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Molecular tools to study the development and organization of the retinal neuronal networks

Summary of the Doctoral Thesis

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Key words

Retina

Retinal Ganglion Cells

Brn3

Development

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Introduction

Vision represents the most important of our senses, providing approximately 80% of the information concerning the elements beings relate with. Although all the structures that form the eye contribute to high quality vision, the retina represents the most complex and essential part.

The Retina

Similar to many other structures of the central nervous system (CNS), the retina is a complex neural circuit responsible for detecting, integrating and transmitting light related information into specific patterns of electric impulses that travel to higher centres of the brain.

The neuronal retina is a laminated structure. It is composed of five different classes of cells (photoreceptors, bipolar, horizontal, amacrine and ganglion cells) organized in three nuclear layers and two plexiform layers where different cell types establish their synaptic connectivity (Fig.1). In addition, photoreceptors are protected by and are in metabolic interactions with an epithelial layer formed by the retinal pigment epithelial cells (RPE) while glial support is provided by Muller cells, astrocytes and resident microglia.

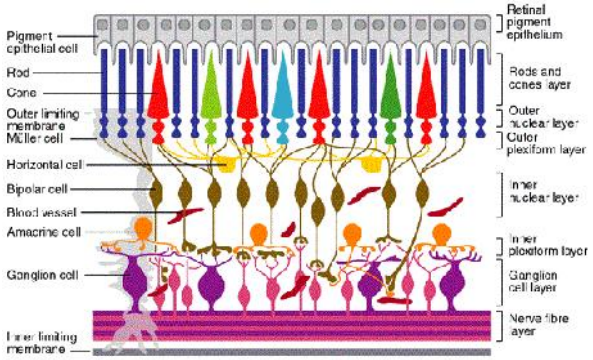


Figure 1. Schematic representation of the mammalian retina (Wilkinson-Berka, 2004).

Retinal Ganglion Cells (RGCs), the main focus of this work, are the output neurons of the retina, collecting information from their pre-synaptic partners (amacrine and bipolar cells) and transmitting it further to the brain by means of their axons, which fasciculate and form the optic nerve. The axons of RGCs coming from both eyes meet at the optic chiasm, and segregate into the optic tracts of the same (ipsi-) or opposing (contralateral) brain side. Similar to other cell types in the retina, RGCs consist of different subtypes, whose number has been changing in the scientific literature. The diversity of RGCs is related to the number of different types of

information streams than need to be transmitted to distinct brain nuclei, and to what extent this is processed at the level of the retina or in the upper visual areas in the brain.

The characterization of mammalian RGCs has initiated with morphological studies on buffalos and dogs carried on by Ramon y Cajal (Kolb, http://webvision.med.utah.edu) and continued by several other groups that described their variety in different species: monkeys (Rodieck and Watanabe, 1993), cats (Boycott and Wassle, 1974), rabbits (Rockhill *et al*., 2002), rats (Sun *et al.*, 2002) and mice (Badea and Nathans, 2004), to mention only a few. In general, morphological characterizations are based on the size of the cell body as well as that of the dendritic arbor and its lamination in the IPL (Coombs *et al.*, 2006). There are 22 subtypes RGCs observed by Cajal in his early studies developed indogs (200 years ago), 13 and 15, respectively in more recent classifications done by Rockhill and collaborators, as well as Badea and collaborators. Besides that, different recent studies carried on in the mouse report different numbers of the RGCs subtypes, ranging from 11 to 22 (reviewed by Masland, 2012).

Functionally, it is clear that RGCs differ among each other, as they receive different synaptic input and, in turn, they project to a diverse palette of structures in the brain. Fibers that reach the superior colliculus (SC) are thought to mainly integrate the head and eye movements, whereas the ones that project to the lateral geniculate nucleus (LGN) – then ultimately, to the visual cortex – are responsible for transmitting information related to contrast, chromatic content, shape and motion, and ultimately contribute to conscious vision. Fibers reaching nuclei like the olivary pretectal nucleus (OPN) and the suprachiasmatic nucleus (SCN) transmit non-image information that is instrumental to pupillary light reflex and circadian photoentraiment, respectively. In addition, medial, lateral and dorsal terminal nuclei process vestibulo-ocular coordination.

Most of our knowledge on the physiology of the different RGC subtypes was gathered during the last decades. RGCs are the only neurons in the retina that respond with action potentials upon stimulation. They are susceptible to different type of stimuli like direction, speed of motion, size, brightness and color (R. Nelson, webvision.med.utah.edu). However, the precise process by which the information received in the retina is transformed by the RGCs and is perceived as visual information by the brain is still under investigation. In addition, subsets of ganglion cells that perform functions that are not related to image formation, called intrinsically photosensitive retinal ganglion cells – ipRGCs (Berson, 2003; Rollag *et al.*, 2003). These

neurons represent a small fraction of the total number of RGCs (1-8%), with wide dendritic arbors that cover the entire retinal surface. They use an opsin-based photopigment called melanopsin and are responsible for tasks like pupillary light responses and circadian entrainment. (Hattar *et al.*, 2002).

The differences in terms of morphology and physiology among various RGCs are generated by differences in their intrinsic programs and the specificity of the underlying retinal synaptic circuitry, shaping ganglion cell physiology. Obviously, this complex architecture is not the simple result of a strict genetic developmental program, but partly a matter of interplay between the ganglion cells themselves. On the other hand, a strict genetic developmental program directs its general phases, with different molecules acting specifically in different cellular subtypes ultimately dictating their identity and function.

What creates diversity in the retina?

In terms of the genetic program leading to the formation of the mammalian retina, many cascades of different genes interplay during development. A very interesting fact is that all five neuronal classes share a common progenitor – the retinal progenitor cell – RPC (Turner and Cepko, 1987). In the mouse, the main model object of this study, histogenesis takes place from embryonic day 11.5 (E11.5) to postnatal day 11 (P11). The first neurons formed are the ganglion cells, followed, in order, by the horizontal cells, cone photoreceptors, amacrine cells, bipolar cells and rod photoreceptors.

In order for all the eye elements to be formed, a hierarchical set of transcription factors become active as early as E8.5 in the anterior neural tube. These are frequently called eye field transcription factors (EFTF). EFTSs are generally highly conserved among the species, from Drosophila, to C. elegans and humans. The most important ones, Otx2, Pax6, Rx, Lhx2 and Six3 are strictly required for eye formation, their miss-expression resulting in severe eye defects, like small eye phenotypes or complete loss of eye formation (Marquardt *et al*., 2001; Porter *et al.*, 1997; Zuber *et al*., 2003; Mawersik and Maas, 2000). In addition to the transcription factors (TFs), signaling molecules like Notch, Wnt and Shh (and their downstream molecules) play an important role in the formation of the eye cup.

In the retina, the RPCs first differentiate into RGCs, as these are the first cells to form. For that task, the first active transcription factor is Math5, a homolog of the Drosophila Athonal

gene (Brown *et al*., 1998). Mutant mice lacking this gene have only less than 1% RGCs (Brown *et al*., 2001; Wang *et al*., 2001). In addition, expression of Math5 is observed in other cell types (Yang *et al*., 2003), including RPCs. The next important transcription factor, this time expressed only at the time when RPCs exit the cell cycle and became post mitotic neurons, is Brn3b/Pou4f2 (Xiang *et al.*, 1995, Xiang *et al.*, 1996, Erkman *et al.*, 1996). Its ablation also affects a majority of RGCs. Later, Brn3a and Brn3c, two other transcription factors from the POU domain family, closely related to the one illustrated above, are also expressed in RGCs selectively. During development, other sets of TFs contribute with their activity to the generation of RGCs and play important roles in their correct patterning: members of the Iroquois family – Irx1, Irx2, to Irx6, lim-homeo-domanin TFs like, Islet1 and Islet2, zinc fingers like Gfi1, Olf 1 and Olf2 (reviewed by Mu and Klein, 2004) as well as Dlx1 and Dlx2 (Eisenstat *et al.*, 1999).

Like in the case of the ganglion cells, all the other cells that are formed at later stages require the activity of transcription factors for lineage commitment. Mash1 (homologue of *Drosophila*'s Achete-Scute) is important for the correct differentiation of bipolar and horizontal cells and to some extent, of photoreceptors (Tomita *et al*., 2000). NeuroD is a regulator of decision of neuronal versus glial fate, as well as of the proper development of amacrine and bipolar cells (Yan *at al*., 2005). Nrl, Crx, Nr2E3 are key transcription factors for the development of photoreceptors (Swaroop *et al*., 1992; Cheng *et al*., 1997; Chen 1999).

Brn3 transcription factors

During past 2 decades, considerable knowledge has been gained on the role of Brn3 transcription factors, and especially of their function in the retina, by means of mouse mutant lines created and analyzed by several groups of researchers: Xiang, Nathans, Erkman, Turner, Rosenfeld, Gan, Klein, Wang, Mu, Badea.

It was shown that Brn3 factors play an important role in the mammalian sensory neuronal development (Xiang *et al.*, 1998), the three of them (Brn3a, Brn3b and Brn3c) being expressed in distinct patterns in the retina, auditory and vestibulary neurons, dorsal root and trigeminal ganglia.

In the retina Brn3s, are expressed in an overlapping pattern (Badea and Nathans, 2011), highly specific for the ganglion cells. Chronologically, Brn3b is the first transcription factor of this family that is expressed, beginning at E11.5, approximately when the RPCs start to become post-mitotic, committing to a ganglion cell fate. As retinal development progresses, Brn3b

expression expands circumferentially towards peripheral retinal regions, peaking at E12.5 to E15.5 when the majority of RGCs are generated. Expression of Brn3a begins one day later, followed by Brn3c, the last of the Brn3 genes to be expressed in the retina (Xiang *et al.*, 1995; Gan*et al*., 1999).

Different mouse lines carrying mutations in the Brn3 genes show interesting phenotypes, in particular the Brn3b mutants. Deletion of Brn3b results in 70%-80% loss of the RGCs, with the remaining 30% suffering severe intra, as well as extra retinal defects, mostly related to their axonal integrity and path finding towards the optic nerve head (Erkman *et al.*, 1996; Gan *et al.*, 1996; Erkman *et al.*, 2000; Badea *et al*., 2009a). Regarding Brn3a, the analysis of a homozygous mutant is impossible in postnatal stages, as they are postnatal lethal, probably due to the severe defects in the trigeminal and dorsal root ganglia (McEvilly *et al*., 1996; Xiang *et al.*, 1996; Eng *et al.*, 2001). However, deletion of the Brn3a specifically in the retina results in effects in dendritic arborisation of the RGCs and distribution in the IPL (Badea *et al*., 2009a). Conversely, the general aspect of the retina appears to be completely unaffected by deletion of Brn3c, the third TF of the family.

