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Commonalities of optic nerve injury and glaucoma-induced neurodegeneration: Insights from transcriptome-wide studies☆

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Abstract

Glaucoma is a collection of diseases that lead to an irreversible vision loss due to damage of retinal ganglion cells (RGCs). Although the underlying events leading to RGC death are not fully understood, recent research efforts are beginning to define the genetic changes that play a critical role in the initiation and progression of glaucomatous injury and RGC death. Several genetic and experimental animal models have been developed to mimic glaucomatous neurodegeneration. These models differ in many respects but all result in the loss of RGCs. Assessing transcriptional changes across different models could provide a more complete perspective on the molecular drivers of RGC degeneration. For the past several decades, changes in the retinal transcriptome during neurodegeneration process were defined using microarray methods, RNA sequencing and now single cell RNA sequencing. It is understood that these methods have strengths and weaknesses due to technical differences and variations in the analytical tools used. In this review, we focus on the use of transcriptome-wide expression profiling of the changes occurring as RGCs are lost across different glaucoma models. Commonalities of optic nerve crush and glaucoma-induced neurodegeneration are identified and discussed.

Keywords

Glaucoma; Optic nerve crush; Retinal ganglion cell (RGC); Transcriptome; RNA sequencing; Microarray; GeneNetwork; Neurodegeneration

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Declaration of competing interest

None.

1. Introduction

Innovations in genetics, genomics and related technologies allow us to define the molecular cascades underlying blinding disease such as glaucoma. Affecting millions of people worldwide, glaucoma can cause an irreversible loss of vision (Leske, 1983; Quigley, 1996; Thylefors and Negrel, 1994). One widely accepted location for axon damage at the optic nerve head, leading to the death of retinal ganglion cells (RGC). Defining the molecular cascades mediating RGC loss could lead to development of valuable therapeutic interventions to treat or even cure glaucoma. Within this diverse set of diseases, there are only few causes that are due to Mendelian inheritance involving a single gene such as Myocilin (Resch and Fautsch, 2009; Sears et al., 2019; Stone et al., 1997). In contrast, most glaucoma cases are due to complex genetic interactions. The most common form of glaucoma is primary open-angle glaucoma (POAG), which is usually accompanied by an increase of intraocular pressure (IOP). In some cases, such as normal tension glaucoma, RGC death occurs even when the IOP is relatively low. Clinically lowering IOP remains the single best treatment for glaucoma; however, lowering IOP does not completely prevent the progression of the disease (Investigators, 2000; Jammal et al., 2021; Leske et al., 2003; Lichter et al., 2001). For most glaucoma including POAG and normal tension glaucoma, multiple risk factors affect the loss of RGCs and the progression of the disease (Aboobakar et al., 2016; Liu and Allingham, 2011; Nickells, 2012; Springelkamp et al., 2017). The Ocular Hypertension Treatment Studies (OHTS) (Gordon et al., 2002) and subsequent independent findings of others (European Glaucoma Prevention Study et al., 2007; Medeiros et al., 2003) defined a number of phenotypic risk factors for POAG, including: age, intraocular pressure (IOP), central corneal thickness, cup-to-disk ratio, and family history (genetic background). Driven by recent advances in genomics technologies, more and more genetic risk factors have been identified in a series of genome wide association (GWAS) studies (Craig et al., 2020; Gharahkhani et al., 2021; Springelkamp et al., 2015; Wiggs et al., 2013). To date, a total of 127 genomic loci associated with the susceptibility to POAG have been recognized (Gharahkhani et al., 2021). In many cases, the relative functional consequences of the genetic risk factors are unknown. Using animal models and a variety of different approaches, many of the molecular interactions leading to glaucomatous injury and RGC death are being defined. In this review, we highlight interesting candidate genes and molecular pathways, looking at cutting edge methods such as single-cell RNA sequencing (scRNA-seq). The advances we are seeing in our understanding of glaucoma are informing us about targets for intervention and treatment for this blinding disease.

When examining the changes occurring in RGCs in glaucoma or after optic nerve damage, it is important to realize that there are several distinct functional compartments within the RGCs (Syc-Mazurek and Libby, 2019). After injury, there is a distinct series of events occurring in the axon distal to the injury as axon death and Wallerian degenerations occurs (Conforti et al., 2014). This portion of the axon is removed from the transcriptional networks in the cell body of the RGC and thus is undergoing an injury process without transcriptional support from the nucleus or trafficking of proteins by axonal transport (Conforti et al., 2014). The proximal portion of the axon, between the site of injury and the RGC somata undergoes a very different response. Initially, there is an axonal regrowth attempt that was

termed abortive regeneration by Ramon y Cajal (Ramón y Cajal, 1926). Growth-cone like structures form and proteins pile up at the site of injury as axonal transport meets the end of the injured axon (Conforti et al., 2014). There are also specific changes within the dendrites of the RGCs that include remodeling and altered protein and mRNA transport (El-Danaf and Huberman, 2015; Risner et al., 2018). Finally, within the RGCs soma itself are dramatic changes in gene transcription and protein production affecting not only the soma, but also the dendrites and proximal axon. Here, we review the changes occurring in the transcriptome and proteome from the whole retina (Freeman et al., 2011; Guo et al., 2010; Howell et al., 2011; Panagis et al., 2010; Sharma et al., 2014; Steele et al., 2006; Ueno et al., 2018; Watkins et al., 2013; Yang et al., 2007; Yasuda et al., 2014a, 2014b, 2016) or isolated RGCs (Belin et al., 2015; Bray et al., 2017; Fischer et al., 2004; Guo et al., 2011; Tran et al., 2019; Williams et al., 2017b) that occur following glaucomatous injury or optic nerve crush. As a proxy to comprehend the changes occurring in glaucoma, we will focus on genome-wide expression profiling defining the changes occurring in the retina or RGCs in response to axon injury by examining microarray and RNA-seq studies, and, to a lesser extent, proteomic studies. In all of these, it is understood that specific methods have strengths and weaknesses. Technical differences and variations in the analytical tools may account for some of the subtle differences in results. Nonetheless, each of these approaches offers valuable insights into the response of the RGC to injury. Our laboratory has examined the response of the retina to optic nerve crush in the mouse, specifically in the BXD recombinant inbred (RI) strain set (Geisert and Williams, 2020; Templeton et al., 2013; Wang et al., 2018) and due to our extensive use of this model system, we have used it to frame some interpretations in this review. The BXD RI set has been used for more than a decade to study the genetic basis of variations in the structure of the eye, retina, and central visual system (Geisert and Williams, 2020). We created large databases with considerable statistical power, among which are Affymetrix Mouse Gene 2.0 microarrays from 55 different normal strains and Illumina Mouse WG-6 v2.0 microarrays from 62 mouse strains following optic nerve crush. In addition, the BXD strains (Fig. 1) are bred to provide a genetic reference panel allowing for the mapping of genetic networks (Geisert et al., 2009). The power of this set of mouse strains lies in the recombination between the two parental genomes. There are over 7000 break points allowing for fine mapping of quantitative trait loci and the identification of genetic networks by correlating expression profiles across the BXD strains (Geisert and Williams, 2020).

2. Mouse models of RGC degeneration

Animal models are one of the most viable tools for researchers to study diseases, especially complex diseases with multiple genetic and environmental risk factors. In general, each model brings a unique benefit to the understanding of the disease process, and in specific cases, the animal models are critical to the development of therapeutics and treatments (Pang and Clark, 2020; Struebing et al., 2016). This is especially the case for glaucoma. Glaucoma is a family of diseases affecting multiple biological systems, all of which result in the loss of RGCs and, if left untreated, blindness. There are purely genetic (inherited) models of glaucoma and experimentally induced models (Calkins, 2012; Geisert and Williams, 2020; Howell et al., 2008; McKinnon et al., 2009; Struebing and Geisert, 2015; Yang and Zack,

2011). Of the genetic models, the most widely used is the DBA/2J mouse. This mouse strain carries two mutations (Tyrp1^{b} and *Gpnmb*^{R150X}), which together cause an inherited disease reminiscent of pigment dispersion glaucoma in humans (Anderson et al., 2002). Mirroring human glaucoma, this model is asynchronous (both eyes are not affected to the same extent) and its occurrence is sporadic. Although these characteristics truly recapitulate many of the epochs of human glaucoma, the DBA/2J model complicates experimentation, simply because it is unpredictable and sporadic in nature. To synchronize the onset of glaucoma, inducible models can be used. This is commonly achieved through IOP elevation (Samsel et al., 2011; Sappington et al., 2010) either by direct or indirect blockage of the trabecular meshwork. Examples include injections of polystyrene or magnetic microspheres into the anterior chamber to block the trabecular meshwork (Samsel et al., 2011; Sappington et al., 2010); other models mimic or cauterization of the episcleral vein (Ruiz-Ederra and Verkman, 2006), which both block the outflow facility further distally, leading to increased IOP. Episcleral and limbal veins can also be photocoagulated with argon laser, leading to similar effects (Gross et al., 2003). Recently, two additional models were developed. One using the photopolymerization of hyaluronic acid glycidyl methacrylate in the anterior chamber to block the trabecular meshwork (Guo et al., 2018). The other injected silicon oil in to the anterior chamber to induce ocular hypertension (Zhang et al., 2019). All of these inducible models cause IOP elevation within a few days after the procedure, and in most models, IOP stays elevated for a couple of weeks, which is enough to compromise RGC health.

The most straight-forward way to damage RGCs directly is optic nerve crush (ONC), where the optic nerve is crushed using self-closing fine forceps(Dietz et al., 2014; Fernandes et al., 2013; Li et al., 2007; Templeton et al., 2009, 2013; Templeton and Geisert, 2012). The advantage of this technique is a dramatic and temporally synchronized injury to all RGC axons (Allcutt et al., 1984). This allows the research scientist to look at the effects of injury in a relatively large number of cells simultaneously. The disadvantage is that this does not represent the type of continuous non-synchronized cell death that is observed in either acute glaucoma or chronic glaucoma. It is worth noting that the response of the retina to elevated IOP is not identical to that of ONC (Yang et al., 2007). These data indicate that there are changes that are common to all injuries independent of the type of insult, and that there are genetic changes that appear to be model specific. A comparison between the models has to consider the staggered cell death of specific cell types in the genetic models of glaucoma as compared to the synchronized death of all RGC subtypes in the ONC model. All of these models are potentially instructive of the changes occurring in human glaucoma.

