

# A novel approach to the functional classification of retinal ganglion cells

Abbreviated title: DREADD for RGC classification

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## 1 Abstract

2 Retinal neurones come in remarkable diversity based on structure, function and genetic  
3 identity. Classifying these cells is a challenging task, requiring multimodal methodology. Here,  
4 we introduce a novel approach for retinal ganglion cell (RGC) classification, based on  
5 pharmacogenetics combined with immunohistochemistry and large-scale retinal  
6 electrophysiology. Our novel strategy allows grouping of cells sharing gene expression and  
7 understanding how these cell classes respond to basic and complex visual scenes. Our  
8 approach consists of increasing the firing level of RGCs co-expressing a certain gene (*Scnn1a*  
9 or *Grik4*) using excitatory DREADDs (Designer Receptors Exclusively Activated by Designer  
10 Drugs) and then correlate the location of these cells with *post hoc* immunostaining, to  
11 unequivocally characterise anatomical and functional features of these two groups. We  
12 grouped these isolated RGC responses into multiple clusters based on the similarity of their  
13 spike trains. With our approach, combined with immunohistochemistry, we were able to extend  
14 the pre-existing list of *Grik4* expressing RGC types to a total of 8 and, for the first time, we  
15 provide a phenotypical description of 14 *Scnn1a*-expressing RGCs. The insights and methods  
16 gained here can guide not only RGC classification but neuronal classification challenges in  
17 other brain regions as well.

18

## 19 Keywords

20 DREADD, *Grik4*, *Scnn1a*, retinal ganglion cells, multielectrode array, classification

21

## 22 Introduction

23 The retina contains two types of photoreceptors, rods for dim light and cones for daylight and  
24 colour vision. Furthermore, cone-contacting bipolar cells can be divided into ON and OFF  
25 types and further subdivided into more than a dozen different subpopulations (1). These  
26 parallel processed channels are further divided into a variety of functional output channels,  
27 so-called retinal ganglion cells (RGCs), which encode different features of the visual

28 environment. There are ~1 million RGCs in the human retina and ~45,000 in the mouse retina  
29 (2,3), integrating visual information processed from photoreceptors down the retinal neural  
30 network. Different types of RGCs extract very specific features from the visual scenery (4).  
31 This code is transmitted to postsynaptic targets in the brain, leading to visual perception. At  
32 present, more than 40 RGC types have been identified in the mouse retina (5,6). RGC  
33 classification is typically based on common anatomical features (7,8), responses to light (5,9–  
34 11) or on shared gene expression (6,12–14). Classification based on gene expression is  
35 relatively recent, and the majority of RGC groups sharing specific genes have not been  
36 phenotyped yet.

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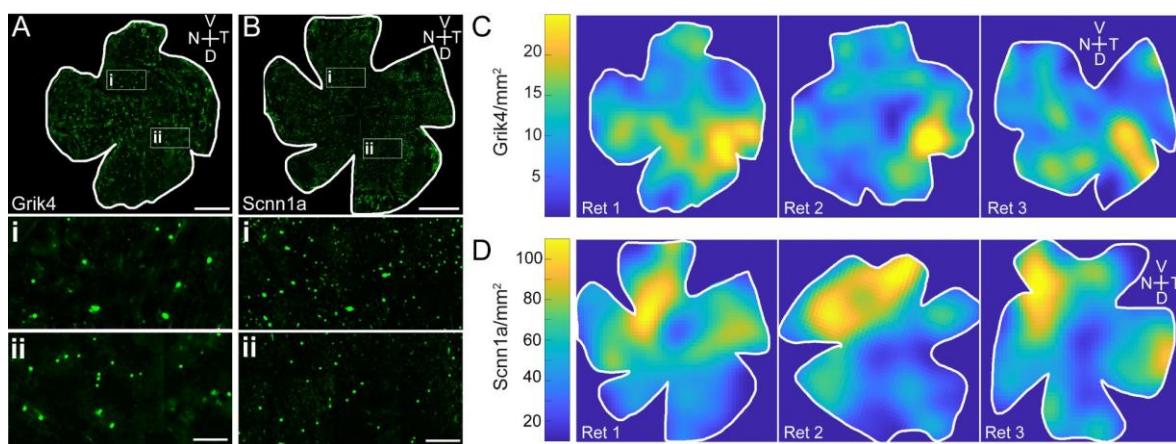
38 Current approaches for functional characterization of RGC subpopulations at pan-retinal scale  
39 are limited. Multielectrode arrays (MEAs) allow high throughput simultaneous electrical  
40 recording from the RGC population at high spatiotemporal resolution (15). Here we use a  
41 CMOS (complementary metal-oxide-semiconductor) MEA system consisting of 4,096  
42 electrodes (2.67 x 2.67 mm), allowing us to record light responses from hundreds to thousands  
43 of RGCs simultaneously at pan-retinal level and near cellular resolution (16,17). We selected  
44 two genes based on Cre lines availability, on the sparse expression distribution across the  
45 RGC layer and on their novelty for phenotypic characterization (Allen Mouse Brain  
46 Connectivity Atlas (2011)). *Grik4* (glutamate receptor, ionotropic, kainate subunit 4, HGNC:  
47 4582) expressing RGCs have already been partially described using a *Grik4* Cre mouse line  
48 (18). The other gene we investigated is *Scnn1a* (non-voltage gated sodium channel, epithelial  
49 1 subunit alpha, HGNC:10599). *Scnn1a* Cre-induced recombination (*Scnn1a*-Tg3-Cre line) is  
50 present in sparse Layer 4 neurones, mostly in the somatosensory cortex (19). Current  
51 knowledge of *Scnn1a* expressing RGCs in the retina is limited to the fact that their dendritic  
52 arbour stratifies in sublaminae S1 and S2 (OFF layers) and in sublamina S4 (ON layer) of the  
53 inner plexiform layer (13). Here, we crossbred *Grik4* and *Scnn1a* Cre mice with DREADD flox  
54 mice to further investigate these RGC types.

55 Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) (20) technology is  
56 a powerful new approach to pharmacologically dissect out the role of specific neuronal cell  
57 classes in network activity (21,22). DREADDs are an engineered version of G-proteins that  
58 allow precise control of G-protein signalling pathways and can be activated with clozapine-N-  
59 oxide (CNO), a “designer drug” with no endogenous receptors in the organism. Most  
60 commonly used DREADDs are excitatory (hM3Dq, triggering release of calcium from  
61 organelles leading to increase of intracellular concentration of free calcium and to membrane  
62 depolarization). In this study, we have generated Cre recombinase-mediated restricted  
63 expression of cell-specific DREADD (23) expression in either *Grik4* or *Scnn1a* reporter lines.  
64 We have successfully isolated light-evoked responses in RGCs sharing either *Scnn1a* or *Grik4*  
65 gene expression by combining excitatory DREADD activation, large-scale retinal CMOS MEA  
66 recording and *post hoc* labelling of DREADD-expressing RGCs. We grouped the RGC  
67 responses into multiple clusters based on the similarity of the spike trains they generate in  
68 response to a series of stationary stimuli. Our method was validated by successfully isolating  
69 existing *Grik4*-RGC types, but it also added several new candidates to the group. It provided  
70 novel classification for 14 subgroups of *Scnn1a*-RGCs.

71

## 72 Results

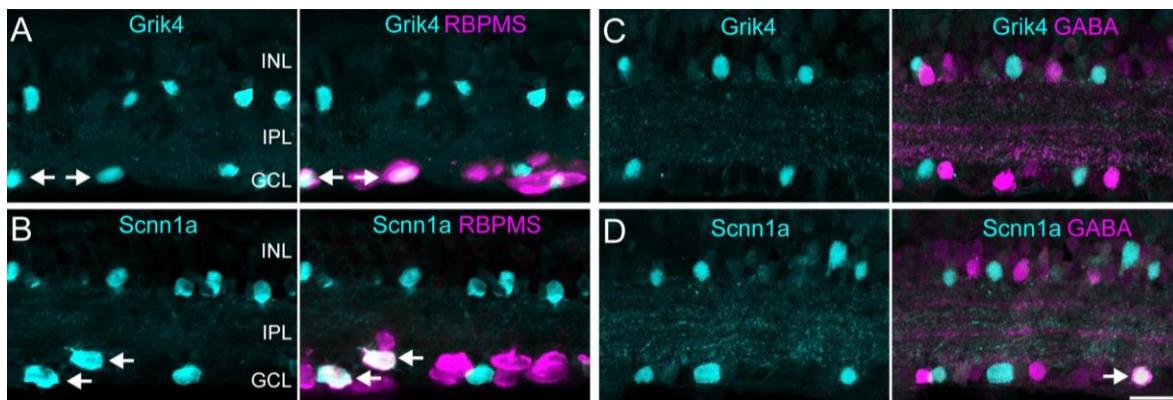
73 To functionally validate RGC subgroups according to shared gene expression, we first  
74 established an immunohistochemical atlas of these cells. Building such a resource for *Grik4*  
75 and *Scnn1a* expressing cells in the mouse retina is important for estimation of the RGC  
76 numbers and types in these two genetic pools. We used the intrinsic fluorescence signal of  
77 *Grik4*-DREADD (hereafter named *Grik4*) and *Scnn1a*-DREADD (hereafter named *Scnn1a*)  
78 cells to provide a detailed IHC map of *Grik4* and *Scnn1a* expressing cells in retinal whole  
79 mounts and vertical sections. Each DREADD is tagged with hemagglutinin (HA) as well as  
80 mCitrine, allowing to visualize DREADD-expressing cells by immunofluorescence. We first  
81 investigated the distribution of *Grik4* and *Scnn1a* cells in the ganglion cell layer (GCL) in retinal



*Figure 1: Grik4 and Scnn1a cells in the GCL are not homogeneously distributed. Whole mount antibody staining against Grik4 (A) and Scnn1a (B) DREADD GFP were imaged at the level of the GCL. All stained GFP cells were counted and the densities were calculated and presented in pseudocolors for 3 Grik4 (C) and Scnn1a (D) retinas. V= ventral, T = temporal, D = dorsal, N = nasal. Scale bar A, B = 1 mm; Scale bar A, B insets II = 100  $\mu$ m.*

82 whole mounts using an antibody against Green Fluorescent Protein (GFP) to amplify the  
83 intrinsic mCitrine signal (Fig 1). Both lines exhibit sparse cellular distribution in the GCL, with  
84 Scnn1a cells significantly more abundant than Grik4 (Fig 1 A, B insets I and II). We calculated  
85 the cell densities in the GCL of three representative Grik4 (Fig 1 C) and Scnn1a (Fig 1 D)  
86 retinas. Grik4 and Scnn1a cell densities respectively vary between 10-25 and 40-100  
87 cells/mm<sup>2</sup>. Both pools exhibit non-even distributions. Grik4 cells are more prominent in the  
88 dorsal-temporal periphery (Fig 1 C, yellow areas), while Scnn1a cells are more prominent in  
89 the ventral-nasal periphery (Fig 1 D, yellow areas).

