京都大学
KYOTO UNIVERSITY

KURENAI 紅
京都大学学術情報リポジトリ
Kyoto University Research Information Repository

PNAS

www.pnas.org

1

2 Main Manuscript for

3 **Rhodopsin-mediated Light-off-induced Protein Kinase A Activation in**
4 **Mouse Rod Photoreceptor Cells.**

5 Shinya Sato[a*], Takahiro Yamashita[b], and Michiyuki Matsuda[a,c]

6 [a]Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Kyoto
7 606-8501 Japan;

8 [b]Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan;

9 [c]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto
10 606-8501 Japan;

11 *To whom correspondence should be addressed: Shinya Sato
12 **Address**: Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University,
13 Kyoto 606-8501 Japan;
14 **TEL:** +81-75-753-9450
15
16 **Email:** sato.shinya.7e@kyoto-u.ac.jp

17 ORCIDs: Shinya Sato, 0000-0002-5978-0975; Takahiro Yamashita, 0000-0002-7956-9288; Michiyuki
18 Matsuda, 0000-0002-5876-9969

25 **Author Contributions**

26 S.S. and M.M. designed the research; S.S. performed the research; S.S. and T.Y. analyzed the data; and

27 S.S., T.Y. and M.M. wrote the paper.

28 **This PDF file includes:**

29 Main Text

30 Figures 1 to 6

31   **Abstract**

32   Light-induced extrasynaptic dopamine release in the retina reduces cAMP in rod

33   photoreceptor cells, which is thought to mediate light-dependent desensitization.

34   However, the fine time course of the cAMP dynamics in rods remains elusive due to

35   technical difficulty. Here, we visualized the spatio-temporal regulation of cAMP-

36   dependent protein kinase (PKA) in mouse rods by two-photon live imaging of retinal

37   explants of PKAchu mice, which express a fluorescent biosensor for PKA. Unexpectedly,

38   in addition to the light-on-induced suppression, we observed prominent light-off-induced

39   PKA activation. This activation required photopic light intensity and was confined to the

40   illuminated rods. The estimated maximum spectral sensitivity of 489 nm and loss of the

41   light-off-induced PKA activation in rod-transducin-knockout retinas strongly suggest the

42   involvement of rhodopsin. In support of this notion, rhodopsin-deficient retinal explants

43   showed only the light-on-induced PKA suppression. Taken together, these results suggest

44   that, upon photopic light stimulation, rhodopsin and dopamine signals are integrated to

45   shape the light-off-induced cAMP production and following PKA activation. This may

46   support the dark-adaptation of rods.

47

48   **Significance Statement**

49   Rod photoreceptor cells mediate scotopic vision using rhodopsin and its downstream

50   signal transduction cascade to degrade cGMP. However, less is known about another rod

51   photoresponse: dopamine-mediated cAMP degradation. Cyclic AMP enhances the rod

52   photosensitivity, and therefore its degradation contributes to the visual desensitization.

53   Here, we show the fine time course of rod cAMP regulation using a two-photon

54   microscope and fluorescent protein probe for cAMP-dependent protein kinase (PKA).

55   Unexpectedly, the light-induced PKA suppression was followed by robust PKA

56   activation upon light-off. Our data strongly suggest the involvement of rhodopsin in this

3

57   activation, and thus the presence of its alternative output toward cAMP. The light-off-

58   induced PKA activation might contribute to an efficient dark-adaptation after the

59   transition from light to dark environment.

60

Main Text

Introduction

Vertebrate photoreceptor cells, i.e., rods and cones, convert light information into electrical signals through an enzymatic process called phototransduction (1-4). In this process, light-activated visual pigments, rhodopsin in rods and cone visual pigments in cones, activate heterotrimeric G-protein transducin and thereby phosphodiesterase, culminating in the degradation of cyclic guanosine-3′,5′-monophosphate (cGMP). The reduction in cGMP concentration causes closure of cGMP-dependent channels in the plasma membrane to evoke an electrical response. For the timely update of visual information, activated visual pigments and their downstream enzymes are then rapidly deactivated (5) and, when back in darkness, the cGMP level is restored by guanylate cyclase (GC) (6, 7).

Ca^{2+} is the primary factor that decelerates the shut off processes of phototransduction and thereby increases photoreceptor sensitivity (7-9). Under scotopic conditions, Ca^{2+} flows into photoreceptor cells through the cGMP-gated cation channel. Ca^{2+} binds to calcium-binding proteins, including S-modulin/recoverin and guanylate cyclase activating proteins (GCAPs). Ca^{2+}-liganded S-modulin/recoverin inhibits rhodopsin phosphorylation through inhibition of G protein coupled receptor kinase 1 (GRK1), to delay the quenching of photoactivated rhodopsin by arrestin (10). On the other hand, Ca^{2+}-liganded GCAPs are negatively modulated and do not activate the cGMP restoration by GC (6). Thereby, high Ca^{2+} in scotopic conditions delays the recovery of the photoresponse and increases the sensitivity of photoreceptor cells (5).

Recently, in addition to Ca^{2+}, cAMP has been suggested to increase photoreceptor sensitivity (11). Similarly to the Ca^{2+} level, the cAMP level in photoreceptors is reduced by light via a dopamine-mediated mechanism (12, 13). Dopamine is released extrasynaptically from dopaminergic amacrine cells in a light-dependent manner (14, 15).

5

87   Photoreceptor cells detect dopamine with the dopamine receptor D4 (D4R) (16, 17),

88   which is coupled with G_i to inhibit adenylate cyclase (AC) and thereby to reduce cAMP

89   (18). A number of previous studies showed that cAMP-dependent protein kinase (PKA)

90   phosphorylates several phototransduction components. PKA phosphorylates GRK1 to

91   reduce its rhodopsin phosphorylation ability (19), regulator of G protein signaling 9-1 to

92   reduce its GTPase activating protein activity (20), phosducin to accelerate the trafficking

93   of transducin toward the outer segment (21), and GC to increase its sensitivity to Ca^{2+}-

94   dependent inhibition (22). These studies suggest a role of PKA in increasing

95   photoreceptor sensitivity. Indeed, Astakhova and colleagues showed that

96   pharmacological activation of the cAMP production induces a two-fold increase in the

97   signal-to-noise ratio in frog rods (11, 23). Furthermore, PKA strengthens the electrical

98   coupling of photoreceptor cells via the phosphorylation of connexin36 (24) to increase

99   the visual sensitivity (25). Taken together, these findings strongly suggest that light

100   reduces cAMP levels to desensitize photoreceptor cells via PKA suppression. However,

101   unlike in the case of cGMP regulation, which has been studied electrophysiologically

102   with single-cell resolution and millisecond precision, there are currently no available

103   methods to monitor cAMP in living photoreceptor cells, which limits the systematic

104   characterization of its spatio-temporal regulation in photoreceptors.

105   Here, we used a new two-photon PKA activity imaging method to address this

106   deficiency. A retinal explant culture for imaging was prepared from PKAchu mice (26-

107   28) that ubiquitously express a Förster resonance energy transfer (FRET)-based PKA

108   activity sensor protein, AKAR3EV (29, 30). Unexpectedly, in addition to the

109   aforementioned light-on-induced PKA suppression, we observed a prominent light-off-

110   induced PKA activation in photoreceptor cells. This PKA activation was induced by

111   photopic light stimulation and confined to rod photoreceptor cells in the illuminated area.

112   Spectral sensitivity data suggested the involvement of rhodopsin in the PKA activation.

6

113 Consistent with this suggestion, the activation was not detected from retinal explants that

114 were deficient in rhodopsin signaling. Interestingly, the rhodopsin-deficient retinas

115 showed only light-on-induced PKA suppression. From these results, we propose that

116 rhodopsin and dopamine signals are integrated to shape the light-off-induced PKA

117 activation in rods.

Results

Ex vivo two-photon imaging delineates various layers of the PKAchu mouse retina.

First, we set up two-photon live imaging of the isolated mouse retina (Fig. 1*A*). A retina from a PKAchu mouse was flat-mounted on a culture insert. The insert was then placed under a two-photon microscope and perfused with oxygenated culture medium. The PKA biosensor AKAR3EV (SI Appendix, Fig. S1*A*) was expressed ubiquitously in PKAchu mice, which allowed us to delineate tissue morphology at the subcellular level in six different layers of the retina (Fig. 1 *B-H*, SI Appendix, Movie S1). The nuclear-sparing pattern was caused by the cytoplasmic localization of AKAR3EV (30). The outer nuclear layer (ONL) thickness at 1.0 mm superior from the optic nerve head (ONH) was 54.3 ± 2.9 μm (mean ± SD, n = 12 retinas), which is comparable to the value for the young-adult wild-type (31). Intriguingly, a subset of cells in the photoreceptor layer (Fig. 1 *E-H*) expressed AKAR3EV much more abundantly (AKAR3EV^{high} cells) than the rest (AKAR3EV^{low} cells). Therefore, to prevent saturation of the detector, we needed to reduce the excitation laser power from the outer plexiform layer (OPL; arrow in Fig. 1*H*). Please note that this intensity difference does not mean high and low PKA activities. Although PKA increases the FRET efficiency of AKAR3EV (SI Appendix, Fig. S1*A*) to induce the spectral shift of the fluorescence, the corresponding intensity change is limited (SI Appendix, Fig. S1B).

In the OPL, the large size and shape of the AKAR3EV^{high} photoreceptor cells closely resembled the cone pedicles (Fig. 1*E*) (32). Moreover, the nuclei of the AKAR3EV^{high} photoreceptor cells (white arrowheads in Fig. 1*H*) were located near the interface between the ONL and photoreceptor segments layer (PRS). These observations strongly suggested that the AKAR3EV^{high} and AKAR3EV^{low} cells are cones and rods, respectively. This was confirmed by using rhodamine labeled peanut agglutinin (PNA-rhodamine), which specifically binds to the extracellular sheath of cones (33). As

8

144   expected, PNA-rhodamine labeled the tips of the AKAR3EV^high cells (Fig. 1*I*, SI

145   Appendix, Movie S2). In conclusion, our two-photon imaging method clearly delineates

146   the layer structure of the PKAchu retina and enables comparison of PKA activities in

147   rods and cones.

148

149   **The PKAchu retina visualizes cell type-specific PKA regulation by dopamine.**

150   To confirm whether AKAR3EV detects PKA activity in the retina, we first examined the

151   response to forskolin, which increases the cAMP level by stimulating AC. The mode of

152   action of AKAR3EV and the method to generate PKA activity images were described

153   previously (34, 35). Briefly, the signal intensity in the FRET-acceptor channel (FRET

154   Ch) was divided by that of FRET-donor channel (CFP Ch) to obtain FRET/CFP

155   pseudocolor images, which depict PKA activity (SI Appendix, Fig. S1*B*). As expected, 20

156   µM forskolin strongly increased FRET/CFP in all layers of the retina (SI Appendix, Fig.

157   S2 *A* and *B*, Movie S3). Interestingly, this elevation was less pronounced in cones (white

158   arrowheads in SI Appendix, Fig. S2*A*) than in rods, which may be ascribable to the

159   difference in AC subtypes (36).

160   Next, we examined the response to dopamine, which is one of the major

161   neurotransmitters that modulates visual processing in the retina (37). Dopamine either

162   activates or inactivates PKA, depending on the type of dopamine receptors: D1-like

163   receptors (D1R and D5R) activate PKA via G_s, whereas D2-like receptors (D2R, D3R

164   and D4R) suppress PKA via G_i (13). In the mouse retina, D1R is expressed in horizontal

165   cells and a subset of bipolar and amacrine cells in the inner retina (38). On the other hand,

166   D4R is expressed in photoreceptor cells (16, 24). In agreement with this layer-specific

167   distribution, 10 µM dopamine increased FRET/CFP values in the inner plexiform and

168   inner nuclear layers, and decreased them in photoreceptor cells (SI Appendix, Fig. S2 *C*

169 and *D*, Movie S4). Therefore, we concluded that AKAR3EV in the PKAchu retina

170 detects both PKA activation and suppression.

171

172 **Light-induced PKA suppression is followed by an unexpected "overshoot" of**

173 **activation in photoreceptor cells.**

174 According to previous studies, light induces extrasynaptic dopamine release from

175 dopaminergic amacrine cells, which reduces cAMP in photoreceptor cells (16, 39, 40).

176 However, previous studies did not elucidate the fine time course of this response;

177 therefore, we time-lapse imaged the light-induced cAMP reduction via PKA activity

178 change. Dark- and light-adapted PKAchu retinas were prepared for imaging (please see

179 *SI Appendix, Supplementary Methods* for details about preparations). As expected from

180 previous studies that reported the elevated photoreceptor cAMP level in darkness (40,

181 41), the basal PKA activity in photoreceptor cells was significantly greater in the dark-

182 adapted retina (Fig. 2 *A* and *B*, PRS). To measure the time course of the photoresponse, a

183 stimulation light was delivered onto the retina (*SI Appendix, Supplementary Methods*).

184 Upon a brief 6 sec light stimulation, photoreceptors in the dark-adapted retina showed a

185 large drop in PKA activity (Fig. 2*C* and *D*, dark). Unexpectedly, however, PKA activity

186 rebounded over the basal level, and the increased activity level continued for 30 min. On

187 the other hand, photoreceptors in the light-adapted retina exhibited only a modest

188 suppression of PKA (Fig. 2*C* and *D*, light), but showed marked PKA activation for 15

189 min. The suppression was detected within 12 sec from the stimulation, and subsequent

190 activation was detected at 50 ± 5 sec and peaked at 214 ± 16 sec (mean $\pm$ SD, n = 5; Fig.

191 2*E*).