3. Changes in protein and gene expression following injury

There are a number of different methods to examine the changes occurring in the retina following injury. Using anatomical methods, investigators would examine the morphology of the retina and could see that RGCs and optic nerve axons degenerated following ONC (Allcutt et al., 1984; Barron et al., 1986) and they were also lost in advanced stages of glaucoma (Kalesnykas et al., 2012; Quigley et al., 1995). As molecular tools advanced, it became possible to look at individual proteins and their altered expression following injury. This allowed for the detection of changes in expression along with the cellular localization

of the proteins. It also provided a means to detecting the temporal changes in protein expression. Both glaucomatous injury and ONC evoke a series of well-characterized changes in protein and gene expression. With the death of RGCs, there are pronounced declines in the expression of many RGC markers, including THY1 (Barnstable and Drager, 1984; Li et al., 1999; Schlamp et al., 2001), POU4F1 (Erkman et al., 1996; Jain et al., 2012; Sajgo et al., 2017; Xiang et al., 1995), POU4F2 (Erkman et al., 1996; Jain et al., 2012; Sajgo et al., 2017; Xiang et al., 1993, 1995), and Class III Beta tubulin (TUBB3) at the protein (Mellough et al., 2004) and message levels (Struebing et al., 2016). These changes are also observed following ONC (Templeton et al., 2013; Templeton and Geisert, 2012), experimentally induced glaucoma (Chen et al., 2011; Huang et al., 2018; Struebing and Geisert, 2015) and in naturally occurring murine models of glaucoma such as the DBA/2J mouse (Anderson et al., 2002; Stone et al., 1997). They represent early signs of injury, reflecting the rapid changes occurring in the RGCs. Another common hallmark of glaucoma and optic nerve injury is reactive gliosis. In this glial response to injury, astrocytes and Müller glial cells hypertrophy and undergo a series of changes that is best characterized by an upregulation of glial fibrillary acidic protein (GFAP) (Bjorklund and Dahl, 1985; Pekny et al., 2014). These changes signify injury not only in the retina but also throughout the central nervous system.

With the advent of high throughput genomic technologies such as microarrays and next generation sequencing, researchers are able to examine systematically the global changes in the transcriptome. These methods, along with sophisticated bioinformatic tools, reveal many significant changes occurring in glaucoma and after ONC. These efforts have generated a rich group of datasets looking at changes in gene and protein expression after glaucomatous injury or optic nerve crush (Table 1 and Supplemental Table S1). We have made comparisons across the studies based on their published DE gene list and/or supplementary data (Guo et al., 2010, 2011; Howell et al., 2011; Panagis et al., 2010; Park et al., 2019; Sharma et al., 2014; Steele et al., 2006; Templeton et al., 2013; Tran et al., 2019; Ueno et al., 2018; Watkins et al., 2013; Williams et al., 2017b; Yang et al., 2007; Yasuda et al., 2014a, 2014b, 2016). For the few studies in which the DE genes were not reported (Howell et al., 2011; Sharma et al., 2014; Williams et al., 2017b), their data stored in Gene Expression Omnibus (GEO) repository. The data was downloaded and re-analyzed to define DE genes. This has allowed us to define the frequency of detecting changes in gene expression across studies of glaucoma and optic nerve crush. By including different models, we are also able to define the changes that are in common in both models representing alterations in the retinal transcriptome due to general injury to the retina. The collective data from this cross-study analysis forms the basis of our approach to this review as we look for the changes that were most frequently observed across the collective group of studies. This includes transcripts that are up-regulated following injury (Fig. 2) as well as transcripts that are down-regulated (Fig. 3). Most of the changes were similar in both glaucoma and optic nerve injury models. There were differences in gene expression which could be due to elevated IOP, asynchronous death associated with glaucoma or the synchronized injury that results from ONC. Both models involve RGC axon injury and both result in a retrograde signal to the RGC somata initiating the death of the cell. The very nature of each injury may underlie differences in the response of the retina. The glaucoma models result in a gradual axonal insult at the optic nerve head and potentially an exaggerated difference in response

of RGC subtypes (see below); while with crush or transection the injury is immediate and synchronized across all RGC subtypes. There are few studies that had few or none up- or down-regulated genes overlapping with other studies (Figs. 2 and 3), mostly due to the limited number of genes (15–40 genes) that they reported in their publication (Panagis et al., 2010; Park et al., 2019; Steele et al., 2006; Ueno et al., 2018). For the study of (Sharma et al., 2014), we re-analyzed their data and selected the top 100 DE genes for analysis, and the results showed only one up-regulated gene (*Atf3*) overlapped with other studies (Fig. 2), while 5 down-regulated genes overlapped with other studies (Fig. 3).

3.1. Transcripts up-regulated after injury

Looking at the changes in the transcriptome, many of the up-regulated transcripts are in common across all of the different injury models. The 57 most frequently identified up-regulated transcripts are shown in Fig. 2. The top 10 up-regulated genes were identified in at least 6 of the studies and include*: Atf3, Tnfrsf12a, Sox11, Lcn2, Jun, Clic1, Hmox1, Gfap, Ecel1* and *Asns*. Examining the pattern of up-regulated transcripts (Fig. 2), most of the genes are up-regulated following injury in both ONC models and glaucoma models. However, there are genes that were identified in either ONC models of injury or in glaucoma models. Two genes (*Ifitm3* and *C3*) were up-regulated in glaucoma models and were not in the most frequently up-regulated genes in models of direct optic nerve injury. There were also many genes that were determined to be up-regulated following ONC and were not observed in glaucoma models, including: *Aars, Adcyap1, Arid5a, Atf5, Cdkn1a, Chac1, Cox6a2, Ddit3, Gars, Mthfd2, Nupr1, Phgdh, Plekho1, Psat1, Rhog. Slc7a3, Srxn1, Stmn4, Tac1, Tes* and *Vgf*. Two of these genes (*Chac1* and *Srxn1*) were observed to be increased in Moderate Glaucoma in the study by Howell et al. (2011). The data is available on GeneNetwork.org (Data set: Howell et al., 2011, DBA/2J Glaucoma Retina M430 2.0 RMA). Five of these genes (*Aars, Adcyap1, Pieklno1, Tac1* and *Vgf*) were slightly down-regulated in the DBA/2J model (Howell et al., 2011); while being up-regulated in after ONC 2 days after crush (Templeton et al., 2009, 2013). In this review we will analyze these changes looking at functional pathways and unique changes in gene expression. Many of these transcripts participate in major common pathways activated by injury and will be considered below.

3.1.1 Immediate early genes—There is a rapid increase in the expression of many immediate early genes in response to injury. The gene with the highest frequency of detection across the glaucoma and ONC studies was *Atf3*, a gene associated with an immediate early response. The expression level of *Atf3* is found to be up-regulated in 11 out of 15 studies (Fig. 2). While its expression is maintained at low levels in normal RGCs, it is significantly upregulated in response to retinal damage, including both ONC and glaucoma. *Atf3* (Activating Transcription Factor 3) is a transcription factor that belongs to the ATF/CREB family, which is known to have different functions in different mammalian cells. Its physiological role in a variety of cells is to relieve the stress by DNA damage repair and regulate cell cycle through activation or repression of cell-cycle regulators such as p21 and p53 (Yan and Boyd, 2006). Enhancement of p21 and reduction of p53 expression following overexpression of ATF3 leads to cell-cycle arrest (Yoshida et al., 2008). The expression of ATF3 can be stimulated by a variety of stress signals including hypoxia, chemokines, and cytokines (Rohini et al., 2018). ATF3 was previously identified as an

important neuronal marker for nerve injury, induced upon cellular stress in the sensory and motor neurons as well as dorsal root ganglions (Braz and Basbaum, 2010; Tsujino et al., 2000). It was not expressed in naive rats' spinal cord but was immediately induced in all DRG neurons following peripheral nerve axotomy (Tsujino et al., 2000). A recent study showed that overexpression of ATF3 promoted RGC survival and preserved RGC function 2 weeks after ONC (Kole et al., 2020). However, overexpression of ATF3 and simultaneous downregulation of PTEN did not provide additional RGC neuroprotection compared with PTEN downregulation alone (Kole et al., 2020). Thus, ATF3 appears to be neuroprotective in the retina following injury and may provide insights into potential treatment for axonal damage in the optic nerve.

The role of *Atf3* in RGCs may potentially be related to its function in innate immunity. The promoter region of ATF3 exhibits numerous transcription factor-binding sites, such as AP-1, ATF/CREB, and NF-κB, suggesting that ATF3 may be induced by stress signals, including cAMP, calcium influx, and cytokines (Liang et al., 1996). Studies have demonstrated that ATF3 homodimer binds to the promoter regions of its target genes and recruits HDAC, which in turn inhibits the transcription of these genes (Cheung et al., 2000). After stress-induced activation of immune cells, *Atf3* is up-regulated and subsequently downregulates the expression of target genes, including cytokines (e.g., IL-1β, IL-4, IL-5, IL-6, IL-12p40, IL-12b, IL-13, TNF, and IFNβ/γ) (Ku and Cheng, 2020) and pro-apoptotic genes *Bak* and *Bax* (Thompson et al., 2013), by binding to their promoters. It also negatively regulates the transcription of pro-inflammatory cytokines that contains ATF/CREB binding sites, such as Toll-like receptor 4 (TLR-4) (Gilchrist et al., 2006; Hoetzenecker et al., 2011). After exposure to injury, TLRs activate the innate immune system signaling cascade and stimulate the release of inflammatory cytokines. Inhibition of the TLR4 receptor has been shown to enhance RGC survival in ONC (Morzaev et al., 2015), ischemic retinal injury (Halder et al., 2015) and ischemic injury to other parts of the brain (Kilic et al., 2008; Poyomtip, 2019). Moreover, Wang et al. (2020) reported that in experimental glaucoma model, targeting TLR4/NF-κB to suppress pro-inflammatory factors (P50, IL-6 and TNF-α). This evidence supports the idea that activation of *Atf3* may be protective to the RGCs after injury (Kole et al., 2020).

When we examine the list of up-regulated genes, other members of the immediate early response genes are present, including: *Jun, Hspb1* (HSP27), *Egr1, Edn2, Gal, Sprr1a* and *Ddit3* (CHOP). ATF3 is known to interact with many of these other genes (Fig. 4). It is down-stream from *Jun* (Chen et al., 1996; Hai and Curran, 1991; Hein et al., 2015; Li et al., 2015). One of the targets of JUN is ATF3 (Guo et al., 2009). Both *Atf3* and *Jun* are up-regulated following different types in injury and play key roles in modulating cell death or cell survival (Fernandes et al., 2013). They have been implicated in both protective and detrimental roles depending on cell type, cellular environment and context of expression. In neurons, ATF3 is induced in response to axotomy and there is a correlation between increased *Atf3* expression and upregulation of *Jun* in surviving neurons following injury (Takeda et al., 2000; Tsujino et al., 2000). There is also evidence that axonal regeneration is initiated when both JUN and ATF3 are up-regulated in neurons in response to axotomy (Pearson et al., 2003). They are also identified to promote neurite outgrowth in both CNS (rat cortical neurons) and PNS neurons (mouse DRG) (Chandran et al., 2016; Danzi et al.,

2018). Down-stream of ATF3, we find a group of genes (*Hspb1, Egr1, Edn2, Gal, Sprr1a* and *Ddit3*) that are involved in the response of the retina to injury and in the regulation of cell death (Fernandes et al., 2013; Giraldo et al., 2012). Overall, the immediate early response appears to play multiple roles in the response of the retina to the insult caused by glaucoma and ONC.

