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2 ***In vivo optogenetic stimulation of the primate retina activates the***
3 ***visual cortex after long-term transduction***

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24

25 **Abstract**

26 Over the last 15 years, optogenetics has changed fundamental research in neuroscience, and is now
27 reaching toward therapeutic applications. Vision restoration strategies using optogenetics are now at
28 the forefront of these new clinical opportunities. But applications to human patients suffering from retinal
29 diseases leading to blindness rise important concerns on the long-term functional expression of
30 optogenes and the efficient signal transmission to higher visual centers. Here we demonstrate in non-
31 human primates, continued expression and functionality at the retina level ~20 months after delivery of
32 our construct. We also performed *in-vivo* recordings of visually evoked potentials in the primary visual
33 cortex of anaesthetized animals. Using synaptic blockers, we isolated the *in-vivo* cortical activation
34 resulting from the direct optogenetic stimulation of primate retina.

35 In conclusion, our work indicates long-term transgene expression and transmission of the signal
36 generated in the macaque retina to the visual cortex, two important features for future clinical
37 applications.

38

39

40 **Introduction**

41 Repairing sensory impairments has always been an overarching goal in medicine. In the
42 particular case of vision loss, considerable progress has been achieved in recent years through the
43 development of various therapeutic strategies, such as retinal prostheses (1-4), stem cell transplantation
44 (5-8) and optogenetic therapies (9-19). All these approaches aspire to restore retinal visual information.
45 Microbial opsin-based optogenetics is one of the most promising of these approaches. It involves the
46 expression of light-sensitive ion channels in preserved inner retinal neurons, restoring the intrinsic light
47 sensitivity of the pathological retina in several types of ocular disease.

48 In inherited forms of retinal degeneration, such as retinitis pigmentosa (RP), the retinal
49 degeneration is progressive, beginning with the photoreceptors and inevitably leading to blindness (20).
50 The choice of target cell type in the retinal circuit should take into account the potential for translation
51 into clinical applications and uses in patients. The accessibility of the targeted cell population, and the
52 maintenance of its structure and integrity after the onset of retinal degeneration are key features. Since
53 the first use of optogenetics to restore vision in blind mice through the expression of channelrhodopsin-
54 2 (Chr2) in RGCs (9), many other studies have been conducted, targeting different cell types in the
55 retina: photoreceptors (10, 21), bipolar cells (15, 16, 18) or retinal ganglion cells (RGCs) (9, 11, 13, 14,
56 17). Importantly, in diseases such as age-related macular degeneration (AMD) and RP, the retinal
57 ganglion cells (RGCs) remain well-preserved during the process of retinal degeneration, even at late
58 stages of the disease, after the death of the photoreceptors (22). Various models, including rodents,
59 non-human primates, post-mortem human retina and human induced pluripotent stem cells (hIPS), have
60 been used for investigations of optogenetic approaches, with promising results (7, 9, 13, 21).

61 Primate models seem to be the best of the animal models for testing optogenetic therapeutic
62 approaches, because they share essential anatomical features and a similar organization of visual
63 pathways with humans (23, 24). However, few studies to date have used this animal model for
64 investigations of the potential of optogenetic therapy for the retina. For example, several opsins targeting
65 RGCs have been tested in *ex vivo* preparations, including the microbial opsin channelrhodopsin 2 (14)
66 in marmosets and CatCh (11), ReaChr (19) and ChR-tdT (13) in macaques. All these opsins were found
67 to be functional in RGCs. Furthermore, the optogenetic responses of RGCs *in vivo* have been recorded
68 with calcium imaging after photoablation of the photoreceptors in the macaque foveal region (17).
69 However, none of these experiments was able to demonstrate the transfer of optogenetic activation
70 from RGCs to higher visual centers, such as the primary visual cortex. Such experiments on retinal
71 optogenetic approaches have been formed only in rodents, and have shown that the optogenetic
72 activation of transduced retina induces specific visual evoked responses (VEPs) in the visual cortex (10,
73 11, 15, 25, 26). Moreover, specific cortical responses were recorded following the activation of RGCs
74 by photovoltaic subretinal implants in rats (27). A recent study on primates fitted with subretinal implants
75 showed that these prostheses induced some behavioral responses (2). However, to our knowledge, no
76 study to date has demonstrated the transmission of information to higher visual areas following
77 activation of the transduced retina in primates.

78 Using this approach to optogenetic therapy, we targeted the retinal ganglion cells (RGCs) in
79 primate retinas through the *in vivo* expression of an ectopic light-sensitive ion channel, ChrimsonR,
80 coupled to the fluorescent reporter tdTomato (13). The possible application of this strategy to blind
81 patients suffering from retinal dystrophies raises important concerns about long-term functional
82 expression to ensure efficient signal transmission to higher brain centers (i.e. the visual cortex) (28). We
83 previously showed that the transduced retina displays a high degree of spatiotemporal resolution *ex*
84 *vivo*, compatible with the perception of highly dynamic visual scenes at light levels suitable for use in
85 humans (13). Here, we demonstrate, in non-human primates, sustained functional efficacy ~20 months
86 after the delivery of an AAV2.7m8-ChrimsonR-tdTomato vector similar to that currently undergoing
87 clinical evaluation. Our results reveal a persistence of expression in the perifovea, mediating information
88 transfer to higher brain centers. Indeed, we recorded visual evoked potentials in the primary visual cortex
89 of anesthetized primates in response to optogenetic retinal activation. We used an intravitreal injection
90 of synaptic blockers to isolate the cortical component resulting from the *in vivo* optogenetic stimulation
91 of primate RGCs. Our findings demonstrate the long-term functional efficacy of optogenetic therapy to
92 restore information transfer from the retina to the brain *in vivo*.

93

94 Results

95 The experiments were conducted on three monkeys (*Macaca fascicularis*); each of them
96 receiving, in one eye, a single intra-vitreal injection of AAV2.7m8-ChrR-tdT at a dose of 5×10^{11} vg/eye,
97 the other eye being kept as a control. More than 20 months later, we performed *in-vivo* cortical
98 experiments to record visually evoked potentials to the retinal optogenetic stimulation. Subsequently,
99 ~24h hours after the euthanasia, we measured directly optogenetic responses on *ex-vivo* retinal foveal
100 explants. For every eye treated with ChrR-tdT, half of the retina was used for single-cell RGC recordings
101 and 2-photon imaging, whereas the other half was used for Multi Electrode Array (MEA) RGCs
102 population recordings and later histology.

