A dedicated circuit links direction-selective retinal ganglion cells to the primary visual cortex

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How specific features in the environment are represented within the brain is an important unanswered question in neuroscience. A subset of retinal neurons, called direction-selective ganglion cells (DSGCs), are specialized for detecting motion along specific axes of the visual field1. Despite extensive study of the retinal circuitry that endows DSGCs with their unique tuning properties2,3, their downstream circuitry in the brain and thus their contribution to visual processing has remained unclear. In mice, several different types of DSGCs connect to the dorsal lateral geniculate nucleus (dLGN)4–6, the visual thalamic structure that harbours cortical relay neurons. Whether direction-selective information computed at the level of the retina is routed to cortical circuits and integrated with other visual channels, however, is unknown. Here we show that there is a di-synaptic circuit linking DSGCs with the superficial layers of the primary visual cortex (V1) by using viral trans-synaptic circuit mapping7,8 and functional imaging of visually driven calcium signals in thalamocortical axons. This circuit pools information from several types of DSGCs, converges in a specialized subdivision of the dLGN, and delivers direction- and orientation-tuned signals to superficial V1. Notably, this circuit is anatomically segregated from the retino-geniculo-cortical pathway carrying non-direction-tuned visual information to deeper layers of V1, such as layer 4. Thus, the mouse harbours several functionally specialized, parallel retino-geniculo-cortical pathways, one of which originates with retinal DSGCs and delivers direction- and orientation-tuned information specifically to the superficial layers of the primary visual cortex. These data provide evidence that direction and orientation selectivity of some V1 neurons may be influenced by the activation of DSGCs.

Visual perception involves the activity of neurons in the cerebral cortex. The most direct route for visual information to reach the cortex is via the ‘retino-geniculo-cortical pathway’ consisting of retinal ganglion cells (RGCs), relay cells in the dLGN and neurons in primary visual cortex (V1) (Extended Data Fig. 1a–c)9. Recently, we and others discovered that direction-selective retinal ganglion cells (DSGCs) project to the dLGN and therein target a specific layer in the lateral shell5–6,10 (Fig. 1a–c). Hereafter we also refer to this layer as the DSGC-recipient zone or ‘DSGC-RZ’ (Fig. 1d).

Previous work showed that the dLGN shell receives input from the superior colliculus1 and thus, like other thalamic compartments12, neurons in the shell/DSGC-RZ may restrict their connections to subcortical networks, rather than participating in the retino-geniculo-cortical pathway. We infected neurons in the dLGN shell and a small portion of the dLGN core by injections of adeno-associated virus (AAV)-tdTomato (Fig. 1e). Within V1, tdTomato+ axons were observed in deeper layers 4 and 6 and superficial layers 1 and 2 (Fig. 1f–i). Thus, neurons in the shell/DSGC-RZ probably include thalamocortical relay neurons, but it was unclear whether they target specific V1 layers.

To determine whether there is laminar specificity of mouse geniculo-cortical connections we injected retrograde tracers into different V1 layers (Fig. 2a–c) and analysed the position of the retrogradely labelled neurons in the dLGN (Fig. 2d–i) (Extended Data Fig. 2a–d). Injections of all V1 layers retrogradely labelled cells across the full width of the dLGN (Fig. 2a, d, g). By contrast, injections directed to V1 layer 4 preferentially labelled neurons in the dLGN core (Fig. 2b, e, h) and injections into superficial V1 layers 1 and 2 preferentially labelled neurons in the dLGN shell/DSGC-RZ (Fig. 2c, f, i) (Extended Data Fig. 3a–c). These laminar-specific patterns of retrograde labelling were independent of retinotopy or eye-specific connectivity (Extended Data Fig. 4) and together they indicate that cells in the dLGN core project to deeper V1, whereas cells in the dLGN shell/DSGC-RZ preferentially target superficial V1 (Extended Data Fig. 3d).

RGC axons form synapses onto the somas and dendrites of dLGN neurons13, the latter of which are not entirely labelled using traditional retrograde tracing methods. Thus, we extended our exploration of the connections between DSGCs and thalamocortical relay neurons by using a glycoprotein-deleted rabies virus expressing mCherry (AG-RABV-mCherry) that infects neurons at the level of their axon terminals, leading to Golgi-like expression of mCherry throughout the infected cell. We note, however, that by itself AG-RABV-mCherry does not pass trans-synaptically7,8. Following injections of AG-RABV-mCherry into superficial V1 (Fig. 3a, b) we observed relay neurons in the dLGN shell expressing mCherry throughout their somas and dendritic arborizations (Fig. 3c, d). By performing these experiments in mice with genetically tagged DSGCs, we determined that the majority
of the mCherry\(^+\) cells resided in the DSGC-RZ (Fig. 3e, f) (Extended Data Fig. 4) (Methods). We observed putative sites of contact between the axon terminals of DSGCs and the dendrites of mCherry\(^+\) dLGN neurons (Fig. 3g), some of which contained VGLUT2 (Fig. 3h–k) (Extended Data Fig. 5), a presynaptic glutamate transporter that, in the dLGN, arises solely from RGC axon terminals\(^14\). Together, these experiments indicate that the neurons in the dLGN shell/DSGC-RZ that project to superficial V1 are contacted by, and probably receive synaptic input from, On-Off DSGCs.

