Expression of Channelrhodopsin-2 in Rod Bipolar Cells Restores ON and OFF Responses at High Spatial Resolution in Blind Mouse Retina

Miriam Reh, Meng-Jung Lee, and Günther Zeck*

Rod bipolar cells (rBCs) represent a promising target for vision restoration as they relay information to the major image processing pathways in the retina. However, the diversity of the retinal output and the resolution obtained by optogenetic actuation of rBCs is unclear. Herein, the photostimulation of rBCs expressing channelrhodopsin-2 is studied in a transgenic, photoreceptor-degeneration mouse (rd10) while simultaneously recording the retinal output using a high-density microelectrode array. After analyzing several hundred retinal output neurons, both optogenetic ON and OFF type responses are identified at similar ratio at stimulation thresholds well below photodamage intensity. The temporal latency of both response types is in the same range (≈ 50 ms) as reported for healthy mouse retinal ganglion cells. The spatial resolution ranges between 0.1 and 0.2 cycles deg⁻¹, which is close to that found in healthy mice. Photostimulation over an extended area (29 deg) but not localized stimuli induces strong bursting activity, which may originate from large-scale activation of a coupled network of retinal cells. The distinct ON and OFF optogenetically induced activity patterns in retinal ganglion cells and the high temporal and spatial resolution demonstrate that near-physiological vision restoration may be achieved under optimal conditions in late stage retinal degeneration.

1. Introduction

Loss of photoreceptors is one of the main causes for retinal degeneration which results in blindness. While retinal diseases affect approximately 285 million people worldwide,[1] there is currently no effective treatment for retinal diseases such as age-related macular degeneration and retinitis pigmentosa (RP). Promising attempts for partial vision restoration comprise retinal implants,[2] gene therapy,[3] stem cell transplantation,[4] and chemical,[5] and optogenetic stimulation.[6]

Optogenetic therapy turns retinal neurons into replacement sensory cells, where microbial light-gated ion channels, like channelrhodopsin-2 (ChR2), allow for a cellular specific and temporally precise control of the membrane potential of individual cells.[7] Depending on the state of retinal degeneration, different cell types are preferable as cellular targets for optogenetic vision restoration. Previous studies investigated optogenetic transduction in remnant photoreceptor somata,[8] bipolar cells (BCs),[9–14] amacrine cells,[15] or retinal ganglion cells (RGCs).[16–20]

Among all the retinal cell types, bipolar cells appear to be particularly interesting targets for two reasons: First, they represent the cell layer adjacent to the lost photoreceptors in RP, and their synaptic connections to the inner plexiform layer (IPL) stay preserved in the advanced degenerative stage.[21,22] Second, because of their diversity of about 14 cell types,[23] BCs decompose the visual signal into many parallel channels, broadly classified into ON- and OFF- pathways.

Here, we use rod bipolar cells (rBCs) as optogenetic actuators, as they are connected via interneurons to almost all of the other bipolar cell types.[24] The elicitation of optogenetic RGC activity results from the fact that the rod pathway couples to cone pathways via AII amacrine cells.[25] As a consequence, by optogenetically targeting the rBC, we envision to restore the response properties known from physiology, most notably the ON- and OFF- dichotomy expressed in different electrophysiological activities of RGCs. Experiments were performed on the ex vivo mouse retina interfaced to a high-density microelectrode array (MEA) based on complementary metal oxide semiconductor technology (CMOS-
MEA). Spatially patterned photostimuli projected onto the CMOS MEA induced RGC responses with different response polarities in the same retina. Using pharmacological treatments, we were able to classify the optogenetically activated cell types into ON and OFF and to determine their temporal and spatial response properties, which were in the same range as reported for healthy mouse vision.

2. Results

2.1. Characterization of RGC Responses

One of the major goals in vision restoration is to obtain functionally diverse RGC response patterns. In this section, we determine the response polarity of RGCs evoked by activation of optogenetically modified rBCs. The response polarity is obtained after photostimulation with Gaussian white noise (gwn) followed by calculation of the spike-triggered average (STA). [26]

We derived the STA for about 500 RGCs under low-intensity visual stimulation and high-intensity optogenetic photostimulation. To avoid any potential influence of remnant photoreceptors, we applied optogenetic photostimulation in the presence of L-AP4, an agonist of mGluR6 expressed at the dendrites of ON BCs.[27] However, this protocol does not discriminate the visual and optogenetic responses for OFF cells. By adding strychnine (stry), the connection between rBCs to OFF BCs was blocked. Thus, if the OFF RGC response initially recorded under intense photostimulation disappeared in the presence of L-AP4 and stry, we concluded that the OFF RGC response recorded under high-intensity photostimulation originates from the activation of rBCs.

We used this elaborate protocol to ensure our detected photostimulation responses originate from optogenetic stimulation of rBCs. Figure 1A shows a simplified schematic with separated ON- and OFF retinal pathways, sandwiched between light stimulation from photoreceptor side and a CMOS-MEA used for RGC activity recording. An exemplary recording, with cell positions on the CMOS MEA and the electrical footprints of two RGCs, is depicted in Figure 1B. The RGCs positions are color-coded indicating the optogenetic ON or OFF polarity, which was inferred as follows. Stimulation snippets and the firing response of an exemplary ON and OFF cell under different conditions are depicted in Figure 1C.D. By averaging over the spike-triggered stimulus ensemble obtained from gwn stimulation, we obtained the STA for the two exemplary cells (see Experimental Section). Figure 1E shows the STA of an optogenetic ON cell (“opto ON”) during the application of pharmacological blockers. This exemplary ON cell did not show any STA during visual stimulation, while after application of L-AP4 and high intensity optogenetic stimulation a clear STA is detected. After further addition of stry, the cell still shows a clear STA with ON polarity (positive peak before t = 0).

Figure 1F shows an STA with OFF polarity (negative peak before t = 0) after addition of L-AP4. Note that no STA was detected for visual stimulation and for photostimulation after adding strychnine. Based on the OFF-pathway sketched in Figure 1A, we will refer to these responses as “opto OFF.” Responses of these cells arise through optogenetic activation of rBCs only under high-intensity photostimulation, without influence of visual stimulation from remnant photoreceptors. For this pure optogenetic OFF cell response, the STA obtained during application of stry was classified as noise.

Based on the criteria described in Experimental Section, we divided cell responses into responsive during application of L-AP4 and responsive during application of L-AP4 and stry (see Table 1). “Opto ON” cells (n = 143) are characterized by an STA with ON polarity during photostimulation in the presence of L-AP4, as well as in the presence of stry. We also recorded cells (n = 83) showing STAs with ON polarity in the presence of L-AP4 but could not recover an STA in the presence of stry. These cells are not analyzed any further. A part of “opto ON” cells (27%) showed an ON-type STA under low-intensity visual stimulation without any pharmacological blockers. These cells most likely receive input from remnant photoreceptors;[28] their significance will be discussed in Section 3.5.

“Opto OFF” RGC responses (n = 103, 30% of them showed an OFF-type STA for low-intensity visual stimulation) are characterized by an STA with OFF polarity in the presence of L-AP4 but spontaneous activity and no STA in the presence of stry.

