Early remodeling of Müller cells in the rd/rd mouse model of retinal dystrophy

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ABSTRACT

We studied the anatomical remodeling and gliosis of retinal Müller cells in the rd/rd mouse model of photoreceptor degeneration. A computational calculation of glutamine synthetase immunoreactivity was developed so we could specifically quantify changes in Müller cell anatomy between control mice (C57Bl/6) and the dystrophic strain. We found no change in number of Müller cell somata between mice strains indicating no cell proliferation as a function of development and degeneration. The retinal area occupied by the total Müller cell body (soma and processes) was significantly less in the rd/rd mouse retina compared with control mice. When only the outer retina was considered, we found rd/rd Müller cell processes were dramatically reduced during the cone phase of photoreceptor degeneration. However, at older ages, an increase Müller cell processes was seen. Conversely, glial fibrillary acidic protein (GFAP) expression showed a significant increase during cone degeneration followed by a reduction in older ages. Müller cell electrophysiology, particularly K⁺ currents and membrane potential was similar between rd/rd and control Müller cells during cone degeneration. Together, these results show that glial remodeling in the rd/rd retina follows separate phases - an initial conservative glial response involving the loss of Müller cells processes, hyper-expression of GFAP and preservation of normal electrophysiology followed by an active growth of Müller cell processes, glial seal formation and attenuation of GFAP expression after complete photoreceptor loss.
INTRODUCTION

Retinal degeneration secondary to inherited mutations or environmental insult results in the rapid loss of retinal neurons as well as anatomical and functional remodeling (Gargini et al., 2007; Marc and Jones, 2003; Marc et al., 2007; Marc et al., 2003; Marc et al., 2008; Pignatelli et al., 2004; Strettoi and Pignatelli, 2000; Strettoi et al., 2003; Strettoi et al., 2002; Sun et al., 2007). Glial cell reactivity, or gliosis, is universal to retinal disease (Bringmann et al., 2006). Müller cells, the major glial cells in the retina become reactive during the early stages of degeneration (Ekström et al., 1988; Felmy et al., 2001; Iandiev et al., 2006) then contribute to the development of a glial seal that envelops the entire retina at late stage degeneration (Jones et al., 2006; Jones et al., 2003; Jones et al., 2005; Marc et al., 2008). Thus, a better understanding of the glial changes that occur during retinal degeneration is crucial for the development of successful treatment strategies.

Müller cell gliosis is described as either proliferative or conservative/reactive (Bringmann et al., 2000). In both types, early stage gliosis is associated with up-regulation of the intermediate filament proteins including, glial fibrillary acidic protein (GFAP), vimentin or nestin thought to support structural integrity or hypertrophy in retinal disease (Bignami and Dahl, 1979; Bringmann and Reichenbach, 2001; Eisenfeld et al., 1984; Lewis and Fisher, 2003). In late stages of proliferative gliosis, glial replication and migration occurs...
and Müller cell electrophysiology, particularly K⁺ currents are reduced (Bringmann et al., 2006; Burke and Smith, 1981). Disruption to K⁺ channels has severe implications for the retina as they facilitate primary Müller cell functions including K⁺ homeostasis (Kofuji et al., 2002; Newman, 1993), osmoregularity (Pannicke et al., 2004) and neurotransmitter recycling (Bringmann et al., 2000; Bringmann et al., 2009). In contrast, conservative reactive gliosis is associated with no cell proliferation and minimal changes in K⁺ conductance (Bringmann et al., 2000; Bringmann and Wiedemann, 2012).

The rd1 or rd/rd mouse is an animal model of Retinitis Pigmentosa (Farber et al., 1994). The rd/rd mouse retina displays a rapid loss of rod photoreceptors beginning around postnatal day 10 (P10), and completed by ~P25. Cone degeneration begins at three weeks of age and is complete by approximately P40, with very few cones remaining after six weeks of age (Farber et al., 1994). Functional remodeling of glutamate receptors occurs during the cone degeneration phase in the rd/rd retina (Chua et al., 2009; Marc et al., 2007).

Gliotic changes including expression of nestin, GFAP and rhodopsin by Müller cells has been shown in the rd/rd retina (Goel and Dhingra, 2012; Strettoi et al., 2003; Strettoi et al., 2002). These observations however, are limited to qualitative analysis of immunolabeled tissue. Furthermore, electrophysiology of rd/rd Müller cells is yet to be described. This study aims to comprehensively characterize Müller cell changes during retinal degeneration in the rd/rd retina. We evaluate Müller cell anatomy including glial seal formation, GFAP expression and electrophysiological characteristics from early
stage (P10) to late stage (P101) photoreceptor degeneration. Glial cell immunoreactivity was assessed with a computational calculation that specifically quantifies changes in Müller cell anatomy in the outer and inner retina allowing us to assess potential hypertrophy (increase in Müller cell processes) and hyperplasia (increase in Müller cell somata) up to the end of Phase 2 of retinal degeneration (Marc and Jones, 2003).