192   In an effort to visualize the light-induced suppression more clearly, we applied a

193 longer 10 min stimulation to the light-adapted retina (Fig. 2 *F* and *G*, SI Appendix,

194 Movie S5). In this case, photoreceptor PKA activity exhibited a transient drop in the first

10

195 2 min, but returned to the basal level for the remaining 8 min. Then, to our surprise, PKA

196 was prominently activated upon light-off. We speculate that, upon the light-on, the

197 dopamine-mediated PKA suppression occurred and was observed as a transient drop at 2

198 min. But simultaneously, an antagonizing light-induced PKA activation occurred with

199 slow kinetics that canceled out the suppression during the subsequent 8 min. Then, upon

200 light-off, the dopamine effect was removed instantly probably due to an efficient

201 dopamine clearance from the extrasynaptic space (42), and only the activation remained

202 to shape the apparent off response. Because such light-induced PKA activation has not

203 been reported in normal photoreceptor cells [but see (43, 44)], hereafter, we focused on

204 this PKA activation using light-adapted retinas.

205

206 **Light-off-induced PKA activation occurs exclusively in rod photoreceptor cells.**

207 To determine cell type specificity of the light-off-induced PKA activation, we first

208 examined spatial confinement of the activation in photoreceptor cells. The light spot was

209 projected at the center of the view field (dashed circle in Fig. 3*A*), and time courses of the

210 PKA activation were compared among four areas: three inside and one outside of the

211 light spot (Fig. 3*B*). The activation was sharply confined to the illuminated area (Fig. 3*C*).

212   We also confirmed that the response was mediated by the phosphorylation on the

213 FRET biosensor AKAR3EV, but not by bleaching/switching of its fluorophores.

214 PKAchu-NC mice were used for this purpose. This negative control mouse strain carries

215 AKAR3EV-NC, which has an alanine substitution at the threonine residue in the PKA

216 substrate motif (SI Appendix, Fig. S1*C*) (29). In PKAchu-NC retinas, light-induced

217 changes were not observed (Fig. 3 *A* and *C*, SI Appendix, Movie S6), confirming that the

218 light response in the PKAchu retina is induced by the phosphorylation of the AKAR3EV.

219   Next, to study the cell-type specificity of the PKA activation, we performed 5D

220 imaging (x, y, z, time and PKA activity). Longitudinal reconstruction images showed that

11

221 PKA was activated exclusively in photoreceptor cells, especially at PRS (Fig. 3 *D* and *E*,

222 SI Appendix, Movie S7). A small number of cells in PRS showed less activation (Fig.

223 3*D*, arrowheads) than did surrounding cells, suggesting that PKA was activated less

224 efficiently in cones than in rods. Indeed, rod-specific PKA activation was visualized in

225 high-magnification cross-sectional images (Fig. 3 *F* and *G*, SI Appendix, Movie S8).

226 Taken together, these results indicated that the light-off-induced PKA activation occurs

227 exclusively in light-exposed rod photoreceptor cells. In the following experiments, we

228 further characterized this PKA activation in rods.

229

230 **Light-induced PKA activation is driven by rhodopsin and transducin.**

231 To identify the photopigment that drives the light-off-induced PKA activation, we

232 analyzed the spectral sensitivity of the response. For this purpose, responses toward

233 various intensities of light were recorded from rods (SI Appendix, Fig. S3*A*).

234 Measurements were performed for seven different wavelengths to obtain a series of

235 intensity-response relationships (SI Appendix, Fig. S3*B*). Then, half-saturating light

236 intensities ($I_{1/2}$) were estimated by fitting. The inverse of $I_{1/2}$ values were plotted as a

237 function of wavelength to obtain the spectral sensitivity (Fig. 4*A*). Assuming that the

238 light-induced PKA activation would be triggered by an opsin-family photopigment, the

239 data was fitted with an A1-pigment spectral template (45). The estimated λ_{max} was 489

240 nm, which suggests the involvement of rhodopsin (500 nm), isorhodopsin (487 nm) (46),

241 and M-cone pigment (508 nm) in the PKA activation. Intriguingly, the estimated

242 maximum amplitude increased as the wavelength became longer (SI Appendix, Fig.

243 S3*C*), which is opposite to the activity regulation of melanopsin (see Discussion for

244 detail).

245   To validate the involvement of rhodopsin in the light-off-induced PKA activation, we

246 crossed PKAchu mice with *Gnat1*$^{-/-}$ mice, which lack the α subunit of the G-protein

12

247 transducin and are deficient in rhodopsin-mediated signal transduction in rods (47). The

248 amplitude of the response in PRS was clearly decreased in PKAchu $Gnat1^{+/-}$ and

249 completely lost in PKAchu $Gnat1^{-/-}$ (Fig. 4*B*), showing the critical roles of rhodopsin and

250 transducin in the light-off-induced PKA activation. Notably, the estimated half-saturating

251 light intensity for 500 nm light was 6.5×10^7 photons μm^{-2} (SI Appendix, Fig. S3*B*),

252 which is $>10^4$-fold greater than that of the visual photoresponse in rods (48). In such a

253 high intensity range, the normal rod phototransduction is completely saturated. Thus,

254 these data suggest the presence of an alternative rod phototransduction mechanism, which

255 is activated exclusively with intense illumination.

256

257 **Both rhodopsin and isorhodopsin were detected in our light-adapted retinal explant.**

258 The estimated λ_{max} of the light-off-induced PKA photoresponse (489 nm) was 11 nm

259 shorter than the absorption maximum of rhodopsin (500 nm). To study the cause of this

260 blue-shift, we analyzed the absorption spectrum of photopigment extracted from our

261 light-adapted retinal explant. Wild-type C57BL6/J (B6J) retinas were flat-mounted using

262 the protocol performed in the PKA activity imaging, then, solubilized for absorption

263 measurements. As a control, retinas from dark-adapted mice were collected under dim

264 red light. Absorption of photopigment was detected from both light- and dark-adapted

265 retinas at around 500 nm (Fig. 5*A*). The peak height of the light-adapted sample was $16 \pm$

266 1% (mean $\pm$ SD, n = 3) of the height observed for the dark-adapted control. Surprisingly,

267 the absorption maximum of the light-adapted preparation was detected at 495 ± 2 nm

268 (mean $\pm$ SD, n = 3; Fig. 5*B*, Light), which was 7 nm shorter than that of the dark-adapted

269 control (504 ± 1 nm; Fig. 5*B*, Dark) and well fitted with the spectral sensitivity plot of the

270 light-off-induced PKA activation (Fig. 5*C*).

271   We speculated that the blue-shifted absorbance was caused by the formation of

272 isorhodopsin, which contains 9-*cis* retinal and shows an absorption maximum at 487 nm

13

273 (46). Indeed, the obtained absorption spectrum was fitted well with the sum of the

274 rhodopsin and isorhodopsin templates (Fig. 5*D*). To verify the presence of isorhodopsin,

275 the chromophore composition was determined by normal phase high performance liquid

276 chromatography (HPLC; Fig. 5*E*). Most of 11-*cis* retinal, which comprises $90 \pm 3\%$

277 (mean ± SD, n=2) of total retinoids in the dark-adapted control (Fig. 5 *E* and *F*, Dark),

278 was converted to all-*trans* retinal and all-*trans* retinol in the light-adapted sample (Fig. 5

279 *E* and *F*, Light). In agreement with the absorbance data, 11-*cis* retinal and 9-*cis* retinal

280 were also detected at comparable levels: $9.0 \pm 2.3\%$ and $8.3 \pm 0.8\%$, respectively (mean ±

281 SD, n = 3; Fig. 5*E* and *F*, Light). Therefore, we concluded that isorhodopsin is formed

282 specifically in the light-adapted retinal explants and produces the blue-shifted spectral

283 sensitivity of the light-off-induced PKA activation.

284

285 **Rhodopsin-deficient albino PKAchu rods show only the light-on-induced PKA**

286 **suppression.**

287 To further investigate the involvement of rhodopsin, we used albino mice, which show a

288 greater reduction in rhodopsin level compared to their wild-type counterparts when kept

289 under bright light (49). Although, rhodopsin content in the dark-adapted albino retina was

290 $51 \pm 5\%$ (mean ± SD, n = 4) to the wild-type level, surprisingly, that of the light-adapted

291 albino retina was less than the detection limit (Fig. 6*A*, Albino), without any thinning in

292 the ONL thickness (53.9 ± 1.3 µm at 1 mm superior from the ONH; mean ± SD, n = 6

293 retinas). The albino retina exhibited markedly higher basal PKA activity in the PRS layer

294 than the wild-type (Fig. 6 *B* and *C*). This high basal PKA activity in PRS was suppressed

295 to nearly the wild-type level in the presence of 100 nM exogenous dopamine (Fig. 6*D*),

296 suggesting a decreased dopamine level in the albino retina. In fact, the albino mice used

297 in this study (B6N-Tyr[c-Brd]/BrdCrCrl; B6 albino) are deficient in tyrosinase, which

298 supports dopamine biogenesis (50).

14

299   We performed the photostimulation experiments with albino PKAchu retinas. In stark

300   contrast to the wild-type, only transient suppression was observed following a brief 6 sec

301   stimulation (Fig. 6*E*, Albino). Moreover, the PKA activity was kept at a low level during

302   a 10 min stimulation and returned to the basal level upon light-off, without a prominent

303   overshoot (Fig. 6*F*, SI Appendix, Movie S9). These data further support the notion that

304   rhodopsin plays a critical role in the light-off-induced PKA activation in rods.

Discussion

305

306   In the present study, we report a two-photon live imaging method to visualize PKA

307   activity in all layers of the retina (Fig. 1, SI Appendix, Fig. S2, Movies S1-S4) and

308   describe the light-off-induced PKA activation in rods (Fig. 3, SI Appendix, Movies S7

309   and S8). The light-on-induced reduction of retinal cAMP was reported in previous studies

310   (16, 41, 51). Here, our live imaging approach with high spatiotemporal resolution has

311   succeeded in characterizing the light-off-induced PKA activation. Application of two-

312   photon microscopy to the retina is technically difficult because visual pigments are

313   substantially activated by both high-power infrared excitation laser and fluorescence

314   emission from probes, which cause a low-photopic level of activation in the visual

315   transduction system (52) (*SI Appendix, Supplementary Text*). The light-off-induced PKA

316   activation was readily observed, probably because this response requires a high-photopic

317   level of illumination from the external light source (SI Appendix, Figs. S3 and S4).

318   The mechanism underlying the light-off-induced PKA activation is still unknown, but

319   we suggest that this mechanism likely involves two different light-dependent pathways: a

320   PKA suppression pathway with fast kinetics and a PKA activation pathway with slow

321   kinetics (Fig. 6*G*). Rhodopsin-containing wild-type PKAchu retinas showed transient

322   PKA suppression during the 10 min light-on period followed by immediate and robust

323   PKA activation upon light-off (Fig. 6*F*, SI Appendix, Movie S9). In contrast, the albino

324   PKAchu explants showed persistent PKA suppression during the 10 min light-on period

325   (Fig. 6*F,* SI Appendix, Movie S9). Because rhodopsin was not detected in the albino

326   retinal explant (Fig. 6*A*), this persistent suppression is likely to be mediated by the light-

327   induced dopamine release that can be triggered by cone visual pigments and/or

328   melanopsin in intrinsically photosensitive retinal ganglion cells (15) [but see (14)]. We

329   speculate that the persistent PKA suppression also operates in wild-type mice during the

330   light-on period, but that its presence is masked by rhodopsin-mediated PKA activation.

16

331 The fast kinetics of the suppression pathway is supported by the observation that the PKA

332 activity is restored immediately after light-off in the albino PKAchu (Fig. 6*F*).

333   Rhodopsin and transducin play critical roles in the light-off-induced PKA activation

334 (Fig. 4). Isorhodopsin is detected in the retina (Fig. 5E), but is not necessarily required for

335 the light-off-induced PKA activation, because the response was also observed in dark-

336 adapted PKAchu retinas devoid of 9-*cis* retinal (Figs. 2*C* and 5*F*). Conventional

337 phototransduction, which utilizes the cGMP system (1-3), is also not likely to underlie the

338 mechanism by the two reasons. First, the working light intensity of the light-off-induced

339 PKA activation (SI Appendix, Fig. S3 *A* and *B*) is >10^4-fold higher than that of the rod

340 visual transduction (48). Second, the duration of the light-off-induced PKA activation is

341 >10 min (Fig. 3); this is clearly longer than that of the visual photoresponse, which ends

342 in a few seconds (48). Therefore, we speculate the presence of an alternative pathway

343 acting downstream of transducin. Because the light-off-induced PKA activation is strictly

344 confined within the illuminated region (Fig. 3 *A-C,* SI Appendix, Movie S6), intercellular

345 signaling mechanisms may be excluded from this process.

346   One possible mechanism underlying the transducin-mediated PKA activation is the

347 light-dependent transducin translocation. The Ca^{2+}-insensitive AC isoforms are enriched

348 in the photoreceptor inner segment (53). It is suggested that transducin stimulates AC in

349 primary culture of tiger salamander rods (43). Rod transducin is localized in the outer

350 segment in darkness, but moves to the inner segment under bright light. This

351 translocation contributes to the rod survival and synaptic transmission to rod bipolar cells

352 (54), and is suggested to be a mechanism to escape from rod saturation (55). The

353 transducin translocation occurs under the light level that saturates rod photoresponse with

354 a half-time of completion of 5 and 12.5 min for α and β subunits, respectively (56) (57).

355 These features coincide with the high half-saturation light intensity (SI Appendix, Fig.

356 S3) and time course (Fig. 2) of the light-off-induced PKA activation. In addition, cone

17

357 transducin does not show the translocation (58), in line with the lack of the light-off-

358 induced PKA activation in cones (Fig. 3F). Interestingly, phosphorylation of phosducin,

359 one of the PKA targets in rods (21), facilitates the recovery from the translocation (59).

360 The transducin-mediated PKA activation may constitute a feedback loop to transducin for

361 the recovery from the translocation to boost the dark-adaptation.

362 The presence of isorhodopsin in our light-adapted retinal explants was unexpected

363 (Fig. 5). As a similar example, photoregeneration of isorhodopsin was reported

364 previously in frog retinal explants (60). In that case, the isorhodopsin was thought to be a

365 product of back photoconversion from a meta-intermediate of rhodopsin. It is tempting to

366 speculate that low-quantum yield isorhodopsin (61) plays a role in rod light-adaptation.

