# Electroretinographic and Histologic Study of Mouse Retina After Optic Nerve Section: A Comparison Between Wild Type and rd1 Mice

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| Keywords:        | Retinal degenerations, Retina, Electroretinogram, Optic neuropathy |
Figure 1. Retrograde labelling of RGCs. Representative images of a whole-mount retina double labelled with DTMR (red) (A), applied directly on the optic nerve stump at the time of axotomy, and FG (blue) (B), applied on both SCI one week before ONS. Axotomy was performed unilaterally on the left optic nerve and tissue was processed for microscopy imaging two days later. The right retina (uninjured) was labelled only with FG (C). To highlight the influence of the labelling technique on RGC counts, numbers are specified in the exact same areas in panels (A) and (B).
Figure 2. Comparison of DTMR- and FG-labelled RGCs. High magnification images of a whole-mount retina showing RGCs retrogradely labelled with FG (blue) or DTMR (red). Example of an uninjured retina (A, B); Representative images from axotomized retinas four (C1, 2), six (D1, 2), fourteen (E1, 2) and twenty-one (F1, 2) days after surgery. All photographs were taken in the dorsal retina, 0.5-mm behind the optic disc. Scale bar, 100 microns.

149x284mm (125 x 125 DPI)
RGC survival kinetics following axotomy. ONS caused a highly significant decrease of the RGC population. Although FG showed larger cell counts than DTMR in all groups, both labelling methods evidenced that the first significant cell loss occurred at six days and that no significant differences were found between fourteen and twenty-one days post-axotomy (one-way ANOVA). Data are summarized in table 1. Values represent mean ± SD. Significance respect to control is indicated as (** p<0.001).
RGC survival after ONS in wild type (CD1 wt) and dystrophic (CD1 rd1) retinas. RGC density in wild type and rd1 did not differ significantly in the uninjured group (control). Nevertheless, a significant difference (**p<0.001) was found when both groups were compared twenty-one days after ONS. Values represent mean ± SD. All measurements were carried out in the mid-peripheral retina.
Electroretinographic responses in axotomized eyes. Electroretinographic trace recordings (A) and averaged data of ERG amplitudes (B) in control and axotomized eyes. The graph in A represents superposed ERG recordings obtained from a single animal. Black traces correspond to control eye and gray traces correspond to axotomized eye. The intensity of the light stimuli is indicated in log cd•s•m•2 to the left of each trace recording. Sample measurements for the a- and b-waves is shown in the upper trace. The graph in B represents the relationship between light intensity (log cd•s•m•2) and ERG response amplitude (µV), for a-waves (squares) and b-waves (circles), in control (solid symbols) and axotomized (empty symbols) eyes. The amplitude values depicted were averaged (mean ± SD) from a total of 12 mice. The most significant differences were observed for wave amplitudes in response to stimuli of low light intensity (pSTR).
Temporal evolution of the pSTR wave after ONS. Histogram representation of the pSTR wave amplitude recorded from control and axotomized eyes in the wild type animals, in response to a light stimulus of \(-5 \log\) cd\(\cdot\)s\(\cdot\)m\(^{-2}\). Values were averaged (\(\pm\) SD) from a total of 8 mice, recorded at increasing time intervals (1, 2 and 3 weeks) after ONS. One week after axotomy no significant difference was observed between ERG waves from control and axotomized eyes; in contrast, two and three weeks after ONS, significant difference was observed in the pSTR amplitude between control and axotomized eyes. (* \(p<0.05\); ** \(p<0.01\)).
Table 1

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<tr>
<th>Days after ONS</th>
<th>Density (RGCs/mm²)</th>
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<tr>
<td></td>
<td>Fluorogold</td>
<td>DTMR</td>
</tr>
<tr>
<td>Ø</td>
<td>3.151 ± 294&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>2.988 ± 268&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.654 ± 162&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>4</td>
<td>2.812 ± 289&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.453 ± 137&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>1.552 ± 310&lt;sup&gt;b&lt;/sup&gt;</td>
<td>984 ± 196&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>14</td>
<td>461 ± 85&lt;sup&gt;c&lt;/sup&gt;</td>
<td>227 ± 57&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>21</td>
<td>381 ± 80&lt;sup&gt;c&lt;/sup&gt;</td>
<td>187 ± 46&lt;sup&gt;c&lt;/sup&gt;</td>
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Different letters indicate statistical difference
Original Article – Laboratory Science