Furthermore, we identified RGC responses (n = 189, 23% showed an OFF-type STA for low-intensity visual stimulation), which are characterized by an STA with OFF polarity in the presence of L-AP4 as well as in the presence of stry (“mixed OFF”), and finally, a part of the RGC responses (n = 47, out of 482 opto responsive cells) were characterized by OFF STAs during L-AP4 application, while addition of stry switched their polarity to ON. These RGCs were categorized as “polarity switching OFF.” We hypothesize that these RGCs are driven by ON- and OFF- BCs, where the ON influence is weaker than the OFF influence. The input of ON-BCs becomes visible only after blocking the OFF pathway with stry. The term “polarity switching OFF” should not be misinterpreted; these cells are optogenetic OFF RGC with partial input from ON BCs.

In summary, out of the identified RGC response types, two of them were unequivocally assigned to optogenetic activation of rBCs followed by signal transmission through the classical retinal circuitry, as shown in Figure 1A. The response type “mixed OFF” may arise from the activation of remnant photoreceptors and incomplete block of the glycine receptors (see Figure S1, Supporting Information), given that the STA under stry has a significantly lower amplitude compared to that during L-AP4 condition.

Table 1. Classification of RGC responses based on the STA obtained from spatially homogeneous temporal gwn stimulation. Stimulation was performed under dim light (“visual”), under intense light and the presence of L-AP4 and under intense light and the presence of L-AP4 and stry. For details, see Experimental Section. Herein, for all drug conditions, the cell was active; X means the measured STA is false with respect to the criteria defined in Experimental Section and therefore discarded.

| Cell response classification | L-AP4 | L-AP4 + stry | Detected cells | Visually active before addition of L-AP4 |
|-----------------------------|-------|-------------|----------------|---------------------------------------|
| opto ON                     | ON    | ON          | 143            | 27%                                   |
| opto OFF                    | OFF   | X           | 103            | 30%                                   |
| Mixed OFF                   | OFF   | OFF         | 189            | 23%                                   |
| Polarity switching OFF      | OFF   | ON          | 47             | 0%                                    |

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Temporal resolution is an important factor determining success in vision restoration. In this section, we assess the temporal resolution of RGCs obtained through activation of optogenetically modified rBCs using gwn stimulation.

To determine the temporal resolution of optogenetic stimulation, we fit an exponentially decaying sigmoidal function to the detected STA $k$:

$$k(t) = a \times \sin(\omega \times t + \phi) \times \exp(-c \times t) + b$$

(1)

with initial amplitude $a$, offset $b$, phase offset $\phi$, and damping factor $c$. The STA cycle duration $T$ is given as $T = \frac{2\pi}{\omega}$, and is used as a measure for temporal resolution. The fit result was discarded, if the root mean square (RMS) value was below 0.7. We compared the cycle duration for different cell types during application of L-AP4 and during application of L-AP4 + stry, with the resulting STA cycle durations being summarized in Table 2.

Figure 2A,B shows histograms depicting the distribution of cycle durations for “opto ON” cell responses, “opto OFF” responses, “mixed OFF” responses, and “polarity switching OFF” responses. Panels 2C and D compare the distributions by application of a Kruskal–Wallis test to detect significant changes. The STA cycle duration for “opto OFF” responses is significantly shorter ($p < 0.01$) than that of “mixed OFF” responses. “Polarity switching OFF” cells show a similar cycle duration as “opto ON” cells after addition of stry. Surprisingly, addition of stry increases the
Figure 2. Temporal resolution (STA cycle duration) of four RGC response types upon rBC optogenetic stimulation. A) Histograms of the STA cycle duration for “opto ON” responses during L-AP4 application and during L-AP4 + stry application. B) Histograms of the STA cycle duration for different response types, p-values are derived from Kruskal–Wallis test. The STA cycle duration for “opto OFF” responses is significantly shorter than that of “mixed OFF” responses. “Polarity switching OFF” cells show a similar cycle duration as “opto ON” cells after addition of stry. Error bars denote the standard error of the mean. We applied the Bonferroni correction, meaning the α-values to determine significance were adapted by the number of performed comparisons (k = 8): ***: p-value < 0.001/k, **: p-value < 0.01/k, ns: not significant.

Table 2. Cycle duration T of STAs for different cell response types under L-AP4 and stry blocking condition. Note that not for all detected cells (Table 1), a fit with an RMS >0.7 was achieved.

| Response type                  | Number of cells | Pharmacological blocker | Cycle duration $T \pm T_{err}$ [ms] |
|-------------------------------|-----------------|-------------------------|-------------------------------------|
| opto ON                       | 117             | L-AP4                   | $123 \pm 22$                         |
|                               |                 | L-AP4 + stry            | $158 \pm 54$                         |
| opto OFF                      | 97              | L-AP4                   | $102 \pm 10$                         |
| Mixed OFF                     | 91              | L-AP4                   | $111 \pm 10$                         |
|                               |                 | L-AP4 + stry            | $183 \pm 57$                         |
| Polarity switching OFF        | 16              | L-AP4                   | $97 \pm 12$                          |
|                               |                 | L-AP4 + stry            | $140 \pm 8$                          |

ON cell response latency, with the stry influence on the ON response being much weaker than for the OFF cells, but still non-negligible. This has also been reported in literature, with previous experiment in the cat eye showing that the light responses of all ganglion cells became more sustained upon the application of strychnine. [29,30] Jiang et al. [31] examined the influence of strychnine on the salamander retina and found that using strychnine to block endogenous glycine feedback reduces the amplitudes of light-evoked responses in both ON and OFF BCS.

The average cycle duration under L-AP4 drug condition was $123 \pm 22$ ms and $102 \pm 10$ ms for “opto ON” and “opto OFF” responses, respectively. These values correspond to STA zero crossings of $61 \pm 11$ ms ($51 \pm 5$ ms), being shorter but in the same range of the STA zero crossings previously reported for visual stimulation of healthy ex vivo mouse retina. [32,33]

To summarize, we detected ON and OFF RGC responses driven by optogenetic activation of rBCs with an observed cycle duration derived from the exponentially decaying oscillating STA corresponding to the spontaneous oscillation frequencies (~5 Hz) observed in rd10 mice. [34,35]

To assure that the “opto OFF” response is not influenced by residual photoreceptors, we used a very sensitive criterion (see Experimental Section), which allows us to conclude that the responses of these cells are not influenced by remaining visual stimulation, which will be important for the following analysis on spatial resolution and intensity dependence. Furthermore, we can conclude that all optogenetic light response are driven through rBCs and not RGCs due to the long response latencies observed here. Figure S3, Supporting Information, opposes STAs observed for different cell type stimulations and compares optogenetic rBC-induced response latencies to visual response latencies observed in the same RGC. In the following chapters, we consider cells that are identified as purely optogenetic responsive (“opto ON”, “opto OFF”) for further analysis.

2.3. Spatial Resolution

Spatial resolution, or visual acuity, describes the ability to discriminate fine patterns and to resolve the details in a visual scene. Furthermore, it provides an indication of the distance from which an animal can see objects. Here, the light-sensitive cells are rBCs,
which constitute about 50% of all BCs in mice.\[^{36}\] In the following, we use an alternating grating stimulus, an approach inspired by in vivo experiments, where the optomotor reflex of animals looking at alternating gratings of different spatial frequencies is recorded and analyzed.\[^{37}\] Therefore, we presented grating patterns with opposite polarity (180° phase shift) to ex vivo flat mount retina interfaced to the CMOS-MEA.

Figure 3A shows the exemplary overlay of an electrical footprint of an identified RGC and the alternating grating stimulus, presented to an RGC population including the exemplary cell. The spiking activity of this cell to gratings of different spatial frequency is shown in Figure 3B.