367 However, we suppose that isorhodopsin is not formed in the mouse retina *in vivo* because

368 9-*cis* retinal is not detected from retinoids directly extracted from light-adapted mouse

369 eyes (62). We speculate that isorhodopsin formation is an artifact caused by our sample

370 preparation, in which retinas were temporarily exposed to light at room temperature.

371 The maximum amplitude of the light-off-induced PKA activation became larger as

372 the light stimulus was shifted to longer wavelengths (SI Appendix, Fig. S3 *B* and *C*). As

373 the spectral sensitivity showed its maxima at 489 nm (Fig. 4*A*), this discrepancy suggests

374 the input from another photopigment(s) that independently regulates the amplitude.

375 Melanopsin displays a tristable photoequilibrium, and the fraction of its signaling form is

376 decreased as the wavelength becomes longer within the 400-600 nm range (63, 64).

377 Therefore, we speculate that red light-induced melanopsin inactivation increases the

378 amplitude of the light-off-induced PKA activation via the aforementioned dopamine-

379 mediated pathway.

380 Lastly, what is the physiological role of the light-off-induced PKA activation?

381 Previous biochemical studies have shown that PKA phosphorylates many

382 phototransduction components (19-22) and gap junction protein (24).

18

383   Electrophysiological studies have shon that cAMP improves the rod photosensitivity (11,

384   23). Taken together, these findings suggest that PKA promotes the dark-adaptation of

385   rods. In support of this notion, recent studies by Kolesnikov and colleagues reported a

386   delayed rod dark-adaptation in mutant mice whose GRK1 is mutated to block its cAMP-

387   dependent phosphorylation (65). PKA phosphorylates GRK1 to reduce its rhodopsin

388   phosphorylation ability (19), which in turn extends the active lifetime of rhodopsin to

389   increase the photosensitivity. They also reported lack of phenotype in cone dark-

390   adaptation, which is consistent with the lack of responses in our PKAchu cones (Fig. 3 *F*

391   and *G*). Therefore, we speculate that the physiological role of the light-off-induced PKA

392   activation is the acceleration of rod dark-adaptation upon the transition from a light to a

393   dark environment. Further physiological studies will be needed to quantitatively describe

394   the contribution of PKA in rod dark-adaptation.

395

396   **Materials and Methods**

397   **Animals.** Colonies of wild-type PKAchu (nbio185; NIBIOHN) and PKAchu-NC

398   (nbio186) transgenic mouse lines were maintained in heterozygous state on the B6J

399   background. Albino PKAchu was obtained by crossing with B6 albino (Charles River

400   Laboratories Japan). PKAchu Gnat1$^{+/-}$ and PKAchu Gnat1$^{-/-}$ were obtained by crossing

401   wild-type PKAchu with Gnat1$^{-/-}$ (B6J background; the kind gift from Dr. Vladimir J

402   Kefalov) for 2-3 generations.

403   **Two-photon live imaging of the retinal explant.** A flat-mounted retina was perfused

404   with oxygenated medium and imaged with an upright multi-photon microscope system

405   (Fluoview FV1000MPE; Olympus) using a water immersion objective lens

406   (XLPLN25XWMP; Olympus). Stimulation light was generated with a custom-made LED

407   system (SI Appendix, Fig. S4) and delivered to the retina through the objective lens.

19

408   All additional experimental details are provided in *SI Appendix, Supplementary*

409   *Methods*.

410

Acknowledgements

422

423   **References**

424   1.   S. Kawamura, S. Tachibanaki, Rod and cone photoreceptors: molecular basis of the
425   difference in their physiology. *Comp Biochem Physiol A Mol Integr Physiol* **150**, 369-377
426   (2008).

427   2.   G. L. Fain, R. Hardie, S. B. Laughlin, Phototransduction and the evolution of
428   photoreceptors. *Curr Biol* **20**, R114-124 (2010).

429   3.   T. D. Lamb, Evolution of phototransduction, vertebrate photoreceptors and retina. *Prog*
430   *Retin Eye Res* **36**, 52-119 (2013).

431   4.   K. W. Yau, R. C. Hardie, Phototransduction motifs and variations. *Cell* **139**, 246-264
432   (2009).

433   5.   L. Astakhova, M. Firsov, V. Govardovskii, Activation and quenching of the
434   phototransduction cascade in retinal cones as inferred from electrophysiology and
435   mathematical modeling. *Mol Vis* **21**, 244-263 (2015).

436   6.   X. H. Wen, A. M. Dizhoor, C. L. Makino, Membrane guanylyl cyclase complexes shape
437   the photoresponses of retinal rods and cones. *Front Mol Neurosci* **7**, 45 (2014).

438   7.   O. P. Gross, E. N. Pugh Jr., M. E. Burns, cGMP in mouse rods: the spatiotemporal
439   dynamics underlying single photon responses. *Front Mol Neurosci* **8**, 6 (2015).

440   8.   F. Vinberg, J. Chen, V. J. Kefalov, Regulation of calcium homeostasis in the outer
441   segments of rod and cone photoreceptors. *Prog Retin Eye Res* **67**, 87-101 (2018).

442   9.   J. Zang, S. C. F. Neuhauss, The binding properties and physiological functions of
443   recoverin. *Front Mol Neurosci* **11**, 473 (2018).

444   10.   Senin, II, K. W. Koch, M. Akhtar, P. P. Philippov, Ca2+-dependent control of rhodopsin
445   phosphorylation: recoverin and rhodopsin kinase. *Adv Exp Med Biol* **514**, 69-99 (2002).

446   11.   L. A. Astakhova, D. A. Nikolaeva, T. V. Fedotkina, V. I. Govardovskii, M. L. Firsov,
447   Elevated cAMP improves signal-to-noise ratio in amphibian rod photoreceptors. *J Gen*
448   *Physiol* **149**, 689-701 (2017).

449   12.   E. Popova, Role of dopamine in distal retina. *J Comp Physiol A Neuroethol Sens Neural*
450   *Behav Physiol* **200**, 333-358 (2014).

451   13.   P. Witkovsky, Dopamine and retinal function. *Doc Ophthalmol* **108**, 17-40 (2004).

452   14.   V. Pérez-Fernández *et al.*, Rod photoreceptor activation alone defines the release of
453   dopamine in the retina. *Curr Biol* **29**, 763-774 e765 (2019).

454   15.   C. L. Prigge *et al.*, M1 ipRGCs influence visual function through retrograde signaling in
455   the retina. *J Neurosci* **36**, 7184-7197 (2016).

456   16.   A. I. Cohen, R. D. Todd, S. Harmon, K. L. O'Malley, Photoreceptors of mouse retinas
457   possess D4 receptors coupled to adenylate cyclase. *Proc Natl Acad Sci U S A* **89**, 12093-
458   12097 (1992).

459   17.   C. R. Jackson, S. S. Chaurasia, C. K. Hwang, P. M. Iuvone, Dopamine D(4) receptor
460   activation controls circadian timing of the adenylyl cyclase 1/cyclic AMP signaling
461   system in mouse retina. *Eur J Neurosci* **34**, 57-64 (2011).

462   18.   K. A. Neve, J. K. Seamans, H. Trantham-Davidson, Dopamine receptor signaling. *J*
463   *Recept Signal Transduct Res* **24**, 165-205 (2004).

464   19.   T. J. Horner, S. Osawa, M. D. Schaller, E. R. Weiss, Phosphorylation of GRK1 and
465   GRK7 by cAMP-dependent protein kinase attenuates their enzymatic activities. *J Biol*
466   *Chem* **280**, 28241-28250 (2005).

467   20.   N. Balasubramanian, K. Levay, T. Keren-Raifman, E. Faurobert, V. Z. Slepak,
468   Phosphorylation of the regulator of G protein signaling RGS9-1 by protein kinase A is a
469   potential mechanism of light- and Ca2+-mediated regulation of G protein function in
470   photoreceptors. *Biochemistry* **40**, 12619-12627 (2001).

471   21.   H. Song, M. Belcastro, E. J. Young, M. Sokolov, Compartment-specific phosphorylation
472   of phosducin in rods underlies adaptation to various levels of illumination. *J Biol Chem*
473   **282**, 23613-23621 (2007).

474   22.   G. Wolbring, P. P. Schnetkamp, Modulation of the calcium sensitivity of bovine retinal
475   rod outer segment guanylyl cyclase by sodium ions and protein kinase A. *Biochemistry*
476   **35**, 11013-11018 (1996).

477   23.   L. A. Astakhova, E. V. Samoiliuk, V. I. Govardovskii, M. L. Firsov, cAMP controls rod
478   photoreceptor sensitivity via multiple targets in the phototransduction cascade. *J Gen*
479   *Physiol* **140**, 421-433 (2012).

480   24.   H. Li *et al.*, Adenosine and dopamine receptors coregulate photoreceptor coupling via
481   gap junction phosphorylation in mouse retina. *J Neurosci* **33**, 3135-3150 (2013).

482   25.   P. H. Li, J. Verweij, J. H. Long, J. L. Schnapf, Gap-junctional coupling of mammalian
483   rod photoreceptors and its effect on visual detection. *J Neurosci* **32**, 3552-3562 (2012).

484   26.   A. Goto *et al.*, Circuit-dependent striatal PKA and ERK signaling underlies rapid
485   behavioral shift in mating reaction of male mice. *Proc Natl Acad Sci U S A* **112**, 6718-
486   6723 (2015).

487   27.   T. Yamaguchi *et al.*, Role of PKA signaling in D2 receptor-expressing neurons in the
488   core of the nucleus accumbens in aversive learning. *Proc Natl Acad Sci U S A* **112**,
489   11383-11388 (2015).

22

490   28.   R. Mizuno *et al.*, In vivo imaging reveals PKA regulation of ERK activity during
491   neutrophil recruitment to inflamed intestines. *J Exp Med* **211**, 1123-1136 (2014).
492   29.   Y. Kamioka *et al.*, Live imaging of protein kinase activities in transgenic mice expressing
493   FRET biosensors. *Cell Struct Funct* **37**, 65-73 (2012).
494   30.   N. Komatsu *et al.*, Development of an optimized backbone of FRET biosensors for
495   kinases and GTPases. *Mol Biol Cell* **22**, 4647-4656 (2011).
496   31.   M. A. Samuel, Y. Zhang, M. Meister, J. R. Sanes, Age-related alterations in neurons of
497   the mouse retina. *J Neurosci* **31**, 16033-16044 (2011).
498   32.   C. Behrens, T. Schubert, S. Haverkamp, T. Euler, P. Berens, Connectivity map of bipolar
499   cells and photoreceptors in the mouse retina. *Elife* **5** (2016).
500   33.   L. V. Johnson, G. S. Hageman, J. C. Blanks, Interphotoreceptor matrix domains ensheath
501   vertebrate cone photoreceptor cells. *Invest Ophthalmol Vis Sci* **27**, 129-135 (1986).
502   34.   J. Zhang, Y. Ma, S. S. Taylor, R. Y. Tsien, Genetically encoded reporters of protein
503   kinase A activity reveal impact of substrate tethering. *Proc Natl Acad Sci U S A* **98**,
504   14997-15002 (2001).
505   35.   K. Aoki, M. Matsuda, Visualization of small GTPase activity with fluorescence
506   resonance energy transfer-based biosensors. *Nat Protoc* **4**, 1623-1631 (2009).
507   36.   D. Mustafi *et al.*, Transcriptome analysis reveals rod/cone photoreceptor specific
508   signatures across mammalian retinas. *Hum Mol Genet* **25**, 4376-4388 (2016).
509   37.   D. G. McMahon, P. M. Iuvone, G. Tosini, Circadian organization of the mammalian
510   retina: From gene regulation to physiology and diseases. *Progress in Retinal and Eye*
511   *Research* **39**, 58-76 (2014).
512   38.   P. Farshi, B. Fyk-Kolodziej, D. M. Krolewski, P. D. Walker, T. Ichinose, Dopamine D1
513   receptor expression is bipolar cell type-specific in the mouse retina. *J Comp Neurol* **524**,
514   2059-2079 (2016).
515   39.   A. I. Cohen, C. Blazynski, Dopamine and its agonists reduce a light-sensitive pool of
516   cyclic AMP in mouse photoreceptors. *Vis Neurosci* **4**, 43-52 (1990).
517   40.   I. Nir *et al.*, Dysfunctional light-evoked regulation of cAMP in photoreceptors and
518   abnormal retinal adaptation in mice lacking dopamine D4 receptors. *J Neurosci* **22**, 2063-
519   2073 (2002).
520   41.   C. R. Jackson *et al.*, Essential roles of dopamine D4 receptors and the type 1 adenylyl
521   cyclase in photic control of cyclic AMP in photoreceptor cells. *J Neurochem* **109**, 148-
522   157 (2009).
523   42.   Z. Cheng, Y. M. Zhong, X. L. Yang, Expression of the dopamine transporter in rat and
524   bullfrog retinas. *Neuroreport* **17**, 773-777 (2006).

525 43. P. D. Alfinito, E. Townes-Anderson, Activation of mislocalized opsin kills rod cells: a
526   novel mechanism for rod cell death in retinal disease. *Proc Natl Acad Sci U S A* **99**, 5655-
527   5660 (2002).

528 44. J. Wang, N. Zhang, A. Beuve, E. Townes-Anderson, Mislocalized opsin and cAMP
529   signaling: a mechanism for sprouting by rod cells in retinal degeneration. *Invest*
530   *Ophthalmol Vis Sci* **53**, 6355-6369 (2012).

531 45. V. I. Govardovskii, N. Fyhrquist, T. Reuter, D. G. Kuzmin, K. Donner, In search of the
532   visual pigment template. *Vis Neurosci* **17**, 509-528 (2000).

533 46. J. Fan, B. Rohrer, G. Moiseyev, J. X. Ma, R. K. Crouch, Isorhodopsin rather than
534   rhodopsin mediates rod function in RPE65 knock-out mice. *Proc Natl Acad Sci U S A*
535   **100**, 13662-13667 (2003).

