Retinoid therapy restores eye-specific cortical responses in adult mice with retinal degeneration

Graphical abstract

Highlights
- A synthetic retinoid was administered to an adult mouse model of retinal degeneration
- Retinoid treatment partially restored retinal light sensitivity and optomotor response
- Retinoid restored eye-specific responses in V1 via ipsilateral-eye signal enhancement
- Retinoid treatment restored arousal-mediated V1 modulation

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In brief
Huh et al. report that a mouse model of retinal degeneration can undergo a significant restoration of vision when treated with retinoid therapy. Several visual properties in V1 are fully restored even when the therapy is started in adulthood, showcasing the potential of retinoid compounds as promising therapeutics for treating retinal diseases.
Retinoid therapy restores eye-specific cortical responses in adult mice with retinal degeneration

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SUMMARY

Despite the recent emergence of multiple cellular and molecular strategies to restore vision in retinal disorders, it remains unclear to what extent central visual circuits can recover when retinal defects are corrected in adulthood. We addressed this question in an Lrat−/− mouse model of Leber congenital amaurosis (LCA) in which retinal light sensitivity and optomotor responses are partially restored by 9-cis-retinyl acetate administration in adulthood. Following treatment, two-photon calcium imaging revealed increases in the number and response amplitude of visually responsive neurons in the primary visual cortex (V1). In particular, retinoid treatment enhanced responses from the ipsilateral eye, restoring the normal balance of eye-specific responses in V1. Additionally, the treatment rescued the modulation of cortical responses by arousal. These findings illustrate the significant plasticity of the adult central visual system and underscore the therapeutic potential of retinoid administration for adults with retinal diseases.

RESULTS AND DISCUSSION

Effective treatment for childhood blinding diseases involving retinal dysfunction remains one of the major unmet needs in medicine.1 Over the last two decades, numerous strategies have emerged, including gene replacement therapy,2 gene editing approaches,3 optogenetics,4 retinal electrical implants,5 induced pluripotent stem cell (iPSC)-derived retinal cell replacements,6 and pharmacological agents.7–9 These approaches can restore light-sensing ability at the retinal level and lead to improvements in visual perception in patients to varying extents. Studies of synthetic retinoid treatment in patients with RPE65 or LRAT mutations have led to improvements in visual field area and visual cortex activation, as seen on fMRI scans.10–12 A separate study using intravitreally administered synthetic retinoid to treat RPE65-mutant dogs found that electroretinogram (ERG) recordings recovered a normal shape and response threshold following treatment, although the duration of this effect was limited to ten weeks. The treatment also improved vision-dependent behavioral tests at low light intensities.13 Clinical trials of gene therapy for recessive RPE65-associated retinopathies have also demonstrated behavioral and visual field area improvements and increases in visual cortex fMRI signals in treated patients and dogs.2,14–16 Indeed, measures of visual cortex activity may provide an objective way to assess long-term treatment efficacy.17 Although some progress has been made,3,18,19 it still remains unclear the extent to which adult visual circuits can be restored to a fully functional state at the level of the visual cortex upon correction of the retinal defect.

It is widely appreciated that visual impairments in early critical periods of postnatal development can lead to lifelong deficits in central visual function, even when the initial abnormality is corrected.20 Recent studies have revealed exceptions to this critical period restriction.21 How the critical period applies to visual circuit remodeling in response to restoration of retinal function has not been defined. This question is crucial, as its answer would inform the optimal timing of visual restoration efforts. We employed an established model of a childhood blinding disease, the lecithin:retinyl acyltransferase knockout (Lrat−/−) mouse, to determine the extent of recovery of the central visual circuit following acute rescue of retinal light sensitivity. The Lrat−/− mouse has a metabolic blockade in the visual cycle
pathway that produces the visual chromophore, 11-cis-retinal, necessary for the formation of photoreceptor visual pigments, thus recapitulating the primary pathology associated with Leber congenital amaurosis (LCA) type 14 (Figure 1A). This blockade can be circumvented pharmacologically by the administration of a retinoid, 9-cis-retinyl acetate (9-cis-R-Ac). 9-cis-R-Ac, a

**Figure 1.** 9-cis-retinyl acetate (9-cis-R-Ac) partially restored retinal light sensitivity and optomotor response in Lrat<sup>−/−</sup> mice

(A) Visual cycle pathway. 9-cis-R-Ac was used to replenish the visual chromophore.

(B) Experimental timeline of drug administration and visual function recording. Each square represents 1 day. DH, dark-housed; OMR, optomotor response; ERG, electroretinography; Euth, euthanized. The treatment consisted of either 40 mg/kg 9-cis-R-Ac in DMSO or DMSO alone (vehicle control) administered via intraperitoneal injection.

(C–E) A-wave (top) and B-wave (bottom) ERG responses obtained at three different time points. 9-cis-R-Ac-treated mice (n = 18 eyes) showed enhanced ERG responses compared with vehicle-treated mice (n = 14 or 16 eyes; two-way repeated-measures ANOVA; ***p < 0.0001 with respect to treatment effect). Insets show representative ERG responses to the 1 cd · s<sup>−2</sup> flash at each time point.

