPE to examine neuroprotective non-cell autonomous effects of OTX2 and OTX2 YL.

A. OTX2 in Interphotoreceptor Matrix.

RPE cells and photoreceptors have high level of OTX2 (Figure 3). We observed endogenous OTX2 in the IPM layer that contains RPE microvilli and PRs OS. We separated by laser capture dissection the IPM layer from RPE and ONL. Lysate from whole wild-type retina show a strong OTX2 band at 38 KDa while HeLa cells do not (negative control) Western blot confirmed the presence of OTX2 in the IPM layer (Figure 18). The OTX2 in the IPM could have been synthesized in RPE and transferred to the PR OS. Alternatively, OTX2 in the IPM could be produced in the PRs and shuttled to their OS. However, our current methods do not allow us to determine the origin (PR and/or RPE) of the OTX2 in the OS layer.

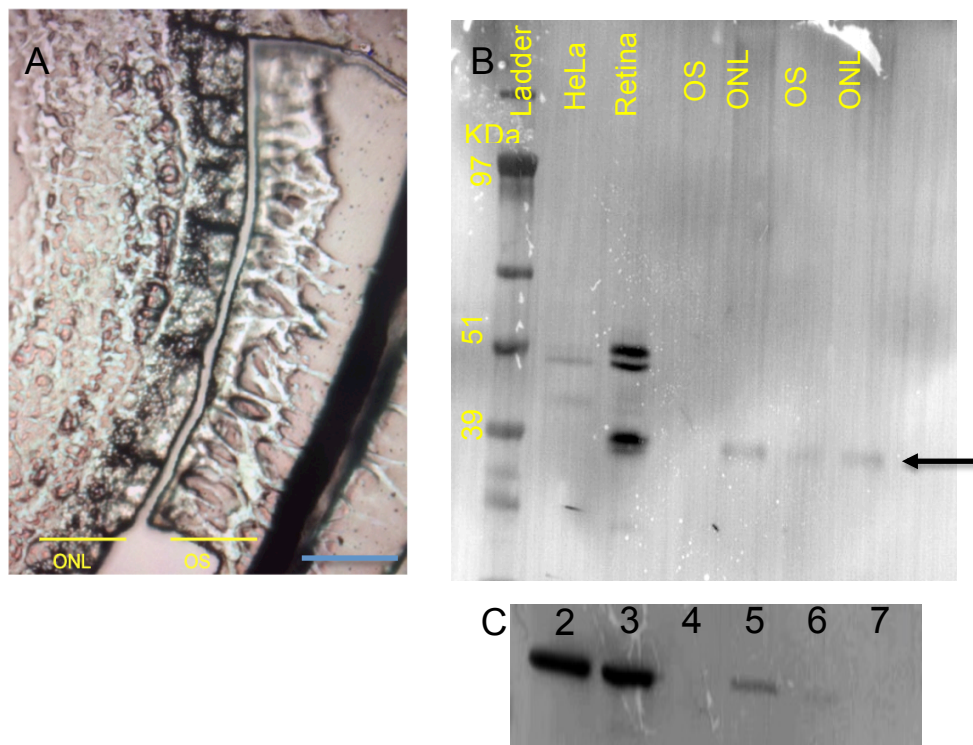


Fig 18. OTX2 in interphotoreceptor matrix.

(A) IPM containing PR OS from fixed retinal tissues were laser dissected and separated from the ONL. (B) Western blots for OTX2 were performed on dissected IPM. Lane 4 and 5 had IPM dissected from 4 sections, while lane 6 and 7 had 6 sections of isolated IPM. OTX2 molecular weight is 38 KDa (black arrow). (C) Cytoplasmic marker beta actin was used. Band visible in lane 6 confirming presence of beta actin. Scale bar 40 μ m.

B. Localization of OTX2 on live RPE cell surface.

We established primary mouse RPE cell cultures to study OTX2 *in vitro*. One-day-old live primary, non-permeabilized RPE cell cultures were exposed to anti-

OTX2 and anti-tubulin antibodies. Our results show that anti-tubulin antibody did not penetrate the living cells or bind to the plasma membrane (Figure 19A). Anti-OTX2 antibody bound to OTX2 on the RPE cell surface (Figure 19B). This was further confirmed by staining permeabilized RPE cells. When RPE cells were permeabilized the anti-tubulin antibody marked cytoskeleton of RPE cells (Figure 19C), whereas anti-OTX2 antibody stained the nucleus (Figure 19D). Taken together our data shows that OTX2 is present in the nuclei of cultured RPE cells as observed in the tissue and also at the cell surface of live primary RPE cells. The presence of OTX2 on the surface of primary RPE cells raises the possibility that OTX2 can be transferred from the RPE.

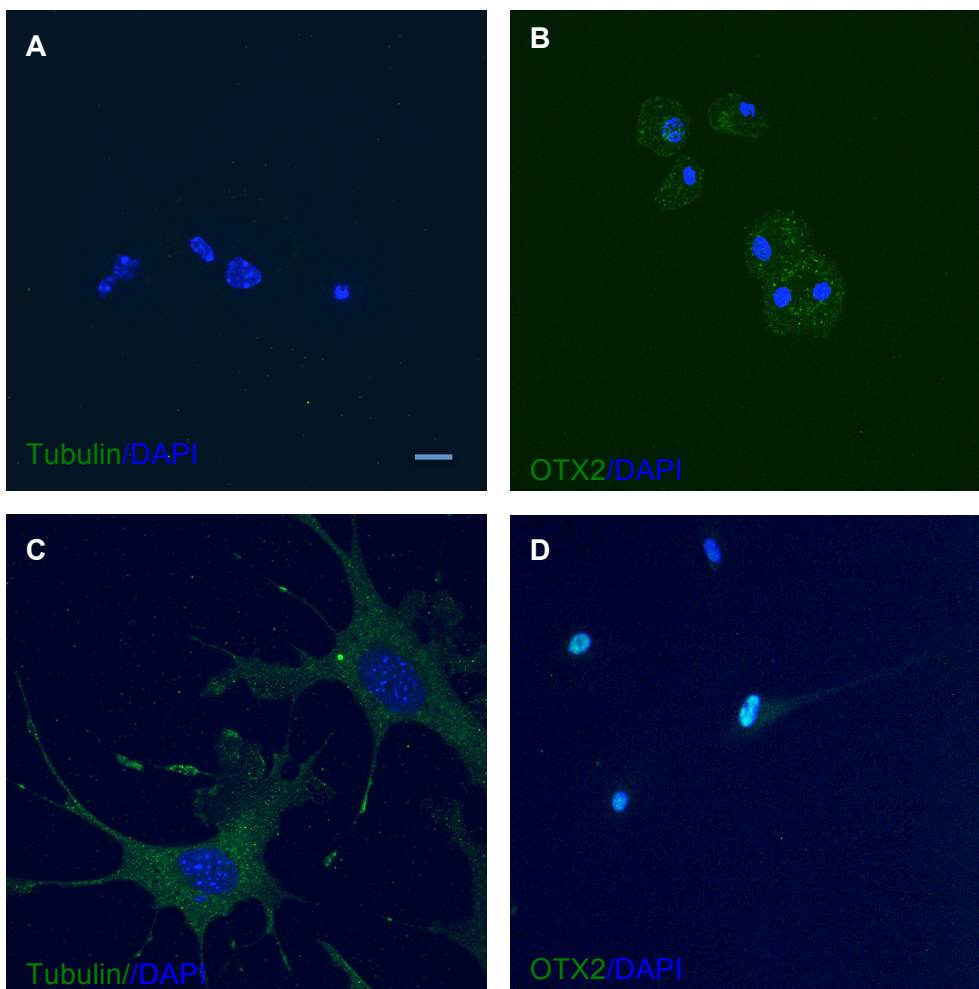


Fig 19. Immunostaining of OTX2 on the surface of live primary non-permeabilized RPE cell.

(A-B) After one day *in vitro* live RPE cells were treated with anti-tubulin and anti-OTX2 antibodies. Live cells treated with anti-tubulin antibody did not reveal the cell cytoskeleton. However, anti-OTX2 antibody labelled OTX2 on the surface of live RPE cells. (C-D) Permeabilized and fixed four-day-old RPE cells reveal cytoplasmic staining for tubulin, while the anti-Otx2 antibody stained OTX2 in the nucleus (green) that co-localized with DAPI (blue). Scale bar 25 μm .

C. Exogenous OTX2 binds to retina *ex-vivo*.

Endogenous retinal OTX2 is present in RPE cells, ONL, INL, and RGCs and cultured RPEs have the protein on their surface. This latter observation is consistent with the possible transfer of OTX2 in the retina and this raises the question of binding sites in the retina.