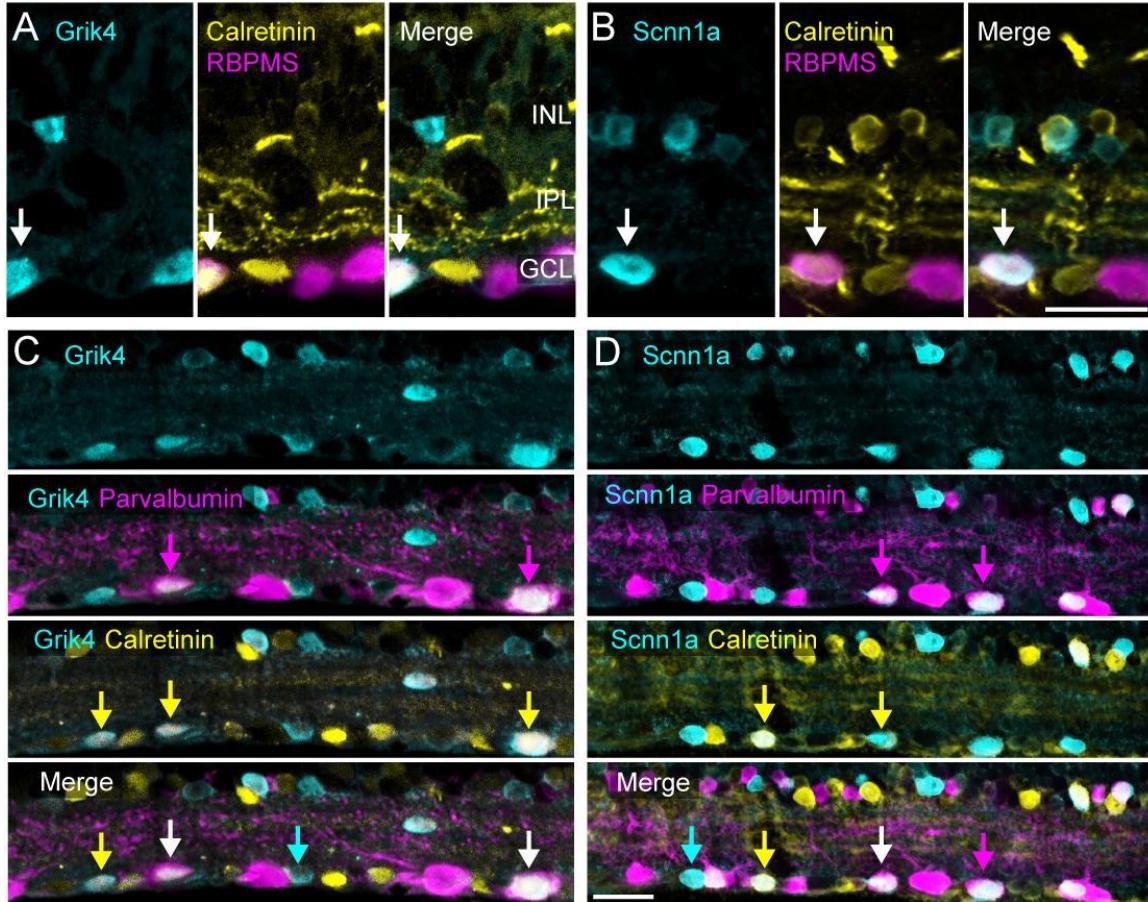
90 The GCL consists mainly of RGCs but it also contains displaced amacrine cells (dACs) and  
91 glial cells. We investigated whether dACs contribute to the pool of Grik4 and Scnn1a cells in  
92 the GCL. We used known specific RGC and amacrine cell (AC) markers to estimate the  
93 contribution of different RGC types in both gene pools. First, we double-labelled Grik4 and  
94 Scnn1a retinal vertical sections against GFP (Fig 2 A, B cyan) and RBPMS (Fig 2 A, B  
95 magenta), a selective marker for RGCs in the mammalian retina (24). More than 50% of the  
96 GFP labelled Grik4 cells in the GCL are RBPMS positive (Fig 2 A, arrows) whereas the other



**Figure 2: Grik4 and Scnn1a DREADD are expressed in RGCs and ACs.** Vertical sections were stained for Grik4 (A, C, cyan) and Scnn1a (B, D, cyan) GFP. Sections were further co-labelled with RBPMS (A, B, magenta), a marker for RGCs, and GABA (C, D, magenta). INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. Scale bar in D = 20  $\mu$ m.

97 cells are likely dACs. In support, the somata of putative dACs in the GCL are relatively small,  
98 which is a key feature of these cells. A similar pattern was found for Scnn1a cells in the GCL  
99 but here, the majority of Scnn1a cells are RGCs (Fig 2 B, arrows). Sparsely distributed cells  
100 expressing either Grik4 (Fig 2 C, cyan) or Scnn1a (Fig 2 D, cyan) were present in the proximal  
101 inner nuclear layer (INL). These cells did not stain for RBPMS (Fig 2 A, B), confirming they  
102 are amacrine cells (ACs). Most AC types are GABAergic, hence we initially stained for GABA  
103 (Fig 2 C, D, magenta) and GFP in Grik4 and Scnn1a vertical sections. Interestingly, we did  
104 not find any GABA-positive Grik4 ACs (Fig 2 C). On the other hand, we occasionally found  
105 GABAergic Scnn1a cells in the INL or GCL (Fig 2 D, arrow). In conclusion, a major proportion  
106 of Grik4 and Scnn1a cells in the GCL are RGCs but both genes are also sparsely expressed  
107 in ACs in the INL and in dACs in the GCL. Further, GABA is not present in Grik4 cells, but it  
108 is in a very small population of Scnn1a expressing cells.

109 To further characterize the expression patterns of Scnn1a and Grik4 in RGCs and ACs, retinal  
110 sections were stained for GFP, RBPMS and for the well-described calcium-binding protein  
111 markers Parvalbumin and Calretinin (Fig 3) (25,26). Briefly, Parvalbumin is found in ACs and



**Figure 3: Grik4 and Scnn1a DREADD are expressed in multiple RGC types.** Vertical sections of Grik4 and Scnn1a retinas were triple stained A, B) for GFP (cyan), RBPMS (magenta) and Calretinin (yellow) or C, D) GFP (cyan), Parvalbumin (magenta) and Calretinin (yellow). INL = inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. Scale bar in D = 20  $\mu$ m.

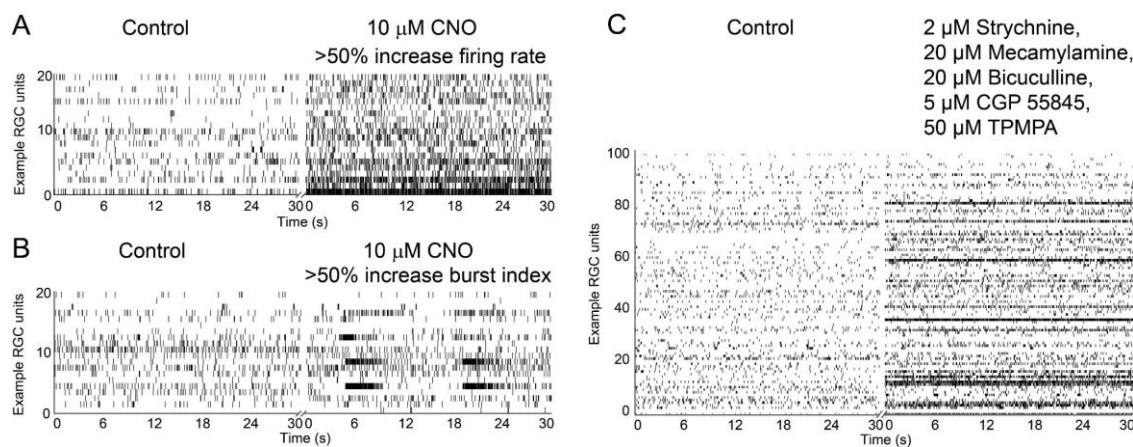
112 in 8-14 different RGC types. Calretinin is also present in ACs and in 10 RGC types. In a first  
113 set of experiments (Fig 3 A, B) we labelled respectively against GFP (cyan), RBPMS  
114 (magenta) and Calretinin (yellow) in vertical sections of Grik4 (Fig 3 A) and Scnn1a (Fig 3 B)  
115 retinas. For both retinas, we found a subset of Grik4 and Scnn1a RGCs that were  
116 GFP/RBPMS/Calretinin positive (Fig 3 A, B arrows) and GFP/RBPMS positive cells, which  
117 clearly demonstrates that there are at least two RGC types for each pool. We did not find any  
118 Calretinin positive Grik4 ACs, but we did find some Calretinin positive Scnn1a ACs. For  
119 technical reasons we were not able to combine RBPMS and Parvalbumin staining (both  
120 antibodies were raised in the same species). To extend on these findings and to further

121 investigate if the genetic pools of Grik4 and Scnn1a consist of more than two RGC types, we  
122 triple-labelled vertical sections (Fig 3 C, D) for GFP (cyan), Calretinin (yellow) and Parvalbumin  
123 (magenta). Our initial findings indicate that the majority of GFP cells in the GCL are RGCs.  
124 These experiments described below thus aimed to find out whether these GFP cells can be  
125 subdivided into smaller groups. We found that the population of Grik4 (Fig 3 C) expressing  
126 cells (cyan) in the GCL consists of at least three different types of cells: only GFP (Fig 3 C,  
127 cyan arrow), GFP/Calretinin (yellow arrow) and GFP/Calretinin/Parvalbumin (white arrows)  
128 cells. It is likely that these three different cell types reflect RGCs and not dACs because we  
129 did not find any putative Grik4 ACs in the INL expressing Calretinin and/or Parvalbumin.

130 For the pool of Scnn1a expressing cells in the GCL (Fig 3 D), we found four different types:  
131 those expressing only GFP (Fig 3 D, cyan arrow), GFP/Calretinin (yellow arrow),  
132 GFP/Parvalbumin (magenta arrow) and GFP/Calretinin/Parvalbumin (white arrow) cells. A  
133 fraction of the GFP positive ACs in the INL were positive for Calretinin, suggesting that some  
134 P/Calretinin and GFP/Calretinin/Parvalbumin cells in the GCL are potentially dACs. In  
135 summary, the pool of Grik4 and Scnn1a cells in the GCL respectively consists of at least three  
136 and four different RGC types. That information is important for the validation of our novel  
137 approach to the functional classification of these same RGCs.