536 47. P. D. Calvert *et al.*, Phototransduction in transgenic mice after targeted deletion of the rod
537   transducin alpha -subunit. *Proc Natl Acad Sci U S A* **97**, 13913-13918 (2000).

538 48. S. Nymark, R. Frederiksen, M. L. Woodruff, M. C. Cornwall, G. L. Fain, Bleaching of
539   mouse rods: microspectrophotometry and suction-electrode recording. *J Physiol* **590**,
540   2353-2364 (2012).

541 49. G. H. Daly, J. M. DiLeonardo, N. R. Balkema, G. W. Balkema, The relationship between
542   ambient lighting conditions, absolute dark-adapted thresholds, and rhodopsin in black and
543   hypopigmented mice. *Vis Neurosci* **21**, 925-934 (2004).

544 50. A. T. Slominski *et al.*, "Sensing the environment: regulation of local and global
545   homeostasis by the skin's neuroendocrine system" in Advances in Anatomy, Embryology
546   and Cell Biology*, P. Sutovsky, Ed. (Springer, 2012), vol. 212, chap. 2, pp. 7-26.

547 51. G. W. DeVries, A. I. Cohen, I. A. Hall, J. A. Ferrendelli, Cyclic nucleotide levels in
548   normal and biologically fractionated mouse retina: effects of light and dark adaptation. *J*
549   *Neurochem* **31**, 1345-1351 (1978).

550 52. T. Euler, K. Franke, T. Baden, "Studying a light sensor with light: multiphoton imaging
551   in the retina" in Multiphoton Microscopy*, E. Hartveit, Ed. (Humana Press, 2019), vol.
552   148, chap. 10, pp. 225-250.

553 53. R. M. Abdel-Majid, F. Tremblay, W. H. Baldridge, Localization of adenylyl cyclase
554   proteins in the rodent retina. *Brain Res Mol Brain Res* **101**, 62-70 (2002).

555 54. A. Majumder *et al.*, Transducin translocation contributes to rod survival and enhances
556   synaptic transmission from rods to rod bipolar cells. *Proc Natl Acad Sci U S A* **110**,
557   12468-12473 (2013).

558 55. A. Tikidji-Hamburyan *et al.*, Rods progressively escape saturation to drive visual
559   responses in daylight conditions. *Nat Commun* **8**, 1813 (2017).

560   56.   E. S. Lobanova *et al.*, Transducin translocation in rods is triggered by saturation of the
561   GTPase-activating complex. *J Neurosci* **27**, 1151-1160 (2007).

562   57.   M. Sokolov *et al.*, Massive light-driven translocation of transducin between the two
563   major compartments of rod cells: a novel mechanism of light adaptation. *Neuron* **34**, 95-
564   106 (2002).

565   58.   E. S. Lobanova *et al.*, Mechanistic basis for the failure of cone transducin to translocate:
566   why cones are never blinded by light. *J Neurosci* **30**, 6815-6824 (2010).

567   59.   M. Belcastro *et al.*, Phosphorylation of phosducin accelerates rod recovery from
568   transducin translocation. *Invest Ophthalmol Vis Sci* **53**, 3084-3091 (2012).

569   60.   T. Reuter, Photoregeneration of rhodopsin and isorhodopsin from metarhodopsin III in
570   the frog retina. *Vision Res* **16**, 909-917 (1976).

571   61.   R. Hubbard, A. Kropf, The action of light on rhodopsin. *Proc Natl Acad Sci U S A* **44**,
572   130-139 (1958).

573   62.   K. A. Lee, M. Nawrot, G. G. Garwin, J. C. Saari, J. B. Hurley, Relationships among
574   visual cycle retinoids, rhodopsin phosphorylation, and phototransduction in mouse eyes
575   during light and dark adaptation. *Biochemistry* **49**, 2454-2463 (2010).

576   63.   T. Matsuyama, T. Yamashita, Y. Imamoto, Y. Shichida, Photochemical properties of
577   mammalian melanopsin. *Biochemistry* **51**, 5454-5462 (2012).

578   64.   A. J. Emanuel, M. T. Do, Melanopsin tristability for sustained and broadband
579   phototransduction. *Neuron* **85**, 1043-1055 (2015).

580   65.   A. V. Kolesnikov, J. D. Chrispell, S. Osawa, V. J. Kefalov, E. R. Weiss, Phosphorylation
581   at Serine 21 in G protein-coupled receptor kinase 1 (GRK1) is required for normal
582   kinetics of dark adaption in rod but not cone photoreceptors. *FASEB J* **34**, 2677-2690
583   (2020).

584

Figure legends

Figure 1 Two-photon imaging of the PKAchu retina. (A) Imaging setup. The isolated PKAchu retina was flat-mounted on a culture insert, perfused with DMEM/F12 medium and imaged using an upright two-photon microscope with a water immersion objective lens. (B-G) Fluorescent images obtained from the PKAchu retina at the ganglion cell layer (GCL; B), inner plexiform layer (IPL; C), inner nuclear layer (INL; D), outer plexiform layer (OPL; E), outer nuclear layer (ONL; F) and photoreceptor segments layer (PRS; G). Images were obtained by averaging CFP Ch and FRET Ch images. (H) Longitudinal view of the PKAchu retina. The image was reconstructed from z-stack images (214 planes with 1 μm z-intervals). White arrowheads indicate cone nuclei. Black arrowheads indicate z-positions of the cross-sectional images in (B)-(G), and an arrow indicates the z position from which the excitation laser power was attenuated to avoid detector saturation. (I) Cone labeling with PNA-rhodamine (magenta) overlaid with AKAR3EV signals (green).

Figure 2 Basal PKA activities and light-induced PKA activity changes in the dark- and light-adapted PKAchu retinas. (A) Longitudinal PKA activity images. Arrowheads indicate the positions of each layer analyzed in (B). (B) FRET/CFP values obtained from each layer of the retina. Mean ± SD, n = 7 and 18 measurements for light- and dark-adapted retinas, respectively. The numbers on the bars are p-values from Tukey-Kramer's test following a two-way ANOVA. (C) Time-lapse PKA activity images from dark- and light-adapted retinas. Images are cross-sectional views obtained at the PRS layer. An arrow indicates the timing of a 6 sec light stimulation (500 nm, 1.0×10^7 photons μm^{-2} sec^{-1}). (D) Time courses of the PKA activity at the PRS layer in dark- and light-adapted retinas. Mean ± SD (n = 3 and 8 measurements, respectively). (E) The early phase of the light response in light-adapted retinas. Mean ± SD (n = 5). (F) Time-lapse PKA activity

26

611 images at the PRS layer in the light-adapted retina. Bars indicate the timing of a 10 min-

612 light stimulation (500 nm, 3.2×10^7 photons μm^{-2} sec^{-1}). (G) Time course of the PKA

613 activity at the PRS layer. A dashed bar indicates the timing of light stimulation. Light was

614 temporarily turned off four times for image acquisitions (every 2 min, <10 sec each).

615 Mean ± SD (n = 4). Data points in (D), (E) and (F) were obtained every 2 min, 12 sec and

616 2 min, respectively.

617

618 **Figure 3** Spatial distribution of the light-off-induced PKA activation. (A) Time-lapse

619 PKA activity images from light-adapted PKAchu and PKAchu-NC retinal explants.

620 Images are cross-sectional views at the PRS layer. Light stimulation (dashed circle,

621 wavelength 500 nm, 1.0×10^8 photons μm^{-2} sec^{-1}, 6 sec) was given just before 0 min

622 (arrow). Scale bar: 400 µm. (B) Positions of four areas analyzed in (C). (C) Time courses

623 of the ΔFRET/CFP in the 6 sec-stimulation experiments. Values are the means of five

624 (PKAchu) and three (PKAchu-NC) measurements. (D) Longitudinal view of the light-

625 induced PKA activation toward 6 sec stimulation (500 nm, 1.0×10^8 photons μm^{-2} sec^{-1}).

626 Images were reconstructed from a z-stack of 61 images with 3 µm z-intervals. White

627 arrowheads show cones. Black arrowheads indicate the layer positions analyzed in (E).

628 (E) Time courses of the light-induced PKA activity change at the four indicated layers.

629 Values are the mean of four measurements. (F) High-magnification cross-sectional view

630 of the light-off-induced PKA activation at the PRS layer. Images were obtained before

631 and after the 6 sec stimulation (500 nm, 1.0×10^8 photons μm^{-2} sec^{-1}). White arrowheads

632 indicate cones. (G) Time courses of the light-induced PKA activity change in rods (black)

633 and cones (cyan), in the 6 sec-stimulation experiments. Rod and cone signals were

634 separated by intensity-based image segmentation. Values are the mean ± SD (n = 8

635 measurements). Data points in (C), (E), and (G) were obtained every 2 min. An arrow in

636 each panel indicates the timing of light stimulation.

27

637

638 **Figure 4** Involvement of rhodopsin and transducin in the light-off-induced PKA

639 activation. (A) Spectral sensitivity plot of the light-induced rod PKA activation. Plots

640 were fitted with the spectral template of the visual pigment to obtain the estimated λ_{max} of

641 489 nm (dashed curve). Data were normalized to the peak of the curve. Values are the

642 mean ± SEM (n = 3 to 7). (B) Time courses of the PKA activity in the 6 sec-stimulation

643 experiments, obtained from rods imaged at PRS in wild-type PKAchu, PKAchu $Gnat1^{+/-}$

644 and PKAchu $Gnat1^{-/-}$. Data for wild-type PKAchu is a replot of Fig. 3*G*. An arrow in

645 each panel indicates the timing of light stimulation (500 nm, 1×10^8 photons μm^{-2}, 6 sec).

646 Values are the mean ± SD (n = 9 for PKAchu $Gnat1^{+/-}$ and n = 18 for PKAchu $Gnat1^{-/-}$).

647

648 **Figure 5** Formation of isorhodopsin in the light-adapted retinal explant. (A) Difference

649 absorption spectra of retinal photopigments measured at 4 °C. Solid curve: light-adapted

650 retinal explants, dashed curve: dark-adapted control. Values are the average of three

651 measurements. Each sample was prepared from one retina. (B) Normalized spectra

652 created from the data in (A). (C) A comparison between the difference absorption

653 spectrum of the light-adapted retina (red) and the spectral sensitivity data (open circles,

654 replot of Fig. 4*A*). Note that the vertical axis is in log scale. (D) Data fitting with

655 rhodopsin (red) and isorhodopsin templates (cyan). The averaged spectrum of the light-

656 adapted retina (solid black curve, replot from (A)) was fitted with the sum of the

657 rhodopsin and isorhodopsin spectra (green). (E) HPLC chromatogram of retinoids

658 extracted from a dark- or light-adapted retina. Retinal isomers were extracted as *syn*- and

659 *anti*-oxime forms, separated by the normal phase HPLC, and detected by their absorbance

660 at 360 nm. (F) Molar ratio of the retinoids estimated by HPLC. Values are the means of

661 two and three measurements for dark- and light-adapted retinas, respectively. 11c: 11-*cis*

662 retinal; at: all-*trans* retinal; 9c: 9-*cis* retinal; 13: 13-*cis* retinal; ROL: all-*trans* retinol.

28

663

664 **Figure 6** Basal PKA activity and light-induced PKA suppression in the albino PKAchu

665 retina. (A) Difference absorption spectra of membrane proteins extracted from a wild-

666 type (black) or an albino (red) mouse retina. Values are the means of three samples. (B)

667 Longitudinal PKA activity images of wild-type and albino PKAchu retinas. Arrowheads

668 indicate the positions of each layer analyzed in (C). (C) FRET/CFP values obtained from

669 each layer of the retina. Mean ± SD, n = 7 and 14 measurements for the wild-type and

670 albino retinas, respectively. The number on the bar is a p-value from Tukey-Kramer's test

671 following a two-way ANOVA. (D) Responses of rod PKA to 100 nM dopamine

672 perfusion in wild-type (black) and albino (red) PKAchu retinas. Light-colored thin curves

673 and dark-colored bold curves are individual and averaged data, respectively. (E, F).

674 Light-induced PKA activity changes in response to 6 sec stimulation (E; 500 nm, 1.0 ×

675 10^8 photons μm^{-2} sec^{-1}) and 10 min stimulation (F; 500 nm, 3.2 × 10^7 photons μm^{-2} sec^{-1}).