We thus studied the binding and distribution of exogenously added OTX2 on fresh frozen retinal sections. Exogenous OTX2 immuno-reactivity was dense in the INL, present in the RGCs, and diffuse in the ONL. Light diffuse staining was also present in the PS (Figure 20). After the addition of exogenous OTX2 to the retinal sections, the immunofluorescent signal was greatly intensified especially in the INL, OPL, and OS (Figure 20).

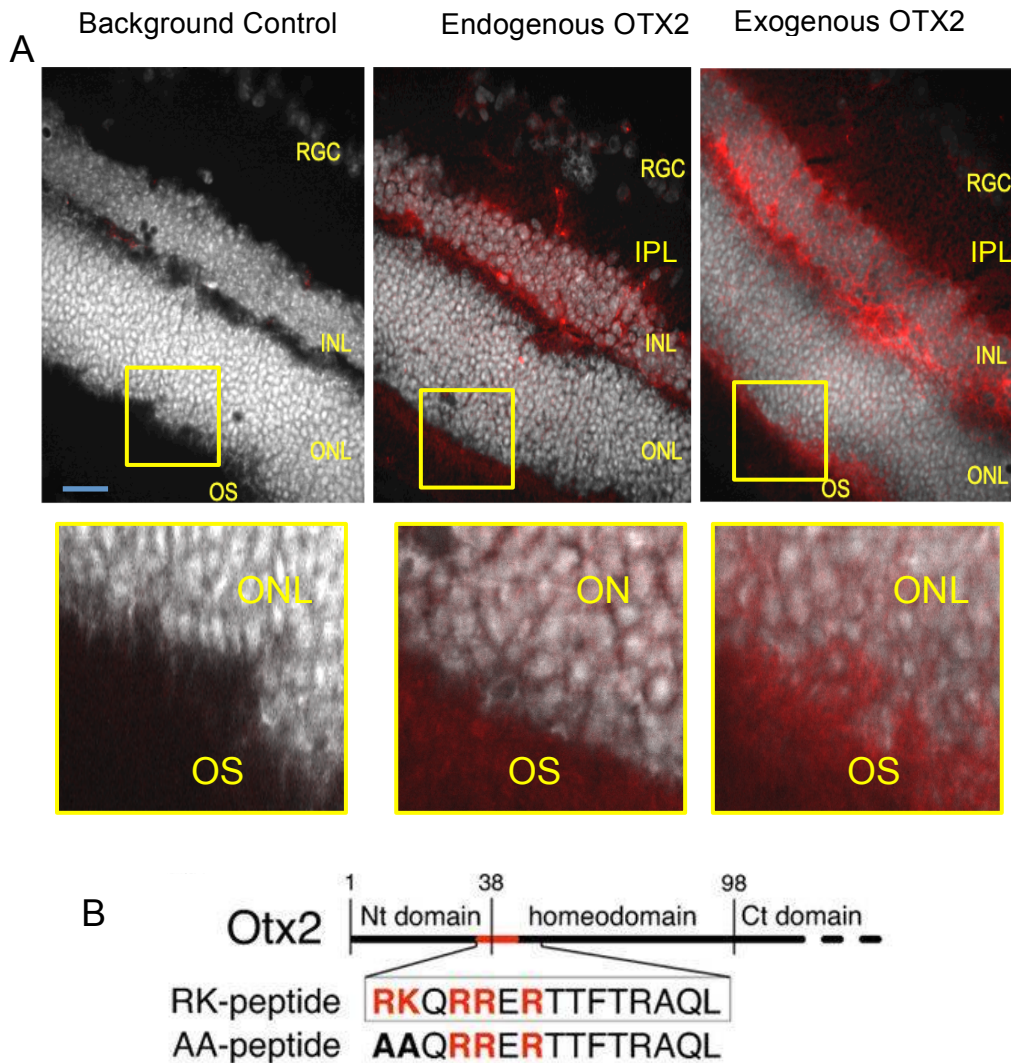


Fig 20. Binding of exogenous OTX2 on fresh frozen sections.

(A) OTX2 was added to fresh frozen sections and OTX2 was revealed using mouse anti-OTX2 antibody under non-permeabilized conditions. Red signal indicates presence of endogenous OTX2. Samples treated with OTX2 showed a sharp increase in the signal around the nucleus of RGCs, INL, ONL and the OS. Demonstrating that OTX2 has extracellular targets in the neural retina. Scale bar 50 μ m. (B) Putative GAG-binding motif (in red) in the Otx2 N-terminal region bears an RK doublet just before the homeodomain. RK and AA peptide sequences are indicated. Taken from Beurdeley et. al., 2012.

Previous work has shown that the RK peptide in the N-terminus of OTX2 is necessary for OTX2 binding to GAG-rich extracellular sites such as GAGs (Beurdeley et. al., 2012). Hence, we added AP-OTX2-Nt-HD on fresh frozen sections to reveal binding sites for OTX2. AP-OTX2-Nt-HD is an alkaline phosphatase fused to the N-terminus and homeodomain of OTX2. The AP-OTX2-Nt-HD has similar binding patterns to full length exogenous OTX2. A strong signal was visible in the RGC, INL, ONL, and the OS of retinal tissue (Figure 21A). This suggests that the N-

terminus containing the RK region and homeodomain was sufficient for OTX2 binding. Mutating the RK doublet to an AA doublet eliminated OTX2 specific binding to PV cells in visual cortex (Beurdeley et al., 2012). Similarly in the retina, we found that AP-OTX2-Nt-HD AA had greatly decreased binding on adult retinal sections (Figure 21B).

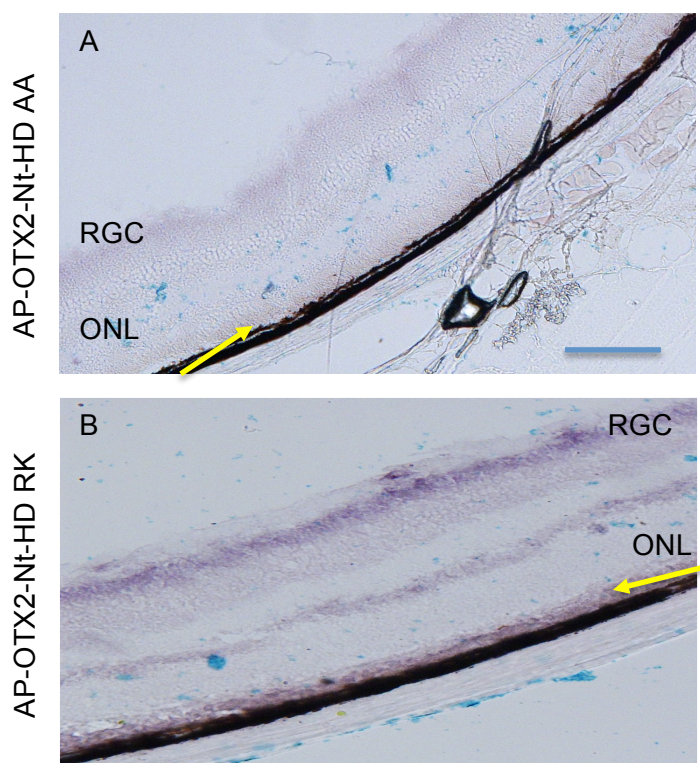


Fig 21. Binding between Nt-HD RK and Nt-HD AA OTX2.

Fresh frozen sections treated with OTX2-Nt-HD RK peptide and mutated peptide OTX2-Nt-HD AA. Mutating RK to AA causes OTX2 to decrease its binding properties. **(A)** OTX2-Nt-HD AA has decreased binding affinity on retinal tissue. **(B)** Alkaline phosphatase staining reveals that OTX2-Nt-HD RK is able to bind to all retinal layers, including the OS (yellow arrows). Scale bar 40 μ m.

D. OTX2 retina binding involves GAGs.

OTX2 binding to neurons in visual cortex involves GAGs (Beurdeley et al, 2012). To determine if OTX2 binding in the retina also involves GAGs, fresh frozen sections were treated with Chondroitinase ABC (ChABC). ChABC is an enzyme that digests polysaccharides containing chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate. It efficiently removes GAGs from the tissue and, along with it, any proteins or growth factors attached to GAGs. In the absence of ChABC, AP-OTX2-Nt-HD bound to the OS layer, ONL, and INL (Figure 22C). However, on ChABC treated retinal sections AP-OTX2-HD RK binding was greatly diminished (Figure

22B). The results thus clearly show that ChABC treatment reduced OTX2-Nt-HD RK binding to neural retina. This mechanism is similar to the visual cortex where OTX2-Nt-HD binds to the GAGs and allows for OTX2 specific internalization.