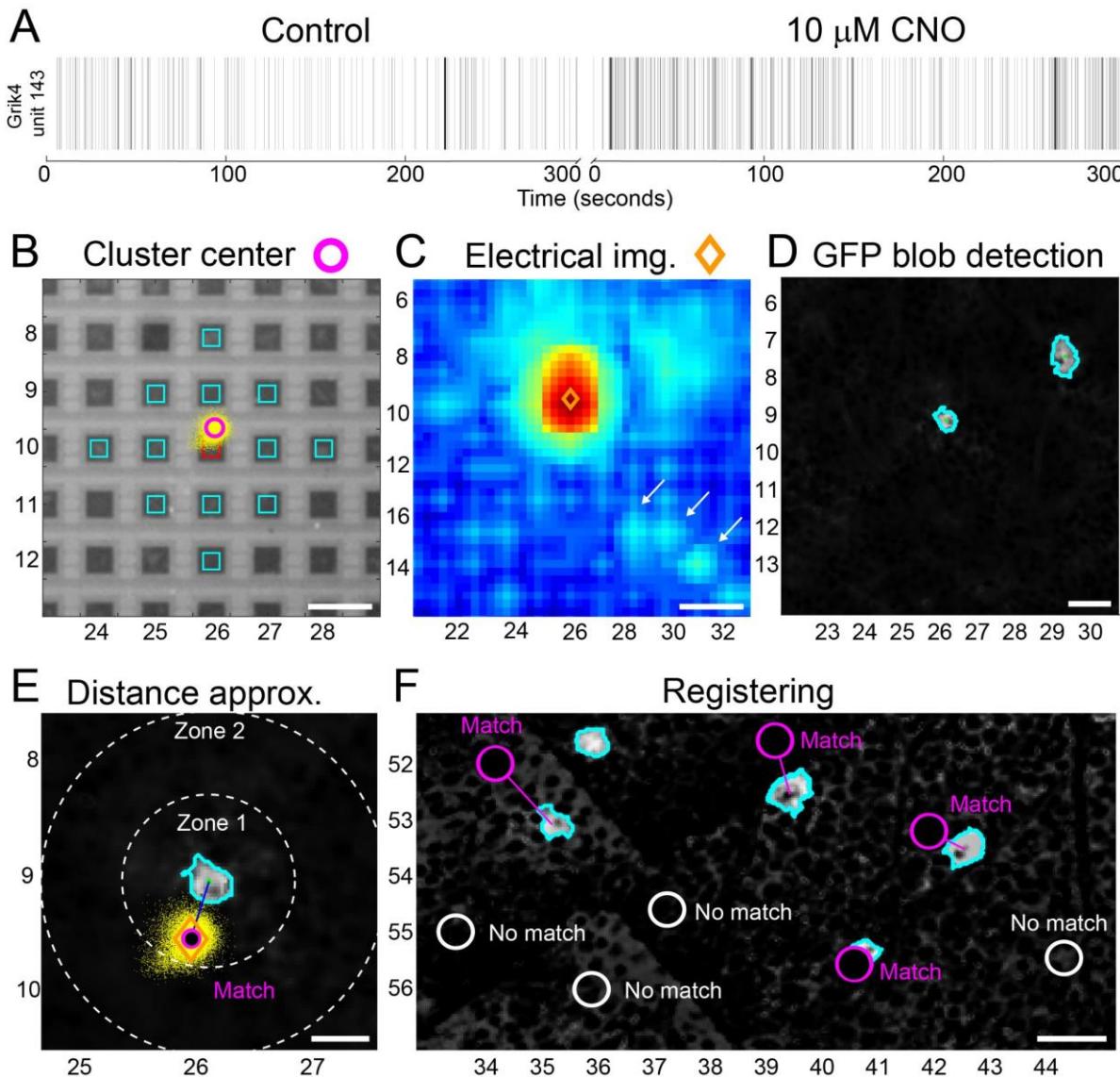
138 We next identified Grik4 and Scnn1a RGCs according to the change of their spiking pattern  
139 following DREADD activation with CNO, and we grouped them into functional clusters based  
140 on response similarity. The IHC experiments revealed that DREADDs are expressed in  
141 several RGC types but also in ACs (albeit more sparsely). Hence, the first step to isolate Grik4  
142 and Scnn1a expressing RGCs is to pre-identify cells that showed either a 50% (arbitrarily  
143 chosen) increase in spontaneous firing rate (Fig 4 A) or a 50% increase of the Burst Index (Fig  
144 4 B) in the presence of CNO. That step is followed by correlating the physical position of these  
145 identified RGCs with micrographs of DREADD-GFP expressing cells in the GCL. This step  
146 was necessary because CNO also evoked activity in ACs which in turn altered spiking  
147 activities in non-DREADD expressing RGCs (see also Fig 6). Broadband blocking of these

148 unknown DREADD ACs with pharmacological agents resulted in up- and down regulated  
149 spiking



*Figure 4: DREADD activation leads to spike pattern changes in RGCs. DREADDs in RGCs can be activated with Clozapine N-oxide (CNO) and lead to an increase in firing frequency (A, right half) and sometimes also in bursting activity (B, right half). Similar activities can be evoked with an “allround” AC neurotransmitter blocker (C) without CNO. The cocktail contains: 2 μM Strychnine (glycine receptor antagonist), 20 μM Mecamylamine (nicotinic acetylcholine receptor antagonist), 20 μM Bicuculline (GABA<sub>A</sub> antagonist), 5 μM CGP 55845 ((2S)-3-[(1S)-1-(3,4-Dichlorophenyl)ethyl] amino -2- hydroxypropyl] (phenylmethyl) phosphinic acid hydrochloride, GABA<sub>B</sub> antagonist), 50 μM TPMPA (1,2,5,6-Tetrahydropyridin-4-yl methylphosphinic acid, GABA<sub>C</sub> antagonist). Plotted in A, B are 20 Grik4 RGCs that showed a minimum of 50% change in firing frequency or bursting activity and in C 100 randomly selected*

150 activities in almost all RGCs (Fig 4 C), hence it is not a suitable approach to isolate the  
151 DREADD RGCs. The process of correlating physical positions with structural imaging allowed  
152 us to unequivocally correlate anatomical and functional features of Grik4 and Scnn1a RGCs.  
153 Here, we provide a brief exemplary description, using an identified Grik4 RGC (Fig 5), to show  
154 how we register GFP labelled cells with their spiking activity for unambiguous identification of  
155 Grik4 RGCs. Further technical details are available in the Method section. The spike  
156 localization cluster centre (magenta circle) is calculated for a pre-identified (see above) Grik4  
157 RGC (Fig 5 B) using the spike interpolation algorithm in Herdingspikes2. In addition, electrical  
158 imaging confirmed the maximal current sink position (Fig 5 C, orange diamond) and visualized



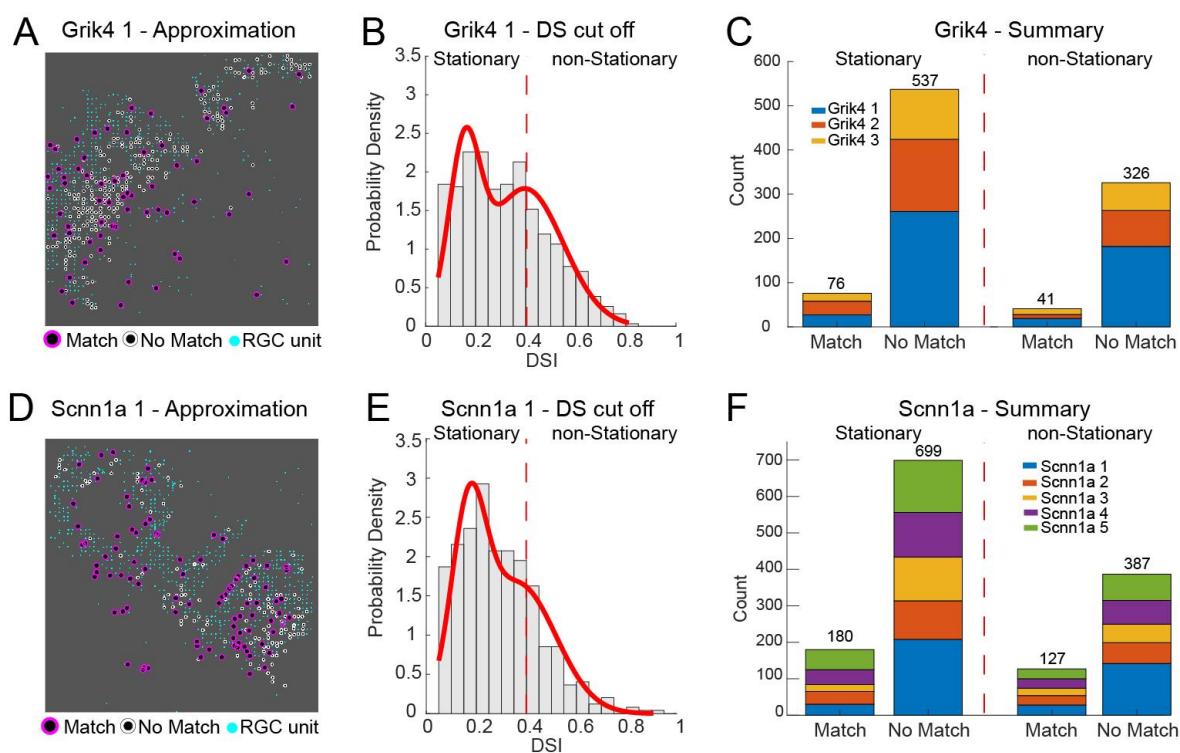
*Figure 5: Registering GFP RGCs with nearby isolated spike centre clusters. The spike cluster centre (B, circle) and the electrical imaging (C, diamond) define the potential electrical source location of the isolated RGCs. Spike centres in close proximity to GFP labelled RGCs (D) were registered as potential DREADD-expressing RGCs (E, F, Match). Scale bars A, C, F = 40  $\mu$ m; B = 80  $\mu$ m; E = 20  $\mu$ m.*

159 putative axonal trajectories (Fig 5 C, arrows), thus helping to align the GFP cell structure with  
 160 the electrical activity more precisely. Image segmentation techniques are used to threshold  
 161 the GFP foreground from the background and a blob detection is applied to detect the outlines  
 162 and centre of Grik4 and Scnn1a GFP positive cells in the GCL (Fig 5 D, green outline). Lastly,  
 163 the x and y positions are converted to match with MEA images dimensions (Fig 5 E). Spikes  
 164 originate from the axon initial segment (AIS) rather than from the cell body. Therefore, we

165 expect the spike cluster centre to be slightly eccentric with respect to the soma itself. The  
166 exact location of the AIS in RGCs can be very close to the soma, or sometimes rather distant  
167 along the axon (27,28). All the information from panels B-D is needed to estimate if a GFP-  
168 expressing cell corresponds to a nearby spike cluster centre. Two virtual circles with a radius  
169 of 30 and 60  $\mu\text{M}$  (arbitrary values) are drawn around a detected GFP blob centre (Fig 5 E),  
170 and spike cluster centres falling within one of these two zones are registered. From  
171 experience, we reached the conclusion that using the 60  $\mu\text{m}$  zone was sufficient to reliably  
172 determine whether an isolated spike cluster corresponds to a specific GFP-expressing cell.  
173 Such cases are referred to as “Match”, and those falling outside these boundaries are  
174 classified as “No Match” (Fig 5 F, Fig 6 A, D). All other recorded spike units, that did not exhibit  
175 changes in spike behaviour in the presence of CNO, were labelled “RGC unit” (Fig 6 A, D) but  
176 not considered for further classification.