MATERIALS AND METHODS

Animals

The rd/rd mice (on a C57Bl/6 background; Farber et al., 1994) retinae were studied at P10, P15, P18, P25, P28, P40 and P101 (n = 35). The oldest rd/rd mice at P101 reflects the time where almost all photoreceptors are lost and thus the animals are in late Phase 2 of retinal dystrophy (Marc and Jones, 2003). The control mice (C57Bl/6) were examined at the same ages (n = 35). Animals were maintained on a 12 h light/dark cycle and had access to standard mouse chow and water ad libitum. From each experiment, five independent samples were selected and only the central third of the retinal region was analyzed. The experimental protocols in this study were approved by The University of Melbourne animal ethics committee.

Fixation, Sectioning and Immunocytochemistry

Retinal pieces were fixed in chilled 4% (w/v) paraformaldehyde and 0.01% (w/v) glutaraldehyde in 0.1 M phosphate buffer for 30 min, cryoprotected and processed for
cryosectioning (16 μm thickness). Non-specific binding sites were blocked with 6% (v/v) goat serum, 1% (w/v) bovine serum albumin, 0.5% (v/v) Triton X-100, 0.05% (w/v) thimerosol in phosphate buffered saline (pH 7.4) for 1 h and subsequently washed in phosphate buffered saline.

The primary antibodies were diluted in 3% (v/v) goat serum, 1% (w/v) bovine serum albumin, 0.5% (v/v) Triton X-100, 0.05% (w/v) thimerosol in phosphate buffered saline (pH 7.4) and applied to the retinal sections overnight at 4°C. Primary antibodies used in this study are described in Table 1. The primary antibodies were visualized using secondary antibodies (goat anti-mouse conjugated to Alexa TM 594 and goat anti-rabbit to Alexa TM 488; Invitrogen, Carlsbad, CA), incubated for 4 h. In double labeling experiments, sections were incubated in a mixture of primary antibodies followed by a mixture of secondary antibodies. Secondary antibody specificity was observed as minimal background fluorescence when the primary antibody was omitted or a secondary antibody from a different species was used. Tissues were incubated for 5 min in the nuclear counterstain, 4',6-diamidino-2-phenylindole (DAPI; S33025; Invitrogen, Carlsbad, CA), diluted 1:300 with phosphate buffered saline. The slides were washed in phosphate buffered saline and coverslipped with an anti-fading medium (Citifluor, Alltech, NZ).

**Antibody characterization**
Calbindin: The specificity of the calbindin antibody was found in Western blots of mouse brain lysate where it presented as a single 28 kDa band (manufacturer’s data sheet; Chalazonitis et al., 2008). The labeling patterns observed in this study also matched previous calbindin immunoreactivity observed in the rd/rd retina (Chua et al., 2009).

Glial fibrillary acidic protein (GFAP): The specificity of the GFAP antibody was observed in Western blots of mouse spinal cord lysate (Darman et al., 2004) and rat retina lysate (Chang et al., 2007) where it presents as a 51 kDa band. In this study, labeling patterns for GFAP matched those previously observed in the C57Bl/6 mouse retina (Acosta et al., 2005).

Glutamine synthetase (GS): The specificity of the GS antibody was confirmed with a Western blot using rat cerebellum lysate where it reacted with a 45 kDa band which corresponds to the expected size of the GS protein, amino acids 1-373 (BD Biosciences technical data sheet). GS immunoreactivity also matched previous results in the C57Bl/6 and rd/rd mouse (Haverkamp et al., 2000; Strettoi et al., 2002).

Image Capture

Frozen tissue sections of the control and dystrophic retinae were processed under identical conditions. For each experiment, five independent samples were imaged using Leica SP 2 confocal microscope (Heidelberg, Germany), where a total of eight stacks were captured under the same magnification to ensure unbiased cell coverage. Images
were adjusted for black levels and contrast. In some cases, images were sharpened using the Unsharpen Mask tool of Adobe Photoshop (Version 8.0 for Windows, Adobe Systems, USA) for final image production. No other digital image manipulation was performed unless otherwise stated.

Quantification of Müller Cell Density

Müller cell density was determined by counting GS-labeled Müller cell somata in a defined retinal length (n = 5 at each conditional age group) and expressed as the number of cells per 100 μm retinal length. Cells were only counted from the central region of the retina (total area = 9 mm²). The sample size for each age group and for each mouse type resulted in <5% sampling error with a 95% confidence interval assuming 90% efficiency and a Müller cell density of 144,783 cells/mm² in the mouse retina (Jeon et al., 1998). Similar calculations comparing total rd/rd and control mice populations resulted in a sampling error of <2%. As per previous studies, only GS positive cells that had distinct soma labeling above background levels were selected (Acosta et al., 2007; Sun and Kalloniatis, 2006).