To determine whether there is a bona fide di-synaptic circuit linking DSGCs to superficial V1, we used rabies-virus trans-synaptic network tracing\(^24\). We injected ΔG-RABV-mCherry into superficial V1 to infect neurons in the dLGN shell/DSGC-RZ via their presynaptic terminals and, in the same mice, we infected dLGN neurons with an AAV expressing the rabies glycoprotein and histone-tagged green fluorescent protein (AAV2-Glyco-hGFP) (Fig. 4a). In this experimental configuration, the double-infected RABV-mCherry\(^+/\)Glyco-GFP\(^−\) dLGN relay neurons produce infectious ΔG-RABV-mCherry that propagates trans-synaptically to infect and label the RGCs that form synapses with them (Fig. 4a) (Methods). A small number of double-infected cells were present in the dLGN and these were always located in the shell/DSGC-RZ (Fig. 4b–e) (8 mice, \(n = 21\) cells). Moreover, we performed these experiments in mice where GFP is selectively expressed by posterior-tuned On-Off DSGCs (Extended Data Fig. 6) and we used immunohistochemical markers that recognize multiple On-Off DSGC types\(^15,16\) to determine (1) whether DSGCs provide di-synaptic input to V1, and (2) if so, whether multiple DSGC types feed this pathway.

In mice with one to three double-infected dLGN neurons we observed 1–3 mCherry\(^+\) RGCs, which is consistent with the convergence of mouse retinogeniculate connections\(^7\). An example of a trans-synaptically labelled RGC is shown in Fig. 4f, g. The cell has thin dendrites and looped arborizations and is bistratified in the On and Off sublayers of the inner plexiform layer (IPL) (Fig. 4g, and inset), features characteristic of On-Off DSGCs\(^2,3,5,6\). Also, the RGC is GFP\(^−\) in a transgenic mouse where GFP is selectively expressed in a posterior-tuned On-Off DSGC (Fig. 4h–j) (Extended Data Fig. 7). Another mCherry\(^+\) DSGC trans-synaptically labelled from superficial V1 is displayed in Fig. 4k–m. The cell expresses cocaine- and amphetamine-regulated transcript (Cart), a marker of On-Off DSGCs\(^13,14\). Interestingly, trans-synaptic tracing from superficial V1 also labelled J-RGCs, which are asymmetric Off-type DSGCs tuned for upward motion\(^4\) (Fig. 4n, o).

Of all the RGCs trans-synaptically labelled from superficial V1 we verified that the vast majority were DSGCs (\(n = 27/28\) RGCs; 8 mice). 17/28 were genetically identified as posterior-tuned On-Off DSGCs\(^2,3,5,6\) and 9/28 were Cart\(^+\) but not GFP\(^−\) and thus are On-Off DSGCs which are probably tuned to other cardinal axes of motion\(^15,16,1\). 1/28 was an upward tuned J-RGC\(^4\), and one could not be classified. Thus, although we cannot conclude that only DSGCs contribute to this pathway, we find that several types of On-Off DSGCs as well as Off-type DSGCs provide di-synaptic input to superficial V1.

Do DSGCs also feed the classic retino-geniculo-cortical pathway into layer 4? We addressed this by injecting mice with ΔG-RABV-mCherry into V1 layer 4 and AAV2-Glyco-hGFP into the dLGN, then examining the RGC types trans-synaptically labelled with mCherry (Fig. 4p). In this regime, the mCherry\(^+/\)Glyco-GFP\(^−\) double-infected neurons resided in the dLGN core (Fig. 4q–t) (Extended Data Fig. 8) (7 mice, \(n = 53\) cells) (Fig. 4t versus Fig. 4e; ***\(p < 0.0001\); two-tailed t-test).