177 We successfully analysed 3 Grik4 and 5 Scnn1a retinas to register “Match” (Fig 6 A, D,  
178 magenta) and “No Match” (Fig 6 A, D, white) cells. Figure 6 A, D are representative examples  
179 from the cohort of all experiments. Note that the recording area is generally not completely  
180 covering the entire 64 x 64 array. In total, we identified 117 “Match” and 863 “No Match” RGC  
181 in the 3 Grik4 retinas (Fig 6 C) and 307 “Match” and 1066 “No Match” in the 5 Scnn1a retinas  
182 (Fig 6 F). We divided these RGCs according to their response preference for stationary (static,  
183 full field) and non-stationary images (moving bars for direction of orientation selectivity)).  
184 Briefly, cells that were exceeding a threshold value (see Methods) for their direction or  
185 orientation selectivity index (DSI and OSI, respectively) were classified as non-stationary (Fig  
186 6 B, E). Figure 6 is showing an example for DSI grouping but we also grouped cells according  
187 to their OSI. In the following, we focus on “Match” Grik4 and Scnn1a RGCs only. A total of 78  
188 Grik4 stationary and 41 non-stationary RGCs were collected (Fig 6 C). For Scnn1a, 180  
189 stationary and 127 non-stationary RGCs were collected (Fig 6 F). However, most RGCs did  
190 exhibit changes in firing rate (increase or decrease) without revealing any GFP signals, hence  
191 they are unlikely to be Grik4 or Scnn1a RGCs (here called “No Match”). These cells are most

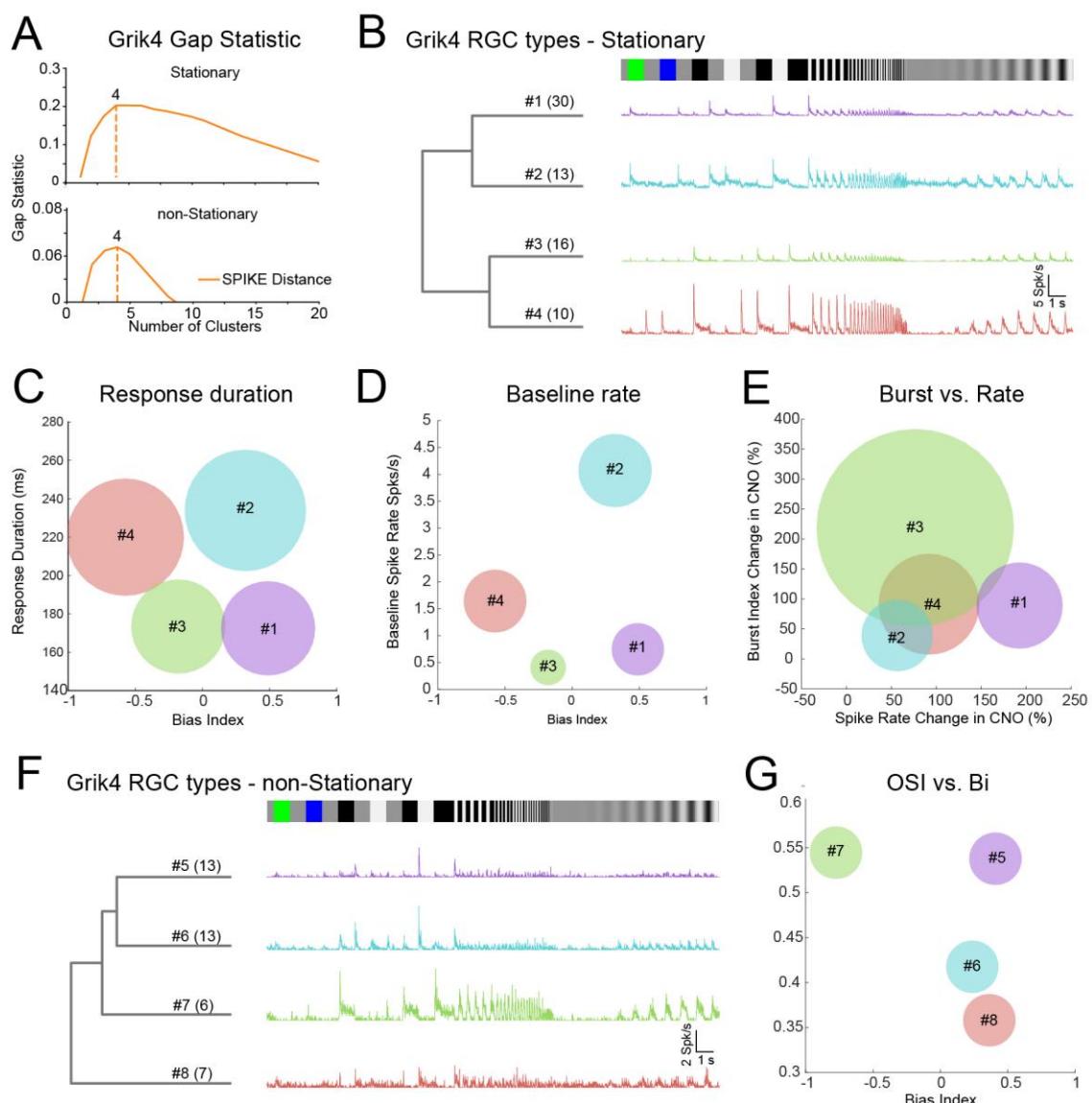
192 likely other RGC types affected by either Grik4 or Scnn1a expressing ACs, and they  
 193 outnumber the “Match” RGCs. In summary, we successfully combined near pan-retinal



*Figure 6: Dividing registered Grik4 and Scnn1a RGCs into stationary and non-stationary responders. Moving stimuli were used to estimate the direction selectivity index (DSI) of registered “Match” RGCs (Grik4 A, Scnn1a D, magenta). The DSI was plotted against the probability density (histogram) and fitted with two gaussians (B, E, red line). RGCs exceeding a set threshold (B, E, dotted red line) were labelled “non-Stationary”. The registered RGCs from all retinas were grouped into “non-Stationary” and “stationary” (C, F).*

194 recordings with *post hoc* anatomical characterization and isolated GFP-positive Grik4 and  
 195 Scnn1a RGCs with increased spiking or bursting activity in the presence of CNO.

196 After the successful registration of spikes with Grik4/Scnn1a GFP RGCs, the final step is to  
 197 classify these cells into functional groups according to the nature of their responses to light.  
 198 We recently described a non-parametric approach for RGC classification by using the SPIKE  
 199 distance (29,30) as a clustering metric (10). In order to use that approach, it is necessary to  
 200 have a stimulus that elicits responses simultaneously over the entire recording area. Here we  
 201 used a chirp stimulus inspired from Baden et al (2016) (5) that elicits responses from all RGCs  
 202 at the same time to pre-sort stationary and non-stationary RGCs (chirp, Fig 7 B, F, top) for



*Figure 7: Clustering of Grik4 stationary and non-stationary RGC responses. RGC responses that showed a high spike train similarity for a chirp stimulus (B, F) were grouped together using gap statistics (A). For each RGC of the groups, the PSTH was calculated and the mean PSTH was plotted (B, F, colored lines). The means for Bias Index (C, D, G), Response Duration (C), Baseline Spiking (D), Burst change (E), Spike Rate change (E) and orientation selectivity index (OSI, G) index were scatter plotted and their standard deviations were used as the circle diameters.*

203 SPIKE distance measure and hierarchical agglomerative clustering. We manually validated  
 204 our detected clusters by grouping several response parameters, e.g. bias index (ON, ON-OFF  
 205 or OFF) or response duration (transient or sustained), from the chirp and moving bars (for  
 206 example Fig 7 C-E, G). Manual grouping was only feasible because the expected cluster

207 numbers for Grik4 and Scnn1a were small (respectively 3 and 4 according to our IHC  
208 analyses). The pairwise SPIKE distances were determined from all trials of the chirp stimulus  
209 and the resulting distance matrix of stationary and non-stationary RGCs was clustered with a  
210 hierarchical clustering algorithm followed by the construction of a dendrogram as shown in  
211 Jouty et al., 2018 (10). To find the optimal number of clusters for stationary and non-stationary  
212 SPIKE distances we used gap statistics (10,31). For stationary and non-stationary RGCs, gap  
213 statistics estimated four response clusters of Grik4 RGCs (Fig 7 A). Additional response  
214 parameters were extracted from the chirp peri-stimulus time histogram (PSTH) e.g., bias index  
215 or response duration of these 4 stationary RGC types. We found potentially ON transient (#1,  
216 n = 30), ON sustained (#2, n = 13), ON-OFF (#3, n = 16) and OFF sustained (#4, n = 10)  
217 response types. Some RGC units were discarded during the process of SPIKE distance  
218 grouping as they showed a low signal-to-noise ratio i.e., high response variability (10). To  
219 validate these four detected clusters, we plotted the mean bias index against the mean  
220 response duration from all RGCs in a cluster (Fig 7 C), revealing four distinct groups that  
221 corresponded to the confirmed stationary RGC types. Moreover, spontaneous activity follows  
222 very specific patterns for certain RGC types and can therefore help classification. Plotting the  
223 mean Bias Index against mean spontaneous firing rate (baseline firing) revealed that the ON  
224 sustained cluster #2 (Fig 7 B, C, cluster 2) exhibited a very strong baseline firing rate (Fig 7  
225 D). The same plot also shows that the three other RGC types have a moderate baseline firing  
226 rate and further confirmed the 4 well separated RGC types. Whether the spontaneous firing  
227 rate becomes burstier or simply increases monotonously in the presence of CNO can be used  
228 to further group RGCs into clusters (Fig 7 E). Interestingly, the effect of CNO on cluster #2 is  
229 minimal (Fig 7 E) whereas it is maximal for cluster #3 (Fig 7 E). The non-stationary RGCs  
230 barely responded to the chirp stimulus (Fig 7 F, scale bar) but that response was, albeit  
231 modest, reliable and unique for the different clusters. We used the orientation (OS) and  
232 direction selectivity (DS) index in addition to the chirp PSTH parameters to define the four non-  
233 stationary Grik4 clusters (Fig 7 F, G). We found ON - OS (#5, n = 13), ON-OFF - DS (#6, n =  
234 13; #8, n = 7) and OFF – OS (#7, n = 6) response groups in the pool of non-stationary Grik4