(F–H) OMR responses. 9-cis-R-Ac-treated mice (n = 9 mice) showed significantly enhanced OMR responses compared with vehicle-treated mice (n = 7–8 mice; two-way repeated-measures ANOVA; ***p < 0.0001 with respect to treatment effect).

In (C)–(H), responses recorded from age-matched wild-type mice (ERG: 8 eyes; OMR: 5 mice) are shown in the leftmost panels for comparison. Dots represent individual data points; open circles represent mean values; shaded regions represent 95% CI in (C)–(E) and standard error of the mean (SEM) in (F)–(H).

See also Figures S1 and S2.
Figure 2. 9-cis-R-Ac restored visual responses in V1 of Lrat<sup>−/−</sup> mice
(A) Cortical responses to contrast-reversing central 30° stimuli were recorded using intrinsic signal optical imaging (ISOI).
(B) The activated area in the binocular zone of visual cortex (V1) is shown in the ISOI amplitude map in example wild-type (top) and Lrat<sup>−/−</sup> mice (bottom).
(C) Experiment timeline. DH, dark-housed.
(D) Amplitude maps in retinoid-treated (top two) and vehicle-treated Lrat<sup>−/−</sup> mice (bottom).
(E) Normalized peak amplitudes. Thin lines, data from the same animal; thick lines, group medians; green triangle, 9-cis-R-Ac-treated; orange circle, vehicle-treated; thick gray line, wild-type median; dotted line, no change from baseline. Shapiro-Wilk normality test: p < 0.05 (data are not normal). Wilcoxon rank-sum tests (retinoid- versus vehicle-treated) with BH multiple-comparison correction.
(F) Peak amplitude values from retinoid-treated Lrat<sup>−/−</sup> mice. Each data point, individual animal; thick gray line, wild-type mean. Error bars: group mean ± SEM. Shapiro-Wilk normality test: p > 0.05 (data are normal). Linear mixed-effects models (baseline versus other time points).

(legend continued on next page)
9-cis-retinal prodrug, is currently under clinical investigation as a visual chromophore substitute.8,10,12 We confirmed that intraperitoneal administration of 9-cis-R-Ac (40 mg/kg in DMSO) to Lrat−/− mice for seven consecutive days led to substantial 9-cis-retinal accumulation in the eye (Figures S1A–S1C) and produced a rapid and prolonged improvement in light-induced ERG activity and optomotor reflexes (OMR) compared with vehicle (DMSO) control (Figures 1B–1H). Prior studies have shown that cone photoreceptors completely degenerate in Lrat−/− mice by postnatal day 42.20,24 Consistently, we observed no M-cone opsin immunostaining in the Lrat−/− mice used for our experiments, irrespective of retinoid treatment (Figure S2), which strongly indicated that the ERG and OMR improvements arose from restoration of rod photoreceptor function. We next employed widefield intrinsic signal optical imaging and two-photon calcium imaging to measure changes in primary visual cortex (V1) neuronal activity following retinoid treatment.

We first investigated the effect of 9-cis-R-Ac treatment on V1 activation in adult Lrat−/− mice using widefield intrinsic signal optical imaging (ISOI), which provides a bulk measure of visual activity (Figure 2A). Prior to treatment at baseline, Lrat−/− mice exhibited poor visual activation of V1 compared with wild-type mice (Figure 2B). Lrat−/− mice were administered either retinoid or vehicle daily for seven days (Figure 2C). We found that 9-cis-R-Ac treatment led to a significant increase in the amplitude of V1 activation (median of 28% increase when using stimuli with max. luminance of 33 cd/m²; Figures 2D–2G). The increased amplitude in retinoid-treated mice was sustained for at least 9 days post treatment and provided therapeutic effects at 27 days post treatment compared with the vehicle (Figures 2E–2G). Behavioral (OMR) improvements were sustained for at least 19–20 days post treatment (Figures 1F–1H). By contrast, vehicle-treated mice exhibited a progressive decrease in response amplitude over time (Figures 2D, 2E, and 2G). To investigate the nature of this progressive decline, we evaluated V1 activation in repeated ISOI imaging recordings in age-matched wild-type mice. Wild-type mice displayed a similar level of reduction in visual response amplitude with time, as we observed with vehicle-treated Lrat−/− mice (Figures 2E–2G). We speculate that these changes reflect an expected level of degradation in signal due in part to visual adaptation to repeated stimuli under awake recording conditions and likely do not indicate further decline of visual function in Lrat−/− mice over a relatively short period of time.