All the RGCs trans-synaptically labelled from layer 4 had large somas and broad, smooth monostratified dendrites, features characteristic of mouse alpha RGCs\(^18,19\) (Fig. 4u, v) and/or they expressed SMI-32, a marker of alpha RGCs\(^4\) (7 mice, \(n = 38\) RGCs) that is not expressed by On-Off DSGCs (Fig. 4w–bb). Moreover, none of the RGCs trans-synaptically infected from layer 4 exhibited DSGC morphologies, stratification patterns or molecular/genetic markers\(^4,6,13,16\) (Fig. 4c–e) (0/38 RGCs examined; 7 mice). Combined with the results in Figs 2, 3, 4a–o, these data indicate that alpha, non-direction-tuned RGCs are
Figure 4 | Synaptic circuit linking DSGCs to superficial V1, and non-DSGCs to L4. a, Trans-synaptic tracing, b, Infected dLGN neurons. Arrow and arrowhead: double-infected cells; arrow is same cell as in c, d. Scale bar, 100 μm. c, d, Cell from b. Scale bar, 15 μm. Dashed line, lateral border. e, Distribution of double-infected dLGN cells = 9.29 ± 1.82% (8 mice, n = 21 cells). f, On-Off DSGC trans-synaptically labelled from superficial V1. g, On (red) and Off (black) dendrites. Arrowhead, axon. Scale bar, 50 μm. h–j, Cell (f) is GFP+ On-Off DSGC. Scale bar, 10 μm. k–m, Trans-synaptically labelled GFP+ and Cart+ DSGCs shown at low (left) and high (right) magnifications. m, Scale bar left, 75 μm; right, 10 μm. n, Trans-synaptically labelled J-RGC. o, Off dendrites (black). Scale, 50 μm. p, Same as a, but layer 4 injection. q–s, Infected neurons in core; q, arrow, arrowhead: double-infected cells. Scale, 100 μm. r, s, Cell from q (arrow). s, Scale bar, 15 μm. t, Distribution of double-infected dLGN cells = 70 ± 2.65% (7 mice, n = 53 cells) (P < 0.0001 versus e, two-tailed t-test). u, v, Alpha RGC labelled from V1 layer 4. u, Scale bar, 100 μm; sideview, 50 μm. w–y, mCherry RGC same as from u, v, is smi-32+. Scale bar, 20 μm. z–bb, smi-32 and cart. Scale bar, 25 μm. cc–ee, ΔG-RABV-mCherry+ alpha RGC; lacks GFP and cart. Scale bar, 150 μm.

poised to influence layer 4 of V1 via neurons in the dLGN core, whereas DSGCs are poised to influence superficial V1 via neurons in the dLGN shell. Next we asked what qualities of visual information are delivered by the dLGN to superficial V1. We forced a subset of dLGN neurons to express the calcium indicator GCaMP6 by injections of AAV2-Syn-GCaMP6 into the shell/DSGC-RZ (Fig. 5a–c) (5 mice). We then imaged the visually evoked calcium dynamics (ΔF/F) in thalamocortical axons that target superficial layers of V1 (Fig. 5d) using in vivo time-lapse two-photon microscopy (Fig. 5e), while presenting the mice with drifting gratings of different orientations and directions (Fig. 5e–g) (Methods).

Figure 5 | In vivo imaging of visually evoked Ca2+ signals in thalamocortical axons. a, AAV2-GCaMP6 injection to dLGN shell. b, GCaMP6+ neurons (arrows). Scale bar, 50 μm; inset, 10 μm. c, GCaMP6+ dLGN axons, superficial V1. Arrows: varicosities. Scale, 50 μm. d, GCaMP6+ axons, superficial V1. Circles, square in d correspond to polar plots l, o, p. Scale bar (d), 5 μm. e, In vivo imaging/visual stimulation. f, Visually evoked Ca2+ signal in thalamocortical axon (top trace: photodiode signal; bottom trace: ΔF/F). g, Directional stimuli (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°). h–j, Directional- (h, i) and orientation-tuned (j) varicosities. 5–8 trial average. k–s, Polar plots of F1 (red) or F2 (black) magnitude responses (Methods). Lower inset, solid ring, average response to mean grey stimulus. Shaded, 3 standard deviations greater than the mean response to grey stimuli. Lower right of each plot, OSI/DSI. Upper right, Fourier amplitudes. t, DSI/OSI, all varicosities (5 mice, n = 58 varicosities). Mean ± s.e.m. u, Cumulative distributions OSI (circles), DSI (squares).
Figure 5f shows an example of visually evoked calcium transients in a thalamocortical axon. Figure 5h, i shows two clear and marked examples of direction-selective signals in thalamocortical axons located within superficial V1. The first example (Fig. 5h), responds only to gratings drifting at 45 degrees (polar plot Fig. 5k). The second example (Fig. 5i) is direction-selective but is more broadly tuned (polar plot Fig. 5l). Interestingly, we also observed thalamocortical axons that were strongly tuned not for direction, but instead for orientation (Fig. 5j and Fig. 5n–q). Overall, a variety of strengths of direction and orientation tuning were observed, including some thalamocortical axons that were not tuned for either feature (Fig. 5r, s). Approximately 60% of all thalamocortical axons imaged had direction selectivity indices (DSIs) \( \approx 0.4 \), a commonly used threshold for categorizing mouse DSGCs\(^{21}\) and approximately \( \sim 85\% \) had orientation selectivity indices (OSIs) of \( \geq 0.5 \) (Fig. 5t, u) (5 mice, \( n = 58 \) varicosities). Thus, the majority of visual information delivered to superficial V1 by neurons in the dLGN shell/DSGC-RZ is direction- and/or orientation-tuned.