Quantification of absolute Müller Cell area

Absolute Müller cell area - defined as the retinal area occupied by the total Müller cell (i.e. soma and processes), was determined by quantifying the total number of GS labeled pixels per 100 μm retinal length. To confirm the location of the outer plexiform layer, we double labeled tissue with GS and the horizontal cell marker, calbindin (Fig. 1A-C). This
was essential at late stages of degeneration when the outer plexiform layer cannot be easily distinguished from other distal structures. Images containing 256 discrete grey pixel levels (0 - 255) were thresholded (Fig. 1D-E), where immunoreactive areas of pixel levels greater than 128 were assigned as 1 (positive labeling), and immunoreactive areas of pixel values lower than 128 were assigned 0 (negative labeling). Threshold images (either black or white) were inverted, and GS (green) and calbindin (red) immunoreactive pattern were inserted into the respective red-green-blue (rgb) channels (Fig. 1F-H). Areas of colocalized GS and calbindin labeling were yellow (Fig. 1I). For illustrative purposes, red calbindin immunoreactivity and yellow co-localized areas used in the methodology have been replaced with magenta and white respectively in Figure 1. The yellow (white in Fig. 1J) labeling at the outer plexiform layer was selected and combined with the green labeling which represented the entire Müller cells (Fig. 1J). The yellow labeling separated the Müller cell processes into either the outer or inner retina (Fig. 1 K-L).

The absolute Müller cells’ area was determined using the ‘magic wand tool’ in Adobe Photoshop. The ‘magic wand tool’ selects a consistently colored area in an image without having to trace its outline. GS immunoreactivity was selected by clicking the green color. The number of selected pixels, pixel intensity and the total number of pixels in the image was obtained from the ‘histogram palette’ in Adobe Photoshop. For relative Müller cell area, immunoreactive Müller cell pixels were divided by the number of pixels across the total retina area (from outer limiting membrane to inner limiting membrane) and data was normalized using P10 as the age point (n = 3 for each age group; P10, P18, P25 and P101).
Quantification of Müller Cell gliosis

Tissue was double labeled GS and GFAP and the gliosis index was derived by counting the number of colocalized GFAP + GS profiles/total GS profiles per 300 μm retinal length (n = 5 at each conditional age group). To differentiate the distal and proximal retina, the inner nuclear layer and inner plexiform layer were separated into five equal regions. Gliosis index was presented for two reference points - the ‘inner retina’, defined as the distal 20% of the entire inner plexiform layer and the ‘outer retina’ as the distal 20% of the entire inner nuclear layer (Figure 7B). This method was used to avoid GS immunoreactivity from Müller cell soma and glial seal and GFAP immunoreactivity in the proximal inner plexiform layer masking gliosis.

Whole-cell recordings

Electrophysiological measurements were obtained in whole-mount retina using a blind approach to target Müller cells (Borowska et al., 2011). Müller cells were recognized by their characteristic IV relationships, which were dominated by K\(^+\) currents. The retina was continuously bathed with a solution of 110 mM NaCl, 2.5 mM KCl, 1 mM CaCl\(_2\), 1.6 mM MgCl\(_2\), 10 mM dextrose and 22 mM NaHCO\(_3\) at 35 - 37°C bubbled with 95% O\(_2\): 5% CO\(_2\) to produce a pH of 7.4. Patch clamp electrodes contained 115 mM K\(^+\) gluconate, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM EGTA, 10 mM HEPES, 4 mM ATP Mg\(_2\), 0.5 mM GTP Na\(_3\), and 7.75 mM Neurobiotin (Vector Laboratories, Burlingame, CA). The pH was adjusted to 7.4 with KOH. Recordings were made with a Multiclamp 700B amplifier (Molecular Devices Inc, Sunnyvale, CA). Analog signals were filtered at 1 kHz and sampled at 2 kHz with the Digitada 1400 A/D board (Molecular Devices Inc,
Sunnyvale, CA) under control of pClamp10 (Molecular Devices Inc, Sunnyvale, CA).

Data in graphs represent mean ± SEM. After physiological recordings, cells were fixed in 4% (w/v) paraformaldehyde in PBST (150 mM NaCl, 10 mM phosphate, 0.3% (v/v) Triton X-100) with 1% (w/v) streptavidin-Cy3 overnight to visualize Neurobiotin loading by confocal microscopy.

Statistical Analysis

For Müller cell density and gliosis index, data were analyzed by using a two-factor analysis of variance (ANOVA; SPSS for Windows 16.0; SPSS Inc., Chicago, IL) and plotted in SigmaPlot 8.02 (Systat Software Inc, Chicago, IL). Condition group (control and dystrophic) and age (seven ages) were the dependent variables. For whole cell recordings, paired t-tests were used to assess statistical significance.

RESULTS

Müller cell morphology in the rd/rd retina

Figure 2 shows the typical Müller cell morphology of the adult control and rd/rd retina. In control mice, GS immunoreactive Müller cell processes spanned the entire retina with their cell bodies located in the middle of the inner nuclear layer (Fig. 2A; upward arrows). In the rd/rd mouse, Müller cells had a similar distribution, with processes visible in the outer and inner retina (Fig. 2B; upward arrows). Distal Müller cell processes in the adult rd/rd mouse retina still ensheathed the few remaining cells in the outer retina (Fig. 2B; downward arrows). The exact identity of these cells is unknown but are likely to be
remaining cone photoreceptors (Carter-Dawson et al., 1978; Lin et al., 2009), or possibly emigrated inner retinal cells (Jones et al., 2003; Marc et al., 2003).