Title: Electroretinographic and Histologic Study of Mouse Retina After Optic Nerve Section: A Comparison Between Wild Type and rd1 Mice

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Running Title: Retinal ganglion cells survival after injury
Abstract

**Background**: Retinal ganglion cell death underlies the pathophysiology of neurodegenerative disorders such as glaucoma or optic nerve trauma. To assess the potential influence of photoreceptor degeneration on retinal ganglion cell survival, and to evaluate functionality, we took advantage of the optic nerve section mouse model.

**Methods**: Surviving retinal ganglion cells were double stained by exposing both superior colliculi to fluorogold, and by applying dextran-tetramethylrhodamine to the injured optic nerve stump. To assess retinal function in wild type animals, electroretinograms were recorded on the injured eyes and compared to the contralateral. Similar labelling experiments were carried out on rd1 mice. Surviving retinal ganglion cells were counted 21 days after axotomy and compared with wild type mice. No functional experiments were performed on rd1 animals, since they do not develop electroretinographic responses due to the photoreceptor degeneration.

**Results**: A significant decrease in retinal ganglion cell density was observed 6 days after axotomy in the wild type. Functional studies revealed that, in scotopic conditions, axotomy induced a significant amplitude decrease in the positive scotopic threshold response component of the electroretinogram. Such decrease paralleled cell loss, suggesting it may be an appropriate technique to evaluate functionality. When comparing retinal ganglion cell densities in wild type and rd1 mice, a significant greater survival was observed on the rd1.

**Conclusions**: After optic nerve section, electroretinographic recordings exhibited a progressive decrease in the amplitude of the positive scotopic threshold response wave, reflecting ganglion cell loss. Interestingly, rod degeneration seemed, at least initially, to protect from axotomy-driven damage.

**Keywords**: Retinal degenerations, retina, electroretinogram, optic neuropathy.
Introduction

A common event in the pathophysiology of optic neuropathies is the death of retinal ganglion cells (RGCs), the neurons responsible for transmitting visual information from the retina to the brain. Since RGCs are post-mitotic neurons, unable to divide in the mature adult retina, their loss irreversibly commits to blindness. Therefore, a big effort is being made nowadays to investigate and develop novel neuroprotection strategies that enhance RGC survival and prevent sight loss. Axonal injury has been proposed as a main cause of RGC death in most optic neuropathies, including glaucomatous, inflammatory, ischemic, infiltrative, or traumatic (Levin, 2007). Experimentally, axonal injury has been often induced by complete transection of the optic nerve, so as to provide a model for the study of RGC death and neuroprotection pathways (Thanos, 1991; Villegas-Pérez et al., 1993; García-Valenzuela et al., 1994; Quigley et al., 1995; Germain et al., 2007; Rodríguez-Muela et al., 2012). However, many of the techniques used to evaluate RGC survival are invasive and, more importantly, do not allow the assessment of RGC functionality.

Non-invasive methods, such as the recording of electroretinographic responses, allow, in contrast, repeated measurement of global retinal functionality over a time period. In the electroretinogram (ERG), different waves are attributed to different cell populations. In scotopic conditions the a-wave corresponds to photoreceptors, whereas the b-wave corresponds to ON-bipolar and Müller cells (Dowling, 1987). The association of other waves to certain neurons has not been so clearly established though. Also in dark-adapted conditions, a very dim light flash stimulus evokes the scotopic threshold response (STR), comprised by a positive (pSTR) and a negative component (nSTR). Although these waves are not definitely identified, research carried out in several animal species suggests they originate in the inner retina (Frishman et al., 1988), specifically in the RGCs (Saszik et al., 2002; Bui and Fortune, 2004; Casson et al., 2004; Alarcón-Martínez et al., 2009, 2010). However, some controversy exists over the origin of the STR, since electrophysiological studies in the Math5 mutant mouse revealed the existence of a STR despite the lack of RGCs (Brzezinski et al., 2005). To address this question and, at the same time, to evaluate the suitability of the STR as a tool for the assessment of RGC functionality, we have made use of the intraorbital optic nerve section (ONS) mouse model.