3.1.2. Up-regulation of Sox11—*Sox11* was another gene frequently up-regulated in glaucoma and after ONC. Following nerve crush in the mouse, *Sox1*1 mRNA increases by 8-fold making it the most highly up-regulated transcription factor two days following optic nerve crush (Li et al., 2018). The SOX11 protein is known to play a significant role in the response of neurons to injury. After injury to the peripheral nerve, there is a dramatic upregulation of SOX11 in the dorsal root ganglion as the axon begins to regenerate (Jankowski et al., 2009; Tanabe et al., 2003). The upregulation and sustained expression of *Sox11* is critical to the survival of the dorsal root ganglion neurons and the regeneration of peripheral axons along the injured nerve. Decreasing levels of *Sox11* in the neuronal cell body results in slower axonal regeneration of peripheral nerves (Jankowski et al., 2006, 2009). When *Sox11* is knocked down in cultured peripheral neurons, there is also a reduction in neurite growth and an increase in apoptosis (Jankowski et al., 2006). Conversely, over-expressing *Sox11* in cultured dorsal root ganglion cells produces an increase in neurite growth, and *in vivo* overexpression of *Sox11* accelerates the growth of regenerating axons (Jing et al., 2012). These data reveal the critical role of *Sox11* in axon regeneration. When we examined publicly available datasets for the changes occurring in the DBA/2J model of glaucoma, *Sox11* was dramatically up-regulated in the early phases of glaucomatous damage (Howell et al., 2011). A similar pattern was observed following ONC in C57BL/6 and DBA/2J mice, where *Sox11* is up-regulated over 2-fold 2 days and over 3-fold 5 days after nerve crush (Templeton et al., 2013). Previous studies have implicated artificially high levels of *Sox11* to promote regeneration in PNS (Chandran et al., 2016; Jankowski et al., 2009; Jing et al., 2012) and corticospinal axons (Wang et al., 2015b).

Further evidence in support of the notion that SOX11 promotes axon growth comes from studies overexpressing (Norsworthy et al., 2017) or knocking-down *Sox11* (Li et al., 2018; Welsbie et al., 2017). Norsworthy et al. (2017) found that *Sox11* upregulation facilitated axon regeneration following ONC, while Li et al. (2018) found that down-regulation of *Sox11* two weeks before ONC injury resulted in no increase in axonal regeneration. This is a clear indication that there is a cell intrinsic effect of *Sox11* on the regenerative capacity of RGCs. The response in RGCs suggests that this may be in part related to the “abortive regeneration” response termed by Ramon y Cajal (Otero, 2018; Ramón y Cajal, 1926). Welsbie et al. (2017) demonstrated that the upregulation of *Sox11* happens down-stream of DLK/LZK. It may be possible to identify the down-stream targets within the transcriptional cascade associated with the regenerative stall of the retina relative to the *Sox11* regeneration associated with the peripheral nerve. Gene profiling showed that *Sox11* overexpression activated a set of developmental genes which are possibly related to axon growth and that it downregulated genes involved in synaptic transmission. This is consistent with a developmental process switch from axon growth mode of immature neurons to dendrite or synapse growth mode in mature neurons.

3.1.3. Innate immune system—Among the most frequently up-regulated genes are members of the complement cascade, part of the innate immune system, including *C1qb, C4b* and *C3*. These genes are consistently observed following glaucomatous or optic nerve injury and are transcribed within retina itself (Ahmed et al., 2004; Howell et al., 2011; Panagis et al., 2010; Steele et al., 2006; Templeton et al., 2013; Vazquez-Chona et al., 2004). Recent studies that examined the molecular responses either following glaucomatous damage (Howell et al., 2011), ONC (Sullivan et al., 2012; Templeton et al., 2013) or even ocular blast injury (Struebing et al., 2018a), confirmed and validated the involvement of the complement cascade (Howell et al., 2011) and the activation of an innate immune network within the retina itself (Templeton et al., 2013). Using a systems biology approach (Templeton et al., 2013), we defined a genetic network modulating the expression of this innate immune system in response to injury. Many of the complement gene expression changes are associated with the activation of microglia and astrocytes (Silverman et al., 2016; Soto and Howell, 2014; Templeton et al., 2013), which are now known to be major players in the response of the retina to glaucoma-relevant insults (Bosco et al., 2011).

We found that the innate immune response is a highly coordinated genetic network that is activated by injury (Templeton et al., 2013), and within this network, we find a microglial gene signature, including *Aif1* (the gene product of the protein IBA1, a marker of increased microglial activity), the lysosomal marker *Cd68*, and *Cd74*. This indicates that at least glial cells intrinsic to the retina are associated with this innate immune network. Single cell RNA-seq data indicates that in the normal retina, Muller glial cells are the major source of *C1q, C3* and *C4* (Pauly et al., 2019). This does not exclude the possibility that these genes are expressed in other retinal cell types.

In the immunologically privileged environment of the retina (Benhar et al., 2012), complement components play a functional role in surveillance, neural development, and the response of the retina to injury (Alawieh et al., 2018; Orsini et al., 2014; Silverman et al., 2016). C1q and C3 are intimately involved in RGC death and survival. C1q deficiency is neuroprotective (Howell et al., 2014; Kumari et al., 2015) and can protect the RGC cell body, dendrites and synaptic connections (Williams et al., 2016). In contrast, deletion of C3 negatively impacts RGC survival (Harder et al., 2017) in glaucoma. Interestingly, inhibition of C3 activation (but not totally eliminating C3 expression) contributes to RGC degeneration (Harder et al., 2017), suggesting that C3 activation is associated with RGC degeneration. All of these data point to the critical role of the complement system in the response of the retina to insult playing a critical role in RGC susceptibility to death. This same system is at work throughout the central nervous system. In the brain, others have found that glial cells are sources of many members of the complement cascade (Trouw et al., 2008; Veerhuis et al., 2011; Walker et al., 1995). Even in other neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease, which directly affect the brain, the involvement of microglia is increasingly appreciated (Mukherjee et al., 2019).

3.2. Global down-regulation of retinal genes following injury

When examining the most frequently identified down-regulated transcripts (Fig. 3), the immediate feature that stands out is that these genes are markers

for RGCs, including: *Kcnd2, Sncg, Pvalb, Pou4f1, Nefl* and *Nefh*. To examine further this possibility, we interrogated the mouse RGC atlas (Tran et al., 2019) (https://singlecell.broadinstitute.org/single_cell/study/SCP509/mouse-retinal-ganglion-cell-adult-atlas-and-optic-nerve-crush-time-series#study-visualize). The top 50 most frequently identified down-regulated genes were placed into the search engine, all of which showed high expression (Fig. 5). Some transcripts, like *Pou4f1* and *Sncg*, were expressed across all RGC subtypes, while other transcripts had restricted expression in only a few RGC subtypes, such as *Ctxn3, Pou4f2* and *Irx2*. These data provide clear evidence that one of the major changes in the transcriptome of the retina following injury is the down-regulation of RGC-specific genes.

Pathway annotation of these gene sets gives an insight of the underlying biology. The top five KEGG pathways of down-regulated genes include: oxidative phosphorylation, CNS degeneration, ubiquitin mediated proteolysis, mRNA surveillance pathways, and adrenergic signaling. One of the more interesting changes is the depression of genes associated with oxidative phosphorylation involving mitochondrial pathways associated with ATP production and NADH dehydrogenase activity. These pathways are also suppressed following blast injury to the retina (Struebing et al., 2018a) and can be thought to reflect a general depression in mitochondrial activity. The mitochondrial gene aldehyde dehydrogenase (*Aldh7a1*) is most strongly associated with the susceptibility of RGCs to experimentally induced glaucoma in mice (Struebing et al., 2018b), and its pathway is known to be associated with increased glaucoma risk in humans (Bailey et al., 2016). The metabolic depression following injury is proving to be increasingly relevant to glaucoma and nervous system injury (Wang and Barres, 2012).

3.2.1. NAD related pathways—NAD + has received a considerable amount of attention due to its potential role in neuronal protection (Howell et al., 2013). The first molecular insights linking NAD^+ to neuronal death and axon degeneration came from the identification of a mutation in the mouse, called “Wallerian degeneration slow”, WLDs (Perry et al., 1991). The mutation results in the production of a chimeric protein made up of UBE4B and NMNAT1 (Lunn et al., 1989; Perry et al., 1991). The WLDs fusion protein localizes not only to the nucleus but also to axons (Wang et al., 2015a), resulting in an increased production of NAD^+ in cellular compartments that it is normally distributed. This increase in NAD^+ has profound effects of axon and neuron survival. In the DBA/2J mouse model of glaucoma, supplementing the diet with NAD+ or overexpressing *Nmnat1* partially protects from glaucomatous degeneration (Williams et al., 2017c). When the WLDs allele is placed on the DBA/2J background and the mouse is also supplemented with NAD+, there is an almost complete rescue from the effects of glaucoma with 94% of the treated eyes not developing glaucoma (Williams et al., 2017a). These data strongly indicate that a critical effect of altered oxidative phosphorylation is associated with neuronal death that can be overcome by supplementing NAD^+. Interestingly, the NAD + pathway has clear relevance to RGC survival in models of glaucoma (Williams et al., 2017b) but does not seem to confer a protective effect in ONC (Fernandes et al., 2018). This is part of the growing evidence that the mechanisms of RGC soma death, and Wallerian degeneration are distinctly different.

Thus, strategies for neuroprotection of RGC somata and axon survival are different, with both being necessary for the retention of visual function.

