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Evaluating Retinal Ganglion Cell Loss and Dysfunction

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Abstract

Retinal ganglion cells (RGC) bear the sole responsibility of propagating visual stimuli to the brain. Their axons, which make up the optic nerve, project from the retina to the brain through the lamina cribrosa and in rodents, decussate almost entirely at the optic chiasm before synapsing at the superior colliculus. For many traumatic and degenerative ocular conditions, the dysfunction and/or loss of RGC is the primary determinant of visual loss and are the measurable endpoints in current research into experimental therapies. To actually measure these endpoints in rodent models, techniques must ascertain both the quantity of surviving RGC and their functional capacity. Quantification techniques include phenotypic markers of RGC, retrogradely transported fluorophores and morphological measurements of retinal thickness whereas functional assessments include electroretinography (flash and pattern) and visual evoked potential. The importance of the accuracy and reliability of these techniques cannot be understated, nor can the relationship between RGC death and dysfunction. The existence of up to 30 types of RGC complicates the measuring process, particularly as these may respond differently to disease and treatment. Since the above techniques may selectively identify and ignore particular subpopulations, their appropriateness as measures of RGC survival and function may be further limited. This review discusses the above techniques in the context of their subtype specificity.

Keywords

Retina; Retinal Ganglion Cells; Electroretinography; Visual Evoked Potential; Quantification; Dysfunction

1. INTRODUCTION

Retinal ganglion cells (RGC) project their axons along the optic nerve and are responsible for the propagation of visual stimuli to the brain. In humans, 50% of RGC decussate within

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the chiasm with 80% of projections terminating in the lateral geniculate nucleus (LGN). In contrast, 96-99% of RGC in the rodent retina decussate within the optic chiasm(Thuen et al., 2005) with the vast majority synapsing in the superior colliculus (SC)(Berry et al., 2008, Nadal-Nicolas et al., 2012, You et al., 2013). The importance of these cells is fast realised when they die or become dysfunctional following injury and disease. The mechanism for RGC apoptosis following damage to the optic nerve has been reviewed previously (You et al., 2013). The development of models of eye disease along with the testing of potential therapies requires accurate and reliable assessment of RGC numbers along with appropriate testing of RGC function. Techniques for calculating RGC loss include: counting using either phenotypic markers of RGC, retrograde labelling, expression of fluorophores under a RGC specific promoter, and retinal nerve fibre layer thickness (RNFL) measurements. The counting method also varies between counting in histological retinal sections, whole mounts, and *in vivo* (with a laser ophthalmoscope). Determining RGC function is done through electrophysiological testing, such an electroretinography (ERG) and visual evoked potentials (VEP).

Although researchers have employed all of the above techniques, the comparable accuracy and reliability, and the relationship between RGC death and RGC dysfunction are poorly understood. It is also often overlooked the large number of different RGC subtypes(Sanes and Masland, 2015) and the inevitable difference in the sensitivity of these subtypes to the various quantitative techniques. This is particularly important when considering that different RGC subtypes are more resilient to optic nerve damage(Muller et al., 2014, Duan et al., 2015, Nadal-Nicolas et al., 2015b), ocular hypertension(Filippopoulos et al., 2006, Li et al., 2006, Zhou et al., 2008), photoreceptor degeneration induced-RGC loss(Lin and Peng, 2013), more resistant to particular neuroprotective strategies(Valiente-Soriano et al., 2015) and equally, may be under or over represented in certain functional assessments. This review aims to provide a discussion on the above techniques, their strengths and weaknesses and accurate quantitative comparisons between RGC counting and RGC functional assessments. For quantifying RGC axonal loss and dysfunction in their axonal transport properties, see recent review(Nuschke et al., 2015).

2. Quantification of RGC – Phenotypic markers and tracers

To accurately count RGC, good phenotypic markers are required that leave no doubt to the identity of the cell in question. The ganglion cell layer (GCL) is occupied not only by RGC but also astrocytes and displaced amacrine cells, potentially leaving RGC at only 40-50% of the total GCL population(Bunt and Lund, 1974, Perry, 1981, Schlamp et al., 2013, Nadal-Nicolas et al., 2015b). Previously used markers such as βIII tubulin(Chou et al., 2013, Leibinger et al., 2013, Jiang et al., 2015) and islet-1(Johnson et al., 2014) stain RGC and amacrine cells(Sharma and Netland, 2007, Mead et al., 2014), calling into question their accuracy at reporting RGC numbers. Despite this, βIII-tubulin is still suggested to be a reliable marker(Jiang et al., 2015). Currently there exist several good phenotypic markers of RGC/fluorescent tracers that are used in multiple laboratories.

2.1 FluoroGold (FG)

FG is a widely used retrograde fluorescent tracer whose excitation wavelength (400nm) is found on most fluorescent microscopes. FG diffuses into vesicles within the axon raising the pH and ultimately trapping itself within(Wessendorf, 1991). The vesicles are transported along the cytoskeletal fibres of the axon towards the soma. Where the FG is administered it can drastically alter which RGC are stained. Due to the fact only 98% of axons project into the contralateral SC, the optic nerve is preferred over SC administration(Salinas-Navarro et al., 2009a, Salinas-Navarro et al., 2009b). Injection into the optic nerve proximal to a lesion will mark every RGC whereas administration after a lesion site will only mark those RGC with axons (spared or regenerating) extending past the lesion site. Although this is typically done in lesioned optic nerves *via* injection into the proximal stump(Mead et al., 2014), FG can be administered into intact nerves(Nadal-Nicolas et al., 2015a).

FG remains a gold standard for quantifying RGC in intact retina to the point that FG^+ cells in the GCL of an intact retina are regarded synonymously as RGC (Figure 1D). However, several issues confound FG's utility in the injured retina. Firstly several labs have reported FG^+ macrophages in the eye, likely *via* phagocytosis(Heiduschka et al., 2010, Mead et al., 2014). Although they can often be morphologically excluded from the count, it will inevitably confound the counting process and their numbers increasing dramatically in injured/treated retinae adds a confounding variable to the comparison. Secondly, FG has been shown to diffuse into amacrine cells, potentially through gap junctions(Abdel-Majid et al., 2005) limiting its long term accuracy in labelling RGC. Labelling amacrine cells with the intention of excluding them is difficult as they are diverse population of cells comprised of over 30 subtypes and requiring different phenotypic marker(Voinescu et al., 2010). Thirdly, FG has been reported to be toxic to neuronal function(Garrett et al., 1991, Hu et al., 2013), further limiting its long-term use and potentially confounding any retinal functional tests performed post-labelling. Finally and perhaps most importantly, since the staining of RGC relies on retrograde transport, accurate counting may be engendered in ocular hypertensive models where the transport process is dysfunctional(Quigley et al., 2000). This adds difficulty in concluding whether differences in FG^+ cells counts are due to RGC loss/neuroprotection or modulation of the axonal transport efficiency, of which FG transit relies on. Retrograde labelling of RGC could be done prior to the injury to avoid this, however, issues mentioned above (uptake into amacrine cells and macrophages) along with photobleaching of the fluorescence in live tissue make this approach inaccurate(Abdel-Majid et al., 2005). Finally, the administration of FG into animal nerves and brain regions is an invasive procedure that should be avoided if possible.

