

Differential stimulation of the retina with glutamate toward a neurotransmitter-based retinal prosthesis

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ABSTRACT

Subretinal stimulation of the retina with neurotransmitters, the normal means of conveying visual information, is a potentially better alternative to electrical stimulation widely used in current retinal prostheses for treating blindness from photoreceptor degenerative diseases. Yet, no retinal stimulation study exploiting the inner retinal pathways exists. Here, we demonstrate the feasibility of differentially stimulating retinal ganglion cells (RGCs) through the inner nuclear layer of the retina with glutamate, a primary neurotransmitter chemical, in a biomimetic way. We show that controlled pulsatile delivery of glutamate into the subsurface of explanted wild-type rat retinas elicits highly localized simultaneous inhibitory and excitatory spike rate responses in OFF and ON RGCs. We also present the spatiotemporal characteristics of RGC responses to subretinally injected glutamate and the therapeutic stimulation parameters. Our findings could pave the way for future development of a neurotransmitter-based subretinal prosthesis offering more naturalistic vision and better visual acuity than electrical prostheses.

26 Introduction

27 Photoreceptor (PR) degenerative diseases such as retinitis pigmentosa and macular degeneration are
28 leading causes of presently incurable blindness. The irreversible vision loss in these diseases is ultimately
29 due to the loss of PR cells, which normally transduce visual information into chemical signals that
30 activate the retinal ganglion cell (RGC) layer through a network of neurons in the inner nuclear layer
31 (INL). Of the various strategies explored for treatment of PR degeneration over the last two decades,
32 including gene therapy and stem cell transplantation, retinal-based prostheses have emerged as the most
33 promising to restore vision in blind patients¹. The treatment strategy of retinal prostheses is predicated on
34 artificial stimulation of either RGCs or INL neurons. Direct stimulation of RGCs with a prosthesis in the
35 epiretinal (front of the retina) configuration bypasses INL neurons, while INL stimulation with a
36 prosthesis in the subretinal (behind the retina) configuration seeks to exploit the inherent visual
37 processing capability of INL neurons, which remain intact and functional despite the significant retinal
38 remodeling subsequent to PR degeneration^{1,2}. The latter scheme offers the possibility of differentially
39 stimulating the OFF and ON pathways of the retina at the synaptic layer where the separation between
40 these parallel pathways originates. These parallel pathways separate visual stimuli into OFF and ON
41 components (e.g. a point of light would excite the ON pathway and inhibit the OFF) and play a major role
42 in processing and mediating contrast sensitivity in visual perception³. Since the distinction between the
43 OFF and ON components is maintained throughout the entire visual system, differential stimulation of
44 these pathways is critical to restoring naturalistic vision to patients with PR degeneration with a retinal
45 prosthesis. In addition, a retinal prosthesis must be able to localize the stimulation ideally to a single cell
46 or a few cells to achieve high spatial specificity, i.e., good visual acuity.

47 Presently, all existing subretinal prostheses designed for stimulating INL neurons employ electrical
48 current to stimulate target neurons. Though electrical stimulation has been optimized to evoke functional
49 RGC responses, electrical current activates all types of retinal cells including their axons
50 indiscriminately^{4,5}. This non-selective nature of electrical stimulation results in the simultaneous

51 excitation of both the OFF and ON pathways in the INL, creating unnatural and confusing perceptions in
52 patients^{5,6}. Although some groups are actively exploring techniques to generate quasi differential
53 stimulation effects in the retina with electrical current^{7,8}, electrical-based prostheses are unlikely to restore
54 naturalistic vision since this form of quasi differential stimulation does not engage INL circuitry. Another
55 major drawback of electrical stimulation is that it requires large-diameter electrodes to safely handle the
56 high currents required to stimulate degenerated retinas without tissue damage^{1,5}. This has limited the
57 visual acuity that electrical prostheses in clinical use today can restore to levels (LogMARs 1.43–2.9)
58 much worse than the legal definition of blindness (LogMAR 1.0)⁹. Thus, at present, restoration of high
59 resolution naturalistic vision with electrical-based prostheses remains elusive despite significant
60 advancements in retinal prosthesis technology over the past two decades^{9–12}, and therefore, it is
61 worthwhile to explore alternative agents that could stimulate the retina more effectively along established
62 visual pathways.

63 A neurotransmitter chemical, particularly glutamate, the main agent of intercellular communication in
64 the normal retina, has long been suggested as a more effective stimulus agent than electrical current for a
65 retinal prosthesis, though very few studies have explored the feasibility of retinal stimulation with
66 neurotransmitters^{13–16}. Two studies^{15,16} that have demonstrated the feasibility of glutamate stimulation of
67 in-vitro preparations of whole retinas focused on direct stimulation of RGCs from the epiretinal side.
68 These studies showed glutamate to be effective and advantageous over electrical current in stimulating
69 RGCs, but since they bypassed INL neurons, they did not take advantage of the intrinsic visual processing
70 capability of INL circuitry. In the normal retina, visual information processed by photoreceptors is
71 separated and transmitted to the OFF and ON pathways at photoreceptor-bipolar cell synapses through the
72 release of glutamate onto a variety of glutamate receptors in the outer plexiform layer (OPL)¹⁷. Evidence
73 from numerous studies on retinal degeneration modeling indicates that INL cell types continue to exhibit
74 glutamatergic responses even in late stages of degeneration, although the glutamate sensitivity may be
75 reduced or eliminated in some cases^{2,18–23}. Theorizing that the loss of glutamate sensitivity is likely caused

76 by the lack of glutamatergic input from PRs, these studies suggest that early therapeutic intervention of a
77 degenerated retina could preserve glutamate sensitivity in INL neurons whereas treating late stage
78 degeneration may be possible, albeit difficult. Based on this evidence, it has been hypothesized that
79 subretinal glutamate stimulation of the INL of a degenerated retina could activate RGCs through the
80 amacrine-bipolar-RGC pathway and allow for differential stimulation of the OFF and ON pathways of
81 visual circuitry²⁴. The possibility of activating RGCs through INL neurons has a paradigm-shifting
82 implication for retinal prosthesis technology because this biomimetic stimulation of the retina could
83 restore high resolution naturalistic vision to patients with PR degeneration. Yet, no experimental study on
84 subretinal chemical stimulation has been reported.

85 Here we address the feasibility of differentially stimulating the retina with glutamate from the
86 subretinal side and present the spatiotemporal characteristics of the glutamate-evoked RGC responses
87 obtained by setting up an experimental platform that allows easy access to both the front and back sides of
88 explanted retinas. Using this platform, we convectively injected controlled volumes of 1.0 mM glutamate
89 70 μm into the subsurface of in-vitro preparations of wild-type rat retinas via 10 μm -diameter tip
90 micropipettes while recording the RGC responses on the other side with a multielectrode array (MEA).
91 Glutamate was focally delivered over each RGC receptive field (RF) center using a pulsatile pressure of
92 0.69 kPa and 10-30 ms pulses. The RGC RF centers were determined a priori by stimulating the retinas
93 with Gaussian white noise (GWN) checkerboard stimuli and conducting spike-triggered averaging (STA)
94 of the recorded spike trains.