4. Selective changes in glaucoma vs ONC

Looking at the genes frequently detected as up-regulated (Fig. 2), there were a group of genes that were up-regulated following ONC that were not detected as up-regulated following glaucomatous injury. These genes include: *Aars, Adcyap1, Arid5a, Atf5, Cdkn1a, Chac1, Cox6a2, Ddit3, Gars, Mthfd2, Nupr1, Phgdh, Plekho1, Psat1, Rhog, Slc7a3, Srxn1, Stmn4, Tac1, Tes* and *Vgf.* As an initial step to evaluate these genes selectively up-regulated following ONC, we examined the glaucoma dataset generated from the DBA/2J glaucoma model (Howell et al., 2011) and the changes occurring 2 days following ONC (Genenetwork. org, HEI ONC vs Control Retina Illumina V6.2 RankInv dataset) (Templeton et al., 2013). Of these genes, all were up-regulated 2 days following ONC. Two of the transcripts, *Chac1* and *Srxn1*, were up-regulated in cases of moderate levels of glaucomatous damage (Genenetwork.org, Howell et al., 2011, DBA/2J Glaucoma Retina M430 2.0 RMA dataset) (Howell et al., 2011). Thus, the majority of these genes are truly differentially up-regulated following ONC (Table 2). To define the potential role of these genes in the injury response of the retina, we turned to the single cell RNA-seq data from Tran et al. (2019). All of these genes, with the exception of *Rhog,* were expressed in isolated RGCs (Supplemental Fig. 1) and were dramatically up-regulated after ONC, beginning 1 day after ONC and extending to at least 2 weeks after ONC (Supplemental Fig. 2).

When we examine the list of genes that appear to be up-regulated selectively in ONC, there are several genes that are associated with stress responses or mitochondria, including: *Charc1, Nupr1, Ddit3* (CHOP), *Cox6a2, Mthfd2 Aars* and *Gars*. The most highly up-regulated gene 2 days after ONC was *Chac1* (Templeton et al., 2013) which was up-regulated nearly 6-fold compared to normal retina and was not up-regulated in the D2 glaucoma dataset (Howell et al., 2011). It is also up-regulated in RGCs (Tran et al., 2019). *Charc1* is a proapoptotic protein and is down-stream of the ATF4-ATF3 CHOP pathway. It is involved in ER stress potentially leading to cell death (Mungrue et al., 2009; Oh-Hashi et al., 2013; Wang et al., 2019). Nuclear protein 1 (*Nupr1)* is the gene with the second largest increase, 3-fold. *Nupr1* is a stress induced chromatin protein involved in ER stress. With the inactivation of ER stress and the induction of function deficits mitochondria decreasing ATP NUPR1 results in ER-stress response (Santofimia-Castano et al., 2018). NUPR1 down-regulation prevents pancreatic cells a normal ER stress response, leading to a programmed cell death (Santofimia-Castano et al., 2018). Nupr1 is involved in neuronal apoptosis and autophagy through the ER stress signaling pathway (Xu et al., 2017). *Ddit3* (CHOP) was up-regulated 2.5-fold in the Templeton et al. database (Templeton et al., 2013). *Ddit3* deletion conferred mild protection to RGC somas, but did not significantly prevent RGC axonal degeneration in the D2 glaucoma model. Together, these data suggest that *Ddit3* plays a minor role in perpetuating RGC soma apoptosis caused by chronic ocular hypertension-induced axonal injury, and thus does not significantly contribute to distal axonal degeneration (Marola et al., 2019). In optic nerve crush, *Ddit3* deletion partially protects the RGC soma following crush (Hu et al., 2012; Syc-Mazurek et al., 2017).

A second set of genes that appears to be selectively up-regulated after ONC is associated with mitochondrial function. These genes include: *Cox6a2, Mthfd2, Aars* and *Gars*. *Cox6a2* codes for an isoform of a cytochrome *c* oxidase subunit that is part of mitochondrial complex IV. Deletion of *Cox6a2* enhances oxidative stress in neurons, which in turn impairs maturation and functional properties (Sanz-Morello et al., 2020). These effects reflect the essential role of COX6A2 in energy balance in neurons. Interestingly, increasing niacin in obese rats causes an upregulation of *Cox6a2* along with oxidative phosphorylation (Ringseis et al., 2013). *Mthfd2* (methylenetetrahydrofolate dehydrogenase 2) is an NAD + dependent enzyme with dehydrogenase and cyclohydrolase activity, and it plays an essential role in mitochondrial one-carbon folate metabolism. Knocking down *Mthfd2* suppresses the trichloroacetic acid cycle (Pikman et al., 2016). Aminoacyl-tRNA synthetase (Aars) and glycyl-tRNA synthetase gene (Gars) are both are essential for protein translation in mitochondria (Boczonadi et al., 2018; Fine et al., 2019). The upregulation of these genes following ONC suggest that neurons are attempting to increase metabolic activity in an attempt to survive.

Are these changes really specific to ONC and glaucoma or are they intermingled, and if so, how can they be deconvoluted? In ONC, there is a synchronized injury induced to all RGCs alike, while in glaucoma and glaucoma models, there is a gradual injury to the axons at the optic nerve head and this injury can occur along different regions, causing wedge-shaped sectors of injured RGCs next to sectors where RGCs are uninjured (Howell et al., 2007). This latter effect may even be further exaggerated by the differential susceptibility of RGC subtypes to insult. Some RGC types, like the heavily labeled POU6F2 RGCs (Li et al., 2019) are very sensitive to glaucomatous injury (King et al., 2018), while other subtypes, like the intrinsically photosensitive RGCs, are resistant to injury (Cui et al., 2015; Perez de Sevilla Muller et al., 2014; Robinson and Madison, 2004). Thus, at any one time in a naturally occurring glaucoma model, a small number of RGCs are undergoing cell death and it is likely that the specific makeup of RGC subtypes differs over time. In fact, CHOP protein is up-regulated in RGCs following ONC (Fernandes et al., 2013; Hu et al., 2012); and CHOP protein is also increased in RGCs after elevated IOP (Doh et al., 2010) or in naturally occurring models of glaucoma (Marola et al., 2019). Although these different mouse models of glaucoma may not accurately reflect the changes occurring in human glaucoma, each of the model may provide unique insights into the disease process. Thus, these changes that appear to be unique to ONC, like the up-regulation of *Charc1, Nupr1, Ddit3* (CHOP), *Cox6a2, Mthfd2, Aars* and *Gars*, may be detected in ONC models, and are critical in the disease process of glaucoma even though they may not be readily detected by monitoring changes in the transcriptome of the retina of mouse glaucoma models.

5. Susceptibility of RGC subtypes to injury

When examining the down-regulation of transcripts after glaucomatous injury or ONC, changes associated with RGC loss are the primary focus, since the disruption of their homeostatic response directly relates to the clinical symptom of progressive blindness. Looking back on Cajal’s early work, there is a tendency to treat all of the RGCs as a single cell type when dealing with the response of optic nerve injury. Cajal himself defined many different RGC subtypes based on their morphology, and yet he ignores them when

examining the effects of injury to the optic nerve (Ramón y Cajal, 1892). It may have been a matter of needing to simplify the system to examine the effects of injury on the axons within the optic nerve, ignoring the fact that many different cell types send their axons down the optic nerve to the brain. Interestingly, to some extent, this approach has continued to the present day. This generalization applies not only to examining the response of axons to injury, but also to the way the RGC somata are responding to insult. When investigating RGC survival, most of the studies lump RGCs into one category, reporting the number of cells dying or the effects of manipulation on the rescue of RGCs (Jakobs et al., 2005; Syc-Mazurek and Libby, 2019).

The first hint that RGC subtypes respond differently to injury came from the Quigley laboratory, where it was found that in the optic nerve of monkeys with elevated IOP there appeared to be a selective loss of large diameter axons (Quigley et al., 1987; Sanchez et al., 1986; Weber and Harman, 2005); similar findings have been reported by the Weber laboratory (Weber and Harman, 2005). In these studies, large diameter axons were selectively lost within the optic nerve following ocular hypertension, suggesting that a subset of RGCs were selectively more sensitive to injury induced by elevated IOP. Recent advances defined RGC subtypes by examining uniquely expressed genes and proteins (Berson et al., 2002; Hattar et al., 2002; Kay et al., 2011; Kim et al., 2010; Munguba et al., 2013; Sanes and Masland, 2015; Tran et al., 2019). These studies form a basis for the identification of the of specific RGC subtypes in the mouse. For example, alpha RGCs selectively express KCNG4 and each of the 4 subtypes can be uniquely identified by specific markers: On-s αRGCs are OPN + Calbindin+, Off-s are OPN + Brn3a + Brn3c-, On-t αRGCs are OPN + Brn3a-/dimCalbindin-, and Off-t αRGCs are OPN Brn3c, respectively (Krieger et al., 2017). Specific sub-classes of ON-OFF directionally selective RGCs can be defined by the expression of CART (Kay et al., 2011) making up 15% of the RGCs. Using single cell RNA-seq on isolated RGCs, 46 specific RGC subtypes were identified based on gene expression profiles (Tran et al., 2019). These subtype specific markers provide the means necessary to track specific RGC subtypes following injury (Agostinone et al., 2018; Duan et al., 2015).

The response of RGC subtypes to injury is dramatically different (Duan et al., 2015; Tran et al., 2019). Cells that were the most resistant to death following ONC were the alpha RGCs: At 14 days following ONC, 80% of the alpha RGCs were still alive in the retina (Duan et al., 2015; Tran et al., 2019). In contrast, the most sensitive cells to ONC were the CART-positive ON-OFF directionally selective RGCs. Only a few CART positive cells remain in the mouse retina 14 days following crush. The most sensitive cells to injury appear to be RGCs heavily labeled with POU6F2 (King et al., 2018; Li et al., 2019). In the DBA/2J mouse model of glaucoma, heavily-labeled POU6F2 RGC subtypes were selective lost early in glaucoma (King et al., 2018; Li et al., 2019). We observed that 16% of the RGCs are POU6F2-positive but not in the CART positive RGCs, indicating that they are a novel ON-OFF directionally selective RGC subtype that is uniquely sensitive to glaucomatous injury (Li et al., 2019). The data clearly showed that *Pou6f2* was heavily expressed in a previously undefined RGC subclass. When looking at changes following ONC, *Pou6f2* is one of the genes that is sensitive to injury being down-regulated within 12 h after optic nerve crush (Tran et al., 2019). These findings demonstrate that some RGC subtypes are more sensitive to injury than

others (Daniel et al., 2018; Kay et al., 2011; King et al., 2018; Tran et al., 2019) and that specific RGC subtypes are relatively resistant to axonal damage.