GS immunoreactive Müller cell processes were divided into distal (Fig. 2C, F; white dotted box) and proximal regions based on calbindin immunoreactivity of the outer plexiform layer. In the control retina, distal Müller cell processes were in close contact with the photoreceptors (Fig. 2C-E). In the rd/rd mouse, thick Müller cell processes were observed in the outer retina (Fig. 2F-H; white dotted box). This is consistent with a fibrotic seal described in retinal degeneration that separates the retina from the underlying retinal pigment epithelia and choroid (Gouras and Tanabe, 2003; Jones et al., 2005; Marc and Jones, 2003; Marc et al., 2003).

We double labeled the control and rd/rd mouse retinae with GS (green) with calbindin (magenta) at various developmental ages to track the evolution of the glial seal (Fig. 3). In control mice, GS was observed in the Müller cells at P10 and progressively increased to appear adult-like by P18 (Fig. 3A-E). In contrast, the outer nuclear layer of the rd/rd mouse gradually thins as a function of development, leaving only the GS immunoreactive processes (Fig. 3F-J). The inner retina of the rd/rd retina remains largely intact and similar to the control retinae.

Müller cell density in the rd/rd retina

We predicted that the glial seal observed in the adult rd/rd retinae could develop via (1) collapsing of existing Müller cell processes or, (2) proliferative gliosis; whereby Müller
cells increase in size and number in response to photoreceptor death. To test these hypotheses, we quantified the density of Müller cell soma and processes in control and rd/rd retinas. The density of Müller cells was determined by the number of GS immunoreactive soma in the control and dystrophic retinae. The mean number of Müller cell bodies across all ages for control retinae was 16 ± 2.0/100μm retina length and 16 ± 2.4/100μm retina length in the rd/rd mouse (n = 5 retina per age group). There was no significant difference with age (ANOVA; p = 0.66), nor mouse type (ANOVA; p = 0.94).

**Absolute Müller cell area in the rd/rd retina**

To assess the possibility of Müller cell hypertrophy, we quantified changes in the total area occupied by Müller cell somata and processes as a function of degeneration. Müller cell area was quantified as the number of GS immunoreactive pixels per 100 μm retina length based on the method described in Figure 1. This value - termed ‘absolute Müller cell area’, gives the absolute area of Müller cells for each animal group irrespective of the total retinal area. Figure 5A shows absolute Müller cell area from P10 - P18 gradually increased in both the control and dystrophic retinae even though the total area of the retina is reduced in the rd/rd retina due to rod degeneration. There was no significant difference between the two mouse strains at these ages (ANOVA; P10: p = 0.31; P15: p = 0.07; P18: p = 0.06). From P25 – P40, absolute Müller cell area was significantly lower in the rd/rd retina than the control retina (ANOVA; p<0.001).
Figure 5B-C shows absolute Müller cell area for the outer retina and inner retina respectively. In the rd/rd mouse, absolute Müller cell area was consistently significantly lower than the control retina at all ages (Fig. 5B; ANOVA; p<0.0001). When only the trend of data was considered, absolute Müller cell area in the rd/rd mouse increased similar to the control from P10 – P18. After P25, absolute Müller cell area of the outer retina of control mice reached a plateau, remaining constant to adulthood. However from P25 - P28 (beginning of cone degeneration phase) in the rd/rd mouse, absolute Müller cell area reduced dramatically (Fig. 5B). This was followed by an increase in absolute Müller cell area in the adult rd/rd animal. When the outer retina was evaluated for the two mouse groups, age has a significant effect on the area devoted to Müller cells (ANOVA; p<0.0001), with absolute area as a function of age being significantly different for the two mouse groups (ANOVA; p<0.0001).

For the inner retina (Fig. 5C), absolute Müller cell area was again lower in the rd/rd mouse compared to the control but was not significantly different as a function of age between the control and rd/rd mice (ANOVA; p = 0.23). The overall trend of the data was similar between the two animal groups with an increase in absolute Müller cell area between P10 – P18 followed by a gradual decrease from P25 to adult. Absolute Müller cell area in the rd/rd inner retina also closely paralleled that of the entire rd/rd retina.

Figure 5D further highlights the differences in absolute Müller cell area for the outer and inner retina with difference plots (control/dystrophic) calculated from Fig. 5B and 5C. For the inner retina, absolute Müller cell area in the control (Fig. 5C, square data points)
was only 10 - 50% greater than the \textit{rd}/\textit{rd} mouse (Fig. 5C, triangle data points) across all ages. In contrast, absolute Müller cell area in the outer retina was \textasciitilde250% higher in the control (Fig. 5B, square data points) compared to the \textit{rd}/\textit{rd} retina (Fig. 5B, triangle data points) during the cone degeneration phase (P25 – P28). Following cone photoreceptor degeneration (from P40 to \textasciitildeP100), the difference ratio for the outer retina is reduced to \textasciitilde100%.