103 The long-term functional expression of ChrimsonR-tdTomato (ChR-tdT) was assessed in the
104 primate retina by *ex vivo* retinal recordings in the presence of glutamate receptor antagonists (see
105 Methods), which were added to the bath solution to suppress any natural light response. Live
106 epifluorescence images revealed a high density of transfected cells localized in the perifoveolar region,
107 forming a torus shape (**Fig. 1A**), mostly in the retinal ganglion cell layer (**Fig. 1B**, **Sup. Fig. 1**). On some
108 of these transfected RGCs, we observed small dendritic arbors suggesting that foveal midget RGCs
109 responsible for high acuity vision (29) were expressing ChR-tdT. Light sensitivity and temporal dynamics
110 were measured, at the single-cell level, with the two-photon guided patch-clamp technique, on ChR-tdT-
111 positive cells (**Fig. 1C**). We found that the mean normalized photocurrent response increased
112 significantly with increasing light intensity (**Fig. 1D**, top), reaching a peak (mean of 143.6 ± 47.4 pA,
113 $n=12$) at a light intensity of 3×10^{17} photons.cm $^{-2}$.s $^{-1}$. In the loose-patch recording configuration, firing
114 rate displayed a similar dependence on light level (99.03 ± 7.59 Hz, $n=32$ cells), also peaking at a light
115 intensity of 3×10^{17} photons.cm $^{-2}$.s $^{-1}$ (**Fig. 1E**, right). We then showed that the highest frequency
116 responses were obtained for light stimuli at 575 nm (**Fig. 1F**), corresponding to the excitation peak of
117 ChrimsonR (590 nm) (30). We obtained reliable firing bursts with fast dynamics during the measurement
118 of RGC responses to stimuli of increasing durations (20 ms to 4 s, $n=9$, **Fig. 1G**) or various flicker
119 frequencies (10 repeats in full duty cycle) up to 28 Hz ($n=9$, **Fig. 1H**). These results demonstrate the
120 ability of these engineered cells to follow and resolve short, long and fast light stimulations accurately
121 up to frequencies very similar to the video-rate frequencies (~25 Hz) required for fluid movement
122 perception and compatible with the limits for flicker perception reported for humans (31).

123 In parallel, we used MEA recordings to investigate the responsiveness of the cell population
124 in the transfected area (**Fig. 2**, **Sup. Fig. 2**). A large proportion of the perifovea contained a high density
125 of ChR-tdT-expressing cells, as indicated by counting TdTomato-positive cells on projections of confocal
126 stacks (**Fig. 2A-B**). The MEA chip (**Fig. 2C**, top left) covered a large area of the hemifoveal retina flat
127 mounts, making it possible to take recordings for a large proportion of the transfected RGCs (**Fig. 2C**,
128 bottom left; **Sup. Fig. 2A**). **Figure 2C** (right) and **Sup. Fig. 2C** show the global recorded activity for
129 RGCs, represented as the firing rate of 256 sites over a period of 4 s in response to a 2 s flash of light.
130 We performed 10 recordings in total, at a wavelength of 595 nm and a light intensity of 7×10^{16}
131 photons.cm $^{-2}$.s $^{-1}$ (**Fig. 2C**, right). A large proportion of the recording sites were responsive, and the
132 response observed was correlated to the degree of optogenetic expression, as determined by measuring

133 tdTomato reporter fluorescence. Overall, in the two retinas for which we were able to obtain spontaneous
134 activity, 45.5% of all active recording sites were also responsive to light stimulation (172 of 378 active
135 sites, **Fig. 2D**). We performed MEA recordings as a function of light intensity (**Fig. 2E**). The results
136 obtained were very similar to those obtained with the single-cell technique (**Fig. 1D-E**). Indeed, the
137 threshold light intensity for a response was found to be 9×10^{15} photons.cm $^{-2}.$ s $^{-1}$. We then analyzed MEA
138 responses as a function of stimulus duration (**Fig. 2F**), at an intensity of 7×10^{16} photons.cm $^{-2}.$ s $^{-1}$. In the
139 two retinas tested, cells responded to stimuli with a duration of at least 5 ms, but not to stimuli of shorter
140 duration.

141 In living animals, we then investigated whether the optogenetic stimulation of these transfected
142 RGCs could activate the visual pathway *in vivo*. To this end, we recorded visual evoked potentials
143 (VEPs) in response to different visual stimuli presented to the anesthetized animals during short-term
144 experiments (**Fig. 3A**). In this way, we were able to compare VEP responses before and after the
145 intravitreal injection of synaptic blockers (PDA and L-AP4) into both eyes (**Fig. 3B**) to block natural
146 retinal responses to light (32). With the synaptic block, light stimulation directly activates the engineered
147 cells rather than the normal retinal pathway. In monkey M1, following stimulation of the ChR-tdT-
148 expressing eye with an orange LED (595 nm) before the injection of the blocker, we recorded typical
149 VEP responses, defined by the presence of four different phases: two negative phases with latencies of
150 28 and 76 ms (blue triangles) and two positive phases (latencies of 51 and 107, red triangles). Similar
151 VEPs were obtained for stimulation of the control eye, confirming that the sequence of orange flashes
152 activated the visual pathway naturally. After the intravitreal injection of synaptic blockers and stimulation
153 of the eye with the same orange LED, we noted major changes in the shape of the recorded VEP. The
154 VEP responses of the ChR-tdT-expressing eye (**Fig. 3B** orange traces) were characterized by an early
155 positive phase (21 ms, orange arrow) and a late sustained negative phase (101 ms, blue arrow).
156 Interestingly, stimulation of the control eye resulted in a stable recording, with no early positive or
157 subsequent negative phase. Similar results were obtained with a second animal (monkey M2, **Fig. 3B**
158 **bottom**), with an early peak response at 24 ms. The latency of the first positive peak was 21-24 ms for
159 both animals, which is much shorter than the latencies classically reported for natural VEP components
160 (positive components occur at 50-81 ms) and observed here. These data are consistent with the notion
161 that these VEP responses in the visual cortex were elicited by the direct activation of ChR-tdT-
162 expressing RGCs.

163 We then investigated the effects of several parameters, such as the light intensity and frequency
164 of the orange LED, on VEP responses (**Fig. 3C**). The amplitude of positive peak responses increased
165 proportionally to light intensity (from 43 to 152 μ V for monkey 1 and from 121 to 221 μ V for monkey 2),
166 and the peak latencies of these responses decreased with increasing light intensity (see inset); from 82
167 to 24 ms for monkey M1 and 28 to 24 ms for monkey M2). No significant VEP response was recorded
168 for the control eye (**Sup. Figure 3**), even at maximal light intensity, confirming that the glutamate
169 receptor antagonists used effectively abolished the natural light response. We then recorded VEP
170 responses in response to an orange LED flashed at a frequency of 4 Hz (125 ms) and 16 Hz (32 ms,
171 **Fig. 3C**) to confirm that activation occurred earlier after the optogenetic stimulation of RGCs. VEP peaks
172 occurred more rapidly after blocker administration (orange traces) than in the absence of blocker (black
173 traces), with similar time periods observed for stimulation at 4 Hz and at 1 Hz (30-37 ms for monkey M1;
174 Fig 3B). For monkey M2, the difference in time to VEP peak was larger at 4 Hz (82-84 ms) than at 1 Hz
175 (~58 ms). After stimulation at 16 Hz, VEP traces followed the train of pulses both before and after the
176 injection of synaptic blocker. Although mean amplitudes of VEP responses were similar for stimulations
177 at 1 Hz and 4 Hz, they decreased drastically after stimulation at 16 Hz for both animals (Fig. 3E). No
178 such activation was observed following stimulation of the control eye, in either of the animals studied
179 (**Sup. Fig. 3**). These results demonstrate that optogenetic activation of the retina can trigger a transfer
180 of information to higher visual centers, providing additional support for the potential of ChR-tdT for future
181 therapeutic applications.