### 2.3.1. Spatial Resolution Determined by Sigmoidal Fitting of the RGC Population

In the following, we compared the RGC population activity in the two phases of the alternating grating stimulus of one given frequency with the aim to identify meaningful parameters which differ between the two phases. The modified bias index (mBI) identifies changes in the RGC firing rate upon phase switches of the grating stimulus, while the reliability index (RI) quantifies how robust an RGC responds to repetitive changes of the grating (see Experimental Section). The spiking response of an exemplary RGC to alternating gratings of three spatial frequencies is shown in Figure 3B. The rBC stimulation evokes strong bursting in the RGC for low spatial frequencies but not for the highest one. The parameters mBI and RI decrease with decreasing width of the grating, indicating that they detect decreasing sensitivity. However, we observed an unexpectedly high value of mBI (0.49) related to sparse spiking at high spatial frequencies.

The estimation of spatial sensitivity could be biased by sparse spiking activity of RGCs, which leads to a high mBI. Conversely, high spontaneous RGC activity may lead to high reliability (RI). To ensure that our result is not affected by these shortcomings, we also calculate the product of the two indices: the weighted mBI (RI×mBI).

In the following, we show how different gratings influence the weighted mBI for pure optogenetic ON and OFF cells (see Figure 4).

Toward a quantitative estimation of the spatial sensitivity, the weighted mBIs (average values for the population of opto ON RGCs and opto OFF RGCs) for each spatial frequency are plotted (Figure 4A) and approximated by a sigmoidal fit:

\[
S(x) = \frac{a}{e^{-(x-\mu)/s}} + b_0 + 1 \tag{2}
\]

with gain factor s, saturation amplitude a, vertical offset b_0, and inflection point \( \mu \) of the sigmoid.

The fit quality is high (RMS > 0.96), justifying the use of this kind of sigmoidal fit. The weighted mBI maximum for optogenetic stimulation increases significantly (Kruskal–Wallis test with p-values < 0.01) in comparison to visual stimulation for opto ON and opto OFF cells (see saturation at 500 \( \mu \)m in Figure 4A). The change from L-AP4 to stry condition is rather insignificant for pure ON cells (see saturation at 500 \( \mu \)m in Figure 4A). For opto OFF cells, the saturation value is significantly reduced after addition of stry, however, not to the very low level observed for visual stimulation (Figure 4B). This could be due to the relatively lower concentration of strychnine (2 \( \mu \)M) we used compare to other studies\[^{11}\] (10 \( \mu \)M).

Figure 4C shows the histograms of the weighted mBIs for a population of 110 opto ON RGCs activated by three exemplary
Figure 4. Sigmoidal increase of the weighted mBI (RI \times mBI) for increasing grating widths for opto ON and opto OFF cells. A,B) RI\times mBI for opto ON (A) and opto OFF (B) cells under visual condition, L-AP4 drug and stry drug condition with overlaid sigmoidal fit (dashed trace). The dependence on the grating width is similar for L-AP4 and additional stry blocker conditions, while for visual stimulation, the saturation maximum of the sigmoid is significantly smaller. Error bars denote the standard error of the mean. C,D) Histograms showing the weighted mBI distribution (mBI \times RI) for grating widths of 20, 100, and 500 \mu m for visual stimulation (optogenetic stimulation under L-AP4 blocker condition) in black (blue) for 110 opto ON (C) and 59 opto OFF (D) cells. The square denotes the mean of the distribution. The x-axis (* label) denotes the number of cells normalized on the maximum of the respective histogram.

gratings. While the weighted mBI is zero for the majority of RGCs for visual stimulation (black histograms), it changes significantly upon photostimulation in the presence of L-AP4 (blue). A qualitatively similar behavior is observed for the RGC population of 59 opto OFF RGCs (see Figure 4D).

To estimate the spatial sensitivity obtained from a population of independent cells, we calculated the inflection point of the sigmoidal approximation to the parameters mBI, RI, and weighted mBI. The resulting fit curves are depicted in Figure S4, Supporting Information, together with a pictorial confrontation of the interpretation of the different parameters. The values range between 85 and 158 \mu m, depending on the selected evaluation parameter.

The spatial sensitivity estimated for opto ON and opto OFF cells is summarized in Table 3. Translation of the spatial frequency to visual acuity in cycles per degree (cpd) is based on findings from Schmucker et al., yielding that 1 deg on the mouse retina equals 35 \mu m.\[18] If the RI parameter is considered, a spa-

| Cell type | Cell nr | Value type | \( \max \pm \max_{\text{err}} \) \([\text{a.u.}] \) | \( \mu \pm \mu_{\text{err}} \) \([\mu m] \) | \( \mu \pm \mu_{\text{err}} \) \([\text{cpd}] \) |
|-----------|---------|------------|-----------------|-----------------|-----------------|
| opto ON   | 110     | mBI \cdot RI | 0.27 \pm 0.07  | 158 \pm 20  | 0.11 \pm 0.01 |
|           |         | RI         | 0.47 \pm 0.08  | 93 \pm 8    | 0.19 \pm 0.02  |
|           |         | mBI        | 0.52 \pm 0.31  | 128 \pm 43  | 0.14 \pm 0.05  |
| opto OFF  | 59      | mBI \cdot RI | 0.12 \pm 0.05  | 123 \pm 23  | 0.14 \pm 0.03  |
|           |         | RI         | 0.35 \pm 0.03  | 85 \pm 3    | 0.20 \pm 0.01  |
|           |         | mBI        | 0.32 \pm 0.28  | 98 \pm 53   | 0.18 \pm 0.1   |
| opto ON/OFF | 169    | mBI \cdot RI | 0.22 \pm 0.07  | 149 \pm 21  | 0.12 \pm 0.02  |
|           |         | RI         | 0.42 \pm 0.06  | 91 \pm 6    | 0.19 \pm 0.01  |
|           |         | mBI        | 0.45 \pm 0.30  | 120 \pm 46  | 0.15 \pm 0.06  |
Figure 5. Logistic regression used to predict the grating phase based on the RGC firing rate. A–I) Examples for the firing response of RGCs for the two phases of the stimulus are presented for grating widths of 30 (A), 70 (D), and 300 μm (G). The illuminated area of the CMOS-MEA is projected onto the y-axes as a black and white grating. Exemplary performances of the logistic regression model accuracy (B,E,H) and the respective weight of the logistic regression coefficient (C,F,I) for ten cross-validated sets of 60% training and 40% test size are shown for the different grating stripe widths. The circled units in (A), (D), and (G) are the most prominent cells for classification of phase 1 (black) and phase 2 (red) of the alternating grating. K) Logistic regression coefficients for different retinas and different grid sizes, where each line represents a different retina. L) The prediction performance for each retina and each grid size is presented by a dot of different color. The average prediction increases to 85% for a grid size of 50 μm and saturates at 100% for grating stripe widths ≥ 100μm.

2.3.2. Spatial Resolution Determined by Logistic Regression (Log Res)

Given that we calculated a parameter for each RGC and each spatial frequency, we asked in the following if a computational model, which selects specific cells, might provide other estimates for the visual acuity. Therefore, we use a logistic regression approach (see Experimental Section) for different grating widths. We trained the model to predict the phase of the alternating grating given the firing responses of the cell population during a given phase. The output of our logistic regression model was compared for five different retinal samples recorded with the CMOS-MEA.