Aims of the thesis

From a functional point of view, the retina is composed by three general parts: the light detectors (the photoreceptors), the circuitry that encodes the information provided by photoreceptors (horizontal cells, bipolar cells and amacrine cells) and the final output (the retinal ganglion cells). The main focus of this work is represented by the ganglion cells, mainly considering three aspects:

- It is known that in a retina in which photoreceptors degenerate, second order neurons undergo regressive remodeling and partial degeneration. This process seems to spare ganglion cells, suggesting their partial independence from the other neuronal types. In this work the opposite situation is investigated, asking what changes occur in a retina that lacks a major fraction of ganglion cells.

- Second, the attention of this thesis is focused on the development of ganglion cells, investigating which are the genes that determine distinct features of retinal ganglion cells, making them different in terms of types and yet homogeneous enough to constitute a discrete class. These molecules, if expressed under controlled conditions, could potentially help to the regeneration of these particular neurons.

- The third objective is the generation of two mouse models using gene targeting to facilitate further high resolution analysis of RGC development and organization. These experiments should provide new notions in perspective instrumental to address pathologies affecting specifically ganglion cells.

- The forth objective of the presented thesis is the development of different cell lines using the optogenetic tool, the light sensitive channel – Channelrhodopsin. Such cell lines should provide useful information, eventually for the investigation of neuronal circuit.

Materials and methods

1. Analysis of the inner plexiform layer in the Brn3b$^{CKOAP/-}$ mutants

Retinas of Pax6αCre;Brn3b$^{CKOAP/-}$ (KO) and Pax6αCre;Brn3b$^{CKOAP/+}$ (WT) adult mice were analyzed by IHC studies. The Pax6αCre;Brn3b$^{CKOAP/-}$ mutant animals present a peculiar recombination pattern, driving a similar expression of the AP reporter, with a wedge shape oriented dorso-ventral in which recombination has taken place at a lower degree. The retinas show a portion with lower recombination and one in which the recombination happens at high intensity, resulting in a complete mutant phenotype (Marquardt *et al.,* 2001). Double immunostaining with different antibodies presented in Table 1 was carried out for the analysis of the retinas of different genotypes presented above.

Table 1. Primary antibodies used for sections

Antigen	Antiserum	Localization	Working dilution	Provider	Cat. number
Brn3b	Rabbit anti-Brn3b	Ganglion cells	1:10	Xiang *et al.*, 1995	
Cabp5	Rabbit anti-Cabp5	Bipolar cells	1:200	Haeseleer*et al*., 2004	
Calbindin	Rabbit anti-calbindin	Horizontal cells	1:500	Sigma	C2724
Calretinin	Rabbit anti- calretinin	Cone bipolar cells	1:1000	Swant	7699/4
Choline acetyl transferase (ChAT)	Goat anti-ChAT	Cholinericamacrine cells	1:1000	Chemicon	AB1144P
Connexin36	Rabbit anti-Cnx36	Electric synapses	1:500	Invitrogen	364600
CtBP2	Mouse anti-Ctbp2	Ribbon synapses	1:500	BD	612044
Neurofilament 200	Mouse anti-NF200	GCs arborization	1:200	Sigma	N0142
PKCα	Mouse anti- PKCα	Rod bipolar cells	1:800	SantaCruz	Sc-208
PSD95	Mouse anti-PSD95	Post-synaptic densities	1:500	Ab Cam	Ab13552
Synaptotagmin	Mouse anti- ZNP1	Cone bipolar cells	1:1000	ZIRC	

2. Expression profile analysis of gene sets downstream of Brn3a and Brn3b from a highly enriched population of RGCs

Retinas extracted from offspring resulting from the mating of Brn3$^{WT/KO}$; Pax6α:Cre x Brn3$^{CKOAP/CKOAP}$ were dissociated either at E15 or at P3. Therefore, the retinal ganglion cells

(RGCs) have either Brn3$^{AP/WT}$ (WT) or Brn3$^{AP/KO}$ (KO) genotype, and thus phenotypically they are similar to WT and KO mutants respectively.

For the dissociation of the tissue a papain based protocol was used. Afterwards, to obtain a highly enriched population of RGCs, the dissociated retinas were processed using an immunomagnetic purification protocol employing the Dynal^{TM} Beads Pan Mouse IgG (Invitrogen, USA). The RNA was extracted from the purifcated RGCs and pooled 3 to 4 samples of the similar genotype for the final experiment – the RNA sequencing.

Data obtained was analyzed by several criteria of selection. To begin with, the "WT samples" are always referred to be the results from the RNA sequencing of RNA samples extracted from Brn3a/b$^{AP/WT}$, animals known to have phenotype similar to their complete WT mates. On the other hand, the "KO samples" represent the results of the RNA sequencing of RNA samples extracted from Brn3a/b$^{AP/KO}$, known to have similar phenotype as a homozygous KO. The "retina samples" represent the information obtained from sequencing of the RNA extracted from supernatants of either WT or KO samples. An additional analysis compared "RGC samples" that are either WT or KO to "retina samples" that are either WT or KO.

From the beginning of the analysis, all the samples with FPKM (Fragments per kilobase of exon per million fragments mapped) values lower than 2 were excluded from the data set. Additionally, a threshold of 2 fold was set when running different comparisons between data sets.

3. Dual recombinase dependent conditional knock-in reporter strategy for the study of development and function of Retinal Ganglion Cells

The development of new mutant mouse models requires extensive analysis of the gene to be replaced and accurate planning. Besides the basic elements that replace the endogenous gene, components like additional terminator signals are also very important. Moreover, screening methods for the integration of the construct into the ES (embryonic stem) cell line require additional attention and planning. The typical workflow for the generation of new mutant models using homologous recombination follows several steps: targeting vector design, transfection of the targeting construct into ES cells, chimera production and heterozygous mutant production. In *Mus musculus* the Brn3a (Pou4f1) gene is composed by 3 exons (358, 1186, 2224 bp long

respectively), whereas Brn3b (Pou4f2) has only 2 exons (294, 942 bp). In the targeting constructs, the first recombination site (RoxP), required for the Dre recombinase activity, is placed 42 bp/ 98 bp upstream the ATG initiator codon in the Brn3a^{CKOCre}/ Brn3b^{CKOCre} respectively. Downstream the 3'UTR, 3 repeats of the SV40 early region transcription terminator were added, 48 bp (for *Brn3a*) and 340 bp (for *Brn3b*) 3′ of the Brn3 translation termination codon, followed by a second RoxP site. Immediately adjacent to the second targeting site one can find the ORF for the Cre recombinase, which, after the recombination of the 2 RoxP sites under the exposure to the Dre recombinase, replaces the Brn3 endogenous gene, and is expressed further on under the Brn3 promoter.

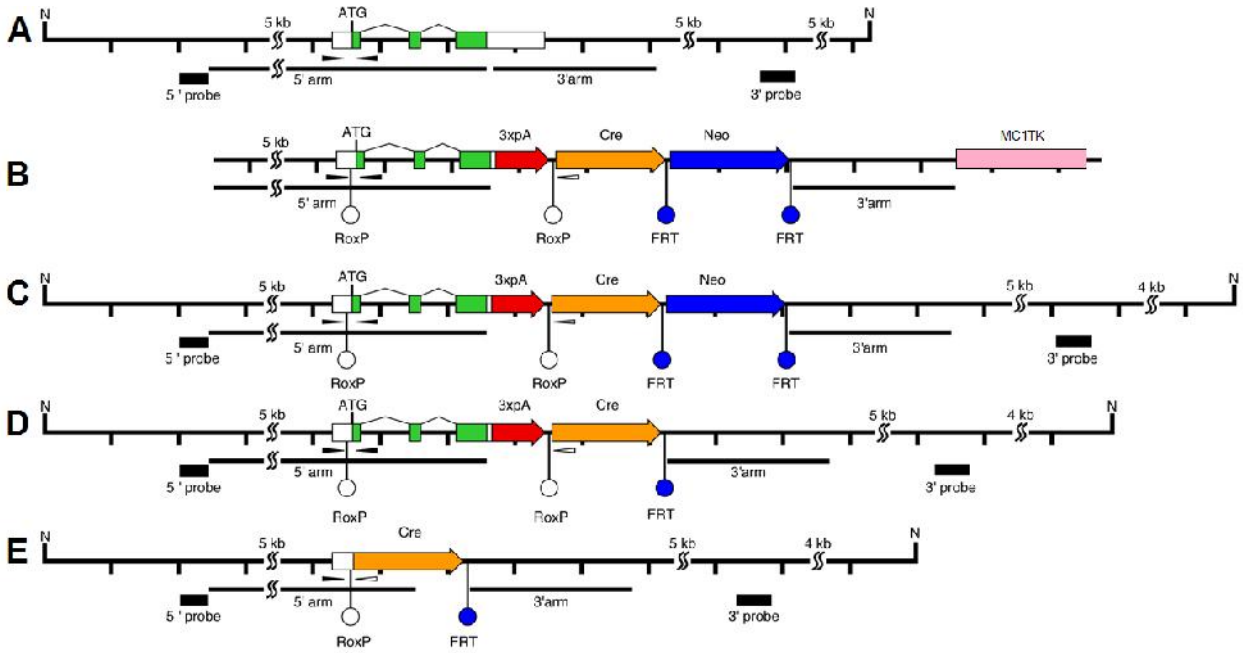


Figure 2. Targeting strategy for the Brn3a^{CKOCre} line. A, Brn3a configuration of the wild type locus. The coding regions are marked by green blocks, while the UTRs are white and splice junctions are represented by thin black lines. Thin lines below represent the homology arms. The probes for the Southern blot detection are represented by black boxes. N – NheI restriction site. B, Brn3aCKOCre targeting construct. The first RoxP site was inserted in the 5'UTR of the gene, before the ATG, and the second one in the 3' UTR, preceded by an extra terminator signal - 3xpA (red arrow). The Cre ORF (orange cassette) follows after the second RoxP, then the positive selection cassette - Neo (blue), flanked by 2 FRT sites. The negative selection cassette – MC1TK (pink) is placed at the end of the 3' targeting arm. C, The Brn3a^{CKOCre} targeted gene, after the homologous recombination. D, The Brn3a^{CKOCre} targeted gene after the removal of the positive selection cassette (Neo) resulted after crossing the line with one carrying the Flp recombinase. E, Brn3a^{Cre}. Upon Dre recombination of the 2 RoxP sited the Cre ORF is expressed at the Brn3a locus. Black segments represent 1kb.

