Optogenetic therapy: high spatiotemporal resolution and pattern discrimination compatible with vision restoration in non-human primates

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Vision restoration is an ideal medical application for optogenetics, because the eye provides direct optical access to the retina for stimulation. Optogenetic therapy could be used for diseases involving photoreceptor degeneration, such as retinitis pigmentosa or age-related macular degeneration. We describe here the selection, in non-human primates, of a specific optogenetic construct currently tested in a clinical trial. We used the microbial opsin ChrimsonR, and showed that the AAV2.7m8 vector had a higher transfection efficiency than AAV2 in retinal ganglion cells (RGCs) and that ChrimsonR fused to tdTomato (ChR-tdT) was expressed more efficiently than ChrimsonR. Light at 600 nm activated RGCs transfected with AAV2.7m8 ChR-tdT, from an irradiance of $10^{15}$ photons.cm$^{-2}$.s$^{-1}$. Vector doses of $5 \times 10^{10}$ and $5 \times 10^{11}$ vg/eye transfected up to 7000 RGCs/mm$^2$ in the perifovea, with no significant immune reaction. We recorded RGC responses from a stimulus duration of 1 ms upwards. When using the recorded activity to decode stimulus information, we obtained an estimated visual acuity of 20/249, above the level of legal blindness (20/400). These results lay the groundwork for the ongoing clinical trial with the AAV2.7m8 - ChR-tdT vector for vision restoration in patients with retinitis pigmentosa.

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Optogenetics has transformed neurobiology, by enabling scientists to control the activity of excitable cells with light\(^1\). Optogenetic therapy has also raised considerable hopes for new forms of brain–machine interfaces, with cell selectivity and distant optical control. Rebuilding vision through optogenetic approaches is conceptually straightforward, as the aim is to restore light sensitivity in the residual retinal tissue after photoreceptor degeneration, in diseases such as retinal dystrophies\(^2\) and age-related macular degeneration\(^3\). These diseases mostly affect photoreceptors, so the remaining retinal layers, including the retinal ganglion cells (RGCs) can still communicate with the brain via the optic nerve. The feasibility of reactivating these retinal layers has already been demonstrated with retinal prostheses\(^1\)\(^,\)\(^2\) despite their major limitations in terms of surgery, spatial resolution, and cell specificity\(^6\).

The use of optogenetics to restore vision was first proposed by Zhao Pan and his colleagues\(^7,\)\(^8\). They expressed the microbial opsin channelrhodopsin-2 (Chr2) in the RGCs of blind mice\(^7\), and subsequently in the retina of normal marmosets\(^8\). These studies led to a clinical trial using this microbial opsin, which began in February 2016, but for which no results have yet been published\(^9\). Other retinal cells (bipolar cells\(^1\)\(^0\)–\(^12\) and dormant cone photoreceptors\(^1\)\(^3\),\(^1\)\(^4\)) were subsequently targeted to restore vision in blind rodents, postmortem retinal tissue, and non-human primates. Clinically, the choice of cell type targeted depends on the stage of tissue remodeling after photoreceptor degeneration\(^1\)\(^5\)–\(^1\)\(^7\). We performed translational studies targeting retinal ganglion cells (RGCs), the neurons projecting their axons out of the retina because this strategy could potentially work in all patients who have lost their photoreceptors, regardless of disease stage\(^1\)\(^8\).

RGCs in the non-human primate retina can be activated with a more sensitive form of Chr2, “CatCh”\(^1\)\(^9\). We tested this approach with an RGC-specific promoter\(^2\)\(^0\). However, the intensity of blue light required was close to radiation safety limits\(^2\)\(^1\). It was therefore of clinical importance to evaluate other opsins potentially conferring a better balance between light sensitivity and channel kinetics\(^2\)\(^2\).

In this study, we used the optimum AAV capsid with the most red-shifted opsin, operating at a wavelength 45 nm longer than ReaChR\(^2\)\(^3\). We demonstrate here that the high spatiotemporal resolution of this system is suitable for use in vision restoration. A single intravitreal injection, at a dose of 5 \(\times\) \(10^{10}\) or 5 \(\times\) \(10^{11}\) eye photons/cm\(^2\) transfects up to 7000 RGCs/mm\(^2\) in the perifovea. Responses were elicited at a stimulus duration of 1 ms and saturated at a stimulus duration of 30–50 ms. Furthermore, using the responses to moving bars and letters generated on a multielectrode array, we obtained an estimated theoretical visual acuity of 20/249, which is above the threshold for legal blindness. These characterizations of the visual response in the non-human primate retina paved the way for the ongoing clinical trial with the AAV2.7m8-ChrimsonR-tdT vector for vision restoration in patients with retinitis pigmentosa.