Figure 5A–I shows examples for the firing response of RGCs to the two phases of the stimulus for grating stripe widths of 30, 70, and 300 μm, with the illuminated area of the CMOS-MEA being projected onto the y-axes as a black and white grating, as well as the performances of the logistic regression model accuracy and the respective weight of the logistic regression coefficient. Figure 5K shows the evolution of the logistic regression coefficients for different grating widths, while Figure 5L depicts the Log Res models’ average performance. For a grating stripe width of 10 or 20 μm, the model prediction is about 60%, meaning the two phases are determined almost randomly. The average prediction...
Figure 6. Estimation of intensity response curves during L-AP4 blocking condition. A) Raster plot for ON cell response to increasing intensity. The spike timestamps are overlaid with the blue stimulus trace. B) Same as (A) for an exemplary OFF cell. C) Absolute bias index (BI) for ON cell response to increasing intensity. The blue line shows the outcome of a sigmoidal fit. D) Same as (C) for an exemplary OFF cell. E) Fit values for sigmoidal saturation amplitude. The response of ON cells saturates at higher BIs as for OFF cells. The saturation value of the bias index for optogenetic stimulation is significantly lower for OFF cells than for ON cells (Kruskal–Wallis test p-value < 0.01). Error bars denote the standard deviation. F) Fit values for stimulation threshold (sigmoidal inflection point μ). The threshold for optogenetic stimulation is significantly lower for ON cells than for OFF cells (Kruskal–Wallis test p-value < 0.01). Error bars denote the standard error of the mean.

increases to 85% for a grid size of 50 μm and saturates at 100% for grating stripe widths ≥ 100 μm. For even broader widths (e.g., 300 μm), the grating dimensions can even be seen in the positional firing rate plot (Figure 5G).

The Log Res model saturates later in comparison to Log Res performed on data derived from recordings of optogenetic stimulation of RGCs[33]; also the derived values for the sigmoidal turning point as an estimate for spatial resolution are surprisingly lower than values derived for optogenetic stimulation in RGCs (87.3 ± 9.9 μm or 0.2 ± 0.02 cpd, respectively).[33] We do not expect the expression ratio playing a mayor role, as ChR2 is only expressed in rBCs, which comprise nearly 50% of the BC population.[36] An explanation for decreased resolution might be the blurred RGC response induced by rBC stimulation, as shown in Figure 3, which is less transient than the RGC response induced by direct RGC stimulation, leading to lower response reliabilities and mBIs. Still, the estimate for the spatial resolution with sigmoidal analyses is 0.117 ± 0.017 cycles deg⁻¹ (see Table 3), which is close to visual acuity for rod and cone vision observed in healthy mice (0.22–0.6 cycles deg⁻¹).[39]

2.4. Stimulus Response Relations

The stimulus response relation is an important feature to quantify the intensity needed for successful stimulation and to estimate, if this stimulation threshold lies below the photodamage threshold. Here, we measure the modulated RGC activity for different stimulation intensities and estimated the threshold intensity for robust optogenetic stimulation.

Figure 6A shows a raster plot for an exemplary opto ON cell with overlay of the stimulus. Here, a clear increase of the ON
firing rate with increasing stimulation intensity is observed. Figure 6C shows the absolute bias index for different intensity steps for the ON cell shown in Figure 6A, where the absolute bias index increases because of the increase of ON response. For all detected ON cells, we also derived threshold values by fitting the pure ON response of the cell to the increase in stimulus intensity.

Figure 6B shows the raster plot for an exemplary opto OFF cell and an overlay of the used stimulus. The firing rate in the ON phase of the stimulus was suppressed by an increase in stimulus intensity. The opto OFF cell showed a high maintained activity, a typical feature of OFF-type RGCs in mouse retina. Figure 6D shows the absolute value of the bias index for increasing stimulation intensity for the opto OFF cell shown in Figure 6B. Note that increasing values of the absolute bias index result from suppression of the spontaneous activity for increasing intensities.

To compare how opto ON and opto OFF cells respond to an increase in stimulation intensity of full-field flicker stimulation, we analyzed the absolute bias index, at which the sigmoidal fit saturates and the inflection point μ of the sigmoid. The sigmoidal fit saturates for opto ON cells at significantly higher values of the bias index than for opto OFF cells, meaning the optogenetic response is easier to differentiate from spontaneous activity. Also, the inflection point of the sigmoidal fit lies at lower intensities for ON driven cells than for OFF driven cells, meaning the optogenetic response of ON cells is already prominent at lower stimulation levels than that of OFF cells. This is apparently correlated to the fact that the change in bias index for OFF cells is more strongly driven by a suppression of the ON response than by an increase of the OFF response. We observed that the OFF RGCs are highly spontaneously active in comparison to ON RGCs, as former described in Margolis et al. The sigmoidal fit we used is described by Equation (2).

Table 4 summarizes the intensity response characteristics for optogenetic ON and OFF cells. Opto ON cells show a significantly higher saturation bias index and a significantly lower stimulation threshold than opto OFF cells. We hypothesize that this deviation comes from synaptic coupling differences from AII to ON-BC or OFF-BC.

For stimulation of RGCs expressing ChR2, we derived in a previous study values of μ = 0.21 ± 0.08 m W mm⁻²,[35]

To estimate if these threshold values lie below the maximum permissible retinal peak irradiation MPE, we calculated the equivalent energy E_equi as explained in Experimental Section. As photodamage thresholds are intensity, duration, and frequency dependent (Table 12 in Yan et al.,[41]), our estimates lie below the photodamage threshold for pulsatile stimulation in RBCs with short pulse durations (< 5.6 ms (ON) and < 4.2 ms (OFF) at a frequency of of 6 Hz).

### 2.5. Bursting Activity

We observed the occurrence of RGC bursting during optogenetic stimulation, which seems to limit the spatial resolution (see Section 2.3). Spike bursts also explain the oscillatory behavior of the calculated STAs (see Section 2.2 and exemplary STAs shown in Figure 1). In the following, we investigated potential sources of this bursting activity.

Figure 7A depicts a simplified circuit diagram for rBC optogenetic stimulation triggering oscillations in rd10 retina (adapted from Trenholm et al.[42]), where arising oscillations are resulting from the coupled network of AII amacrine cells and ON cone BCs. Figure 7B shows the RGC responses as a raster plot of ON and OFF driven RGCs. Classification into ON and OFF cells was obtained via the polarity of the detected cells STA. It can be seen that the ON and OFF cells form two clusters: the OFF cells respond strongly during the OFF phase of the stimulus, while showing slight bursts during the ON phase of the stimulus. The ON-driven RGCs spike in a strong bursting pattern during the ON phase, while mainly silent during the OFF phase. When the ON population is active, the OFF population seems to be suppressed and vice versa.

To understand if the stimulation size is affecting the bursting activity, we performed localized stimulation with a pattern of nine spots with the size of 110 μm × 110 μm, separated by 220 μm. An exemplary stimulus pattern and responsive RGCs are depicted in Figure 8A, where the color of the cells denotes the bias index.

Localized stimulation triggers fewer bursts (exemplary data are shown for one retina in Figure 8). We used the burst detection algorithm described in Experimental Section to estimate the number of bursts in a cell response. Histograms in Figure 8B show that full-field stimulation triggers up to two times more bursts than localized stimulation. Raster plots in Figure 8C,D show cell responses to localized and full-stimulation, where the black dot and black line denote the center of the detected burst and its duration. Additionally we performed experiments, where only one half of the retina is stimulated. In this case, the non-stimulated part of the retina also showed bursting activation, though less than the directly activated part, motivating that the bursting is caused by the network activation (see Figure S6, Supporting Information).