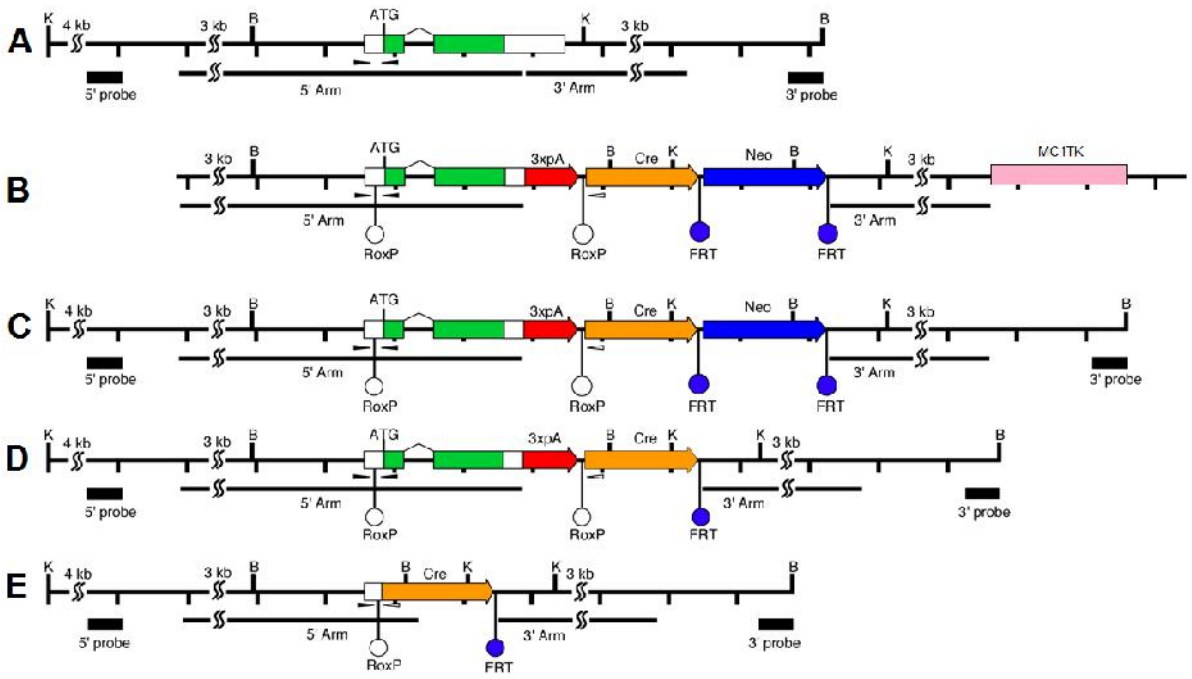


Figure 3. Targeting strategy for the Brn3b^{CKOCre} line. A, Brn3b configuration of the wild type locus. The coding regions are marked by green blocks, while the UTRs are white and splice junctions are represented by thin black lines. Thin lines below represent the homology arms. The probes for the Southern blot detection are represented by black boxes. K – KpnI, B – BamH restriction sites. B, Brn3b^{CKOCre} targeting construct. The first RoxP site was inserted in the 5'UTR of the gene, before the ATG, and the second one in the 3' UTR, preceded by an extra terminator signal - 3xpA (red arrow). The Cre ORF (orange cassette) follows after the second RoxP, then the positive selection cassette - Neo (blue), flanked by 2 FRT sites. The negative selection cassette – MC1TK (pink) is placed at the end of the 3' targeting arm. C, The Brn3b^{CKOCre} targeted gene, after the homologous recombination. D, The Brn3a^{CKOCre} targeted gene after the removal of the positive selection cassette (Neo) resulted after crossing the line with one carrying the Flp recombinase. E, Brn3b^{Cre}. Upon Dre recombination of the 2 RoxP sited the Cre ORF is expressed at the Brn3b locus. Black segments represent 1kb.

4. New optogenetic tools for the future study of the retinal circuits

Three cell lines were created. First, the CaPoChR2 cell line consists of three elements, a Calcium, a Potassium channel, and a cationic channel, sensitive to light – the Channelrhodopsin (ChR2). All elements were inserted at a single location in the HEK293 FlpIn cell's genome by the means of the pcDNA5/FRT vector. Secondly, the ChR2 coding sequence was expressed in the HEK293 FlpIn cells following the same strategy. Finally, the third cell line consists in the first one, CaPoChR2 line, in which the ChR2 coding sequence was overexpressed by random insertion.

Results and discussion

1. Analysis of the inner plexiform layer in the Brn3b$^{CKOAP/-}$ mutants

This subchapter presents the analysis of retinal neurons, pre-synaptic to ganglion cells in the inner plexiform layer (IPL), in the context of a major absence of RGCs themselves. Using cell specific markers we performed a quantitative as well as qualitative analysis of different retinal cell populations.

Initially, different markers widely used for staining bands in the IPL were analyzed. The first general analysis confirmed a result reported by Badea and coworkers (Badea *et al.,* 2009a) and namely that, apart from a minimal decrement in the thickness in the IPL of the mutants, no changes occur in the lamination staining (Fig. 4.A).

Whole mount staining was used to study retinal neurons which provide synaptic input to RGCs performing a quantitative and qualitative analysis. Starburst/cholinergic amacrine cell number shows no significant differences between the two data sets compared (Fig. 4.B). Bipolar cells were analyzed using Cabp5 and ZNP1 for cone bipolar cells and PKCα for rod bipolar cells (Fig. 4.C). Examination of vertical sections showed that the general morphology and pattern of stratification of these neurons as well as the position of their synaptic terminals are not disturbed by the absence of 70% RGCs.

In addition, to assess the integrity of the synaptic components of the IPL two different markers were used – CtBP2 (Ribeye) a constituent of ribbon synapses, and Cnx36, a component of a subset of gap junctions in the IPL. No differences were observed in the numbers of the Cnx36 and Ribeye puncta between the WT and KO retinas.

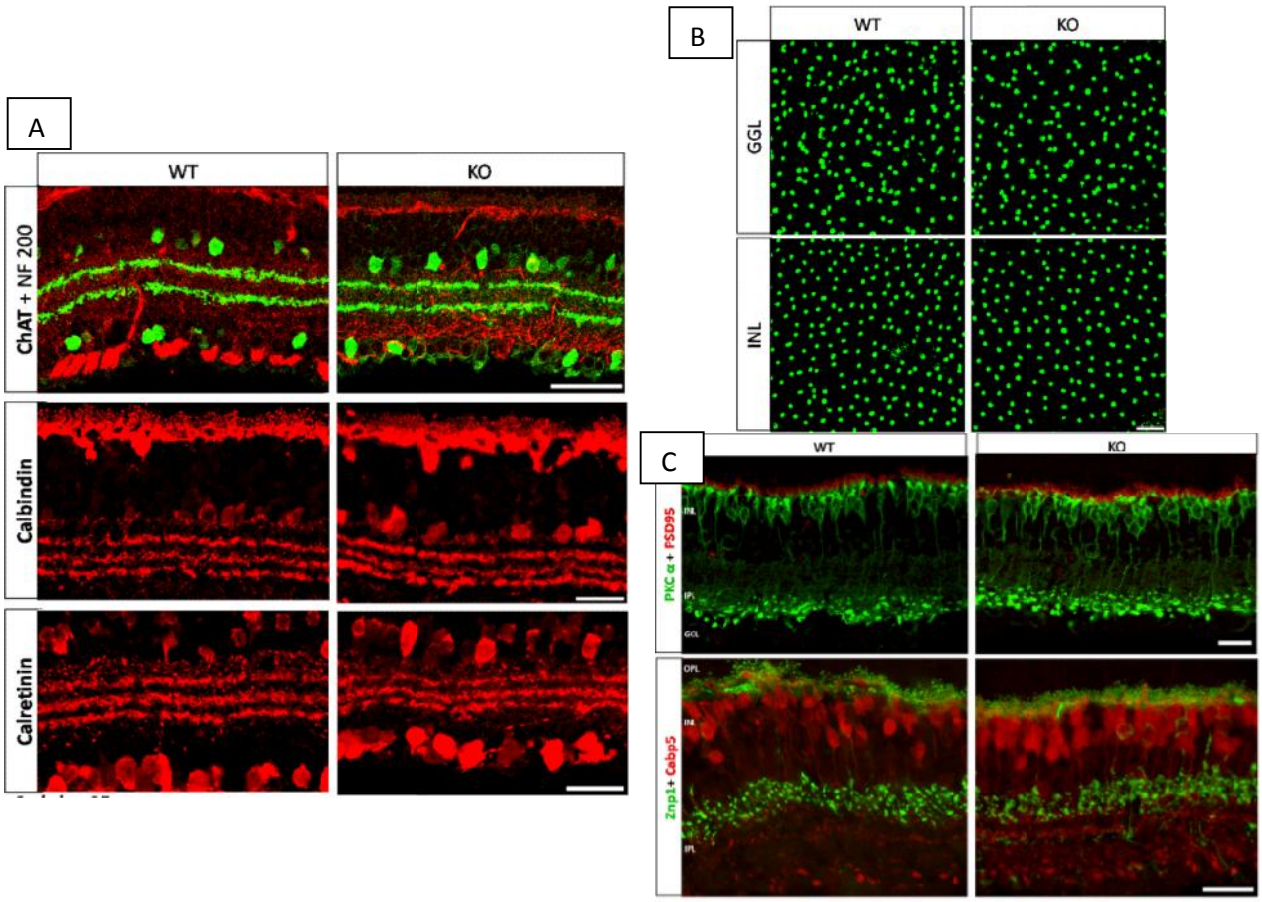


Figure 4. Markers of lamination, amacrine cells and bipolar cells show neither quantitative nor qualitative difference in the Brn3b KO. (A) The thickness of the inner plexiform layer is not changed in the KO. NF200 staining in the top-right panel shows only few fibers in the layer of the ganglion cell, indicating that this is a retinal area of complete recombination. Scale bar: 25 µm. (B) Starburst amacrine cells stained in whole-mount for ChAT. Cellular bodies were imaged in both ganglion cell layer (GCL) as well as in the inner nuclear layer (INL). Scale bar: 50 µm. (C) Bipolar cell markers. No difference is visible between WT and mutants regarding the rod bipolar cells labeled with PKCα (top panels). Similar results were obtained when analyzing two different cone bipolar markers (Znp1 and Cabp5; bottom panels). The post-synaptic density marker (PSD95) that labels synaptic terminations in the OPL shows no difference between WT and KO. Scale bar: 25 µm.