95 **Results**

96 **Differential stimulation of RGCs through INL neurons**

97 To investigate if subretinally injected glutamate stimulated RGCs through INL neurons, we analyzed
98 multiple sets of MEA-recorded data comprising RGC spikes evoked by GWN and glutamate stimuli from
99 18 retina samples. First, we identified a total of 1654 spiking units from all retina samples through spike

100 sorting and then classified populations of RGC subtypes viz., OFF, ON, ON-OFF, or visually
101 nonresponsive, on the basis of their spike responses to GWN stimulation. Next, we examined RGC spike
102 responses to glutamate stimulation from all retinas and found that 551 (of the total 1654) units were
103 responsive to glutamate. The glutamate-responsive units were found to be present across all RGC
104 subtypes, including cells that did not exhibit robust visual responses to GWN (Table 1a), which indicates
105 that glutamate likely stimulated some RGC subtypes, such as directionally selective cells, that could not
106 be easily identified through GWN stimulation²⁵. An analysis of the peristimulus time histograms (PSTHs)
107 of glutamate-responsive units revealed that glutamate evoked both excitatory and inhibitory responses in
108 RGCs (see Supplementary Fig. S1) with some RGC subtypes showing a preference toward one or the
109 other (Table 1b). ON RGCs exhibited almost exclusively excitatory responses, while OFF RGCs
110 displayed a mix of purely excitatory, purely inhibitory or both excitatory and inhibitory glutamate
111 responses. Furthermore, OFF RGCs switched their response behavior (i.e., from excitatory to inhibitory
112 or vice versa) as the glutamate injection site moved relative to their RF centers (Fig. 1), with significantly
113 ($p < 0.01$, two-proportion z-test) higher likelihood of cells expressing inhibitory responses to injections
114 close to their RF centers and excitatory responses to injections farther away.

115 The presence of inhibitory responses to glutamate, primarily from OFF RGCs, demonstrates indirect
116 stimulation of RGCs through other presynaptic neurons because RGCs have strictly excitatory responses
117 to glutamate¹⁷. Excitatory responses, however, might result from either direct activation of RGC synapses
118 and/or those of presynaptic neurons. Furthermore, we observed differential responses (i.e., simultaneous
119 excitatory ON and inhibitory OFF RGC responses to a given stimulus) in about 60% (66 out of 114 sets)
120 of the glutamate injections that elicited both OFF and ON RGC responses (see Fig. 2) and purely
121 excitatory responses suggestive of direct activation of RGCs in the remaining 40% of glutamate
122 injections. These observations, specifically the high proportion of simultaneous differential responses,
123 strongly demonstrate that exogenous glutamate can differentially stimulate the OFF and ON subsystems

124 and imply that glutamate can modulate RGC firing through different retinal neural pathways, most likely
125 INL neurons.

126 To confirm our inference that subretinal glutamate injections elicited differential responses through
127 INL neural stimulation, we also examined local field potentials (LFPs) recorded with MEA electrodes
128 near injection sites and found large amplitude transients (Fig. 3a). Previous research indicates that LFPs
129 originate from INL neurons^{26,27}. The glutamate-evoked LFP (gLFP) transients we observed were triphasic
130 with an initial rising phase (Fig. 3b) and usually preceded (Fig. 3c) significant spike rate responses (gSRs)
131 in nearby units. We recorded LFP data in a subset of recordings and identified 127 sets of glutamate
132 injections that yielded significant transient gSRs, of which 89 (70%) also induced significant gLFPs. The
133 presence of substantial gLFP activity, combined with the high proportion of inhibitory gSRs, supports the
134 conclusion that subretinally injected glutamate modulates INL neurons, such as bipolar or amacrine cells,
135 because RGC activity has been shown to only induce slight amplitude and temporal alterations to light-
136 evoked LFPs, also known as electroretinograms (ERG)²⁷.

137 **Spatial resolution of glutamate-evoked responses**

138 As mentioned above, achieving stimulation with high spatial specificity (i.e., high spatial resolution) is
139 vital to restoring good vision with a retinal prosthesis. We define spatial specificity as the spatial extent
140 over which a bolus of glutamate stimulates RGCs, evoking a significant response in either gSRs or
141 gLFPs. We investigated the spatial specificity of subretinal glutamate stimulation by quantifying the
142 distances of RF centers of all glutamate responsive RGCs from the injection site. Fig. 4a shows a color
143 plot of the spatial distribution of gSRs to injections, where the midpoint represents the injection site,
144 warmer colors indicate higher densities of cells responding, and cooler colors indicate regions with fewer
145 responsive cells. The plot reveals the median distance between RF centers and the injection site to be 180
146 μm and half of all gSRs were confined to distances ranging between 75-310 μm (lower-upper quartiles)
147 from the glutamate injection site. Because the MEA permits only a sparse sampling of RGCs, we fit the
148 data of Fig. 4a with a 2D Gaussian spread function to create a more continuous and presumably accurate

149 estimate of the spatial distribution of stimulation (Fig. 4b). This spread function fits the data well ($r^2 =$
150 0.99) and predicts that half of all glutamate-responsive RGCs lie within 230 μm of the injection site.
151 Similarly, we plotted the spatial distribution of gLFP responses (Fig. 4c) and a Gaussian distribution (Fig.
152 4d) of all gLFP responses to the same glutamate stimulation. The gLFP spatial distribution plot (Fig. 4c)
153 reveals the median distance between electrodes with gLFP responses and the injection site to be 245 μm
154 with half of all gLFPs confined to distances ranging between 110-575 μm (lower-upper quartiles) from
155 the glutamate injection site. The LFP data were also well fit by a Gaussian spread function (Fig. 4d) and
156 revealed the median distance of all glutamate-responsive LFPs to be 105 μm .

157 While characterizing the spatial specificity of glutamate responses, it is important to also consider the
158 response amplitudes as the amplitudes vary with the distance from the injection site. To investigate the
159 efficacy of glutamate stimulation around the injection site, we compared the gSR amplitudes normalized
160 by the peak amplitude of full-field flash-evoked responses for all RGCs in the vicinity of the chemical
161 delivery site. The amplitudes were normalized to allow for population-level comparisons across RGCs
162 because the amplitudes of spike rate responses to glutamate and light stimuli can vary widely from cell to
163 cell. Unsurprisingly, we found that RGCs expressed the largest normalized response amplitudes to
164 injections close to their RF centers ($< 200 \mu\text{m}$), where they exhibited response amplitudes roughly
165 equivalent to their peak full-field flash amplitudes. Injections farther from the RF center yielded
166 progressively and significantly ($p \ll 0.001$, Kruskal-Wallis) smaller normalized response amplitudes
167 (Supplementary Fig. S2a). An example of this relationship can be seen in Supplementary Fig. S2b, which
168 shows PSTH and raster plots for a representative RGC in response to injections at several distances from
169 the injection site, indicating the decrease in response amplitude with distance. A similar trend was
170 observed for gLFP amplitudes (Supplementary Fig. S2c). These data suggest that effective RGC
171 responses evoked by 1 mM glutamate injected into the retina at 0.69 kPa for 10-30 ms is spatially
172 localized within a distance of about 200 μm from the injection site. The stimulation effect decreases with

173 increasing distances of RGCs from the injection site, possibly due to decrease in lateral spread of
174 glutamate as well as robust natural mechanisms for glutamate removal in the retina²⁸.

175 **Temporal resolution of glutamate-evoked responses**

176 Any functional retinal prosthesis must be able to provide stimulation that is temporally naturalistic to
177 allow patients to detect motion, especially during activities like locomotion. Here, we define temporal
178 resolution as the highest rate of subretinal glutamate injections that evokes a significant response in either
179 gSRs or gLFPs. We studied gSR responses of 21 RGCs and gLFP responses at three electrodes
180 corresponding to varying injection frequencies from 0.25 Hz to 6 Hz. Fig. 5 shows PSTH plots for a
181 representative RGC (Fig. 5a) and the corresponding LFPs (Fig. 5b) for multiple frequencies of injections.
182 RGCs responded with gSRs to injection frequencies up to 3.5 Hz, but robust gLFP responses
183 corresponding to injection frequencies as high as 6 Hz were recorded. The results suggest that glutamate
184 may modulate the activity of INL neurons at higher frequencies, but cannot drive the RGC firing rates at
185 those higher frequencies. We speculate that this difference between gSR and gLFP response frequencies
186 may be due to the relatively low temperature (22°C) at which this study was conducted because previous
187 work has shown that the latencies of LFP responses are less affected by shifts in temperature than spike
188 rate responses²⁹. Extrapolating our data to body temperature (37°C) using the Q10 values reported by
189 Schellart *et al.*²⁹, we predict that gSR temporal resolutions as high as 14 Hz may be achievable.