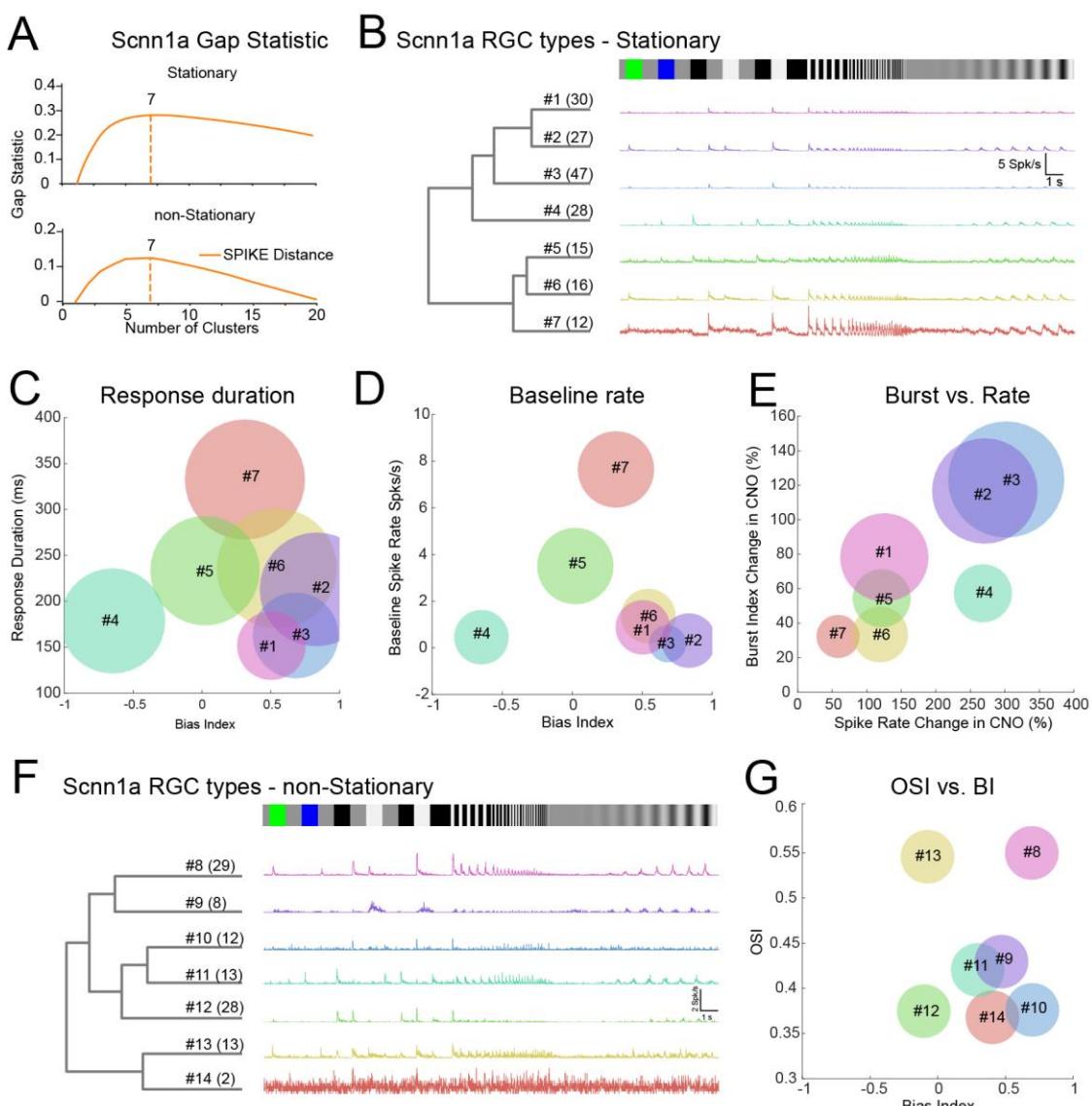
235 RGCs. The mean DS index was almost identical for these 4 groups; hence we used the OS  
236 index for manual validation. While the ON – OS (#5) and the OFF – OS (#7) are clearly  
237 distinguishable from each other, the difference is less pronounced between the two ON-OFF  
238 – DS clusters (#6 & #8). They have a similar mean BI, but Cluster 6 tends to be marginally  
239 more OS (Fig 7 G). Clusters 6 and 8 would therefore need further evaluation to determine  
240 whether they are truly different RGC types. In summary (Table 1), we found 4 stationary RGCs  
241 types and most likely 3, non-stationary RGC types that share the Grik4 gene pool.

Grik 4 Cluster	Grik 4 stationary	Grik 4 Cluster	Grik4 non-stationary
#1	G20 ON high freq after Baden et al., 2016	#5	G17 ON local trans OS after Baden et al., 2016
#2	G22 ON sustained after Baden et al., 2016; Putative PixON after Johnson et al., 2018	#6	G12 ON-OFF DS1 after Baden et al., 2016; Vaney, Sivyer & Taylor, 2012; Temporal, dorsal cluster 10, 16, 24 after Tran et al., 2019
#3	G11 ON-OFF local after Baden et al., 2016; Putative HD1/2 after Jacoby & Schwartz 2017	#7	G4 OFF slow after Baden et al., 2016; Putative F-midi OFF after Rousso et al., 2017; Cluster 28 after Tran et al., 2019
#4	G5 OFF alpha sustained after Baden et al., 2016; Krieger et al., 2017 Cluster 42 after Tran et al., 2019	#8 (#5?)	G12 ON-OFF DS1 after Baden et al., 2016; Vaney, Sivyer & Taylor, 2012

Table 1: Summary of identified Grik4 clusters and potential matches with existing literature. NB these are suggestions.

242 We proceeded in a similar way with stationary and non-stationary registered Scnn1a RGCs  
243 (Fig 8). Gap statistics suggested 7 clusters for both groups (Fig 8 A). The dendrogram of the  
244 chirp PSTH consists of ON transient (#1, n = 30; #2, n = 27; #3, n = 47), ON sustained (#6, n  
245 = 16; #7, n = 12), ON-OFF (#5, n = 15) and OFF (#4, n = 28) like response for Scnn1a RGC  
246 types (Fig 8 B). Further analysis revealed that the ON sustained (#7), OFF (#4) and ON-OFF  
247 (#5) response parameters (Fig 8 C - Bias Index vs Response Duration; Fig 8 D – Bias Index  
248 vs Baseline) were clearly distinct from each other. At the same time, the distinction is less

249 clear between clusters #1, #2, #3, #6 (Fig 8 C, D). The effect of CNO on the different Scnn1a  
 250 RGCs was also diverse (Fig 8 E) with #7 barely affected and #2 & #3 massively affected. The



*Figure 8: Clustering of Scnn1a stationary and non-stationary RGC responses. RGC responses that showed a high spike train similarity for a chirp stimulus (B, F) were grouped together using gap statistics (A). For each RGC of the groups, the PSTH was calculated and the mean PSTH was plotted (B, F, colored lines). The means for Bias Index (C, D, G), Response Duration (C), Baseline Spiking (D), Burst change (E), Spike Rate change (E) and orientation selectivity index (OSI, G) index were scatter plotted and their standard deviations were used as the circle diameters.*

251 non-stationary Scnn1a RGCs (Fig 8 F) clustered into OFF-DS (#11, n = 13), ON-OFF– DS  
252 (#12, n = 28; #14, n = 2), ON-OFF – OS (#13, n = 13), ON – DS (#9, n = 8; #10, n = 12) and  
253 ON - OS (#8, n = 29) like responses. Although there was some overlap between certain  
254 clusters (#9, #10, #11, #14) when plotting the Bias Index and OS index means (Fig 8 G), their  
255 chirp PSTH plots were substantially different, suggesting that these cells do belong to distinct  
256 functional groups. Therefore, it is likely that the Scnn1a RGC pool consists of several DS and  
257 OS cells which will necessitate further investigation to establish their basic functional  
258 differences. In summary (Table 2), we found a minimum of four stationary and a maximum of  
259 seven non-stationary Scnn1a RGC types.

Scnn1a Cluster	Scnn1a stationary	Scnn1a Cluster	Scnn1a non-stationary
#1	G18 ON trans after Baden et al., 2016	#8	G17 ON trans (OS) after Baden et al., 2016
#2	G18 ON trans after Baden et al., 2016	#9	G26 ON DS sustained 2 after Baden et al., 2016
#3	G18 ON trans after Baden et al., 2016	#10	?
#4	G8 OFF alpha trans after Baden et al., 2016; Krieger et al., 2017; Cluster 45 after Tran et al., 2019	#11	G2 OFF DS after Baden et al., 2016; Putative F-mini OFF after Rousso et al., 2017; Cluster 4 after Tran et al., 2019
#5	G10 W3 local edge detector after Baden et al., 2016; Zhang et al., 2012; Cluster 6 after Tran et al., 2019	#12	G12 ON-OFF DS1 after Baden et al., 2016; Nasal, ventral cluster 12, 16 after Tran et al., 2019
#6	G18 ON trans after Baden et al., 2016	#13	G14 ON-OFF local OS after Baden et al., 2016
#7	G22 ON sustained after Baden et al., 2016; Putative PixON after Johnson et al., 2018	#14	?

Table 2: Summary of identified Scnn1a clusters and potential matches with existing literature. NB these are suggestions.

260 Discussion

261 We established a novel approach for RGC classification, based on grouping of cells sharing  
262 gene expression and similarities in their light response patterns. A previous study used  
263 pharmacogenetics in combination with MEA recordings for Parvalbumin RGC classification  
264 (33). PV expressing cells in the retina are manifold with at least 8 RGC types (26) and a distinct  
265 number of amacrine cells. Other approaches combined MEA recordings with anatomical  
266 imaging (34,35). We successfully used a combination of all three methods, excitatory  
267 DREADD activation in retinal cells, immunohistochemistry and large-scale retinal  
268 electrophysiology to provide a significantly more comprehensive pan-retinal phenotypical  
269 description of Grik4- and Scnn1a-expressing RGCs in the mouse retina. Indeed, we extended  
270 the pre-existing list of Grik4 expressing RGC types, which validates our approach, and  
271 indicates that it has more powerful detection potential than previous ones. For the first time,  
272 we also provide a functional description of Scnn1a-expressing RGCs. We also showed that  
273 certain types of ACs express Grik4 and Scnn1a and that exciting these cells with DREADDs  
274 activation leads to spike firing change in many undefined RGCs. Our approach is not restricted  
275 to retinal cells but is widely applicable to other neurons from other brain regions. It is a scalable  
276 multimodal approach and can provide fast grouping of large cohorts of neurons with similar  
277 gene expression.