6. Dendritic changes in RGCs following injury

In addition to monitoring the survival of RGCs, many investigators examine the morphological changes occurring in the RGC soma and dendrites following optic nerve crush (Daniel et al., 2018; Kalesnykas et al., 2012; Williams et al., 2013) or glaucoma (Agostinone et al., 2018; El-Danaf and Huberman, 2015; Kalesnykas et al., 2012; Puyang et al., 2015; Risner et al., 2018). Early on in the process of glaucomatous damage, the dendrites of RGCs become labeled with complement factor C3 (Bosco et al., 2018; Harder et al., 2017; Templeton et al., 2013). This is believed to be an early indicator of dendritic remodeling. Several labs have examined dendritic remodeling in the retina during ONC and glaucoma. In general, it is believed that dendrites are pruned in early phases of the RGC response to injury and that over time they become less and less branched and begin to retract (Risner et al., 2018). Many of these studies have attempted to delineate this response across all RGC subtypes. Thus, these findings could be confounded by examining different RGC subtypes with dendritic morphologies that are different even in the normal retina (Coombs et al., 2006; Dhande et al., 2015). The exception was the study from the Di Polo lab (Agostinone et al., 2018) that used a specific alpha RGCs marker to define RGCs and to track their dendritic changes following optic nerve damage.

The dendrites of the RGCs ramify in the inner plexiform layer in distinct sublaminae. The most inner portion of the inner plexiform layer is occupied by dendrites of ON RGCs and the outer portion of the plexiform layer is where dendrites of the OFF RGCs ramify. When examining the effects of dendritic remodeling in the ON and OFF sublamina of the inner plexiform layer, distinct differences in dendritic remodeling following injury were seen. In mouse models of glaucoma, the dendrites ramifying in the OFF sublamina are lost before those in the ON sublamina of the inner plexiform layer (Della Santina et al., 2013; El-Danaf and Huberman, 2015; Puyang et al., 2017). It appears that these OFF dendrites are more sensitive to the changes occurring following injury. The dendritic arbors within the OFF sublamina of the inner plexiform layer are more affected than those in the ON sublamina (Della Santina et al., 2013; El-Danaf and Huberman, 2015). In agreement with the anatomical changes, the OFF response was more severely affected than the ON response of RGCs, even in ON-OFF RGCs (Puyang et al., 2017). This differences between sublaminae is also observed in the ON-OFF directionally selective cells that have dendrites ramifying in both the ON and OFF sublaminae, where the dendrites of these cells in the OFF sublamina are more affected than those of the same RGC in the ON sublamina (Kay et al., 2011). Furthermore, the OFF component is functionally diminished before the ON component (Puyang et al., 2017). Taken together, these studies have provided a wealth of information about the response of the RGC to injury, indicating that OFF responses are lost early relative to ON responses.

7. RGC transcriptome networks

The differential susceptibility to injury of histologically distinct RGC subtypes led to attempts to identify subtype-specific, unique gene expression profiles that may account for the difference in RGC viability. A good start to define RGC subtypes would be to look at retinal gene expression differences in a genetically and phenotypically diverse population such as the BXD recombinant inbred strains. In this model, the BXD strains show differences in RGC specific gene expression along with variations in RGC number. This type of strain specific difference in cellular distribution occurs in photoreceptors number (Keeley et al., 2014) and horizontal cell number. By counting RGC axons in 17 strains, Williams et al. (1996) found a striking strain-specific difference with a range of 32,000 to 87,000 RGCs per retina. Recently, we extended RGC counts for 48 BXD strains, with a similar distribution as described before (Struebing et al., 2018b). The heritability of this trait was determined to be very high, with an h^2 of ~0.8. While no attempts were undertaken to distinguish between different RGC subtypes, it is conceivable that BXD strains also differ therein. In a recent study, we examined variance in between-strain gene expression as a proxy for RGC subtype susceptibility to increased IOP. First, we used the DoD CDMRP Retina expression microarray dataset (55 strains, 4 replicates per strain) to examine correlates of known RGC markers, which were compiled after a thorough literature review, across the BXD RI strain set. By examining their regulatory loci using expression quantitative locus mapping (eQTL mapping), we were able to associate almost all of the general RGC markers described in the literature selectively with one of two genetic networks, the *Thy1*-network or the *Tubb3*-network. Both *Thy1* (Barnstable and Drager, 1984) and *Tubb3* (Snow and Robson, 1994) are believed to be generalized markers for RGCs, and antibodies against their protein products (THY1/CD90 and TUJ1/Class III Beta tubulin) typically stain the entire RGC population. Nonetheless, these “pan-RGC markers” segregated into two distinct genetic networks with common upstream regulators, while subtype-specific RGC markers would contain regulatory signatures from either one or both “pan-RGC” networks. For example, this comprehensive analysis revealed that two pairs of subtype-specific genes were regulated in a similar fashion: *Cartpt* and *Jam2* as well as *Kcng4* and *Opn4* showed very similar heat maps to each other. *Cartpt/Jam2* shared the trans-band on distal Chromosome 1 with the *Thy1*-network, whereas *Kcng4/Opn4* shared the *Thy1*-network trans-band from Chromosome 13.

To define the susceptibility of RGC subtypes to glaucomatous injury, we used the glaucoma severity score (GSS) of publicly available microarray data generated from DBA/2J glaucomatous eyes (Howell et al., 2011) and correlated this with expression levels of RGC markers. As expected, expression levels of almost all RGC markers decreased as nerve damage increased. This was also the case for two subtype-specific markers, *Jam2* and *Cartpt*, but not for *Kcng4* and *Opn4*. While *Kcng4* is a known alpha-RGC marker, and *Opn4* is a specific marker for ipRGCs, our results suggest that these two RGC subtypes are differentially susceptible to nerve damage. This confirmed experimental data from other labs, where ipRGCs and alpha-RGCs were found to be exclusively resistant to optic nerve axotomy (Duan et al., 2015). This may be due to upstream modulators that share trans-bands and confer protection.

8. Defining RGC subtypes expression profiles

The most recent method for defining cellular subtypes is to profile the transcriptomes of individual cells, followed by clustering based on similar gene expression profiles. The advent of single cell RNA-seq protocols has opened this powerful approach to study the cell composition of the entire retina (Clark et al., 2019; Jaitin et al., 2014; Macosko et al., 2015; Norrie et al., 2019; Peng et al., 2019), and also specifically RGCs (Rheaume et al., 2018; Tran et al., 2019). These studies characterize RGCs from the C57BL/6J mouse: isolated at postnatal day 5 (Rheaume et al., 2018) and isolated from the adult mouse retina (Tran et al., 2019). Profiling single cells at different time points enables the establishment of subtype-specific trajectories. By tracing a specific marker's gene expression changes over time, developmental programs can be revealed. For example, when comparing the expression of *Pou6f2* across all three time points, the expression profiles within the population of RGCs differs significantly. There were considerably more *Pou6f2*--positive cells in the post-natal day 5 (P5) retina (Rheaume et al., 2018) as compared to the adult (Tran et al., 2019). To determine if this difference reflected the biology of the retina or technical differences, we stained retinas for POU6F2 at these different ages. At postnatal day 5, 51% of the RGCs were positive for POU6F2 (Geisert and Wang, unpublished observation), while in the adult of both C57BL/6J and DBA/2J mice, 32% of the RGC were labeled as POU6F2-positive. Identifying individual RGC subtypes is greatly facilitated by single-cell RNA-seq studies. Tran et al. (2019), characterized six RGC subtypes expressing relatively high levels of *Pou6f2* (termed 7-Novel, 8-Novel, 10-Novel, 18-Novel, 37-Novel and 44-Novel). If we examine the retina for heavily labeled POU6F2 positive cells, 7% of the cells were POU6F2 positive, while 7% were positive for POU6F2 and Cadherin 6 (*Cdh6*) (Li et al., 2019). The single-cell RNA-seq data (Tran et al., 2019) reveals that these two classes of RGCs are in fact six different RGC subtypes: three express only *Pou6f2* (18-Novel, 37-Novel and 44-Novel), and three express *Pou6f2* and *Cdh6* (7-Novel, 8-Novel and 10-Novel). These findings demonstrate the ability of single-cell RNA-seq in classifying cells in a complex structure like the mammalian retina.

Given the power and recent dependence on single-cell RNA-seq, there are several caveats that must be considered when examining single cell RNA-seq results (Mathieson et al., 2018). In general, when we examine the biology of the retina and RGCs, we look for and think of proteins. When examining data from single cell RNA-seq studies, each cell is captured at a single time-point in its life. It is assumed that the transcriptome profile captured at that time represents the profile of every gene expressed in the cell. This may or may not be the case. It depends on the expression level of specific messages and the half-life of that message (Leng et al., 2015; Lugowski et al., 2018; Sharova et al., 2009). For example, we know that some messages have very short half-lives in the range of a few hours (Lugowski et al., 2018; Sandoval et al., 2013). These include many transcription factors as well as cell cycle genes (Whitney et al., 2006). For the retina this is particularly important for the regulation of circadian rhythms (McMahon et al., 2014). Other transcripts have very long half lives in the range of days and these include many structural genes and cytoskeletal genes (Yang et al., 2003). The relevance to the biology of the system is further exacerbated by the half-lives of the protein products of the genes (Mathieson et al., 2018). Many of the

proteins made by short half-life mRNAs also have relatively short half-lives. For example, the half-life of many transcription factors is in the range of hours, so that the duration of action of that gene at the protein level is really rather short. This obviously is exactly what one would want from a protein that should be there to activate the genome on demand and then disappear when the particular function is no longer needed. On the opposite end of the spectrum are cytoskeletal proteins (Boumil et al., 2018; Yuan et al., 2009). Tubulin (class III beta tubulin specifically) for example can have a relatively long half-life, 200 days or more, and its message is also long lived. For the nervous system this long half-life is very important, for the cytoskeleton supports dendrites and axons that extend over considerable distances and in general need to be stable. While these considerations also apply to bulk RNA-seq data, the analytical problems are mitigated by profiling large numbers of cells. Independent of these caveats, the data generated by singe cell RNA-seq provides insights into RGC transcriptomes that really cannot be revealed by examinations of the whole retina transcriptome where the gene expression of individual cells types can be masked by the complexity of the whole transcriptome.

9. Changes occurring in RGCs following ONC

The analysis of the changes in transcriptome profiles of isolated RGCs is complicated by several factors. In the Tran et al. study (Tran et al., 2019), 46 subclasses of RGCs were identified in the mouse retina. When examining the changes occurring in the mouse retina from 12 h up to 14 days after ONC, changes in specific RGC transcripts were clustered and demonstrated a systematic progression of gene changes following crush. The temporal pattern of changes in expression allowed for the grouping of genetic changes resulting in 8 Modules (Fig. 6) with different patterns of up-regulation and down-regulation (Tran et al., 2019). The changes in RGC gene expression were compared to those observed in studies in ONC across the BXD strains (Templeton et al., 2013) (Fig. 6). In general, the changes in transcript levels in single cell RNA-seq for isolated RGCs (Tran et al., 2019) are similar to those observed in whole retina microarray studies of ONC (Templeton et al., 2013). In the whole retina transcriptome analysis, it is difficult to determine the cell type in the retina that these changes are occurring in; however in this specific case there is a remarkable similarity in RGC specific changes to those occurring in the whole retina samples.