We also estimated relative Müller cell area as a ratio of Müller cell immunoreactive pixels to the total number of pixels in the retina and normalised to P10 for each mouse group. In the control retina, relative Müller cell area increased by approximately 18% from P10 to the adult mouse (P10 = 1.0, P18 = 1.42, P25 = 1.49, Adult (P101) = 1.18). In contrast, there was a 215% increase in relative Müller cell area in the \textit{rd}/\textit{rd} retina (P10 = 1.0, P18 = 2.58, P25 = 2.28, Adult (P101) = 2.15). Since the absolute area of Müller cells in the dystrophic retina decreases with degeneration, the increase in relative Müller cell coverage suggests a disproportionately greater decrease in total retinal area compared to the small change in absolute Müller cell area.

\textit{Müller cell gliosis in the \textit{rd}/\textit{rd} retina}

Gliotic changes were characterized as a function of degeneration using the gliosis marker, GFAP (Fig. 6). In the adult control retina, GFAP expression was only found in the nerve fiber layer (Fig. 6K, L). In the \textit{rd}/\textit{rd} retina, GFAP labeling was also restricted to the nerve fiber layer at P10 but by P28, GFAP labeling progressed distally through the \textit{rd}/\textit{rd} mouse retina (Fig. 6A-F). GFAP labeling then regressed later in the degeneration process (Fig. 6G-J).
We determined the gliosis index - the number of GFAP immunoreactive processes within GS immunoreactive Müller cell profiles of the outer and inner \textit{rd/rd} retina (Fig. 7). To differentiate outer and inner retina, the inner nuclear layer and inner plexiform layer were separated into five equal regions and only the most distal part of each region (the 20\% component) was used (Fig. 7B). This method was used to avoid GS immunoreactivity from Müller cell soma and glial seal and GFAP immunoreactivity in the proximal inner plexiform layer masking gliosis. No gliosis occurred within the outer or inner \textit{rd/rd} retina until P18 (Fig. 7A). Gliosis peaked at P28 and a subsequent reduced in the adult (P101). The change in the gliosis index was significantly different for the outer and inner retina in the \textit{rd/rd} mouse retina (ANOVA; p<0.0001). When the gliosis index was evaluated for the two retinal locations, age had a significant effect (ANOVA; p<0.0001), with gliosis as a function of age being significantly different for the two locations (ANOVA; p<0.0001).

\textit{K$^+$ currents of Müller cells in \textit{rd/rd} retina}

Müller cell electrophysiology was assessed as gliosis is associated with decreased K$^+$ currents in various retinopathies (Hirrlinger et al., 2010; Kuhrt et al., 2008). Whole-cell K$^+$ currents of control Müller cells were compared with \textit{rd/rd} Müller cells at P30 during the cone degeneration phase when GFAP expression was at its highest. Figure 8A illustrates the morphology of a neurobiotin-filled \textit{rd/rd} Müller cell recorded in a whole-mount preparation. Large currents were evoked by brief voltage pulses (-100 to +40, in 20 mV increment) from a holding potential of -80 mV for control and \textit{rd/rd} Müller cells (Fig. 8B). The average resting potential for \textit{rd/rd} Müller cell was -81 ± 1.5 mV (n=10),
similar to that observed in control animals \((-76 \pm 2 \text{ mV}; n=5\)). The amplitude of K\(^+\) currents were also similar in \textit{rd/rd} and control Müller cells. The average slope of the voltage-current relationship was not significantly different (\(p>0.05\)) between \textit{rd/rd} (83 \pm 10 \text{ M\Omega}) and control Müller cells (65\pm13 \text{ M\Omega}; Fig. 8C). Overall, K\(^+\) currents in \textit{rd/rd} Müller cells remain largely unaltered during the cone photoreceptor degeneration.

**DISCUSSION**

Retinitis Pigmentosa results in photoreceptor cell death, functional remodeling of glutamate receptors and the formation of a glial seal at late stages of degeneration (Chua et al., 2009; Jones et al., 2003; Marc et al., 2007; Strettoi and Pignatelli, 2000). Our results, summarized in Figure 9, demonstrate structural remodeling and gliosis of Müller cells from an early stage of retinal degeneration in the \textit{rd/rd} mouse. In particular, Müller cell anatomy in the outer retina undergoes significant changes concurrently with photoreceptor degeneration highlighting the close relationship between glia and neurons in the retina. Understanding these changes is essential for successful therapeutic intervention in inherited retinal dystrophies.