182

183 Discussion

184 We previously showed that a single intravitreal injection of AAV2.7m8.CAG.ChrimsonR-tdT can
185 efficiently target the foveal region of the retina, which is responsible for high-acuity vision (13). Using *ex*
186 *vivo* live imaging and histology, we show here that expression persists in cells in the perifovea region
187 more than 20 months after the injection, and we demonstrate, with electrophysiological recordings, that

188 these transfected RGCs remain functional, displaying rapid, robust responses. Previous studies based
189 on *ex vivo* retinal recordings in NHP models have demonstrated efficient optogenetic expression for up to
190 six months (11, 13, 14), and a study based on retinal imaging *in vivo* extended this functional window
191 up to 14 months (17). This maintenance of activity so long after the injection is consistent with the notion
192 that gene therapy can lead to long-term gene expression. Furthermore, it provides additional evidence
193 that the microbial opsin ChR-tdT does not induce an immune response that might eventually destroy
194 the engineered RGCs. Indeed, the RGC responses were in a range similar to that recorded in our initial
195 experiments (13), and transfected cell density was also at similar levels. However, given the small
196 number of replicates in this study and the considerable inter-subject variability previously observed after
197 two or six months, it is difficult to make quantitative comparisons with earlier expression time points.

198 We show here, for the first time, through *in vivo* VEP recordings, that the selective stimulation
199 of transfected RGCs induces specific cortical responses. Based on our retinal observations, we can
200 interpret these VEP recordings as reflecting the activation of cortical neurons due to the direct functional
201 optogenetic activation of RGCs, leading to the synaptic transfer of information to cortical neurons. All
202 previous studies on optogenetic RGC activation in the primate retina were performed on RGCs either
203 *ex vitro* (11, 13, 14) or *in vivo* (17). The recorded rates of RGC firing activity and the reported increases
204 in calcium indicator fluorescence were highly suggestive of potential information transfer to the higher
205 visual centers, but no experimental demonstration for the existence of this communication was provided.
206 Here, following a blockade of glutamatergic synaptic transmission in the retina, the direct optogenetic
207 activation of RGCs elicited specific VEP responses with an earlier peak response than normal VEP
208 responses. Such early cortical responses were recorded in blind rodents during optogenetic activation
209 of either the dormant cones (10), the bipolar cells (15, 16) or the RGCs (11, 26). The short VEP response
210 latencies recorded are probably the signature of direct optogenetic stimulation within the inner retina,
211 occurring more rapidly than natural responses, due to the slowness of the phototransduction cascade
212 and of synaptic information transfer between the different retinal layers. We showed that cortical VEP
213 responses increased with light intensity. This result is highly consistent with the RGC spike recording
214 on the isolated retina, with a clear relationship between RGC activity and light intensity. Given the high
215 light levels used in our VEP experiments, the observed increases in VEP peak amplitudes with
216 increasing light intensity are consistent with an optogenetic origin, because any residual natural light
217 responses would be fully saturated at such light intensities. Finally, these VEP recordings show that
218 optogenetic responses can follow frequencies of at least 16 Hz, as expected from the high temporal
219 resolution achieved with RGCs in *ex vivo* single-cell recordings (Fig. 1H). All these VEP recordings
220 validate the therapeutic potential of ChR-tdT for restoring vision in blind patients.

221 In conclusion, this study extends existing data for vision restoration strategies tested in NHPs,
222 and raises hopes of long-term functionality for optogenetic approaches in blind patients, who may benefit
223 from this therapy. It also opens up new avenues of research into the neural integration and computations
224 occurring at the cortical level in NHPs, with a view to restoring the sensitivity of sensory organs through
225 optogenetics.

226

227 **Materials and Methods**

228 *Animals*

229 Data were collected for three captive-born macaques (*Macaca fascicularis*; 2 males, monkey M1,
230 monkey M2, weighing 3.2, and 3.9 kg, respectively; one female, monkey M0, 4.1 kg). Monkeys were
231 housed in pairs and handled in strict accordance with the recommendations of the Weatherall Report
232 on good animal practice. Monkey housing conditions, surgical procedures and experimental protocols
233 were performed in strict accordance with the National Institutes of Health Guidelines (1996), and after
234 validation of the European Council Directive (2010/63/EU), and the study was approved by the French
235 government and institutional and regional committees for animal care (Committee C. Darwin,
236 registration #9013). Our routine laboratory procedures included an environmental enrichment program,
237 in which the monkeys were allowed visual, auditory and olfactory contact with other animals and, when
238 appropriate, could touch and groom each other.

239 *AAV production*

240 ChrimsonR-tdTomato was inserted into an AAV backbone plasmid. The construct included a WPRE
241 and bovine growth hormone polyA sequences. Recombinant AAVs were produced by the plasmid
242 cotransfection method (32), and the resulting lysates were purified by iodixanol gradient
243 ultracentrifugation, as previously described. Briefly, the 40% iodixanol fraction was concentrated and
244 subjected to buffer exchange with Amicon Ultra-15 Centrifugal Filter Units. Vector stocks were then
245 titered for DNase-resistant vector genomes by real-time PCR relative to a standard (33).

246 *Gene delivery*

247 Primates were anesthetized with 10:1 mg/kg mixture of ketamine and xylazine. We injected 100 μ L of
248 viral vector suspension into the vitreous of one eye in each animal. Following the injection, an ophthalmic
249 steroid and an antibiotic ointment were applied to the cornea. Experiments were conducted 21, 20 and
250 22 months after injection for M0, M1 and M2, respectively. None of the treated animals displayed any
251 sign of photophobia or vision-related behavioral changes during housing.

252 *Recording of VEPs*

253 We performed *in vivo* VEP recordings 20 to 22 months after AAV injection, during a terminal experiment
254 in two animals (M1 and M2). Briefly, anesthesia was induced with ketamine (0.2 mg/kg, intramuscular:
255 i.m.), and dexmedetomidine (0.015 mg/kg, i.m.) and maintained with alfaxan (0.1 mg/kg/min, i.v.). The
256 monkey was placed in a stereotaxic frame and heart rate, temperature, respiration, and peripheral
257 oxygen saturation were monitored throughout the experiment. We placed four electrodes in
258 subcutaneous positions: two at each temple and two at each occipital operculum (left and right), and we
259 set the high- and low-path filters to 50 Hz and 0.05 Hz, respectively. Light stimuli were generated with
260 two different LEDs: a blue LED (M470L3 Thorlab) and an orange LED (M595L3 from Thorlab 595 nm).
261 VEP responses were recorded before and after the intravitreal injection of synaptic blockers — 2,3-
262 piperidine dicarboxylic acid (PDA) and L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) — used to block
263 natural light responses (34). The eyes of the monkeys were subjected to flash stimuli at various
264 intensities (from 0.02 to 33.3 mW/cm²) and frequencies (1, 4 and 16 Hz). The duration of the stimuli was
265 200, 125 and 32 ms for frequencies of 1, 4 and 16 Hz, respectively. We made 300 consecutive
266 recordings of each VEP response and then averaged response waveforms for each VEP measurement.
267 VEP responses were similar for the ipsilateral and contralateral electrodes. We therefore present VEP
268 responses for the electrode contralateral to the injected eye only.