10. Common changes in the retinal response to injury

There is a number of different retinal injury models (experimental glaucoma, genetic glaucoma and ONC) that share similar changes in gene expression, independent of the methods used to monitor the changes. Within the modules of genes from the Tran et al. study (Tran et al., 2019), we compared the changes in gene expression in the retina of the DBA/2J model of glaucoma (Howell et al., 2011) to changes occurring in isolated RGCs (Williams et al., 2017b). We also compared these changes to those following ONC (Templeton et al., 2013; Tran et al., 2019). Gene regulation could be different across studies given different injuries or different techniques. Across these mouse injury models, there is a considerable number of transcript changes that are in common (Fig. 3). For the up-regulated genes (Modules 5–8) there were 77 that were up-regulated in all three datasets and for the down-regulated genes (Modules 1–3) 158 were found in common (Table 1), such as *Sox11*

upregulation in ONC model across multiple studies (Li et al., 2018; Norsworthy et al., 2017; Tran et al., 2019; Welsbie et al., 2017).

It is imperative to consider these changes in transcriptome relative to recent studies on optic nerve regeneration(Leon et al., 2000; Park et al., 2008; Yin et al., 2006, 2009). Knocking down *Pten* (Park et al., 2008) or inducing a mild immune reaction (Leon et al., 2000; Yin et al., 2006, 2009) can aid in promoting axonal regeneration down the injured optic nerve. Combining these two treatments can further improve the amount of regeneration observed following nerve injury (de Lima et al., 2012; Wang et al., 2018). Interestingly, in these dual treated animals, most of the regenerating axons arise from a single RGC subtype, the alpha RGCs (Bray et al., 2017; Watanabe and Fukuda, 2002). One of the genes that facilitates axonal growth in the peripheral nervous system is *Sox11*. Overexpressing *Sox11* (Norsworthy et al., 2017; Welsbie et al., 2017) has dramatic effects on the response of the retina to injury, increasing the ability of RGCs in general to regeneration. Unfortunately, *Sox11* overexpression also causes selective death of the alpha RGC subtype, while retaining regeneration of some axons in the optic nerve, suggesting that a different RGC subtype was now regenerating. Thus, over-expressing *Sox11* kills the RGC subtype that normally regenerates but causes other RGC subtype(s) that usually do not regenerate to regenerate and survive. The complexities of gene expression and RGC subtype susceptibility to injury offer an interesting ground for understanding the molecular cascades that lead to cell death and could potentially be targeted for neuroprotection. Furthermore, the differential effects of regenerative capacity of RGC subtypes will provide a basis for developing strategies to facilitate the survival of all RGCs and the regeneration of their axons to CNS targets.

11. Future directions and conclusions

The response of RGC subtypes to injury is varied and complex. It is clear that there are different transcriptome responses depending on the mouse model used; from the mild, gradual injury observed in the DBA/2J glaucoma model, to the dramatic response seen after ONC. A comprehensive analysis of the transcriptome changes following injury is complicated by the fact that there are over 40 RGC subtypes and that each of these subtypes may respond differently to the injury. Future studies must analyze the response of each RGC subtype to glaucomatous and crush injury. These data may serve to inform novel treatment strategies for neuroprotection of all RGC subtypes along with the potential for axon regeneration and functional recovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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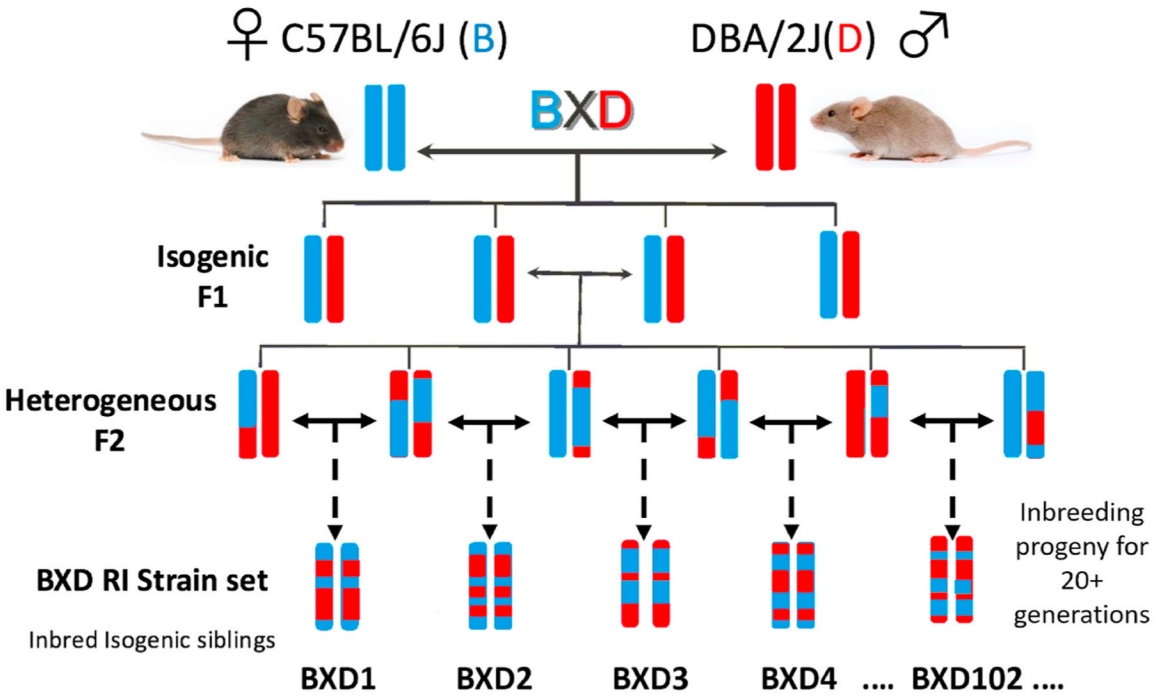

Fig. 1.

The breeding scheme for the production of the BXD recombinant inbred strains is illustrated. The parental strains were a female C57BL/6J mouse and a male DBA/2J mouse. The mice were crossed to produce an F1 generation and the F1 mice were crossed to yield the F2 generation where recombination events occurred. These F2 mice were inbred through brother-sister matings for at least 20 generations to generate inbred sub-strains. These BXD sub-strains provide for a powerful mapping panel with all strains being fully mapped and the parental strains being fully sequenced. Currently, there are over 150 BXD strains available.

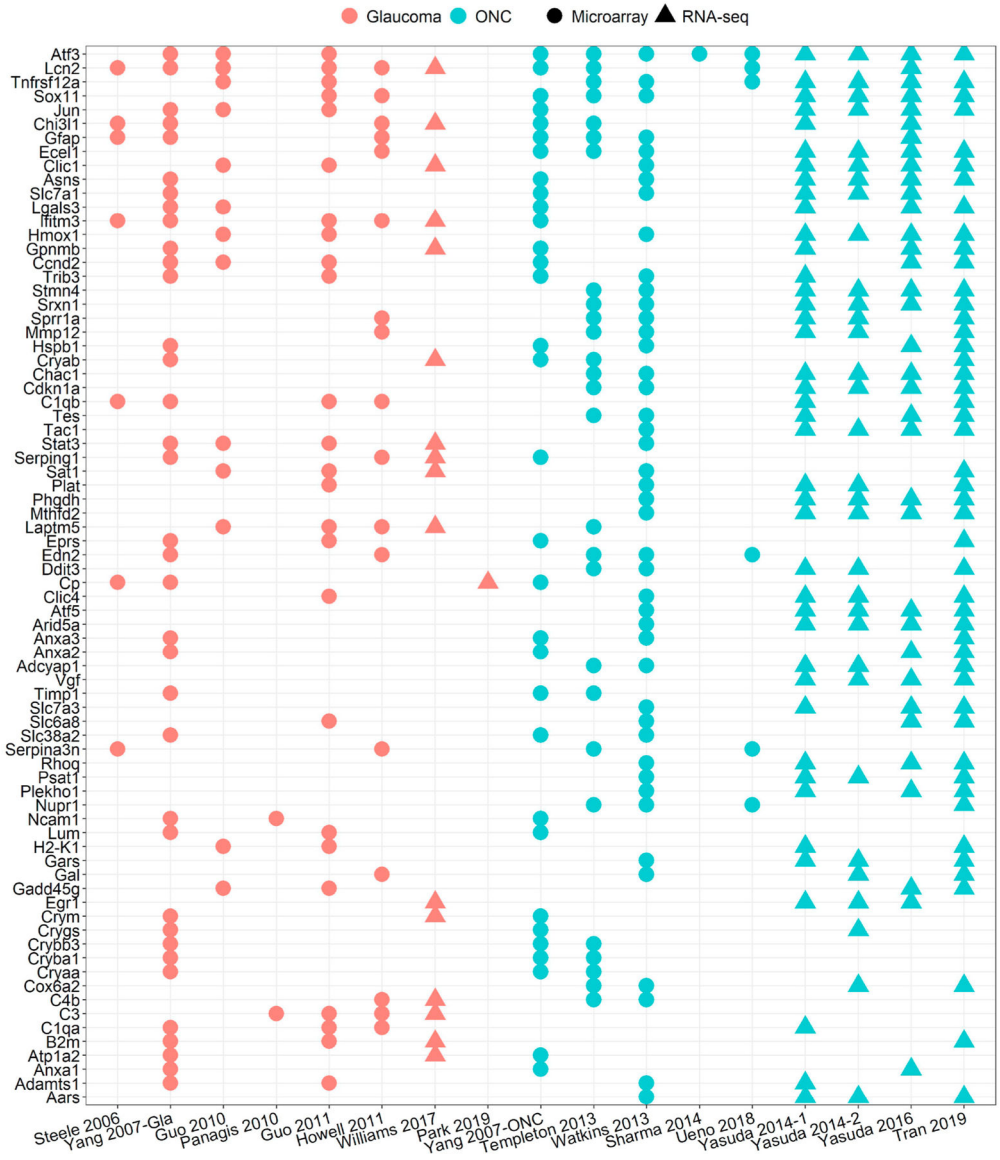

Fig. 2.