These results demonstrate that OTX2 binds to GAGs in the OS, ONL, and INL of the retina. The RK peptide adjacent to the homeodomain is necessary for OTX2 to bind to retinal GAGs that are present in the extracellular matrix of the adult retina.

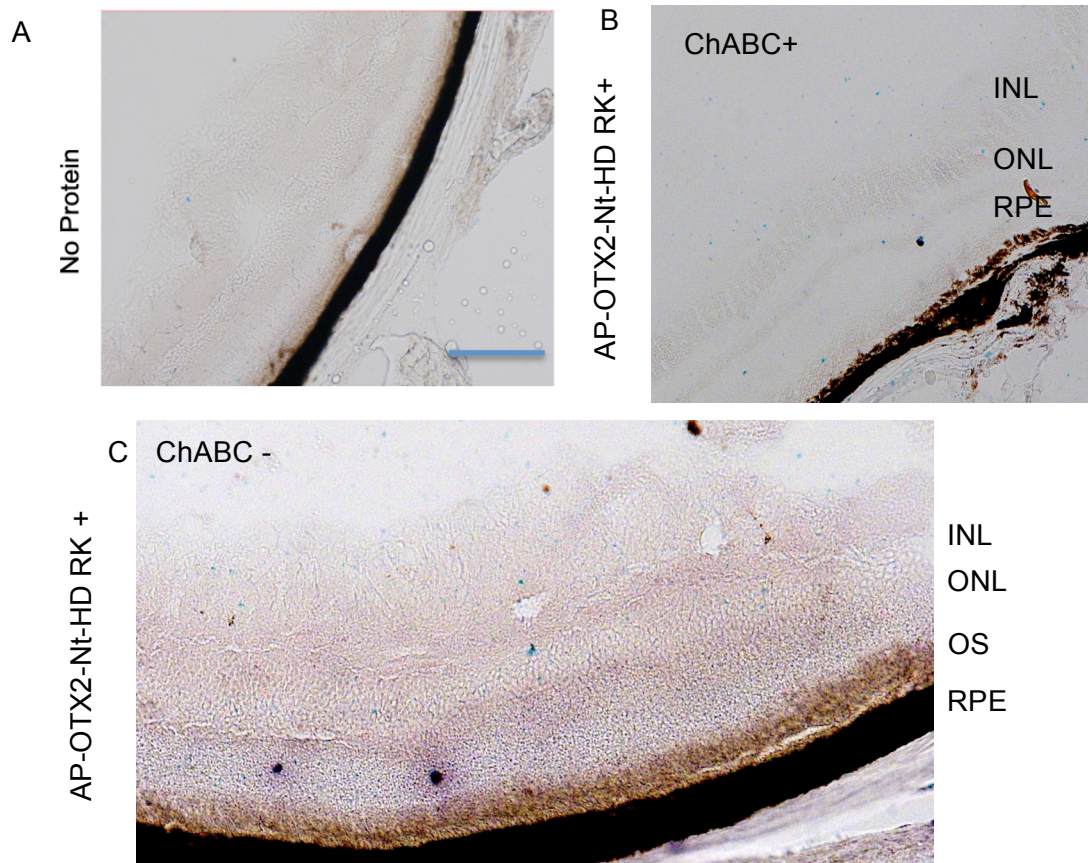


Fig 22. AP-OTX2-Nt-HD RK binding to GAG.

(A) Inactivation of endogenous alkaline phosphatase by heat. (B) Sections treated with ChABC lose their GAGs and have reduced binding affinity for OTX2-Nt-HD. (C) On untreated fresh frozen sections AP-OTX2-Nt-HD RK is able to bind to extracellular GAGs. Purple-violet color indicates presence of alkaline phosphatase from AP-OTX2-Nt-HD RK. Endogenous alkaline phosphatase was inactivated in all samples by heat. Scale bar 40 μ m.

E. Transfer of tagged OTX2 from RPE to IPM/OS.

To verify if OTX2 can transfer from RPE to IPM *in vivo*, we constructed viruses that express tagged OTX2 (mVMD2-HA-Otx2-M2) and hBEST-iCre driven by an RPE specific promoter. We validated the tissue specificity of these promoters by injecting

them in the subretinal space of $Otx2^{flox/flox};Rosa^{Ai14/Ai14}$ mice. Viral mediated recombination was observed only in the RPE layer and not in other retinal layers. Immunohistochemistry confirming that the hBEST1 or mVMD2 promoters were specific to RPE layer (Figure 23).

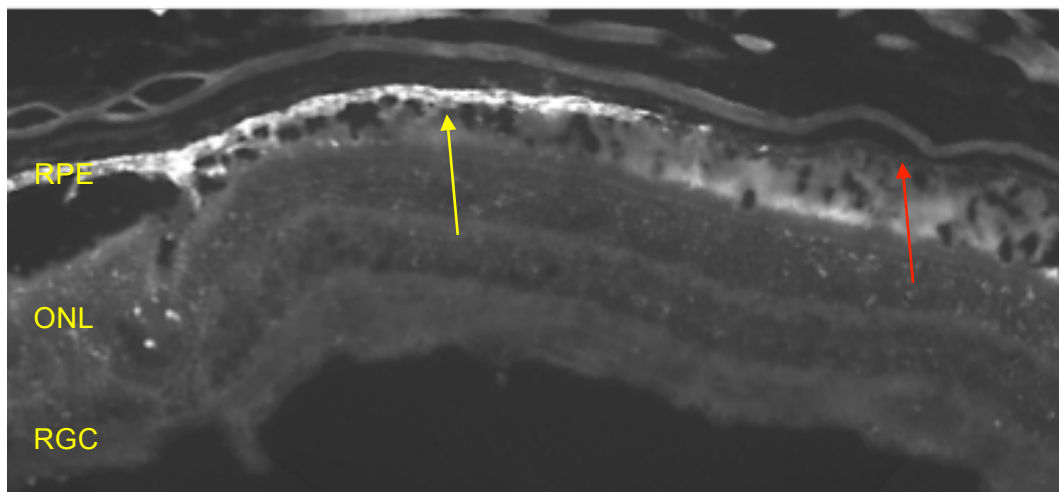


Fig 23. Subretinal injection of AAV2/5-hBEST1-iCre in $Otx2^{flox/flox};Rosa^{Ai14/Ai14}$ mice.

Subretinal injection of AAV5 virus expressing iCre under the control of RPE specific promoter hBEST1. Expression of *iCre* specifically in the RPE layer recombines the ROSA locus allowing for *tdTomato* expression. Yellow arrow indicate RPE cells expressing tdTomato (no antibody used) and red arrow indicates an area lacking recombination. This virus can be used in the $Otx2scFv$ mice to specifically block OTX2 secretion from the RPE cell layer. (Courtesy of Pasquale Pensieri, Annabelle Mantilleri and Thomas Lamonerie)

To detect the presence of tagged OTX2 (OTX2-M2) in the IPM, we considered two complementary approaches. In the first approach, RPE specific promoter, mVMD2, drove expression of tagged OTX2-M2 only in the RPE. And we tried to detect the M2 tag in and around the IPM via IHC. Different conditions for IHC were used to detect OTX2-M2. But we were unable to visualize the tagged protein in the IPM of the injected eye (not shown). We reasoned that our inability to visualize the OTX2-M2 in sections of retina could be due to low amounts of transferred OTX2-M2 from the RPE to IPM and we needed to do this with higher sensitivity. In a second approach, we repeated the experiment and used laser capture microdissection to isolate infected IPM in the region around the AAV infection. We performed western blot to detect the M2 tag fused to the OTX2 (Figure 24). Figure 24A shows that no tagged OTX2 was detected in the IPM. The very faint bands of actin as a loading

control indicate only trace levels of protein were recovered in the laser dissection capture (Figure 24B).