Since 9-cis-R-Ac partially restores photoreceptor function in Lrat−/− mice25 (Figures 1C–1E), the effect on visually evoked activity in V1 as observed using ISOI may be explained by improved retinal signals leading to (1) the activation of a greater number of V1 neurons, (2) increased response amplitude per neuron, or (3) both. To examine these possibilities, we measured neuronal activity in V1 at cellular resolution using two-photon calcium imaging. Adult Lrat−/− mice were injected with a syn-GCaMP6s virus into the binocular zone of V1, implanted with a cranial window, and treated with seven days of 9-cis-R-Ac. Typically, the same sets of V1 neurons were recorded at baseline, within one day following the end of treatment and nine days post treatment (Figure 3A). Neuronal activity was measured while mice viewed stimuli through both eyes, the contralateral (“contra”) eye or the ipsilateral (“ipsi”) eye, relative to the recorded hemisphere (Figure 3B).

Two-photon calcium imaging revealed that 9-cis-R-Ac treatment led to a significant increase in the response amplitude of individual V1 neurons (mean of 2-fold increase when using stimuli with max. luminance of 33 cd/m²) and that this effect reversed during the post-treatment period (Figures 3C and 3D). The number of neurons visually responsive to both- and contra-eye stimulation doubled after treatment, an effect that was largely reversed during the post period (Figure 3E). Strikingly, the number of neurons activated by ipsi-eye stimulation increased by nearly 5-fold after treatment, an increase that was sustained during the post period (Figure 3E). We confirmed using ISOI that the effect of treatment on V1 activation through the ipsilateral eye was observed at both low and high visual luminance levels (max. luminance of 33 and 330 cd/m²; Figure S3), indicating that even at high luminance levels, the cortical response to the ipsilateral eye stimulation is enhanced by the treatment.

Two-photon calcium imaging further revealed that V1 neurons displayed broader orientation tuning, preference for higher spatial frequencies (SFs) and larger SF bandwidth following treatment (Figure S4). The higher SF preference and broader SF tuning effects were transient, whereas the broader orientation tuning effect remained at post (Figure S4). These results indicate that Lrat−/− V1 increased in terms of response strength, number of activated neurons, and stimulus bandwidth. Although the enhancement of the contralateral-eye signal was transient, the ipsilateral eye signal underwent a particularly prominent and long-lasting improvement.

Given that 9-cis-R-Ac led to differential effects on Lrat−/− V1 responses depending on which eye was stimulated, we examined the effects on V1 neurons’ ocular dominance properties. Ocular dominance index (ODI) values near 1 indicate contralateral eye dominance, values near −1 indicate ipsi-eye dominance, and values near zero indicate significant responses to both eyes (binocular). At baseline, V1 neurons in Lrat−/− mice displayed severely abnormal ODI with mostly contra-dominant cells (Figures 3F–3H). Immediately after retinoid treatment, ODI distributions shifted such that greater proportions of neurons displayed ipsi-dominant or binocular responses (Figures 3F–3H). However, ODI distributions in Lrat−/− mice did not fully shift to normal until the post period, becoming indistinguishable from those found in wild-type mice (Figures 2F–2H), indicating that binocular circuits in Lrat−/− mice take time to restore to normal following treatment.

One explanation for the increased ipsi-dominant population after treatment of the Lrat−/− mice is that newly ipsi-dominant neurons arise from a pool previously non-responsive to visual stimuli. Or, the newly ipsi-dominant neurons are originally contra-dominant or binocular neurons and switch their OD...
properties after treatment. To distinguish between these mechanisms, we tracked the activity of the same neurons over time. We designated neurons as “ipsi-destined,” “contra-destined,” or “binocular-destined” based on their post-period responses and compared them with their prior OD identity at baseline and after treatment. Although the majority of ipsi-destined neurons originated from a previously non-responsive pool of neurons (44/68 or 65%; Figure 3J), we found an unexpectedly large
proportion of ipsi-destined neurons that were previously contra-
dominant (18/68 or 26%; Figure 3I,J). This form of OD plasticity—
monocular neurons switching OD to the other eye—is surprising,
as it rarely occurs during juvenile development.26 The small
remainder of the ipsi-destined population were either already
ipsi-dominant neurons (4/68 or 6%) or neurons that switched
OD status between baseline and treatment (”other,” 2/68 or
3%; Figure 3J).

Although some visual properties in V1 were restored by 9-cis-
R-Ac treatment in adult Lrat−/− mice, it is unclear whether the
recovery extends to functions involving multiple visual pathways.
Arousal-mediated modulation of the visual system involves the
coordination of multiple circuits and undergoes prolonged pe-
riods of development.27,28 To investigate arousal-mediated V1
modulation, we performed pupil tracking during two-photon cal-
cium imaging of V1 neurons (Figure 4A). At baseline,
Lrat−/−/−/− mice
displayed large pupils with minimal size modulation compared
with those found in wild-type mice, suggesting that their pupils
are constantly enlarged with little arousal-mediated modulation
(Figure 4B). Following retinoid treatment, mean pupil size was
reduced but remained larger than that of wild-type mice under similar light conditions (Figures 4B and 4C). The amplitude of pupil size modulation was abnormally low in Lrat−/− mice, and following treatment, pupil size modulation became larger and was indistinguishable from wild-type values by the end of the post-treatment period (Figure 4D). However, we note that pupil size modulation may have been low at baseline in Lrat−/− mice partially due to ceiling effects of their abnormally large pupil sizes.