676 Albino data (red, mean ± SD, n = 4 and 5, respectively) are adjusted 0.16 upward from

677 the wild-type data (dashed black) with consideration for the average difference in the

678 basal FRET/CFP (PRS in (C)). Wild-type data are replots of Fig. 3*G* and Fig. 2*G*,

679 respectively. Data points were obtained every 2 min. (G) A hypothetical model of the

680 light-off-induced PKA activation. Green bars show the timing of a light stimulation.
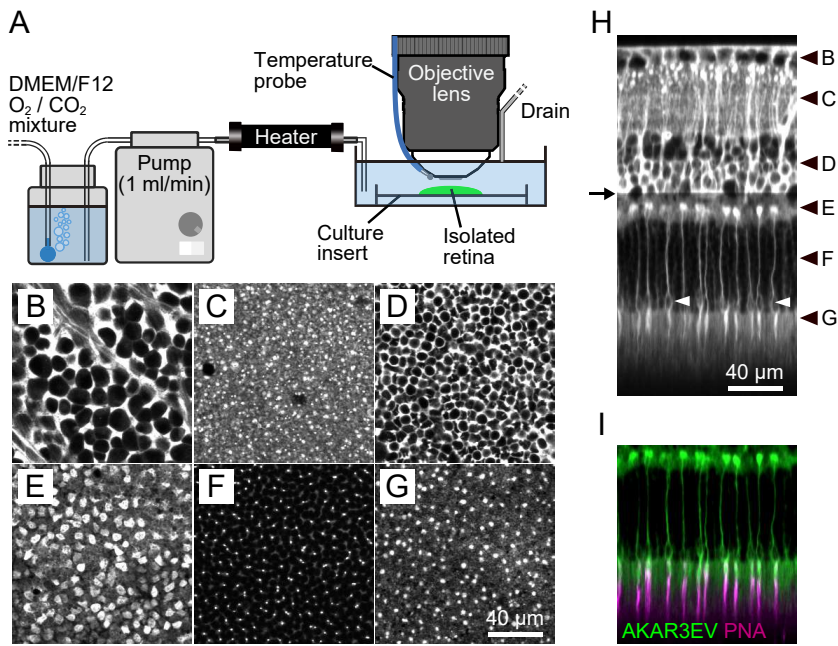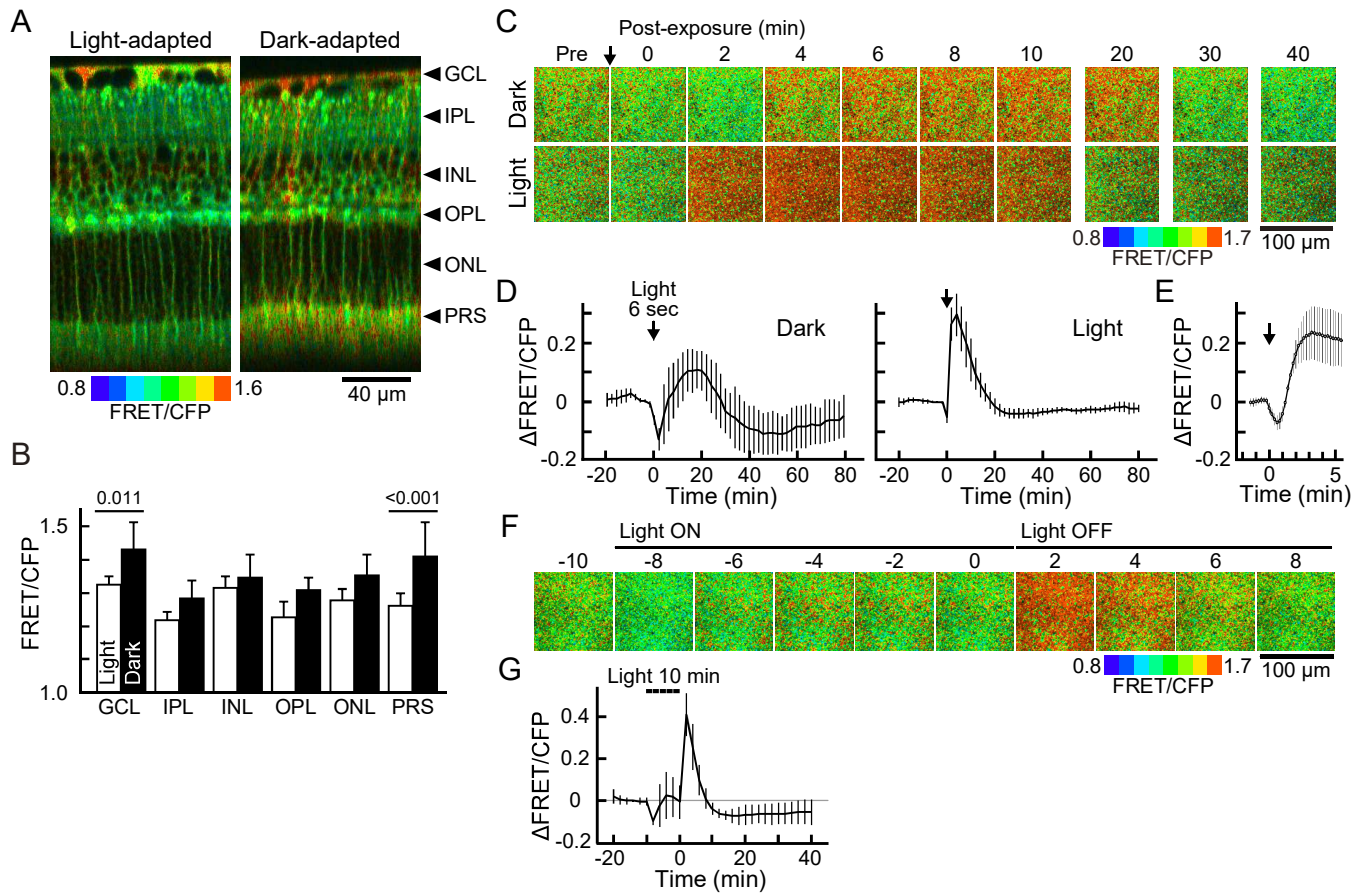
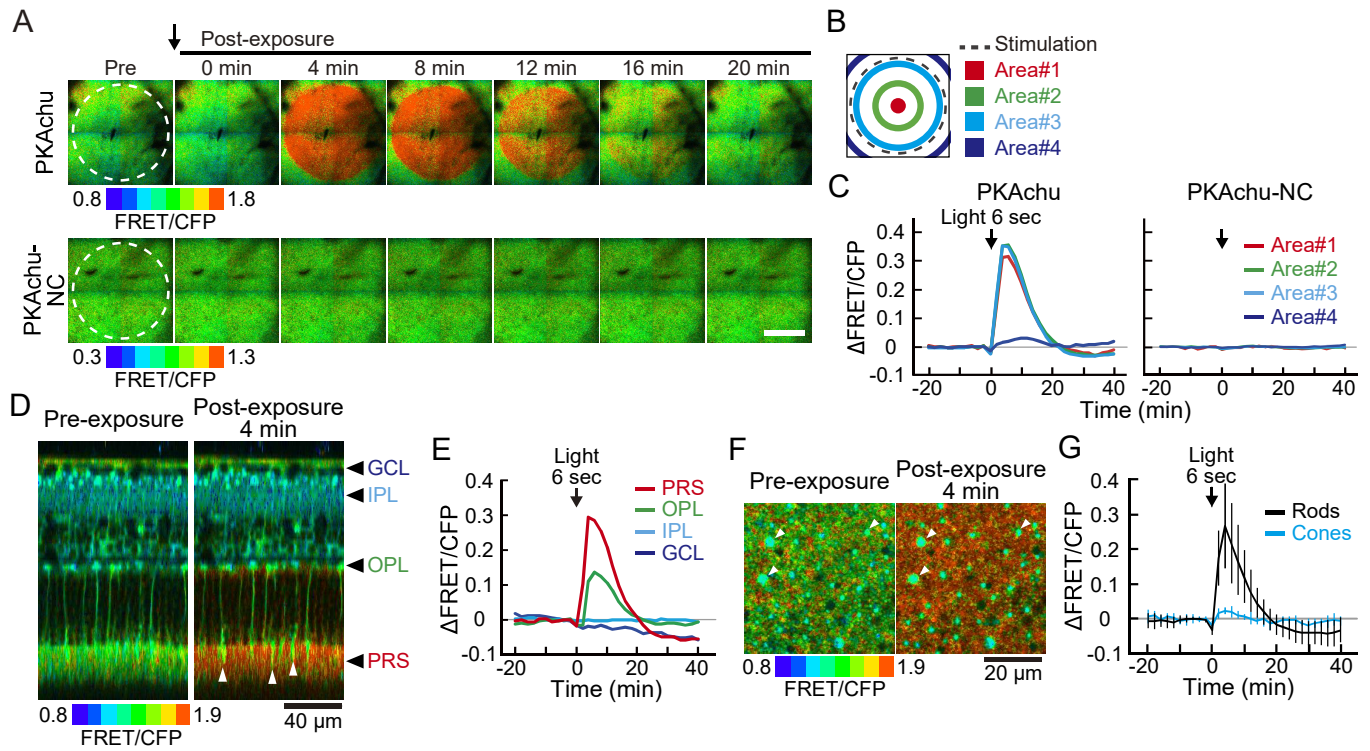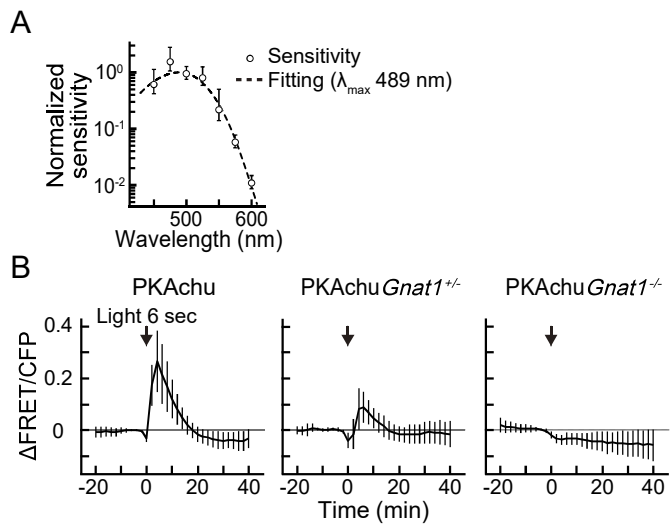681

Figure 1

Figure 2

Figure 3

A

Post-exposure

PKAchu
Pre   0 min   4 min   8 min   12 min   16 min   20 min

0.8 — 1.8
FRET/CFP

PKAchu-NC

0.3 — 1.3
FRET/CFP

B
Stimulation
Area#1
Area#2
Area#3
Area#4

C
PKAchu   PKAchu-NC
Light 6 sec

ΔFRET/CFP
Area#1
Area#2
Area#3
Area#4
Time (min)

D
Pre-exposure   Post-exposure 4 min
GCL
IPL
OPL
PRS
0.8 — 1.9
FRET/CFP
40 μm

E
Light 6 sec
ΔFRET/CFP
PRS
OPL
IPL
GCL
Time (min)

F
Pre-exposure   Post-exposure 4 min
0.8 — 1.9
FRET/CFP
20 μm

G
Light 6 sec
ΔFRET/CFP
Rods
Cones
Time (min)

Figure 4

A

B

Figure 5

Figure 6

A

B Wild-type   Albino

◀ GCL
◀ IPL
◀ INL
◀ OPL
◀ ONL
◀ PRS

0.8 ████████████ 1.6
FRET/CFP

40 μm

C

D 100 nM Dopamine

E Light 6 sec

F Light 10 min

G

Intense light → Rhodopsin → Transducin → (unknown) → → → Activation slow kinetics

Intense light → Cone pigment and/or Melanopsin → Dopamine → Suppression fast kinetics

Light-off-induced PKA activation

PNAS

www.pnas.org

1

2   Supplementary Information for

3   **Rhodopsin-mediated Light-off-induced Protein Kinase A Activation in**

4   **Mouse Rod Photoreceptor Cells.**

5   Shinya Sato[a*], Takahiro Yamashita[b], and Michiyuki Matsuda[a,c]

6   [a]Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University, Kyoto

7   606-8501 Japan;

8   [b]Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502 Japan;

9   [c]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto

10   606-8501 Japan;

11   *To whom correspondence should be addressed: Shinya Sato

12   **Address**: Laboratory of Bioimaging and Cell Signaling, Graduate School of Biostudies, Kyoto University,

13   Kyoto 606-8501 Japan;

14   **TEL:** +81-75-753-9450

15   **Email:** sato.shinya.7e@kyoto-u.ac.jp

16

17   **This PDF file includes:**
18   Supplementary Text
19   Supplementary Methods
20   Figures S1 to S4
21   Legends for Movies S1 to S9
22   SI References
23

24   **Other supplementary materials for this manuscript include the following:**
25   Movies S1 to S9
26

54   comparable to $(1.6 \times 10^{12}) \times (2.4 \times 10^{-9}) \approx 3.8 \times 10^3$ photons μm^{-2} flash of 500 nm light.

55   By multiplying the collecting area of a mouse rod, 0.47 μm^2 (4), rhodopsin activation was

56   estimated as 1.8×10^3 R* rod^{-1}, when the retina is dark-adapted and contains ~100%

57   rhodopsin. However, in our light-adapted retinal explant, unbleached photopigments were

58   approximately 8% rhodopsin and 8% isorhodopsin (Fig. 5*D*). Quantum yield of

59   isorhodopsin is about 1/3 of rhodopsin (5). Therefore, estimated 1P excitation in our

60   light-adapted retinal explant is $(1.8 \times 10^3) \times (0.08 + 0.08/3) \approx 1.9 \times 10^2$ R* rod^{-1}.

61   Assuming that 1P and 2P excitations have comparable contributions, we estimated the

62   total direct excitation rate as approximately 4×10^2 R* rod^{-1}.

63   Next, we estimated the indirect activation. Because imaging was mainly done from

64   PRS, we assumed that the AKAR3EV fluorescence is absorbed by rhodopsin without any

65   attenuation. To obtain the relationship between photon emission and the pixel intensity of

66   fluorescent image, calibrated 475 nm or 535 nm light from the light stimulation device

67   (Fig. S4) was injected directly into the objective lens (Fig. S5*B*), and the light was

68   detected with the CFP Ch detector (460-500 nm) or FRET Ch detector (520-560 nm),

69   respectively, with an 8 μsec of the pixel dwelling time (Fig. S5*C*). Then, mean intensities

70   of images were plotted as a function of the photon number per pixel (Fig. S5*D*). Using

71   this relationship, a mean CFP Ch pixel intensity in our typical imaging, 300, was

72   converted to 6×10^2 photons pixel^{-1}. The FRET Ch sensitivity was 94% of the CFP Ch

73   sensitivity; this value will be used later for a calibration.

74   In the real specimen, fluorescent emission is supposed to be radiated toward random

75   directions so that only a part of them are collected by the objective lens. Based on the

76   numerical aperture of the objective lens (1.05) and the refractive index of the DMEM/F12

77   medium (1.34), the objective aperture angle is calculated as 0.90 radians, and the fraction

78   of the photon collection was estimated as $(0.90 \times 2) / (2\pi) \approx 0.29$. Thus, the estimated

79   total photon emission from the AKAR3EV is $(6 \times 10^2) / 0.29 \approx 2 \times 10^3$ photons pixel^{-1}.