This retinal axotomy model meets the purpose of our study, as cell death is induced selectively in RGCs and not in other retinal cell types. In this paradigm, we have found that, after ONS, standard electroretinographic recordings showed similar responses to those registered in uninjured mice, except for the STR wave, which exhibited a progressive amplitude decrease, mainly reflecting the RGC loss.

In addition, we have been searching for factors that may influence the process of RGC degeneration. Among the ones described to date, we may find; the distance from the lesion to
the optic nerve head (Villegas-Pérez et al., 1993); the type of lesion, being damage more severe after section than after crush (Parrilla-Reverter et al., 2009); the developmental stage of the animal (Allcutt et al., 1984; McKernan et al., 2006); the participation of microglial cells in the process (Thanos, 1991; Thanos et al., 1993); or even the differences between RGC classes (Allcutt et al., 1984; Robinson and Madison, 2004). In order to move further in the determination of factors involved in RGC survival capacity, we have compared the effects of ONS in sighted vs. blind mice. One of the most widely studied retinal degeneration models is the rd1 mouse, whose blind phenotype is due to a non-sense mutation in exon 7 of the Pde6b gene, encoding the beta subunit of the cGMP phosphodiesterase from rod photoreceptors (Pittler and Baehr, 1991). In the rd1 model, second order neurons show abnormalities that culminate with the atrophy of dendrites in cone bipolar cells and horizontal cell remodelling (Strettoi et al., 2003), but despite inner retinal remodelling associated to photoreceptor degeneration, RGC structure, survival, and projections to higher visual centres seem to be remarkably preserved (Mazzoni et al., 2008; Damiani et al., 2012). In the present study, we defined the influence of photoreceptor degeneration on RGC survival, statistically showing that an ongoing retinal degenerative process was able to delay axotomy-induced RGC loss.

Materials and methods

Animals

All experimental procedures were carried out in accordance with the European Union statement for the use of laboratory animals (Directive 86/609/CEE). Two-month old, wild type or dystrophic rd1 CD1 (ICR) mice (Charles River Laboratories), weighing 20-30g, were used in these studies. The genotype of rd1 mice was assessed by polymerase chain reaction as previously described (Gimenez and Montoliu, 2001). Animals were reared in an artificial 12-hour light/dark cycle with food and water ad libitum. Illumination was 30 cd·m⁻².

Surgical Procedures and retrograde labelling of RGCs

Mice were anesthetized with an intraperitoneal injection of saline solution (NaCl 0.9%), containing ketamine (70 mg/kg; Ketalar, Parke-Davis) and xylazine (7 mg/kg; Rompun, Bayer Leverkusen, Germany). For comparison, RGCs were labelled with the retrograde tracers Fluorogold (FG) (2-hydroxystilbene.4,4´-dicarboxamid bis (methasulfonate)) and dextran-tetramethylrhodamine (DTMR) (Molecular Probes, Eugene, OR). FG was applied to both superior colliculi (Sci) following previously described methods (Salinas-Navarro et al., 2009a). Briefly, after exposing the midbrain, a small pledget of gelatine sponge (Espungostan Film, Ferrosan A/S, Denmark), soaked in saline solution containing 3% FG and 10% DMSO, was applied over the entire surface of the Sci. Previous studies in control rats have shown that FG
application to both SCi, which are the main retino-recipient target regions in the brain, results one week later in the labelling of approximately 96.6% of the RGC population in C57 adult mice (Salinas-Navarro et al., 2009a). One week later, the left optic nerve was intraorbitally transected 2 mm behind the eyecup, taking care not to injure blood vessels. This was confirmed by fundus ophthalmoscopy and electroretinography. During axotomy, RGCs were retrogradely labelled by applying a piece of spongostan soaked in 0.1% DTMR at the optic nerve stump. After surgery, an antibiotic solution containing polymyxin B, neomycin and gramicidin (Oftalmowell, Medeva Pharma, Spain) was applied to the orbital space.