278 Grik4 expression in the retina has been described (13,35–38) but there is functional  
279 characterization only for two RGC types (37,38). Johnson et al., (38) reported that the Grik4-  
280 expressing PixON RGC type is an ON sustained type and has strong spontaneous activity.  
281 Our Grik4 cluster #2 from the stationary RGCs (Tab 1) has the same key phenotypic features  
282 - sustained responses and strong spontaneous activity. Cluster #2 also matches with the ON  
283 sustained group (G) 22 from Baden et al. 2016, (5). Grik4 clusters #1, #3 and #4 potentially  
284 resemble G20 (ON high freq), G11 (ON-OFF local) and G5 (OFF alpha sustained) from that  
285 study. Cluster #3 is potentially also resembling HD1/2 after Jacoby & Schwartz 2017 (39) and

286 the #4 OFF alpha sustained is further described in Krieger et al. 2017, (40) The latter one is  
287 parvalbumin positive (5,14,40) and in line with our IHC results that revealed Grik4 and  
288 parvalbumin expressing RGCs. Baden et al. (2016) found many more parvalbumin-expressing  
289 RGC types rather than the traditionally known 8 types (26). We suspect that there is some  
290 overlap between Grik4 and yet undescribed parvalbumin, potentially also calretinin, RGC  
291 types. We currently investigate the functional features of parvalbumin and calretinin co-  
292 expressing RGCs using a similar methodology (41). Further, the existence of Grik4-expressing  
293 ON-OFF – DS RGCs has been demonstrated in the mouse retina [22]. Cluster #6 of our non-  
294 stationary RGCs also show ON-OFF DS RGCs (42) and potentially resemble G12 (ON-OFF  
295 DS1) from Baden et al. (2016) and the dorsal-temporal clusters 10, 16 and 24 after Tran et  
296 al., 2019 (6). Grik4 cluster #8 is also G12 ON-OFF DS though the responses are less  
297 pronounced than in cluster #6. Our non-stationary Grik4 cluster #5 has similarities with G17  
298 (ON local trans - OS) and cluster #7 with G4 (OFF slow) from Baden et al. (2016). The latter  
299 one also shows similarities to the F-midi OFF cell responses described in Roussou et al. 2017,  
300 (43) and Tran et al. 2019, (6). It would also explain the findings of parvalbumin and calretinin  
301 positive Grik4 RGCs in our experiments (Fig 3 C) since F-midi OFF cells are supposed to  
302 express both (43).

303 For Scnn1a, we found seven stationary and seven non-stationary RGC response groups (Tab  
304 2). Interestingly, the Scnn1a ON sustained Cluster #7 showed remarkable similarity with the  
305 PixON characteristics described earlier - sustained responses and high baseline firing. There  
306 are no reports about Grik4 and Scnn1a co-expressing cells but it is not uncommon that certain  
307 RGC types share the same genes e.g. *Foxp* and *Brn3* (43) or *Pvalb* and *Calb2* (41,43,44).  
308 Our Scnn1a stationary clusters #1, #2, #3 and #6 were grouped by gap statistics into 4 different  
309 groups but our post-hoc analysis (Fig 8 C, D) suggests one group. Therefore, these four  
310 clusters may reflect just one, or maybe two functional groups. They all exhibit ON transient  
311 responses but are not able to follow high frequencies in the chirp (like cluster #1 in Grik4),  
312 thus they resemble G18 (ON trans) in Baden et al. (2016). Cluster #4 has OFF transient

313 response characteristics and could resemble G8 (OFF alpha trans, see also (40)) after Baden  
314 et al., 2016 and cluster 45 after Tran et al. 2019, (6). The Scnn1a stationary cluster #5 shows  
315 weak ON and OFF responses to chirp and could potentially be the W3 local edge detector  
316 (G10 in Baden et al. 2016) (45). The grouping of the non-stationary Scnn1a clusters #8 - #14  
317 shows a variety of response types. Scnn1a cluster #8 shows strong ON transient and slight  
318 OS responses and may relate to G17 (ON trans OS) in Baden et al. (2016). Scnn1a cluster  
319 #9 shows very weak and sluggish ON DS responses and is not really following the oscillating  
320 components in the chirp. It shows similarities to G26 (ON DS sust 2) from Baden et al. (2016).  
321 Our Scnn1a OFF DS cluster #11 is most likely G2 (OFF DS) in Baden et al. (2016) and/or  
322 potentially the F-mini OFF described in Roussou et al. (2016) and Tran et al., 2019. The F-  
323 mini-OFF is calretinin (occasionally parvalbumin as well) positive which would fit with our IHC  
324 results. The non-stationary Scnn1a ON-OFF DS cluster #12 may relate to G12 (ON-OFF DS  
325 1) and nasal-ventral clusters 12 &16 after Tran et al., 2019. Cluster #13 is potentially G14  
326 (ON-OFF local OS) from Baden et al. 2016. Clusters #10 and #14 show very weak chirp  
327 responses and it is difficult to assign them to any existing response type. In summary, our  
328 approach is able to group individually MEA recorded RGC response types into established  
329 response clusters (Baden et al. (2016 and Tran et al., 2019)) obtained with  $\text{Ca}^{2+}$  imaging or  
330 genetically, respectively.

331 DREADDs were also expressed in one or several yet undescribed AC types. For Scnn1a, we  
332 found few GABAergic ACs but most GFP-positive ACs were not characterized further. For  
333 Grik4, we did not find any GABAergic ACs, nor were we able to define the AC type with our  
334 experimental means. Excitatory activation of Grik4 and Scnn1a ACs does have a profound  
335 effect on postsynaptic RGC activity. A single AC affects nearly every RGC within reach of its  
336 dendrites (46). Briefly, assuming that CNO acts simultaneously on some ACs and RGCs, we  
337 are confronted with the problem of untangling the underlying circuits. For example, let us  
338 consider a simple network with one population of DREADD ACs which modulate one  
339 population of bipolar cells and one population of DREADD-RGCs. Adding CNO will have a

340 direct effect on RGCs, increasing their activity (firing and/or bursting rate). At the same time,  
341 ACs activity increases, thereby reinforcing lateral inhibition. This situation is already complex  
342 enough if we consider non linearities in the cell's responses. But as those cells are embedded  
343 in a network, the net effect of CNO can have many forms leading to a priori undetermined  
344 situations. For example, it is known that cascade of inhibition can result in excitation (e.g.  
345 push-pull effect) (47). A possible solution to this problem relies on a quantitative analysis that  
346 requires considering the factors constraining individual cell responses, without and with CNO,  
347 and the network connectivity. It is possible to propose a map of CNO induced scenarios in  
348 simple situations, with a suitable space of relevant biophysical parameters. These questions  
349 will be addressed on modelling and mathematical grounds in a forthcoming paper.

350 Finding DREADD expression in ACs was initially challenging. We managed to circumvent the  
351 interference of DREADD activated ACs by combining MEA recordings with IHC, which is not  
352 a straightforward solution and renders experiments much more complex. Our experimental  
353 design was based on Cre-Lox recombination, resulting in DREADD expression in all cells with  
354 the same promoter gene in the organism. In future work, DREADD expression should be  
355 targeted only to RGCs via intraocular viral transfection in order to avoid such strong side  
356 effects (48). Blocking AC input onto postsynaptic targets (bipolar cells or RGCs) would be  
357 another solution but has a big caveat: such disinhibition leads to massive increase in activity  
358 levels in all RGCs, hence masking RGC specific DREADD activation in specific subgroups  
359 where DREADDs are directly expressed on RGCs.

360

## 361 Methods

362 *Animals and retina preparation:*

363 All experimental procedures were approved by the ethics committee at Newcastle University  
364 and carried out in accordance with the guidelines of the UK Home Office, under control of the  
365 Animals (Scientific Procedures) Act 1986. Grik4 (C57BL/6-Tg(Grik4-cre)G32-4Stl/J, the  
366 Jackson Laboratory, MA, JAX Stock No: 006474) and Scnn1a (B6;C3-Tg(Scnn1a-cre)3Aibs/J,

367 JAX Stock No: 009613) mice were cross-bred with Gq-DREADD mice (B6N;129-Tg(CAG-  
368 CHRM3\*,-mCitrine)1Ute/J, JAX Stock No: 026220) to generate a strain of mice with the  
369 excitatory Gq-DREADD expressed in Grik4 and Scnn1a expressing cells (from here on  
370 named: Grik4-DREADD and Scnn1a-DREADD, respectively). In addition, we crossbred the  
371 Grik4 and Scnn1a lines with an inhibitory DREADD (B6.129-Gt(ROSA)26Sortm1(CAG-  
372 CHRM4\*,-mCitrine)Ute/J, JAX Stock No: 026219) but the effect on RGC firing rate was  
373 negligible and the litters were used only for immunofluorescence studies. Male and female  
374 wild-type, Grik4-DREADD and Scnn1a-DREADD mice, housed under a 12-hour light-dark  
375 cycle and aged between postnatal days (P) 53-148 were used for the experiments. Mice were  
376 dark-adapted overnight and killed by cervical dislocation. Eyes were enucleated, and following  
377 removal of the cornea, lens, and vitreous body, they were placed in artificial cerebrospinal fluid  
378 (aCSF) containing the following (in mM): 118 NaCl, 25 NaHCO<sub>3</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 3 KCl, 1 MgCl<sub>2</sub>,  
379 2 CaCl<sub>2</sub>, 10 glucose, and 0.5 l-Glutamine, equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The ventral  
380 and dorsal orientation was marked after enucleation. The retina was isolated from the eye cup  
381 and flattened for MEA recordings. For vertical cryosections, mouse eyecups were fixed in 4%  
382 paraformaldehyde (PFA; Alfa Aesar, MA) in 0.1 M phosphate buffer solution (PBS) for 2 x 20  
383 minutes at room temperature and washed with PBS several times. For whole mounts, retinas  
384 were isolated from the eye cup and mounted on nitrocellulose paper (Sartorius, Germany) and  
385 transferred to 4% PFA in PBS (2 x 20 min), rinsed in PBS and prepared for further procedures.  
386 All procedures involving live animals and retinas were performed in dim red light and the room  
387 was maintained in darkness throughout the experiment.