The genes most frequently detected as up-regulated in studies of glaucoma and ONC are displayed. To the left the individual genes are listed with the most frequently detected gene at the top and least frequently detected change on the bottom. There were 57 genes that were identified as up-regulated in at least four separate studies. The studies that identified the changes are listed across the bottom of the plot. Glaucoma studies are represented by pink and ONC studies are indicated by light blue. The microarray studies are indicated by dots and RNA-seq studies by triangles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

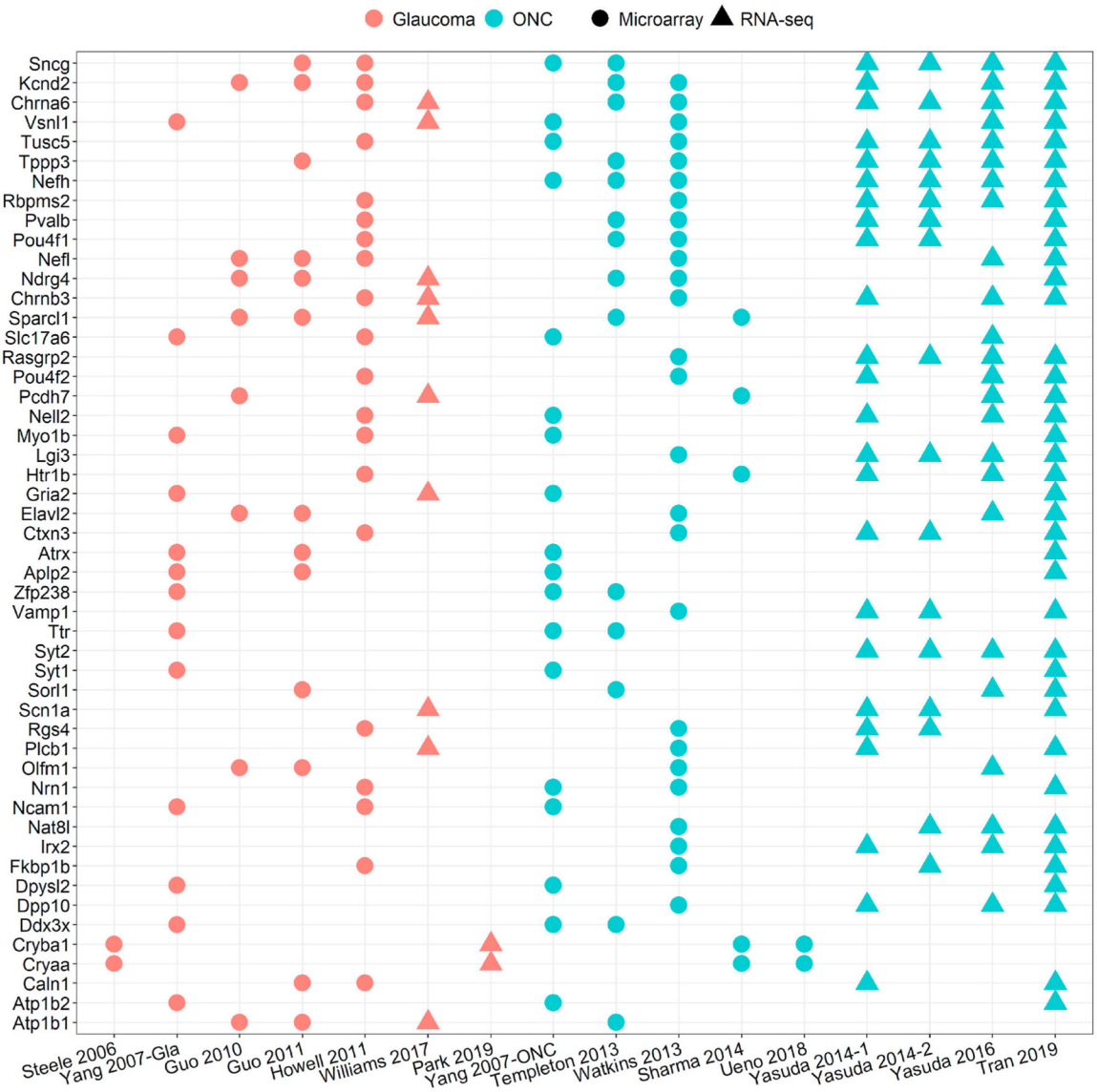

Fig. 3.

The genes most frequently detected as down-regulated in studies of glaucoma and ONC are displayed. To the left the individual genes are listed with the most frequently detected gene at the top and least frequently detected change on the bottom. There were 50 genes that were identified as down-regulated in at least four separate studies. The studies that identified the changes are listed across the bottom of the plot. Glaucoma studies are represented by pink and ONC studies are indicated by light blue. The microarray studies are indicated by dots and RNA-seq studies by triangles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

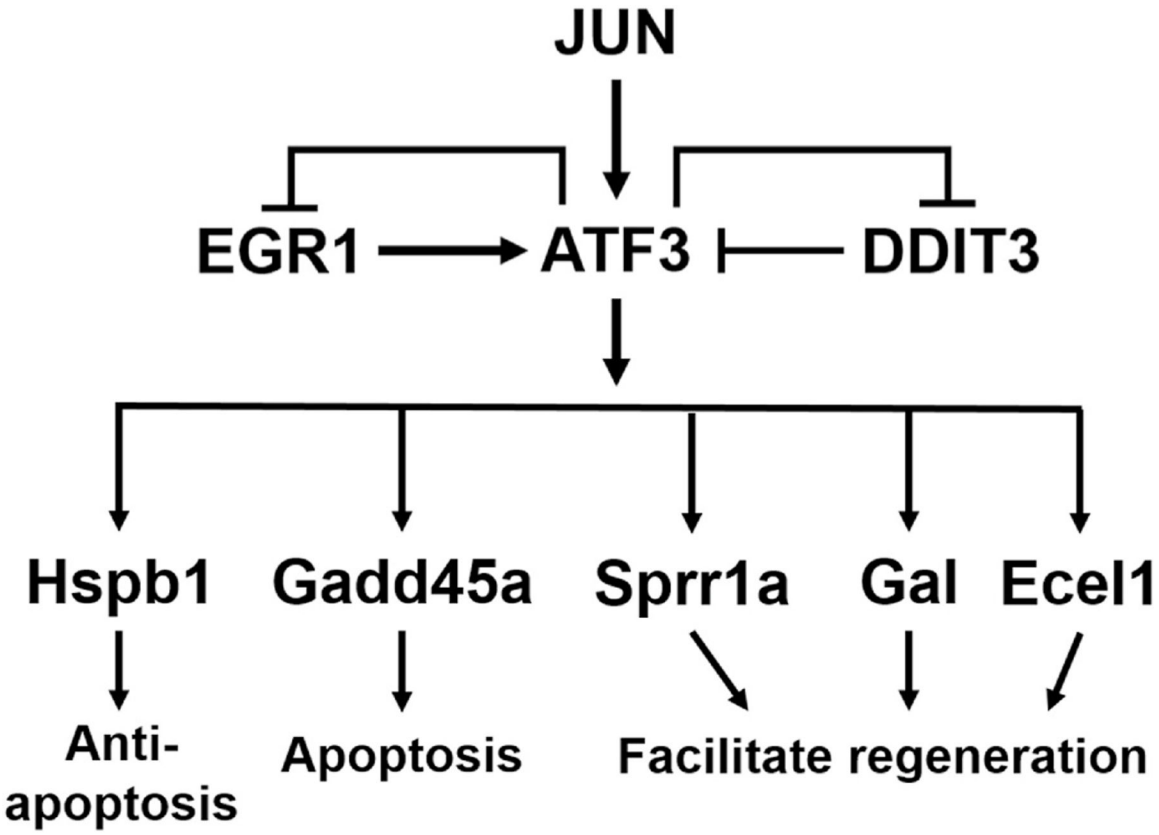

Fig. 4.

Signaling pathway of ATF3 in regulating RGC response to injury. Protein expression of ATF3 can be activated by JUN and EGR1. The activation of ATF3 then leads to multiple genes activation including *Hspb1*, *Gadd45a*, *Sprr1a*, *Gal* and *Ecel1* (Gey et al., 2016; Kaneko et al., 2017; Nakagomi et al., 2003; Tanaka et al., 2011). ATF3 and EGR1 form a negative feedback loop (Giraldo et al., 2012). ATF3 and DDIT3 suppress each other's expression (Jauhiainen et al., 2012).

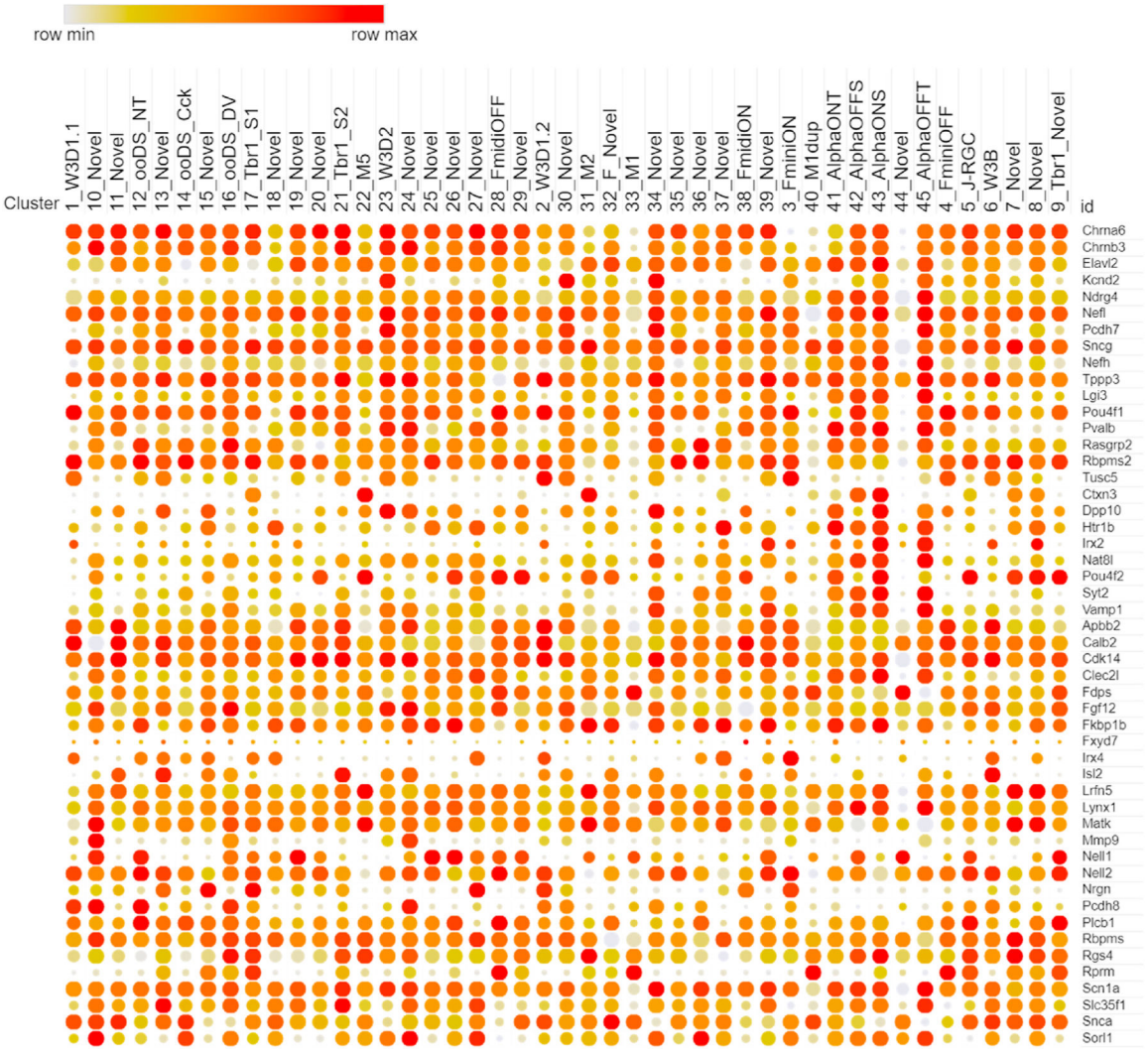

Fig. 5.