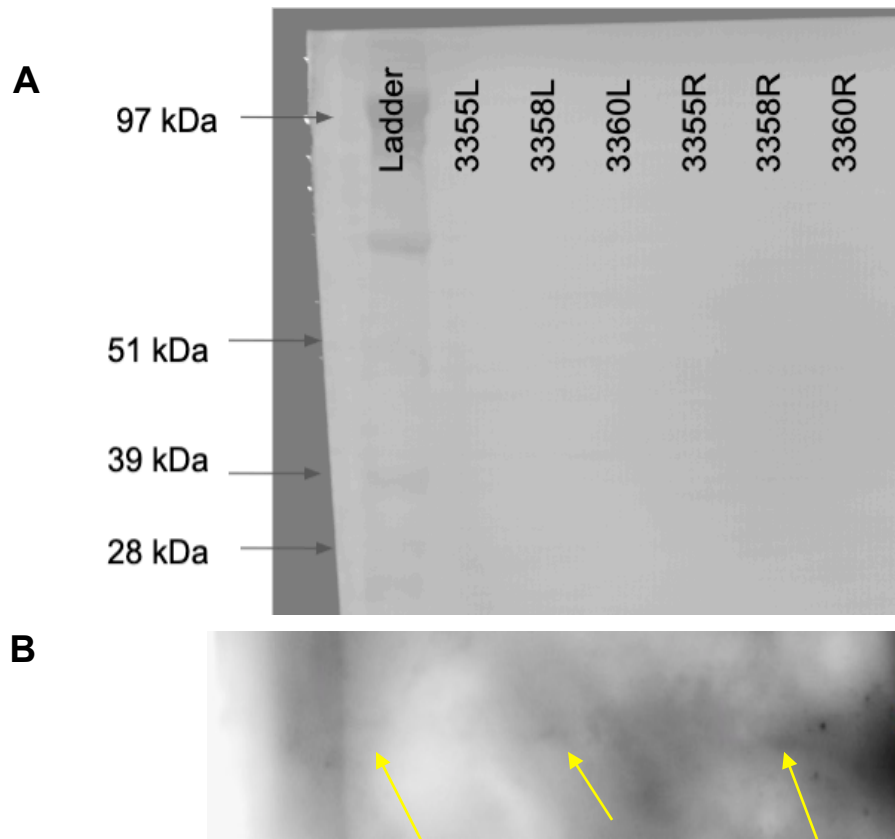


Fig 24. Detecting tagged OTX2 by WB in IPM.

(A) IPM layers were laser captured from control (3355L, 3358L, 3360L) and injected retinas (3355R, 3358R, 3360R). No signal for M2 was detected at 42 kDa in the injected retina. (B) Loading Control Actin. Due to a small sample size a faint band is detectable in the lanes. Arrows point to bands

F. Survival of Photoreceptors due to OTX2 YL.

To examine protective effects OTX2 and OTX2 YL (a non-internalized OTX2 variant), a sub-retinal injection of an AAV expressing *Otx2* and *Otx2* YL under VMD2 promoter at P30 in *Otx2^{flox/flox}* mice was performed. This approach specifically expressed *Otx2* and *Otx2* YL in the RPE cells. At P37, endogenous OTX2 in these mice was floxed by injecting tamoxifen. Immunohistochemistry against OTX2 showed that *Otx2* in the RPE, and ONL layers was successfully floxed by tamoxifen (Figure 25 top panel). Moreover, the viral introduction of *Otx2* and *Otx2* YL specifically in RPE showed the protein expressed in the RPE of the injected eye (Figure 25 bottom

panel). Typically, floxing OTX2 in the retina leads to PR degeneration (Béby et. al. 2010) and re-expression of the protein in RPE prevents PR loss (Housset et. al., 2013). In a preliminary analysis expression of wild-type OTX2 in RPE resulted in greater ONL thickness compared to contra eye resulting in a reduction of PR. The quantification of the number of photoreceptors nuclei bodies revealed that retinas where OTX2 or OTX2 YL were re-introduced specifically in the RPE had elevated number of surviving photoreceptor nuclear bodies (Figure 26). This was unexpected since OTX2 YL cannot be internalized and had no neuroprotective effect on RGC (Torero Ibad et. al., 2011). This experiment should be repeated but if shown reproducible would suggest that secreted extracellular OTX2 might have a trophic activity independent of its internalization by PRs.

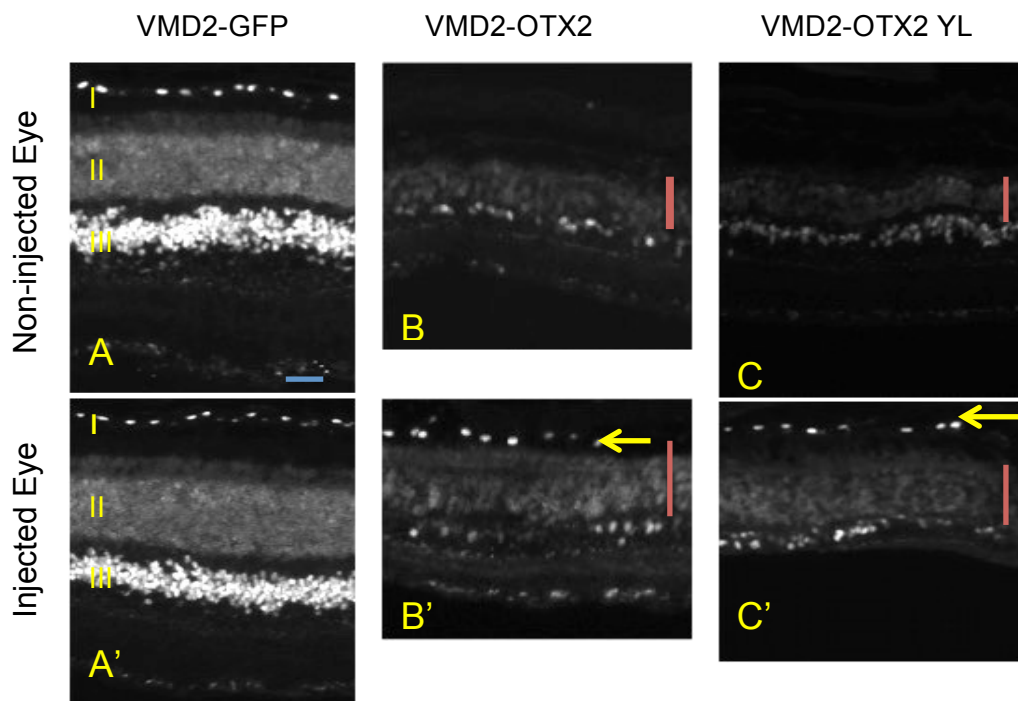


Fig 25. IHC for OTX2 reveal RPE specific viral expression of OTX2 and OTX2 YL.

The top panel shows non-injected eyes from *Otx2^{fllox/fllox}* mice. At P37, tamoxifen was injected to flox endogenous *Otx2*. RPE did not reveal presence of OTX2 after tamoxifen injection. The bottom panel shows virus-injected eyes from *Otx2^{fllox/fllox}* mice. (**A** and **A'**) GFP expressed in the RPE as a control. Injected retinas have viral mediated expression of OTX2 (**B'**) and OTX2 YL (**C'**) in the RPE, whereas these expressions are absent in the non-injected eye (**B** and **C**). Yellow arrows indicated injected RPE. Red bars indicate the measured thickness of ONL. (I = RPE; II = ONL; III = INL). Scale bar 50 μ m. (Courtesy of Pasquale Pensieri, Annabelle Mantilleri and Thomas Lamonerie).

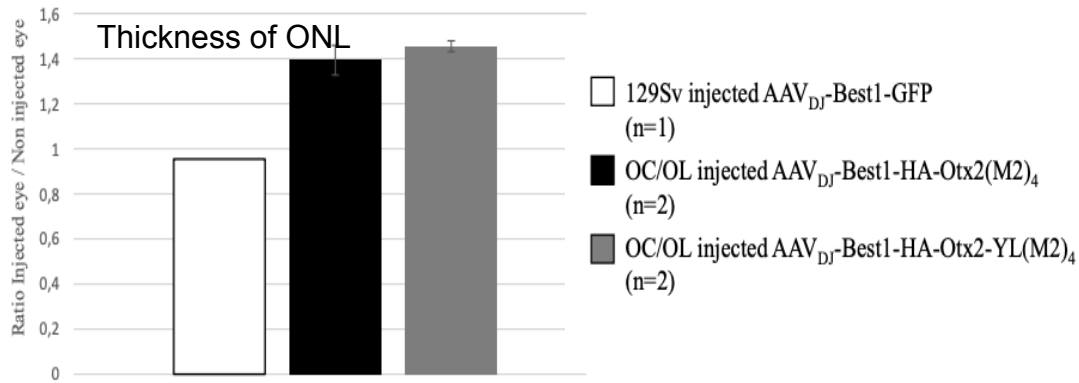


Fig 26. Survival of PRs due to viral expression of *Otx2*, and *Otx2* YL in the RPE.

RPE specific expression of GFP in *Otx2*^{flox/flox} mice had thinner ONL. Where as RPE expressing wild type OTX2 and OTX2 YL had increased number of PRs. (Courtesy of Pasquale Pensieri, Annabelle Mantilleri and Thomas Lamonerie).

Discussion

The *Otx2* locus is active in RPE, ONL, and INL, however, the protein can be also be found the RGCs. In retinal sections, OTX2 is strongly localized to the nuclei of RPE and in a perinuclear ring around PR nuclei. In addition, we observed OTX2 to be present in the IPM that contains PR OS and RPE microvilli. Since homeoproteins including OTX2 can transfer between cells we considered the possibility that OTX2 could accumulate in or around the OS in the IPM.