**Tissue preparation**

Animals were killed with an overdose of sodium pentobarbital. To maintain retinal orientation, a suture was placed on the superior pole of each eye previously to enucleating. The retina was dissected out from the eyecup, flat-mounted vitreous side up and fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), for 1 h at room temperature.

**Cell counting**

Left (injured) retinas were studied for both, DTMR and FG labelling, while right (uninjured) retinas were only studied for FG labelling (Fig. 1). Thirteen different areas (214 x 161 µm²) were selected for RGC scoring; Four in the central retina, 0.5 mm away from the optic disc, and nine in the peripheral retina, more than 1 mm away from the optic disc. Any cell with a rounded soma and a diameter exceeding 8 µm was regarded as a RGC (Boycott and Hopkins, 1981; Maffei et al., 1990; Provis, 1979). Cell counting was performed in control (n=7) and injured retinas at different times after ONS (2 days, n=5; 4 days, n=5; 6 days, n=6; 14 days, n=5; 21 days, n=5). All cell counts were performed in a blind fashion by the same investigator.

**Data analysis**

Data were compared by one-way ANOVA with Tukey-Kramer post-test or Student’s t-test, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA, www.graphpad.com).

**Electroretinography**

ERG recordings were performed only on wild type animals, both in the axotomized and control eyes. Full-field ERG was the technique of choice. For low intensity stimuli (log -6 to log -2 cd·s·m⁻²) a Ganzfeld dome, which ensures a homogeneous illumination of 120° in central retina (Dieterle and Gordon, 1956), was used, whereas for high intensity stimuli (log -2 to log 2 cd·s·m⁻²) we used one single light emitting diode (LED) placed close to the eye. Illumination was measured by a photometer (Mavo Monitor USB, Gossen, Nürnberg, Germany). Mice were
kept at 37º C on a heating pad (Hot-Cold, Pelton-Sherpherd Industries, CA) during the entire procedure. Dark-adapted (12 h) animals were anesthetized and, before recording, pupils were dilated with 1-2 drops of 1% tropicamide (Alcon Cusi S.A., El Masnou, Barcelona, Spain). To preserve corneal surface from desiccation, a drop of 2% methyl-cellulose was applied (Methocel, Ciba Vision, Hetlingen, Switzerland). Three recording electrodes, ground, reference and corneal, were disposed (Burian-Allen, Hansen Ophthalmic Development Lab, Coralville, IA). The corneal electrode (contact lens type) was placed in the visual axis 5 mm from the cornea. The recorded electrophysiological response was amplified and filtered (CP511 AC amplifier, Grass Instruments, Quincy, MA), and digitalized (AD Instruments Ltd., Oxfordshire, United Kingdom), the whole process controlled by Scope version 6.4 software (Power Lab, AD Instruments Ltd.).

Protocols of stimulation and recording
First, the scotopic response was registered after dark adaptation. Next, a series of increasing intensity stimuli were applied to sequentially record rod responses, mixed responses and oscillatory potentials. Finally, mice were adapted to a light background higher than 100 cd·m⁻² during 5-10 min, and then cone and flicker responses were recorded. Protocols of stimulation were designed according to the International Society for Clinical Electrophysiology of Vision (ISCEV). Six types of standard ERG responses were recorded:

1) STR response: A series of very low intensity stimuli (6 to -5 log cd·s·m⁻²) were applied using the Ganzfeld dome.

2) Rod response: A series of low intensity stimuli (4 to -2 log cd·s·m⁻²) were applied using the Ganzfeld dome. After 100 ms of basal line recording, a 5 ms stimulus was applied and the retinal response was recorded for 500 ms. High-pass (1 Hz) and low-pass (1000 Hz) filters were used. Rod response was averaged from enough recordings to achieve a stable response.