The expression of down-regulated genes (listed to the right) across all RGC subtypes (top row) is shown. The percentage of RGC subtypes expressing the gene is indicated by the size of the dot, with smaller dots having a lower percentage of cells expressing the gene and larger dots representing a higher percentage of cells expressing the gene. The level of expression is color coded with the light yellow representing low expression and red representing high expression. Notice that virtually all of the down-regulated genes are expressed in at least one RGC subtype and many of the genes are expressed in all RGC subtypes. These data were taken from the Single Cell Portal website (https://singlecell.broadinstitute.org/single_cell), and the result were of a published study (Tran et al., 2019). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

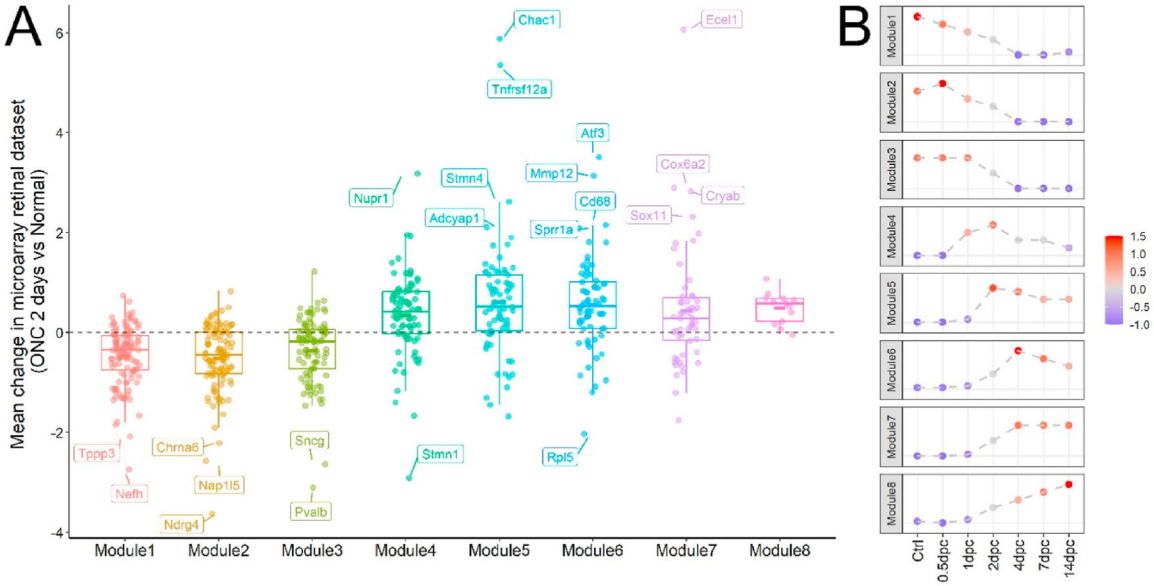

Fig. 6.

Comparison of changes in gene expression following ONC between microarray retinal dataset and single-cell RNA-seq data. The microarray retinal dataset (Templeton et al., 2013) (A) documents the changes occurring two days after crush. The gene changes from the single-cell RNA-seq data are shown in B with 8 different clustered modules across 6 different post crush time points. The genes in module 1–3 are down-regulated at two days (A) and in module 4–8 are up-regulated at 2 days (A). These data from the changes occurring in whole retina are similar to that observed in scRNAseq of RGC dataset (Tran et al., 2019) (B). Thus, similar changes can be detected in the whole retina as is observed in isolated RGCs.

Table 1

Studies for transcriptional changes after glaucomatous injury or optic nerve crush in rodents

Study	Animal	Model	Timepoint	Method	Platform	Sample
Steele MR, 2006	Mice (DBA/2J and C57BL/6J)	Glaucoma (DBA/2J)	3,5,8 months of age	Microarray	Affymetrix Mouse Genome 430 version 2.0 GeneChip arrays	Retina
Yang Z, 2007	Rats (albino Wistar)	ONC	1 and 3 days, 1, 2, 4, and 8 weeks	Microarray	Affymetrix Rat Genome 230 2.0;	Retina
Yang Z, 2007	Rats (albino Wistar)	Glaucoma (Translimbal laser photocoagulation)	1 and 3 days, 1, 2, 4, and 8 weeks	Microarray	Affymetrix Rat Genome 230 2.0;	Retina
Panagis L, 2010	Mice (DBA/2J)	Glaucoma (DBA/2J)	11- to 15-month-old DBA/2J female mice	Microarray	Affymetrix Mouse Genome 430 version 2.0 GeneChip arrays	Retina (RGC loss area vs RGC preservation area)
Guo Y, 2010	Rats (Brown Norway)	Glaucoma (episcleral vein injection of hypertonic saline)	Early-injury group and the advanced injury group.	Microarray	SMCmou8400A and SMCmou6600A cDNA microarrays	Retina and RGC
Guo Y, 2011	Rats (Brown Norway)	Glaucoma (episcleral vein injection of hypertonic saline)	Early-injury group and the advanced injury group.	Microarray	SMCmou8400A and SMCmou6600A cDNA microarrays	RGC
Howell GR, 2011	Mice (DBA/2J)	Glaucoma (DBA/2J)	NOE, moderate, severe	Microarray	Affymetrix Mouse Genome 430 version 2.0 GeneChip arrays	Retina and optic nerve head
Templeton JP,2013	Mice (BXD Recombinant inbred strains)	ONC	2 days	Microarray	Illumina Mouse WG-6 v2.0 (GPL6887)	Retina
Watkins TA, 2013	Mice (DLK-inducible KO mice)	ONC	3 days	Microarray	Agilent's Whole Mouse Genome 4 × 44Kv2 arrays	Retina
Yasuda M, 2014	Mice (C57BL/6J)	ONC	2 days	RNA-seq	Illumina HiSeq2000	Retina
Yasuda M, 2014	Mice (C57BL/6J)	ONC	2 days	CAGE	Illumina HiSeq2000	Retina
Sharma TP, 2014	Mice (BALB/c)	ONC	Naïve (0), 3, 7, 14, 21, 28 days	Microarray	Affymetrix Mouse Gene 1.0 ST arrays	Retina and optic nerve
Yasuda M, 2016	Rats	ONC	2 days	RNA-seq	Illumina Hiseq2500	Retina
Williams PA, 2017	Mice (DBA/2J)	Glaucoma (DBA/2J)	DBA/2J at 4 month and 9 month	RNA-seq	Illumina Hiseq2500	RGCs
Ueno S, 2018	Mice (C57BL/6J)	ONC	1&4 days	Microarray	3D-Gene Scanner 3000 (Toray, Kanagawa, Japan)	Retina
Tran NM, 2019	Mice (C57BL/6J)	ONC	Naïve (0), 0.5, 1, 2, 4, 7, 14 days	Single cell RNA-seq	NextSeq 500 or Illumina HiSeq 2500 platforms	Single RGCs
Park YH, 2019	Mice (C57BL/6J)	Glaucoma (Intracameral injection of polystyrene microbeads and sodium hyaluronate)	2 weeks	RNA-seq	Illumina Hiseq2500	isolated RGCs

Table 2

The expressional changes of genes that are up-regulated following ONC but not glaucomatous injury at 2 days after ONC.

Gene	Location (Chr, Mb)	Expression level		Fold Change[c]
		Naïve[a]	ONC 2 days[b]	
Chac1	Chr2: 119.354235	9.56	11.95	5.88
Nupr1	Chr7: 126.623477	12.41	13.75	3.09
Cox6a2	Chr7: 128.205647	7.21	8.70	2.88
Stmn4	Chr14: 66.357960	8.49	9.84	2.77
Cdkn1a	Chr17: 29.100547	7.86	9.37	2.74
Ddit3	Chr10: 127.295816	11.61	12.78	2.48
Srxn1	Chr2: 152.111000	11.11	12.23	2.22
Adcyap1	Chr17: 93.205227	7.76	8.83	2.10
Tes	Chr6: 17.105678	9.69	10.66	2.01
Vgf	Chr5: 137.033011	9.90	10.79	1.76
Mthfd2	Chr6: 83.305825	10.09	10.83	1.61
Slc7a3	ChrX: 101.079353	9.58	10.33	1.58
Phgdh	Chr14: 95.419888	8.05	8.92	1.55
Tac1	Chr6: 7.556724	10.36	11.03	1.46
Psat1	Chr19: 15.905452	11.43	11.97	1.37
Aars	Chr8: 111.055496	13.23	13.58	1.32
Arid5a	Chr1: 36.322759	6.97	7.39	1.25
Atf5	Chr7: 44.812359	9.94	10.60	1.16
Gars	Chr6: 55.079417	14.97	15.34	1.12
Rhog	Chr7: 102.239421	7.84	7.99	0.36
Plekho1	Chr3: 95.989335	6.81	6.80	0.11

[a] These data are extracted from HEI Retina Normal Illumina V6.2 (Apr10) RankInv dataset hosted on GeneNetwork.org.

[b] These data are extracted from ONC HEI Retina (April 2012) RankInv dataset hosted on GeneNetwork.org.

[c] These data are extracted from HEI ONC vs Control Retina Illumina V6.2 (Sep11) RankInv dataset hosted on GeneNetwork.org.