190 **Discussion**

191 Pulsatile injections of glutamate, the primary retinal neurotransmitter chemical, delivered into explanted
192 rat retinas at the PR-bipolar cell synapse layer (approximately 70 µm below the PR surface) where the
193 ON and OFF pathways are first established, elicited robust physiological responses from RGCs. After
194 exploring the therapeutic ranges of the key stimulation parameters, viz., the glutamate concentration,
195 injection pressure, pulse width and frequency, we determined an optimal set of the stimulation parameters
196 to accomplish the three most important stimulation traits for a neurotransmitter-based subretinal

197 prosthesis: biomimetic differential stimulation, spatially localized stimulation and temporally naturalistic
198 stimulation. We found that small boluses of 1.0 mM glutamate convectively released into the INL of the
199 retina using 0.69 kPa and 10-30 ms pressure pulses can differentially stimulate retinal OFF and ON
200 pathways, eliciting colocalized inhibitory and excitatory responses in OFF and ON RGCs, respectively.

201 While exogenous glutamate released in the OPL was theorized to mimic the retina's natural synaptic
202 communication and differentially activate the ON and OFF pathways, the therapeutic set of parameters
203 necessary for practically achieving differential stimulation were unexpected given the multiplicity of
204 glutamate-responsive neuron types throughout the retina¹⁷. To examine the pathways by which glutamate
205 differentially stimulated OFF and ON RGCs, we sought to narrow down the cellular targets of the
206 exogenous glutamate in the INL. OFF and ON bipolar cells, which establish the distinction between these
207 pathways in the OPL, were the ideal intended targets to produce differential stimulation, but the presence
208 of almost exclusively excitatory ON responses combined with a mix of excitatory and inhibitory OFF
209 responses suggests that the injected glutamate most likely engaged an unexpected and more complex set
210 of retinal circuits. Based on our results, we can exclude stimulation of cell types that cause inhibitory ON
211 responses such as ON bipolar, photoreceptor, and horizontal cells^{17,30,31}. In fact, an examination of the
212 retinal pathways reveals that glutamate should only yield inhibitory OFF responses (without inhibitory
213 ON responses) if it acted on amacrine cells, most likely the AII cell that is particularly dominant in the
214 rodent retina^{32,33}. The AII cell bridges the rod and cone pathways by receiving glutamatergic input from
215 rod bipolar cells and conveying this information to ON bipolar cells through sign-conserving electrical
216 gap junctions and OFF bipolar cells via inhibitory (sign-inverting) glycinergic synapses³³. When excited
217 with glutamate, AII cells would therefore cause excitatory responses in neighboring ON bipolar cells and
218 inhibit nearby OFF bipolar cells, which corresponds well with our differential response data. In the
219 remainder of stimuli where differential responses were not observed, the excitatory OFF responses could
220 be the result of OFF bipolar cell stimulation, but the simultaneous and colocalized excitation of ON RGCs
221 is interesting and suggests either direct RGC stimulation or a mix of OFF bipolar and AII cell stimulation.

222 Additional work would be required to convincingly identify the cellular targets for differential stimulation
223 and localize glutamate delivery to those target cells. Nevertheless, our results demonstrate that subretinal
224 glutamate injections can reliably elicit differential responses in the OFF and ON pathways through
225 stimulation of INL neurons, with evidence pointing towards AII cell activation.

226 The spatial specificity (lower and upper quartile distributions of glutamate-responsive RGCs)
227 achieved with single-port glutamate stimulation of rat retinas in this study, if reproduced in human
228 retinas, correspond to visual acuities in the range 1.2-1.8 LogMAR, which compare favorably with those
229 reported for electrical prostheses in clinical use today. We speculate that even better visual acuities closer
230 to the legal definition of blindness may be possible with multi-site glutamate stimulation using a large
231 array of independently addressable injection ports, each providing spatially localized stimulation.
232 Furthermore, the maximum temporal stimulation rate of 3.5 Hz demonstrated at 22°C with pulsatile
233 glutamate injections is comparable to what has been deemed adequate for enabling rudimentary reading
234 and daily functions in patients using electrical prostheses in clinical trials¹². Higher temporal stimulation
235 rates are likely achievable with finer on-chip actuation of glutamate in a device interfaced with the retina
236 at physiological temperatures³⁴⁻³⁶.

237 Although an exogenous glutamate stimulation strategy is ultimately targeted for retinas with
238 degenerated PRs, we purposely conducted this study on wild-type rat retinas (with functional PRs) in
239 order to systematically guide the study by identifying RGC subtypes and their RF centers with GWN
240 checkerboard stimulation. Determination of RGC subtypes via GWN checkerboard stimulation facilitated
241 the analysis of gSRs to confirm whether or not differential stimulation was achieved with a particular
242 glutamate injection event. Identification of RGC RF centers enabled fine characterization of the spatial
243 localization of glutamate responses in terms of distances between the glutamate-responsive RGCs and the
244 injection site. Further work would be required to reproduce the findings of this study in degenerated
245 retinas. Nevertheless, since both bipolar and AII cells are known to preserve some level of glutamate
246 sensitivity even in late stages of degeneration², we conjecture that the results and conclusions of this work

247 apply to a degenerated retina, though glutamate stimulation efficacy may be reduced depending on the
248 level of retinal degeneration.

249 Our findings are encouraging for advancing the development of a neurotransmitter-based subretinal
250 prosthesis as a serious and more effective alternative to electrical-based prostheses for restoring useful
251 vision to patients with PR degenerative diseases. A neurotransmitter-based subretinal prosthesis could
252 potentially not only reduce the burden for external visual signal processing electronics, but also restore
253 more natural vision to patients by taking advantage of the retina's inherent visual processing circuitry. We
254 envision that a neurotransmitter-based retinal prosthesis could be enabled by fabricating a MEMS
255 (microelectromechanical-systems)-based microfluidic device featuring a high density array of
256 independently addressable hollow microneedles in the subretinal space that store and release pulsatile
257 boluses of neurotransmitters near target INL synapses (see Supplementary Fig. S3). To develop such a
258 device, a number of key parameters, including the device geometry (e.g., port diameters and spatial
259 resolution) and chemical delivery parameters (e.g., range of pressures and pulse widths of glutamate
260 injection), must be optimized. Although the stimulation parameters would have to be optimized for the
261 type and stage of retinal degeneration, the optimal set of glutamate stimulation parameters determined
262 here offers baseline parameters to guide future work towards the development of a neurotransmitter-based
263 retinal prosthesis.

264 **Methods**

265 **Study Design**

266 The study design was based on controlled laboratory experiments involving in-vitro stimulation of
267 explanted rat retinas with glutamate. The objective of the study was to convectively inject pulsatile
268 boluses of glutamate chemical into the subsurface of retinas to evoke physiological responses comparable
269 to the normal light-evoked responses. Based on the findings of several pilot experiments carried out to
270 investigate therapeutic ranges of various stimulation parameters (glutamate concentration, injection

271 pressure, pulse width and frequency), an optimal set of therapeutic stimulation parameters (1 mM
272 glutamate, 0.69 kPa, 10-30 ms, 0.25-6 Hz) that elicited robust glutamate-evoked retinal responses was
273 employed in this study.

274 Neurotransmitter-based stimulation was systematically investigated using a sample size of 18 retinas,
275 which was determined by the total number of glutamate-responsive RGCs identified across all stimulation
276 trials in this study. Data collection was completed once a total of at least 500 glutamate-responsive RGCs
277 were identified to provide a sufficiently large representative dataset for meaningful analyses and
278 conclusions. The only data excluded from data analyses were responses from RGCs with abnormal spike
279 shapes (i.e. responses not quantitatively determined to be from either somal or axonal).

280 All animal experiments were conducted in accordance with the guidelines outlined by the National
281 Research Council's *Guide for the Care and Use of Laboratory Animals*. Animal handling and euthanasia
282 protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the
283 University of Illinois at Chicago.