3) Mixed response: High intensity stimuli (2 to 2 log cd·s·m⁻²) were applied using only one LED, with the same latency, stimulus, recording time, low and high pass filters, and average described for rod response.

4) Oscillatory potentials: A high intensity 5-ms stimulus (2 log cd·s·m⁻²) was applied. High-pass (30 Hz) and low-pass (10000 Hz) filters were used.

5) Cone response: A series of high intensity stimuli (-2 to 2 log cd·s·m⁻²) were applied. After 100 ms of basal recording, a stimulus of 5 ms was applied and the retinal response was recorded for 500 ms. Sixty responses were averaged with an inter-stimuli of 1 s. High-pass (1 Hz) and low-pass (1000 Hz) filters were used.

6) Flicker response: A high intensity 5-ms stimulus (2 log cd·s·m⁻²) was applied every 50 ms (frequency 20 Hz), with the same response average and filters as described for cone response.
We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

**Results**

*Global cell loss*

No evidence of general damage to the retina, due to surgical procedures or ischemia, was noticed in axotomized retinas. Since tracers’ retention within the cytosol implies the presence of a functional plasma membrane (Garcia-Valenzuela et al., 1994), we identified such labelled cells as intact RGCs (figure 2). Early after injury, labelled microglial cells and axonal bundles were observed and differentiated by morphological criteria. The section of the optic nerve induced a decrease in the density of FG and DTMR-labelled RGCs. As represented in figure 3, quantification of surviving RGCs revealed statistical differences for each labelling type (one-way ANOVA; p<0.001).

Four days after ONS, FG-labelled RGCs showed no significant difference with control eyes. Six days after axotomy, a significant difference was found with control eyes, density representing 49% of the original population. Fourteen days after ONS, RGC density decreased to 15% indicating that RGC death was massive between days 4-14 post-axotomy. Twenty-one days after ONS, RGC density decreased to 12% of control. A significant difference was found between six and fourteen days, but not between fourteen and twenty-one days post-axotomy. See table 1 for RGC densities.

To compare surviving DTMR-labelled cells, we took as control the data from retinas 2 days after ONS, because it is the time necessary for the tracer to reach the somas from the optic nerve stump (Vidal-Sanz et al., 1988), and at this time point there is not a significant decrease yet, as we have confirmed with FG labelling and is also reported in the literature (Galindo-Romero et al. 2011). Appearance of these DTMR-labelled RGCs was similar to FG-labelled control cells (Fig 2A and B). Four days after axotomy no significant differences were found in DTMR-labelled RGCs. However, six days after ONS a significant difference was observed with control, as well as between six and fourteen days, but not between fourteen and twenty-one days after ONS (see table 1 for statistical significance).

*RGC survival after axotomy in wild type vs. rd1 mice.*

In order to study if other degeneration processes, occurring simultaneously in axotomized retinas, could change the course of RGC death, the same procedures of FG labelling and axotomy were carried out on a group of CD1 mice (n=12), which carried the Pde6b<sup>rd1</sup> mutation. By comparing both groups with Student’s t-test, a significant difference was found (p<0.001) (figure 4). Control retinas of rd1 mice presented 3282±182 cells/mm<sup>2</sup> (n=5), whereas twenty-
one-day axotomized retinas displayed 1017±108 cells/mm² (n=5), which is a 31% survival, and represents a significant increase in comparison with wild type (12% survival). Although RGC population in rd1 undamaged control retinas showed to be slightly larger than in the wild type, this difference was statistically non-significant. However, the differences between injured rd1 and wild type retinas were significant (p<0.001).