For OTX2 to accumulate in the IPM it has to be secreted either from the RPE or the ONL. We demonstrated that primary RPE cultures expressed OTX2 and that OTX2 was present on the cell surface. However, we were unable to detect secreted OTX2 in the culture media. Our inability to detect OTX2 could be due to limitations in our methods. Since a small amount of OTX2 is secreted into the culture medium, it could be diluted and undetectable by western blot.

To further examine the transfer of OTX2 from the RPE to the PRs *in vivo*, we drove the expression of M2-tagged OTX2 selectively in the RPE cells, but were unable to detect the tagged protein in the IPM. Either RPE-originated OTX2 does not transfer to IPM, or the transferred OTX2 is in levels below our threshold of detection. We are now considering more sensitive methods such as mass spectrometry of laser dissected IPM to be able to detect OTX2-M2. A second method would be to visualize the M2 tag by electron microscopy.

Transferred OTX2 would require its binding to target cells followed, or not, by its internalization. Hence, we showed that exogenous OTX2 binds to OS, ONL, INL and the RGCs. In addition, the RK region of OTX2 is required to bind to GAGs on the extracellular surface of the retinal tissue. ChABC treatment of sections diminishes OTX2 binding. This suggests that transferred OTX2 either from the RPE or the PRs utilizes the RK region to bind GAGs localized at the IPM surface.

Expression of wild-type OTX2 in RPE of floxed *Otx2*^{flox/flox} was associated with a thinning of ONL compared to the eye in which OTX2 was not re-expressed, in line with previous reports (Béby et. al. 2010; Housset et. al., 2013). OTX2 YL cannot be internalized and lacks neuroprotective activity for RGCs *in vitro* and *in vivo* (Torero Ibad et. al., 2011). If internalization of OTX2 by PRs is required for PR survival, we anticipated OTX2 YL expression in RPE to be without effect in the OTX2 floxed retina. To our surprise the ONL thickness of the retina in which OTX2 YL was expressed in RPEs was similar to that expressing wild-type OTX2 and greater than the retina that did not express endogenous OTX2. This must be taken with caution since it is based on a single experiment containing a single mouse per treatment. If a neuroprotective effect of OTX2 YL on PRs is established this would indicate that internalization of OTX2 is not necessary for an effect. This would favour an action of OTX2 in the RPE, perhaps in association with other survival factor(s) in PR survival. More experiments will be needed to explore this further.

Résumé en Français

Le locus *Otx2* est actif dans l'EPR, couche nucléaire extérieur (CNE) et couche nucléaire intérieur (CNI), mais la protéine peut également être retrouvée dans les CGRs. Sur des coupes rétinienne, OTX2 est fortement localisée dans les noyaux de EPR et dans un anneau périnucléaire dans les PRs. De plus, nous avons observé la présence d'OTX2 dans la MIP qui contient les microvillosités des segment externes des PRs et des cellules de l'EPR. Comme OTX2 peut transférer entre les cellules, nous avons envisagé la possibilité qu'OTX2 puisse s'accumuler dans ou autour du segment externe dans la MIP.

Pour qu'OTX2 s'accumule dans la MIP, il doit être sécrété soit par l'EPR, soit par la CNE. Nous avons démontré que les cultures primaires d'EPR exprimaient OTX2 et qu'il était présent à la surface des cellules. Cependant, nous n'avons pas pu détecter OTX2 sécrété dans le milieu de culture. Notre incapacité à détecter OTX2 dans le milieu de culture pourrait être due aux limites de nos méthodes. Comme une petite quantité d'OTX2 est sécrétée dans le milieu de culture, elle pourrait être diluée et rendue indétectable par Western blot.

Pour examiner plus en détail le transfert d'OTX2 de l'EPR aux PR *in vivo*, nous avons stimulé l'expression d'OTX2 marquée avec M2 de manière sélective dans les cellules de l'EPR, mais nous n'avons pas pu détecter la protéine marquée dans l'EPR. Soit OTX2 issue de l'EPR ne transfère pas à la MIP, soit la quantité d'OTX2 transféré est inférieure à notre seuil de détection. Nous envisageons maintenant des méthodes plus sensibles telles que la spectrométrie de masse de la MIP disséquée au laser microdissecteur pour pouvoir détecter l'OTX2-M2. Une deuxième méthode consisterait à visualiser le marqueur M2 en microscopie électronique.

OTX2 transféré nécessiterait sa liaison aux cellules cibles suivie, ou non, de son internalisation. Ainsi, nous avons montré qu'OTX2 exogène se lie au segment externe, à la CNE, à la CNI et aux CGRs. En outre, la région RK d'OTX2 doit se lier aux GAGs sur la surface extracellulaire du tissu rétinien. Le traitement des sections avec la ChABC, une enzyme qui digère les carbohydrates complexes, diminue la liaison d'OTX2. Cela suggère qu'OTX2 transféré soit de l'EPR ou des PRs utilise la région RK pour se lier aux GAGs localisés à la surface de l'EPR.

Une suppression de l'expression d'OTX2 dans la rétine est associée avec une perte des PRs lorsque la réexpression d'OTX2 dans l'EPR protège contre cette

dégénération (Béby et. al. 2010 ; Housset et. al., 2013). Une forme mutée d'OTX2, OTX2 YL, ne peut pas être internalisé et manque d'activité neuroprotectrice pour les CGR *in vitro* et *in vivo* (Torero Ibad et. al., 2011). Si l'internalisation d'OTX2 par les PRs est nécessaire pour la survie de ceux-ci, nous anticipons que l'expression d'OTX2 YL dans l'EPR serait sans effet dans la rétine de souris OTX2 flox/flox. À notre grande surprise, l'épaisseur de la rétine pour laquelle OTX2 YL a été exprimée dans les RPE, était similaire à celle exprimant OTX2 de type sauvage et supérieure à celle de la rétine n'exprimant pas OTX2 endogène. Cette constatation doit être prise avec précaution car elle est basée sur une seule expérience contenant une seule souris par traitement. Si un effet neuroprotecteur de OTX2 YL sur les RP est établi, cela indiquerait que l'internalisation de OTX2 n'est pas nécessaire pour obtenir un effet. Cela favoriserait une action d'OTX2 dans l'EPR, peut-être en association avec un ou plusieurs autres facteurs de survie, dans la survie des PRs. D'autres expériences seront nécessaires pour approfondir cette question.

Chapter 5. General Discussion

OTX2 is a homeoprotein transcription factor expressed in the CNS including the retina. In mouse, at E5 *Otx2* is expressed in the visceral endoderm and epiblast. By E7 it is expressed throughout the embryo (Acampora et al., 2009; Acampora et al., 2001). However, by late embryonic and early postnatal stages *Otx2* is dominantly expressed in the neural retina, retinal pigment epithelium (RPE), and choroid plexuses (Nothias et al., 1998). In the neural retina *Otx2* is expressed in the RPE, ONL, and the INL, but not in GCL.

OTX2 is critical for retina development and it regulates maturation and homeostasis of photoreceptors. In the photoreceptors, genes such as *Vsx2* and *Blimp1* are directly regulated by OTX2. In fact OTX2 is a key modulator for differentiation of progenitor cells to photoreceptor or bipolar cells. For instance, *Otx2* expression initiates *Blimp1* expression causing an increase in *Blimp1*. In turn this increased expression of *Blimp1* causes *Vsx2* expression to increase. In this case the cell is presented with two possibilities for cell determination (Chapter 1, Figure 4). If *Vsx2* and *Otx2* are highly expressed in a progenitor cell, it is fated to become a bipolar cell. But if *Otx2* expression is low and *Vsx2* is absent the cell differentiates into a rod photoreceptor (Kim et al. 2008, Brezinski et al., 2013; Emerson & Cepko 2011). By regulating expression of *Vsx2*, OTX2 prevents maturation of bipolar cells and cone cells. Hence, interfering with *Otx2* expression during maturation of the retina will prevent maturation of photoreceptor and bipolar cells (Cepko., 2015).

In postnatal and adult mice OTX2 is also essential. OTX2 binds to promoter regions of *Crx* and *Nrl* (White et. al., 2016). Once *Crx* expression is initiated, CRX can substitute for OTX2 in binding to the *Nrl* promoter and maintaining the photoreceptor cell fate (Samuel et. al., 2014; Montana et al., 2011). Initially, both CRX and OTX2 are needed for photoreceptor development because they bind to promoter regions of *Nrl* in postnatal mice (Furukawa et al., 1999; Nishida et al., 2003). In adulthood, only the expression of *Crx* is necessary to maintain photoreceptor cell fate (Cepko., 2015). A loss of both *Crx* and *Otx2* results in dramatic loss of *Nrl* activity leading to photoreceptor degeneration. This suggests that while OTX2 is important in the adult retina to maintain photoreceptor cells it can be substituted by CRX, but only after OTX2 has initiated *Crx* expression. All of the activities of OTX2 discussed above are considered to be cell autonomous.