**Anatomical remodelling**

Müller cell density remained constant as a function of age in the \textit{rd/rd} retina and was similar to control animals indicating no hyperplasia during glial remodeling in the \textit{rd/rd} mouse up to late Phase 2 (Marc and Jones, 2003). Absolute Müller cell area in the \textit{rd/rd} retina however was significantly less than control retina at all ages indicating no growth.
of Müller cell processes. This was particularly pronounced in the outer retina of the \textit{rd/rd} mouse during the late rod/early cone degeneration suggesting shortening, thinning or dropout of Müller cell processes during this stage. Strettoi et al. (2002) also noted dendritic pruning of the Müller cells in the \textit{rd/rd} retina (Strettoi et al., 2002).

Alternatively, Müller cell expression of GS may be reduced during this phase. Previous work suggests major Müller cell hypertrophy in the dystrophic retina beyond Phase 2 of retinal degeneration (Marc et al., 2002). We confirm that there is no net increase in the area encompassing Müller cell processes up to P101 in the \textit{rd/rd} mouse. The increase in relative Müller cell coverage is likely the result of a disproportionate decrease Müller cell area compared to an even greater decrease total retina area. It is therefore important to determine absolute area devoted to glial and neuronal space to confirm hypertrophy.

Our data suggests that formation of the glial seal after total photoreceptor loss could be from the structural compression of remnant Müller cell processes in the outer retina.

Absolute Müller cell area increased by 60\% (p<0.03; ANOVA) in the adult \textit{rd/rd} retina suggesting the distal glial seal contains some new Müller cell processes. Even with this increase, absolute Müller cell area was still less overall in the \textit{rd/rd} retina compared to normal. Müller cell processes have been previously been reported in the choroid of an aged ambient-light model of retinal degeneration (Sullivan et al., 2003) as well as outgrowth of Müller cell processes into the subretinal space of human patients and animal models of retinal detachment (Fan et al., 1996; Fisher and Lewis, 2003; Lewis and Fisher, 2000; Lewis et al., 1994; Sethi et al., 2005). Quantification of absolute Müller cell area beyond Phase 2 of retinal degeneration will confirm hypertrophy.
Mechanisms behind the distinct glial remodeling process of the rd/rd retina (i.e. initial reduction of absolute Müller cell area in the outer retina followed by Müller cell growth after total photoreceptor loss; Fig. 9) are unknown. Strettoi and co-workers proposed that neurons in retinal degeneration undergo dendritic pruning and later, sprouting due to loss of their afferent synapses (Strettoi and Pignatelli, 2000; Strettoi et al., 2003; Strettoi et al., 2002). This is supported by Lee et al. (2011) who showed Müller cell processes remodel to match photoreceptor rearrangement in a rat model for Retinitis Pigmentosa (Lee et al., 2011). Altered glutamate signaling during retinal degeneration may also play an indirect role. Down-regulation of GS in other animal models for retinal degeneration is thought to be a glial response to decreased glutamate from fewer photoreceptors (Grosche et al., 1995; Hartig et al., 1995; Lewis et al., 1989). This is reasonable considering the functional role of glutamate recycling held by Müller cells (Pow et al., 1994). Transition of Müller cells to a proliferative state may occur after complete degeneration of the outer retina when the blood-brain barrier is compromised (Bringmann et al., 2000). Indeed, a range of glutamate receptors on Müller cells are activated by blood derived molecules (Newman and Reichenbach, 1996; Puro and Stuenkel, 1995; Schwartz et al., 1993; Wakakura and Yamamoto, 1994).

Müller cell gliosis

Up-regulation of GFAP and other intermediate filament proteins in retinal astrocytes and Müller cells are hallmarks of gliosis (Eisenfeld et al., 1984; Ekström et al., 1988; Eng and Ghirnikar, 1994; Goel and Dhingra, 2012; Grosche et al., 1995; Hartig et al., 1995; Lewis
and Fisher, 2003; Strettoi et al., 2003). In the rd/rd retina, up-regulation of GFAP in the outer retina was seen during cone photoreceptor degeneration (P18 - P30) and corresponded with the reduction of absolute Müller cell area (P25 - P40). GFAP expression later declined in the adult rd/rd retina, analogous to previous studies (Strettoi et al., 2003; Strettoi et al., 2002). Neurochemical remodeling, specifically aberrant expression of functional glutamate receptors by inner retinal neurons has also been reported in the rd/rd retina at these ages (Chua et al., 2009). This overlap suggests interactions between neuron and glia remodeling processes.

Up-regulation of GFAP only during cone photoreceptor degeneration suggests its function is critical during periods of retinal stress. This would explain decreased GFAP in the adult rd/rd mice as most photoreceptors are already lost and remaining retinal degeneration is slow. The exact role of GFAP in gliosis remains unclear but several studies suggest a protective role; forming barriers, increasing rigidity and maintaining structural integrity of compromised tissues (Lewis and Fisher, 2003; Liedtke et al., 1996; Lu et al., 2011). Mice lacking GFAP demonstrate increased susceptibility to prion infection, cerebral ischemia and blood brain barrier defects indicating the neuroprotective role of GFAP (Gomi et al., 2010; Nawashiro et al., 2000; Otani et al., 2006; Pekny et al., 1998). However GFAP is also associated with the disease process including increased neuron dysfunction and scar tissue formation (DiLoreto et al., 1995; Kinouchi et al., 2003; Lewis and Fisher, 2003; Marc et al., 2008). In vivo, reactive Müller cells and astrocytes prevent retinal neural grafts from growing, integrating and regenerating neurites (Kinouchi et al., 2003). Current theories point to beneficial effects of GFAP up-
regulation in the short term but detrimental effects in the long term as it interferes with neuron regeneration (Bringmann and Wiedemann, 2012; Pekny et al., 2007; Vazquez-Chona et al., 2011).