**Standard ERG recordings in control animals**

Light stimuli of very low intensity (-6.0 to -5.0 log cd·s·m⁻²) evoked positive (pSTR) and negative (nSTR) deflexions in dark-adapted control mice. By increasing light intensity, larger amplitude waves were obtained. The maximum amplitude of a- and b-waves was reached after stimuli of -2 log cd·s·m⁻² (maximum scotopic response), whereas higher intensity stimuli gave way to a mixed retinal response, generated by cones, rods and their postsynaptic cells. After retinal adaptation to light, we recorded photopic responses for light intensity stimuli of -1.5 log cd·s·m⁻². For larger intensity stimuli an increase of the b-wave was recorded, reaching a maximum for intensities of 2 log cd·s·m⁻².

**Standard ERG recordings after ONS**

The light intensity-voltage response relationship of control and injured eyes in a group of animals, two weeks after ONS, is illustrated in figure 5. Both control (right) and injured (left) eyes were compared in each animal. In scotopic conditions, the recordings corresponding to injured eyes showed a significant decrease in the amplitude of the positive deflexion (pSTR and b-wave) in response to very low light stimuli (-6.0 to -5.0 log cd·s·m⁻²). However, neither responses to high intensity stimuli (rods and mixed response), nor ERG recordings after light adaptation were altered, suggesting that pSTR wave amplitude could be an index of RGC death.

**Evolution of ERG waves after ONS**

To find out if the progressive RGC loss correlated to a decrease in the pSTR wave amplitude, we compared pSTR in control and injured eyes of CD1 mice one, two and three weeks after ONS (figure 6). One week after axotomy no significant decrease was observed. Two weeks after axotomy, a decrease of 30% in the pSTR wave amplitude resulted significant (p<0.05, Student’s t-test). Three weeks after ONS, the pSTR wave amplitude reached only 50% of control eyes and was highly significant (p<0.01, Student’s t-test). All these data suggest that the progressive loss of RGCs induced a parallel decrease in the pSTR wave amplitude. Therefore, we conclude that the pSTR is the ERG wave that better represents electrical activity of RGCs.

**Discussion**
The first goal of this study was to compare RGC survival after axotomy in wild type and dystrophic mice (carrying the rd1 mutation), so as to deepen in the understanding of factors potentially involved in the death/survival balance subsequent to injury. The second goal was to study the pSTR wave capacity to evaluate RGC death.

Mouse is a largely used model for morphological and functional studies of degenerative neurological processes, ever since current research has provided with techniques to generate genetically manipulated animals. One difficulty is that the numbers of ganglion cells are highly variable among diverse types of mice, subspecies and strains (Williams et al., 1996; Salinas-Navarro et al., 2009a), as well as in rats (Salinas-Navarro et al., 2009b). For this reason, it becomes strictly necessary to know the specific number of RGCs in the mouse model of study. In agreement with previous reports (Agudo et al., 2008; Villegas-Pérez et al., 1993), the decrease in RGC density began to be significant at similar post-axotomy time, six days (Galindo-Romero et al., 2011).

Quantitative differences were observed at any time after axotomy between DTMR and FG labelling. These differences suggest that not only active retrograde axonal transport (FG) is impaired, but also passive diffusion (DTMR) along the axon is early altered after RGC axotomy. An axonal collapse may be occurring in optic nerve stump after transection, so that DTMR does not penetrate into all the axons.

Some RGCs were DTMR, but not FG labelled. It may be explained because, although mouse RGC projections are directed mainly to the contralateral SCi, a little percentage of the RGC population send their axons to other targets different from the SCi, this is to the dorsal lateral geniculate nucleus (Quina et al., 2005).