**Results**

**AAV2.7m8-Chr-tdT provides the highest transduction efficiency.** Our primary objective was to determine the best genetic construct for expressing ChrimsonR in primate RGCs. The intravitreal delivery of AAV vector in non-human primates (NHPs) has been shown to lead to transduction of the ganglion cell layer in the perifoveal ring\(^2\)\(^0\),\(^2\)\(^4\). The mutated capsid AAV2.7m8 has been demonstrated to yield stronger transduction of the perifovea\(^2\)\(^5\). We, therefore, decided to compare the efficiency of ChrimsonR (Chr) expression from the AAV2.7m8 vector with that of the wild-type AAV2. Chr is often fused to the fluorescent protein tdTomato for visualization of its expression within cells. We therefore also investigated whether the native Chr protein and the ChrimsonR-tdTomato (Chr-tdT) fusion protein were produced in similar amounts in primate RGCs. The four selected constructs (AAV2 and AAV2.7m8 vectors encoding either Chr or Chr-tdT) were each injected into four eyes, at the same concentration (5 \(\times\) \(10^{11}\) eye photons); eight animals in total were used for this experiment (Supplementary Table S1). While no in-depth behavior analysis was performed, none of the treated animals displayed signs of photophobia or vision-related changes in behavior under normal lighting in the animal house. The level of microbial opsin expression was assessed in functional analyses two months after the intravitreal injection in vivo. The transduced retinas were isolated ex vivo and divided into hemifovea for extracellular large-scale 256-multielectrode array (256-MEA) recordings for one hemifovea and two-photon targeted patch-clamp recordings for the other hemifovea (Fig. 1). No natural light responses were recorded in our experimental conditions, but we nevertheless added synaptic blockers to the bath to suppress any residual natural light responses (see Supplementary Materials and Methods). For the quantitative measurement of functional efficacy, the results shown are the multiunit activity on all electrodes following full-field stimuli; the use of this approach may have amplified the differences between results (see below). 256-MEA recordings revealed large differences in the ability to generate functional ChrR expression between vectors (Fig. 1a–d). Recording quality was defined as the number of electrodes for which spontaneous spiking activity could be measured (active electrodes: 152 ± 46 electrodes per retina explant, on average), whereas ChrR efficacy was defined as the number of electrodes displaying an increase in activity during the presentation of light flashes (responsive electrodes, SN ratio >4). This quantification revealed the existence of a significant difference between the constructs, with the highest efficacy for AAV2.7m8–Chr-tdT (Fig. 1c, 64.4% of active sites responsive vs. 13.4%, 10.6 and 0% for AAV2.7m8–Chr-tdT, AAV2.7m8–Chr, AAV2–Chr-tdT, and AAV2–Chr, respectively, \(P < 0.001\)). For all constructs, the foveal area was identified and selected for recording. The corresponding retinal explant was positioned on the MEA before confirmation of the eventual presence of fluorescence. If no light response was measured, we repositioned the tissue on the array to increase the sampling area. Light sensitivity was measured with a range of light intensities, from 1.37 \(\times\) \(10^{14}\) to 6.78 \(\times\) \(10^{16}\) photons cm\(^{-2}\) s\(^{-1}\) on all responsive retinas (Fig. 1b, d). Responses were obtained with AAV2.7m8–Chr-tdT, in all four retinas tested with this construct (verses 2, 1, and 0 for AAV2.7m8–Chr-tdT, AAV2.7m8–Chr, and AAV2–Chr, respectively). This vector also yielded the highest light sensitivity, with responses recorded for 2.34 \(\times\) \(10^{15}\) photons cm\(^{-2}\) s\(^{-1}\), at a frequency higher than for the other constructs.

Consistent with its optogenetic origin, the spiking activity had a short latency, was activated for the whole duration of stimulation and its frequency was modulated by light irradiance. Furthermore, an increase in the number of responsive electrodes with increasing irradiance was clearly observed (Fig. 1b). We recorded the action spectrum of the responses (Fig. 1e), and the measured peak was consistent with the known spectral sensitivity of ChrR, at about 575 nm\(^2\)\(^3\).

The results of 256-MEA experiments were confirmed in two-photon targeted patch-clamp recordings (Fig. 1f–h) on the other hemifovea from the same eye. At the highest irradiance, AAV2.7m8–Chr-tdT elicited robust responses, with a typical photocurrent shape, consisting of a fast transient followed by a steady-state current (Fig. 1g, 12 to 375 pA, mean: 88.7 ± 25.5 pA, \(n = 1\) ). These currents increased steadily with increasing light intensity, from 5.8 \(\times\) \(10^{14}\) to 3.15 \(\times\) \(10^{17}\) photons cm\(^{-2}\) s\(^{-1}\) (Fig. 1h).
Fig. 1 Higher transfection efficiency with AAV2.7m8-ChR-tdT in NHP retinas. a Images of a primate retina expressing AAV2.7m8-ChR-tdT as observed during MEA recordings. Top: Infrared image, electrodes can be seen as black dots; the retina explant in gray, and its limit is shown as a dashed line. An asterisk indicates the center of the fovea, the circle indicates the example electrode in b. Bottom: Epifluorescence image of the same piece of the retina. The strong perifoveal expression can be observed in the mounted hemifovea thanks to tdT fluorescence. b, top: Raw signal recorded from one sample electrode (circled electrode in a) in response to stimuli of increasing intensities (7 × 10^14, 2 × 10^15, 9 × 10^14, and 7 × 10^16 photons cm^-2 s^-1). Light to dark orange rectangles indicate the temporal duration (2 s) of the different intensities of light stimulation delimited by dashed vertical lines. Voltage and temporal scale on the left (bottom) Spike density function for all the active electrodes of the hemifovea shown in a (gray lines, n = 197 lines) as a function of time, before, during, and after a two-second stimulus. Firing rates were averaged over ten repetitions. Black lines show the mean firing rate for the electrode displayed in the upper panels and circled in a. The numbers at the top indicate the number of responsive electrodes per intensity compared to total active electrodes (i.e., electrodes where spikes are recorded). c Total of active electrodes recorded for the four different constructs (four experiments per construct, theoretical maximum: 1024 electrodes per construct). Data are then split for each construct between active and responsive electrodes (white) and active but unresponsive electrodes (black). The proportion of active and responsive electrodes is maximal for AAV2.7m8-ChR-tdT (n = 4, Fisher contingency test, P < 0.0001). d Mean additional firing rate per responsive retina for the four constructs ± SEM (four responsive retinas for AAV2.7m8-ChR-tdT, two for AAV2-ChR-tdT, one for AAV2.7m8-ChR, zero for AAV2-ChR). Stimulation at 590 nm ±15 nm. The inset shows a zoomed image around the first responsive intensity: 2.34 × 10^15 photons cm^-2 s^-1. e Mean normalized action spectrum for three retinas expressing AAV2.7m8-ChR-tdT ± SEM. f Infrared image of the perifoveal region from a retina treated with AAV2.7m8-ChR-tdT and recorded by two-photon targeted patch clamp. The patch-clamp electrode is indicated with a white asterisk, the clivus oculus is indicated, separating the fovea from the parafovea. g, h Whole-cell patch-clamp recordings of ChR-tdT-expressing macaque perifovea neurons. g Photocurrent traces from one recorded cell at different light intensities. h Mean normalized photocurrents peaks are represented as a function of light intensity for each individual recorded cell (dashed lines, n = 17), the solid line represents the population-averaged photocurrent after normalization to maximal peak value +/− SEM. Light stimulation intensity ranged from 5.8 × 10^14 to 3.2 × 10^17 photons cm^-2 s^-1.