Our results are the first to define a circuit relationship between DSGCs and visual cortex and they suggest that, in the mouse, direction- and orientation-tuned V1 neurons may inherit their characteristic receptive field properties from DSGCs. Notably, such influence may be specific to neurons receiving thalamic excitation within superficial V1. Indeed, our results of trans-synaptic labelling of RGCs from V1 layer 4, combined with recent studies that recorded thalamic excitation in layer 4 (refs 22, 23), suggest that orientation and direction selectivity of neurons in deeper V1 arises from the convergence of retinal and thalamic afferents with centre-surround receptive fields, as opposed to direction- or orientation-tuned receptive fields.

Interestingly, we discovered that several different types of On-Off DSGCs as well as upward-tuned Off-DSGCs feed the retino-geniculo-direction- or orientation-tuned receptive fields. Layer 4, combined with recent studies that recorded thalamic excitation within superficial V1 by neurons in the dLGN shell/DSGC-RZ is direction- and/or orientation-tuned.  

METHODS SUMMARY

All experimental protocols were conducted according to the National Institutes of Health (NIH) guidelines for animal research and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego, USA.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions A.D.H., A.C.-M., A.G. and R.N.E. designed the experiments. A.D.H., A.C.-M., R.N.E. and P.L. carried out and analysed the circuit connectivity experiments. A.C.-M. carried out the in vivo imaging experiments. B.S. and A.C.-M. analysed imaging data. S.D. collected data on molecular markers of cell types. E.M.C. and F.O. designed and made the rabies viruses. A.D.H. and A.C.-M. wrote the paper in collaboration with the other authors. A.D.H. and A.C.-M. prepared the figures. A.D.H. oversaw the project.

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METHODS

All experimental protocols were conducted according to the National Institutes of Health (NIH) guidelines for animal research and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego, USA.

Mice. Pigmented, wild-type, DRD4-GFP, Trhr-GFP mice of both sexes (40 to 60 days old) were used. Animals from different litters and parent mice were used for each experiment. Sample sizes (number of mice and/or cells) for each experiment are stated in main text and figure legends.

Retrograde tracer injection. Mice were anesthetized using an isoflurane–oxygen mixture (4.0% (v/v) for induction and 1.5% (v/v) for maintenance) and given the analgesic buprenorphine (SC, 0.3 mg kg⁻¹). After a midline scalp incision, a small burr hole was made with a pneumatic dental drill (Henry Schein) over V1. For tracer injections into cortex we used either red fluorescent-latex beads (Lumaflo) that travel strictly in retrograde fashion from axon terminals at the injection site to cell bodies of origin, or cholera toxin beta (CTb) that travel retrogradely through dendrites and axons (for example, Fig. 2a, d). All injections were targeted stereotaxically to V1 using the following coordinates: 2.0–2.5 mm anterior to Lambda and 2.5 mm lateral from midline. For superficial injections we penetrated the cortex to a final destination of 150 μm and for deep injections to a depth of 650 μm. Full-depth experiments were accomplished by injecting tracers at multiple cortical depths.

Viruses. For injections of AAV2 expressing histoamine-tagged GFP and rabies glycoprotein B19G linked by the F2A element the F2A element was amplified by PCR30. These PCR fragments were inserted into the AAV vector containing woodchuck hepatitis virus posttranscriptional regulatory element. Every plasmid was sequenced before virus production. AAV were generated with transfection of HEK293 cells, purified by iodixanol gradient centrifugation and titred in HEK293 cells with quantitative PCR. The titre of the AAV used in this study was 10¹⁰ genomic titre per ml (ref. 31). AAV-α-dsRed-mCherry was amplified in B7G cells, concentrated by two rounds of centrifugation, and titred in HEK293 cells as described previously. The titres of the rabies viruses were 8.0 × 10¹⁰–2.0 × 10¹¹ infectious units per ml. The viruses were stored at −80 °C until use.

Retinal physiology. Trhr-GFP mice (6–8 weeks old) were used for targeted recordings of GFP⁺ DSGCs using methods described previously. GFP⁺ RGCs were identified using two-photon microscopy to minimize photopigment bleaching. Loose patch recording was used to measure spike responses to moving bar stimuli projected onto the photoreceptor layer from an OLED array (eMagin) with mean light levels of approximately 70 rhodopsin isomerizations per rod per second and bar contrasts of 200–300%.