80   Because the photon number so far is estimated only for those detected by the CFP Ch

81   detector (i.e. photons in 460-500 nm range), we expanded this for the whole emission

82   spectrum of the AKAR3EV. The emission spectrum of the AKAR3EV was predicted by

83   summing up those of mTurquoise and YPet (Fig. S5*E*) which comprise AKAR3EV. The

84   YPet spectrum was then scaled based on the basal FRET/CFP of PRS layer (1.24; Fig.

85   2*B*, PRS of the light-adapted retina). Considering the detector sensitivity ratio above, the

86   ratio of the area corresponding to FRET Ch and CFP Ch was adjusted at 1.24 / 0.94 =

87   1.32. The whole spectrum was further scaled up to set the area for the CFP Ch to the

88   above estimated photon number, 2×10^3 (Fig. S5*F*). Finally, the effect of the AKAR3EV

89   fluorescence on the rhodopsin activation was estimated by multiplying the normalized

90   absorbance spectrum of rhodopsin (Fig. S5*G*), which converts the photon number of each

91   wavelength into the corresponding number at 500 nm. The area of this calibrated

92   spectrum is comparable to 5×10^3 photons pixel^{-1} of 500 nm light (Fig. S5*H*). Using the

93   pixel size (0.17 μm^2), this value was converted to $(5 \times 10^3) / (0.17) \approx 3 \times 10^4$ photons μm^{-}

94   ^{2}. Using the collecting area (0.47 μm^2) and the fractions of rhodopsin and isorhodopsin in

95   our light-adapted retinal explant (8% each), the value was further converted as described

96   earlier, to obtain the total indirect excitation rate of 1×10^3 R* rod^{-1}.

97   The photon density estimated for direct and indirect activations are in the order of 10^3

98   and 10^4 photons μm^{-2}, respectively, at 500 nm. On the other hand, the half saturating light

99   intensity of the light-off-induced PKA activation was 6.5×10^7 photons μm^{-2} at 500 nm

100   (Fig. S3*B*). Therefore, the impact of imaging technique is thought to be negligible, at

101   least on the PKA activation.

102

103 **Supplementary Methods**

104 **Ethical approval.** The animal protocols were reviewed and approved by the Animal Care

105 and Use Committee of Kyoto University Graduate School of Medicine (MedKyo17539,

106 17539-2, 18086, 19090, and 20081) and methods were carried out in accordance with the

107 relevant guidelines and regulations.

108

109 **Reagents.** Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham

110 (DMEM/F12; D2906, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% (v/v)

111 Penicillin-Streptomycin (26253-84; Nacalai Tesque, Kyoto, Japan) was prepared just

112 before the imaging experiment. The medium was warmed up in a 40 °C water bath and

113 bubbled with an O_2 and CO_2 gas mixture. The pH was monitored with a pH tester

114 (HI98100; HANNA Instruments, Woonsocket, RI, USA), and the CO_2 concentration was

115 adjusted to obtain pH 7.2-7.4. For the cone-specific staining in Fig. 1*I*, PNA-rhodamine

116 (RL-1072; Vector Laboratories, Burlingame, CA, USA) was added to DMEM/F12

117 medium at 0.2% (v/v). Forskolin (F-500; Alomone Labs, Jerusalem, Israel) was dissolved

118 in dimethyl sulfoxide (DMSO) at 50 mM and kept at -20 °C until used. Dopamine (040-

119 15433; FUJIFILM Wako Pure Chemical, Osaka, Japan) was dissolved in ultrapure water

120 at 1 mM and kept at -80 °C until used. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

121 acid (HEPES) and NaCl were purchased from Nacalai Tesque. NaOH and hydroxylamine

122 hydrochloride were from FUJIFILM Wako Pure Chemical. *n*-Dodecyl-β-D-maltoside

123 (DDM) was from Dojindo (Kumamoto, Japan). Reagents for retinoid extraction and

124 HPLC analysis (dichloromethane, *n*-hexane, Na_2SO_3, diethyl ether, isopropanol and

125 benzene) were from FUJIFILM Wako and Nacalai Tesque. The genomic DNA extraction

126 kit (69504; DNeasy Blood and Tissue Kit) was from Qiagen (Hilden, Germany). PCR

127 reagents were from TaKaRa Bio (Kusatsu, Japan) and Toyobo (Osaka, Japan). PCR

128 primers were from Fasmac (Atsugi, Japan).

129

5

130   **Animals.** Mice (*Mus musculus*) were housed in a specific-pathogen-free facility with a

131   14/10 hour light-dark cycle, fed a standard laboratory chow diet and water *ad libitum,* and

132   used at the age of 1–6 months. Both male and female mice were used. Two or more

133   animals were used for every experiment to confirm the reproducibility. PKAchu

134   (C57BL/6J (B6J) background, nbio185; NIBIOHN, Osaka, Japan) and PKAchu-NC

135   (C57BL/6J (B6J) background, nbio186) transgenic mice were backcrossed with B6

136   albino mice (Charles River Laboratories Japan, Yokohama, Japan) for more than 9

137   generations and used as albino PKAchu and albino PKAchu-NC, respectively.

138   Transgene-positive, heterozygous mice were selected by visual inspection of their body

139   fluorescence and used for both breeding and imaging. Wild-type PKAchu and PKAchu-

140   NC were generated by crossing those albino mice with normal pigmented B6J mice

141   (Charles River Laboratories Japan) for 3-7 generations, except for PKAchu used in the

142   experiment shown in Fig. 1, which was crossed for only 1 generation. Removal of both

143   the *rd8* mutation (a single base deletion at nt3481 on *Crb1*) (6) and the albino mutation (a

144   G291T point mutation on *Tyr*) (7) was verified by Sanger sequencing of genomic DNA

145   extracted using the DNeasy Blood and Tissue Kit (Qiagen). The following primers were

146   used: for rd8 genotyping, 5'-ACCTGATGGGTTCCCAATTG-3' (forward) and 5'-

147   AACCAGCCTTGTTTAGCACC-3' (reverse); for albino genotyping, 5'-

148   GGGGTTGCTGGAAAAGAAGTCTGTG-3' (forward) and 5'-

149   TGTGGGGATGACATAGACTGAGCTG-3' (reverse). PKAchu *Gnat1*$^{+/-}$ and PKAchu

150   *Gnat1*$^{-/-}$ were obtained by crossing wild-type PKAchu with *Gnat1*$^{-/-}$ mice (B6J

151   background; the kind gift of Dr. Vladimir J. Kefalov) (8) for 2-3 generations. For

152   genotyping *Gnat1*, genomic DNA was extracted by incubating a biopsy sample in 50 μL

153   of 0.1 M NaOH (1 hour, 95 °C). The sample was then neutralized by adding 50 μL of 1

154   M Tris-HCl (pH 7.5) and centrifuged (15300 ×g, 1 min, 25 °C). The resulting supernatant

155   was used as a template for a genotyping PCR. The following primers were used: for the

156   *Gnat1* wild-type allele, 5'-GGCTTTCTTCAGGGGTCTTA-3' (forward) and 5'-

6

157   GGCAGGGTAGTGGTTGTGAA-3' (reverse); for the *Gnat1* knockout allele, 5'-

158   CATTCGACCACCAAGCGAAACATC-3' (forward) and 5'-

159   ATATCACGGGTAGCCAACGCTATG-3' (reverse). Taken together, the genetic

160   background of PKAchu and PKAchu-NC used in this study is normal pigmented B6J

161   except for albino PKAchu and PKAchu-NC that have the B6 albino background.

162

163   **Preparation of the retinal explants for imaging.** The following tools were used in the

164   retina dissection: a stereomicroscope (SZX16; Olympus, Tokyo), curved forceps (91197-

165   00; Fine Science Tools, Foster City, Canada), micro-scissors (15009-08; Fine Science

166   Tools) and two fine forceps (11251-20; Fine Science Tools). For dark-adapted retinas, a

167   mouse was kept in darkness overnight and the preparation was done under dim red light.

168   For light-adapted retinas, a mouse was kept under normal room illumination (white light,

169   200-300 lux) for more than 1 hour and the explants were prepared under the same

170   lighting condition. The relative amount of unbleached photopigments in this explant was

171   constantly about 16% to that of rhodopsin in the dark-adapted control for wild-type (Fig.

172   5A) and was below the detection limit for albino (Fig. 6A). If not otherwise specified,

173   imaging data were obtained from light-adapted retinal explants. A mouse was killed

174   under isoflurane anesthesia. Eyes were enucleated with curved forceps and hemisected to

175   remove the cornea, vitreous and lens. Eyecups thus obtained were transferred into a 35-

176   mm culture dish filled with 2 mL DMEM/F12 medium and cut radially from three or four

177   points. The retina was peeled off with fine forceps and transferred onto a culture insert

178   (PICMORG50; Merck Millipore, Billerica, MA, USA) which was pre-wet with

179   DMEM/F12. The retina was flat-mounted ganglion cell-side up and tightly adhered onto

180   the culture insert with suction (9) to prevent positional drift and detachment during time-

181   lapse imaging. The culture insert was then fixed in a custom perfusion chamber made of

182   acrylic glass (width 40 mm, depth 33 mm, height 10 mm) or a square polystyrene dish (1-

183   4698-01; As One, Osaka, Japan). The entire preparation process was completed in less

184   than 20 min after administration of anesthesia. For the cone-labeling in Fig. 1*I*, the

185   culture insert with a retinal explant was placed onto 1 mL DMEM/F12 supplemented

186   with 0.2% (v/v) PNA-rhodamine, pre-incubated in a humidified CO_2 incubator at 37 °C

187   for 1 hour and then washed once with DMEM/F12 just before the imaging (10).

188

189   **Perfusion equipment.** The perfusion chamber was placed on an electromotive

190   microscope stage (MPT-AS01-FV; SIGMA KOKI, Hidaka, Japan) with a heating pad

191   (MP-908-N; Makami Denshi, Kishiwada, Japan). The retina in the chamber was perfused

192   with oxygenated DMEM/F12 medium delivered at 1 mL/min using a double-head

193   peristaltic pump (AC-2110 II; ATTO, Tokyo) and warmed up with an inline heater (an

194   SF28 heater controlled by a TC344B temperature controller; Warner instruments,

195   Hamden, CT, USA) (Fig. 1*A*). Medium was continuously drained from the chamber with

196   the same pump. The drain rate was adjusted to be slightly higher than 1 mL/min to

197   prevent overflow. The heater voltage was adjusted manually to maintain the temperature

198   in the dish at 33-35 °C (11), which was monitored by a K-type thermocouple probe (AD-

199   1214; A&D, Tokyo) attached near the tip of the objective lens. Temperature data was

200   imported to a computer via a breakout board (MAX31855; Adafruit, New York, NY) and

201   an Arduino UNO microcontroller board (Arduino, Turin, Italy). Temperature was

202   recorded using a program modified from the MAX31855 library (Adafruit;

203   https://github.com/adafruit/Adafruit-MAX31855-library) or the Tera Term software

204   (https://ttssh2.osdn.jp/index.html.en).

205

206   **Two-photon microscopy.** The retina in the chamber was perfused with oxygenated

207   DMEM/F12 medium using the above-mentioned perfusion system. Imaging was done

208   with an upright multi-photon microscope system (Fluoview FV1000MPE; Olympus,

209   Tokyo) with a water immersion objective lens (XLPLN25XWMP; Olympus). A

210   Ti:Sapphire femtosecond laser (Mai-tai DeepSee HP; Spectra-Physics, Santa Clara, CA,

8

211 USA) was tuned at 840 nm and used for excitation. Laser power at the specimen was

212 adjusted within 5-10 mW. The specimen was XY-scanned at 256×256 pixels with 8

213 μsec of pixel dwelling time. Fluorescent emission was collected by the objective lens,

214 filtered through a dichroic mirror RDM690 (Olympus) to block the returned excitation

215 light, split with a set of dichroic mirrors (DM505, DM450 and DM570; Olympus) and

216 finally filtered through bandpass filters (FF01-425/30-25; Semrock, Rochester, NY,

217 USA; BA460-500, Olympus; BA520-560, Olympus; 645/60, Chroma, Bellows Falls, VT,

218 USA) to separate blue (not used in this study), cyan (CFP Ch), yellow (FRET Ch) and red

219 fluorescence (Red Ch; used only for the rhodamine-labeled image in Fig. 1*I*),

220 respectively. Fluorescent signals were detected and amplified with multi-alkaline

221 photomultiplier tubes to reconstruct images on FV10-ASW software version 4.2

222 (Olympus). Images in Fig. 3*A* and Movie S5 were created by combining four 509 μm

223 square images which were obtained sequentially within 30 sec.

224

225 **Light stimulation.** Stimulation light was generated with a custom-made LED system

226 (Fig. S4). Light intensity and duration were controlled by the Arduino UNO

227 microcontroller board connected to the LED driver, using a program written with

228 Arduino IDE. When necessary, calibrated neutral density filters (ND filters, Fig. S4;

229 Edmund Optics, Barrington, NJ, USA) were additionally used to attenuate the light

230 intensity. Light wavelength was controlled by a bandpass filter (Fig. S4; Edmund Optics).

231 Light was delivered to the retina through the objective lens. To avoid light attenuation,

232 the dichroic mirror RDM690 before the objective lens was manually changed to a normal

233 mirror during the stimulation. Light intensity was calibrated based on power

234 measurements with an optical power meter (TQ8210; Advantest, Tokyo). The power in

235 Watts was divided by the energy of a photon and illuminated area (0.68 mm^2 circle) to

236 obtain photon density. The energy of a photon was derived from

9

237
$$E = \frac{h\nu}{\lambda}$$

238 where E is photon energy, h is the Planck constant ($6.62606957 \times 10^{-34}$ m^2 kg sec^{-1}), c is

239 the speed of light in a vacuum (2.99792458×10^8 m/sec) and λ is wavelength (m). For

240 short stimulations, light was delivered in 6 sec just before an image acquisition, except

241 for 6×10^{10} and 1.9×10^{11} (photons μm^{-2} sec^{-1}) stimulation at both 575 and 600 nm, in

242 which the maximal light intensity was used for 6-30 sec to deliver the indicated numbers

243 of photons. For long stimulations of 10 min (Figs. 2E and 6F), light was delivered only

244 during the intervals of time-lapse imaging, and thus the light was temporarily turned off

245 every 2 min during the image acquisition (< 10 sec). View fields for the light response

246 measurements were always selected from areas where the stimulation light spot had not

247 been projected yet.