With the AAV2.7m8–ChR-tdT combination, we recorded 18 responsive cells (5, 0, 7, 6 cells/retina), whereas only four responsive cells (0, 3, 1, 0 cells/retina) were obtained with the AAV2–ChR-tdT construct. In the absence of tdT fluorescence, for AAV2.7m8–ChR and AAV2–ChR, extracellular recordings were performed on random healthy RGCs in the perifoveal area (>40 cells per condition). In these conditions, none of the RGCs for which recordings were made displayed light-evoked responses, even under conditions known to activate ChR. We cannot exclude a potential bias in favor of the construct including tdTomato, particularly in
the patch-clamp experiments, but the positioning of the MEA based on foveal identification probably rule out such a bias in MEA recordings. These MEA recordings were consistent with greater efficacy of the AAV2.7m8–ChR-tdT constructs; this construct was therefore used in all subsequent experiments.

**AAV2.7m8–ChR-tdT provides greater light sensitivity at a dose of 5 × 10^{11} vg/eye.** Once the capsid and genetic payload had been selected, we assessed transgene stability over time (6 months). In the same set of experiments, we optimized virus load, using three different amounts of vector for intravitreal delivery: 5 × 10^9, 5 × 10^{10}, and 5 × 10^{11} vector genomes per eye (vg/eye), for a total of six animals (four eyes per dose). Additional results were obtained with four more retinas treated with the high dose. After the injections, we examined the eyes of the animals monthly for posterior uveitis and vitreal haze. Clinical evaluation showed no significant immune response following ChR-tdT expression (Supplementary Fig. S1). The success of our vision restoration strategy depends on: (1) a large, dense area of transfected cells and 2) high light sensitivity per cell. For correct estimation of the number of cells transfected and of the retinal coverage of expression, we performed manual cell counts on RGC layers in the confocal stack of images for hemifoveas. We used these counts to establish density maps (Fig. 2a) and density profiles (Fig. 2b). The number of ChR-tdT-expressing cells increased with increasing vector dose (mean total number of transfected cells: 491 ± 64, 4395 ± 631, and 5935 ± 715, for ChR-tdT at 5 × 10^9, 5 × 10^{10}, and 5 × 10^{11} vg/eye, respectively, see “Methods”). The local densities achieved with the two highest concentrations were not significantly different (Fig. 2a), but eyes receiving 5 × 10^{11} vg expressed ChR-tdT with a moderately higher eccentricity (Fig. 2b), resulting in expression over a potentially larger area for this dose. Based on the automatic counting of DAPI-stained nuclei in the same samples, we estimated the peak density at ~40,000 RGCs/mm², with an eccentricity of 0.4 mm (Supplementary Fig. S2), consistent with previous RGC density maps. Based on this number, we estimated that ~20% of RGCs expressed ChR-tdT. Before fixation these hemifoveas were used for MEA recordings, to assess light sensitivity following long-term expression (Fig. 2c). In terms of the fraction of responsive electrodes, there was no clear difference between 5 × 10^{10} and 5 × 10^{11} vg/eye, but the number of retinas with responsive electrodes was smaller for the lower dose (only one of four retinas with responsive electrodes, Fig. 2d). More importantly, the different viral doses led to different levels of light sensitivity, with 5 × 10^{11} vg/eye yielding the strongest overall responses and the lowest response threshold (Fig. 2e, see Supplementary Table S2 for Tukey’s multiple-comparison test). We cannot exclude the possibility of a decrease in the number of ChRtdT-expressing cells between 2 and 6 months, as we were unable to obtain cell counts for both time points. However, we observed no major differences in the expression profile on the fovea and no changes in the subcellular pattern of expression (Supplementary Fig. S3). Furthermore, we observed no difference in the fraction of responsive electrodes (Fig. 2d, 102 ± 58 vs. 73 ± 65 for 2 months and 6 months, respectively, for 5 × 10^{11} vg/eye), or light sensitivity (Figs. 1d and 2e). Based on these findings and the absence of a significant immune response to viral load or ectopic gene expression (Supplementary Fig. S1), we selected 5 × 10^{11} vg/eye as the most appropriate dose for our therapy. Thus, all the data presented hereafter are for a dose of 5 × 10^{11} vg/eye after 6 months of expression.