2.2 Brn3a

Brn3 is a family of transcription factors important to RGC development and includes Brn3a (also known as Pou4f1), Brn3b and Brn3c. Of the population of $Brn3^+$ cells, 96% express Brn3a (alone or co-expressed with Brn3b/c) whereas the remaining 4% express Brn3b alone(Nadal-Nicolas et al., 2012). For this reason, Brn3a is typically used as a phenotypic marker of RGC with approximately 80%(Xiang et al., 1995, Rodriguez et al., 2014)-90% (Nadal-Nicolas et al., 2009, Mead et al., 2014) of FG back-labelled RGC expressing Brn3a (Figure 1C, E). In rodent retina however, the expression of Brn3a is not seen in several

subtypes of RGC, including intrinsically photosensitive RGC (ip-RGC) and RGC projecting to the ipsilateral SC(Nadal-Nicolas et al., 2012), suprachiasmatic nucleus, accessory optic system and pretectal nuclei(Quina et al., 2005). This preferential staining and ultimately, exclusion of entire subpopulations of RGC renders Brn3a liable to substantial under or over estimates. For example, the preferential survival of a subtype of RGC known as ip-RGC(Muller et al., 2014) after optic nerve crush (ONC) is not apparent when staining RGC for Brn3a and this misrepresentation of surviving RGC increases as more non-ip-RGC die. Double staining the rat retina with Brn3a and melanopsin 6 months after ONC demonstrates that Brn3a$^+$ RGC numbers decrease by 99% whereas melanopsin$^+$ RGC fall by only 60% (Nadal-Nicolas et al., 2015b).

One characteristic of Brn3a is an apparent down-regulation that precedes RGC loss(Nadal-Nicolas et al., 2009, Nadal-Nicolas et al., 2012). A recent study showed that 3 day post-ONC, the number of Brn3a$^+$ cells did not significantly decrease while the expression of the protein, measured by Western blot, had significantly decreased(Nuschke et al., 2015). This poorly understood down-regulation of Brn3a coupled with its variable expression (80-90% of RGC in the healthy rat/mouse retina(Nadal-Nicolas et al., 2009, Rodriguez et al., 2014)) suggests Brn3a is a flawed phenotypic marker of RGC. Previous studies have demonstrated that Brn3a and FG counts are comparable after ONC, suggesting that the down-regulation is either only partial or is so closely followed by RGC death that it does not hinder accurate counting (Nadal-Nicolas et al., 2009, Nadal-Nicolas et al., 2012, Mead et al., 2014). In models such as experimental glaucoma where dysfunction of the RGC is an early stage of the disease relative to the cells eventual death, Brn3a, which is known to be down-regulated following elevations in intraocular pressure (IOP)(Guo et al., 2009), is likely unsuitable as marker of RGC numbers.

2.3 RNA-binding protein with multiple splicing (*RBPMS)*

RBPMS belongs to a family of RNA-binding proteins involved in post-transcriptional modifications of mRNA(Kwong et al., 2010). It is found predominately, although not exclusively in the nucleus (Figure 1E). In the GCL of the rat retina, 99.5% of FG$^+$ cells were RBPMS$^+$ demonstrating its accuracy as a phenotypic marker of RGC. Of the RBPMS$^+$ cells, 97% were also FG$^+$ (Kwong et al., 2010) and the lack of RBPMS expression in syntaxin$^+$ amacrine cells strongly suggests RBPMS does not mark displaced amacrine cells(Rodriguez et al., 2014). RBPMS$^+$ cells followed the same progressive loss characteristics of FG$^+$ RGC following ONC with 90% lost 2-4 weeks post-injury(Berkelaar et al., 1994, Kwong et al., 2011, Rodriguez et al., 2014). After both ONC and ocular hypertension, the number of FG$^+$ cells that co-stained for RBPMS remained a high 96-99% demonstrating RBPMS affinity for both healthy and injured RGC.

Although all Brn3a$^+$ cells are RBPMS$^+$, only 80% of RBPMS$^+$ cells express Brn3a in mouse and rat retina, suggesting that RBPMS stains a subpopulation of RGC that are not identified by Brn3a staining(Rodriguez et al., 2014). These subpopulations of RGC that are Brn3a$^-$/RBPMS$^+$ appear to be ip-RGC which can be divided into 5 groups (M1-M5) (Sanes and Masland, 2015). These RGC are identified by their melanopsin expression as well as their potential SMI-32 expression as there is a tentative overlap between ip-RGC and SMI-32$^+$

αRGC.(Nadal-Nicolas et al., 2015b, Sanes and Masland, 2015). However, ip-RGC only account for 5-10% of RGC in mouse/rat retina and thus do not completely account for the 20% of RBPMS^{+} RGC that do not express Brn3a (Rodriguez et al., 2014). Previous reports demonstrate that Brn3a stains only 85-90% of RGC (Nadal-Nicolas et al., 2009, Nadal-Nicolas et al., 2012, Mead et al., 2014, Rodriguez et al., 2014) with Brn3a^{-} RGC being responsible for innervation of the suprachiasmatic nucleus, accessory optic system and pretectal nuclei(Quina et al., 2005), likely candidates for the RBPMS^{+}Brn3a^{-} population. Finally another advantage of RBPMS is its consistent expression in RGC both before and after injury(Rodriguez et al., 2014), in contrast to Brn3a, which is down-regulated after injury(Sanchez-Migallon et al., 2011, Nuschke et al., 2015). Despite RBPMS being a strong candidate as a robust phenotypic marker of RGC, relatively few laboratories have published with it and it therefore warrants further study into its suitability, particularly in different models of RGC loss.