388

389 *Immunohistochemistry and image acquisition:*

390 After enucleation and fixation, the retinal tissue was processed in different ways for vertical  
391 and whole mount immunohistochemistry (IHC). For vertical sections, eyecups were  
392 cryoprotected in 30% sucrose in PBS overnight at 4°C and embedded in OCT Tissue Tek  
393 (Sakura, NL) at -20°C on the following day. Vertical sections (15-20 µm) were cut on a  
394 OTF5000 cryostat (Bright Instruments, UK) and collected on Superfrost microscope slides

395 (Thermo Fisher). Vertical sections and whole mounts were blocked with 10% normal goat  
396 serum (NGS) and/or 10% normal donkey serum (NDS) in PBS for at least 30 minutes at room  
397 temperature.

398 After the blocking procedure and a short rinse in PBS, Vertical sections were incubated with  
399 primary antibodies in 5% NGS (and/or NDS) + 1% Triton X-100 + PBS overnight at 4°C. Whole  
400 mounts were incubated free-floating with primary antibodies in 5% NGS (and/or NDS) + 1%  
401 Triton X-100 + PBS for 4-5 days at 4°C. Incubation with secondary antibodies in 1% Triton X-  
402 100 in PBS was carried out for 2 hours at room temperature for vertical sections or overnight  
403 at 4°C for whole mounts. Details of the primary antibodies are as follows: anti-GFP (chicken,  
404 Abcam 13970, 1:500-1000), anti-RBPMS (rabbit, Phosphosolutions 1830, 1:1000), anti-GABA  
405 (mouse, Sigma Aldrich A0310, 1:1000), anti-Calretinin (mouse, Swant 6B3, 1:1000) and anti-  
406 Parvalbumin (rabbit, Swant PV27, 1:1000). Secondary antibodies are as follows (all  
407 concentrations 1:500): goat anti-chicken CF488, goat anti-rabbit Alexa568/Alexa647 and  
408 donkey anti-mouse Alexa647. After washing several times with PBS, sections and whole  
409 mounts were mounted in Vectashield (Vector Laboratories, UK). All incubations and washing  
410 procedures were performed in the dark.

411 Images were captured using either a Zeiss Axio Imager upright microscope with Apotome  
412 structured illumination fluorescence (using 20x/40x air objectives) or a Zeiss LSM800 confocal  
413 microscope with 40x oil objective (Zeiss, Germany) operated with Zen software. Whole mount  
414 images were stitched together using Zen software, all other image post processing was done  
415 with Fiji (<https://fiji.sc>), Adobe Photoshop (Adobe, CA) and MATLAB (Mathworks, MA). The  
416 steps between single sections of confocal stacks were not exceeding 1  $\mu\text{m}$  and 3 - 5 sections  
417 were superimposed with Fiji for presentation. For the cell density maps, cells were manually  
418 counted using the “Cell Counter” plugin in Fiji and a bivariate histogram (MATLAB *hist3*, bin  
419 size 60 x 60  $\mu\text{m}$ ) was calculated for the cell densities. For visualizing purposes, a 2-D Gaussian  
420 filtering was applied (MATLAB *imgaussfilt*, sigma 3).

421

422 *Large-scale, high-density multielectrode array recordings and light stimulation:*  
423 Recordings were performed on the BioCamX platform with high-density-multielectrode array  
424 (HD-MEA) Arena chips (3Brain GmbH, Lanquart, Switzerland), integrating 4096 square  
425 microelectrodes in a 2.67 x 2.67 mm area and aligned in a square grid with 42  $\mu\text{M}$  spacing.  
426 The isolated retina was placed, RGC layer facing down, onto the MEA chip and flattened by  
427 placing a small piece of translucent polyester membrane filter (Sternitech Corp., Kent, WA,  
428 USA) on the retina followed by a home-made anchor. Retinas were maintained at 33°C using  
429 an in-line heater (Warner Instruments LLC, Hamden, CT, USA) and continuously perfused  
430 using a peristaltic pump ( $\sim 1 \text{ ml min}^{-1}$ ). Retinas were left to settle on the MEA for at least 2  
431 hours before recording. The platform records at a sampling rate of  $\sim 18 \text{ kHz/electrode}$  when  
432 using the full 64x64 array. Recordings are filtered at 50Hz high-pass filter using BrainWaveX  
433 software (3Brain) and stored in hdf5-based data format. Spikes were detected and sorted  
434 using Herdingspikes2 (<https://github.com/mhhennig/HS2>) as in (16). Briefly, spikes were first  
435 detected as threshold crossings individually on each channel, and then merged into unique  
436 events based on spatial and temporal proximity. For each detected spike, a location was  
437 estimated based on the signal centre of mass. Spike sorting was performed by clustering all  
438 events using a feature vector consisting of the locations and the first two principal components  
439 of the largest waveform.

440

441 Light stimuli were projected onto the retina as described elsewhere (17). Briefly, the projector  
442 irradiance was attenuated using neutral density filters to mesopic light levels (white  $4 \mu\text{W/cm}^2$ ).  
443 For stimuli we used a full field ‘chirp’ stimulus consisting of various 1-sec contrast steps,  
444 increasing frequency (1-15Hz) and contrast modulations (1-93 Michelson contrast) which was  
445 repeated 5 times. We also used random black and white moving bars (width 100  $\mu\text{M}$ , 12  
446 directions ( $30^\circ$  separation)), 800 mm/s, and the whole sequence repeated 5 times. For the  
447 chirp and motion stimuli, we estimated each unit’s instantaneous firing rate for the different  
448 stimuli by convolving its spike train with a Gaussian kernel smoothing function (standard  
449 deviation (SD) = 25 ms). We then averaged the trials and extracted several features including

450 the Bias Index and the response duration (see (16)). On average, recording from one retina  
451 yields light responses from hundreds to thousands of individual RGCs.

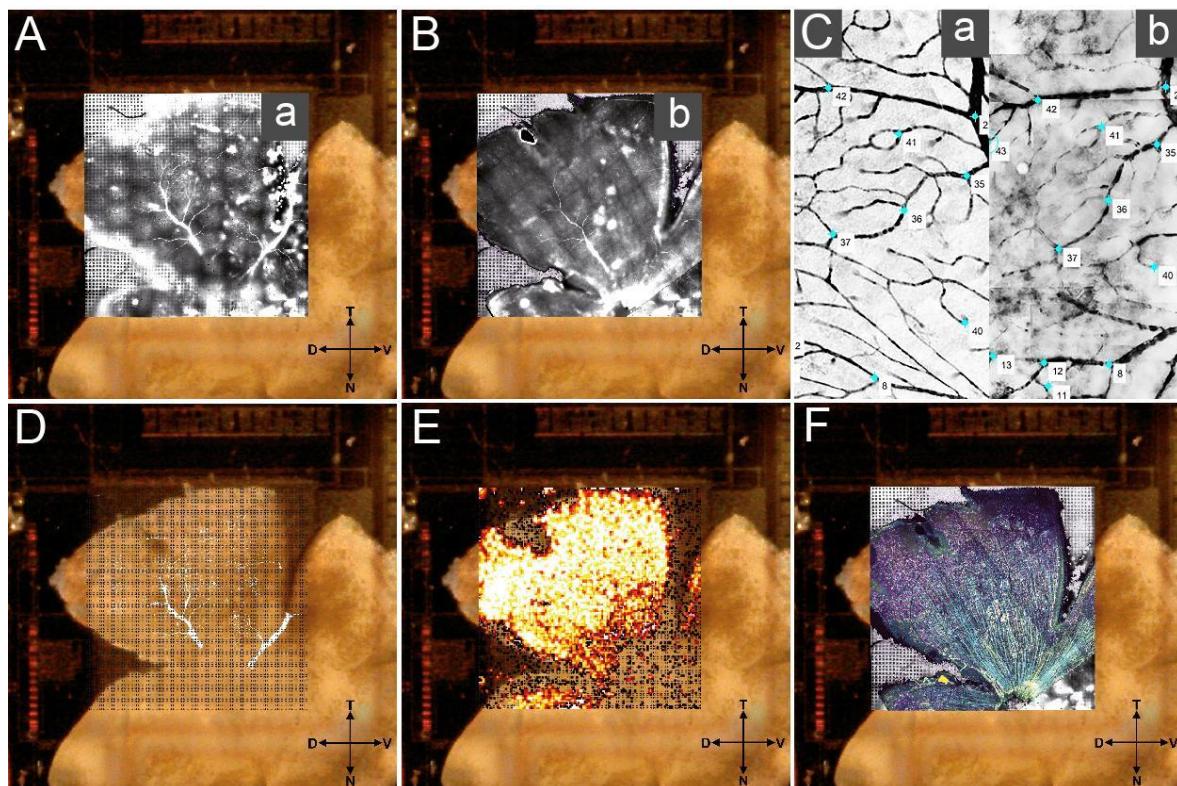


Figure 9: Registering spike location with cell labelling using blood vessel landmarks. Control points in the fixed pre-labelled (A) and moving post-labelled (B) blood vessel image were aligned (C, insets a & b from A and B, respectively) and the resulting transformation matrix (D) was fixed aligned with the spike locations (E) and additional immunostainings (F). D = dorsal; V = ventral; N = nasal; T = temporal; Grid electrode size 2.65 x 2.65 mm

452 *Registering RGC activity with IHC – blood vessels as reference marker:*

453 To register RGC activity with IHC, we used pre- and post-labelled blood vessels as reference  
454 markers (Fig. 1). For pre-labelled blood vessel staining, the eye cup was incubated for 1 hr in  
455 aCSF + 5  $\mu$ M Sulforhodamine 101 (SR101, Sigma Aldrich, MO) and afterwards transferred to  
456 aCSF + 0.06  $\mu$ M SR101 for MEA recording procedures. After recording, the weight and the  
457 polyester membrane were carefully removed to expose the retina, and the MEA well rinsed  
458 with aCSF. If the blood vessels were not visible, a 50% Optiprep (Sigma Aldrich, MO) /50%  
459 aCSF solution was used for rinsing and further clearing. The MEA chip was mounted on a  
460 microscope stage (Olympus AX70, CoolLED pE fluorescence) while continuing rinsing every

461 2-3 min with oxygenated aCSF to ensure the tissue remains healthy throughout the procedure.  
462 The best stained blood vessel layer (either superficial or deep vascular plexus) was meander-  
463 like imaged (Fig. 9 A). For every captured blood vessel, images were acquired in the same  
464 location all the way down to the electrodes as well. Neighbouring images were manually  
465 stitched together in Adobe Photoshop (Adobe, CA) later. The retina was then carefully  
466 removed from the MEA, flattened on a nitrocellulose membrane for IHC procedure. Blood  
467 vessel staining was enhanced by using an anti-mouse secondary antibody in the same  
468 excitation spectrum (normally Alexa568) and blood vessels were imaged again (same plexus  
469 as while the retina was imaged on the MEA (Fig. 9 B). Both blood vessel staining images, pre-  
470 and post-tissue fixation were correlated with each other using the Control Point Selection Tool  
471 in Matlab (Fig. 9 C). Briefly, the images taken in the live retina on the MEA were used as the  
472 fixed (reference) image and images acquired post fixation as the moving image. Minimum 50  
473 reference points were picked for the fixed and moving images to create a geometric  
474 transformation (Matlab *fitgeotrans*, *lwm*, 50). That geometric transformation was applied to the  
475 GFP image. A stack (hereafter called MEA images) with the MEA electrode image, blood  
476 vessels pre- and post-fixation and GFP image was created in Adobe Photoshop (Fig. 9 F).