269 *Primate retina isolation and preservation*

270 After the *in vivo* VEP recordings, the primates received a lethal dose of pentobarbital. Their eyeballs
271 were removed, perforated with a sterile 20-gauge needle and placed in sealed bags with CO₂
272 independent medium (Thermo Fisher scientific) for transport. The retinae were isolated, and the retinal
273 pigment epithelium was removed and stored as retinal explants in an incubator for ~24 hours before
274 recording. Hemifoveal retina fragments were transferred to Neurobasal + B27 medium in polycarbonate
275 Transwells (Corning) for conservation in the cell culture incubator. These hemifoveal regions were
276 subsequently used for simultaneous single-cell and MEA recordings. In these conditions, natural
277 photoreceptor responses were abolished and did not recover. We abolished all natural responses
278 entirely, by applying pharmacological blockers (see below).

279 *Two-photon live imaging and single-cell electrophysiological recordings*

280 A custom-built two-photon microscope equipped with a 25x water immersion objective (XLPLN25xWMP,
281 NA: 1.05, Olympus) with a pulsed femtosecond laser (InSight™ DeepSee™ - Newport Corporation) was
282 used for imaging ChR-tdT-positive retinal ganglion cells. AAV-treated macaque retinas were imaged in
283 oxygenated (95% O₂, 5% CO₂) Ames medium (Sigma-Aldrich). For live two-photon imaging, whole-
284 mount retinas (without the retinal pigment epithelium attached) were placed in the recording chamber
285 of the microscope (ganglion cell layer side up), and images and z-stacks were acquired with the
286 excitation laser at a wavelength of 1050 nm. Images were processed offline with ImageJ.

287 We used an Axon Multiclick 700B amplifier for whole-cell patch-clamp and cell-attached recordings.
288 Patch electrodes were made from borosilicate glass (BF100-50-10, Sutter Instruments) and pulled to 7-
289 9 M Ω . Pipettes were filled with 115 mM potassium gluconate, 10 mM KCl, 1 mM MgCl₂, 0.5 mM CaCl₂,
290 1.5 mM EGTA, 10 mM HEPES, and 4 mM ATP-Na₂ (pH 7.2). We clamped the cells at a potential of -60

291 mV, to isolate excitatory currents. Recordings were also performed in the loose-patch configuration with
292 the pipettes filled with Ames medium, to record spiking activity. The retinae were dark-adapted for at
293 least 30 minutes in the recording chamber before recordings. AMPA/kainate glutamate receptor
294 antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 25 μ M, Sigma-Aldrich), NMDA glutamate
295 receptor antagonist, [3H]3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP, 10 μ M, Sigma-
296 Aldrich) and a selective group III metabotropic glutamate receptor agonist, L-(+)-2-amino-4-
297 phosphonobutyric acid (L-AP4, 50 μ M, Tocris Bioscience, Bristol, UK) were diluted to the appropriate
298 concentration from stock solutions and added to the Ames medium before recordings.

299 **MEA**

300 Multielectrode array (MEA) recordings were obtained for retinal fragments (without the retinal pigment
301 epithelium attached) placed on a cellulose membrane that had been incubated with polylysine (0.1%,
302 Sigma) overnight. Once on the micromanipulator, the piece of retina was gently pressed against a MEA
303 (MEA256 100/30 iR-ITO; Multi-Channel Systems, Reutlingen, Germany), with the retinal ganglion cells
304 towards the electrodes. We measured tdTomato fluorescence to check that the retina was correctly
305 positioned before making recordings under a Nikon Eclipse Ti inverted microscope (Nikon, Dusseldorf,
306 Germany) with the MEA system mounted on the stage. The retina was continuously perfused with Ames
307 medium (Sigma-Aldrich, St Louis, MO) bubbled with 95% O₂ and 5% CO₂ at 34 °C, at a rate of 1–2
308 ml/minute during experiments. AMPA/kainate glutamate receptor antagonist, 6-cyano-7-
309 nitroquinoxaline-2,3-dione (CNQX, 25 μ M, Sigma-Aldrich), NMDA glutamate receptor antagonist, [3H]3-
310 (2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP, 10 μ M, Sigma- Aldrich) and a selective group
311 III metabotropic glutamate receptor agonist, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4, 50 μ M,
312 Tocris Bioscience, Bristol, UK) were diluted to the appropriate concentration from stock solutions and
313 added to the bath through the perfusion system for 10 minutes before recordings. Action potentials were
314 identified on the filtered electrode signal (2nd-order high-pass Butterworth, cutoff frequency 200 Hz), with
315 a threshold of 4 x the SD of the signal. Spike density function was calculated, averaged over repeat
316 stimulations and used to determine the maximal firing rate over a time window corresponding to the
317 duration of the stimulus plus 50 ms. In comparisons of responses between light intensities, we
318 calculated, for each electrode, the added firing rate as the maximal firing rate minus the spontaneous
319 firing rate for the electrode concerned, which was calculated as the mean firing rate in the 2 seconds
320 before stimulation.

321 **Photostimulation for ex vivo experiments**

322 For single-cell electrophysiological recordings, full-field photostimulation was performed with a
323 Polychrome V monochromator (Olympus, Hamburg, Germany) set to 595 nm (\pm 10 nm), and output light
324 intensities were calibrated and ranged from 5.8×10^{14} to 3.15×10^{17} photons.cm².s⁻¹. For spectral
325 sensitivity experiments, stimulation wavelengths between 400 and 650 nm were tested, in 25 nm steps.
326 For flicker stimulation, 10 repeats were used in full duty cycle, at frequencies ranging from 2 Hz to 28
327 Hz. For MEA recordings, full-field light stimuli were applied with another Polychrome V monochromator
328 set to 595 nm (\pm 10nm), driven by a STG2008 stimulus generator (MCS). Output light intensities were
329 calibrated, and ranged from 1.37×10^{14} to 6.78×10^{16} photons.cm².s⁻¹. For intensity curves, we used
330 two-second flashes at five intensities (1.37×10^{14} , 6.56×10^{14} , 2.34×10^{15} , 8.82×10^{15} , 6.78×10^{16}
331 photons.cm².s⁻¹), each repeated 10 times, with a five-second interval between stimuli. For the duration
332 of stimulation assay, we used 12 different durations (ranging from 1 to 2000 ms), each repeated 10
333 times, with a five-second interval between stimuli. Calibrations were performed with a
334 spectrophotometer (USB2000+, Ocean Optics, Dunedin, FL).