Brain histology. Following retrograde or trans-synaptic tracer injection, mice were deeply anesthetized with pentobarbital (35 mg kg⁻¹) and perfused transcardially with 0.9% saline followed by 4% PFA. Brains were removed, fixed overnight in PFA and then transferred to a 30% (w/v) sucrose solution and stored at 4 °C. Brain slices were collected at 40–60 μm thickness in the coronal plane using a sliding microtome. Brain sections containing dLGN and visual cortex were washed with PBS and incubated for 1 h at room temperature in a blocking solution (10% goat serum, 0.25% Triton-X). This was followed by an overnight incubation with rabbit anti-GFP (1:1,000, Invitrogen) and guinea-pig anti-VGLUT2 (1:1,000, Millipore) primary antibodies in blocking solution at room temperature. Sections were then rinsed with PBS (3×, 30 min), incubated for 2 h at room temperature with Alexa Fluor 488 goat anti-rabbit (1:1,000, Invitrogen) and Alexa Fluor 647 goat anti-guinea-pig (1:1,000, Invitrogen) secondary antibody. Lastly, sections were washed with PBS and mounted with Vectashield with DAPI (Vectorlabs).

Targeted filling of genetically tagged RGCs. Targeted RGC filling was conducted using methods described previously. Retinas from mice aged between postnatal day 25–30 (P25–30) mice were dissected and kept in an oxygenated (95% O₂/5% CO₂) solution of Ames’ medium (Sigma-Aldrich) supplemented with 23 mM NaHCO₃. Borosilicate glass sharp electrodes were used to fill GFP⁺ RGCs with 10 mM solution of Alexa 555 hydrazide (Invitrogen) in 200 mM KCl, with the application of hyperpolarizing current pulses ranging between 0.1–0.9 nA for 5–20 ms. After cell filling, the retinas were fixed for 1 h with 4% PFA and were processed for immunocytochemistry using the methods described above. The following primary and secondary antibodies were used: rabbit anti-GFP (1:1,000, Invitrogen), guinea-pig anti-VACHT (1:1,000, Millipore), Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 647 goat anti-guinea-pig (1:1,000, Invitrogen). Sections were rinsed with PBS and mounted with Prolong Gold with DAPI (Invitrogen).

Confocal imaging. Fluorescence images were acquired on a laser scanning confocal microscope (Zeiss LSM 710 and 780) equipped with 405, 488, 561 and 633 nm laser lines. Image stacks with a scanning resolution of 1.024 × 1.024 pixels were collected using the following objective lenses: ×10/0.45 Plan-Apochromat (step size of 3.7–4.0 μm), ×20/0.8 Plan-Apochromat (step size of 1.0 μm) and LD C-Apochromat ×40/1.1 water immersion (step size of 0.5–1 μm).

Quantification. For calculating cortical intensity profiles for superficial and deep injections of tracers, the location of the injection was identified from epifluorescent images of tissue sections taken from the entire brain. We evaluated tracer spread along the length of the injection site (from pia to white matter) and the width (parallel to the pial surface). Background fluorescence was subtracted using the contralateral non-injected cortex as the baseline control. After background subtraction, the peak fluorescence along the injection site was quantified by dividing the site into equidistant areas along the injection height and width from pia to the white matter (100 × 800–1,000 μm). For each fluorescence intensity profile, the peak and background levels were identified as maximum fluorescence. The fluorescence signal was normalized to this value. Four to five intensity profiles were analysed per tissue section. All tissue sections surrounding the injection site were included in the analysis. We confirmed superficial injections as those with peaks ≤150 μm from the pia and deeper injections as those with peaks >300 μm from the pia.

For measuring the location of retrogradely labelled cells along the medial–lateral axis of dLGN, we obtained fluorescent confocal images (×10 and ×20) of coronal brain tissue sections. The boundaries of the dLGN were identified using two stable features: the optic tract, which defines the lateral dLGN border, and the intralaminar white matter (WM) which defines the dLGN’s medial border. Four measurements were taken per tissue section from locations that spanned the entire rostral–caudal length of the dLGN and thus, the entire retinotopic map. These measurements were placed at equidistant points along the width of the dLGN and an average distance of each labelled cell along the axis from the optic tract (0%) to the medial border (100%) was obtained.