**Müller cell electrophysiology**

Changes in Müller cell electrophysiology, specifically the loss of K⁺ channels is limited to proliferative gliosis, an aggressive form of gliosis characterised by Müller cell hypertrophy and dedifferentiation. Alternatively, conservative reactive gliosis (or atypical gliosis) results in minimal changes in Müller cell K⁺ channels (Bringmann et al., 2000; Iandiev et al., 2006). The rd/rd retina, we saw no significant changes in Müller cell membrane potential or K⁺ conductance compared to normal mice during active photoreceptor degeneration. Other animal models of retinal degeneration including the Royal College of Surgeons (RCS) rat and the rds mouse also maintain normal Müller cell electrophysiology during retinal degeneration (Bolz et al., 2008; Felmy et al., 2001; Iandiev et al., 2006; Kacza et al., 2001). Maintenance of normal electrophysiology and the absence of Müller cell proliferation are highly indicative of conservative reactive gliosis in the rd/rd retina during stages of active photoreceptor degeneration. In the RCS rat however, changes in K⁺ currents occurred in very late stages of degeneration suggesting the conservative gliosis can convert to proliferative gliosis in animal models of Retinitis Pigmentosa (Zhao et al., 2012). Additional cell recordings are needed to determine if electrophysical changes occur in rd/rd Müller cells after complete photoreceptor loss.
CONCLUSIONS

Here, we show glial remodeling in the rd/rd retina changes as a function of degeneration. During early cone degeneration, electrophysiological properties are maintained but GFAP expression is up-regulated and Müller cell processes are reduced in the outer retina. After complete photoreceptor degeneration, Müller cell processes expand into the outer retina and contribute to formation of a glial seal whilst GFAP expression decreased. These glial changes occurred concurrently with previously described neurochemical remodelling in the rd/rd mouse. This suggests both retinal neurons and glial cells need to be considered when identifying suitable intervention periods for therapeutic approaches.

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Conflict of interest statement

All the authors certify that there is no conflict of interest with any regarding the material presented in this manuscript.

Role of authors
All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: J. Chua, E. L. Fletcher, M. Kalloniatis

Acquisition of data: J. Chua, S. Trenholm

Analysis and interpretation of data: J. Chua, L. Nivison-Smith, S. Trenholm, M. Kalloniatis

Drafting of the manuscript: J. Chua, L. Nivison-Smith

Critical revision of the manuscript for important intellectual content: L. Nivison-Smith, E. L. Fletcher, G. Awatramani, M. Kalloniatis

Statistical analysis: J. Chua, S. Trenholm

Obtained funding: E. L. Fletcher, G. Awatramani, M. Kalloniatis

Administrative, technical, and material support: E. L. Fletcher, G. Awatramani

Study supervision: M. Kalloniatis
FIGURE LEGENDS

Figure 1. Method for quantifying absolute Müller cell area using glutamine synthetase immunoreactivity. A-C: Retinal tissue was double-labeled with glutamine synthetase (GS; green) and calbindin (CalB; magenta). The distal area of Müller cells is indicated by the white dotted box. Confocal images were then D-E: threshold and F-G: inverted. GS and CalB images were overlaid to produce H: an rgb format in the green and red channels respectively and I: co-localized areas in white. The white band (horizontal cells processes) was used to define J: entire Müller cells, K: Müller cell distal processes in the outer retina and L: Müller cell proximal processes in the inner retina. Scale bar = 50μm.

Figure 2. Confocal micrographs of Müller cells in the adult control and rd/rd mouse retina. DAPI (magenta) was used to label retinal cell nuclei and glutamine synthetase (GS; green) was used to label Müller cells in A: the control retina, and B: the rd/rd retina. Upward arrows indicate Müller cell bodies located in the middle of the INL and the white dotted boxes indicate distal processes in the outer retina. For the adult rd/rd retina, Müller cell distal processes are localized in the region where the outer retina was situated sometimes ensheathing sparse somata (downward arrows). Müller cells were also assessed with calbindin (magenta) and GS (green) in C-E: the control retina, and F-H: the rd/rd retina. The white dotted boxes indicate distal processes of the Müller cells separated from the remainder of the Müller cell body based on labeling at the OPL from
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**Figure 3.** Confocal micrographs of the A-E: developing control (con) mouse retina and F-J: the rd/rd mouse retina from P10 - P28. Müller cells are labeled with glutamine synthetase (green) and the outer retina delineated by labeling of the OPL with calbindin (magenta) Adult-like GS immunoreactivity is evident from ~P18 in the control retina. GS immunoreactivity was intense in the distal retina of the rd/rd mouse as photoreceptors degenerate and Müller cell processes collapsed in the space previously occupied by the ONL. Abbreviations are as in Fig. 1. The lines on the left of each image delineate the IPL. Scale bar = 25μm.