335 **Confocal imaging and quantification**

336 After MEA experiments, the tissue was recovered and fixed by incubation with 4% PFA for 30 min at
337 room temperature, rinsed with PBS and stored at 4 °C in sodium azide solution. Hemifoveas were then
338 mounted in Vectashield containing DAPI (H-1000, Vector Laboratories) on slides and covered with a
339 coverslip (18 x 18 mm, Biosigma), using a 100 μ m spacer (Secure-seal space S24735, Thermo Fisher
340 Scientific), which was sealed with nail polish. The retinas were imaged on an inverted confocal
341 microscope (Fluoview 1200, Olympus), with a 20x objective (UPLSAPO 20XO, NA: 0.85, Olympus),
342 voxel sizes of 0.265 to 0.388 μ m/pixel in the x and y directions and 1.64 μ m/pixel in the z direction. For
343 each hemifovea, we recorded multiple stacks and reconstituted an automatic stitch (10% overlap). Using
344 Td-tomato fluorescence, we performed manual 3D counts of the transfected cells in ImageJ

345 (http://imagej.nih.gov/ij) with the cell counter plugin. The results were then processed with custom-
346 developed matlab analysis software for the calculation of *local density*.

347

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355

356 **Competing Interests:** S.P. is a consultant for *Gensight Biologics*, J-A.S. and S.P. have financial
357 interests in *Gensight Biologics*. G.G., J-A.S and S.P. have filed a patent application relating to the
358 gene therapy construct presented here.

359 **Author contributions:** Conceptualization, A.C., S.P., G.G. and F.A.; methodology & performance of
360 experiments, A.C., M.P., C.J., K.B., G.L., R.G., E.Bu., E.Br., G.G. and F.A.; formal analysis, A.C., C.J.,
361 G.G. and F.A.; writing – original draft, A.C., F.A., G.G. and S.P.; writing – review & editing, A.C., F.A.,
362 G.G., P.P. and S.P.; supervision and funding, J-A.S., S.P., G.G. and F.A.

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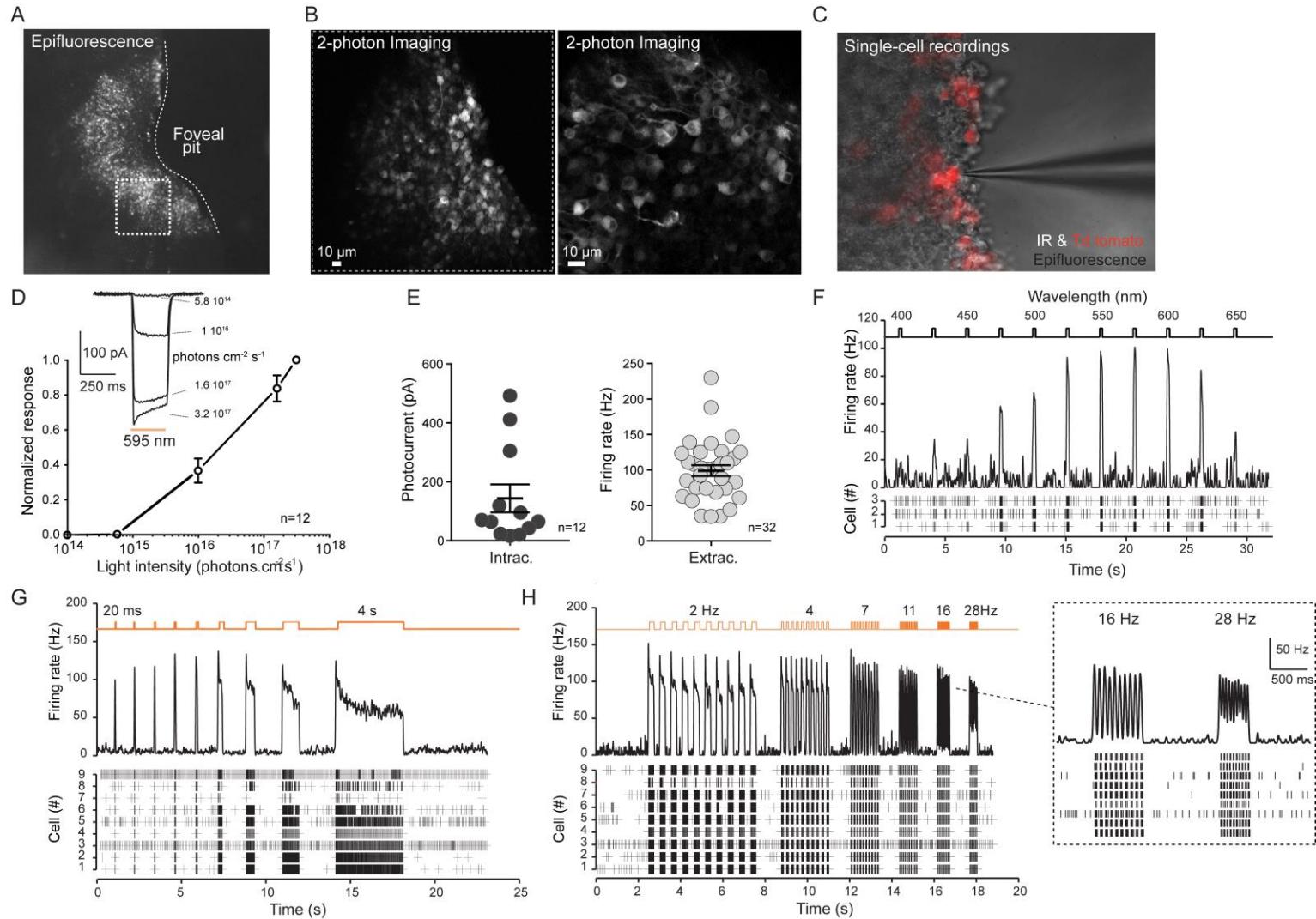
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448 **Figures and Tables**



449

450 **Figure 1. Long-lasting expression of the optogenetic construct in the macaque perifoveal ring and two-photon guided single-cell recordings**

452 (A) Epifluorescence image of an ex vivo primate hemifovea expressing tdTomato-ChrimsonR (perifoveal ring) 20 months after the injection of AAV2.7m8 - ChR-tdT at a dose of 5×10^{11} vg/eye

454 (B) Two-photon images of tdTomato-positive cells in the retinal ganglion cell layer at two different magnifications, taken in the dotted square shown in (A).

456 (C) Combined infrared and epifluorescence image of tdTomato-expressing fluorescent cells during single-cell recordings with a glass electrode (patch-clamp or extracellular recordings).

458 (D) Photocurrent response as a function of the intensity of the light stimulus in transfected retinal ganglion cells 20 months post-injection, and comparison with earlier time points. (top) Example of photocurrents recorded in cells stimulated with light at 595 nm for 250 ms at intensities of 5.8×10^{14} to 3.2×10^{17} photons.cm⁻².s⁻¹. (bottom) Normalized intensity curve for 12 patched cells.