477

478 *Registering RGC activity with IHC – Grik4/Scnn1a DREADD RGC identification:*

479 The main procedure to identify and register RGC activity (spike clusters) with the MEA images  
480 is described in the main results (Figures 5, 9). Only units that did show a significant change in  
481 spontaneous firing rate (sampled during at least 5 min) before and after adding CNO to the  
482 chamber were selected for Grik4/Scnn1a RGC identification and response clustering. Units  
483 with either >50% or <50% change in firing rate in CNO were considered as potential  
484 Grik4/Scnn1a candidates. We also used the Burst Index (32) to look at potential changes in  
485 activity levels induced by CNO, with potential Grik4/Scnn1a cells having changes in their Burst  
486 Index exceeding +/- 50%. Only these selected RGCs were further identified in the next steps.

487

488 During the spike sorting process with Herdingspikes2, the physical x and y spike localization

489 for each detected spike from a single RGC unit is calculated (Figure 5 B). The spike location  
490 coordinates are given for a 64 x64 grid (the electrode layout of our MEA). Occasionally, we  
491 confirmed the cluster centre (e.g., the centre of mass of negative spike amplitude peaks)  
492 calculated by Herdingspikes2 by overlaying the x and y localization for the negative voltage  
493 peak (Figure 5 C magenta diamond) obtained through electrical imaging. For electrical  
494 imaging, the voltage fluctuations from the spike origin electrode and the neighbouring 7  
495 electrode rows and columns were read out. For a minimum of 1000 spikes per RGC unit,  
496 voltage fluctuations before and after (here 5 ms) each spike were normalized, and maximally  
497 projected with absolute values (Figure 5 C) to visualize the current sink and the axonal  
498 trajectories. The spike localization and current sink coordinates were multiplied using a scaling  
499 factor to match the MEA images stack. GFP images were transformed to 8-bit images, a  
500 threshold (Matlab *max entropy*) was applied, potential gaps filled (Matlab *imfill 'holes'*) and  
501 binarized (Matlab *bwareaopen 50*). Binary images were analyzed (Matlab *regionprops*),  
502 centroids and diameters for every potential GFP blob were calculated and the pairwise  
503 distance between centroids and spike locations was measured for all combinations.

504

505 *Registering RGC activity with IHC – Grik4/Scnn1a response clustering:*

506 All potential Grik4/Scnn1a RGC units linked to a GFP blob (see Results) were further analyzed  
507 and grouped according to their response to a chirp stimulus. But before that grouping, cells  
508 were pre-classified into motion sensitive (responding preferably to either direction or  
509 orientation of motion) or cells that respond best to static stimuli (full field, chirp). We calculated  
510 the direction (DSI) and orientation (OSI) selectivity index as described elsewhere (16) but used  
511 a cut-off value for every experiment independently. Briefly, we fitted the tabulated data with a  
512 mixture of two gaussians (Figure 6 B, E). The fit parameters were computed using maximum  
513 likelihood parameter estimates (Matlab *mle*) and mu2 (the second peak, Figure 6 B, E) was  
514 used as cut-off value. The trial variability and signal to noise ratio was calculated (16) for each  
515 stationary/non-stationary RGC, and units with a value below the 25<sup>th</sup> percentile were  
516 discarded. The chirp responses of the remaining Grik4 and Scnn1a stationary/non-stationary

517 RGC units were used to establish response groups. We used a similar approach to calculate  
518 the SPIKE distance as described in our earlier work (10). Briefly, the SPIKE distances were  
519 computed using the open-source package PYSPIKE (30). The pairwise distances between  
520 two units were determined by computing the pairwise distances of all trials of the chirp  
521 stimulus. The resulting distance matrix was then clustered with a hierarchical clustering  
522 algorithm (Python, *Scipy library dendrogram*). Matlab and Python scripts and other extended  
523 material will be made available via [https://github.com/GerritHilgen/DREADD\\_RGCclass](https://github.com/GerritHilgen/DREADD_RGCclass).

524

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532

## 533 Authors' contributions

534 Conceptualization: G.H., E.S., B.C.; Software: G.H. Methodology: G.H., E.S.; Formal Analysis:  
535 G.H., Investigation, G.H., V.K., E.K.; Writing – Original Draft: G.H., E.S., B.C., V.K., J.K.;  
536 Project administration: E.S., B.C.; Funding Acquisition: E.S., B.C.

537

## 538 Legends

539 Figure 1: Grik4 and Scnn1a cells in the GCL are not homogeneously distributed. Whole mount  
540 antibody staining against Grik4 (A) and Scnn1a (B) DREADD GFP were imaged at the level  
541 of the GCL. All stained GFP cells were counted and the densities were calculated and  
542 presented in pseudocolours for 3 Grik4 (C) and Scnn1a (D) retinas. V= ventral, T = temporal,  
543 D = dorsal, N = nasal. Scale bar A, B = 1 mm; Scale bar A, B insets II = 100  $\mu$ m.

544 Figure 2: Grik4 and Scnn1a DREADD are expressed in RGCs and ACs. Vertical sections were  
545 stained for Grik4 (A, C, cyan) and Scnn1a (B, D, cyan) GFP. Sections were further co-labelled  
546 with RBPMS (A, B, magenta), a marker for RGCs, and GABA (C, D, magenta). INL = inner  
547 nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. Scale bar in D = 20  $\mu$ m.  
548 Figure 3: Grik4 and Scnn1a DREADD are expressed in multiple RGC types. Vertical sections  
549 of Grik4 and Scnn1a retinas were triple stained A, B) for GFP (cyan), RBPMS (magenta) and  
550 Calretinin (yellow) or C, D) GFP (cyan), Parvalbumin (magenta) and Calretinin (yellow). INL =  
551 inner nuclear layer, IPL = inner plexiform layer, GCL = ganglion cell layer. Scale bar in D = 20  
552  $\mu$ m.

553  
554 Figure 4: DREADD activation leads to spike pattern changes in RGCs. DREADDs in RGCs  
555 can be activated with Clozapine N-oxide (CNO) and lead to an increase in firing frequency (A,  
556 right half) and sometimes also in bursting activity (B, right half). Similar activities can be evoked  
557 with an "allround" AC neurotransmitter blocker (C) without CNO. The cocktail contains: 2  $\mu$ M  
558 Strychnine (glycine receptor antagonist), 20  $\mu$ M Mecamylamine (nicotinic acetylcholine  
559 receptor antagonist), 20  $\mu$ M Bicuculline ( $\text{GABA}_\text{A}$  antagonist), 5  $\mu$ M CGP 55845 ((2S)-3-[(1S)  
560 -1- (3,4-Dichlorophenyl)ethyl] amino -2- hydroxypropyl] (phenylmethyl) phosphinic acid  
561 hydrochloride,  $\text{GABA}_\text{B}$  antagonist), 50  $\mu$ M TPMPA (1,2,5,6-Tetrahydropyridin-4-yl  
562 methylphosphinic acid,  $\text{GABA}_\text{C}$  antagonist). Plotted in A, B are 20 Grik4 RGCs that showed a  
563 minimum of 50% change in firing frequency or bursting activity and in C 100 randomly selected  
564 Scnn1a RGC units. Control in A & B means without CNO, in C without blockers. Vertical lines  
565 represent spike times plotted as a function of time.

566  
567 Figure 5: Registering GFP RGCs with nearby isolated spike centre clusters. The spike cluster  
568 centre (B, circle) and electrical imaging (C, diamond) define the potential electrical source  
569 location of the isolated RGCs. Spike centres in close proximity to GFP labelled RGCs (D) were  
570 registered as potential DREADD-expressing RGCs (E, F, Match). Scale bars A, C, F = 40  $\mu$ m;  
571 B = 80  $\mu$ m; E = 20  $\mu$ m.

572 Figure 6: Dividing registered Grik4 and Scnn1a RGCs into stationary and non-stationary  
573 responders. Moving stimuli were used to estimate the direction selectivity index (DSI) of  
574 registered “Match” RGCs (Grik4 A, Scnn1a D, magenta). The DSI was plotted against the  
575 probability density (histogram) and fitted with two gaussians (B, E, red line). RGCs exceeding  
576 a set threshold (B, E, dotted red line) were labelled “non-Stationary”. The registered RGCs  
577 from all retinas were grouped into “non-Stationary” and “stationary” (C, F).

578

579 Figure 7: Clustering of Grik4 stationary and non-stationary RGC responses. RGC responses  
580 that showed a high spike train similarity for a chirp stimulus (B, F) were grouped together using  
581 gap statistics (A). For each RGC of the groups, the PSTH was calculated and the mean PSTH  
582 was plotted (B, F, coloured lines). The means for Bias Index (C, D, G), Response Duration  
583 (C), Baseline Spiking (D), Burst change (E), Spike Rate change (E) and orientation selectivity  
584 index (OSI, G) index were scatter plotted and their standard deviations were used as the circle  
585 diameters.

586

587 Figure 8: Clustering of Scnn1a stationary and non-stationary RGC responses. RGC responses  
588 that showed a high spike train similarity for a chirp stimulus (B, F) were grouped together using  
589 gap statistics (A). For each RGC of the groups, the PSTH was calculated and the mean PSTH  
590 was plotted (B, F, coloured lines). The means for Bias Index (C, D, G), Response Duration  
591 (C), Baseline Spiking (D), Burst change (E), Spike Rate change (E) and orientation selectivity  
592 index (OSI, G) index were scatter plotted and their standard deviations were used as the circle  
593 diameters.

594

595 Figure 9: Registering spike location with cell labelling using blood vessel landmarks. Control  
596 points in the fixed pre-labelled (A) and moving post-labelled (B) blood vessel image were  
597 aligned (C, insets a & b from A and B, respectively) and the resulting transformation matrix  
598 (D) was fixed aligned with the spike locations (E) and additional immunostainings (F). D =  
599 dorsal; V = ventral; N = nasal; T = temporal; Grid electrode size 2.65 x 2.65 mm

600 Table 1: Summary of identified Grik4 clusters and potential matches with existing literature.  
601 NB these are suggestions.  
602  
603 Table 2: Summary of identified Scnn1a clusters and potential matches with existing literature.  
604 NB these are suggestions.  
605

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