**Activity modulation at the millisecond scale.** Natural vision is dependent on a highly dynamic temporal range of information for the perception of moving objects. In virtual reality goggles, the minimum information transfer mode seems to be dependent on the video rate (30 Hz). Vision restoration for locomotion or for the perception of dynamic scenes should therefore restore light sensitivity to at least this temporal scale. We, therefore, measured the temporal dynamics of our optogenetic responses with full-field monochromatic stimuli (2 × 10^{17} photons cm^{-2} s^{-1} at 600 nm ± 10 nm) of increasing duration (1–2000 ms). This light intensity was selected because it generated the highest firing rates while remaining below radiation safety limits for continuous eye exposure (~6 × 10^{17} photons cm^{-2} s^{-1}) (28,29). Significant light responses were detected for stimulus durations as short as a few milliseconds (Fig. 3a). Interestingly the firing rate of RGCs reached a plateau for durations of 30–100 ms, depending on the retina tested (Fig. 3b). We defined the minimal stimulus duration generating a reliable response, by calculating the time to the first spike after the onset of stimulation, for all responsive electrodes (Fig. 3c). For stimuli lasting 5 ms or more, we observed a median time to a first spike of about 9 ms. Stimulation for 5 ms is, therefore, sufficient for the reliable activation of RGCs reliably, and the intracellular integration of the ChR-tdT photocurrent initiated spiking in less than 10 ms for most of the responsive electrodes. Furthermore, for a stimulation duration of 20 ms, the time to first spike was between 3 and 11 ms for 50% of the responsive electrodes. We then looked at the distribution of firing rates following stimulations of increasing duration (Fig. 3d). Even for 1 ms stimuli (Fig. 3d, c, dark-blue curves), 12% of electrodes measured a peak firing rate exceeding 100 Hz. We considered multiunit recording, but this observation indicates that, for some RGCs, a 1 ms stimulus was sufficient to elicit a strong response, as clearly seen in Fig. 3a. For stimuli lasting 5 and 20 ms, 48% and 69%, respectively, of the responsive electrodes had firing rates above 100 Hz. Finally, for the longest stimulus duration tested (2 s), peak responses and the sustained firing rate decreased during consecutive stimulations (Fig. 3a, b, d), but both these parameters subsequently recovered. We investigated this effect further for long stimulation durations, by calculating the Fano factor, a measurement of the variability of spike number relative to the mean number of spikes, for all electrodes, as a function of stimulation duration. The Fano factor was below 1 for the short duration (1–200 ms), indicating a lower variability than for the Poisson distribution, but we observed a large increase in spike train variability for stimulations lasting 2 s (Fig. 3e). Most of this effect can be attributed to stimulus hysteresis, as retinal sensitivity subsequently recovered. Consistent with this observation, recordings of activity in response to achromatic binary white noise with a 50% pseudorandom selection rate revealed a gradual decline of evoked activity. The underlying mechanism of this modulation may involve an inactivated state of the microbial opsin (30) or the inactivation of the voltage-gated channels in the ganglion cells. A simple monochrome transformation of natural images would result in a large number of pixels with high values (i.e., light gray) potentially leading to rapid deactivation of retinal ganglion cells. The goggles used for visual stimulation include an event-based asynchronous camera outlining object contours (31). It should therefore be possible to overcome the problem of retinal ganglion cell deactivation by reducing the number of active pixels in a projected frame through the limitation of active pixels to object contours. Flickering stimuli should be used, to reduce the total amount of light and the risk of an increase in spike train variability. For a light pulse width between 5 and 20 ms, stimulation frequencies between 100 and 25 Hz could be used.

**ChR-tdT can produce a high temporal photocurrent and spiking modulations.** In parallel with our population study on MEA, we investigated temporal dynamics, at the single-cell level, by recording photocurrent modulation in single cells. In all recorded hemifovea, fluorescent transfected cells could be seen in the characteristic half-torus shape (Fig. 4a, b). Using...
two-photon guided patch-clamp techniques, we obtained recordings for ChR-tdT-expressing RGCs in the perifoveal area with a cell-attached or voltage-clamp intracellular configuration (Fig. 4c). We first replicated the analysis of photocurrent modulation by light intensity, comparing responses at 6 months (Fig. 4c–e) and 2 months of expression (see Fig. 1f–h). The mean normalized photocurrent followed a similar photosensitivity curve at two and six months (Fig. 4d), with an activation threshold in the $10^{15}$ photons cm$^{-2}$ s$^{-1}$ intensity range, and robust responses to light stimuli at a wavelength of 600 nm (±10 nm) well below the illumination radiation safety limits for the human eye ($\sim 6 \times 10^{17}$ photons cm$^{-2}$ s$^{-1}$)\cite{21,29,32}. 

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In comparisons of peak photocurrent or peak firing rate at maximal light intensity \((3.15 \times 10^{17} \text{ photons cm}^{-2} \text{s}^{-1})\), we found no significant difference between the two durations of expression (Fig. 4c, 2 and 6 months), suggesting that ChR-tdT expression remained stable for as long as 6 months.