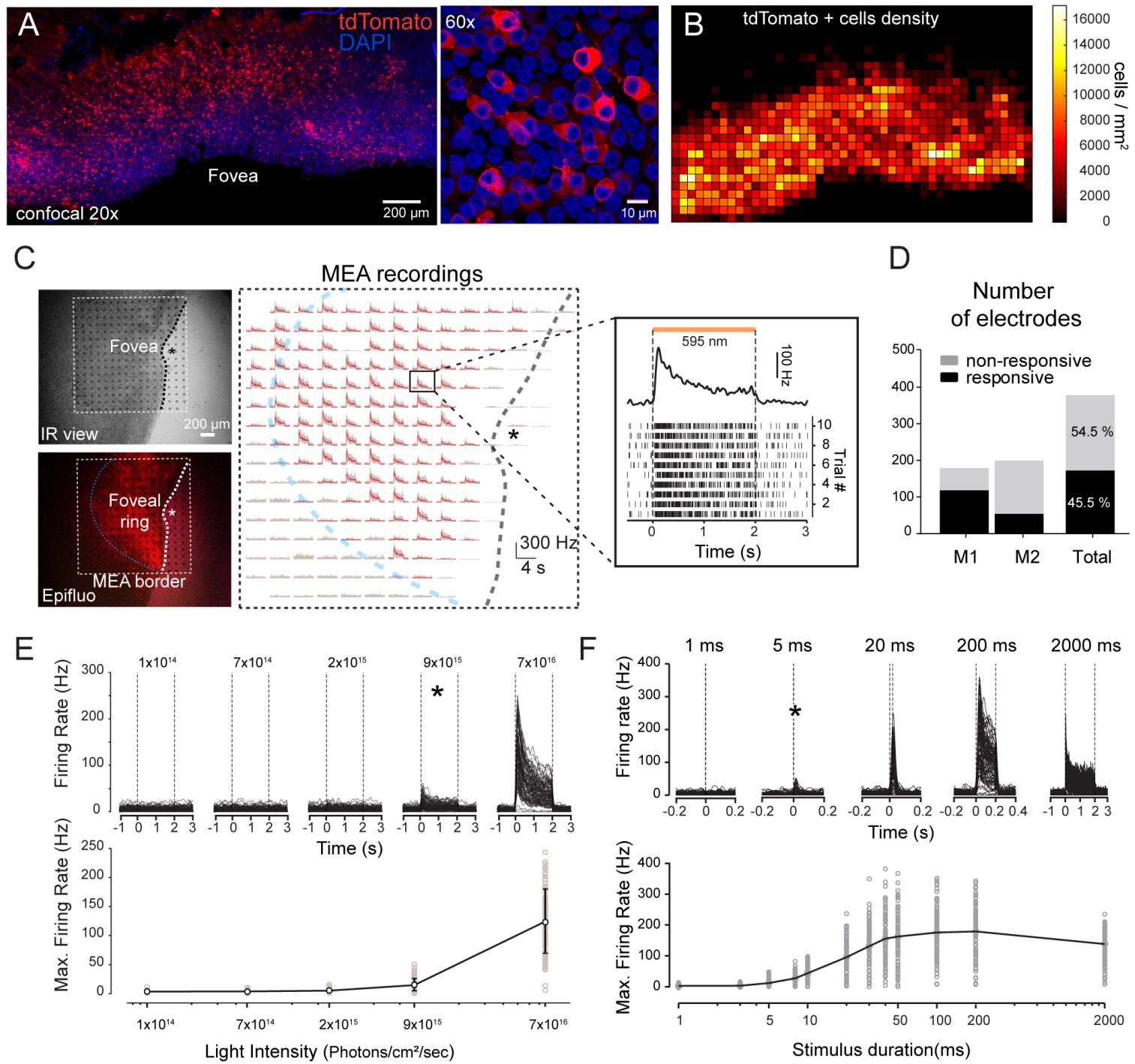
462 (E) Peak responses for photocurrent (left, patch-clamp data, $n = 12$ cells) and firing rate (right, 463 extracellular recordings, $n = 32$ cells) recorded in RGCs for a light stimulus at a wavelength of 595 nm 464 and an intensity of 3.2×10^{17} photons.cm 2 .s $^{-1}$.

465 (F) Spectral tuning. RGC response (raster plot and firing rate) as a function of the stimulus wavelength 466 tested, for wavelengths between 400 and 650 nm ($n = 3$ cells). The peak response (asterisk) was 467 observed at 575 nm. The black curve represents the mean cell response.

468 (G) RGC response as a function of stimulus duration. Raster plot (bottom) for nine cells and firing rate 469 (top), with stimuli of increasing duration, from 20 ms to 4 s. The black curve represents the mean cell 470 response.

471 (H) Temporal resolution. RGC activity as a function of stimulation frequency, represented as a raster 472 plot (bottom) and firing rate (top), for 10 flicker repeats at frequencies from 2 Hz to 28 Hz ($n = 9$ cells). 473 The black curve represents the mean cell response.

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477 **Figure 2. Expression and functionality: multi-electrode array recordings**

478 (A) Projection of confocal stack stitches (20x objective) showing the perifoveal area of M1 retina 20
479 months after treatment with a vector dose of 5×10^{11} vg/eye (left). Chr-tdT-expressing cells are shown
480 in red, and the nuclei (in blue) are stained with DAPI. Scale bar: 200 μ m. (Right) Higher magnification
481 image (60x objective). Scale bar: 10 μ m.

482 (B) Density map of ChR-tdT-positive RGCs for the hemifovea shown in (A).