Further evidence for a role in adult retina comes from *in vitro* and *in vivo* studies on RGCs. Internalized exogenous OTX2 by the RGCs can be trans-synaptically transferred to parvalbumin cells in the visual cortex (Sugiyama et al., 2008). OTX2 is not expressed in RGCs but exogenous protein added to cultures of dissociated adult retina greatly enhances RGC survival (Torero Ibad et. al., 2011). OTX2 acts directly on RGCs since purified adult rat RGCs respond to OTX2. *In vivo*, exogenous OTX2 is taken up by the RGCs where it protects them against NMDA excitotoxicity and prevents visual dysfunction (Torero Ibad et. al., 2011). When OTX2 internalization is abolished, OTX2 no longer promotes survival of adult RGCs. Thus, OTX2 neuroprotection of RGCs occur non-cell autonomously.

The *Otx2* locus is active in RPE cells and they contain the protein (Chapter 1, Figure 3). When OTX2 is conditionally knocked-out in the RPE, PRs degenerate (Béby et. al. 2010). Importantly, OTX2 expressed in RPE in mouse retinas depleted of endogenous OTX2 is sufficient for PRs survival (Housset et al., 2013). This shows that OTX2 expression in the RPE is necessary for PR survival, but it remains unknown if OTX2 acts cell autonomously in RPE cells to provide survival factors/signals, and/or if OTX2 is transferred to PRs to act non-cell autonomously to ensure long-term survivability of photoreceptors.

The objective of this thesis was to study the non-cell autonomous effects of OTX2 in the retina and in particular examine any neural protective effect of OTX2 on PRs.

The approach used in a first set of studies consisted of an *Otx2scFv* plasmid construct expressing the single chain antibody with six MYC-tags and GFP. *Otx2scFv* expression is induced by CRE recombinase that recombines at Lox-p sites to remove a stop cassette allowing the OTX2scFv to be transcribed. The construction contains a 2A skipping peptide sequence between OTX2scFv-myc and GFP allowing for the separation of the two proteins. The single chain antibody against OTX2 is secreted into the extracellular milieu; where it binds to non-cell autonomous OTX2. It is important to note that this mouse has been used to block OTX2 transfer in the mouse visual cortex (Bernard et al., 2016). A similar genetic strategy was used with success to block ENGRAILED transfer in the *Drosophila* wing disk and optic tectum (Layalle et al. 2011; Wizenmann et al. 2009) and PAX6 transfer in the zebrafish eye anlagen (Lesaffre et al. 2007) and embryonic cerebral cortex.

To study the effects of non-cell autonomous OTX2 in the retina cells we crossed *Otx2scFv* with *PV-Cre* mice. PV is expressed by amacrine and RGC cells, so that in *PVCre::scFvOTX2* mice OTX2scFv would only be expressed and secreted from these cell types. In the retina, *Otx2-scFv* mRNA expression was detected by RT-qPCR and fluorescent in situ hybridization (RNAscope). Full length OTX2-scFv expression was confirmed by immunoprecipitation and western blot of retinal lysates.

Parvalbumin starts to be expressed in RGCs around P11, after the birth of all retinal cell types (Cepko et al., 1996; Young, 1985). And PV gene expression is reported in the amacrine cells and RGCs (Haverkamp and Wässle, 2000; Haverkamp et al., 2009). We used RNAscope to validate the expression of PV gene and *Otx2-scFv* in the amacrine and RGC cells. To our surprise not all RGC and amacrine cells expressed *pv* and *Otx2-scFv* mRNA (Chapter 3 Figure 10). There are three possible explanations for our observation. Firstly, the CRE recombination event was not efficient and may have removed genetic elements necessary for *Otx2-scFv* expression in some cells. Though the CRE recombination system is accurate, it is reported to making errors, since recombination depends on the length of DNA between loxP sites (Zheng et. al., 2000; Liu et. al., 2013). Secondly, it is possible that our mice have heterozygous population of RGC and amacrine cells (Volgyi et. al., 2009; Bailes et. al., 2006). This could result in some cells not expressing *pv*. Finally, it is possible that PV expression peaks between P11 and P30, but at P30 cells lower their PV expression. This would explain why not all of our amacrine and RGCs show *pv* and *Otx2-scFv* mRNA (Chapter 3 Figure 10). Nonetheless, expression of OTX2-scFv was sufficient to produce a reliable retinal phenotype.

In the *PVCre::scFvOTX2* mice, we observed a significant loss of visual acuity. The loss of visual activity occurred in the absence of changes in retinal structure or anatomical organization. All layers were present and RT-qPCR for different retinal cell types suggest that all retinal cells were present and in appropriate numbers. Electrophysiology revealed no dysfunction of cones, rods or bipolar cells. Interestingly the flicker response was altered in the *PVCre::scFvOTX2* mice. The flicker response arises in the GCL (amacrine and RGCs) and reflects cone OFF pathway function (Tanimoto et. al., 2015). Amacrine cells and RGCs do not express OTX2, but they take it up from the inner nuclear layers (Sugiyama et al. 2008; Torero Ibad et. al., 2011). Here we showed that this secreted extracellular OTX2 from the inner retina has functional effects on GCL cells and on vision.

This is the first evidence for OTX2 non-cell autonomous activity in a primary sensory function and the second example in the retina, the first being RGC survival. It is interesting to note that OTX2scFv was low and in fewer cells than expected. And the amount of full length OTX2scFv immunoprecipitated for the retina was low. Yet, the results show that even low levels were enough to produce functional and visual changes.

How can a small quantity of OTX2-scFv have an apparent effect? Previous work has estimated that less than five percent of homeoprotein is transferred between cells (Wizenmann et al., 2009). But this is sufficient for non-cell autonomous activity on axon guidance by ENGRAILED (Brunet et. al., 2005; Wizenmann et al. 2009; Stettler et. al., 2012). Another study showed that non-cell autonomous OTX2 had mitochondrial protective effect on bipolar cells (Kim et. al., 2015). Thus, it appears that even though OTX2 is highly expressed in ONL and in INL, the small amount of transferred protein has important functional consequences. Thus, even low expression of OTX2scFv is enough to alter the non-cell autonomous OTX2 activity in the retina and produce the phenotype observed in *PVCre::scFvOTX2* mice.

Our results using *PVCre::scFvOTX2* mice demonstrate another non-cell autonomous activity in the retina. Coupled with results showing that OTX2 expression in RPE cells is necessary for PR survival we started to explore if this was yet a third non-cell autonomous activity of OTX2.

We demonstrated that exogenous OTX2 was able to bind the IPM, ONL, and INL on sections of retina. Previous studies on cortex demonstrated that OTX2 bound to GAGs around PV-cells (Beurdeley et. al., 2012). Similarly, we observed that OTX2 binding to retina depends on GAGs and the RK peptide N-terminal to OTX2. Thus the same mechanism for OTX2 binding in non-cell autonomous activity in the visual cortex also applies to the retina. RPE OTX2 may stimulate PR survival by cell autonomous activity in RPE cells or by transfer and non-cell autonomous action on PRs. For the second possibility we focused on PR outer segments in the IPM. First, we detected endogenous OTX2 in the IPM and observed exogenous OTX2 binding to this layer. Second, RPE microvilli interdigitate with the OS and if RPE is a source of OTX2, they are in close proximity to the OS domain of PRs, allowing for effective transfer. Third, we considered that the three dimensional structure of the PR/OS might minimize any OTX2 synthesized by the PRs from reaching the OS.

The OS is attached to the PR soma by a connecting cilium at the PR soma basal body. The connecting cilium has a diameter about 0.3 μm in which microtubules are the densely organized (Chen and Sompath 2013). Typically, this is too small for mitochondria to pass and they densely accumulate at the basal body level. It is thus a possibility that OTX2 from the PR cytoplasm does not reach the OS and that OTX2 observed in the IPM originates from the RPE.