To quantify overlap between retrogradely infected dLGN relay neurons and GFP⁺ DSGC axons we obtained fluorescent confocal stacks of coronal tissue sections through the dLGN. For the GFP channel a median filter (2 pixels) was applied and then background subtracted using a rolling ball radius algorithm (50 RPB pixels). After processing, profiles, maximum intensity projections were generated for both channels and the GFP channel was subsequently thresholded using a triangular algorithm. The thresholded image was then binarized to create a mask representing the area populated by GFP⁺ DSGC axons. This mask was superimposed on the red channel. Every mCherry⁺ cell body was identified and its

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location assessed for overlap with the DSGC-RZ. We note that modified rabies viruses may underestimate total numbers of presynaptic inputs to double-infected cells.

To determine the area of contact between dLGN neurons and VGLUT2 containing DSGC axons, we quantified the percentage of the total somatodendritic area of the mCherry expressing neuron that co-localized with GFP signal from the genetically tagged On-Off DSGC, and that were also immunoreactive for VGLUT2. First, GFP and VGLUT2 puncta were identified by a series of filtering (median = 2 pixels) and erosion steps. This image was then thresholded to identify regions of interest (ROIs) that contained VGLUT2 and GFP puncta, and this was divided by the total measurement of the mCherry labelled area. Images were processed and analysed using algorithms in ImageJ (NIH).

For retinal co-localization experiments, the maximum intensity projections for confocal stacks were obtained for red (mCherry), green (GFP) and far-red channel (Caret). Confocal sections were background subtracted and a maximum projection of the stack was obtained. Channels were merged and markers were considered co-localized if signals from different channels coincided within the same plane.

Statistical analysis. Analysis of fluorescent intensity profiles, normalized distances across the cortex and dLGN and axonal overlap of labelled relay cells and axons was carried out using custom routines in MATLAB (Mathworks) and ImageJ (NIH). All statistical analyses were performed with GraphPad Prism (GraphPad Software), and represented as averages. Error bars in graphs represent the s.e.m. for post hoc tests involving multiple comparisons were adjusted using the Tukey’s method. No randomization method was used to assign mice to the experimental groups and investigators were blinded to the original group allocation or when assessing an outcome. Variance and normality were tested to ensure the data set was chosen. The variance was similar between groups that were statistically compared. Normality testing in groups with a small number of samples was done with quantile-quantile plots.

For anatomical tracing experiments samples sizes were determined based on preliminary experiments that tested the labelling/infection efficiency of retrograde tracers and/or viral trans-synaptic tools. For imaging experiments no statistical test was used to determine sample size a priori. Experiments were included in the study if they were deemed successful. For the trans-synaptic studies, the experiment was discarded if there were no rabies infected RGCS as assessed by fluorescence. For experiments involving two-photon imaging of axons, data belonging to non-visual axons was not included in the experiment. In addition, mice in which we found no visually driven axons were also excluded from the study.

Imaging of visually evoked calcium signals in DSGC-RZ thalamocortical axons. For monitoring the activity of dLGN axons in V1 either the slow (GCaMP6F, titre, 3.04 × 10^{11} genomic titre per ml) or the fast (GCaMP6F, titre, 2.96 × 10^{11} genomic titre per ml) version of the genetically encoded Ca^{2+} sensor GCaMP6 (AAV1 Syn. GCaMP6.WPRE.SV40) were injected into the dLGN using the following coordinates: 2.10 mm posterior to Bregma and 2.2 mm lateral from midline. A small glass pipette (−0.5 ΜΩ) was then lowered 2.30 mm from the pial surface and using a Nanoject II (Drummond) 0.04-0.20 µl of virus was inoculated into the brain.

Three weeks after the injection of the calcium sensor, mice were deeply anae-

Imaging acquisition and presentation of stimulus grating. Mice were transferred and head-fixed to a stage while under isoflurane anaesthesia and kept at 37 °C using a temperature control device (Harvard Apparatus). Anaesthesia was maintained at 0.8–1.0% (v/v). Imaging was performed using a two-photon microscope system (Sutter) controlled by ScanImage software^{35} written in MATLAB (MathWorks). Excitation light from a Mai Tai Deepsee laser (Newport Corp.) with group delay dispersion compensation was scanned by galvanometers (Cambridge Technologies). Images were collected through a x20 objective (1.0, N.A., Olympus) and the laser power was maintained well below 50 mW at the sample. GCaMP6F was excited at 910 nm and emission was collected with a green filter (535 nm centre; 50 nm band; Chroma) via photomultiplier tubes (PMT) (H7422PA-40, Hamamatsu Photonics). Images were collected (3×3, digital zoom) at 8 Hz (256×128 pixels) or 16 Hz (256×64 pixels). For each experiment the optical axis was adjusted to be perpendicular to the cranial window. Imaging was stopped between trials and during this time slow drifts in the optical focus were corrected using a template image. Bleaching of GCaMP6F was not evident during experiments. Because of the limitation of current genetically encoded Ca^{2+} sensors our experiments were not sensitive enough to detect single action potentials, and thus, biased to sample from axons with high firing probability.