Within normal mice, V1 neuronal activity is tightly correlated with pupil size fluctuations.27,29 Accordingly, we found that the population calcium trace (average of individual traces) closely recapitulated the minute-to-minute pupil size fluctuations in wild-type mice (Figure 4E, the rightmost panel, top 2 traces). In wild-type mice, we found high cross-correlation between the pupil diameter trace and individual neurons’ calcium traces, with neural signals leading pupil fluctuations (negative pupil-neural lag; 4th panel in Figure 4F). By contrast, Lrat−/− mice displayed little correlation between pupil and neural signals before treatment (Figures 4E–4G, baseline). Following retinoid treatment, pupil and neural traces for Lrat−/− mice became more correlated such that correlation values became similar to wild-type values (Figure 4G). Cross-correlation values also became higher in negative pupil-neural lag windows compared with positive lag windows, indicating that neural signals led pupil traces in treated Lrat−/− mice (Figure 4F).

The extent of central vision recovery observed in our study is notable, given that Lrat−/− mice have dramatically reduced photoreceptor function,25 virtually no cone outer segments, and only ~30% of the rod outer segments remaining by one month of age.23 We found that at baseline, adult Lrat−/− mice display poor activation of V1 by visual stimuli and ocular dominance distributions in V1 neurons that are abnormally skewed toward contralateral-eye dominance. Following 9-cis-R-Δ, some LCA patients reported photophobia,11 which may be consistent with V1 over-activation.

Visual function changes that take longer to manifest and are longer-lasting (e.g., increases in the number of ipsilateral eye responsive neurons and changes in ocular dominance distribution) may reflect new synapses being formed. With stronger input from retinae, new synapses may be added or existing synapses recruited to relay signals from the ipsilateral eye. Interestingly, we found that the majority of the newly ipsi-dominant neurons arose from previously non-responsive or contra-dominant neurons, which suggest not only a de novo cortical activity but a shift in visual tuning properties. The locus of these changes in the visual pathway (in thalamus31 or V1) and underlying mechanisms remain to be investigated in future studies. It also remains to be determined the extent to which the increase in light sensitivity in the retina after the treatment contributes to recruitment of a greater number of V1 neurons. Our immunohistochemistry results indicate that all visual responses in Lrat−/− mice at the adult age we examined, even in those that were treated, originate from rod photoreceptors. It remains to be explored whether a similar extent of central circuit restoration is possible in other models of retinal degeneration.

Our ISOI results show a sustained increase in V1 response that lasts until 9 days post-treatment, but two-photon calcium imaging results show a transient increase that is not sustained until 9 days post-treatment. These differences may reflect the nature of the two signals. ISOI measures oxygenated blood in a large volume of tissue resulting from a combination of neuronal firing and neuropli activity (input from other cortical layers and brain areas). In contrast, two-photon calcium imaging reports activity at the level of single neurons in the specific area and layers that were imaged (V1 L2/3 in our case). It may be that activity in circuits other than V1 L2/3 are restored in a sustained manner (e.g., deeper-layer neurons in V1, projection neurons in higher visual areas), whereas V1 L2/3 neurons show a transient increase, and ISOI amplitude may reflect increased inputs from these sources.

In our study, measures of subcortical visual functions were only partially rescued; however, many of the higher-level (V1) visual functions indicated restoration to normal levels. Previous studies that administered retinoid therapies to LCA patients reported that many experienced long-lasting improvements in visual acuity and visual field area.10,11 In these and other studies,10,32,33 no changes in ERG measurements were observed, despite significant subjective improvements in visual perception. Likewise in our study, improvements at the retina and those reflecting subcortical changes (e.g., ERG, pupil size, and OMR responses) indicated partial recovery, whereas many properties in the visual cortex were fully restored to normal levels (visual response amplitude, ocular dominance, and pupil-neural correlation). Together, these findings suggest that therapeutic interventions can lead to a much greater extent of central vision recovery than expected from retinal readings alone.

In summary, our findings indicate that a mouse model of retinal degeneration can undergo a significant restoration of vision when treated with retinoid replacement therapy, even in adulthood. V1 visual responses, both in terms of the number of...
visually responsive neurons and the strength of visual response per neuron, were strongly enhanced by the treatment. Ipsilateral eye visual processing, arousal-mediated modulation of neuronal activity in V1, and optomotor behavior showed marked improvements that lasted well beyond the end of the treatment. Although our finding of restored ocular dominance distribution suggests at least a partial restoration of binocular circuits, future studies using behavioral assays will be needed to fully assess whether binocular (depth) vision is restored by retinoid treatment in adulthood. Our results reveal an unexpected and promising plasticity of the visual circuit, suggesting that therapeutic strategies for childhood blinding diseases can be highly effective even if intervention is started in adulthood. Despite demonstrated efficacy, ease of oral administration, and excellent safety profile, retinoid therapies have remained relatively under-explored until recent years.\textsuperscript{34} Our findings showcase the potential of retinoid compounds as promising therapeutics for treating numerous retinal diseases involving defects in the visual cycle.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.09.005.