284 **Retinal Sample Preparation**

285 Retinas were explanted from Hooded Long-Evans rats (PND 25-35, $N = 18$, either male or female;
286 Charles River Laboratories, Wilmington, MA) after they were dark-adapted for at least one hour and
287 euthanized by carbon dioxide followed by cervical dislocation. The sample size (18 retinas) was
288 determined by the total number of glutamate-responsive RGCs identified across all stimulation trials in
289 this study. Data collection was completed once a total of at least 500 glutamate-responsive RGCs were
290 identified to provide a sufficiently large representative dataset for meaningful analyses and conclusions.
291 The explanted retinas were placed onto a perforated MEA (pMEA200/30iR-Ti, Multichannel Systems,
292 GmbH) with ganglion cell side towards the electrodes. The MEA chamber was perfused (flow rate 3
293 mL/min) with Ames medium, which was oxygenated with a medical-grade gas mixture of 95% oxygen
294 and 5% carbon dioxide at room temperature (22°C). The retinas were left to stabilize from surgical
295 trauma on the MEA perfused with oxygenated Ames medium for at least 30 minutes before being

296 stimulated visually or chemically. A slight suction was applied at the bottom of the perforated MEA to
297 prevent the retina from moving due to the perfusion and to maintain a firm contact with the electrodes
298 throughout the entire duration of the experiment. All sample preparation work and stimulation
299 experiments in this study were conducted under dim red illumination to preserve the light sensitivity of
300 photoreceptors during data collection.

301 **Experimental Setup**

302 A special experimental platform (schematically illustrated in Supplementary Fig. S4) was built to enable
303 chemical injections into the retina tissue from the photoreceptor (top) side while simultaneously recording
304 the responses of retinal neurons at multiple sites around the injection site on the other (bottom) side
305 contacting the perforated MEA electrodes. The perforated MEA electrodes, each 30 μm in diameter, were
306 laid out in a grid (8 \times 8) pattern with an inter-electrode spacing of 200 μm . The extracellular voltages of
307 activated retinal neurons picked up by 60 electrodes of the perforated MEA were amplified and acquired
308 into a computer through an MEA system with MC-Rack software (MEA1060, Multichannel Systems,
309 GmbH).

310 Pre-pulled glass micropipettes (10 μm tip diameter and 1 mm outer diameter; World Precision
311 Instruments, Sarasota, FL) held in a standard microelectrode/micropipette holder (QSW-A10P, Warner
312 Instruments, Hamden, CT) were used to both store glutamate chemical and serve as a reference electrode.
313 The pressure port of the micropipette holder was coupled with a pressure injector system (PM-8, Harvard
314 Apparatus, Holliston, MA), which was used to inject pulses of glutamate into the retina tissue. The
315 micropipette tip was filled with glutamate buffered in Ames Medium (including NaCl and KCl for
316 impedance measurements) and electrically coupled to a patch clamp amplifier (Axopatch 200b, Molecular
317 Devices, Sunnyvale, CA) with a silver/silver chloride wire to form an electrode. Deviations in the
318 electrode impedance from the initial impedance, monitored through the patch-clamp amplifier output
319 signal, were used to determine contact of the micropipette tip with the retinal surface as the tip was
320 inserted into the retina and for any blockage of the pipette tip (by extracellular material) during an

321 experiment. The spatial position of the micropipette tip with reference to fixed reference points on the
322 MEA was controlled by a three-axis, motorized, precision manipulator (MP-285, Sutter Instruments,
323 Novato, CA) with sub-micron positioning accuracy.

324 The set-up also included a small liquid crystal display (Ruby SVGA Color Microdisplay, 800×600
325 pixels, Kopin, Westborough, MA) driven by a computer running Psychophysics Toolbox (PTB-3) in
326 MATLAB (MathWorks Inc., Natick, MA) to enable GWN checkerboard stimulation of the retina from
327 the bottom side and a green (570 nm) light-emitting diode (LED) to stimulate the retina with full-field
328 flashes of light from the top.

329 To achieve repeatable, high accuracy spatiotemporal modulation and synchronization of the chemical
330 and visual stimulus events and precise positioning of the micropipette tip in the subsurface of the retina in
331 each experiment, all of the main instruments (pressure injector, light sources, and the manipulator) in the
332 set-up were computer controlled via a digital-to-analog DAQ board (PCI-6251, 16-bit, National
333 Instruments) and custom scripts coded in LabView (National Instruments, Austin, TX). In addition, the
334 set-up was also equipped with a stereomicroscope (SMZ 745T, Nikon) supported on a gantry and a digital
335 camera (Moticam 5, Motic, Richmond, BC, Canada) to enable observations of the region near the
336 micropipette during the experiments.

337 **Stimulation Protocol**

338 Before starting the stimulation protocol, the health of the retina sample following the surgical procedure
339 was monitored by observing the spontaneous and light-evoked responses of RGCs for about 30 minutes.
340 Once stabilized, the retina was investigated according to the following major protocol steps: 1. The
341 locations and extent of RGC RF centers as well as RGC subtypes were identified using responses evoked
342 by a GWN checkerboard stimulus projected onto the RGCs from below, 2. The functional health of the
343 retina was assessed, and in some samples LFP responses were recorded, using responses evoked by a full-
344 field visual stimulus aimed at the photoreceptor-surface from above, and, 3. gSRs were recorded by
345 injecting boluses of glutamate targeted at the RF centers of RGCs, which had robust visual responses,

346 predominantly cells identified as OFF or ON RGCs. The details of the visual and chemical stimuli and the
347 stimulations performed in each of the protocol steps are described below.

348 The GWN checkerboard stimulus was a grayscale 24×32 pixel image generated with the LCD and
349 focused onto the retina using a camera lens such that the image projected by each checker on the retina
350 measured 100 μm × 100 μm corresponding to a viewing angle of 0.33° × 0.33°. The LCD frame rate was
351 60 Hz and checkerboard patterns were presented at a rate of 15 Hz. The mean illuminance of the LCD on
352 the epiretinal surface was 4.9 lm/m². An initial 25 min period of GWN checkerboard stimulation was used
353 to quickly characterize and locate RGC RFs for targeted stimulation, followed by a comprehensive 60
354 min stimulation to better characterize RF properties³⁷. The full-field flash stimulus was generated by the
355 green LED and comprised a series of flashes (5 lm/m², 1-2 sec ON and 1-2 sec OFF).

356 L-glutamic acid was mixed with Ames Medium (1 mM; Sigma-Aldrich, St. Louis, MO) and loaded
357 into glass micropipettes to permit glutamate stimulation. The micropipette tip was positioned near target
358 RFs with a micromanipulator (see Supplementary Fig. S5a). Once positioned above a target location, the
359 pipette was lowered until contact with the photoreceptor-surface was detected¹⁶ through an increase in the
360 impedance of the electrode and then inserted 70 μm below the surface to place the aperture near the OPL.
361 After positioning the pipette, glutamate was injected at the target location using pressure pulses of 0.69
362 kPa applied through the pressure injector system. Glutamate injection trials at each target location
363 comprised three sets of 30 pulses at a constant interpulse duration of 3 s, where the pulse width within
364 each set was maintained to be 10 ms, 20 ms, and 30 ms, respectively (Supplementary Fig. S5b). Similar
365 glutamate injection trials were repeated at multiple locations on each of the retina samples tested. Periods
366 of spontaneous activity and further full-field flashes were recorded between injections to track the health
367 of retinal neurons during and after glutamate stimulation.

368 In a subset of recordings (2 of 18 retinas), the frequency of glutamate injections was varied while
369 maintaining a constant pulse duration of 10 ms to investigate the temporal resolution of glutamate
370 stimulation. Interpulse durations ranging between 150 and 4000 ms, resulting in stimulation frequencies

371 ranging from 0.25-6 Hz, were tested. In these stimulation trials, the number of injection cycles was varied
372 in proportion to the stimulation frequency, i.e., fewer (minimum 30) cycles for low frequencies and larger
373 (maximum 200) cycles for high frequencies, in order to collect a sufficiently large quantity of data for
374 comparisons across all stimulation frequencies.