2.4 Thy-1

Thy-1, also known as CD90 is a cell surface immunoglobulin found on RGC along with other cell types such as fibroblasts(Perry et al., 1984). Several transgenic mouse models expressing fluorophores under the Thy-1 promoter exist(Feng et al., 2000) however, the differential expression between one fluorophore compared to another (expressed under the same Thy-1 promoter but with different integration sites) lends confusion to the accuracy of Thy-1 as a phenotypic marker of RGC. For example, Thy-1-yellow fluorescent protein-H/16 (YFP) mice express fluorescence in less than 1% of RGC whereas Thy-1-cyan fluorescent protein-23 (CFP) mice(Raymond et al., 2008) label a large population of RGC (see below) and are predominately used when attempting to quantify the total RGC population. The selective labelling of RGC by Thy-1-YFP however has proved useful in studying dendritic changes and it is suggested that the population of labelled RGC are actually αRGC, as evident by their positive staining for SMI-32(Leung et al., 2011).

Expression of CFP under a Thy-1 promoter (in mice) demonstrates that 84% of Thy-1^{+} cells express RBPMS. Thy-1 is found not only in RGC but amacrine cells, specifically the cholinergic syntaxin^{+} amacrine cells of the inner nuclear layer, inner plexiform layer (IPL) and also displaced into the GCL(Raymond et al., 2008). It is thus expected that the 16% of Thy-1 positive cells in the GCL that are negative for RPBMS are likely displaced cholinergic amacrine cells. Only 61% of Thy-1^{+} cells in the GCL express Brn3a, suggesting that 39% of Thy-1^{+} cells are amacrine cells and ip-RGC, both of which express Thy-1(Hartwick et al., 2007, Raymond et al., 2008).

Of the FG^{+} cells in the GCL of the retina, 80% are Thy-1^{+}, suggesting a population of RGC in the GCL (20%) are not detected by Thy-1 (Wang et al., 2010). Similarly, of the RBPMS^{+} cells in the GCL of the retina, 82% express Thy-1 with the remaining 18% Thy-1^{-}. This 18-20% of Thy-1^{-} RGC are thus a RGC subtype separate from the melanopsin^{+} ip-RGC (which do express Thy-1 (Hartwick et al., 2007)).

Evidence suggests that Thy-1 down-regulation precedes RGC loss making it a poor marker of RGC death and protection. For example, 10 days after ocular hypertension in rats, the number of RGC (FG^{+} cells in the GCL) was decreased by 30% whereas the levels of Thy-1

protein decreased by 60%(Huang et al., 2006). One week after ONC, 80% of Thy-1^{+} cells are lost(Leung et al., 2008), a significantly more rapid loss than the 50% FG^{+} RGC lost after 1 week(Berkelaar et al., 1994).

2.5 ϒ-synuclein

Synucleins are small, unfolded proteins expressed in a number of tissues including the retina(Surguchov et al., 2001). While α- and β-synuclein are expressed in the IPL, ϒ-synuclein is expressed in the RNFL and GCL. ϒ-synuclein stained Brn3a^{+} RGC in the GCL but stained the cytoplasm rather than the nucleus(Surgucheva et al., 2008). All Brn3a^{+} RGC are positive for ϒ-synuclein whereas a minority of ϒ-synuclein^{+} RGC are Brn3a^{-}. Two weeks after ONC in rat, retrogradle tracers report 70% RGC death whereas ϒ-synuclein counts report only 50% RGC death, suggesting ϒ-synuclein marks not only RGC but also other GCL localized cells not affected by ONC(Sun et al., 2014). Previous studies using ϒ-synuclein as a marker of RGC noted FG^{-}/ϒ- synuclein^{+} cells(Soto et al., 2008). In a mouse model of ocular hypertension, Brn3a* cells in the GCL were reduced by 33% whereas ϒ-synuclein^{+} cells did not significantly reduce until several months later(Domenici et al., 2014). Indeed these ϒ-synuclein^{+} cells were later identified as amacrine cells displaced into the GCL and, since these make up 50% of the cells populating the GCL, bring into question its feasibility as a marker of RGC numbers(Nadal-Nicolas et al., 2015b).

3. RNFL assessment by optical coherence tomography (OCT)

OCT can be used to produce a cross sectional image of the retina, from which the thickness of various retinal layers can be measured. RNFL, which is comprised of the RGC axons, is an easily distinguishable layer whose thickness can be measured (Figure 2). Although this technique does not measure RGC numbers directly, axonal loss precedes RGC loss (Buckingham et al., 2008, Soto et al., 2011) and so it is predicted to be a reliable surrogate marker of RGC numbers due to the approximate 1:1 relationship between axonal and RGC numbers. The benefit of using this method is that the procedure is non invasive and can be done *in vivo*, monitoring RGC loss in real time. The measurement also does not discriminate between or ignore RGC subtypes. Finally, it is the only quantitative technique that is currently applied to human patients. The negatives are that it requires a relatively expensive OCT machine and it is much less sensitive than microscopy, due to the lower resolution.

Finally it is not completely clear what the relationship is between RNFL thickness and RGC number. Previous work has shown that in an animal model of glaucoma, the pathways responsible for axonal and RGC loss are distinct. Authors used BCL-2-assosciated X protein (BAX) deficient mice and demonstrated axonal but not RGC loss(Libby et al., 2005). The decoupling of axon and RGC loss makes RNFL thickness measurements a potentially poor surrogate marker of RGC numbers.

Comparing RNFL thickness measurements to Brn3a^{+} RGC counts after ONC, we demonstrated a 40% RNFL thinning 7 days after ONC(Mead et al., 2013) which closely follows the typical 50% RGC loss at this time point (Berkelaar et al., 1994). By 21 days post-ONC, we found RNFL had thinned by 70%, which underestimated the 90% Brn3a^{+} RGC loss(Mead et al., 2013). We surmised that as the RNFL became progressively thinner,

the resolution of OCT was no longer able to accurately determine changes in RNFL thickness and thus, report on RGC numbers.

More recent studies also suggest this, reporting a 70% decrease in RNFL thickness at 4 months post-optic nerve transection (ONT) despite a 98% loss in Brn3a$^+$ RGC numbers(Rovere et al., 2015). Other groups when measuring the RNFL demonstrated an even more marginal change in the thickness following injury.

Recordings at 2 and 3 weeks post-ONT demonstrate a RNFL thinning of 17% and 30% respectively with an RGC loss of 69% and 85% (retrograde labelled RGC), respectively(Choe et al., 2014). In mice, the RNFL only thinned by 20% 3 weeks after ONC although authors did show that the thickness change per week correlated with RGC loss(Munguba et al., 2014). The underestimation of RGC loss by RNFL thickness measurements is likely explained by the presence of blood vessels that populate the RNFL (Figure 2) and, along with macrophages, astrocytes and displaced amacrine cells, contribute to the thickness and become more predominant as more RGC are lost. At a thickness <20μm the RNFL becomes substantially more difficult to distinguish from other retinal layers and thus, combined with the above deficiencies, suggests RNFL is not suitable in models where substantial RGC loss is expected. A separate study used RNFL thickness combined with the GCL and IPL, demonstrating that the majority of the retinal layer thinning occurred between 3 and 7 days post-ONC with very little to no thinning occurring after (Liu et al., 2014). This technique avoids the issues of the RNFL being too thin to measure and the blood vessels being the predominant entity. Since RGC somas and dendritic arbors are included in the quantification, the technique may indeed provide a more accurate measure of RGC damage but likely unreliable as a readout of RGC numbers.