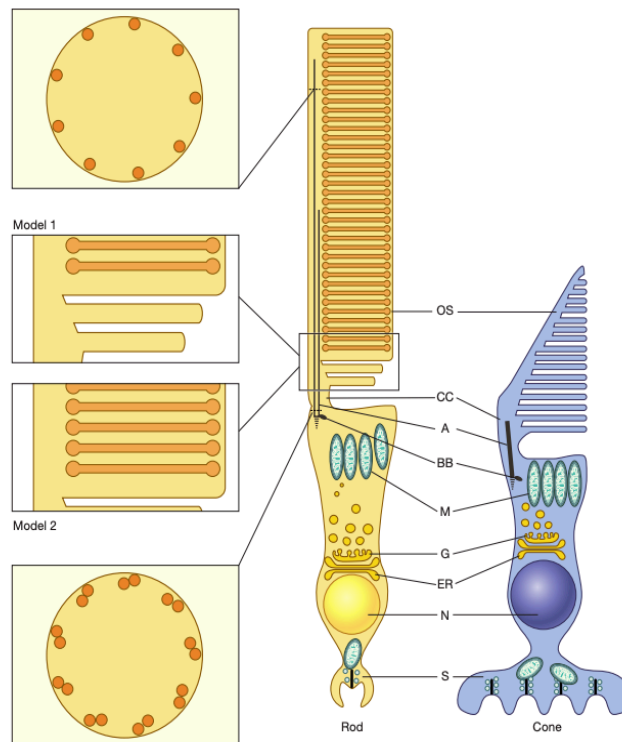


Fig 27. Organization and structure of Photoreceptor.

OS, outer segment; CC, connecting cilium; A, axoneme; BB, basal body; M, mitochondria; G, Golgi apparatus; ER, endoplasmic reticulum; N, nucleus; and S, synaptic terminal. Insets depict the structure of the axoneme at the level of the outer segments where microtubule structure is $9 \times 1 + 0$ (top), and where the inner and outer segment join the microtubule structure is $9 \times 2 + 0$ (bottom). In model 1, outer-segment discs originate from evaginations of the plasma membrane but become closed off and separate from the plasma membrane. In model 2, newly generated outer-segment discs are already closed and separate from the plasma membrane. Taken from Chen and Sampath 2013.

Since there is no known protein translation machinery in the PR outer segments, any OTX2 present would have an alternative source. To answer the question if OTX2 from RPE is transferred to PRs, we infected RPE cells with tagged OTX2. Immunohistochemistry staining failed to visualize the M2-tagged OTX2 in the IPM or elsewhere in the retina. We then tried using another approach to detect tagged OTX2 from the RPE in the IPM. Using laser capture dissection we isolated IPM from infected retinas expressing tagged OTX2, followed by western blot. This

approach also did not detect tagged OTX2 in the IPM. This could be because either OTX2 from RPE does not transfer to PRs, or we are limited by our methods. Hence, another possible approach would be to use mass spectrometry on laser dissected OS to detect tagged OTX2.

When studying the internalization of non-cell autonomous OTX2 it is important to keep in mind that the protein may internalize quickly and not remain at the surface of the cell for long. In addition, in all studies from the laboratory on homeprotein transfer, it was estimated that less than five percent of the nuclear content are found extracellularly making extracellular HPs difficult to detect if rapidly internalized. We thus do not preclude, in spite of our negative results that a small amount of tagged OTX2 is that we were unable to detect.

To study the effects on PRs of any non-cell autonomous OTX2 originating from the RPE, we could use the single chain antibody mice (*Otx2scFv*). In order to recombine and express the *Otx2scFv*, we have developed viruses expressing the Cre recombinase under the control of RPE-specific promoters. This technique would allow us to express and secrete OTX2scFv from the RPE and block non-cell autonomous OTX2 in the IPM. This may allow us to demonstrate a direct non-cell autonomous neuroprotective effect of OTX2 in the IPM and on the photoreceptors, without disturbing cell autonomous OTX2 in the PR. These experiments are being planned. One possible limitation of this approach in the eye is a limited availability of OTX2scFv. The mouse eye has only fifty thousand RPE cells for about 6.5 million PRs (Jeon et. al., 1998). Whether expression of the scFv in the RPE will regionally block all RPE-originated OTX2 is unknown (Fernandez-Godino et. al., 2016). However, based on our *PVCre::scFvOTX2* experiments (Chapter 3), a small amount of secreted OTX2scFv may be sufficient to alter non-cell autonomous OTX2 activity in PRs as we show for GCL function (Chapter 3).

We hypothesise that internalized non-cell autonomous OTX2 could increase PR survival by neutralization of ROS. Previous experiments conducted in the lab demonstrated that OTX2 protects RGCs against NMDA excitotoxicity and embryonic midbrain and retinal cells against H₂O₂ oxidative stress (Torero Ibad et. al., 2011; Abonce et. al., 2019). Our results show that exogenous OTX2 reduces ROS of oxidative stress *ex vivo*. Here we show that at least one protein involved in neutralizing H₂O₂ is maintained by exogenous OTX2. Retinas treated with OTX2 had elevated expression of GPX1 protein, which neutralizes hydrogen peroxide (Figure 28). These preliminary data suggests that OTX2 has an effect on ROS pathway.

However, extensive experimentation is required to further validate the role of OTX2 in ROS pathway and concretely demonstrate that OTX2 PR neuroprotection occurs via modulation of the ROS pathway.

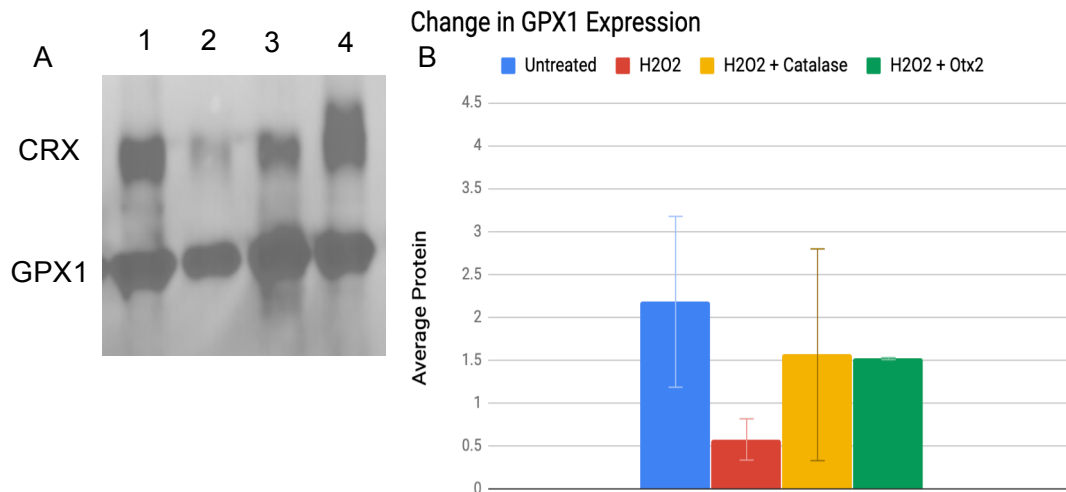


Fig 28. Ex-vivo Retina Treated H2O2 and OTX2.

(A) Western blot for GPX1 protein. CRX used as a loading control. (1) Untreated, (2) H₂O₂ (500mM), (3) H₂O₂+ Catalase (500U), (4) H₂O₂ + OTX2 (300 ng/ul). **(B)** Shows the quantification of the western blot. Retinas treated with OTX2 have larger amount of GPX1 relative to retinas treated only with H₂O₂. This implies that OTX2 is involved in the modifying ROS pathway genes. n=3

Another hypothesized role of non-cell autonomous OTX2 can be its effect on ribbons synapses of the IPL. The *PVCre::scFvOTX2* study demonstrated that secreted OTX2scFv is sufficient to impair visual acuity (Chapter 3). In addition, we demonstrated that exogenous OTX2 could bind the retina IPL (Chapter 4 Figure 20). The IPL contains specialized ribbon synapses formed between bipolar and RGCs (Dembla et. al., 2018). Ribbon synapses transmit visual information from the photoreceptors to the bipolar neurons and bipolar to RGCs (Assawachananont et. al., 2018). The homeoprotein, CRX (OTX3) regulates the development of cytomatrix active zone and anchoring of ribbons in the OPL (Assawachananont et. al., 2018). Mice deficient in *Crx* are unable to anchor ribbons synapses in the presynaptic photoreceptor terminals and PRs degenerate (Assawachananont et. al., 2018).

Based on this study it is plausible to suggest that OTX2 could also be essential for maintenance of ribbon synapses in adulthood. OTX2 and CRX share common characteristics. For instance, both are within the OTX homeoprotein family, they structurally resemble each other, they both function synergistically in adult

photoreceptors, and both can be transferred between cells, although secretion of CRX is low (Furukawa et. al., 1997; Cepko., 2015; Assawachananont et. al., 2018; Lee et. al., 2019 Figure 4). These similarities raise a possibility that OTX2 may act at the ribbon synapses. We can speculate that blocking extracellular OTX2 from reaching IPL prevented OTX2 internalization at the ribbon synapse. This lack of OTX2 could have affected the function of ribbon synapses in the IPL and cause a decrease in visual acuity. To generate this loss of visual acuity only a small amount of non-cell autonomous OTX2 may need to be blocked from reaching its target in the IPL. For future studies, it would be interesting to examine the interaction between OTX2 and ribbon synapses in the *Otx2scFv* mice or *Otx2^{+/-}* mice.