375 **Data Acquisition**

376 RGC spikes were recorded using MC_Rack (Multichannel Systems, 10 kHz sampling, after high pass
377 filtering, 200 Hz cutoff) with an amplitude threshold of approximately $-16 \mu\text{V}$. In a subset of our
378 recordings (9 of 18 retinas), raw voltage data (sampled at 10 kHz with no filtering) were recorded for
379 each electrode in addition to the filtered spike data acquired through MCRack. The raw voltage data were
380 processed in MATLAB by low pass filtering (20 Hz cutoff) and averaged across trials to produce average
381 LFP waveforms^{38,39}.

382 **Data Analysis**

383 ***Spike Sorting***

384 Spikes were sorted into individual units using Offline Sorter (Plexon Inc., Dallas, TX) and MATLAB.
385 Offline Sorter was employed to quickly identify units through principal component analysis (PCA) to
386 enable targeted injections at RF centers in subsequent recordings using data from the initial short GWN
387 stimulus. After concluding an experiment, data from all recordings were combined in MATLAB and
388 sorted into units using the wavelet clustering package, Wave_clus, developed by Quiroga et al.⁴⁰. Once
389 sorted, PSTHs were calculated from unit spike trains using Gaussian kernel density estimation to average
390 spike rates across trials^{41,42} with custom MATLAB code. Spike rate amplitudes were calculated as the
391 difference between extrema and the mean spike rate, with negative and positive amplitudes characterizing
392 inhibitory and excitatory phases, respectively (Supplementary Fig. S6a). If responses contained more than
393 one phase with amplitudes exceeding a spike rate threshold of 3 Hz, the largest amplitude was assumed to
394 be the dominant response.

395 Responsive units were identified by comparing fano factor derived response variables (FRVs) from
396 glutamate-evoked and spontaneous PSTHs as expressed in Eq. (1). Units were deemed responsive to a
397 glutamate stimulus if their glutamate FRV was greater than three standard deviations of its spontaneous
398 FRVs. Responsive units were also required to have spike rate amplitudes of 3 Hz or greater.

$$399 \quad \text{Fano factor response variable (FRV)} = \frac{f_{t1}}{f_{t2}} * f_{all} \quad \text{where } f_x = \frac{\sigma_x}{\mu_x} \quad (1)$$

400 Each fano factor (f_x) is calculated using different parts of the PSTH. The numerator (f_{t1}) is calculated
401 using the standard deviation (σ) and mean (μ) from the initial segments of the PSTH, usually $t < 0.5$ (0.1
402 for high frequency stimulation) seconds. The denominator (f_{t2}) is derived from the remainder of the PSTH
403 ($t > 0.5$ or 0.1 seconds) while the last term (f_{all}) is the fano factor using all time points. Responsive units
404 are identified by finding glutamate-evoked FRVs that exceed three standard deviations of the cell's
405 spontaneous FRVs. Implementation of this metric allowed fast detection of transiently responsive units
406 and was insensitive to the basal spiking deviations of individual units. Equation (1) was also used to
407 detect transient LFP responses using the LFP data in place of spike rate data with a threshold of 6
408 standard deviations (see below).

409 **LFP Analysis**

410 The raw recorded LFP data, which represent the responses of non-spiking retinal neurons, obtained for
411 full-field flash, glutamate injections, and spontaneous conditions were inverted compared to transretinal
412 ERG waveforms (since the reference electrode was placed above the distal retina). As such, all LFP
413 records shown have been inverted to facilitate comparison between each other and previously reported
414 ERGs^{26,43}. A modified FRV (Eq. (1) used in conjunction with LFP data instead of spike rate data) was
415 used to determine responsiveness, where responsive cells displayed glutamate LFP FRVs outside of 6
416 standard deviations of their spontaneous LFP FRVs.

417 **Receptive Field Analysis**

418 Following spike sorting, the location and extent of each RGC RF center was characterized using spike-
419 triggered averaging (STA) with the spikes recorded during GWN stimulation utilizing techniques
420 described by Cantrell *et al.*³⁷. The 30 GWN frames (2 s) preceding each spike were gathered into a large
421 array to assemble a spike-triggered ensemble (STE). The pixel values of the STE were normalized to a
422 grayscale with limits of -1 (black) to 1 (white). The STA was calculated by performing a vector average
423 of the STE, producing a set of 30 frames representing the average visual stimulus that generated spikes.
424 RGCs with substantial visually-evoked responses yielded STA frames showing large deviations (close to
425 1 or -1) from a mean background of 0 . The frame displaying the largest deviation from background was
426 identified and fitted with a 2D Gaussian to produce an estimated size and location for the RF center. The
427 extent of the RF center was assumed to be the area within a 1-standard deviation ellipse centered on the
428 RF midpoint and a threshold of 6 standard deviations from background was used to eliminate RGCs with
429 weak visually-evoked responses.

430 **STC-NC Analysis and RGC Subtype Identification**

431 Non-centered spike triggered covariance (STC-NC) analysis was completed following the techniques
432 described by Cantrell *et al.*³⁷. STC-NC analysis was initiated by implementing a spatial window of 5×5
433 pixels on the STE, aligned with the central checker derived from the STA. The windowed STE was then
434 examined with a non-centered PCA to find the eigenvectors with the greatest eigenvalues that maximize
435 the second moment of the STE around zero, thus estimating the linear filter that best predicts the
436 responses elicited. The output of this linear filter was used as an input for a static nonlinearity
437 representing the preference for an RGC to respond nonlinearly to certain stimuli (i.e. ON, OFF, or both)
438 and this combination (a linear-nonlinear model depicted in Supplementary Fig. S6b) was used to predict
439 spike probability. STC-NC analysis identifies RGC subtypes by estimating the static nonlinearity using
440 the measured linear filter (STC-NC eigenvectors) and the observed spike probability. The static
441 nonlinearity was thus calculated as the element-by-element quotient of the output of the linear filter for

442 the STE divided by the linear filter output for all stimulus frames. The resulting static nonlinearity vector
443 represents the probability for RGC spikes in response to light (stimulus standard deviations > 0) and dark
444 (stimulus standard deviations < 0) stimuli presented to the RF. The positive and negative segments of this
445 static nonlinearity vector were integrated to obtain scalar probabilities of responses to light (P_{ON}) and dark
446 (P_{OFF}) stimuli. These probabilities were used in Eq. (2) to determine the cell's bias, which allowed
447 identification of cells as either ON-center (bias ≥ 0.3), OFF-center (bias ≤ -0.3), or ON-OFF-center (-0.3
448 $< \text{bias} < 0.3$). RGCs with weak light responses and those lacking RFs were unclassified.

$$Bias = \frac{P_{ON} - P_{OFF}}{P_{ON} + P_{OFF}} \quad (2)$$

$$RGC \text{ Subtype} = \begin{cases} ON & Bias \geq 0.3 \\ ON - OFF & -0.3 < Bias < 0.3 \\ OFF & Bias \leq -0.3 \end{cases}$$

450 The bias was determined using the probabilities of light (P_{ON}) or dark (P_{OFF}) stimuli causing spike
451 responses, which were calculated from the static nonlinearity. RGCs with positive biases respond strongly
452 to light stimuli in their RF centers while those with negative biases respond strongly to dark. RGCs with
453 biases close to zero respond to both light and dark stimuli.

454 ***Spatial Properties of Glutamate Stimulation***

455 Although glutamate injections were specifically targeted at the RF centers of cells with robust ON- and
456 OFF-center biases, it is very likely that convective diffusion of glutamate resulted in stimulation of
457 surrounding cells as well. Therefore, to estimate the spatial localization of glutamate stimulation, the
458 vectors separating glutamate responsive RGC RF centers from the injection site were measured. These
459 vectors were spatially binned to form a 2D histogram showing the approximate location of responsive
460 RGC RF centers with respect to the injection site. These data were also fit with a 2D Gaussian spread
461 function to produce a more continuous representation of spatial localization.

462 The spatial properties were further investigated by binning cells based upon the distances separating
463 their RF centers from the injection site using 100 μm wide bins. For each bin, the normalized spike rate
464 amplitude (glutamate-evoked amplitude divided by peak full field flash amplitude) was calculated to
465 enable comparison across cells with widely different basal spiking rates.