In models of ocular hypertension where the axonal pathology precedes RGC loss, RNFL thickness over estimates RGC death post-injury. In a model utilizing injection of microbeads and laser photocoagulation of the trabecular meshwork, Brn3a$^+$ RGC numbers decreased by 26.7% whereas RNFL thickness had reduced by 54%(Chen et al., 2015). In a transforming growth factor-β model of glaucoma, we found that Brn3a$^+$ RGC numbers decreased by 33% whereas RNFL thickness had reduced by 51%(Mead et al., 2016). It is however equally possible that the supposed overestimates in RGC loss detected by RNFL is in fact due to the detection of Brn3a$^-$ RGC loss. Since RNFL is a measure of axonal density, it is possible that proven neuroprotective strategies may not include axonal protection. Indeed in our most recent study we demonstrated a 100% neuroprotection of Brn3a$^+$ RGC in a rat model of glaucoma yet the RNFL was still significantly (25%) thinner compared to healthy controls(Mead et al., 2016).This suggests that in some animal models, particularly those characterised by axonal injury (Berry et al., 2008), the RGC and RGC axon numbers become decoupled(Libby et al., 2005) and renders OCT measures of RGC numbers inaccurate.

Although RNFL is comprised of RGC axons and retinal blood vessels, there are likely other cells present that may further contribute to the overall RNFL thickness. This becomes an even greater issue if this cell population number changes throughout the study in response to the injury or treatment. One such example is the infiltration of microglia, which have been

shown to contribute to the RNFL thickness measurement and increase in response to ONT and even increase significantly in the uninjured fellow eye. This resulted in a prominent increase in RNFL thickness by 50% up to 3 weeks after ONT(Choe et al., 2014).

OCT provides a good initial assessment of RNFL thickness, which is strongly correlated with RGC number, however it is not ideal as the sole means of assessing RGC loss/survival. Substantial variation is seen throughout the literature because thickness measurements are inherently more subjective than counting cells and is confounded further by the resolving power of different OCT machines and variable techniques in how the experimenter measures the RNFL thickness. Adoption of a consistent measuring technique that avoids inclusion of blood vessels as well as other layers would not only improve accuracy but also allow valid comparisons between studies.

4. Counting techniques

Whereas accuracy relies on the marker used, reliability is more dependent on the technique the investigator uses to count the cells. While power calculations are informative of the number of animals required(Mead et al., 2014) , the ideal sampling technique would be one that is neither time nor equipment intensive and represents the total number of RGC.

4.1 Retinal wholemounts

The most reliable technique is to simply mount the entire retina and count every single RGC present. This is impractical unless specialised software is used such as has been demonstrated in rats(Salinas-Navarro et al., 2009b) and mice(Salinas-Navarro et al., 2009a). It is more efficient and more often employed to sample the retina at well-defined locations to provide an estimate of RGC numbers and this is typically done by making 4 radial incisions and laying the retina out in a “petal” (Figure 1A) (Salinas-Navarro et al., 2009a, Choe et al., 2014, Mead et al., 2014). For example, 12 sample counts ($0.260mm^2$) taken at 1.5, 2.5 and 3.5mm from the optic nerve head along the superior, inferior, temporal and nasal radii, estimate similar numbers of RGC to the total RGC count(Mead et al., 2014). However RGC density varies with a declining density at increasing radial distance. Equally, different animal models can result in RGC death in a non-linear fashion with preferential RGC death at the peripheral or central retina, with some glaucomatous models reporting sectorial RGC death(Zhou et al., 2008, Soto et al., 2011, Agudo-Barriuso et al., 2013). Thus although this sampling technique has proven reliability, the accuracy is reliant on ensuring the sample represents the RGC population at all distance from the optic nerve head.

4.2 Radial retinal sections

Since the majority of studies have multiple endpoints, wholemounting the retina, which renders it unusable for further analysis, becomes a significant issue. In depth morphological and immunohistochemical analyses are no longer possible meaning other sampling techniques must be explored. A parasagittal section through the eye allows visualisation of the front and back of the eye with visible retinal layers and both peripheral and central retina included (Figure 1B) (Mead et al., 2014, Munguba et al., 2014). Since >100 sections can be obtained from the rodent eye, multiple proteins and cellular responses can be analysed in the

same animal along with the tracking of administered treatments/cells. By sampling the number of RGC in the GCL at a standard length and a standard section (e.g. when the optic nerve head is visible), RGC death can be estimated.

To determine if this technique is as reliable as wholemounts in quantifying RGC loss we compared it to wholemounts and demonstrated that the 90% RGC death 3 weeks after ONC in rats can be reported with equal fidelity in either wholemounts or radial retinal sections(Mead et al., 2014). However, this sampling technique relies on a relatively uniform spread of RGC, which is not always seen in glaucomatous eyes(Salinas-Navarro et al., 2010, Soto et al., 2011, Agudo-Barriuso et al., 2013).

4.3 in vivo

Using a confocal scanning laser ophthalmoscope (CSLO), the numbers of labelled RGC can be quantified *in vivo.* Although equipment intensive, this approach is non-invasive with animals able to be scanned without the need for anaesthesia(Leung and Weinreb, 2009). Although not appropriate for predicting total RGC numbers, CSLO *in vivo* measurements have also been used to visualize RGC apoptosis through the use of caspase-activated probes(Qiu et al., 2014) or fluorescently tagged annexin 5, which externalizes to the outer membrane in the early phase of RGC apoptosis(Schmitz-Valckenberg et al., 2008).