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AUTHOR CONTRIBUTIONS

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Opsin 1, Medium Wavelength antibody | Novus | Cat# NB110-74730; RRID:AB_1049390 |
| Alexa Fluor 594-conjugated goat anti-rabbit IgG | Thermo Fisher Scientific | Cat# A-11037; RRID:AB_2534095 |
| Bacterial and virus strains |        |            |
| pAAV.Syn.GCaMP6s.WPRE.SV40(AAV1) | Chen et al. | RRID: Addgene_100843 |
| Chemicals, peptides, and recombinant proteins |        |            |
| retinyl acetate | Sigma-Aldrich | Cat# R3250 |
| 9-cis-retinyl acetate | Gao et al. | N/A |
| Deposited data |        |            |
| Data | GitHub | https://github.com/careyhuh/retinoid-therapy |
| Experimental models: Organisms/strains | | |
| Mouse: B6;129S6-Lratm1Kpal/J | The Jackson Laboratory | RRID: IMSR_JAX:018866 |
| Mouse: C57BL/6NCrl | Charles River Laboratories | RRID: IMSR_CRL:027 |
| Mouse: C57BL/6J | The Jackson Laboratory | RRID: IMSR_JAX:000664 |
| Software and algorithms | | |
| Python PsychoPy v1.9 | Python Software Foundation | https://www.python.org |
| Matlab R2015a | The MathWorks | https://www.mathworks.com |
| GraphPad Prism, v 9.4.1 | Dotmatics | https://www.graphpad.com/ |
| R, v 3.6.3 | The R Foundation for Statistical Computing | https://www.r-project.org/ |
| Analysis code | GitHub | https://github.com/careyhuh/retinoid-therapy |
| Analysis code | GitHub | https://github.com/careyhuh/rPacu |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sunil P. Gandhi (spgandhi@uci.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Data have been deposited at GitHub and are publicly available as of the date of publication. DOIs are listed in the key resources table.
- All original code has been deposited at GitHub and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals
Animal procedures were approved by the Institutional Animal Care and Use Committees at the VA Long Beach Healthcare System and the University of California Irvine. All experimental protocols were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, the recommendations of the American Veterinary Medical Association Panel on Euthanasia, and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research.
For all experiments, both sexes of mice were used. All mice were housed in a standard 12/12-h light/dark cycle environment or in a devoted dark room when being used for experiments. Animals were fed standard soy protein-free rodent chow diet (Envigo Teklad 2020X), provided water ad libitum, and housed in plastic cages with standard corn cob rodent bedding and 6 g nestlets (Ancare).

Pigmented Lrat<sup>−/−</sup> mice<sup>25</sup> (RRID:IMSR_JAX:018866) or wild-type C57BL/6 mice (RRID:IMSR_CRL:027; RRID:IMSR_JAX:000664) were used for experiments. For experiments in Figure 1, Lrat<sup>−/−</sup> and wild-type mice at ages of postnatal days (P) 70 – 77 were used. For V1 imaging experiments, Lrat<sup>−/−</sup> and wild-type mice were P60-78 at the time of baseline imaging sessions (mean age: P69).

**METHOD DETAILS**

**Retinoid synthesis and treatment**

9-cis-retinyl acetate (9-cis-R-Ac) was prepared as described previously.<sup>36</sup> Briefly, 0.1 mg of bis(benzonitrile)palladium chloride was added to a solution of all-trans-retinyl acetate (100 g) in hexanes (200 ml) and trimethylamine (55 μl), and the mixture was stirred overnight in the dark at 65 °C. The resulting solution was cooled to –80 °C overnight and filtered by suction using a precooled filter. Sixty microliters of the filtrate were injected into an Agilent 1100 HPLC system (Santa Clara, CA) and chromatographed on a silica Luna column (10 μ, 100 Å, 250 x 21.2 mm, Phenomenex, Torrance, CA) to afford 10 mg of 9-cis-R-Ac. The injection and separation were repeated until sufficient amounts of pure 9-cis-R-Ac were obtained. Purified 9-cis-R-Ac was dried in vacuo and then reconstituted in DMSO for administration to mice.

Prior to the first injection, Lrat<sup>−/−</sup> mice were individually housed, weighed, and dark adapted for >12 hr. Under dim red light, Lrat<sup>−/−</sup> mice were administered 9-cis-R-Ac at a dose of 40 mg/kg in DMSO, or DMSO only (vehicle control), via intraperitoneal injection once daily for seven consecutive days. This dosage was well tolerated by adult (≥ P60) mice.