We then investigated response kinetics, by recording responses to light stimuli of increasing durations (Fig. 4f) in the cell-attached mode (spikes) and in the whole-cell configuration (photocurrent). The photocurrent and the spike rate both precisely followed stimulus components, such duration (Fig. 4f) and frequency (Fig. 4g, h) precisely. Interestingly, the decrease in photocurrent amplitudes, from the initial peak to the lower sustained amplitude, was paralleled by a similar decrease in firing rates. We further investigated the effect of flicker stimuli in a 50% duty cycle (half the stimulus period with the light ON, at 2–28 Hz) or at a specific stimulus duration (5 ms, from 10 to 100 Hz) (Fig. 4g–i). For full duty-cycle stimulation (Fig. 4g), the photocurrent closely followed the stimulus frequency, for flicker stimulations of up to 30 Hz. These results are consistent with the rapid opening and closing kinetics of the ChR channel in RGCs (10 to 90% rise time, 5.2 ± 1.7 ms; decay time, 27 ± 2.9 ms for a stimulus duration of 250 ms at 3.15 × 10^{17} \text{ photons cm}^{-2} \text{s}^{-1}, n = 5, Fig. 4c). The fast photocurrents allow neurons to translate each light pulse robustly into a burst of spikes, but the decay time of the photocurrent does not allow a complete return to the resting level during trains of the stimulus (e.g., 30 Hz flicker, Fig. 4g). We then used a lower duty cycle, consisting of 5 ms stimuli (20 pulses at frequencies between 10 and 100 Hz), which has been shown to activate ChR-tdT-expressing RGCs in MEA experiments (Fig. 3). With such short stimuli, our recordings showed that photocurrent could be modulated at high frequencies, with large amplitudes (50–100 pA), generating periodic spiking activities. The cells for which recordings were obtained followed the stimulus train precisely, even at 100 Hz, but current deactivation was incomplete between light pulses (Fig. 4h, i). Recordings in cell-attachment mode confirmed the ability of neurons to follow stimulus frequencies of up to 66 Hz, despite incomplete current deactivation (Fig. 4i). This 60 Hz range is compatible with the flicker perception limits observed for natural vision in human subjects\(^3\)\(^3\)\(^4\) and could potentially be used for fast video rate stimulation in human patients. Finally, we activated cells with a stimulus simulating natural properties: a one-dimensional random walk, and consisting of a rapidly changing contrast stimulus (full-field stimulus with intensities ranging from \(3 \times 10^{14}\) to \(3 \times 10^{17} \text{ photons cm}^{-2} \text{s}^{-1}\)). Response reliability was strikingly high across trials \((n = 4)\) for both current and firing rate activities (Fig. 4j). Together, these results demonstrate that RGCs expressing ChR-tdT can follow a high dynamic range of light stimulation compatible with human perception.

ChR-tdT can generate a high spatial precision for visual perception. Having shown that the RGC responses precisely follow the temporal resolution of optogenetic stimuli we then tested the spatial sensitivity of optogenetic responses, by stimulating the retina on the MEA using circular spots of various sizes (25 µm, 50 µm, and 100 µm) centered on the MEA electrodes (100-µm electrode pitch, 10 µm diameter) at a light intensity of 2.10 \(\times 10^{17}\) photons cm\(^{-2}\) s\(^{-1}\) (600 nm ± 10 nm) (Fig. 5). The multiunit electrode-based analysis showed that even the electrodes far away (up to 1 mm) from the stimulated spot elicited an increase in spiking frequency (Fig. 5a). For identification of the electrode closest to the recorded cell, we performed spike sorting on the electrode signals, to obtain single-cell activity with an unsupervised sorting algorithm (Supplementary Fig. S4). This spike sorting indicated that individual spikes were recorded on several electrodes, as a consequence of spike propagation in the RGC axons running along the surface of the retina toward the optic disk (Fig. 5a and Supplementary Fig. S5). The increase in latency with distance to the stimulated area was consistent with an anterograde propagation of spikes along axons. We made use of this spike propagation to measure the spike velocity in ChR-tdT-expressing cells (Supplementary Fig. S5). The unimodal distribution peaking at 0.5 ms/s (Supplementary Fig. S5H) suggests that the ChR-tdT-expressing population of RGCs contains a majority of midget RGCs\(^3\)\(^5\). This conclusion concerning cell identity is consistent with the midget cell morphology of tdTomato-expressing cells observed on two-photon microscopy (Supplementary Fig. S5A–E). However, a very small number of cells \((n = 9)\) had faster velocities of axonal spike propagation (>1 m/s), indicating the possible presence of parasol RGCs among the ChR-tdT-expressing RGCs.

As tdTomato fluorescence was detected in RGC axons, we investigated whether light stimulation could elicit spikes directly in ChR-tdT-expressing axons, with anterograde and/or retrograde propagation. When a spot of light was centered on an electrode in contact with ChR-tdT-positive axons but not ChR-tdT-expressing soma, we observed no increase in spike activity in any neighboring or distant electrodes (Fig. 5b). Thus, the optical stimulation of ChR-tdT expressed in axons was not sufficient to trigger spikes. Indeed, a high degree of correspondence was found between the area containing cell bodies expressing TdTomato and the location of electrodes with optogenetic responsive cells (Fig. 5c). When spot size and presentation duration were varied, we observed single-cell activation for spots as small as 50 µm (Fig. 5d–f and Supplementary Movie S1). The number of responsive cells and their spiking frequencies depended on spot size and stimulus duration (Fig. 5g, h and Supplementary Fig. S6). It should be noted that our stimulations were centered on the opaque MEA electrodes (10 µm diameter), potentially greatly decreasing light intensity for the smallest spot (25 µm in diameter). Nevertheless, these observations are consistent with the notion that optogenetic stimulation can provide a high spatial resolution in RGC activation.
**Fig. 3 Millisecond activation of ChR-tdT-expressing primate RGCs.** 