Spiking activity of RGCs to optogenetic stimulation of rBCs is characterized by strong bursting. We hypothesize this induced bursting comes from the large scale network activation of amacrine cells. Application of mecamylamine did not considerably influence the bursting activity (data not shown). To improve visual restoration, this unexpected strong RGC bursting activity needs to be reduced, potentially using gap-junction blocker.[43]

### 3. Discussion

In this study we identified the two major retinal response properties—ON and OFF—in late-stage photoreceptor-degenerated mouse retina (rd10) expressing ChR2 in rBCs (see Figure 9). The temporal and spatial resolution obtained from the population activity of opto ON and opto OFF RGCs recorded in ex vivo retina is in the same range as the resolution reported for healthy retina. In the following, we compare our results with previous work and provide an outlook for

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**Table 4: Intensity response relation characteristics: Threshold μ and saturation bias index B_{sat} for opto ON and opto OFF cells.**

| Cell type | μ [m W mm⁻²] | E_{equi} [m W mm⁻²] | Saturation type | Saturation |
|-----------|--------------|----------------------|----------------|-----------|
| opto ON   | 0.082 ± 0.063| 0.65 B_{sat} 60%       |
| opto OFF  | 0.144 ± 0.083| 1.1 B_{sat} 40%       |
Figure 7. Burst activation by optogenetic stimulation of rBCs driven by large scale network activation of amacrine cells. A) Simplified circuit diagram for rBC optogenetic stimulation triggering oscillations in rd10 retina. Anising oscillations are resulting from the coupled network of AII amacrine cells and ON cone bipolar cells. AII amacrine cells inhibit OFF bipolar cells with glycine (Gly). ON and OFF RGCs are activated via glutamate (Glu) release by ON and OFF BCs. Therefore, neighboring ON and OFF RGCs oscillate out of phase with one another. Modified based on an initial drawing by Trenholm et al.[42] B) RGC responses to a full-field 2.5 Hz flicker stimulus. ON and OFF responses were labeled with respect to the polarity of the cell derived STA. The stimulus is projected onto the x-axis, where the black rectangle shows the off-phase, while the white rectangle represents the on-phase of the stimulus.

Figure 8. Bursting activity in RGCs is driven by spatially extended photostimulation but not by local stimuli. A) Active RGCs during spatially extended (≈29 deg) and by localized stimulation. The cell position is represented by colored dots, which correspond to the cell’s bias index. Red color marks opto ON cells; blue color shows opto OFF cells. Localized stimulation spots are denoted as black squares. Example cells shown in (C) and (D) are labeled in black for full-field and dim gray for localized stimulation. B) Number of detected bursts for full-field versus localized stimulation. Error bars denote the standard error of the mean (SEM). C) Exemplary raster plots for cells active during localized stimulation, as labeled in (A). D) Exemplary raster plots for cells active during full-field stimulation, as labeled in (A). Burst time occurrence is detected by black dots, where the length of the black line denotes the duration of the burst.

vision restoration, with Table 5 representing an overview on the literature.

The major advantage of targeting BCs in vision restoration is the potential to rescue the major visual computation in the retina. Previous studies reported the occurrence of ON and OFF RGC responses.[9–12,44] However, in the cited studies, no detailed spike sorting or quantification was performed. Here, we conclusively demonstrate the occurrence of pure opto ON and of pure opto OFF activity in RGCs (see Table 1). However, we also identified additional response types, which are partially explained by the activation of remnant photoreceptors but also indicate non-classical signal processing in photoreceptor-degenerated retinas.
Figure 9. Conditional ChR2 expression in retinal bipolar cells. Fluorescent image of the central area of 30 μm, cross-sectioned A) rd10-Pcp2-ChR2 retina (P5, age: 36 days), B) rd10-Pcp2-ChR2 retina (P4, age: 174 days), and C) Pcp2-tdTomato retina (TR, age: 92 days). Images (A) and (B) overlay ChR2-EYFP expressed rBCs (green) with DAPI (blue) for rd10-Pcp2-ChR2 retina. Image (C) overlays tdTomato expressed rBCs (red) with DAPI (blue) for Pcp2-tdTomato retina. The fluorescent labeling demonstrates homogeneous transduction of tdTomato in rBCs. D) Upper: Table depicting genotypes of the TR mouse line and the P mouse line. Lower: 1% agarose gel electrophoregram of DNA from ChR2(+) (ChRM), ChR2(-) (ChRW), and Pcp2-cre (Pcp2) of exemplary mice labeled P1-P6. In this example, only mice P4 and P5 show the desired expression of ChR2 and Pcp2-cre simultaneously; thus, only these two mice were chosen for the performed experiments. E) Cross-breeding diagram.

Table 5. Summarized results for optogenetic stimulation of BCs: Estimated spatial resolution and temporal resolution for opto ON RGC (ON latency) and OFF RGC responses (OFF latency).

| Animal model | in vivo | Targeted cells | Optogenetic transducer | Method | Spatial resolution [cdp] | Detected cell polarities | ON latency [ms] | OFF latency [ms] | Stimulation intensity [μW mm⁻²] |
|--------------|--------|----------------|------------------------|--------|--------------------------|--------------------------|-----------------|-----------------|---------------------------|
| Batabyal[14] | Mouse (rd10) | √ ON-BCs | vMCO1-mgluR6 | OMR | ≥ 0.1 | | | | < 10 | @continuum |
| Mulliger[13] | Mouse (rd1) | √ ON-BCs | Opn1mw | OMR | 0.28 ± 0.02 | | | | 10⁻¹¹ | @screen |
| van Wyk[10] | Mouse (rd1) | √ ON-BCs | Opto-mGluR6 | OMR, patchclamp | 0.17 ± 0.04 | ON+OFF | 25–50 | 100 | 2 · 10⁻¹³ | @473nm |
| Lu[12] | Mouse (TKO) | X ON-BCs | CoChR | OMR, MEA | ≥ 0.2 | ON+OFF | | | 6 · 10⁻¹³ | @480nm |
| Cehajic[47] | Mouse (rd1) | X ON-BCs | RHO | OMR, MEA | 0.04 | mainly ON | 150–2000 | 2000 | 8 · 10⁻¹³ | @410nm |
| Gaul[44] | Mouse (rd1) | X ON-BCs | RHO | MEA | ON+OFF | 1000–3000 | 1000–3000 | | 8 · 10⁻¹³ | @510nm |
| Lagali[46] | Mouse (rd1) | X ON-BCs | ChR2 | OMR, MEA | 0.26 | On | 110 | | 2 · 10⁻¹³ | @470nm |
| Maceli[11] | Mouse (rd1) | X ON-BCs | ChR2 | MEA | ON+OFF | 20.3 ± 0.6 | 55.2 ± 1.5 | | 0.5 · 10⁻¹³ | @480nm |
| Cronin[9] | Mouse (rd1) | X ON-BCs | CatCh | MEA | ON+OFF | 20.3 ± 0.6 | 55.2 ± 1.5 | | 10⁻¹⁴ | @DLP projector |
| This work | Mouse (rd10) | X rBCs | ChR2 | CMOS-MEA | 0.12 ± 0.02 | ON+OFF | 61 ± 11 | 51 ± 5 | 2 · 10⁻¹⁴ | @460nm |

We also derived the stimulation intensity for optogenetic stimulation in photons (μ) cm⁻²s⁻¹ at the used wavelength. Note that a comparison of threshold stimulation values is difficult, as individual studies used different parameters and different cut-off values to determine this value. OMR, optomotor response; MEA, microelectrode-arrays.