466 **Statistical Analyses**

467 A one-sided, two-proportion z -test ($\alpha = 0.05$) was used to statistically compare the proportions of
468 inhibitory OFF RGC responses to glutamate injected inside the RF centers of cells with the proportion of
469 inhibitory responses to injections outside RF centers (Eq. (3)). A one-sided test was chosen to test the
470 hypothesis that inhibitory OFF responses were predominantly confined to injections inside the RF center.
471 This comparison showed that OFF RGCs with both excitatory and inhibitory responses possessed
472 significantly (p -value = 0.026) more inhibitory responses to injections inside their RF centers compared to
473 injections outside (difference in proportions = 0.18; z -statistic of 1.94).

$$474 \quad p_{\text{inside}} = \frac{N_{\text{inhibitory responses inside}}}{N_{\text{total responses inside}}} = \frac{38}{50} = 0.7$$
$$p_{\text{outside}} = \frac{N_{\text{inhibitory responses outside}}}{N_{\text{total responses outside}}} = \frac{27}{47} = 0.57$$
(3)

475 The normalized spike rate amplitudes described in the previous section were analyzed ($\alpha = 0.05$, $p <$
476 0.0001, Kruskal-Wallis test) to examine the differences between bins. This test was utilized because the
477 normalized spike rate amplitudes were non-normally distributed. The sample sizes of each bin is reported
478 in Supplementary Fig. S2a. A similar test was used to compare the non-normally distributed LFP
479 amplitudes ($\alpha = 0.05$, $p = 0.0489$, Kruskal-Wallis test) in Supplementary Fig. 2c.

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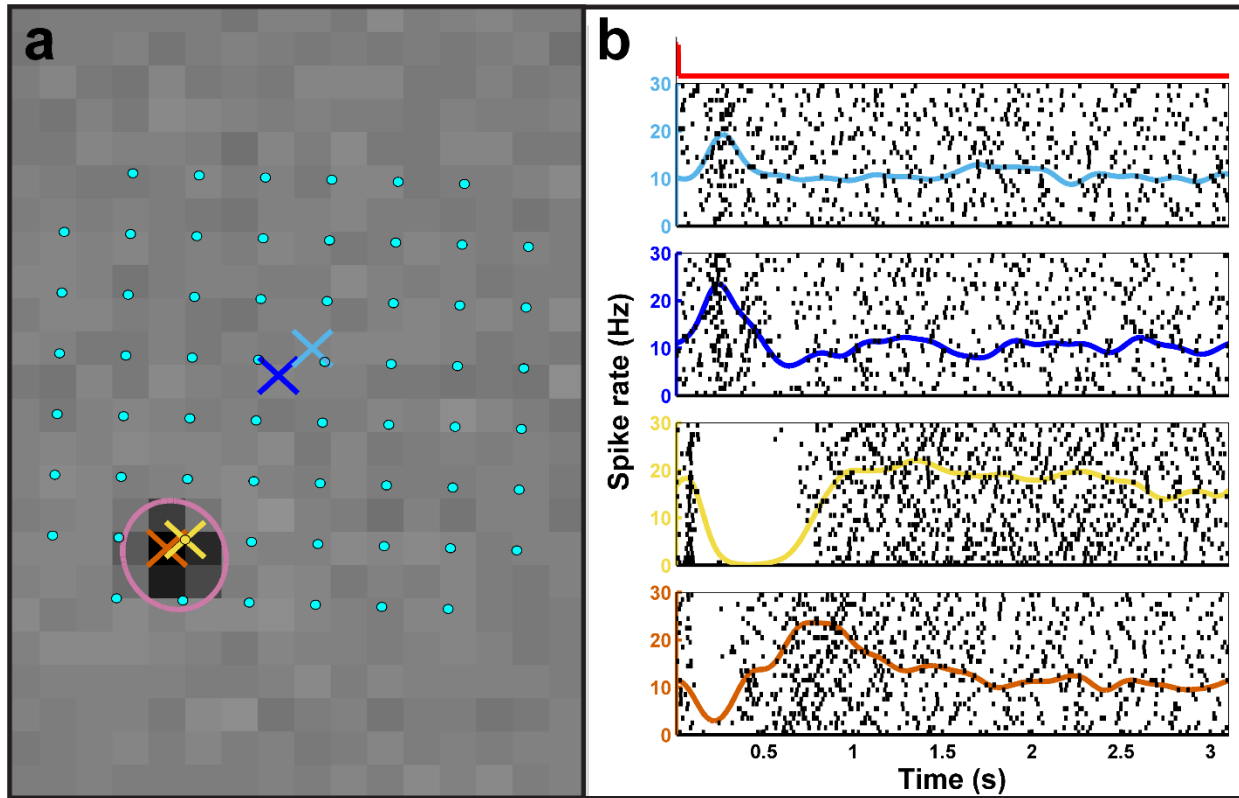
581 **Author Contributions**

582 All authors contributed to the design of the experiments and data analysis. C.M.R. and S.I. developed the
583 experimental setup with oversight from L.S. C.M.R. conducted the experiments, recorded and analyzed
584 the electrophysiological data. J.B.T. oversaw the visual receptive field and electrophysiological data
585 analysis. L.S. initiated and managed the project. All authors discussed the results and prepared the
586 manuscript.

587 **Additional Information**

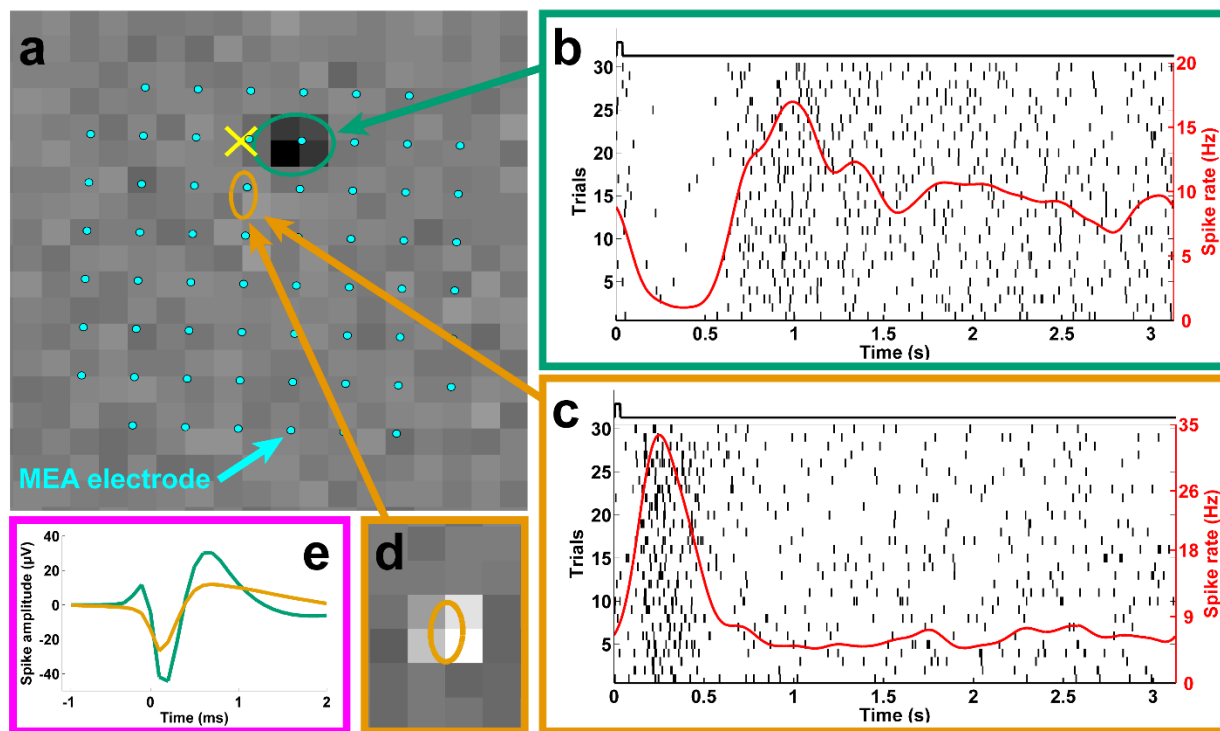
588 **Competing financial interests:** The authors declare no competing financial interests.