**a** Spike density function for all responsive electrodes (n = 66) of one retina treated with for 5 × 10^{11} vg in response to stimuli of increasing duration (1–5–20–200 ms, and 2 s, left to right with different colors) and constant light intensity (2 × 10^{17} photons cm^{-2} s^{-1}, 600 nm ± 10 nm). **b** Mean maximal firing rate ± SEM measured for retinas treated with 5 × 10^{11} vg/eye for all tested stimuli duration and constant light intensity (n = 6). **c** Horizontal box plot displaying time from the onset of stimulation to the first spike, as a function of stimulus duration. Recordings from the different retinas are pooled, such that each electrode has the same weighting. Medians are displayed as an open circle, box edges indicate the 25th and 75th percentiles, whiskers extend to the maximum and minimum, and outliers are plotted individually. **d** Cumulative plot of maximal firing rate per electrode versus stimulus duration, with duration color-coded as in c. **e** Distribution of Fano factor as a function of stimulation duration, for all responsive electrodes. A value of 1 corresponds to the Poisson distribution, and values below 1 indicate an increase in information content (c, d, e: n = 488 electrodes from six retinas expressing ChR-tdT).
The optogenetic stimulation of ChR-tdT-expressing RGCs can support pattern discrimination. We assessed the functional impact of our visual restoration strategy by evaluating the ability of treated retinas to encode information about the direction and speed of motion and to discriminate patterns. We first presented moving bars (75 µm wide), at various velocities (2.2 mm/s or 4.4 mm/s), and with different orientations and directions across the treated retina (Supplementary Movie S2). Based on the known retinal magnification factor, 1 arc-degree of visual size corresponds to 211 µm on the retina. Urban stimuli, therefore, corresponded to a visual field angle of 0.375° moving at 11 or 22 °/s. For calculation of the visual flow elicited, we used spike sorting on the recorded activity, followed by plane fitting to the peak of the cell responses, to estimate the direction of the bar (Fig. 6a and Supplementary Fig. S7) and its speed (Fig. 6d). The plane fitting method made it possible to identify, for each direction, the unique succession of cells activated along the path of the bar (Fig. 6a–c). This temporal response of the cells was found to be sufficient for correct estimation of the direction and velocity of the bar over the retina, despite the discrete spacing of electrodes.
We show here that the AAV2.7m8–ChR-tdT construct is more efficient than the wild-type capsid AAV2 and the non-fused CrimsonR protein in primate retinal ganglion cells. Furthermore, the therapeutic vector dose was defined as 5 × 10^{11} vg/eye, which allows greater light sensitivity, with expression in more cells and over a wider area. We explored key parameters of optogenetic activation (i.e., light intensity, temporal, and spatial modulation) and demonstrated an ability to decode the direction and speed of a moving bar and the identity of different stimulus shapes from the recorded cell activity. These data supported the application to launch the ongoing clinical trial in patients with retinitis pigmentosa.

**Vector optimization for a high level of functional efficacy.** For the successful achievement of our therapeutic goals, the viral optogenetic construct must have a large functional impact on a large proportion of the cells in the RGC population. Our results confirm the higher transduction efficiency of the AAV2.7m8 variant in the retina relative to AAV2.20,20 Surprisingly, we also found that efficacy was greater for the ChR-tdT fusion protein than for ChR alone. We cannot entirely rule out an experimental bias, as ChR expression cannot be localized by fluorescence imaging as for ChR-tdT. However, the expected location of the transduced gene at the fovea greatly decreases the chances of missing expression clusters during tissue isolation and positioning on the MEA. Differential protein trafficking, with td Tomato working as a trafficking helper and possibly also preventing protein aggregation, appears more likely. The result would be a higher level of opsin construct targeting to the membrane. We demonstrated a greater efficacy for both the mutated AAV capsid, AAV2.7m8, and for the ChR-tdT fusion protein, in non-human primates. Given the high degree of structural similarity between the eyes of NHPs and humans, we would expect intravitreal AAV2.7m8–ChR-tdT injection to transduce the retina effectively in blind patients too.

**Dose selection.** Once the construct has been selected, the next critical issue is AAV safety. Previous studies of gene therapy within the eye used AAV doses of 1.5 × 10^{11} vg in the subretinal space and of up to 1 × 10^{11} vg/eye or 1.8 × 10^{11} vg/eye for intravitreal injections. No adverse effects were reported at these doses, but other delivery methods use much higher doses (up to 10^{14} vg) with potentially disruptive effects. In this study, none
of the eyes treated displayed an inflammatory response (Supplementary Fig. S1), with only a few cells in the vitreous and one eye displaying vitreal haze (associated with light hemorrhaging during the IVT procedure). Doses of $5 \times 10^{10}$ and $5 \times 10^{11}$ vg/eye induced ChR-tdT expression and strong functional responses highly efficiently in NHPs. The highest dose used here ($5 \times 10^{11}$ vg/eye) appears to provide more extensive retinal coverage and higher light sensitivity (Fig. 2). This greater coverage would enlarge the patient’s visual field, translating to $\sim 6^\circ$ in the visual field (211 µm on the primate retina per degree angle$^{36}$). A visual field of this size may appear rather limited, but it should be borne in mind that the fovea is the center of high visual acuity. This high visual acuity results from the high density of cone photoreceptors, and the marked predominance of midget RGCs, which
receive inputs from a single photoreceptor and have a very small receptive field. We showed, by spike propagation speed analysis (Supplementary Fig. S5) and morphology examinations, that most of the ChR-tdT-expressing RGCs were, indeed, midget RGCs, likely to mediate visual perception with a high level of acuity. Specific activation of this midget RGC population in the foveal area could potentially provide patients with high-acuity vision.

Effective and safe stimulation intensity. We show here that the stimulation of ChR-tdT, as for most microbial opsins, is effective from $10^{15}$ photons cm$^{-2}$ s$^{-1}$. Given that only a quarter of visible light effectively stimulates any given light-sensitive channel, it would be hard to find situations in everyday life in which stimulation would occur. Outside on a bright day, the effective light intensity on the retina would be around $10^{14}$ photons cm$^{-2}$ s$^{-1}$, and this would fall to $2 \times 10^{12}$ photons cm$^{-2}$ s$^{-1}$ in an office. We would not, therefore, expect transfection with opsins to yield useful levels of perception in isolation. Our strategy will need to include an external photostimulation device for converting images into tailored patterned photostimulation of the optogenetically engineered retina.