2. Expression profile analysis of gene sets downstream Brn3a and Brn3b in a highly enriched population of RGCs

Generation of a highly enriched population of Retinal Ganglion Cells

With the purpose of understanding the gene regulatory networks that lead to the formation and the specification of individual cell types in a tissue, it is very important that the population of the cells studied is highly enriched if not pure for the cell type of interest. In the mouse retina, ganglion cells represent less than 1% of the total number of cells, making the purification difficult.

To overcome this difficulty, an immuno-magnetic purification technique was used. By using the $Brn3^{CKOAP/WT}$ and $Brn3^{CKOAP/KO}$ mutant animals it was possible to isolate only the cells expressing the reporter gene. The alkaline phosphatase (AP) used as reporter is a GPi-linked protein that is expressed on the membrane of the cells (Berger *et al.,* 1987). Another advantage of the use of this mouse model is given by the fact that AP labels only retinal ganglion cells, the cell type in which Brn3a and Brn3b are known to be expressed (Badea *et al.,* 2004).

This highly enriched population of RGCs was then processed for the RNA extraction and expression profile analysis using RNA sequencing.

Brn3 gene expression

One of the initial validations of the RNAseq data was to analyze the expression of the Brn3a and Brn3b genes in the different samples. The results of the analysis are shown in Fig.5. Expression values obtained for Brn3a/b in RGCs versus retina samples of WT animals again confirm the efficiency of the isolation protocol. Both RGC specifically expressed transcription factors gave high values in the WT RGCs. Expression in WT retina supernatants is low. When comparing expression of Brn3a/b in WT versus KO RGCs expression in the respective KO is, as expected, always lower. Interestingly, Brn3a expression appears to be downregulated in the Brn3b KO background, whereas Brn3b expression is slightly upregulated in the Brn3a KO. Based on these findings it can be assumed that regulation of Brn3a/b expression is influenced by the respective other transcription factor. Exactly how this occurs remains to be elucidated.

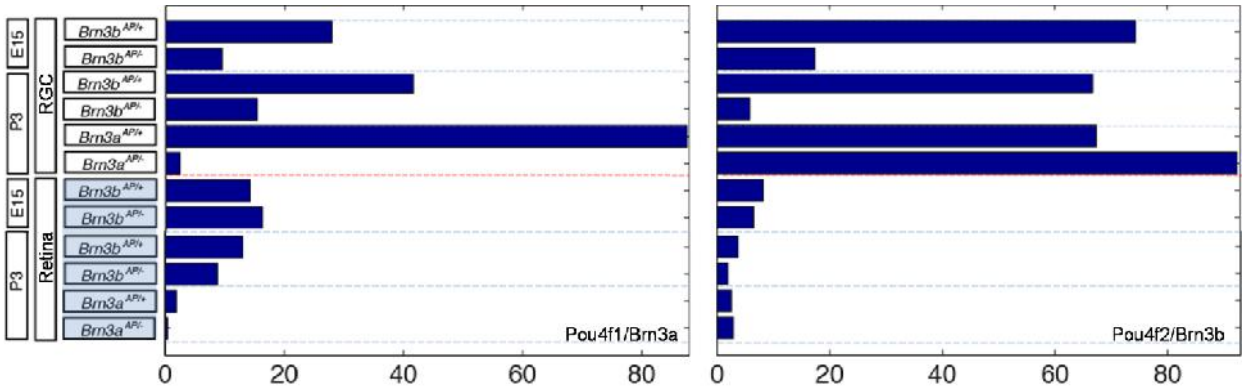


Figure 5. Analysis of Brn3a and Brn3b expression in RGC and retina (supernatant) samples. The top half of the figure represents reads from the "RGC samples" whereas in the bottom half the "Retina samples" are represented. Expression values are expressed in FPKMs

Validation based on published expression data

As a further validation step the results obtained were compared to published expression data with special emphasis on genes that are specifically and/or highly expressed in RGCs at the time points analyzed.

Figure 6 lists the genes together with their expression levels in RGCs, shown in the left-hand panel, and those in the retina (supernatant) in the right-hand panel. High expression levels are represented by "hot" colors (yellow to red); low expression levels by "cold" colors (blue). It is immediately obvious that all genes – that were selected for their RGC specific expression – show low expression levels in the retina supernatant in both Brn3 WT and KO animals.

In RGCs, the two transcription factors under investigation Brn3a (Pou4f1) and Brn3b (Pou4f2) show, as expected, low expression levels in their respective knock-outs.

Even from this general analysis, genes tend to cluster in a few categories. Genes like Snca, Sncg, Nefm, IslI, Pou6f2 show higher expression at E15 compared to the Brn3b samples at P3, suggesting that their activity is more likely required during the first days of the development of the Brn3b RGCs. On the other hand, even in the case of the genes that are highly expressed at E15 but with lower expression at P3 in the "Brn3b" samples, an increased expression is observed in the "Brn3a RGC" samples, suggesting the interplay of the two transcription factors for the specification and correct development of the RGCs. Moreover, for this particular set of genes, Brn3b appear to be the master regulator at P3 and, in cases like Sncg and Nefm, even at E15, as the Brn3b$^{AP/KO}$ columns show cold represented expression values.

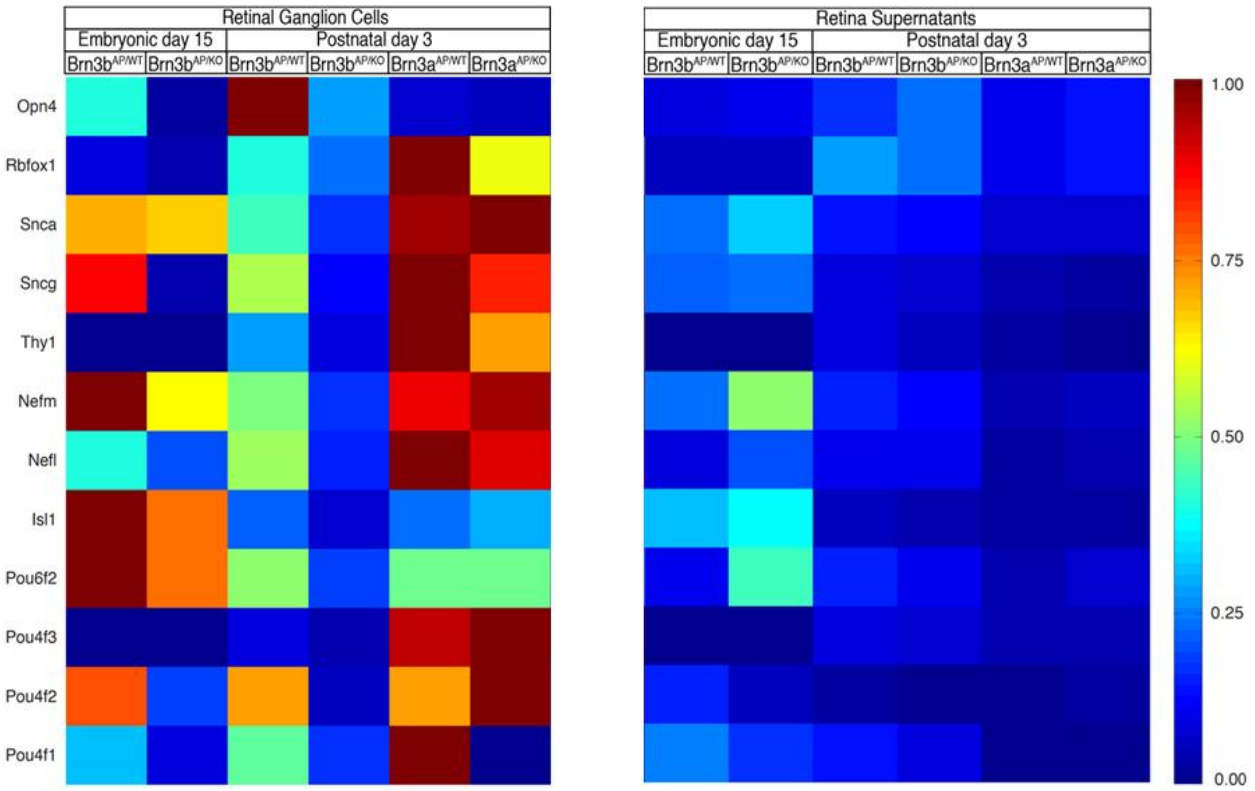


Figure 6. Expression profiles of several RGC specific genes in different samples. Representation of the "RGC" samples (left) and the corresponding "retina" samples (right). The FPKM values are represented as heat map, on a colored scale, cold colors corresponding to low values and hot to high values, respectively. Expression levels are normalized to the maximum level for the each gene.

Gene classification

By sample specificity

Subsequently, the entire set of genes expressed either at P3 or E15 in the Brn3b AP/WT RGC samples was analyzed. These genes can potentially distinguish Brn3b expressing neurons from other cell types in the retina during development. Initial analysis showed that a similar number of genes were expressed in the "Brn3b RGC" samples at the 2 data points analyzed – 1424 genes at E15 and 1348 genes at P3 (blue and yellow circles, respectively in Figure 7, left panel). However, less than 50% of them (i.e. 615 genes) exhibit the same expression level at the two time points (overlap between blue and yellow circles, Figure 7, left panel).

Next, gene sets differentially expressed in Brn3b KO versus WT RGCs were identified. At E15 there are 156 differentially expressed genes (blue circle in Fig. 7, right), whereas at P3 540 genes were found to be expressed differentially (yellow circle in Fig. 7, right). Out of the

139 genes differentially expressed at both time points, the vast majority was found to be downregulated in the KO.