To assess the possible influence of photoreceptor loss on RGC survival, we compared RGC numbers in wild type vs. rd1 mice three weeks after axotomy. A significantly greater cell loss was detected in wild type mice, suggesting that protective mechanisms are activated in response to the photoreceptor degeneration suffered by rd1 animals, which in turn promote RGC survival. A similar response has been observed in other retinal injury models, for instance, RGC axotomy attenuates light-induced degeneration of the outer retina (Bush and Williams, 1991; Casson et al., 2004), which is attributed to trophic factor (bFGF, CNTF) upregulation in photoreceptors and glial cells following optic nerve damage (Kostyk et al., 1994; Casson et al., 2004). Although RGC death subsequent to photoreceptor loss has already been described in inherited retinal dystrophies (Villegas-Pérez et al., 1996; Wang et al., 2000, 2003; Garcia-Ayuso et al., 2010), our results do not contradict these observations. Transneuronal degeneration may be expected after a primary injury, however, it is also possible that protective retinal responses are found at initial stages. In fact, recent investigations suggest that the Wnt signalling pathway,
which is activated in the degenerating retina of the rd1 mouse (Yi et al., 2007), protects primary retinal cell cultures from oxidative stress (Hackam, 2005; Seitz et al., 2010). Wnts comprise a family of genes that control the expression of pro-survival growth factors in mammalian tissues. An altered expression of the Wnt pathway genes, including the anti-apoptotic regulator Dickkopf 3 (Dkk3), is observed during photoreceptor loss (Hackam, 2005). Dkk3 localizes to Müller glia and RGCs in developing and adult mouse retina, and is upregulated during photoreceptor death in the rd1 model of retinal degeneration. Therefore, Wnt activation, alone or in combination with growth factors, may increase the threshold for apoptosis and halt or delay further retinal degeneration (Yi et al., 2007).

The second aim of this study was to analyze whether, in spite of cell membrane changes showed by RGCs after ONS (Germain et al., 2004, 2007), surviving cells were able to maintain electrical responses. Non-invasive methods (ERG) were used to allow a temporal evaluation. Two weeks after ONS, a significant decrease in the pSTR wave amplitude of the injured eye correlates with the greatest amount of death occurring between days 4 to 14 post-axotomy. Similar decreases in the pSTR wave amplitude after ONS have been reported in adult pigmented rats (Bui and Fortune, 2004; Alarcón-Martínez et al., 2009), adult albino rats (Alarcón-Martínez et al., 2009) and mice (Alarcón-Martínez et al., 2010), also coinciding in time with the major RGC loss. In this last study, the amplitudes of the pSTR and nSTR were recorded both, in albino and pigmented mice 2, 4 and 12 weeks after axotomy. They found a similar response to ours 2 weeks after ONS in pigmented mice; and globally, reported that the nSTR amplitude was more variable between animals (especially in albinos) than the pSTR, but this component resulted more affected after RGC damage, better showing a clear and persistent diminution. They also found a decrease of the implicit time of both STRs in the operated eyes.

Nevertheless, contributions of other cells to this wave cannot be discarded. For example, those cells unaffected by ONS, like amacrines, which contribute to pSTR waves (Saszik et al., 2002); additionally, it has been demonstrated that it is possible to record the pSTR wave in transgenic mice lacking RGCs (Brzezinski et al., 2005). These origins could explain why at early time-points (one week after ONS) the pSTR wave does not present significant changes in spite of the fact that half of the original RGC population has already been lost. In any case, the highly significant difference in the pSTR wave amplitude found three weeks after axotomy respect to control eyes, in coincidence with the remarkable decrease of RGC density, suggests the importance of RGCs in the generation of pSTR.

It is interesting that the inner plexiform layer remodelling (Germain et al., 2003) and the down-regulation of ionic currents in retinal neurons (Blanco et al., 2002; Germain et al., 2006),
described in the rabbit retina one month after axotomy, are structural and functional changes
that are not be able to induce a decrease in major (a and b) ERG waves (Winkler, 1972).
However, pSTR wave amplitude suffers a highly significant decrease after massive RGC. These
findings bring to light the novel possibilities that the study of pSTR offers for analyzing RGCs
degenerative processes.

Conclusions

After ONS, ERG recordings showed similar responses to those found in uninjured mice but for
the STR wave, which exhibited a progressive amplitude decrease, mainly reflecting the RGC
loss. Interestingly, the simultaneous incidence of rod degeneration seems to protect RGCs from
axotomy-driven damage. Our work provides new insights on the pathophysiology of RGC
degeneration-related diseases, as well as a convenient non-invasive tool for the assessment of
RGC functionality.