**Figure 4.** The number of glutamine synthetase positive Müller cell bodies in the developing control and rd/rd retina. Each bar represents the mean + SD of 5 independent retinae. The total number of glutamine synthetase immunoreactive Müller cells counted is shown above each column. The y-axis reflects the density of Müller cells, shown as cell numbers per 100 μm of retinal length.

**Figure 5.** Absolute Müller cell area in A: the entire retina, B: the outer retina (i.e. area occupied by distal Müller cell processes) and C: the inner retina (i.e. area occupied by proximal Müller cell processes) in the developing control and rd/rd retina. For A-C,
data is expressed as number of GS labeled pixels per 100 μm of retinal length in the defined area. **D:** The percentage change in area of Müller cell processes between the control and the rd/rd retinae. The different phases of photoreceptor degeneration in the rd/rd mouse retina are shown in the various shaded boxes: rod cell death phase (dark grey), cone cell death phase (light grey) and final phase were there are few remaining cone photoreceptors (white). Each data point represents the mean ± SD (n = 5 retinae from different animals).

**Figure 6.** Confocal micrographs of GFAP expression in the A-J: developing rd/rd mouse retina and K-L: adult control (con) retina. For each age, data are presented as a pairwise comparison of DAPI (blue)/GFAP (green) and GFAP (green)/GS (magenta).

In the rd/rd retina, GFAP immunoreactivity rapidly spreads from the nerve fiber layer to the outer retina by P28 then reduces in the later stages of degeneration (beyond P40). In the control retina, GFAP immunoreactivity is always restricted within the nerve fiber layer. Abbreviations are as in Fig. 1. The lower two lines on the left of each image delineate the IPL and the upper single line, the OPL. Scale bar = 25μm.

**Figure 7.** A: Gliosis index for Müller cells in the rd/rd retina as a function of development. B: Schematic of the retina indicating the 20% range for both the IPL and INL used to determine the gliosis index. Each bar represents the mean ± SD (n = 5 retinae from different animals). Abbreviations are as in Fig. 1.
Figure 8. Electrophysiological properties of Müller cells in control and rd/rd mice at P30. A: Confocal image stack of a neurobiotin-filled rd/rd Müller cell recorded in a whole-mount preparation. Scale bar is 20 μm. B: Example traces recorded from Müller cells in an rd/rd (left) and control retina (right). Large K⁺ currents were evoked when the membrane is briefly pulsed (-100 to +40, in 20 mV increment) from a holding potential of -80 mV. C. Averaged steady state amplitude of K⁺ currents plotted as a function of voltage in rd/rd (n = 10) and control (n = 5) Müller cells.

Figure 9. Schematic representation of Müller cell changes in the rd/rd mouse retina at different stages of photoreceptor degeneration. In the early stages of rod degeneration, Müller cells are present in the outer and inner retina and there are low levels of GFAP expression. As degeneration progresses towards cone loss, Müller cell processes are reduced, particularly in the outer retina whilst GFAP expression is up-regulated. The main apical and basal processes of Müller cells have been drawn straighter at this stage to represent the increased cell rigidity likely to occur from GFAP expression. Once most of the photoreceptors have been lost, the area covered by Müller cells processes in the outer retina increases and a glial seal is formed. GFAP expression, on the other hand is reduced.
**Table 1**: Description of primary antibodies used for this study.

*Abbreviations – Rb, rabbit; Ms, mouse*

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<th>Antigen</th>
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<td>BD Biosciences Pharmingen; 610517</td>
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Chua et al 2012: Müller cells in the rd/rd mouse retina


Fisher SK, Lewis GP. 2003. Muller cell and neuronal remodeling in retinal detachment and reattachment and their potential consequences for visual recovery: a review and reconsideration of recent data. Vision Research


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190x185mm (300 x 300 DPI)
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151x218mm (300 x 300 DPI)
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154x92mm (300 x 300 DPI)
Figure 5
116x230mm (300 x 300 DPI)
Figure 6
171x160mm (300 x 300 DPI)
Figure 7
180x76mm (300 x 300 DPI)
Figure 8
186x105mm (300 x 300 DPI)
Figure 9
160x141mm (150 x 150 DPI)
In the rd/rd mouse, Muller cell processes are reduced in the outer retina during early degeneration but increase after total photoreceptor loss. Conversely, GFAP expression increases at early stages then decreases at older ages. Muller cell electrophysiology remains normal during early degeneration. Thus, rd/rd gliosis has an initial conservative response followed by active remodeling.
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