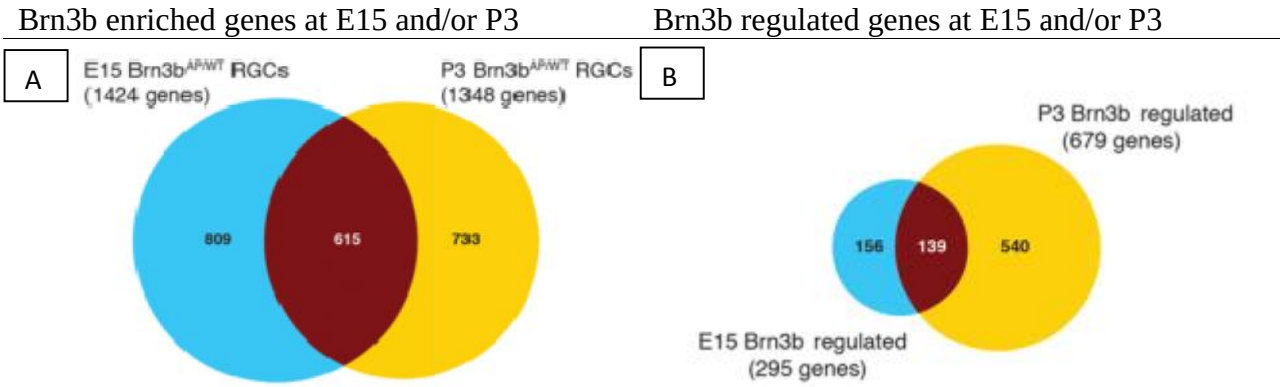


Figure 7. Sets of genes expressed at E15 versus P3 (A) and sets of genes potentially regulated by Brn3b (B). All selected genes have an expression level equal or greater than 2 FPKM. The fold difference between RGC and retina samples analyzed, as well as between Brnb$^{AP/WT}$ and Brn3b$^{AP/KO}$ is equal or greater than 2.

Another interesting aspect considered among the genes in the data set was to identify genes that distinguish Brn3a and Brn3b expressing neurons from other cell types in the retina at P3. For that, genes that are specific for RGC were clustered according to whether they are specific for either Brn3a and/or Brn3b expressing RGCs. Genes expressed in the Brn3a$^{AP/WT}$ RGCs and the Brn3b$^{AP/WT}$ RGCs were compared with the ones expressed in the retina samples.

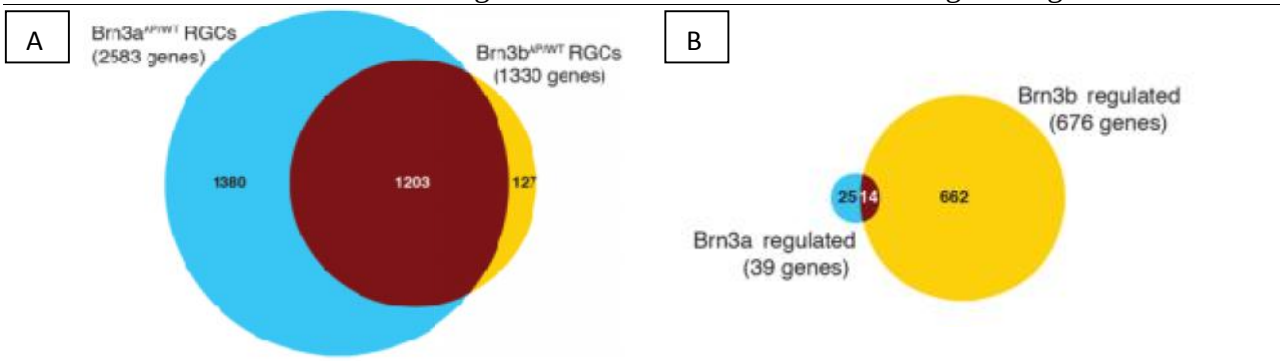


Figure 8. Sets of genes at P3. All selected genes have an expression level equal or greater than 2 FPKM. The fold difference between "RGC" and "retina" samples, as well as Brn3$^{AP/WT}$ and Brn3$^{AP/KO}$ is equal or greater than 2.

Applying these criteria a number of 3913 genes that appear to be specifically expressed in both Brn3a and Brn3b RGCs could be identified. Interestingly, the number of genes that are specific for Brn3a RGCs (1380) is approximately ten times that of Brn3b RGC specific genes (127). A considerable fraction of genes is found to be expressed in both Brn3a and Brn3b RGCs or in Brn3a/Brn3b "double positive" cells (overlap in Fig.8).

Important information can emerge from the analysis of genes that are regulated by either Brn3a or Brn3b or both at P3 where data are available for both. Such genes can be assumed to mediate the functions of Brn3a and Brn3b in RGCs at P3. For this purpose, the pool of genes specifically expressed in RGCs was analyzed to identify genes that are either up- or down-regulated by Brn3a/Brn3b, comparing the data obtained from WT and KO, respectively. Interestingly, out of the number of genes that are specific for Brn3a or Brn3a/Brn3b positive cells in RGCs (2583), only 39 are regulated by the transcription factor. On the other hand, Brn3b appears to regulate a larger number of genes specific for RGCs. The number of genes apparently under the control of Brn3b is approximately half of those found to be expressed specifically in Brn3b or Brn3a/Brn3b positive cells (676 versus 1330). Only 14 genes are regulated by both Brn3a and Brn3b at P3.

By function exhibited in the tissue

After the analysis of the gene sets based on their specificity, we performed a macro-analysis to find out which types of molecules are encoded by the genes in the different categories. We performed an extended research in the available literature looking for evidence that assigned different molecules to neuronal cell type differentiation, morphogenesis and cell-cell interaction, as well as to synaptic connectivity. Results are presented in Table 2.

As expected, transcription factors are highly represented among the expressed genes identified in the RGC populations at the E15 and P3 time points, being important for cell specification and their correct localization at the tissue level.

Table 2. Representation of genes with functions in neuronal development, morphogenesis and cell-cell interaction, as well as in synaptic connectivity in the data set. Functional categorization was based on data obtained from the literature.

Molecular families	Number of members
Transcription factors	81
Integrins	48
Cadherins	11
Protocadherins	18
Ephrins (ligands and receptors)	3
Semaforin-Plexin-Neuropilin	13
Robo-Slit	2
Ig domain	115
Small GTPases (GAP, GEF, GDI)	87
Cell structure adaptors (actin filament, dinein, kinesin, myosin)	50
Synaptic molecules (receptors, transporters, ion channels)	40

Validation of gene expression pattern determined by RNAseq

To validate the finding that the different genes identified by the screening are indeed expressed in the RGC layer, In-Situ Hybridization (ISH) was performed.

To confirm the data obtained for E15, initially we took advantage of the body of knowledge available from the Allen Brain Atlas (ABA) database. 210 genes that are specific and/or regulated by Brn3b from the present data set appear to be expressed in the GC layer of the C57BL/6 mouse strain at E15 according to ABA.

With regard to the P3 data, extended ISH screening was developed to confirm the expression of some of the genes of particular interest at this stage of development. Selection criteria were high levels of expression in “RGC samples” and low in the “retina”; and/or regulated by either Brn3a or Brn3b.

RGC enriched genes with potential importance for neuronal development

Subsequent to the general analysis of the different sets of genes falling under the 2 fold/2 FPKM criteria we carried out an analysis using a higher threshold for the -fold difference between “RGC” and “Retina” samples. In addition changes in expression dependent on the presence/ absence of Brn3a/Brn3b were analyzed by comparing the “RGC WT” and “RGC KO” samples. The analysis focused on 14 genes that were not previously analyzed as RGC markers in the literature, namely Slc11a1, Slc17a6, Dpp10, Chl1, Adap1, Clstn2, Htr1b, Epb4.9, Slc6a4,

Igf1, Kitl, Ctxn3, Synpr and Trhde. However, the analysis presented here yielded -fold differences higher than 10 between RGC and retina for the expression of these molecules. The majority of the expression data was confirmed using the Allen Brain Atlas ISH data base (for E15), for part of them in addition ISH was performed at P3.

Table 3. Selected genes under more stringent selection criteria

	Brn3a P3	Brn3b E15	Brn3b P3
Fold difference WT vs KO >5	0	**Slc6a4**, **Ctxn3,**Synpr, **Igf1**, Trhde, Kitl, Slc17a6, Chl1	Ctxn3, Clstn2, **Synpr**
Fold difference RGC vs Ret >10	**Slc11a1**, **Slc17a6,**Dpp10, **Chl 1**, **Adap1**, Clstn2, Htr1b, Epb4.9, Slc6a4	**Ctxn3**, **Slc11a1**	Ctxn3, **Slc11a1**, **Slc6a4**

Finally, it is worthwhile noticing that part of the 14 genes not previously analyzed as RGC markers were, however, mentioned in the literature in the context of expression profile studies as genes that are specific for RGCs or other sensory organs, at different stages of development (Eng*et al.,* 2007; Ivanov *et al.,* 2008; Lu *et al.,* 2011; Qiu *et al.,* 2008; Siegert *et al.,* 2012). Part of these genes were studied in more detail (but not in the RGCs) and proved to be important for neuronal development and interactions (Matsuyoshi *et al.,* 2012; Moore *et al.,* 2007; La Torre *et al.,* 2012; Rodriguez-de la Rosa *et al.,* 2012; Upton *et al.,* 1999; Upton *et al.,* 2002; Venkateswarlu *et al.,* 2007).

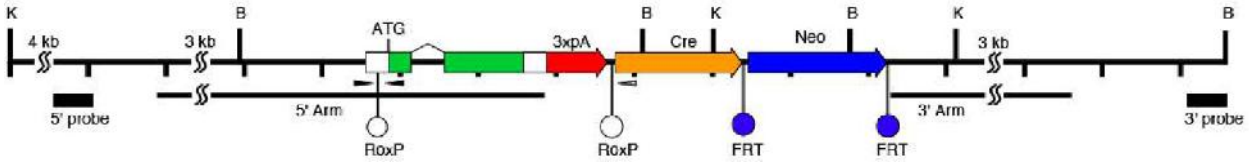
3. Dual recombinase dependent conditional knock-in reporter strategy to study the development and function of Retinal Ganglion Cells

Generation of the Brn3a^{CKOCre} and Brn3b^{CKOCre} mutant lines

Confirmation of targeted integration into ES cells

The DNA samples extracted from Brn3a/b$^{CKOCre/+}$individual ES cell colonies were analyzed, being tested for the 5' and 3' probe, respectively. Initial Southern Blot (SB) assay carried on 96 different clones for each line showed positivity in 68% of the clones, indicating a very good yield of the integration and selection. Integration is represented by the presence of

both a WT band (19 kb for Brn3a and 6 kb for Brn3b) and a mutant one (23.5 kb and 10 kb respectively). Following the propagation of 12 clones, a second SB screening was carried out for the confirmation of the insertion. In addition, after being crossed with a Flp recombinase carrier line, DNA samples from the F1 generation of mutants were SB tested for the removal of the PGK-Neo cassette, showing a 100% removal yield.