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(RD07/0062/0008) to PdlV.
References


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Figure legends

**Figure 1. Retrograde labelling of RGCs.** Representative images of a whole-mount retina double labelled with DTMR (red) (A), applied directly on the optic nerve stump at the time of axotomy, and FG (blue) (B), applied on both SCi one week before ONS. Axotomy was performed unilaterally on the left optic nerve and tissue was processed for microscopy imaging two days later. The right retina (uninjured) was labelled only with FG (C). To highlight the influence of the labelling technique on RGC counts, numbers are specified in the exact same areas in panels (A) and (B).

**Figure 2. Comparison of DTMR- and FG-labelled RGCs.** High magnification images of a whole-mount retina showing RGCs retrogradely labelled with FG (blue) or DTMR (red). Example of an uninjured retina (A,B); Representative images from axotomized retinas four (C₁, 2), six (D₁, 2), fourteen (E₁, 2) and twenty-one (F₁, 2) days after surgery. All photographs were taken in the dorsal retina, 0.5 mm behind the optic disc. Scale bar, 100 microns.

**Figure 3. RGC survival kinetics following axotomy.** ONS caused a highly significant decrease of the RGC population. Although FG showed larger cell counts than DTMR in all groups, both labelling methods evidenced that the first significant cell loss occurred at six days and that no significant differences were found between fourteen and twenty-one days post-axotomy (one-way ANOVA). Data are summarized in table 1. Values represent mean ± SD. Significance respect to control is indicated as (*** p<0.001).

**Figure 4. RGC survival after ONS in wild type (CD1 wt) and dystrophic (CD1 rd1) retinas.** RGC density in wild type and rdI did not differ significantly in the uninjured group (control). Nevertheless, a significant difference (**p<0.001) was found when both groups were compared twenty-one days after ONS. Values represent mean ± SD. All measurements were carried out in the mid-peripheral retina.

**Figure 5. Electoretinographic responses in axotomized eyes.** Electoretinographic trace recordings (A) and averaged data of ERG amplitudes (B) in control and axotomized eyes. The graph in A represents superposed ERG recordings obtained from a single animal. Black traces correspond to control eye and gray traces correspond to axotomized eye. The intensity of the light stimuli is indicated in log cd·s·m⁻² to the left of each trace recording. Sample measurements for the a- and b-waves is shown in the upper trace. The graph in B represents the relationship between light intensity (log cd·s·m⁻²) and ERG response amplitude (µV), for a-waves (squares) and b-waves (circles), in control (solid symbols) and axotomized (empty symbols).
symbols) eyes. The amplitude values depicted were averaged (mean ± SD) from a total of 12 mice. The most significant differences were observed for wave amplitudes in response to stimuli of low light intensity (pSTR).

Figure 6. Temporal evolution of the pSTR wave after ONS. Histogram representation of the pSTR wave amplitude recorded from control and axotomized eyes in the wild type animals, in response to a light stimulus of -5 log cd·s·m⁻². Values were averaged (± SD) from a total of 8 mice, recorded at increasing time intervals (1, 2 and 3 weeks) after ONS. One week after axotomy no significant difference was observed between ERG waves from control and axotomized eyes; in contrast, two and three weeks after ONS, significant difference was observed in the pSTR amplitude between control and axotomized eyes. (* p<0.05; ** p<0.01).

Table 1. Ganglion cell counts following ONS in fluorogold and DTMR labelled retinas. While statistical analysis (one-way ANOVA) of fluorogold labelled retinas evidenced non-significant differences between undamaged control retinas and 4 days axotomized retinas (p>0.05), data from 6, 14, or 21 days axotomized groups were highly significant (p<0.001) when compared to control. Two or 4 days axotomized. The comparison of data from 6-days with data from retinas at 14 and 21 days were also highly significant (p<0.001). However, 14 days compared to 21 days no showed to be statistically significant (p>0.05). Mean ± SD is represented. N, sample size. DTMR labelled RGCs show similar results. Statistical significance is grouped by upper letters.