Following ONT in rats, the number of back-labelled RGC decreased by 18%, 69%, 85% and 92% at 1,2,3, and 4 weeks post-injury(Choe et al., 2014). This is in disagreement with previous studies that show a reproducible 50% loss of (FG^+) RGC 7 days after ONC/ONT(Berkelaar et al., 1994, Berry et al., 2008). Authors demonstrated that CSLO underestimates the number of RGC (determined post-mortem immunohistochemically) by one third and that the discrepancy increases with increasing density of RGC. Another group demonstrated a different problem, that macrophages would become stained upon phagocytosis of the labelled RGC following ONC(Higashide et al., 2006). As CSLO has a lower resolving power compared to microscopy, these labelled macrophages could not be discriminated from RGC without comparing to the original image of the uninjured retina and excluding based on a prior lack of staining. A separate group using Thy-1-CFP mice demonstrated a 68% RGC loss 1 week after ONC with a further 22% and 10% reduction at 2 and 3 weeks post-ONC, respectively(Munguba et al., 2014). However these numbers may be exaggerated as Thy-1 has been shown to be down-regulated following injury(Huang et al., 2006). As mentioned previously Thy-1 also stains amacrine cells which can be found displaced into the GCL and thus will appear in the same plane and compromise the accuracy of the count. In a Thy-1-YFP mouse model, in which αRGC are selectively labelled (evident by SMI-32 positive staining), despite dendritic atrophy the cells and their respective axons survived over 6 months after ONC(Leung et al., 2011). While imaging RGC *in vivo* has many advantages, it is currently not used with many of the more accurate phenotypic markers (Brn3a/RBPMS).

4.4 Conclusion

Despite a wide a variety of phenotypic markers, many have considerable issues that perturb their accuracy. Retrograde tracers such as FG have been the gold standard but conditions that

affect the optic nerve and thus axon transport mechanics limit their use. While each phenotypic marker has certain strengths, RBPMS currently appears to be the marker that stains every RGC and thus, is the ideal phenotypic marker for a global count of RGC numbers. However, variations in experimental hypotheses, immunohistochemical staining techniques, antibody suppliers, animal models as well as animal strains will all affect the antibody affinity as well as the antigen expression level, thus precluding the claim that RBPMS is a “gold-standard” RGC marker for every study/research group. While there is always a need for more reliable phenotypic markers for RGC, the identification of discrete subtypes of RGC is still a current challenge. Discrimination between subtypes is done typically by morphology(Sanes and Masland, 2015) or more recently, the use of transgenic mouse lines(Duan et al., 2015) yet would benefit greatly from the identification of subtype specific phenotypic markers.

5. Functional assessments

As opposed to quantifying the number of living RGC, electrophysiological techniques are also employed to give a readout of the function of the retina and in particular, the function of the remaining RGC. This has been demonstrated to not be a simple 1:1 ratio with RGC surviving injury without functional restitution. Equally, in many instances, dysfunction is an early marker and preceding factor to RGC death and thus, neuroprotective treatments may merely prevent a “sick” cell from dying but ultimately, do nothing for its function. The mechanisms responsible for rendering an RGC dysfunctional, yet viable are poorly understood. Evidence suggests that synaptic loss and dendritic pruning contribute to RGC dysfunction, and are a demonstrable consequence of both the genetic (DBA/2J mice) and inducible (rats receiving intracameral microbeads) glaucoma rodent models, as well as after ONC(Berry et al., 2015, Williams et al., 2016). Just as different RGC subtypes are more susceptible to death, RGC dysfunction is also subtype dependent(Chen et al., 2015). For example, mice intracamerally injected with microbeads to model glaucoma demonstrated a more significant decline in function in the OFF-transient RGC subtype compared to other RGC(Della Santina et al., 2013). Although techniques can now accurately measure RGC function, how the different sub types contribute to these ganglion cell dependent waveforms has not been considered, neither has addressing how this may impact the disparity between RGC survival and RGC function.

5.1 Flash ERG

The ERG is a non-invasive technique to record the function of discrete types of retinal neurons. By subjecting the retina to known intensities of light, an electrical response can be elicited and recorded. Alterations in the stimulus conditions will affect which retinal cell types the compound potential is dependent on. For example, standard full field flash ERG elicits and a- and b-wave, which are photoreceptor(Baylor et al., 1984) and ON bipolar cell(Stockton and Slaughter, 1989) dependent, respectively (Figure 3). To obtain a waveform that is ganglion cell dependent, ERG must be performed in dark (scotopic) conditions and the intensity of light must be reduced considerably to a point near to the behavioral threshold, hence the term, scotopic threshold response (STR) (Sieving et al., 1986, Bui and Fortune, 2004). In rodents, the emergence of a positive and negative STR (pSTR/nSTR)

begins with light intensities of −6.64 log (cd s) m^{-2}, the pSTR peaking first at 100-120ms and the nSTR at 200-220ms (Figure 3). Increasing the intensity of light increases the amplitude of the STR, although predominantly the pSTR. This is likely because the nSTR is also dependent on amacrine cells (Fortune et al., 2004), suggesting the pSTR may be a more accurate measure of RGC function. Corroborating this, selective destruction of RGC (ONT) ablates pSTR completely but not nSTR. Light intensities above −4 and −2 log (cd s) m^{-2} initiated b- and a-waves (respectively), whose amplitudes at 10-20 times greater than STR, dominate the ERG trace(Fortune et al., 2004) (Figure 3).

ERG recordings of the pSTR have been used to record function in animal models of ONC/ONT and glaucoma. Two weeks after ONT in mice, 82% of RGC (identified *via* retrograde labelling) were lost and pSTR amplitude had decreased by 60%(Alarcon-Martinez et al., 2010). Interestingly, at 12 weeks post-ONT, the pSTR had increased to only 50% of that of control, despite further loss in RGC. While the origin of the pSTR is uncertain, the recoverable nature of the pSTR in this study suggests its utility in measuring RGC functional return, such as when using neuroprotective treatments after established retinal injury. It may also suggest that RGC subtypes that are more resilient to the detrimental effects of ONT contribute more to the pSTR amplitude than other less resilient RGC subtypes. For example, αRGC and melanopsin$^+$ RGC make up 40% of the surviving RGC 28 days after ONC(Duan et al., 2015) and melanopsin$^+$ RGC, despite representing only 2% of the RGC population, represent 83% 60 days after ONT(Muller et al., 2014). This is corroborated by a similar study in mice in which pSTR had decreased by 50-60% 4 weeks post-ONC despite >90% death of RGC(Liu et al., 2014), strengthening the idea that RGC survival and function of the ganglion cell population is not a 1:1 relationship. It is also a strong possibility that in mice, pSTR is not completely generated by RGC(Smith et al., 2014). As opposed to mice, ONT in rats has a more significant effect on pSTR amplitude with an almost complete ablation 9 weeks post-ONT, providing a more accurate representation of the number of surviving and thus functional RGC (Bui and Fortune, 2004).