589 **Figures and Tables**



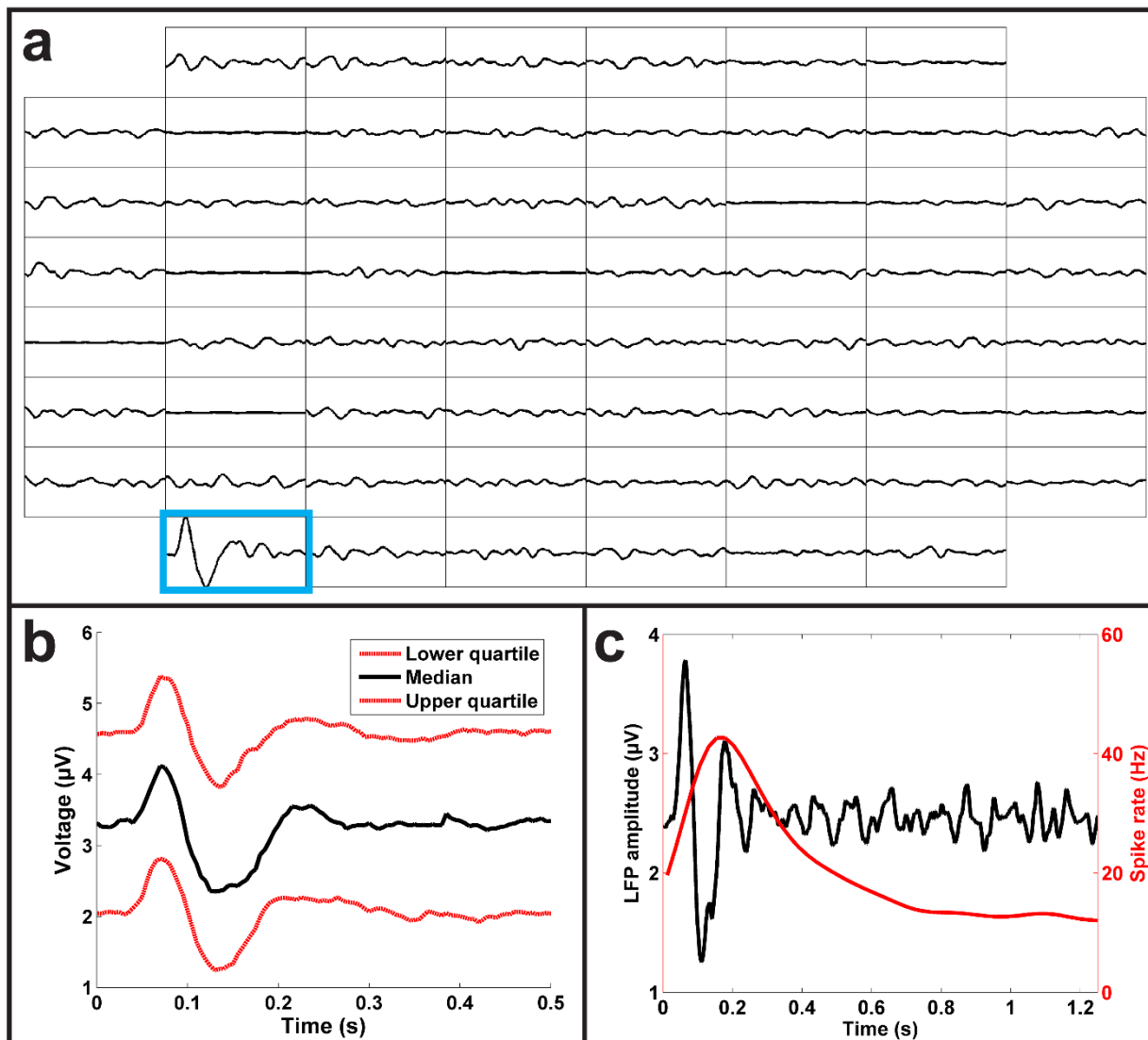
590

591 **Figure 1.** A subset of OFF RGCs responding to glutamate injections displayed both excitatory
592 and inhibitory responses dependent on the distance separating the injection site from their RF
593 centers. (a) A map showing the locations of MEA electrodes (cyan dots; 200 μm separation) and
594 the locations of four glutamate injections (colored 'x's) with respect to the RF center of a
595 representative OFF RGC (magenta ellipse) superimposed over this cell's peak STA frame.
596 (b) The spike rate and raster plots showing this cell's responses to the glutamate injections
597 corresponding to the 'x's in the spatial map arranged in descending order from farthest to
598 closest. The injection timing and duration for all glutamate injection events is indicated by the
599 red square wave above the topmost plot on the left. As can be seen, injections far from the RF
600 center elicited excitatory responses while those in or near the RF center evoked inhibition.
601 Similar patterns were seen in approximately 25% of glutamate-responsive OFF RGCs.