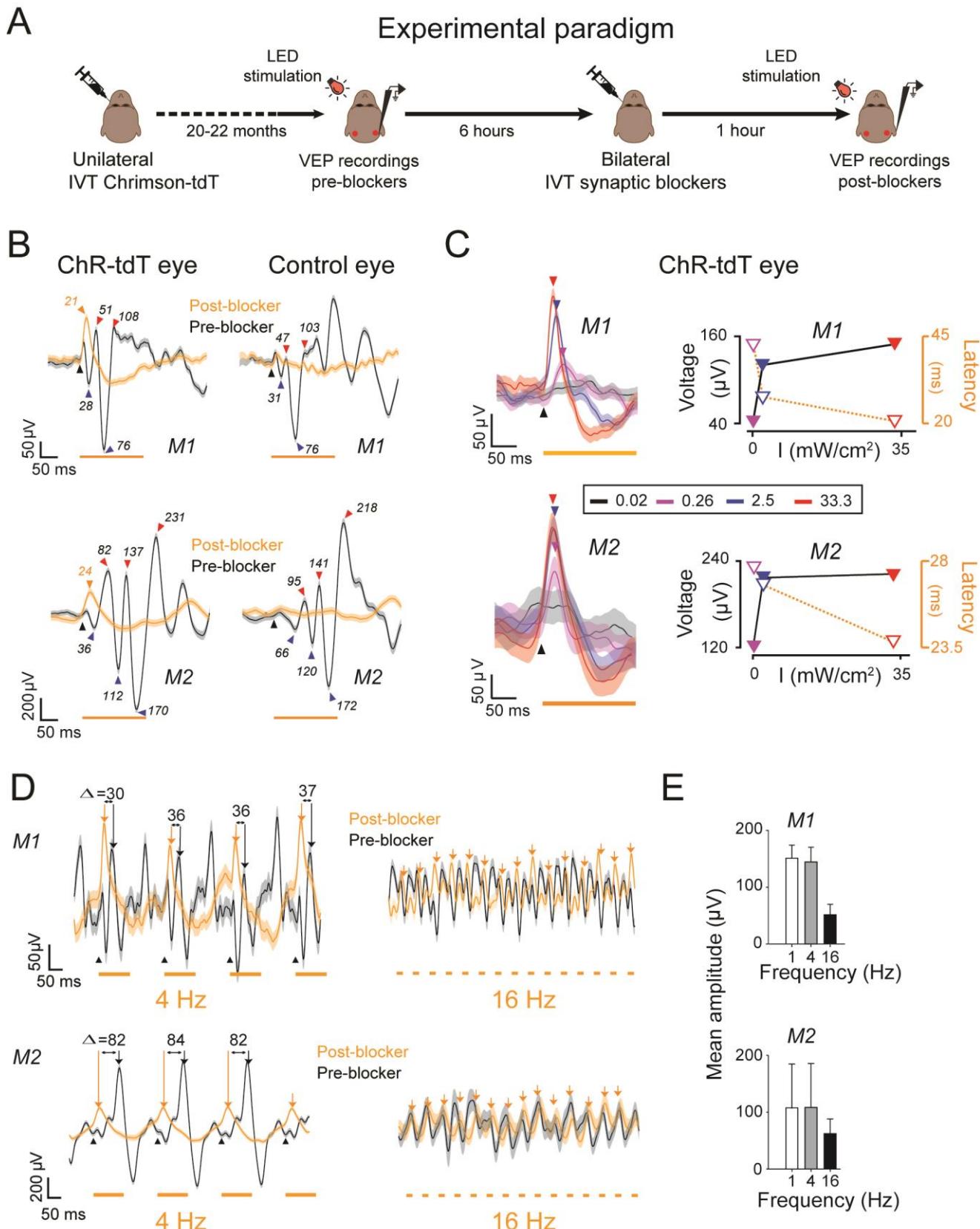
483 (C) Hemifovea RGC population recordings with a MEA (monkey M1). *Top left*, infrared view of the
484 hemifovea with a 256-site MEA placed on the side of the RGC layer. *Bottom left*, epifluorescence image
485 of the same retina showing tdTomato-ChrimsonR fluorescence. *Right*, global representation of the
486 recorded RGC activity (firing rate) of the 256 sites during a 4 s period, with the application of a 2 s flash
487 of light (at a wavelength of 595 nm). *Right, inset*, Raster plot (bottom) and spike-density function (top)
488 of a representative recording site with 10 repeats. The dotted squares represent the MEA borders, the
489 blue curves represent the approximate limits of Chrimson-tdTomato expression (half-donut shape) and
490 the asterisks represent the center of the fovea.

491 (D) Proportion of responsive versus non-responsive MEA electrodes for the retinae of the two primates
492 (NHPs M1 and M2).

493 (E) Firing rate (top) of 118 responsive electrodes (monkey M1 shown as an example) as a function of
494 light stimulus intensity (black curves) and mean response intensity curve for these neurons (bottom).
495 The asterisk represents the threshold light intensity for the elicitation of light responses. Light intensities
496 of between 1×10^{14} and 7×10^{16} photons.cm $^{-2}$.s $^{-1}$ were tested.

497 (F) Firing rate (top) of RGCs shown in (F) as a function of stimulus duration (black curves), and the
498 mean population response curve (bottom). The asterisk represents the threshold light duration required
499 to elicit light responses. Stimulus duration ranged from 1 ms to 2000 ms.

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503 **Figure 3: Cortical responses after retinal ChrimsonR optogenetic stimulation**

504 (A) Experimental set-up. Both animals received injections of AAV-ChrimsonR-Tdtomato into one eye.
505 We recorded VEP responses 20-24 months after the injection, following various protocols of LED flash
506 stimulation of the anesthetized animal. We then injected a synaptic blocker to block photoreceptor
507 transmission. Finally, we recorded VEP responses for the same LED flash stimulation protocols.

508 (B) VEP responses to the orange LED before (black curve) and after (orange curve) the injection of
509 synaptic blocker. The left column shows VEP responses following stimulation of the ChR-Tdt eye, and
510 the right column shows the response to stimulation of the control eye. VEP responses are shown in the
511 top row for monkey M1 and the bottom row for monkey M2. Black inverted triangles indicate the start of
512 LED stimulation, orange triangles indicate the peak latencies of VEP responses after treatment with a
513 synapse blocker. Red triangles indicate positive VEP peaks before injection, and blue triangles indicate
514 the negative phases. Numbers indicate peak latencies in ms.

515 (C) VEP responses to stimulation of the ChR-Tdt eye with different light intensities and frequencies. We
516 tested four different intensities at a frequency of 1 Hz (33.3, 2.5, 0.26 and 0.02 mW/cm²), as indicated
517 by the color code.

518 (D) We also tested two different light frequencies (4 and 16 Hz). We report the differences between
519 peak latencies in ms (e.g. Δ30). Orange arrows indicate positive phases of VEP responses.

520 (E). Mean amplitudes of positive phases of VEP responses for different light frequencies (1, 4 and 8 Hz)
521 for monkey 1 (*top, M1*) and monkey 2 (*bottom, M2*). Errors bars indicate SEM.

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523 **Supplementary Data**

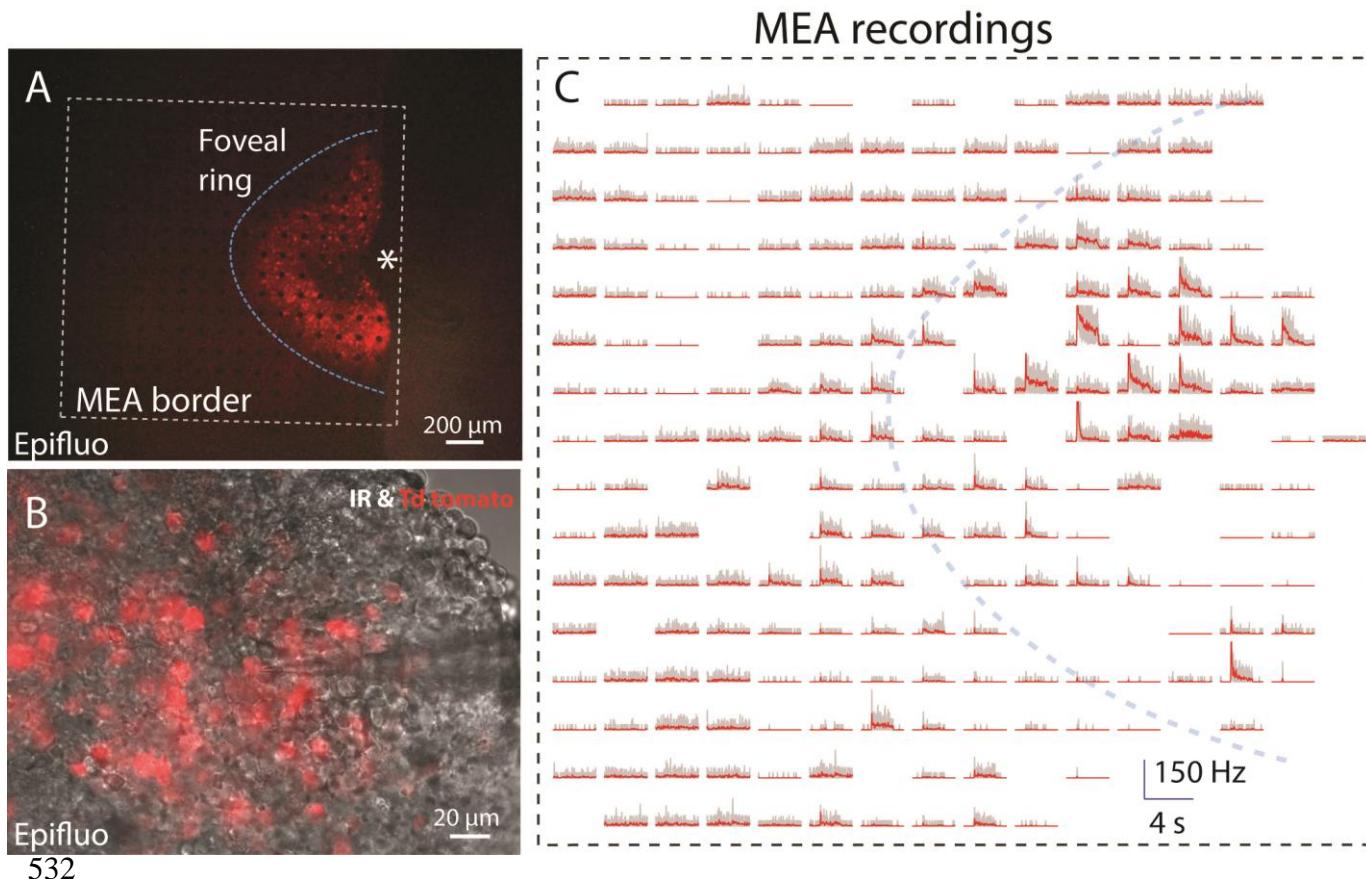
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525 Movie S1 (separate file). **2-Photon live imaging of a large volume of M1 hemifovea.**