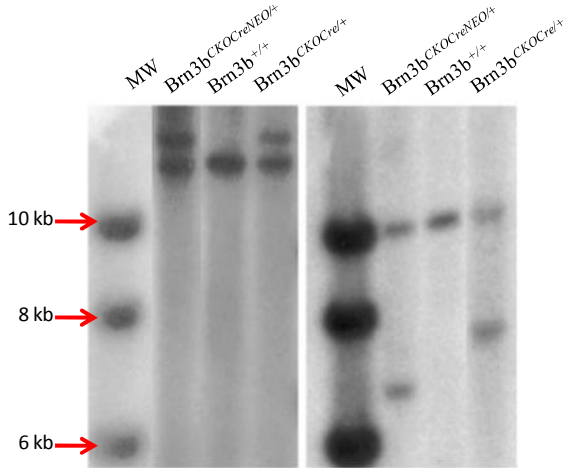


Figure 9. Removal of the PGK-Neo (positive selection) cassette revealed by SB in the Brn3b$^{CKOCre/+}$ line for the 5' (left) and 3' (right) probes. For the 5' probe, the *Kpn I* fragment generated after digestion is 14 kb before and after the Neo removal. The 3' fragment revealed with *Bam HI* digestion shows a 6 kb mutant band before and 7.6 kb after the removal of the positive selection cassette (Ghinia *et al.*, submitted).

Triple transgenic mice: CAG:Dre; Brn3^{CKOCre}; ROSA26$^{iAP/+}$

First, to test the recombination of Dre – RoxP system, we took advantage of the CAG:Dre transgene (Anastasiadis *et al.*, 2009) that expressed ubiquitous the Dre recombinase. As for the reporter line, the ROSA26^{iAP} (Badea *et al.*, 2003) was used for the expression of the AP (Alkalin Phosphatase) reporter upon the Cre recombination. The sequence of the recombination events starts with the expression of the Dre recombinase early in development or in the germline, followed by conditional ablation of the Brn3 gene that induces the expression of the Cre recombinase under the control of the Brn3 promoter. At the reporter locus, the AP will be expressed upon the recombination on the two LoxP sites present in the ROSA$^{iAP/+}$ construct by the Cre recombinase, now expressed from the Brn3 locus.

In the ROSA26^{iAP} line, the second exon followed by additional termination signals is reversed and flanked by two LoxP sites, therefore in the absence of the recombination the transcription of the AP ORF is not possible. After the Cre recombination of the two LoxP sites,

the inverted components will be placed in normal orientation allowing the correct splicing between the two exons and the transcription of the AP reporter (Badea *et al.,* 2009b).

Genotyping PCRs were run for the detection of DNA segments characteristic for different elements – AP, Dre and Cre reombinases (Fig. 10). As expected, the CAG:Dre; ROSA26$^{iAP/+}$ showed the predicted PCR product for the AP as well as Dre primers, the Brn3a/b$^{CKOCre/+}$; ROSA26$^{iAP/+}$ for AP and Cre recombinase, while in the case of the the triple transgenics CAG:Dre; Brn3a/b$^{CKOCre/+}$; ROSA26$^{iAP/+}$for the three primer pairs (1, 2, 3) yielded PCR product.

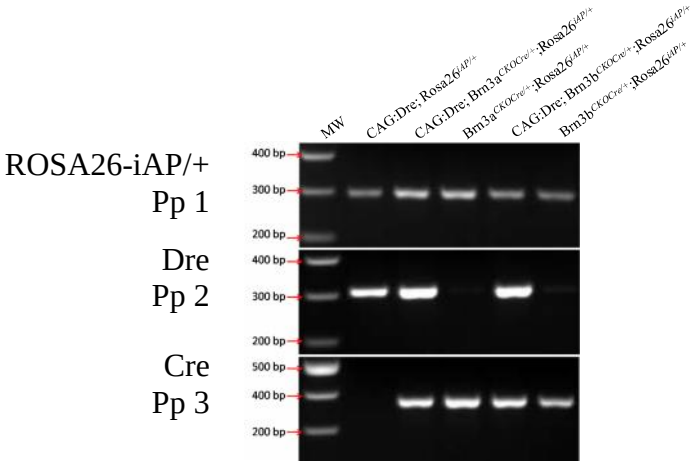


Figure 10. Genotyping PCR of animals carrying different elements. The DNA from the CAG:Dre; ROSA26$^{iAP/+}$ show AP as well as Dre bands. The three pairs of primes (1, 2 and 3) yielded a PCR product in the CAG:Dre; Brn3a/b$^{CKOCre/+}$; ROSA26$^{iAP/+}$ and no Dre correspondent band in the case of the Brn3a/b$^{CKOCre/+}$;ROSA$^{iAP/+}$.

Dre to Cre recombination in the adult mouse

Retinas as well as brain preparations carrying the three different genotypes for each mouse line were analyzed for the expression pattern of the AP reporter. Surprisingly, the retinas as well as the brain sections of the triple transgenic animals were completely stained for the AP, indicating a ubiquitous recombination of the Cre reporter.

Tissues histological processed in identical conditions that were extracted from CAG:Dre; ROSA26$^{iAP/+}$ mice were completely negative for the AP reaction, confirming that the Dre recombinase cannot mediate recombination between two LoxP sites.

In addition, the retinas of Brn3a/b$^{CKOCre/+}$; ROSA26$^{iAP/+}$ animals show several AP positive RGCs, suggesting Dre independent expression of Cre from the Brn3a/b^{CKOCre} allele, possibly due to the read-through the Cre ORF, despite the additional terminator signals added in the two constructs.

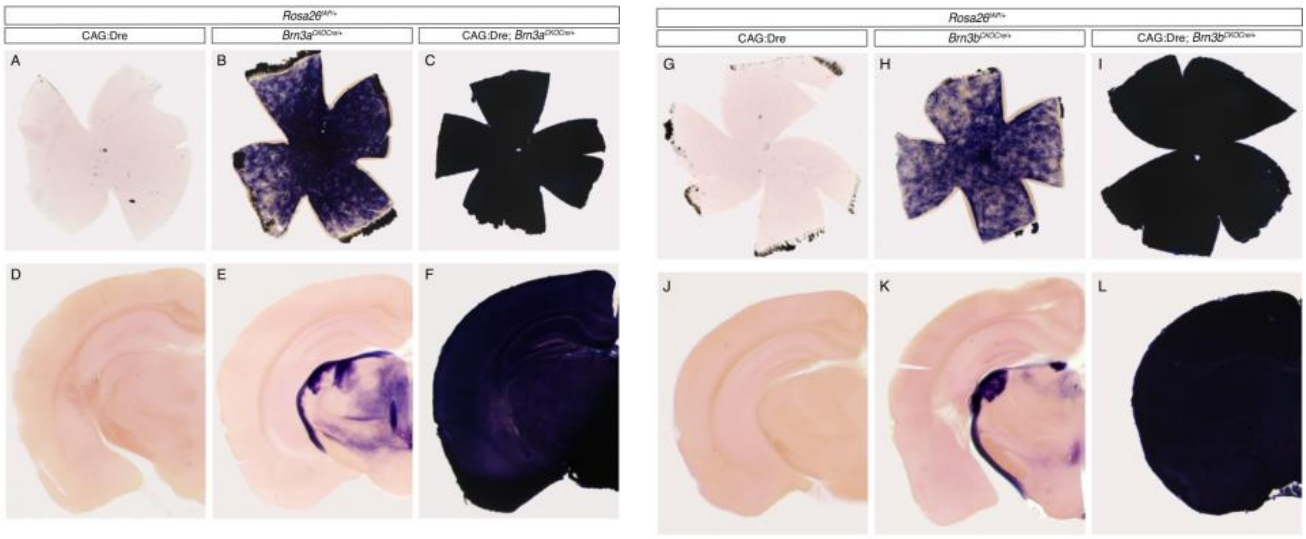


Figure 11. Analyses of tissues derived from mice carrying the dual recombinase system, suggesting early ubiquitous expression of Cre recombinase. Whole mount retinas from adult animals (A-C, G-I) as well as hemispheres from coronal brain sections (D-F, J-L) were stained for AP activity. In the case of the CAG:Dre; ROSA26$^{iAP/+}$ mutant (A, D, G, J), the expression of Dre recombinase in the germline does not result in AP expression from the Cre dependent ROSA26$^{iAP/+}$. Brn3$^{CKOCre/+}$; ROSA26$^{iAP/+}$ tissues show background AP expression, in the absence of Dre recombination. In CAG:Dre; Brn3$^{CKOCre/+}$; ROSA26$^{iAP/+}$ animals AP is found to be expressed ubiquitously as a result of cre-mediated recombination at the ROSA26$^{iAP/+}$ locus in the entire (or vast majority) of the tissue. A, C, D, F n=4, G, J n=6, B, E n=9, H, I, K, L n=13 (Ghinia *et al.*, submitted).

Early/ubiquitous expression of the Cre recombinase from the endogenous Brn3a and Brn3b loci

To further characterize the expression of Cre recombinase from the Brn3a/b^{CKOCre} alleles several mutants carrying the three different transgene combinations were analyzed at E9.5. The CAG:Dre; Brn3$^{CKOCre/+}$; ROSA26$^{iAP/+}$ triple transgenics show homogeneous expression of the AP reporter – indicative of either ubiquitous or germline expression of Cre under control of the Brn3a/ Brn3b promoters.