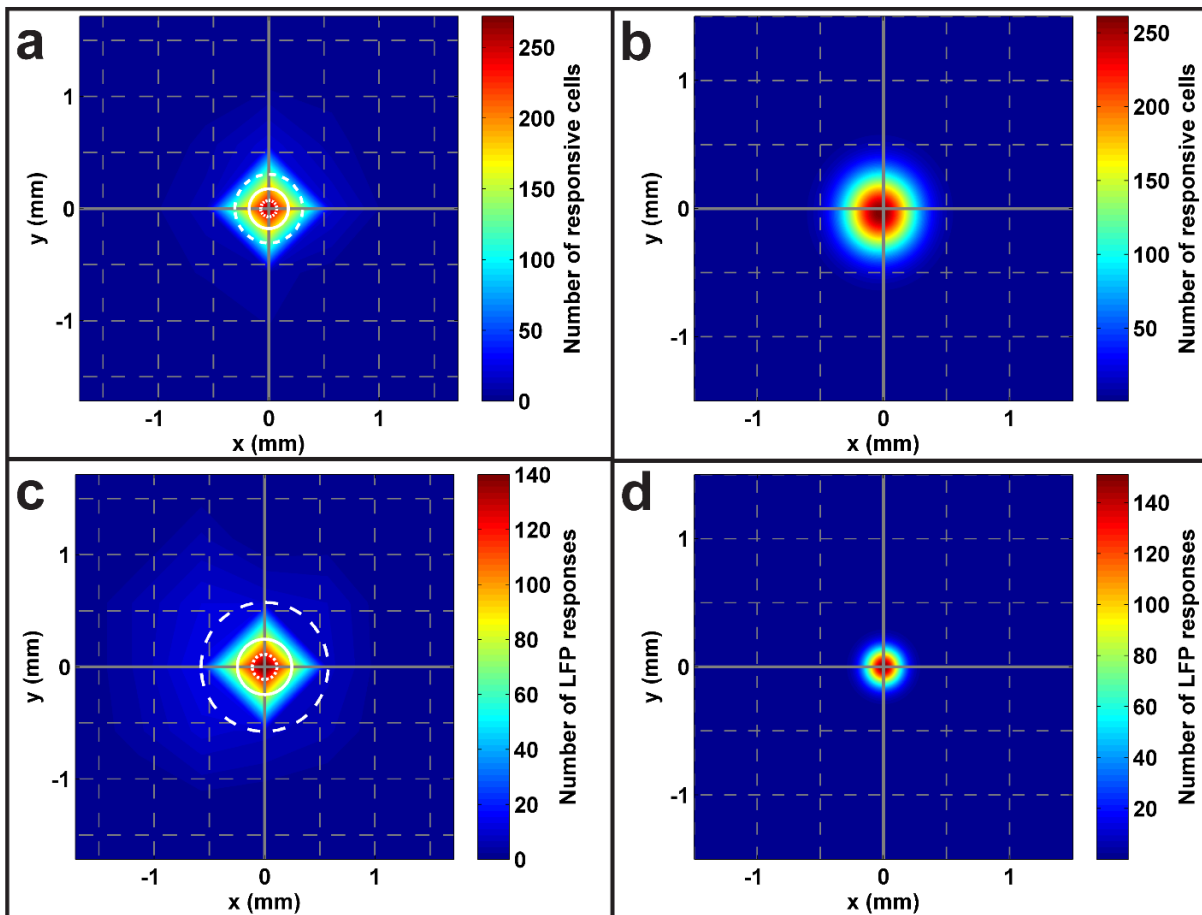


602

603 **Figure 2.** Subretinal glutamate injections elicit colocated and differential responses in the OFF
604 and ON pathways. (a) The locations of the receptive field centers for an OFF (green ellipse) and
605 an ON (orange ellipse) RGC relative to the injection site (yellow 'x') overlaid with 60 electrodes
606 of the MEA (cyan dots). The background displays the visually-evoked spike-triggered-average
607 (STA) for the OFF RGC. (b) and (c) The raster (vertical black lines) and spike rate (red traces)
608 for each cell. The black square waves above each plot indicate the timing and duration of each
609 glutamate injection event. The ON RGC (c) displayed excitation while the OFF RGC (b)
610 exhibited an inhibitory response to the same stimulus. (d) The STA for the ON RGC. (e) Two
611 unique spike shapes corresponding to the two units, which confirms that these are two distinct
612 cells. The different responses of OFF and ON RGCs to a common stimulus strongly suggests
613 differential stimulation of the OFF and ON pathways.

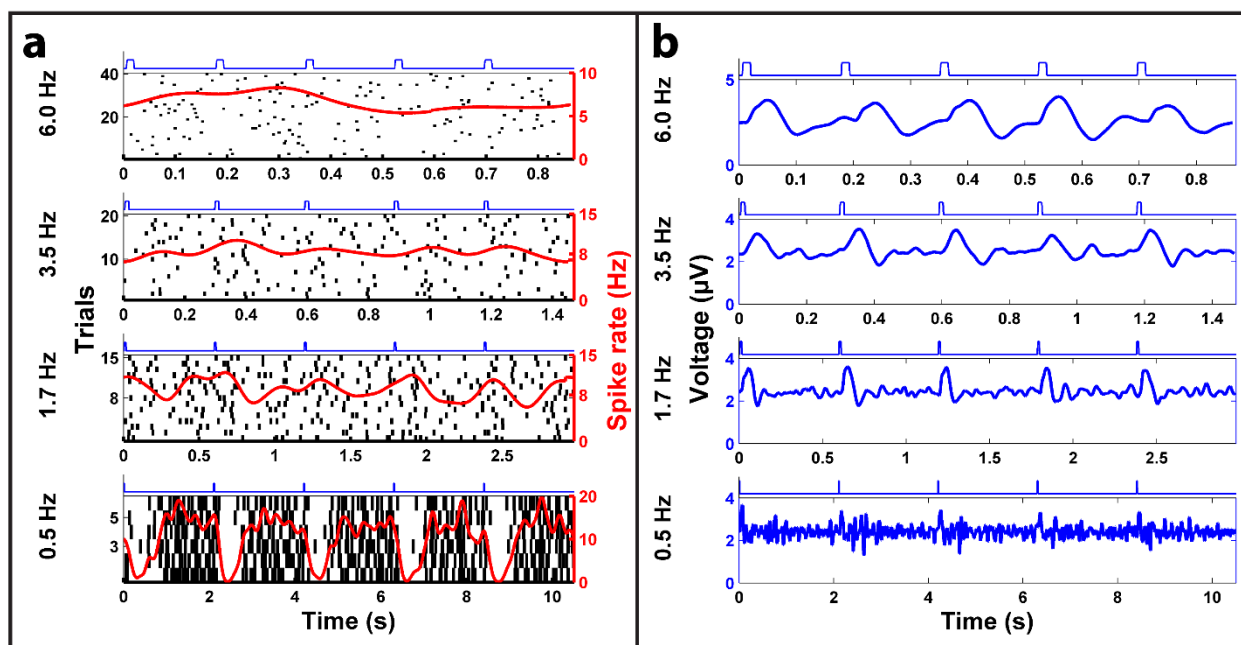


614
615 **Figure 3.** Subretinal glutamate injections elicited robust LFP responses. (a) Representation of
616 LFPs recorded from each of the 60 electrodes (interelectrode distance of 200 μm). The only trace
617 that shows a significant LFP response (outlined in blue) corresponds to the electrode where
618 glutamate was injected. (b) The lower (bottom red), median (black), and upper (top red) quartile
619 gLFP responses show a characteristic triphasic shape. (c) An overlay of the LFP (black) and
620 spike rate (red) responses from a representative ON RGC, demonstrating that LFP responses
621 preceded spike rate responses.



622

623 **Figure 4.** Subretinal injections elicit localized RGC responses. **(a)** A two-dimensional histogram
624 displaying the location of all RGC receptive field centers relative to the injection site (midpoint)
625 that were responsive to glutamate injected below the photoreceptor surface. The solid white
626 circle indicates the median distance of 180 μm while the inner and outer dotted/dashed circles
627 specify the lower (75 μm) and upper (310 μm) quartiles, respectively. Warmer colors represent
628 higher densities of RGCs as quantified by the color bar on the right. The grey gridlines are
629 separated by distances of 500 μm . **(b)** The data in (a) were fitted with 2D Gaussian functions to
630 produce a more continuous spatial distribution than predicted in (a). The Gaussian fit predicts a
631 median spread of 230 μm for subretinally injected glutamate. **(c)** and **(d)** Two-dimensional
632 histograms showing the spatial distribution and Gaussian fit for LFP responses, which yielded
633 median distances of 245 and 105 μm , respectively.



634

635 **Figure 5.** Frequency responses of subretinal glutamate stimulation. (a) Representative raster
636 plots of spike discharge for an OFF RGC. (b) LFP plots recorded from an electrode near the OFF
637 RGC in response to glutamate injections delivered at four different frequencies (indicated on the
638 far left). Each plot shows the average responses to multiple trials, where each trial consisted of 5
639 consecutive 10 ms glutamate injections whose timing is represented by the blue square waves
640 above each subplot. Glutamate injections delivered as fast as 3.5 Hz produced discernable spike
641 rate responses, while injections at the highest frequency (6 Hz) tested elicited robust LFP
642 responses.

643

a.	RGC subtypes		
	GR	GNR	Total
ON	60	53	113
OFF	86	101	187
ON-OFF	221	458	679
Unclassified	184	491	675
Total	551	1103	1654

b.	Response polarity		
	Excitation	Inhibition	Both
ON	55 (91.7%)	4 (6.7%)	1 (1.7%)
OFF	42 (48.8%)	26 (30.2%)	18 (20.9%)
ON-OFF	166 (75.1%)	30 (13.6%)	25 (11.3%)
Unclassified	125 (67.9%)	35 (19.0%)	24 (13.0%)
Total	388 (70.4%)	95 (17.2%)	68 (12.3%)

644 **Table 1.** Populations of RGC sub-types responsive to subretinal glutamate injections and their
645 response polarity. **(a)** Number of glutamate-responsive (GR) and glutamate-nonresponsive
646 (GNR) RGCs classified into subtypes. GNR units were mostly recorded on electrodes far from
647 the injection site. **(b)** Classification of GR RGCs based on their response polarity (excitation,
648 inhibition, or a mix of both, i.e., excitation to one set of injections and inhibition to another). ON
649 RGCs showed a strong preference towards excitatory responses while roughly half of all OFF
650 RGCs displayed some inhibition. ON-OFF and unclassified cells exhibited a strong preference
651 toward excitation though also presented inhibitory or mixed responses. When mixed, the change
652 in polarity creates a center-surround receptive field, with inhibitory responses evoked centrally
653 and excitatory responses evoked peripherally.