Previous studies have shown that virus-mediated reintroduction of wild-type *Otx2* in the RPE is sufficient to rescue PRs (Béby et. al. 2010; Housset et. al., 2013). In addition, other studies have demonstrated that OTX2 rescues RGC against NMDA and protects bipolar cells against oxidative stress, while OTX2 YL did not provide neuroprotection (Torero Ibad et. al., 2011; Kim et. al., 2015), probably because OTX2 YL, although it can be secreted, cannot be internalized (Torero Ibad et. al., 2011; Kim et. al., 2015). In the present work we used a viral based approach to express *Otx2 yl* specifically in the RPE. In a very preliminary study OTX2 YL in the RPE may have a possible effect on the PRs (Courtesy of Pasquale Pensieri, Annabelle Mantilleri and Thomas Lamonerie, Chapter 4 Figure 25 and 26). This could be because OTX2 YL has normal transcriptional activity in RPE. This RPE specific expression of *Otx2 yl* could still act cell autonomously on other non-cell autonomous targets of OTX2, similar to those of wild-type OTX2 to provide neuroprotection to PRs. Another intriguing possibility is that non-internalized extracellular OTX2 may be protective in this system, suggesting a novel signaling pathway. However, the latter results were obtained only once and need to be replicated before one envisages a further dissection of the mechanism.

In conclusion, this thesis shows that blocking non-cell autonomous OTX2 in the retina alters inner retinal function and impairs vision. We moved to extend this to study the effects of non-cell autonomous OTX2 from RPE to PRs and establish neuroprotective effects of this OTX2. Indeed, we were able to demonstrate that OTX2 is present in the IPM layer and that exogenous OTX2 binds to GAGs around the cells that are best candidates for its internalization. In terms of neuroprotective effects of OTX2, *ex vivo* exogenous OTX2 elevated GPX1 levels, indicating that OTX2 could aid in neutralizing oxidative stress. However, we have not yet been able

to demonstrate transfer of OTX2 from RPE to IPM, but this is still ongoing work in the laboratory.

An interesting experiment that can further reveal the roles of GAGs in OTX2 capture and retinal degeneration would be to study cKO model for *Chpf*^{-/-} mice. *Chpf* is a chondroitin polymerization enzyme and cKO mice do not produce GAGs. In the retina of *Chpf*^{-/-} mice, non-cell autonomous OTX2 would not be able to bind to GAGs and internalize. If these mice exhibit retinal degeneration it would support the idea that GAGs are required for non-cell autonomous OTX2 to be captured and internalized.

Whether OTX2 has a non-cell autonomous effect on PR survival or not, we have to consider cell autonomous activities at the level of the RPE. OTX2 in RPE cells may regulate the expression of growth/survival factors that then act on PRs. A first approach to investigate this could be the use of the RPE cell culture model established in this work. RPE from *Otx2*^{flox/flox} mice could be treated to remove *Otx2* expression. RNAseq comparison of the non-floxed and floxed RPE cells may reveal candidate genes for PR survival factors. These candidates could then be tested *in vivo* with the constructs developed in the course of my studies.

Résumé en Français

En conclusion, cette thèse montre que le blocage d'OTX2 secrété au niveau de la rétine altère la fonction rétinienne interne et nuit à la vision. Nous avons décidé d'étendre cette étude pour étudier les effets d'OTX2 non autonome produit par des cellules de l'EPR sur les PRs et d'établir les effets neuroprotecteurs de cet OTX2. En effet, nous avons pu démontrer qu'OTX2 est présent dans la MIP et qu'OTX2 exogène se lie aux GAGs autour des cellules qui sont les meilleures candidates pour son internalisation. En termes d'effets neuroprotecteurs d'OTX2, *ex vivo*, l'ajout d'OTX2 exogène a élevé les niveaux de GPX1, ce qui indique qu'OTX2 pourrait aider à neutraliser le stress oxydatif. Cependant, nous n'avons pas encore pu démontrer le transfert d'OTX2 de l'EPR à la MIP, mais ce travail est toujours en cours au laboratoire.

Une expérience intéressante qui peut révéler davantage les rôles des GAGs dans la capture d'OTX2 et la dégénérescence rétinienne serait d'étudier le modèle cKO pour les souris Chpf^{-/-}. La Chpf est une enzyme de polymérisation de la chondroïtine et les souris cKO ne produisent pas de GAGs. Dans la rétine des souris Chpf^{-/-}, l'OTX2 non cellulaire autonome ne serait pas capable de se lier aux GAGs et d'être internalisé. Si ces souris présentent une dégénérescence rétinienne, cela conforterait l'idée que les GAGs sont nécessaires pour qu'OTX2 non autonome soit capturé et internalisé.

Qu'OTX2 ait ou non un effet non autonome sur la survie de l'EPR, nous devons considérer les activités autonomes dans les cellules au niveau de l'EPR. OTX2 dans les cellules de l'EPR peut réguler l'expression des facteurs de croissance/survie qui agissent ensuite sur les PRs. Une première approche pour étudier cela pourrait être l'utilisation du modèle de culture cellulaire de l'EPR établi dans ce travail. Les EPRs de souris *Otx2*^{flox/flox} pourraient être traités pour éliminer l'expression d'*Otx2*. La comparaison du RNAseq des cellules de l'EPR non floxées et floxées pourrait révéler des gènes candidats pour les facteurs de survie des PRs. Ces candidats pourraient alors être testés *in vivo* avec les constructions développées au cours de mes études.

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RÉSUMÉ

L'objectif de cette thèse est d'examiner l'activité cellulaire autonome et non cellulaire de l'OTX2 dans la rétine. L'OTX2 est un facteur de transcription homéopathe exprimé dans la rétine qui peut se transférer entre les cellules et qui peut agir de manière autonome ou non. Nous avons utilisé une approche génétique pour bloquer le transfert de l'OTX2 dans la rétine et avons constaté une dégradation de la fonction visuelle sans altération de la structure ou de l'organisation de la rétine. De plus, la perte d'acuité visuelle a été associée à un changement de l'activité rétinienne interne. Ces résultats fournissent la preuve d'une activité non autonome des cellules OTX2 sur la fonction rétinienne.

Dans la deuxième partie, nous avons émis l'hypothèse que l'OTX2 de la couche RPE est transférée à la matrice interphotoréceptrice (IPM) contenant les segments externes des photorécepteurs, et que cette OTX2 transférée joue un rôle essentiel dans le maintien de l'homéostasie des photorécepteurs ou de la neuroprotection. Nous montrons que l'OTX2 endogène se lie à la matrice interphotoréceptrice *ex vivo* et que cette liaison se produit avec les GAG. Enfin, les résultats préliminaires montrent que l'OTX2 exogène réduit les niveaux de ROS nocifs dans la rétine *ex-vivo*. interphotoréceptrice, mais nous n'avons pas pu déterminer si cette OTX2 provenait de cellules RPE. Nous montrons que l'OTX2 endogène se lie à la matrice interphotoréceptrice *ex vivo* et que cette liaison se produit avec les GAG. Enfin, les résultats préliminaires montrent que l'OTX2 exogène réduit les niveaux de ROS nocifs dans la rétine *ex-vivo*.

MOTS CLÉS

Rétine, L'activité cellulaire autonome OTX2, Cellules ganglionnaires rétinienne, Photorécepteurs, Neuroprotection et scFvOTX2

ABSTRACT

The objective of this thesis is to examine the cell autonomous and non-cell autonomous activity of OTX2 in the retina. OTX2 is a homeoprotein transcription factor expressed in the retina that can transfer between cells and that can act cell autonomously and non cell autonomously. We used a genetic approach to block OTX2 transfer in the retina and found a degradation of visual function with no alteration in retinal structure or organization. Furthermore, the loss of visual acuity was associated with a change in inner retinal activity. These results provide evidence for OTX2 non cell autonomous activity on retinal function.

In the second part we explored if OTX2 also has neuroprotective effect on photoreceptors. We established that OTX2 is present in the interphotoreceptor matrix of WT mice, where photoreceptor outer segments are located. We also found that exogenous OTX2 is able to bind glycosaminoglycans (GAGs) in the interphotoreceptor matrix. We also found that in *ex vivo* retinas, exogenous OTX2 is able to modulate mitochondrial reactive oxygen species pathway gene to protect retinal cells against oxidative stress.

KEYWORDS

Retina, Non-Cell Autonomous OTX2, Retinal Ganglion Cells, Photoreceptors, Neuroprotection, and scFvOTX2.