**GCaMP6s virus delivery and cranial window implantation**

For two-photon calcium imaging, mice (age: P39-57, mean: P43) were injected with viral vectors to express GCaMP6s (pAAV.Syn.G-GCaMP6s virus delivery and cranial window implantation

For experiments shown in Figure 2, widefield ISOI was performed in awake animals through the cranial window after ≥ 6 days of recovery following craniotomy. Mice were habituated on the setup for 0.5 - 1 hr prior to the first imaging session. Mice were placed on a smooth platform and head-fixed, and a full-contrast noise stimulus was shown to the mice that spanned the central 30° of visual field. The stimulus was a band-limited (<0.05 cpd, ≥2 Hz) binarized spatiotemporal noise movie with a one-dimensional Gaussian spatial mask (30°), generated using custom Python scripts. The contrast-reversing stimulus moved either up or down periodically every 20 sec, presented on a gamma-corrected 24-inch LED monitor (ASUS VG248, 60 Hz refresh rate, 20 cd/m<sup>2</sup> mean luminance, 33 cd/m<sup>2</sup> maximum luminance) at a viewing distance of 25 cm. Widefield images were acquired using a SciMedia THT microscope (Leica PlanApo 1.0X, 6.5 x 6.5 mm imaging area) with an Andor Zyla sCMOS camera. Using a green (530 nm) LED, the camera view was centered on the cranial window and focused ~600 μm into the brain under the surface vasculature. ISOI signals were acquired with a red (617 nm) LED. Under binocular viewing conditions, the stimulus was presented for 5 min and typically 2 - 3 repeats were run for each direction (up or down).

For experiments shown in Figure S3, widefield ISOI was performed through intact skull under isofluorane anesthesia (2% for induction, <1% for maintenance). Body temperature was maintained at ~37 °C by a feedback-controlled heating pad and eyes
were covered with silicone oil to prevent drying. The stimulus presented was the same as for awake recordings, except that two different maximum luminance levels were used: 33 and 330 cd/m². Recordings were performed under monocular viewing conditions, alternating between the eyes ipsilateral and contralateral to the recorded hemisphere. Other details of data acquisition were the same as for awake recordings.

Data were analyzed using custom MATLAB (MathWorks) software to output amplitude and phase maps of cortical activation by extracting signals that varied at the frequency of stimulus repetition (0.05 Hz). Peak amplitude was computed by taking the maximum of the Fourier amplitude map.

Two-photon calcium imaging and pupil imaging

*In vivo* two-photon calcium imaging was performed on awake head-fixed mice sitting on a smooth surface. Mice were habituated on the imaging setup for 0.5 - 1 hr prior to the first imaging day. Typically, the same fields of view in the binocular zone of V1 were recorded over several sessions, for 2 - 3 hr per day. If the sets of neurons from a previous session could not be identified, different fields of view in V1 were recorded. The average time interval between GCaMP6s virus injection and the first two-photon imaging session was 23 days (age at baseline imaging session: P60-78, mean: P69).

GCaMP6s imaging was performed using a resonant two-photon microscope (Neuroabware), 920 nm excitation laser (Mai Tai HP, Spectra-Physics) and a Nikon 16X (NA = 0.8) water-immersion objective. Typical fields of view were acquired at 4x zoom, covering a rectangular field of view of ~450 μm x 550 μm and image sequences were acquired at 8 Hz (990 lines) at approximately 200 μm below the brain surface, corresponding to cortical layers 2/3. Data acquisition was controlled by Scanbox software (Neuroabware).

Visual stimuli were presented on a gamma-corrected 24-inch LED monitor (Asus VG248, 60 Hz refresh rate, 20 cd/m² mean luminance), placed at 25 cm from the mouse’s eyes. Stimuli were generated by custom Python software using the PsychoPy 1.9 library. They were spherically corrected, and included full-field drifting sinusoidal gratings (contrast: 99%) of 6 spatial frequencies (SF; 0.03 - 0.96 cpd, spaced logarithmically) and 8 directions (0 - 315°, in 45° steps) at a temporal frequency of 2 Hz, a blank (uniform luminance) stimulus, and a full-field flicker (2 Hz) stimulus. Each trial consisted of a uniform gray screen for 2 sec and a visual stimulus for 2 sec. Eight repeats per stimulus were presented in a random order without replacement. For monocular viewing, stimuli were presented to one eye at a time, either first to the contralateral or ipsilateral eye using an occluder. The order of eye presentation was chosen randomly for each session. For binocular viewing, no occluder was used.

Two-photon calcium movies were motion-corrected and processed for extracting fluorescence traces using custom Python software. Motion leading to translational artifacts was corrected using a Fourier transform approach. Using a summed intensity projection of the motion-corrected movies, we applied morphological criteria to manually identify regions of interest (ROIs) that correspond to neuronal somata. Pixel values within the ROI were averaged to yield the fluorescence trace of the ROI. The fluorescence signal of a ROI at time \( t \) was determined as follows: \( F_{\text{cell}}(t) = F_{\text{soma}}(t) - (R \cdot F_{\text{neuropil}}(t)) \). We empirically determined R to be 0.7 by comparing blood-vessel intensity of the GCaMP6s signal to that in the neuropil. The neuropil signal was estimated by averaging values in pixels within ~3 μm radius outside the cell’s outline.