A visual stimulus containing a full-field two-dimensional moving grating or bar was generated using Psychtoolbox^{36}–38 in Matlab version 2009. The monitor screen was positioned approximately parallel to, and 29 cm from the right eye of the mouse. The visual angle subtended by the monitor (± 35° azimuth × ± 23.5° elevation) ensured that projective distortions were small. Both drifting sinusoidal gratings and square wave gratings were used. For sinusoidal gratings, each presentation of a grating started with a grey screen for 3 s, followed by the drifting gratings (0.02 cycles per degree (CPD), 2 Hz, 3 s). In the case of square wave gratings, each trial started with a grey screen for 3 s, followed by the drifting bars (0.01 CPD, 0.33 Hz, 12.75 s). Each trial consisted of presenting eight gratings of different orientations. Within a trial, the orientation of the grating was randomly interleaved. Five to eight trials were recorded to measure average axonal responses to different orientations. Simultaneously, we captured the on-screen luminance by recording the output of a photodiode (Thorlabs) placed at the corner of the screen.

Analysis of time-lapse images and visually evoked fluorescent responses. Frames from time-lapse imaging were registered using template matching (normalized correlation coefficient) and alignment plugins coded in ImageJ (NIH; https://sites.google.com/site/zingzonteng/template-matching-j-plugin). To align images we first generated an average image from a small number of frames within a trial where there was minimal movement. A region of interest (ROI) was then selected within this average template image and was used to register all the frames within that repetition.

To extract fluorescence signals, ROIs were drawn over GCaMP6^{+} varicosities identified by using the mean intensity and standard deviation values of all the repetitions. The pixels in each ROI were averaged to estimate fluorescence corresponding to a single varicosity. Calcium signals were expressed as relative fluorescence changes (ΔF/Fo = ((F1-F0)/F0)) corresponding to the mean fluorescence from all pixels within specified ROIs.

For each experiment, stimulus temporal frequency was measured using the photodiode signal. Putative visually responsive axons were chosen and the calcium response power at the stimulus frequency (F1) and response power at twice stimulus frequency (F2) were measured. To gauge if the measured power was significant, we compared each visually evoked response power with the mean (μ_{avg}) and the standard deviation (σ_{avg}) of calcium response powers to the grey stimulus that immediately preceded each grating. Axons were deemed visually responsive, and included for further analysis, if the F2 power of the visually evoked calcium signal was greater than μ_{avg} + 3σ_{avg}, for at least two of the orientations probed. Most (4 out of 5 mice) of the calcium imaging experiments were done using GCaMP6F. For the experiment where we used GCaMP6F we were able to measure frequency doubling responses to the stimulus presentation. Axons were classified as ‘linear’ if at the orientation corresponding to peak F1 power, the F2 power was smaller than the F1 power. If the F2 power was greater, the axon was judged as ‘nonlinear’. In experiments where we used GCaMP6F in combination with lower temporal frequency drifting grating stimulus, we observed two GCaMP6^{+} varicosities with On-Off responses (Fig. 5i and corresponding polar plot 5l, and polar plot in Fig. 5q; and see imaging methods above). We did not observe differences in the percentage of varicosities that were visually responsive using GCaMP6S compared to GCaMP6F (data not shown); therefore both sets of data are included.

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Extended Data Figure 1 | The retino-geniculo-cortical pathway links retinal cells and circuits to the brain.  

**a**, Diagram of retina, dorsal lateral geniculate nucleus (dLGN) and primary visual cortex (V1). The optic tract which carries retinal ganglion cell (RGC) axons and thalamocortical (dLGN to V1) pathway also shown.  

**b**, Diagram of retinal layers: PRL, photoreceptor layer; opl, outer plexiform layer; INL, inner nuclear layer; ipl, inner plexiform layer; GCL, ganglion cell layer; nfl, nerve fibre layer.  

**c**, Retina diagram with cells shown (labels same as in **b**).
Extended Data Figure 2 | Approach for assessing laminar specificity of mouse geniculocortical projections. 