3.1. Temporal Resolution upon Optogenetic Stimulation of rBCs

In optogenetic therapeutic strategies, there is a trade-off between temporal resolution and sensitivity of the optogenetic actuator. In Figure S3, Supporting Information, we show additional STAs observed for different cell types and compare optogenetic rBC induced response latencies to visual response latencies observed in the same RGC. Although the values are in the same range (50–100 ms), visual responses were significantly slower. The STA for visual stimulation and thereby the response latency, however, depends on the contrast of the stimulus.[45]
In the following, we compare the temporal resolution obtained for different optogenetic transduction methods in BCs of degenerated mouse retina for fast ChR2-variants \(^{[9,12,14,46]}\) and slower but more light sensitive opsins. \(^{[10,13,14,44,47]}\)

In an approach related to ours, Macé et al. targeted all ON BCs through an intravitreal injection of AAV-Chr2/H134R in photoreceptor-degenerated rd1 mice and reported ON and OFF RGC activity.

Response latencies in the visual cortex were estimated as 20.3 ± 0.6 ms for ON responses and 55.2 ± 1.5 ms for OFF responses. \(^{[11]}\) The OFF response latency of 55 ms is in the same range as our estimate (see Table 2). The ON response latency reported by Macé et al. is two to three times faster than our estimate (see Table 2), which in turn corresponds to the latency measured in wild-type retina. The fast response of \(\approx 22\) ms may be attributed to the expression of ChR2 in all ON BCs, including ON cone BCs. These cells connect directly to ON RGCs, bypassing the synaptic pathway through AII amacrine cells.

The majority of optogenetic approaches targeting BCs focused on low-sensitive transducers with the caveat of long response latency (several 100 ms) and thus a low temporal resolution below 10 Hz. \(^{[10,13,14,44,47]}\)

### 3.2. Spatial Resolution upon Optogenetic Stimulation of rBCs

The spatial resolution is usually assessed either in vivo via optomotor response (OMR) experiments or by analyzing the RGC response upon projection of alternating grating patterns onto ex vivo retina. The spatial resolution derived from OMR experiments in blind mice with optogenetically reactivated BCs ranges between 0.04 \(^{[47]}\) and 0.28 cpd. \(^{[13]}\) depending on the optogenetic transducer and the light intensity. \(^{[10,12,14,46,47]}\) The estimated spatial resolution in our study shows some variability, depending on the parameter and method used to calculate it. If we assume the spatial resolution to be determined by the population of independent RGCs and consider the inflection point of a sigmoidal approximation, we obtained a resolution of 0.1 cpd or 0.2 cpd for the weighted mBI or the RI parameter, respectively (see Table 3). If we consider the spatial resolution to be determined by few “active” RGCs in a logistic regression approach, even higher acuity may be inferred (see Figure 5). The spatial resolution for healthy mouse retina has been reported in the range between 0.22 cpd under scotopic condition and 0.6 cpd under photopic condition. \(^{[11,39]}\) Thus, targeting BCs appears to resolve spatial resolution to a large degree. In a recent study, the visual acuity upon AAV-mediated expression of ChR2 in ON-BCs was lower as inferred for direct RGC activation at the same photostimulation intensity. \(^{[12]}\) A similar conclusion can be drawn from the present study and recent work, evaluating the spatial resolution in ex vivo photoreceptor-degenerated retina expressing ChR2 in PV-positive RGCs using the same experimental setup and the same set of stimuli and evaluation methods. \(^{[13]}\) One explanation for the higher spatial resolution inferred from RGCs expressing ChR2 as compared to retinas expressing ChR2 in rBCs might be the occurrence of strong bursting following optogenetic stimulation of rBCs. This bursting leads to a decreased mBI parameter, which was used to infer spatial resolution.

### 3.3. Stimulation Threshold for Optogenetic Stimulation of rBCs

The stimulation thresholds reported previously cover several orders of magnitude, with the highest intensities obtained for Chr2 \(^{[9,14,44,46]}\). All stimulation thresholds, including the one determined here (0.082 m W mm\(^{-2}\)) for opto ON cells and 0.144 m W mm\(^{-2}\) for opto OFF cells, see Table 4) are below the safety limits for pulsed stimulation (see Table 12 in Yan et al. \(^{[41]}\)). However, Lu et al. estimated the threshold light intensity to elicit responses from BCs with 1 log unit higher than that of direct RGC stimulation. \(^{[12]}\) A factor influencing this outcome may have been the lower expression of ChR2 in BCs compared to RGCs. We note that time-continuous stimulation used here for the estimation of temporal filters (see Figures 1 and 2) is not relevant from an application perspective, and therefore, the intensity used there is not considered. We refrain from detailed comparison of stimulation threshold intensities, as the individual studies used different parameters and different cut-off values.

### 3.4. BCs versus RGCs as Potential Targets for Vision Restoration

The number of ChR2 expressing cells is about ten times higher for expression in rBCs than in PV-RGCs (13,000 PV-RGCs \(^{[48]}\) compared to 200,000 rBCs \(^{[49]}\)). In the following (see Table 6), we compare the results on vision restoration success of this study, using transgenic mice expressing ChR2 in rBCs to former results obtained using transgenic mice expressing ChR2 in PV-positive RGCs. \(^{[33]}\)

Optogenetic transduction of BCs in photoreceptor-degenerated retinas has been commonly considered advantageous over RGC transduction. \(^{[6,46,50,51]}\) In a recent comparative study, \(^{[12]}\) this hypothesis has been questioned. Performing experiments on AAV-mediated expression on ChR2 in ON-BCs and RGCs, they state, that although stimulation of BCs is able to evoke ON and OFF RGC responses, severe drawbacks are the decreased visual acuity of BC stimulation in comparison to RGC stimulation at the same level of light. The estimated threshold light intensity to elicit responses from BCs was 1 log unit higher than that of direct RGC stimulation. A factor influencing this
outcome may have been the lower expression of ChR2 in BCs compared to RGCs due to the used promoter and restricted efficiency of virus delivery.

In this work, we also observe that visual acuity was higher, with stimulation threshold in a similar range upon transduction of RGCs as compared to rBCs. One drawback of optogenetic BC activation appears to be the strong bursting behavior for stimulation with large stimuli (see Figure 7), which has not been reported so far. Small stimuli, in contrast, do not evoke bursting behavior. Spampinato et al. used AAV-driven CoChR expression in rBCs and stimulated them with a spatially restricted stimulus, which did not trigger any bursting activity. A similar result had been shown for electrical stimulation of late-stage rd10 retina, where stimulation with large electrodes evoked non-localized, oscillatory spike bursting in the range of 5–10 Hz. Oscillatory bursting in RGCs of photoreceptor-degenerated mouse retina is explained by the activation of the coupled network of AII amacrine cells and ON-BCs. Thus, a reduction of the oscillatory bursting behavior either by pharmacological treatment or by avoiding large stimuli seems necessary for vision restoration through optogenetic stimulation of rBCs.

Bursting activity in rd mouse lines has been reported previously. Here, based on pharmacological blocker testing with mecamylamine, a nicotinic antagonist, we hypothesize the bursting activity being mediated through AII-AII and AII-rBCs couplings. Inhibition of bursting activity in the presence of gap junction blockers is difficult to interpret given that there are multiple independent oscillating/bursting networks in rd retinas. Future experiments with focus on gap junction blockers could yield a more in-depth analysis of bursting and oscillating activity under different pharmacological conditions.