In a rat model of glaucoma, we found that a 35% loss in RGC coincided with a 72% loss in pSTR amplitude(Mead et al., 2016). Even when 100% neuroprotection was afforded in this model, pSTR was still reduced by 50% compared to uninjured controls. In a separate study on genetic mouse models of glaucoma, authors found a 72% decrease in pSTR despite no consistent decrease in RGC numbers (Brn3a$^+$ or FG$^+$)(Perez de Lara et al., 2014). The slow progressive loss of RGC that is characteristic of glaucoma and unlike ONC/ONT makes it unsurprising that dysfunction precedes RGC loss. The dysfunction (as measured by pSTR) is transient and its reversal coincides with a normalisation of IOP (Kong et al., 2012). Comparing RGC numbers to function can be difficult as certain RGC phenotypic markers are more intimately linked to dysfunction than others. For example, whereas RBPMS is strongly associated with functional and dysfunction RGC alike(Rodriguez et al., 2014), Brn3a down-regulates following injury, even preceding the reduction in pSTR amplitude and thus presumably marks the early stages of RGC dysfunction(Yukita et al., 2015). Neuroprotection of RGC does not reverse RGC dysfunction, particularly if the IOP is still raised(Mead et al., 2016). Although limited studies conduct in depth quantitative and functional assessments, it is very likely that many neuroprotective agents may simply be keeping dysfunctional RGC alive, ultimately providing no visual benefit. Another peculiarity

is the differential effects of ocular hypertension and ONC on subtypes of RGC. Subtypes of RGC have demonstrated substantial resilience or susceptibility to ocular injury (Li et al., 2006, Zhang et al., 2013, Duan et al., 2015) and dysfunction(Chen et al., 2015) whereas subtypes of RGC such as melanopsin^{+} respond uniquely to ERG light stimuli(Fukuda et al., 2010). If pSTR does not represents all RGC subtypes such as seen with phenotypic markers, ERG readouts are at risk of grossly under or overestimating the retinal function.

Another less widely used RGC-dependent waveform is the photopic negative response (PhNR), that follows the b-wave following stimulation under photopic conditions. In glaucoma patients, PhNR can be used diagnostically(Viswanathan et al., 2001) and has shown to be as reliable as pattern electroretinography (PERG; discussed below)(Preiser et al., 2013). In a rat ONT model, PhNR drops significantly 1 week post-injury but remains consistent between 1 and 15-weeks post injury(Li et al., 2005). Since only half of RGC die after the 1 week time point, this suggests that PhNR acts as a measure of RGC function independent of RGC death. Interestingly PhNR only decreased by 55%, despite almost all RGC dying 15 weeks post-ONT (Li et al., 2005). A separate study similarly demonstrated that PhNR is not altered by ONC in mice suggesting that PhNR is not solely dependent on RGC and not a reliable readout of their function(Miura et al., 2009). Indeed previous studies have demonstrated that amacrine cells contribute significantly to the negative potential seen in rat photopic ERG, overlapping with and continuing passed the b-wave. Thus it seems likely that PhNR has an amacrine dependent component(Machida et al., 2008). A separate study found that PhNR increased rather than decreased following ONT and loss of Brn3a^{+} RGC(Smith et al., 2014). Under scotopic conditions, b-wave was shown to have partial RGC dependency(Bui and Fortune, 2004, Smith et al., 2014) and decayed faster after ONT, artificially increasing the amplitude of PhNR.

5.2 Pattern electroretinography (PERG)

Like ERG – STR, PERG uses stimuli in the form of light to elicit an electrical response that is recorded on the cornea. The stimulus however is not a flash of light but a flickering brought about by a contrast-reversing checkerboard. The generated waveform has 3 distinct components, a negative trough at 35ms (n35) a positive peak at 50ms (p50) and a large negative trough at 95ms (n95)(Holder, 2004, Porciatti, 2015). The electrical response generated is ganglion cell dependent because other cellular responses (i.e. photoreceptors) are in opposing phases and are cancelled out upon recording(Porciatti, 2015).

PERG is suggested to be a more sensitive measure of RGC function than pSTR. In a study assessing pSTR and PERG in mice after ONC, PERG responses were completely ablated by 7 days post-ONC whereas pSTR showed a moderate 50% decrease in amplitude(Liu et al., 2014), which is more representative of RGC numbers. Separate studies on mice 1 month post-ONC demonstrated a 75-100% reduction in PERG amplitude(Miura et al., 2009, Chou et al., 2013) following a 88% loss of RGC, although this RGC loss is likely an over exaggeration as βIII-tubulin was used as a marker(Chou et al., 2013). In transgenic mice expressing mutated myocillin and thus modelling ocular hypertension, attenuation of PERG amplitude either coincides with (Zode et al., 2011) or precedes any morphological changes, RGC degeneration or IOP elevations(Chou et al., 2014). This association between PERG

amplitude and RGC degeneration is seen in both rodents and primates(You et al., 2013, Porciatti, 2015).

PERG appears acutely sensitive to the functionality of RGC, as several studies have demonstrated an almost immediate effect on the PERG recording. For example, RGC in rodent rely on a retrograde supply of neurotrophic factors derived from the SC which when blocked, induces a 50-60% reduction in PERG amplitude within 60 minutes(Chou et al., 2013, Yang et al., 2013). Interestingly, VEP (and flash ERG) was unaffected by this, demonstrating a distinction between these tests and PERG at quantifying retinal function. Authors also showed there was no loss of RGC numbers and no thinning of RNFL(Yang et al., 2013). In mice after ONC, PERG decreased by 50% after 3 days (earliest time point measured) without any evidence of significant RGC loss(Liu et al., 2014).

Interestingly, the RGC dysfunction, as measured by PERG appears to be reversible with lowering of the IOP restoring PERG amplitude to normal(Feghali et al., 1991) similar to as seen with pSTR(Kong et al., 2012). This suggests the dysfunctional RGC are still viable and suggests why patients who receive IOP lowering surgery claim to have visual improvements. However even when the injury is transient and retrograde transport mechanics are no longer impaired, RGC still remain dysfunctional (as measured by PERG) for many days after, implying that the return of function to RGC is a complex and poorly understood process(Fahy et al., 2016). Equally it could mean that PERG is just as poorly understood as a measure of RGC function. For example, by tilting the head, PERG (as well as IOP) are significantly affected despite vision being unaffected(Porciatti and Nagaraju, 2010). Thus, although evidence supports its correlation with RGC activity in some circumstances, it is not a measure of visual function. Another negative of PERG is the significantly increase complexity compared to flash ERG and the need for a specialised PERG machine. It also typically had a lower signal to noise ration and a very small dynamic range with an amplitude of 5.9 μV ± 2.2 μV in healthy retina and 0 μV in ONC animal retina. In contrast, pSTR was 30.3 μV ± 9.4 μV in healthy retina and 13.6 μV ± 4.4 μV in ONC retina(Liu et al., 2014) and can be as high as 100 μV in healthy rats, particularly albino(Alarcon-Martinez et al., 2010, Mead et al., 2016). However, the use of non-corneal electrodes allows PERG recording as high 20-30μV, increasing the dynamic range(Chou et al., 2014, Dutca et al., 2014). While PERG can be detected in albino mice, it is much more difficult due to a small response amplitude and increased delay with respect to the stimulus(Liu et al., 2014). Despite this, PERG recording with amplitudes of 20μV have been reported in albino BALB/C mice(Xia et al., 2014), suggesting that these difficulties can be overcome. Despite the great sensitivity of PERG, it is unclear if it is ignorant to particular RGC subtypes, which is possible given its complete absence 7 days post-ONC(Liu et al., 2014) despite certain RGC subtypes (ip-RGC) demonstrating impressive resistance to ONC(Muller et al., 2014).