To calculate a response amplitude of an ROI to the stimulus (ΔF / F₀), the calcium trace during the stimulation period was normalized to the baseline value (0.5 sec preceding the stimulus). The mean ΔF / F₀ was generated by averaging normalized responses across all the repeats of the stimulus. The mean response amplitude of the ROI during blank stimulus presentation was used to estimate the spontaneous calcium fluctuation. For each SF, the visual responsiveness of an ROI was determined using a one-way ANOVA (p < 0.01) across responses for all orientations for that SF against responses for the blank condition. For analysis of visual response properties (with the exception of pupil-neural cross-correlation analysis), we used responsiveness criteria where only ROIs whose responses at the preferred SF (SF that gave the strongest response) reached statistical significance at p < 0.01 were examined. For calculation of SF bandwidth, considering only neurons with preferred SF between 0.06 and 0.48 cpd (inclusive), the SF tuning curve was fitted with a Gaussian function, and the bandwidth was calculated by taking the square root of the width at half the maximum of the fit. For statistical analysis of preferred SF and response amplitude values, log-transformed values were used. Global orientation selectivity index (gOSI) was determined using a method based on circular variance of the cell’s response as follows.

\[
gOSI = \frac{\sqrt{\left(\sum F(\theta) \sin 2\theta\right)^2 + \left(\sum F(\theta) \cos 2\theta\right)^2}}{\left(\sum F(\theta)\right)}
\]

where \( F(\theta) \) is the averaged amplitude of a cell’s response to a stimulus orientation \( \theta \).

Ocular dominance index (ODI) for each ROI was calculated as \((C - \ell) / (C + \ell)\), where C is contralateral-eye response amplitude and \( \ell \) is ipsilateral-eye response amplitude; response amplitude is the mean ΔF / F₀ as described above. For ROIs that did not show a significant response for one of the eyes according to the responsiveness criteria described above, response amplitude for that eye was set to zero. Such neurons were deemed functionally monocular and described as contralaterally (contra)-dominant or ipsilaterally (ipsi)-dominant (ODI values of 1 or -1, respectively). We note that other studies typically have not probed spatial frequencies above 0.48 cpd as we have done here and we have found that probing at higher SF’s is important because many high-SF responsive V1 cells are monocular. The wild-type ODI distribution we report here is consistent with those from previous studies from our and other groups.
Eye tracking was achieved using IR-compatible GigE cameras (Allied Vision Mako-131B) and illumination by the infrared laser was used for two-photon imaging. Eye tracking movies were acquired simultaneously with two-photon calcium imaging. To identify the pupils, contours were extracted from each movie frame using routines from the Python OpenCV library (version 3.2.0). Accuracy of pupil detection was visually checked and frames with artifacts and/or blinks were removed by an experimenter naive to the purpose of the experiment. For pupil-neural correlation analysis, calcium traces from all ROI’s were included, regardless of passing the responsiveness criteria. The mean and standard deviation of pupil diameter were calculated from recordings that were typically 26 min long and acquired at 8 frames/sec. Although eye tracking movies were acquired for both eyes, only one (left) eye recording was used for pupil-neural correlation, as preliminary analysis showed that pupil diameter was essentially indistinguishable between the two eyes. Pupil diameter values were first normalized and values at 5 z-scores away from the mean were excluded. Calcium traces from neural data were first de-trended and normalized. To de-trend, the lm function in the stats package in R was used to fit a linear model to the calcium trace and residuals were computed to obtain de-trended data, where residuals were the difference between the data and fitted values. Cross-correlation values between the pupil diameter trace and individual calcium traces were calculated at different pupil-neural lags, using the ccf function in the stats package in R. The pupil-neural lag is negative when neural signal is leading; it is positive when pupil signal is leading. The maximum cross-correlation value from each cell was used for statistical comparison.

**Electroretinography**

Electroretinography (ERG) was performed in a dark room using a Diagnosys Celeris rodent ERG device (Diagnosys, Lowell, MA), as described previously. Under dim red light, the dark-adapted mice were anesthetized with ketamine (100 mg/kg, KetaVed; Bioniche Teoranta, Inverin, Galway, Ireland) and xylazine (10 mg/kg, Rompun; Bayer, Shawnee Mission, KS) by intraperitoneal injection, and their pupils were dilated with 1% tropicamide (Tropicamide Ophthalmic Solution USP 1%; Akorn, Lake Forest, IL) and thereafter kept moist with 0.3% hypromellose gel (GenTeal; Alcon, Fort Worth, TX). An additional 3 min were allowed in full dark adaptation before recordings were initiated. Light stimulation was produced by an in-house scripted stimulation series in Espion software (version 6; Diagnosys). The eyes were stimulated with a green light-emitting diode (LED) (peak 544 nm, bandwidth 160 nm) using a 11-step ascending flash strength series between 0.0002 and 100 cd·s/m². The ERG signal was acquired at 2 kHz and filtered with a low-frequency cutoff at 0.25 Hz and a high-frequency cutoff at 300 Hz. Espion software automatically detected the ERG a-wave (first negative ERG component) and b-wave (first positive ERG component) amplitudes; a-wave amplitude was measured from the signal baseline, whereas b-wave amplitude was measured as the difference between the negative trough (a-wave) and the highest positive peak.