3.5. Limitations of the Study and Implications for Future Vision Restoration

Although we were able to identify optogenetically driven ON and OFF responses in many RGCs of photoreceptor degenerated mice, we note several limitations of the study. This study represents a proof-of-principle study using a triple transgenic mouse line. For clinical applications, appropriate AAV vectors are necessary, which transport the optogenetic transducer into the rod bipolar cell. We further note that optogenetic ON or optogenetic OFF RGC activity may not correspond to the original polarity (i.e., visual ON or OFF RGC activity). To evaluate a potential correspondence, younger animals may be investigated. However, our pharmacological protocol used here to identify optogenetic ON and optogenetic OFF RGCs responses (L-AP4 and L-AP4 + strychnine, respectively) relies on the physiologic circuitry in the retina (Figure 1). Thus, even without visual responses, the identification of optogenetic-ON and optogenetic-OFF RGC spiking suggests that the signals originate from physiologic circuitry. Amacrine cells in old rd10 retinas appear to be functional to a certain degree, as demonstrated by the results obtained upon application of the glycinegic blocker strychnine (see Figures 1E and 4B). However, as part of the receptive field surround, they do not prevent the strong bursting detected in RGCs upon stimulation with large stimuli (see Figure 8). A related induction of RGC bursting in one third of RGCs in old rd10 has been reported for artificial electrical stimulation. The missing receptive field center-surround in rd10 retinas and eventually in RP patients may be responsible for poor contrast sensitivity of restored vision. For electrical stimulation of ex vivo blind retina, the reported contrast sensitivity of 12% is well above the value obtained for normal vision. However, the brain may take advantage of the diverse retinal input and learn to see again, as suggested by fMRI studies in late blind patients with retinal prosthesis. Second, the fluorescent images we acquired from the rd10-Pcp2-ChR2 mouse line raise the doubt that the ChR2 might not only be expressed in rBCs but also in some cone BCs (see Experimental Section: Mouse strains and identification of rBCs, Figure 9). The mouse BAC-Pcp2-ires-Cre transgenic line used in this study has been reported to express cre-recombinase specifically in retinal rod BCs in the mouse retina. Despite the agreement that the majority of the Pcp2-protein-expressing BCs were identified as rBCs, there are studies reporting the expression of Pcp2 proteins in other ON-cone BC types with axon terminals stratified mainly in stratum 4 of the IPL. However, it is noteworthy that these studies were done either on a different Pcp2-cre mouse line or on the variant of the Pcp2. Hence, the above-mentioned studies might derive different results and may not be directly applied to the current study. Note that the studies above show that not all the rBCs express Pcp2, leading to the relatively sparse labeling of the EYFP in the stratum 5 of IPL. The expression of EYFP found in stratum 4 of IPL (see Figure 9B) might imply the expression of ChR2 in other cone BC types that was not reported before in this mouse line, though the proportion may be very low among all the BCs that express ChR2. Therefore, we cannot completely exclude the possibility that the ON response recorded in this study could include a certain level of ON responses from some ON-cone BCs. All reported optogenetically induced RGC responses were obtained after blocking mGluR6 receptors, which are expressed on the postsynaptic side of the cone-rBC synapse. Here, we used a complex analysis based on the detected STAs of a respective RGC, as we were not able to block photoreceptor input to OFF cone BCs. Future studies should consider a specific block of the cone OFF bipolar cell synaptich transmission, which may ease the classification of rBC-mediated ON and OFF responses (see Supporting Information for details). The estimation of non-vanishing visually evoked temporal filters (STAs) in 23% of RGCs is unexpected, given the late retinal degeneration stage. However, Barone et al. reported a positive visual behavior for 1-year-old rd10 mice. Residual vision, which may originate from ectopic synapses between cones and rBCs needs to be considered when evaluating artificial vision, since the inner retina relays information between the naturally activated retinal circuitry and prosthetic percepts. Long-range information transmission in the retina, together with non-homogeneous optogenetic transduction achieved in humans, may lead to a complex visual percept.

4. Conclusion

Our study shows that optogenetic activation of rBCs leads to ON and OFF response patterns at similar ratios in photoreceptor-degenerated mouse retina. While the temporal response latency (51–61 ms) is close to that of healthy mice, the spatial resolution (0.1–0.2 cpd) is slightly decreased. Comparing rBC stimulation...
to RGC stimulation shows that although rBC stimulation leads to ON and OFF optogenetic driven responses and similar stimulation intensity thresholds, the spatial resolution is deteriorated, most likely due to the observed bursting activity triggered by the network activation of AII amacrine cells following optogenetic stimulation of rBCs. Future vision restoration strategies based on optogenetic actuation will consider this approach but need to prevent the aberrant bursting.

5. Experimental Section

Mouse Strains and Identification of RBCs: To selectively express ChR2 in rBCs in the blind retina, the Pde6brd10 line ("rd10", JAX 00429, The Jackson Laboratory, Bar Harbor, US) was first crossbred with the transgenic lines A132(RCL-Chr2(H134R)/EYFP) ("Chr2", JAX 024109) and Pcp2-Cre ("Pcp2-Cre", JAX 010536) to create rd10-Chr2 and rd10-Pcp2-Cre lines. The rd10-Chr2 line was then crossbred with a rd10-Pcp2-cre line to create the rd10-Pcp2-Chr2 mouse line. RGC data derived from 11 distinct retinal pieces from seven adult mice of either sex were used. Experiments were performed at a late stage of photoreceptor degeneration[72] (mouse age: 182–215 days, average: 210 days).

Figure 9A, B shows fluorescent images of the central area of 30 μm, cross-sectioned rd10-Pcp2-Chr2 (P) retina of two mice from the same litter. While mouse P5 (Figure 9A) showed clear EYFP expression located in the proximal margin of the IPL, where the typical location of rBCs are, mouse P4 (Figure 9B) showed EYFP expression near stratum 4 of IPL. To verify the reliability of the Pcp2-chr2 mouse line, the same Pcp2-cre mouse line was cross-bred with another cre-reporter line B6.Cg-Tg(ROSA)26Sortm9(CAGtdTomato)Hze/J (from Jackson Laboratory, 007909), which had a much stronger fluorescent signal than EYFP. Both P and TR mouse lines expressed cre-recombinase under the control of the mouse Purkinje cell protein (Pcp2) with loxP-anked STOP cassette. Expression of tdTomato from the central area of 30 μm, cross-sectioned Pcp2-tdTomato (TR) retina (Figure 9C) showed a clear label of rBCs, confirming the low possibility of modification in Pcp2-crc gene. The imaged mice were further assured with genotyping results from Pcp2-cre, ChR2, and tdTomato (Figure 9D). Every mouse in this study was confirmed with the correct genotype before usage. Figure 9E provides a scheme of the cross-breeding for the rd10-Pcp2-Chr2 and Pcp2-tdTomato mouse lines.