In a previous experiment ROSA26rtTACreER; Brn3bCKOAP mice (Badea *et al.*, 2009b) were used to investigate tissue and developmental stage specificity of the Brn3b promoter. In these mice induction of Cre expression from the ROSA26 locus results in recombination at the Brn3b locus giving rise to an allele from which AP is expressed under the control of the Brn3b promoter. As can be seen in Fig. 12 (A), AP activity in embryos of this mouse line can be

detected in the germinal ridges. This finding suggests that the Brn3b promoter is active in the germline. The homogeneous expression of the AP reporter in CAG:Dre; Brn3$^{CKOCre/+}$; ROSA26$^{iAP/+}$ triple transgenics is, therefore, likely to be the result of recombination mediated by Cre expressed in the germline.

The double transgenics (CAG:Dre; ROSA26$^{iAP/+}$ and Brn3$^{CKOCre/+}$; ROSA26$^{iAP/+}$) revealed patterns similar to the ones found in adult tissues, and namely no AP expression and sparse recombination, respectively. In all these crosses, both female and male carried the Brn3CKOCre allele.

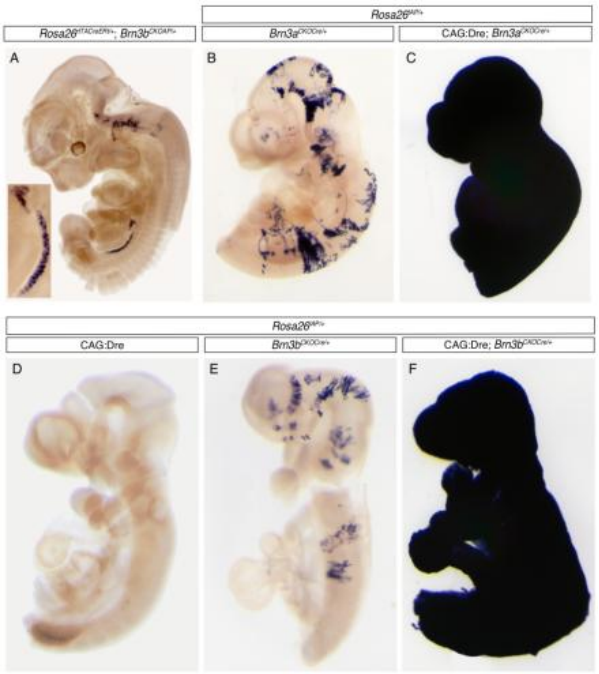


Figure 12. Early Dre to Cre recombination event in the CAG:Dre; Brn3^{CKOCre}; ROSA26$^{iAP/WT}$. Whole mount staining of E12.5 ROSA26$^{rtTACreER;}$ Brn3b^{CKOAP} embryo showing AP expression in the germinal ridges (A, left). E9.5 embryos show complete AP expression in the CAG:Dre; Brn3^{CKOCre}; ROSA26$^{APi/WT}$ and sparse recombination in the Brn3^{CKOCre}; ROSA26$^{iAP/WT}$. As in the retinas and brain sections, the CAG:Dre; ROSA26$^{iAP/WT}$ embryos show no AP expression. B n=9, C n=6, D, E n=4, F n=1 Ghinia et al., submitted).

4. New optogenetic tools for the future study of the retinal circuits

In this project, three cell lines were created. First, the CaPoChR2 cell line, consisting of three elements, a Calcium channel, a Potassium channel, and a light sensitive cationic channel– Channelrhodopsin (ChR2). All elements were inserted at a single location in the genome of the HEK293 FlpIn cells by the means of the pcDNA5/FRT vector. Secondly, the ChR2 coding sequence was cloned in the pcDNA5/FRT vector following the same strategy. Finally, the third cell line is based on the CaPoChR2 line, in which the ChR2 cDNA was overexpressed by random insertion.

Genotyping of different elements in the cells

To check the presence of each coding sequence (CDS) into the cell lines, different cellular clones were dissociated, the genomic DNA was extracted and several sets of PCR were run.

For the CaPoChR2 cell line, as well as for the gChR2 cell line, the correct integration of the elements was checked using P1-P2, R-C1 pairs of primers. By using these combinations, it was possible to check first, the 3'end of the CaV1.2 CDS and the 5' end of the ROMK1, and, in the second case, the 3' of the ROMK1 CDS and the 5' of the ChR-EYFP CDS.

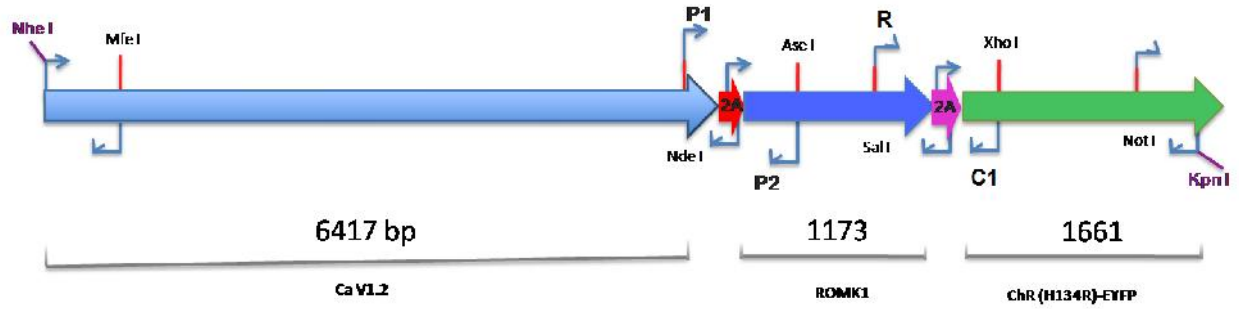


Figure 13. The CaPoChR2 construct. The three elements – Ca channel, K channel and Channelrhodopsin were linked by the 2A peptide viral sequences. Primer pairs P1-P2 and R-C1 were used for the amplification of the linkers as well as for the genotyping of the cells carrying different elements.

Moreover, as the gChR2 cell line was developed based on the HEK293-CaPoChR2 cell line when it reached advanced passage number (15-20), the clones were further tested to confirm the absence of any type of rearrangements and the correct orientation of each element. For that, different clones were tested for the presence of the 5' end of the CaV1.2 CDS, between Ca1 and Ca2 primers as well as for the presence of the ChR2-EYFP 3' end, between C2 and C3.

For the HEK293-ChR2 cell line, the one that expresses only ChR2-EYFP CDS, the only control PCR was for the 3' end, portion that flanks completely the EYFP CDS.

YFP fluorescence of the different cell lines

Initial control of the YFP intensity in the first created cell line – CaPoChR2 – showed YFP positive cells in comparison with the negative control, the HEK293 Flp In cells, which were completely negative fluorescently. However, a test experiment, in which the HEK293 cells

overexpressed ChR2-EYFP transiently, brought in attention the fact that the fluorescence potential of the ChR2-EYFP vector is higher than the one obtained in the CaPoChR2 cell line.

This fact led to the development of the ChR2 cell line, in which the ChR2-EYFP coding sequence is expressed at one single locus by the means of the Flp In recombination system. The YFP intensity in the ChR2 only cell line is at least 10 fold increased comparing with the CaPoChR2 cell line.

However, the fact that the second cell line expresses only ChR2 was not sufficient for the initial purpose of the project.

The gChR2 cell line represents the improved version of the other 2 cell lines, created previously. It expresses all the 3 elements: the CaV1.2, ROMK1 and the ChR2-EYFP CDS, as well as many copy additionally overexpressed of the ChR2-EYFP fusion-protein. Fig. 14 shows a screening through representative cellular clusters for the intensity of the YFP.

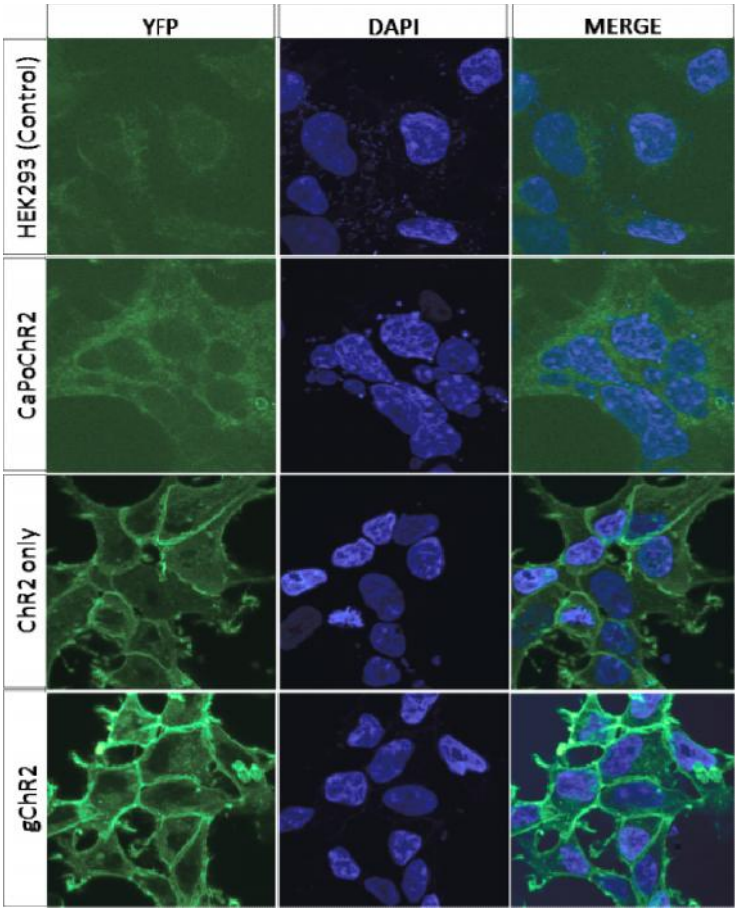


Figure 14. YFP fluorescence in representative samples of each cell line. CaPoChR2, cell line including Calcium, Potassium and Channelrhodopsin channels. ChR2 – Channelrhodopsin, gChR2 – cell line in which ChR2 was overexpressed by random insertion. YFP – yellow fluorescence protein, DAPI – nuclear staining.

Patch Clamp analyzes of the CaPoChR2 and ChR2 cell lines

Cells carrying the CaPoChR2 construct respond to light under different conditions with current waves that reach 150 pA when constant light was used delivering 0.2 s long stimuli for 10 times. In contrast, by delivering constant as well as varying light for 10 times, 2 s, the current obtained is lower, yet the time of response is higher. In general the same pattern was detected in the case of the cells that carry only the ChR2 gene. However, the depolarization response in this case reached 8 nA, equally similar under the three conditions tested, corresponding to a 53 fold increase in comparison with the previously tested cell line.