**Optomotor reflex test**

The optomotor reflexes (OMR) were assessed using a commercial OMR platform (Phenosys qOMR, PhenoSys GmbH, Berlin, Germany) that utilizes automated head tracking and behavior analysis, following protocols described earlier. The OMR arena was dimmed using neutral density filters in front of the stimulus displays. The ambient luminance was measured at ~1 lux, corresponding to roughly twilight light level. Rotating (12° sec⁻¹) vertical sinusoidal grating stimuli at changing contrast levels were presented for 12 min per trial to initially dark-adapted mice. The spatial frequency of the grating was set at 0.1 cycles per degree of the visual angle whereas the pattern contrast (5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 37.5, 50, or 100%) changed every 60 sec at random order, except that each session was always started with 100% pattern contrast to facilitate acclimatization to the task. Each mouse was tested in at least four trials. The performances across the trials were averaged for analysis, excluding those 60-sec stimulus periods that led to a correct/incorrect ratio smaller than 0.8.

**Retinoid analysis**

Following ERG and OMR experiments, mice were placed in darkness for 24 hr and then sacrificed. Eyes were excised under dim red light, placed in Eppendorf tubes, flash frozen in liquid nitrogen, and stored at -80 °C. Retinoids were extracted from the whole eyes and analyzed by high-performance liquid chromatography (HPLC) as described previously. 9-cis-retinal oxime standard was generated by reacting 9-cis-retinal in 50% methanol with excess hydroxylamine, pH 8, and then extracting the resulting 9-cis-retinal oximes with hexanes. The retinoid concentration was determined spectrophotometrically using an extinction coefficient (absorptivity $A_{342}$ nm) of 54,119 cm⁻¹ M⁻¹. Known amounts of 9-cis-retinal oxime were injected onto the HPLC column to allow generation of a standard curve relating syn-9-cis-retinal oxime mass to elution peak area under the curve. All procedures were performed under dim red light.

**Immunohistochemistry**

Following euthanasia, mouse eyes were enucleated and fixed for 30 min in PBS containing 4% (w/v) paraformaldehyde (Electron Microscopy Sciences) at room temperature. After fixation, the eyes were incubated at room temperature sequentially in PBS containing 10%, 20%, and 30% (w/v) sucrose (MilliporeSigma) for 30 minutes at each concentration. Then, the eyes were incubated for 30 minutes at room temperature with a 4:1 mixture of PBS containing 30% sucrose and O.C.T. compound (Fisher Scientific). The eyes were then incubated overnight at 4 °C with a 2:1 mixture of PBS containing 30% sucrose and O.C.T. compound and frozen by immersion in dry ice the next day. Retinal sections were cut at a thickness of 10 μm at -20 °C using a Leica CM1850 cryostat and stored at -80 °C until use. The retinal sections were rehydrated with PBS and then blocked and permeabilized for 30 minutes with 0.2% (w/v)
Triton X-100 (Millipore Sigma) and 5% normal goat serum (Abcam) in PBS. The sections were then incubated overnight with anti-Opsin 1, Medium Wavelength primary antibody (1:250; Novus, NB110-74730) in a solution containing 0.2% Triton X-100 and 5% normal goat serum in PBS. The sections were then washed with PBS 3 times, and then incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:250; Thermo Fisher Scientific, A11037) in PBS. The sections were then washed with PBS 5 times and mounted with VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories) for imaging. Fluorescence images were acquired with a Keyence BZ-X800 All-in-One fluorescence microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

For ISOI, two-photon calcium and pupil imaging experiments, statistical analyses and data plotting were performed using custom software in R. For all other experiments, statistical analysis was carried out using GraphPad Prism. When comparing groups, Shapiro-Wilk normality test was used to test for normality of data. For non-normal data, non-parametric tests such as the Wilcoxon rank sum test were used. In addition to conventional statistical tests, multilevel statistics were employed to take the hierarchical nature of our data into account (e.g., neurons nested inside recording fields, fields nested inside mice). Multilevel linear mixed-effects models with Satterthwaite’s approximation were used, with experimental variables (e.g., treatment) as fixed variables and mouse ID and/or field ID as random variables. For each analysis, the exact statistical tests used are described in the figure legends. A p value < 0.05 was considered significant.