5.3 VEP

VEP is a functional test that unlike ERG, is recorded post-retinal. It is slightly more invasive; requiring exposure of visual brain centres and implantation of electrodes in, or on the visual cortex. An injury to any part of the visual pathway will cause perturbations in the VEP response, e.g. loss of rod and cone photoreceptors cause a loss in the recorded scotopic

and photopic VEP, respectively. Equally, damage to the optic nerve also ablates VEP recordings(Ridder and Nusinowitz, 2006). The standard flash VEP elicits a reproducible waveform characterised by positive (p) and negative (n) deflections. Both amplitude and latency can be measured with latency being a measure of speed of conduction, and is thus affected by demyelination whereas amplitude correlates with axonal (and thus RGC) loss(You et al., 2011).

In a genetic mouse model characterised by degeneration of RGC (without prior degeneration of their axons), VEP amplitude decreased by 37% while FG^+ RGC counts decreased by 35% (Heiduschka et al., 2010). In a separate study of the same mouse model, $Brn3a^+$ RGC numbers decreased by 33% after 7 months whereas VEP amplitude decreased by 70% (Domenici et al., 2014), suggesting that VEP measurements are more dependent on FG^+ RGC, potentially since Brn3a staining excludes ipRGC. Neuroprotection of RGC improves VEP amplitude but not back to normal levels. In a rat glaucomatous model characterised by ocular hypertension, VEP amplitude along with $Brn3a^+$ RGC numbers decrease by 35%(Hill et al., 2015). Along with the invasiveness of this technique another suggested negative is the propensity for plasticity and functional reorganization within the visual cortex. These compensatory mechanisms following retinal injury may impede on the accuracy of VEP to report on RGC numbers and function(Keck et al., 2008).

6. Behavioral testing- Optokinetic reflex

The use of behavioral testing and in particular, the optokinetic reflex (OKR) to assess retinal function has been recently reviewed(Grillo and Koulen, 2015). The OKR allows the eyes to follow an object in motion while maintaining a stabilized image. Detection of the velocity of the moving object in reference to the eye tracking velocity is reliant on a subtype of RGC known as direction-selective RGC (DS-RGC) as well as their inputs into the accessory optic system(Pinto and Enroth-Cugell, 2000, Grillo and Koulen, 2015). Therefore the OKR can be used as a global measure of retinal and sub cortical function and in particular, DS-RGC survival/function, which make up 50% of the RGC population(Huberman and Niell, 2011). Currently few studies have examined OKR in rodent glaucoma models and due to the requirement of an intact retino-cortical connection; OKR has limited use in ONC rodent models. In a mouse model of glaucoma (DBA/2J), progressive elevations in IOP are correlated with a progressive decline in OKR responses(Burroughs et al., 2011) which based on previous studies on the same model, occurred before any significant RGC loss occurred(Schuettauf et al., 2004). Similar results have been seen in a rat model of ocular hypertension induced by saline injection into the episcleral vein(Prokai-Tatrai et al., 2013). In mice, ONC led to a significant decline in OKR after just 1 week and remained consistent for the 4 weeks of the study(Zuo et al., 2013). Neuroprotective strategies that yielded 100% protection of FG-labelled RGC at 2 weeks and 50% at 4 weeks had no significant effect on OKR, likely due to the axonal injury and lack of complete regeneration. A recent study demonstrated a complete loss of OKR 21 days after ONC and an almost complete return when RGC axons were promoted to regenerate and re-innervate the brain(Lim et al., 2016), demonstrating the requirement of functional retino-cortical connections. Further studies are required to determine the utility of OKR in monitoring RGC, and particularly DS-RGC

function in rodent glaucoma models and how it correlates with electrophysiological, morphological and immunohistochemical assessments of healthy and injured retina.

7. Conclusions

The determination of RGC function and numbers is important for research into retinal disease and a multitude of techniques can be employed. Disadvantages however must be carefully considered along with the understanding that retinal function and RGC numbers are not always correlated. Currently the most appropriate RGC marker is RBPMS, which provides an accurate measure of the entire RGC population. RGC functional assessments (pSTR, PERG) are equally important, as a surviving RGC are not necessarily functional. Indeed any neuroprotective pre-clinical trial should endeavor to measure both retinal function and RGC numbers as together they are the strongest indicators of whether a treatment will yield a visual benefit to the patient. Delaying treatment to measure functional restitution after an established retinal injury/disease is equally important and more clinically relevant. Although the phenotypic and functional characteristics of different RGC subtypes is still poorly understood, their differential response to disease and therapies may further complicate quantitative and functional evaluatory techniques, which may be insensitive to particular subtypes and thus, affect the conclusions that can be drawn.

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Abbreviations

RGC	retinal ganglion cells
LGN	lateral geniculate nucleus
SC	superior colliculus
RNFL	retinal nerve fibre layer
ERG	electroretinogram
VEP	visual evoked potential
GCL	ganglion cell layer
FG	FluoroGold
ip-RGC	intrinsically photosensitive retinal ganglion cells
RBPMS	RNA-binding protein with multiple splicing
CFP	cyan fluorescent protein
IPL	inner plexiform layer

OCT	optical coherence tomography
ONC	optic nerve crush
ONT	optic nerve transection
CSLO	confocal scanning laser ophthalmoscope
IOP	intraocular pressure
pSTR/nSTR	positive/negative scotopic threshold response
PERG	pattern electroretinography
PhNR	photopic negative response

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- Research requires accurate counts and functional measures of retinal ganglion cells
- Many different techniques are employed, all with strengths and weaknesses
- Each technique has different selectivity for some of the up to 30 RGC subtypes
- Here we review different techniques in the context of their subtype specificity