248

249 **Image analyses.** Images were analyzed on Fiji (https://fiji.sc). Positional drift in the time-

250 lapse imaging was corrected with the Correct 3D drift plug-in (12). The minimum pixel

251 intensity obtained from a blank image was used as the background signal and subtracted

252 from every pixel. Pseudocolor PKA activity images were generated by multiplying an 8-

253 color FRET/CFP ratio image and the corresponding intensity-normalized grayscale image

254 (Fig. S1B). Longitudinal images of the retina were created by reconstruction from z-stack

255 images. Sixteen y-slice images were averaged for noise reduction and better visualization

256 of cellular morphology. When comparing rods and cones (Fig. 3 F and G), intensity-

257 based segmentation was performed using a mask image that covered the cone or rod

258 region. The mask image was created by superimposing two binary images. The first

259 image was created using the Auto threshold function with the MaxEntropy method (13).

260 To create the second image, a local background image was created by applying a median

261 filter of 10 pixels diameter, and this background image was subtracted from the original

262 image. The subtracted image was then binarized into the top 1% highest intensity pixels

263 and others, to obtain the second binary image. Two mask images were overlaid using the

264 Image calculator function with the OR method. The thus-obtained mask image or its

265 inverted image was applied to the original image to extract cone or rod signals,

266 respectively.

267

268 **Photopigment analyses.** The absorbance spectrum of photopigments in the retina was

269 obtained as described previously (4). A dark-adapted control retina was transferred into

270 500 μL collection buffer (140 mM NaCl, 50 mM HEPES, pH 6.5 NaOH) immediately

271 after its collection and frozen with liquid nitrogen. A light-adapted retina was flat-

272 mounted as described above, transferred into 500 μL collection buffer and frozen. A

273 frozen retina was thawed and homogenized using a 1 mL syringe (SS-01T; Terumo,

274 Tokyo) by passing through a 23-gauge needle (NN-2325R; Terumo) and centrifuged

275 (17,400 ×g, 15 min, 4°C). After removing the supernatant, the membrane precipitate was

276 solubilized in 300 μL solubilization buffer (collection buffer supplemented with 1%

277 (w/v) DDM), mixed on a rotator at 4°C for 30 min, and centrifuged (17,400 ×g, 15 min,

278 4°C). The supernatant was collected, supplemented with 16 μL of 5 M NH_2OH (pH 6.8,

279 NaOH; final concentration of 100 mM) and analyzed with a spectrophotometer (UV-

280 2400PC; Shimadzu, Kyoto) at 4°C. The difference spectrum of photopigment was

281 obtained from absorbance data before and after an irradiation with intense yellow light

282 passed through a Y52 filter. Molar extinction coefficients of rhodopsin (40,200 M^{-1} cm^{-1})

283 (14) and isorhodopsin (43,000 M^{-1} cm^{-1}) (15) were used to estimate the molar amount of

284 each pigment after the fitting analysis in Fig. 5*D*.

285   Equivalent DDM-solubilized samples were also used for the HPLC analysis. Retinoid

286 extraction and HPLC were performed as described previously (16) using an HPLC

287 system (LC-10AT VP; Shimadzu) with a silica column (150 × 6.0 mm, A-012–3; YMC,

288 Kyoto, Japan) and a solvent composed of 98.8% (v/v) benzene, 1.0% (v/v) diethylether,

289 and 0.2% (v/v) 2-propanol. Each retinoid in eluate was detected by its absorbance at 360

11

290   nm. Molar ratios of retinoids were estimated by dividing the peak area with the

291   corresponding molar extinction coefficient at 360 nm (M^{-1} cm^{-1}): 35,000 for *syn*-11-*cis*

292   retinal oxime; 29,600 for *anti*-11-*cis* retinal oxime; 54,900 for *syn*-all-*trans* retinal oxime;

293   51,600 for *anti*-all-*trans* retinal oxime; 39,300 for *syn*-9-*cis* retinal oxime; 30,600 for

294   *anti*-9-*cis* retinal oxime; 49,000 for *syn*-13-*cis* retinal oxime; 52,100 for *anti*-13-*cis*

295   retinal oxime; and 10,900 for all-*trans* retinol.

296

297   **Data fittings.** Rhodopsin absorption (Fig. 5*D*) was fitted with rhodopsin and

298   isorhodopsin templates from 450 to 600 nm by the least squares method. The rhodopsin

299   template was experimentally obtained from the dark-adapted control retina, and the

300   isorhodopsin template was obtained from the literature (17).

301   Intensity-response relationships (Fig. S3*B*) were fitted using Origin 9 (OriginLab,

302   Northampton, MA, USA) with the Naka-Rushton equation:

303
$$R = R_{max} \frac{I^n}{(I^n + I_{1/2}{}^n)}$$

304   where R is the peak amplitude, R_{max} is the amplitude of the saturated response, I is light

305   intensity, n is the Hill coefficient, and $I_{1/2}$ is the light intensity to generate a half-maximal

306   response.

307   A spectral sensitivity plot (Fig. 4*A*) was fitted with the spectral template for the α-
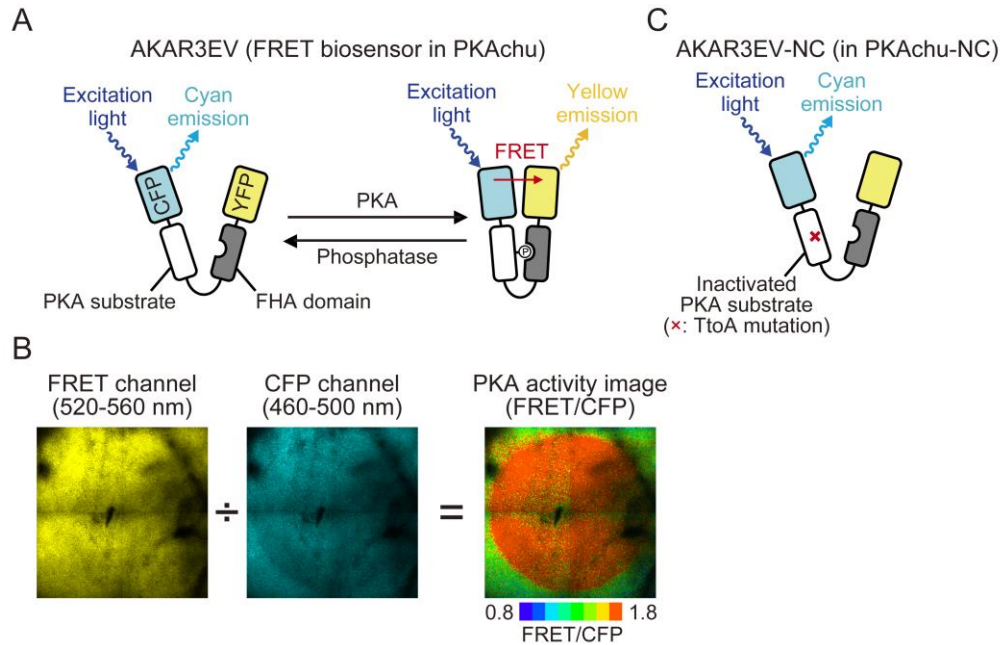
308   band of A1 pigments:

309
$$S(x) = \frac{1}{\exp[A(a - x)] + \exp[B(b - x)] + \exp[C(c - x)] + D'}$$

310   where $x = \lambda_{max}/\lambda$, $A = 69.7$, $a = 0.88$, $B = 28$, $b = 0.922$, $C = -14.9$, $c = 1.104$, and $D =$

311   0.674 (3).

312

313

12

314   **Supplementary Figures**

315   **Fig. S1.** Visualization of PKA activity using AKAR3EV FRET-biosensor.

316   (A) Predicted protein structure and mode of action of AKAR3EV. Activated PKA

317   phosphorylates the threonine residue in the PKA substrate motif. Phospho-threonine is

318   then recognized by the forkhead-associated (FHA) domain to promote FRET from cyan

319   fluorescent protein (CFP) to yellow fluorescent protein (YFP), which increases yellow

320   fluorescence. (B) Generation of a FRET/CFP ratio image to visualize PKA activity. The

321   fluorescent emission from the PKAchu retina is separated into yellow and cyan light and

322   received by two detectors, the FRET and CFP channels, respectively. The intensity of

323   each pixel in the FRET channel image is divided by that of the CFP channel image to

324   generate an 8-color FRET/CFP ratio image (see the *Supplementary Methods* for detail).

325   Sample images are obtained from light-stimulated photoreceptor cells (Fig. 3A). (C)

326   AKAR3EV-NC for the negative control. The PKA phosphorylation site, threonine, is

327   substituted to alanine so that FRET efficiency is not affected by PKA.

328

13
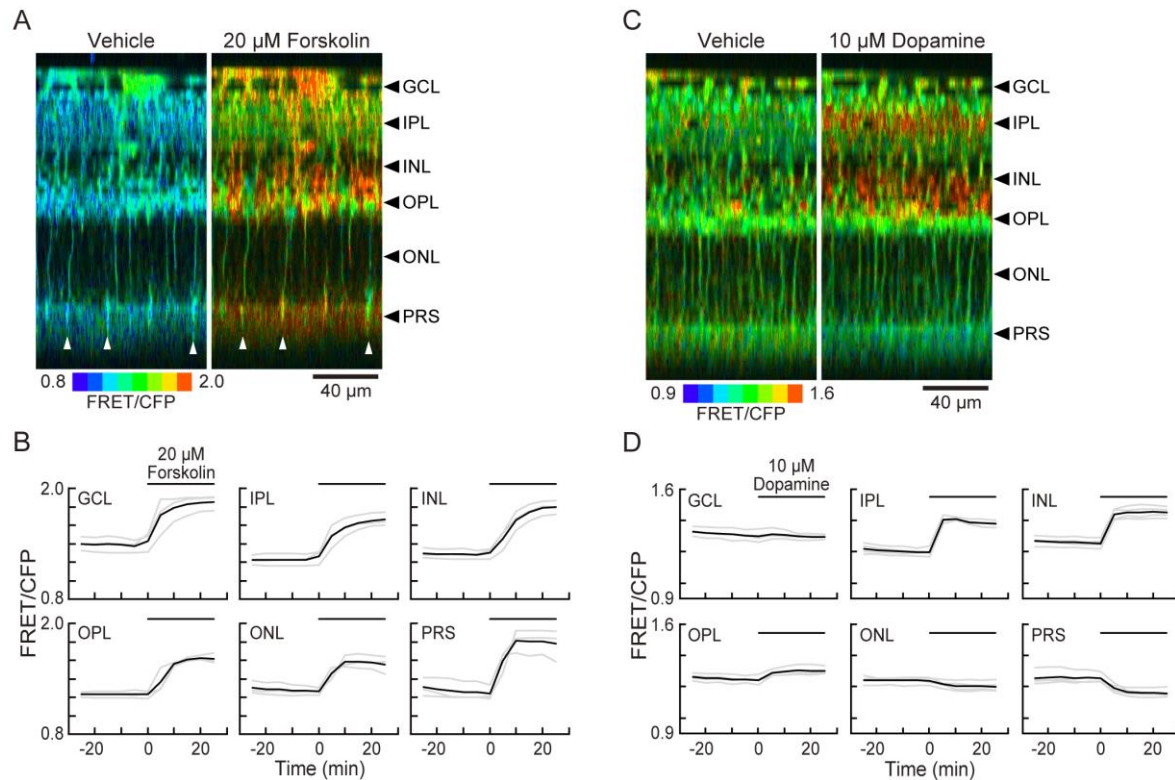
329 **Fig. S2**. Forskolin and dopamine responses in the PKAchu retina.

330　(A) Longitudinal images of the PKA activity before and after the perfusion of 20 μM

331　forskolin. Images were reconstructed from a z-stack of 43 images with 4.5 μm z-

332　intervals. White arrowheads indicate cones. Black arrowheads indicate the positions of

333　the layers analyzed in (B). (B) Time courses of forskolin-induced PKA activations at

334　each layer. (C) Longitudinal images of the PKA activity before and after the perfusion of

335　10 μM dopamine. Images were reconstructed from a z-stack of 40 images with 5 μm z-

336　intervals. (D) Time courses of dopamine-induced PKA activity changes at each layer. For

337　both (B) and (D), data points were obtained every 5 min and horizontal bars show the

338　timing of drug perfusion. Light-colored and dark-colored curves are individual and

339　averaged data, respectively. n = 3 and 4 for forskolin and dopamine, respectively.
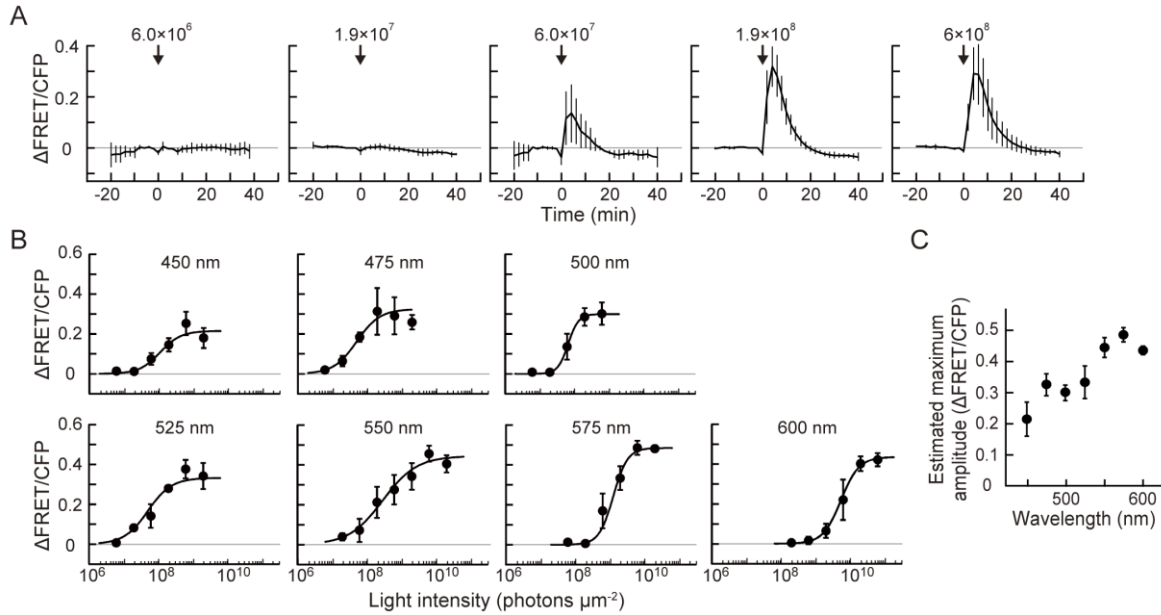
340   **Fig. S3.** Intensity-response relationships of the light-off-induced PKA activation.