These findings suggest that the ChR2 protein is not expressed in the CaPoChR2 line as intense as in the one carrying only the ChR2 gene. This effect might be due to the impossibility of the 2A linkers to offer equal molar ratio between the linked proteins.

General Discussion

The central nervous system (CNS) is known to adapt plastically and to reorganize during development, learning as well as in pathological conditions. As a part of the CNS, the retina is a very dynamic tissue and therefore also undergoes various degrees of rearrangement beginning with its development. Ganglion cells go through different types of changes from their decision to become a particular class, to the process of adopting specific dendritic patterns and interacting with neighboring cells (Sernagor *et al.*, 2001).

Pathologic changes in the retina have been characterized in the last years particularly for diseases affecting photoreceptors in the first place. The so-called retinal remodeling consequent to photoreceptor degeneration is characterized by severe changes in the integrity of second-order neurons including death of some of them, like bipolar and amacrine cells. However, ganglion cells tend not to be affected suggesting that ganglion cells represent an intrinsically stable population of neurons.

The first part of this thesis actually considered the opposite situation – and namely what are the overall retinal effects of a dramatic reduction in the number of ganglion cells. For that, the experimental model used was a mouse in which the RGC population was reduced by the ablation of the Brn3b transcription factor, a leading molecule responsible for the correct development of this particular cell class and strictly specific for them in the mouse retina.

Different markers analyzed to assess the lamination of the IPL – labeling pre-synaptic partners of RGCs like cone bipolar cells and amacrine cells, as well as molecules that assessed the density of the ribbon synapses and gap junctions in the IPL. All these markers showed no modifications in the Brn3b mutants compared with their WT counterparts.

The relative independence of inner retinal cell types has been demonstrated by many studies. For instance, it is known that amacrine cells are largely independent from RGCs; despite the latter represent a major post-synaptic partner. Studies in which the optic nerve was severed leading to RGCs death proved that amacrine cells were able to remain intact, maintaining their stratification in the IPL (reviewed by Chalupa and Gunhan, 2004). Genetic ablation of a high number of RGCs in the Math5 mutant animals shows a similar result (Brown *et al.*, 2001; Wang *et al.*, 2001).

The results illustrated in the first part of this thesis reinforce the findings presented above, suggesting that retinal neurons are rather autonomous during their phase of patterning and lamination and, later, of survival. This aspect might be exploited in the field of the retinal repair approaches, from two points of view. First, it gives a certain hope regarding diseases affecting the optic nerve such as optic neuritis. Approaches like stem cell based therapies designed to rescue ganglion cells can rely on an intact rest of the retina. On the other hand, in the case of photoreceptor degeneration that affects several types of downstream neurons besides the ganglion cells, the field of prosthetic implantation can always rely upon a large preservation of ganglion cells.

To further investigate the genetic program underlying the normal development of retinal ganglion cells the effect of targeted deletion of the genes encoding two transcription factors, Brn3a and Brn3b, was investigated in the framework of the thesis presented here. During the analysis of Brn3 phenotypes, it was brought to light part of the genetic programs underlying the normal development of the ganglion cells. Using a particular type of Brn3b, as well as Brn3a knock-out mouse model, it was possible first to purify a population of RGCs at specific developmental time points, then to carry out a gene expression analysis on this highly enriched population of RGCs.

The reporter, alkaline phosphatase (AP) that replaces the Brn3 gene after the removal (Badea*et al.,* 2009a) allows the staining of the cells as well as their purification by using a specific immune-magnetic purification method that targets the AP linked to the membrane of the cells. This resulted in a highly enriched population of RGCs from which RNA was extracted and performed RNA-seq experiments.

This screening was designed to reveal changes in the genetic patterns of developing RGCs; therefore, cells were analyzed at 2 different time points – E15 and P3 for Brn3b expressing RGCs and only at P3 for the ones expressing Brn3a. The increased activity that the cells exhibit at specific time points corresponding to phases of axonal pathfinding, dendritic development and neighbour interaction, respectively, require the activation of different sets of molecules. The different sets of data obtained allowed the analysis of genes that are specific for Brn3a and/or Brn3b at P3, as well as of genes specific for Brn3b at E15.

First, as illustrated in the Results section, the number of genes expressed only in the Brn3a expressing RGCs is 10 times higher than the ones in the Brn3b only expressing RGCs.

The most majority of the genes that are specific for Brn3b are shared by Brn3a as well, in proportion of 90%. However, this is not the case for the Brn3a specific genes, only 47% of the genes being shared with the Brn3b expressing neurons. In is known that the RGCs that are only Brn3a positive are characterized by small, very dense dendritic arbours. In contrast, the Brn3b positive RGCs have wide and less branched dendritic arbours (Badea and Nathans, 2011). At the functional level, the size of the dendritic arbour represents a very important characteristic, as it reflects the size of the dendritic field (Masland, 2012).

It is very difficult to state that a specific functional subtype is specifically associated to Brn3a but not to Brn3b or vice versa. If we consider a characterization based upon morphology and site of projection, it can be observed that cells that are exclusively Brn3b positive include the melanopsin positive GCs, projecting to areas like the OPN (olivarypretectal nucleus), the IGL (intergeniculate leaflet) and to the SCN (suprachiasmatic nucleus) (Badea and Nathans, 2011). However, most part of the other functional classes of RGCs, like ON-OFF DS, OFF alpha or other types homologous to types found in other mammalian retinas, like the uniformity detectors GCs of the rabbit or the blue-yellow colour opponent GCs of the primate retina, these can be found among both Brn3a and Brn3b positive RGCs (Badea and Nathans, 2011).

There are additional aspects concerning RGCs specification that are different between Brn3a and Brn3b positive RGCs. Several groups claim that, besides dictating differences among cell types, Brn3 factors have generic functions, and namely that Brn3b plays an important role in the development of the axons, while Brn3a is required for the correct development of the dendritic arbors (Erkman *et al.*, 1996; Gan *et al*., 1996; Erkman *et al.*, 2000; Wang *et al*., 2000; Badea *et al*., 2009a; Badea and Nathans, 2011). The main question is, how exactly the genetic programs controlled by the various Brn3 confer precise morphological and functional characteristics to the neurons that express them.

The screening we performed provides an enormous set of data that can be explored in the future. The general idea is to be able to find among these genes functional connections that were not discovered before, especially related to the execution of programs driving cells to the acquisition of a specific identity and function. Indeed, an important aspect is to recognize how different genes are instrumental to the establishment of functional subtypes of RGCs, possibly also using them in gene therapies to restore a specific function.

This thesis presents also the development of genetic tools in which the particular role these genes play in RGCs development can be studied at high resolution. We created 2 mutant lines, in which, by using the dual systems of recombination, we can ensure that a gene is expressed only in a particular cell type. As a theoretical example of a rescue of a phenotype, I will use gene X, found downstream of Brn3b in the set of the RGCs specific genes, and regulated by Brn3b. Let us assume that experimental data has shown that indeed the gene X has a highly specific sequence on which the TF Brn3b links directly. One can design a construct in which a reporter is flanked by two LoxP sites and followed by the CDS of gene X, construct that can be expressed into the $Brn3b^{CKOCre}$ mouse retina upon viral delivery. The result of recombination between the two LoxP sites will ensure the elimination of the reporter and the expression of the gene X directly under the Brn3 promoter, therefore only in certain cell types influenced directly by Brn3b. At this moment this represents the only available possibility to test the influence of gene X in a limited subset of RGCs. However, as stated in Chapter III, this system has some limitations. First, in order to have that desired result at the end of the recombination, the lines need to be crossed with a retinal-specific expressed Dre recombinase. Indeed, the one used so far, which expresses Dre in the germ-line (CAG:Dre) provides the expression of the reporter in the entire organism. For example, transgenes can be developed using the promoter of retinal specific genes (like, for example, the Pax6) to drive the Dre recombinase.

Finally, three different cell lines were generated in which the optogenetic tool ChR was differently expressed, together with additional calcium and potassium channels. These lines are designed in a manner to make them a suitable tool for electrophysiology of a neuron, without damaging any valuable neuronal tissue.

Related to the design of the cell lines, in specially the CaPoChR2 line that carries the 3 elements – Ca and K channels, as well as the ChR2 – was designed in a manner to express the 3 elements at a unique locus in the cell's genome. However, fluorescence analysis showed that the expression of the ChR-EYFP is more reduced in the variant with Ca and K comparing with the cell line in which only the ChR was expressed in the pcDNA5/FRT vector. That suggests the limitation of the 2A linker regarding the ration of expression of the elements that are linked. An explanation can be in the fact that all the 3 channels are complicated proteins that need different rearrangements in order to exert the designated function, more in the case of the ChR, which is a 7TM protein.

Conclusions

This thesis provides new information about the biology of retinal ganglion cells, clarifying different aspects.

First, it is shown that patterning of retinal neurons of all four classes (photoreceptors, horizontal, bipolar and amacrine cells), as well as density of ribbon synapses and of gap junctions are not significantly affected by a severe reduction in the number of RGCs. This evidence supports the notion that the general retinal architecture and connectivity are dictated by master genetic programs that are largely cell-autonomous and not specifically affected by manipulations of these transcription factors.

Secondly, the genetic program that leads the correct development and function of different sets of RGCs was investigated. We were able to obtain a highly enriched population of RGCs using a rather quick protocol, optimal for downstream experiments based on RNA analysis. Expression profile analysis was performed using high throughput analysis of data obtained by next-generation sequencing (RNA-seq), providing solid information regarding different sets of genes that determine distinct features of retinal ganglion cells; large set of them were validated by is-situ hybridization.

Two conditional KO mouse lines were developed, in which the Brn3a and Brn3b endogenous genes were replaced with the ORF of the Cre recombinase. In these lines, the expression of the Cre is conditioned by the recombination of the two RoxP sites that flank the endogenous gene and are highly specific for the Dre recombinase. This dual recombination system allows the analysis of different events that target individual subsets of RGCs.

In addition, stable HEK293 cell lines were generated that express different combination of a light sensitive cation channel (ChR), a voltage dependent Calcium channel (CaV1.2) and a potassium channel with reversal potential close to neuronal levels (ROMK1). This type of biological reporter will find broad application in the study of processes during which accurately timed membrane depolarization is required.