This study focused on the red-shifted opsin ChrimsonR, which has a reported peak sensitivity at 575 nm, a result confirmed here by MEA recordings (Fig. 1e). This is a much safer wavelength than highly phototoxic blue-light wavelengths, making it possible to expose the retina to higher light intensities safely. For the clinical trial, we opted for a middle ground between optimal opsin sensitivity and the lower phototoxicity of higher wavelengths. We decided to use a 595 nm LED (Cree XP-E2, Lumitronix) as the light source for external photostimulation. For this reason, we mostly used light at a wavelength of 600 nm ($\pm 10$ nm) in this study, and this is the wavelength considered for the safety evaluation.

The International Commission on Non-Ionizing Radiation Protection published limits for ocular exposure to visible and infrared radiation in 2013. These limits were translated into retina irradiance by Sengupta et al., using published conversion rates. The resulting threshold for continuous exposure was $5.6 \times 10^{17}$ photons cm$^{-2}$ s$^{-1}$ at 590 nm and $5 \times 10^{15}$ photons cm$^{-2}$ s$^{-1}$ at 500 nm. In 2016, Yan et al. published a review of the 2014 ANSI Z136.1 exposure limits for laser illumination of the retina with ophthalmic instruments ($8.3$) and the potential use of these limits in optogenetic systems. For a full-field continuous stimulus applied for 8 h during a 48-h period, the maximal permissible retinal peak irradiance is $1.1 \times 10^{17}$ photons cm$^{-2}$ s$^{-1}$ at 600 nm and $8 \times 10^{15}$ photons cm$^{-2}$ s$^{-1}$ at 505 nm.
Fig. 7 The measured activity can be used to discriminate stimulation with different shapes and to predict visual acuity. a Responses of cells to three different shapes (rows: X, circle and square, columns: six different times) moving from the center towards the bottom of the retina. The colored disks indicate the electrodes where cells are recorded spiking. The size of the disks show the number of spikes detected in a 10-msec time window centered on the time indicated at the figure top. Color of the disks changes with timing in the sequence. b Raster plots showing the activity of the cells over time. The three shapes (X: left, circle: center and square: right) triggered different patterns of activity in the cell population. Color of the action potential on the raster change with time, fitting the color code in a. The six different time windows used in a are shown as the gray area over the right panel (square). c Discriminability (as a %) of the features of the spatiotemporal spike response obtained from population spike trains for different sizes. Discriminability increased with letter size. “No-stim” discrimination was performed with the spontaneous activity pattern just before the start of stimulation. The no-stim discrimination rate was about 33%, as would be expected by chance alone for three shapes. d Normalized mutual information from the confusion matrix obtained from the discrimination rates in (c). The information tends to increase with the size of the shapes, with saturation occurring at 220 µm. The information and discriminability at 220 µm indicate a visual acuity above the defined threshold for legal blindness.
Both sets of proposed exposure limits are higher for the 600-nm wavelength used here than for the blue light used for opsins such as Chr2. Importantly, we demonstrate here that light intensities below the 600 nm radiation safety limit can activate the transfected retina very efficiently.

Exposure limits are, by nature, conservative. They are set for continuous illumination, as the photochemical hazard, which depends on the total illumination received per 48 h, is the highest risk. However, our strategy will involve stimulation with light patterns extracted from an event camera, reducing moving faces and objects to their outlines\textsuperscript{31,52}. The scarcity of stimulation will increase the maximal retinal peak irradiance permitted\textsuperscript{21}.

The risk of photochemical injury in the retina of patients with advanced RP lacking functional photoreceptors remains unclear, but the build-up of potentially toxic retinoids in the RPE would, presumably, be minimal.

For further guarantees of patient safety during the clinical trial, it will be important to limit the initial duration of exposure and to monitor the state of the retina after each stimulation period very closely.

**Comparison with other studies and constructs.** A previous clinical trial of optogenetic therapy for visual restoration focused on the blue-sensitive microbial opsin, Chr2\textsuperscript{23}. This first clinical study built on preclinical studies in mice\textsuperscript{2} and marmosets\textsuperscript{8}. In the marmoset study, a single MEA electrode recorded spike trains reaching >300 Hz at 6.6 × 10\textsuperscript{16} photons cm\textsuperscript{−2} s\textsuperscript{−1}. More recently, using the human codon-optimized Ca\textsuperscript{2+}-permeable Chr2, (CatCh), which is 70 times more efficient than Chr2, we recorded multiunit spiking frequencies in a similar range in macaque foveal RGCs (∼300 Hz at 8 × 10\textsuperscript{15} photons cm\textsuperscript{−2} s\textsuperscript{−1} \textsuperscript{20}). These studies focused on opsins sensitive to blue-light wavelengths and they reported results at intensities above safety limits. In this study, we observed multiunit spiking frequencies above 700 Hz (Fig. 3a) at 2 × 10\textsuperscript{17} photons cm\textsuperscript{−2} s\textsuperscript{−1} and a peak firing rate above 300 Hz for light intensities well below the safety limits (9 × 10\textsuperscript{15} photons cm\textsuperscript{−2} s\textsuperscript{−1}, Fig. 2c).