Interfacing of Ex Vivo Retina: The experimental procedures for preparation of the ex vivo retina were approved and reported to the local authorities (Regierungspräsidium Tübingen). The preparation of ex vivo retina was performed under dim red light in carboxenated (95% O2, 5% CO2) Ames’ medium (Ames A1420, Sigma Aldrich + NaHCO3) at room temperature. The retina was isolated from the retinal pigment epithelium, the vitreous was removed, and the edges of the isolated retina were trimmed with a scalpel[33]. As the adult, photoreceptor-degenerated retina was relatively fragile, it was drawn into a pipette and released gently into the CMOS-MEA chamber. Using two forceps, the retina was placed ganglion cell side down on the coated (Cell-Tak, Corning) recording electrodes, and the liquid in the CMOS-MEA chamber was removed to enhance adhesion of the retina to the electrodes. The chamber was filled with fresh Ames’ medium immediately after depletion and was perfused at a constant rate of 2–4 mL min−1 and a temperature of 33–35 °C.

Light Stimulation: A patterned light stimulus created by a set of LEDs (CoolLED pE-4000) combined with a digital mirror device (RappOptoelektronik μ-Matrix) allowed to precisely stimulate ChR2-expressing rBCs.

Gaussian White Noise (gwn) Stimulation: gwn stimuli were used with a frequency of 33 Hz presented for a duration of 30 s at n=3 replications with light intensities of 0.435 m W mm−2 at 460 nm for optogenetic stimulation. For visual stimulation, gwn stimuli with a frequency of 33 Hz presented for a duration of 30 s at n=3 repetitions and three different wavelengths (460 nm, 635 nm, 525 nm) with light intensities < 0.003 m W mm−2 were applied.

Alternating Grating Stimulation: To investigate the spatial selectivity of optogenetic stimulation, alternating gratings of dark and blue bars (0.435 m W mm−2 at 460 nm) with spacings between 10 and 1000 μm were projected onto the retina with a stimulus frequency of 2.5 Hz and n = 10 repetitions. A spacing of 1000 μm is equal to full-field illumination of the CMOS-MEA recording site.

Intensity Step Stimulation: A stepwise incremented stimulus with intensities ranging from 0.043 to 0.435 m W mm−2 was presented onto the retina with a frequency of 2.5 Hz at 460 nm for a number of n = 10 repetitions.

Localized versus Full-Field Stimulation: A 2.5 Hz ficker stimulus was presented for 1) a full-field 1 mm−2 (location and 2) a set of 9 separated sub-field square stimuli each 110 μm × 110 μm large and separated by 220 μm from each other to the retina at 460 nm and 0.435 m W mm−2 for a number of n=10 repetitions.

Pharmacology: The elicitation of optogenetic responses is driven by the fact that the rod pathway uses the underlying cone pathway via AII amacrine cell coupling.[23] The ON or OFF response depends on the bipolar cell’s glutamate receptors on the dendrites: metabotropic type 6 (mGluR6) for ON BCs and ionotropic (iGluR) for OFF BCs.[73] To differentiate between remnant visual responses and optogenetic responses, the ON- and OFF-pathways were examined individually (see Figure S1, Supporting Information). To prevent visual ON-responses while stimulating optogenetically, transmission between photoreceptors and ON-BCs was blocked by addition of L-AP4 (20 μM, Tocris Cat. No. 0103), an mGluR6 agonist. Optogenetic OFF-responses were blocked by addition of strychnine (2 μM), a glycine receptor antagonist. Additional experiments with the addition of mecamylamine hydrochloride (10 μM, Sigma M9020) were performed to investigate the bursting of RGCs.

Extracellular Recording Using CMOS-MEAs: Here, CMOS-MEAs[74] (CMOS-MEA 5000, Multi Channel Systems MCS GmbH, 65x65 electrodes on 1 mm2) were used without the top dielectric layer or with hafniumoxide[75] to image retinal activity. In addition, a so-called sensor reset was implemented to prevent electrodes from drifting out of their working range. During reset (frequency: 2–10 Hz), the gate of the sensor transistor was not floating but was connected for a short time (200μs) to a fixed voltage. Recorded data were processed by performing light artifact reduction and filtering as described previously.[35]

Reliability Index of RGC Responses: The reliability index quantifies the reliability of an RGC response for a given number of repetitions.[33] The individual RGC responses from n-1 repetitions (n is the total number of repetitions) were binned into 2-ms-wide bins resulting in histogram H. The same binning was applied to the n-th repetition, leading to histogram h. Repeating this procedure for all possible combinations, the RI index can be derived as follows:

$$RI = \left( \frac{C_{11}}{C_{00}} \right)$$

with C being the absolute value of the off-diagonal element of the symmetric 2x2-covariance matrix of the indexed histograms and angular brackets ⟨⟩ denoting the mean across all possible combinations. A value of 1 indicates identical response patterns across all repetitions, while a value close to 0 indicates no reliability of the RGC responses.

Bias Index: The bias index introduced in Farrow et al.[76] estimates whether the cell responded best to increments or decrements in light intensity. For a symmetric full-field light stimulus consisting of two phases, one with light ON and one with OFF is determined as:

$$BI = \frac{f_{ON} - f_{OFF}}{f_{ON} + f_{OFF}}$$

where f denotes the integrated firing rate in the respective i.
ing phase 2° (c,d). Using the firing rates in the four intervals (a,b,c,d), a modified bias index for each RGC was determined as:

$$mBI = 2 \times \left( \frac{\int_{f_d} f_{d-1} + f_{d-1} f_d}{f_d + f_{d-1}} \right)$$

(5)

where $f_d$ denotes the integrated firing rate in phase $i$. The mBI thus takes values between 0 (no change at the phase transition borders) and 1 (strong change at phase transition borders). The modified Bias Index avoids the misleading underestimation of cell responses (i.e., if an RGC responds at phase transition borders). The modified Bias Index avoids the

values between 0 (no change at the phase transition borders) and 1 (strong change at phase transition borders). The modified Bias Index avoids the misleading underestimation of cell responses (i.e., if an RGC responds at phase transition borders). The modified Bias Index avoids the

mBI calculated. As pupil factor and transmission did not deviate significantly for $\lambda = 460$ nm compared to $\lambda = 505$ nm, the only parameter influenced significantly by the wavelength was the wavelength-dependent correction factor $C_{\text{E}}(\lambda)$:

$$C_{\text{E}}(\lambda) = 10^{-0.02(\lambda-450)}$$

(11)

Therefore, $E_{\text{equi}}$ can be derived as $8 \times E_{460\text{nm}}$.

** Burst Detection:** To determine burst activation, a two-step method burst detection algorithm was relied on. Instead of iterating over multiple channels (electrodes), multiple repetitions of a stimulated RGC were iterated over and the two-step method repeated for twice. A return map was generated for each of the n stimulus repetitions. In step 1, spikes with $\Delta s > \theta_1$ and $\Delta s_{\text{total}} > \theta_1$ were separated for each repetition. In step 2, all the repetitions were merged and spikes with $\Delta s_{\text{max}} > \theta_2$ and $\Delta s_{\text{total}} > \theta_2$ separated. This procedure was repeated twice. Here, the authors chose $\theta_1 = 50$ ms and $\theta_2 = 1.5$ ms and the minimum number of spikes in a final RGC burst to be kept as $0.3 \times n_{\text{total}}$ with $n_{\text{total}}$ being the number of measured stimulus repetitions (for $n_{\text{total}} = 100$, this is at least 33 spikes per burst). The steps of the algorithm are depicted in Figure S5, Supporting Information. The number of bursts per RGC using this definition ranged between 0 and 5 in a time interval of 400 ms.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**

channelrhodopsin-2, complementary metal oxide semiconductor–microelectrode array, mouse retina, optogenetic actuation, retinal bipolar cell, vision restoration
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