a, Focal retrograde tracer injection to V1. Scale bar, 3 mm. 
b, Diagram of the three different injection depths used to generate data in Fig. 2. 
c, Percentage of fluorescence in V1 from superficial (black line) versus deep (grey line) injections. Superficial, peak intensity occurs at 25 μm from pial surface (4 mice). Deep, peak intensity occurs at 350 μm from pial surface. Gray shaded regions, s.e.m. (superficial vs deep = ***P < 0.0001; two-way ANOVA). 
d, Assessment of retrogradely labelled cells across the width of the dLGN. 0% is at optic tract, 100% is at medial border (see Fig. 2g–i).
Extended Data Figure 3 | Retrograde tracers to superficial V1 label cells in the DSGC-RZ. a–c, Same dLGN as in main Fig. 2f but with GFP On-Off DSGC axons shown. a, most of the retrogradely labelled cells (magenta/dashed circles) reside in the DSGC-RZ (green terminals). Asterisk, labelled cell outside the DSGC-RZ. Scale bar, 200 μm. b, c, High magnification views of retrogradely labelled dLGN neuron cell bodies with potential contact from GFP On-Off DSGC axons (arrow in b); c, this cell is in vicinity of DSGC axonal boutons (arrowheads). b, c, Scale, 15 μm. d, Diagram of laminar-specific connections between DSGC-RZ and superficial V1 and dLGN core and deeper V1 layers 4 and 6.
Extended Data Figure 4 | Analysis of dLGN neurons retrogradely infected from superficial V1. a–f, Example serial sections of anterior, middle and posterior portions of dLGN in a mouse with GFP expressing On-Off DSGC axons that was injected with ΔG-RABV-mCherry in superficial layers of V1. a, DAPI to show cytoarchitectural landmarks and dLGN borders. b, GFP+ DSGC axons and AAV2-Glyco-hGFP-infected cell bodies (see main Fig. 4 and text). c, Mask of GFP+ DSGC axons (Methods). d, ΔG-RABV-mCherry+ dLGN relay neurons. e, GFP+ DSGC axon mask superimposed with mCherry signal; this was used to determine colocalization. f, mCherry and GFP signals merged. Scale bar, 200 μm.
Extended Data Figure 5 | Putative sites of contact between DSGC axons and a dLGN neuron retrogradely infected from superficial V1. a–i, GFP+ On-Off DSGC axons (green in all panels except black in b) and mCherry+ dLGN relay neuron (magenta in all panels except white in c) infected by injection to superficial V1. Framed region in a is shown at higher magnification in b–d. Arrowhead (a), thalamocortical axon of mCherry+ dLGN cell. Scale bar in a, 50 μm. Yellow boxed region in c, d, is shown at higher magnification in e–i. Scale bar in d, 15 μm. e–i, Some DSGC axon–dendrite contacts contain VGLUT2 (blue). f–i, Arrowhead, site of GFP/mCherry co-localization that does not contain VGLUT2; arrow, GFP/mCherry/VGLUT2+ contact.
Extended Data Figure 6 | The axons of GFP+ On-Off DSGCs and dLGN neurons infected with AAV2-Glyco-hGFP can be distinguished on the basis of their cellular localization. High magnification view of DSGC-RZ in mouse with GFP+ posterior-tuned On-Off DSGCs that was injected 14 days earlier with AAV2-Glyco-hGFP. Glyco-hGFP+ neurons have nuclear GFP labelling (arrows), whereas DSGCs have GFP in axon terminals (arrowheads). Dashed line, lateral border of dLGN. OT, optic tract. Scale bar, 50 μm.
Extended Data Figure 7 | Signature anatomical and physiological characteristics of GFP-tagged On-Off DSGCs. a, b, Flat-mount retina with GFP^+ On-Off DSGCs (a) and co-stained with DAPI (b). c, Positions of GFP^+ RGCs. Scale bar in c, 150 μm. d-f, High magnification views. Scale bar, 12 μm. g, Targeted fill of a GFP^+ DSGC. Scale bar, 50 μm. h, Schematic of On-Off DSGC stratification and starburst amacrine cells (magenta). Labelling as in Extended Data Fig. 1. i, j, Higher magnification of framed region in g stained for VACHT (starburst amacrine processes). Asterisk, ‘looping arborizations’; dashed line, GFP arborization, which matches VACHT plexus. Scale bar, 10 μm. k, l, Side (x-z plane) views of cell in g. GFP^+ dendrites co-stratify with both the On and Off sublayers. Scale bar, 5 μm. m, Direction-tuned response of a GFP^+ On-Off DSGC targeted for recording and receptive field characterization. The spike count is highest for bars moving towards ~270° in the cardinal axes.
Extended Data Figure 8  | Injections of ΔG-RABV-mCherry into both superficial and deep V1 combined with AAV2-Glyco-hGFP infection of dLGN core. 

**a.** mCherry neurons in the DSGC-RZ and the core of the dLGN. **b.** AAV2-Glyco-hGFP: many neurons throughout the dLGN, but mostly along the medial border and not in the shell/DSGC-RZ express Glyco-hGFP. **c.** Merged of a, b. Scale in a, 100 μm. Boxed regions with arrows: two dLGN neurons; both RABV-mCherry and AAV2-Glyco-hGFP. DSGC-RZ marked by axons of GFP On-Off DSGCs. One or both of these cells infected their presynaptic partner, the RGC shown in Fig. 4 (panels cc-ee) of the main text. Scale bar, 15μm.