Chrimson\textsuperscript{R} is currently the most red-shifted opsin available, with peak sensitivities shifted by ∼100 nm for Chr2 and 45 nm for ReaChr\textsuperscript{23}, but future work may even result in the development of infrared-sensitive opsins, as in snakes\textsuperscript{53,54}. Alternatively, mutagenesis could be used to enhance the properties of existing opsins: Chrimson\textsuperscript{R} was generated by the site-directed mutagenesis of Chrimson\textsuperscript{2}, and was further modified to drive neuron firing rates to higher spiking frequencies\textsuperscript{52}. This kinetic enhancement was achieved at the expense of light sensitivity, and this new variant is not, therefore, relevant for vision restoration, as it would reduce the safe range for stimulation. Based on previous studies aiming to develop an optogenetic vision restoration strategy, we show here a highest level of evoked activity at a wavelength of 600 nm, with a temporal resolution of milliseconds, at intensities below the safety threshold.

**Pattern discrimination for the restoration of vision.** The restoration of vision with retinal prostheses is classically assessed by stimulating individual electrodes\textsuperscript{55,30}, defining object positions and shape\textsuperscript{56–58}, identifying bar orientation\textsuperscript{53,57}, and reading letters or words. An issue encountered with epiretinal prostheses is that single-electrode stimulations activate RGCs axons on their way to the optic nerve, leading to patients perceiving an arc, rather than a point\textsuperscript{59}. In our optogenetic approach, RGCs axons expressed Chr-tdT, as indicated by tDT fluorescence, but the light stimulation applied did not trigger spikes (Fig. 5b, c). This specificity of activation is a requirement for spatially restricted stimulation. We show here through spot stimulation that responses can be elicited with a spot diameter of 50 µm, even for a duration of 10 ms (Fig. 5 and Supplementary Fig. S6). These findings are encouraging, but probably represent an underestimate of actual spatial resolution, for two reasons. First, the circular spot used in these experiments was centered on the 10 µm opaque electrodes, reducing photon flux, especially for smaller stimuli (i.e., 25 µm, Fig. 5). Second, due to the limitations on sampling during MEA recordings, it is unlikely that we recorded the activity of all responsive cells. It is, therefore, difficult to compare our results with those for subretinal implants, because most of these prosthetic devices activate RGCs indirectly through bipolar cells. However, electrodes have a 70–100-µm pitch that can be activated only in a stepwise manner, as all or most of the implanted electrodes must be stimulated for an activation current to be generated\textsuperscript{52,60–61}. Whereas optogenetic therapy can activate RGCs with smaller spot size, and increasing the size of the stimulation spot increases the number of cells recruited in an almost linear manner (Fig. 5b).

Visual acuity is considered normal for a value of 20/20, corresponding to the ability to identify an object of 5 arc-minutes, with a critical gap of 1 arc-minute that needs to be resolved. The best reported visual acuity achieved with current retinal prostheses was 20/546, when assessed with Landolt C-rings\textsuperscript{5}. In our optogenetic strategy, using an approach similar to the Snellen chart, we obtained correct shape discrimination (Fig. 7; 83% discrimination for symbols of 220 µm with 44-µm edges). For adult Macaca fascicularis monkeys, 1 arc-degree in the visual field corresponds to a size of 211 µm on the retina\textsuperscript{36}, to obtain 20/20 acuity, a gap of 3.6 µm must be resolved. In our case, the size of the gap correctly discriminated (edge of the symbol: 44 µm) represents a visual acuity of 20/249, which is above the legal threshold for blindness (20/400\textsuperscript{2,62,63}). This value is consistent with the predictions of simulations\textsuperscript{54} and better than any acuity reported to date with visual prosthetics (20/546\textsuperscript{5}). The impossibility of recording all cells from the ChR-tDT-expressing population would clearly have underestimated visual acuity, which could probably be enhanced even further. One important perifoveal feature not taken into account here is the lateral displacement of the RGC cell body with respect to its receptive field. Indeed, to ensure minimal optical aberration for the light hitting the photoreceptor in the most central part of the fovea, other retinal layers are displaced centrifugally around the fovea pit. Due to this displacement, the direct stimulation of RGCs should occur through a corrected image of the visual field. Finally, degenerated retinas have a much lower signal-to-noise ratio than our pharmacologically isolated RGCs, due to abnormal circuit reorganization. Furthermore, RGCs in the degenerating retina have abnormally high spontaneous firing rates, which may have a large effect on their response to optogenetic stimulation, decreasing visual acuity in patients. However, a recent study has suggested treating this consequence of tissue reorganization with retinoic acid blockers\textsuperscript{65}.

We showed, by making use of the reliability of spike train generation, that pulse width modulation might be preferable to continuous illumination (Fig. 4). Evaluations in patients will define the best duration of stimulation, but we can infer, from our data, that RGCs are reliably activated in the 5–20 ms range (Figs. 3 and 4). We hypothesize that this ability to evoke high-frequency modulation would help (1) to reduce the total amount of light entering the eye, and (2) to maintain precise control over cell activity, potentially improving the outcome of this strategy.

**Conclusion.** We describe here the initial selection process for genetic content, vector serotype, and vector dose in an ambitious “two-prong” vision restoration strategy involving a biological
component and an external light stimulation device. While we could not assess thoroughly the behavioral impact of our strategy on sighted or blind macaques, the results for the biological component presented here provide all the essential information required for the design of the external light stimulation device necessary for the conversion of visual scenes into a stimulation pattern. Both the biological component and external light stimulation device are now in use, together, in the recently launched phase I/II clinical trial of AAV2.7m8-ChR-tdTomato for five-year safety and performance results from the Argus II Retinal Prosthesis System clinical trial.

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