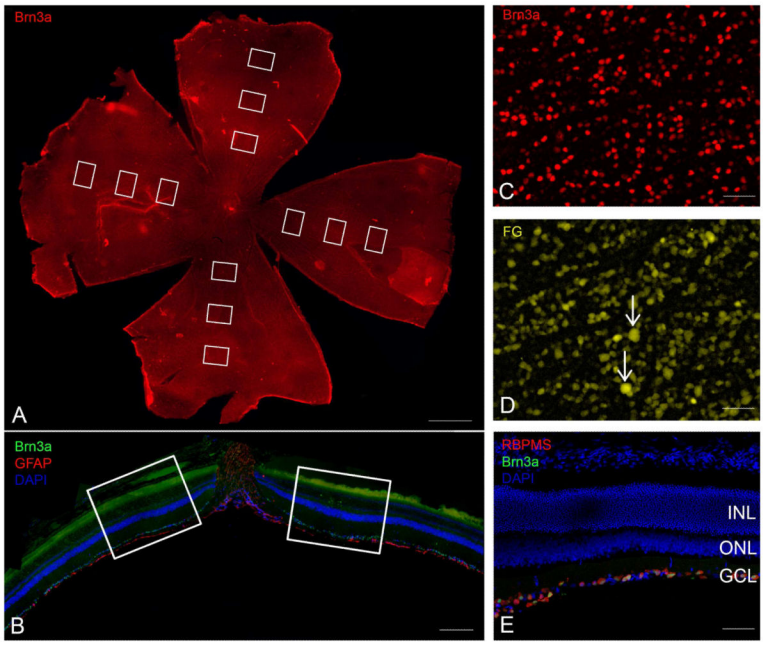

Figure 1. Quantification of RGC in adult rat wholemounts and retinal sections

Immunohistochemical analysis of RGC counts can be done in retinal wholemounts (A) or radial retinal sections (B). The phenotypic markers Brn3a and RBPMS label the nucleus of RGC, which can be easily discerned and quantified in wholemounts (C) and retinal sections (E). The retrograde marker FG in comparison is considered to label all projecting RGC but labels the entire cell, making individual counts more difficult (D). Along with RGC, the presence of FG^+ macrophages (*white arrows*) are often present. (scale bar panel A: 1mm; scale bar panel B: 250μm; scale bar panel C-E 50μm). Adapted from Mead et al., 2014.

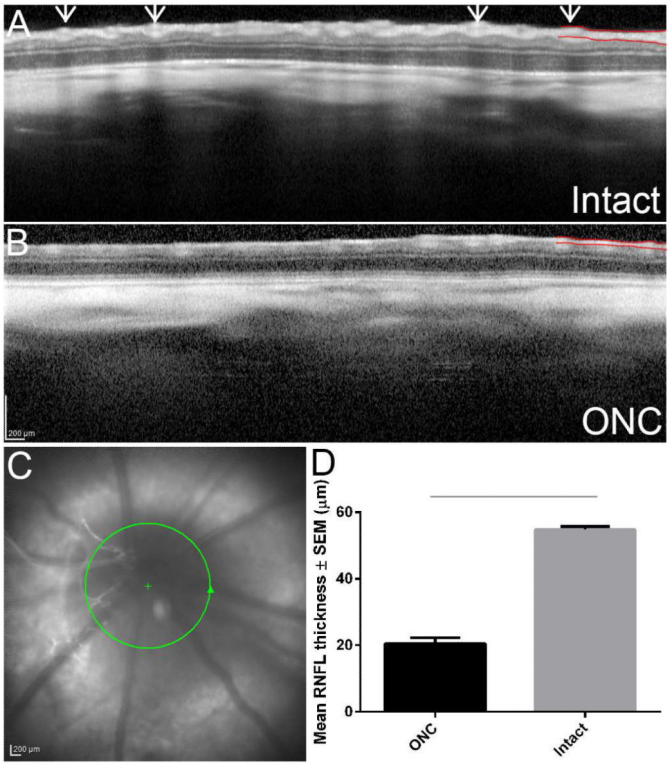

Figure 2. Determination of RNFL thickness using OCT in uninjured and 21 day post-ONC rats

In uninjured retina (A), the RNFL is an easily discernable layer (*red lines*) whose thickness can be measured using OCT, focussing measurements around the optic nerve head (C). Three weeks after ONC, RNFL becomes significantly thinner (B, D; *grey line* indicates significant difference at $p<0.05$) however, blood vessels (*white arrows*) occupying the RNFL becomes more much predominant in the RNFL of injured retina, compromising the accuracy of RNFL thickness measurements (scale bar: 200µm).

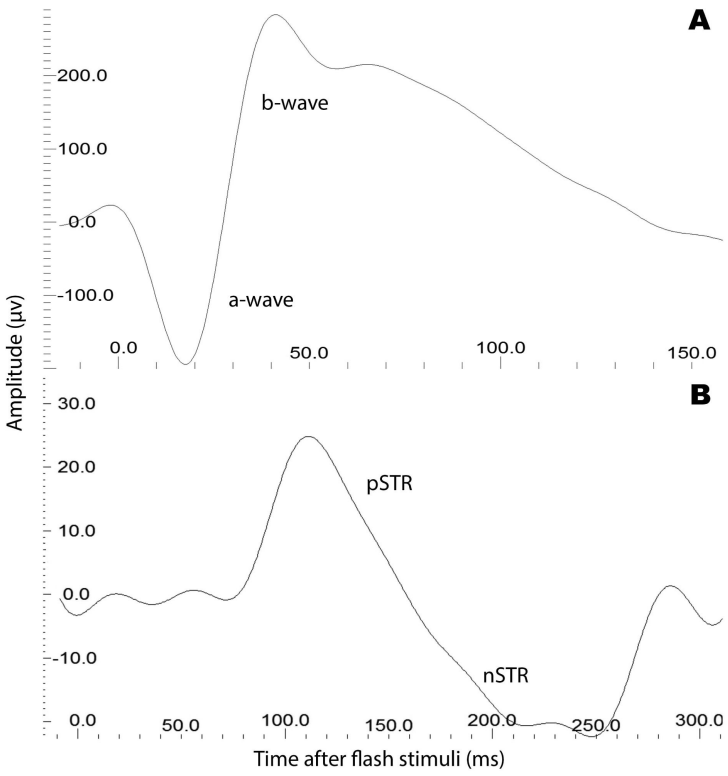

Figure 3. ERG recording from uninjured adult rats

ERG can be used to measure the function of discrete cells of the retina. Amplitudes of the waveforms are primarily used as readout of the respective cells function. Using a relatively high flash intensity (−1 log (cd s) m^{-2}), photoreceptor and bipolar cell dependent responses; the a-wave and b-wave respectively, can be elicited (A). At a much lower intensity (−5 log (cd s) m^{-2}), RGC dependent responses (pSTR and nSTR) are elicited (B). Note the substantial difference in amplitudes between the pSTR and b-wave and thus explains why pSTR is not identifiable in compound potentials including the b-wave.