341   (A) Rod responses toward 6 sec stimulation of various intensities of light at 500 nm.

342   Numbers indicate the light intensity (photons μm^{-2}) and arrows indicate the timing of the

343   stimulation. Data were obtained every 2 min. Values are the mean ± SD (n = 3). (B)

344   Intensity-response relationships of the light-off-induced PKA activation at each indicated

345   wavelength. Values are the mean ± SEM (n = 3 to 7). (C) Relationship between the

346   estimated maximum response amplitude and wavelength of the stimulation light. Values

347   are the mean ± SEM (n = 3 to 7).
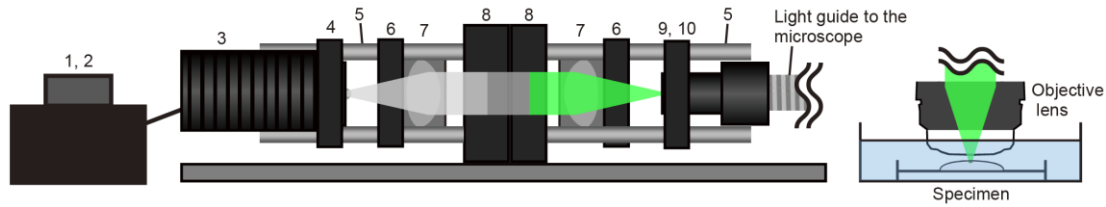
Components

Number	Component	Catalog number*	Quantity
1	Power supply	TPS001	1
2	LED driver	LEDD1B	1
3	White LED	MNWHL4	1
4	Cage plate	CP12	1
5	Cage assembly rods	ER3-P4	2
6	Cage plate	CP02/M	2
7	Lens	AC254-030-A-ML	2
8	Sliding filter mount	CFS1/M	2
9	Optic mount	CXY1	1
10	Adaptor	AD15F	1

*Thorlabs

**Filters used in the component #8**

Product name	Catalog number*
400, 450, 500, 550, 600, 650 and 700 nm bandpass filter kit	#88-300
425 nm bandpass filter	#87-787
475 nm bandpass filter	#87-788
525 nm bandpass filter	#87-789
575 nm bandpass filter	#87-790
625 nm bandpass filter	#87-791
675 nm bandpass filter	#87-792
ND1 filter	#48-090
ND2 filter	#48-092
ND3 filter	#48-093
ND4 filter	#36-273

*Edmund optics

348 **Fig. S4.** Custom-made LED light stimulation system.

349 (A) Layout of the system. Numbers indicate the components listed below. Filters used in

350 component #8 are also listed.
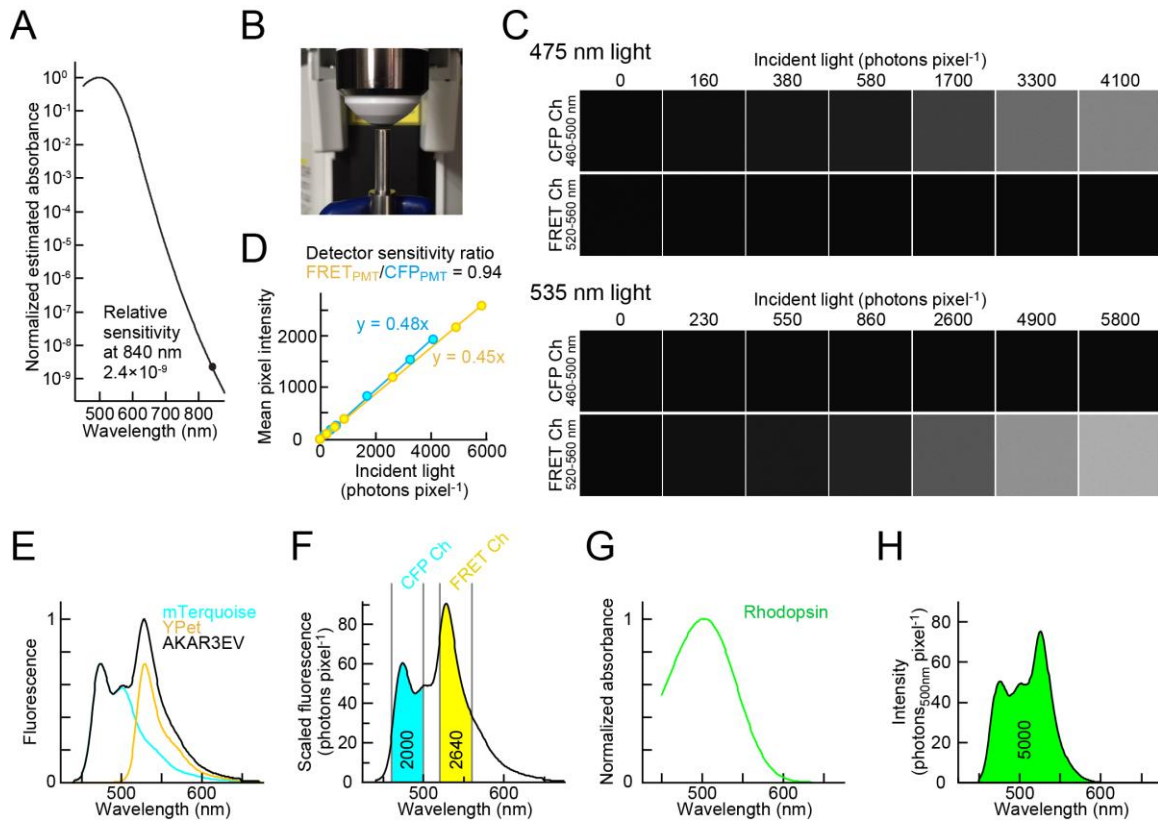
Fig. S5. Estimation of the amount of photopigment activation during two-photon imaging.

(A) The rhodopsin absorbance template extended to the infrared wavelength. (B) A picture of the photon injection setup. (C) Images obtained with CFP Ch and FRET Ch detectors when injecting indicated number of photons at 475 nm (upper panels) or 535 nm (lower panels). (D) Relationships of incident light intensity and mean pixel intensity of images. (E) A predicted emission spectrum of AKAR3EV. (F) A scaled spectrum. Numbers show areas of colored regions. (G) The normalized absorbance spectrum of rhodopsin. (H) A spectrum obtained by multiplying (F) by (G). The number is the area of green region.

362   **Legends for Supplementary Movies**

363

364   **Movie S1** (separate file) Z-stack images of the PKAchu retina. Two hundred cross-

365   sectional images were obtained in a 105 μm square area with 1 μm z-intervals.

366

367   **Movie S2** (separate file) Three-dimensional reconstruction of PNA-labeled

368   photoreceptors. PNA-labeled cone sheaths (magenta) and AKAR3EV^{high} cells (green)

369   were imaged.

370

371   **Movie S3** (separate file) Forskolin response of the PKAchu retina. Longitudinal images

372   were reconstructed from 43 cross-sectional images with 4.5 μm z-intervals. The time-

373   lapse interval was 5 min. Forskolin was continuously delivered from time 0.

374

375   **Movie S4** (separate file) Dopamine response of the PKAchu retina. Longitudinal images

376   were reconstructed from 40 cross-sectional images with 5 μm z-intervals. Dopamine was

377   continuously delivered from time 0.

378

379   **Movie S5** (separate file) The light-off-induced PKA activation after a 10 min-stimulation.

380   Data were obtained from a retinal explant prepared from the light-adapted PKAchu.

381   Images are cross-sectional views from the PRS layer. Light stimulation (500 nm, $3.2 \times$

382   10^7 photons μm^{-2} sec^{-1}) was given from -10 to 0 min. The time-lapse interval was 2 min.

383

384   **Movie S6** (separate file) Responses in PKAchu and PKAchu-NC retinal explants toward

385   6 sec-light stimulation. Images are cross-sectional views from the PRS layer. Stimulation

386   light (500 nm, 1.0×10^8 photons μm^{-2} sec^{-1}) was projected just before time 0 at the center

387   of the view field indicated as dashed circles. The time-lapse interval was 2 min.

388

18

389 **Movie S7** (separate file) Cell-type specificity of the light-off-induced PKA activation.

390 Longitudinal images were reconstructed from 35 cross-sectional images with 5 μm z-

391 intervals. Light stimulation (500 nm, 1.0×10^8 photons μm^{-2} sec^{-1}, 6 sec) was given.

392

393 **Movie S8** (separate file) High-magnification cross-sectional images of the light-off-

394 induced PKA activation at the PRS layer. Light stimulation (500 nm, 1.0×10^8 photons

395 μm^{-2} sec^{-1}, 6 sec) was given just before time 0. Note that bright spots are cones.

396

397 **Movie S9** (separate file) Comparison of the light-induced PKA activity changes between

398 wild-type and albino PKAchu photoreceptor cells. Images are cross-sectional views from

399 the PRS layer. Wild-type data are those used in Movie S5. Light stimulation (500 nm, 3.2

400 $\times 10^7$ photons μm^{-2} sec^{-1}) was given from -10 to 0 min.

401

402 **SI References**

403

404 1.   T. Euler, K. Franke, T. Baden, "Studying a light sensor with light: multiphoton imaging in
405   the retina" in Multiphoton Microscopy, E. Hartveit, Ed. (Humana Press, 2019), vol. 148,
406   chap. 10, pp. 225-250.
407 2.   G. Palczewska *et al.*, Human infrared vision is triggered by two-photon chromophore
408   isomerization. *Proc Natl Acad Sci U S A* **111**, E5445-5454 (2014).
409 3.   V. I. Govardovskii, N. Fyhrquist, T. Reuter, D. G. Kuzmin, K. Donner, In search of the
410   visual pigment template. *Vis Neurosci* **17**, 509-528 (2000).
411 4.   K. Sakurai *et al.*, Physiological properties of rod photoreceptor cells in green-sensitive
412   cone pigment knock-in mice. *J Gen Physiol* **130**, 21-40 (2007).
413 5.   P. H. M. Bovee-Geurts, J. Lugtenburg, W. J. DeGrip, Coupled HOOP signature correlates
414   with quantum yield of isorhodopsin and analog pigments. *Biochim Biophys Acta*
415   *Bioenerg* **1858**, 118-125 (2017).
416 6.   M. J. Mattapallil *et al.*, The Rd8 mutation of the Crb1 gene is present in vendor lines of
417   C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant
418   phenotypes. *Invest Ophthalmol Vis Sci* **53**, 2921-2927 (2012).

419　7.　N. Le Fur, S. R. Kelsall, B. Mintz, Base substitution at different alternative splice donor
420　　　sites of the tyrosinase gene in murine albinism. *Genomics* **37**, 245-248 (1996).

421　8.　P. D. Calvert *et al.*, Phototransduction in transgenic mice after targeted deletion of the rod
422　　　transducin alpha -subunit. *Proc Natl Acad Sci U S A* **97**, 13913-13918 (2000).

423　9.　E. Ivanova, A. H. Toychiev, C. W. Yee, B. T. Sagdullaev, Optimized protocol for retinal
424　　　wholemount preparation for imaging and immunohistochemistry. *J Vis Exp*
425　　　10.3791/51018, e51018 (2013).

426　10.　L. Bhatt, G. Groeger, K. McDermott, T. G. Cotter, Rod and cone photoreceptor cells
427　　　produce ROS in response to stress in a live retinal explant system. *Mol Vis* **16**, 283-293
428　　　(2010).

429　11.　P. R. Williams, J. L. Morgan, D. Kerschensteiner, R. O. Wong, In vitro imaging of retinal
430　　　whole mounts. *Cold Spring Harb Protoc* **2013** (2013).

431　12.　A. Parslow, A. Cardona, R. J. Bryson-Richardson, Sample drift correction following 4D
432　　　confocal time-lapse imaging. *J Vis Exp* 10.3791/51086 (2014).

433　13.　J. N. Kapur, P. K. Sahoo, A. K. C. Wong, A new method for gray-level picture
434　　　thresholding using the entropy of the histogram. *Comput Vision Graph* **29**, 273-285
435　　　(1985).

436　14.　H. Imai *et al.*, Molecular properties of rhodopsin and rod function. *J Biol Chem* **282**,
437　　　6677-6684 (2007).

438　15.　T. Yoshizawa, G. Wald, Pre-lumirhodopsin and the bleaching of visual pigments. *Nature*
439　　　**197**, 1279-1286 (1963).

440　16.　K. Sato *et al.*, Opn5L1 is a retinal receptor that behaves as a reverse and self-regenerating
441　　　photoreceptor. *Nat Commun* **9**, 1255 (2018).

442　17.　J. Fan, B. Rohrer, G. Moiseyev, J. X. Ma, R. K. Crouch, Isorhodopsin rather than
443　　　rhodopsin mediates rod function in RPE65 knock-out mice. *Proc Natl Acad Sci U S A*
444　　　**100**, 13662-13667 (2003).

445