526 A 500 x 500 x 150 μm volume of M1 retina was imaged at an excitation wavelength of 1050 nm to
527 visualize cells expressing the fluorescent reporter TdTomato. ImageJ and the 3Dviewer plugin were
528 used to create a 360° video of the imaged area, revealing a high density of fluorescent cells in the RGC
529 layer.

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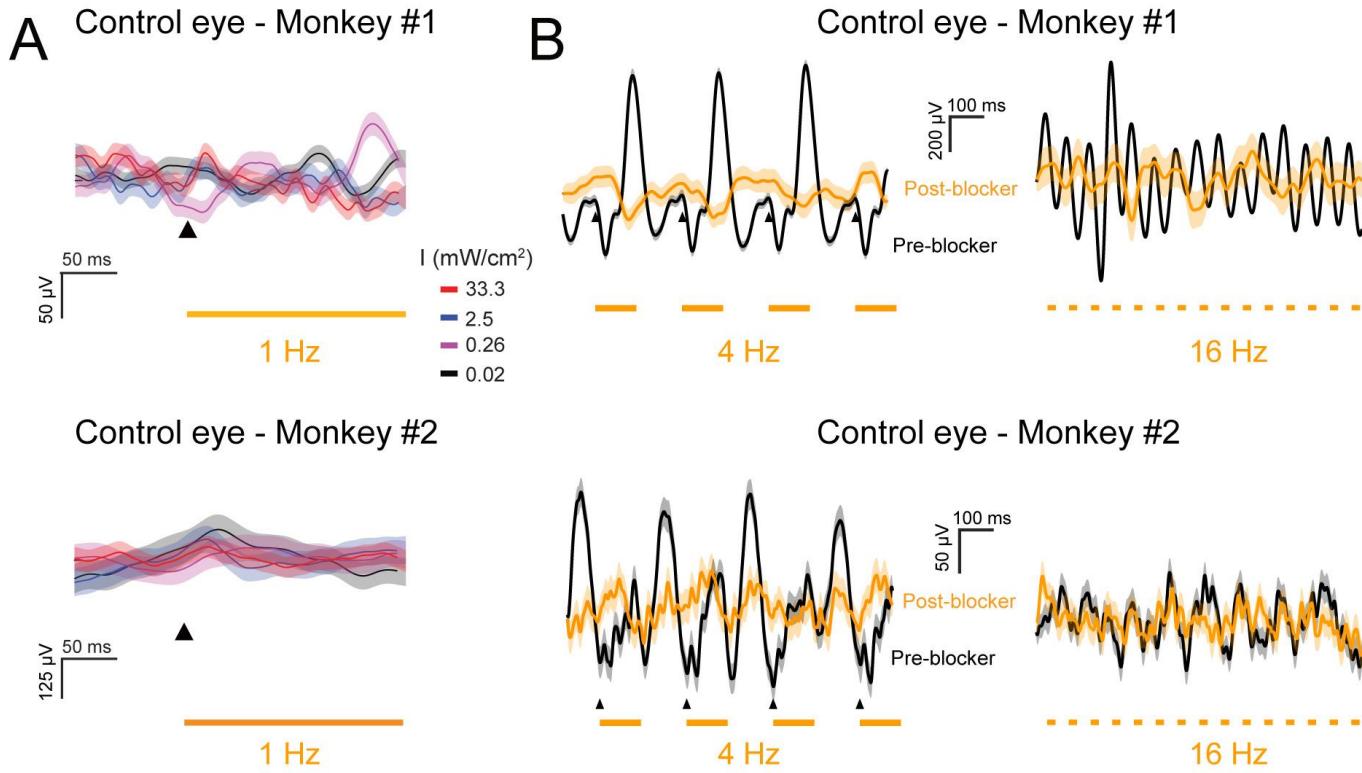


533 Fig. S2. **Expression and functionality: monkey M2.**

534 (A) Epifluorescence image of monkey M2 hemifovea showing tdTomato-ChrimsonR fluorescence, and
535 with a 256-site MEA placed against the RGC layer.

536 (B) Combined infrared and epifluorescence image of tdTomato fluorescent cells during single-cell
537 recordings with a glass electrode (patch-clamp or extracellular recordings). Images were acquired from
538 the second hemifovea.

539 (C) Global representation of the recorded RGC activity (firing rate) for the 256 MEA recording sites
540 shown in (A) over a period of 4 s, with the application of a 2 s flash of light (at a wavelength of 595 nm).
541 The dotted squares represent the MEA borders, the blue curves represent the approximate limits of
542 Chrimson-tdTomato expression (half-donut shape) and the asterisk represents the center of the fovea.
543



544
545 Fig. S3. **VEP recordings in the control eye.**

546 (A) VEP responses to the orange LED when the control eye was stimulated with light at different
547 intensities (top: monkey M1 and bottom: monkey M2). We tested 4 different light intensities at a
548 frequency of 1 Hz (33.3, 2.5, 0.26 and 0.02 mW/cm^2), as indicated by the color code.

549 (B) VEP responses to the orange LED when the control eye was stimulated at two different frequencies
550 (4 and 16 Hz). Orange traces represent VEP traces recorded after the injection of synaptic blocker;
551 black traces were recorded before the injection of synaptic blocker.