Use of a tissue clearing technique combined with retrograde trans-synaptic viral tracing to evaluate changes in mouse retinorecipient brain regions following optic nerve crush

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Abstract
Successful establishment of reconnection between retinal ganglion cells and retinorecipient regions in the brain is critical to optic nerve regeneration. However, morphological assessments of retinorecipient regions are limited by the opacity of brain tissue. In this study, we used an innovative tissue clearing technique combined with retrograde trans-synaptic viral tracing to observe changes in retinorecipient regions connected to retinal ganglion cells in mice after optic nerve injury. Specifically, we performed light-sheet imaging of whole brain tissue after a clearing process. We found that pseudorabies virus 724 (PRV724) mostly infected retinal ganglion cells, and that we could use it to retrogradely trace the retinorecipient regions in whole tissue-cleared brains. Unexpectedly, PRV724-traced neurons were more widely distributed compared with data from previous studies. We found that optic nerve injury could selectively modify projections from retinal ganglion cells in the hypothalamic paraventricular nucleus, intergeniculate leaflet, ventral lateral geniculate nucleus, central amygdala, basolateral amygdala, Edinger-Westphal nucleus, and oculomotor nucleus, but not the superior vestibular nucleus, red nucleus, locus coeruleus, gigantocellular reticular nucleus, or facial nerve nucleus. Our findings demonstrate that the tissue clearing technique, combined with retrograde trans-synaptic viral tracing, can be used to objectively and comprehensively evaluate changes in mouse retinorecipient regions that receive projections from retinal ganglion cells after optic nerve injury. Thus, our approach may be useful for future estimations of optic nerve injury and regeneration.

Key Words: histology; image analysis; light-sheet imaging; optic nerve crush; pseudorabies virus; retinal ganglion cells; three-dimensional imaging; tissue clearing; viral tracing; whole brain study

Introduction
The optic nerve transports visual messages from retinal ganglion cells (RGCs) into the brain. As neurons specific to the central nervous system, RGCs lack the ability to regenerate. Thus, the optic nerve crush (ONC) procedure, in which RGCs are seriously damaged, is widely used to investigate nerve degradation and regeneration in mice (Li et al., 1999; Donahue et al., 2020; Patel et al., 2020). The reformulation of eye-to-brain connections is an important historical and functional indicator of successful optic nerve regeneration (Laha et al., 2017; Yang et al., 2020). According to our findings and those of others, the ONC alters synaptic plasticity, astrocyte activity, and the cortical network within the visual cortex contralateral to the ONC side in mice (Groleau et al., 2020; Zhan et al., 2020). However, whether the ONC leads to changes in the retinorecipient regions related to RGCs in the whole mouse brain remains unclear.

Tissue clearing techniques may be a practicable way to scan retinorecipient regions in the brain after ONC. Combined with whole-organ antibody labeling, viral encoding with fluorescent protein tracing, or the use of transgenic animals, the tissue clearing technique is preferable for visualizing the neural circuits and connectome (Tian et al., 2020; Ueda et al., 2020; Lee et al., 2021; Wang et al., 2021; Li et al., 2022). This is preferable to whole brain imaging of the rodent brain, which can require hundreds or even thousands of sections, can take more than a month to complete, is prone to errors associated with misreading the regions on the different planes, and is vulnerable to missing data because of the large amount of information. A previous study indicated that tissue clearing may enable a fast and comprehensive scan of the spinal cord, dorsal root ganglia, and sural nerve in terms of nerve regeneration (Daeschler et al., 2022). Tissue clearing combined with light-sheet imaging

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can be used to evaluate whole body pancreatic innervation in diabetic mice and patterns of cancer metastasis (Pan et al., 2019; Alvarsson et al., 2020). Thus, this approach has great potential for use in neuroscience and pathology. With large numbers of samples, it will be possible to conduct comprehensive clinical and pathological examinations in the future. However, few studies have examined the possibility of applying the tissue clear and technical assessment of central nervous system regeneration.

Two strains of the pseudorabies virus (PRV) are used in laboratory research: PRV-Becker and PRV-Bartha. PRV-Becker is more similar to wild-type PRV, infects neurons, and is used for both anterograde and retrograde experiments (Quan et al., 1991). In contrast, PRV-Bartha is used more widely because it comes from a live vaccine and is transported retrogradely (Card et al., 1991; Pickard et al., 2002; Card and Enquist, 2014; Xu et al., 2020). PRV-Bartha has mutations in the Us genes (gE, gI, and Us9). For example, Us9 is a structural protein found in all α-herpesviruses. It appears in infected cell membranes and near the trans-Golgi network, and enables the spread of PRV-Bartha infection from axons to cell bodies (Brideau et al., 2000). Hence, PRV-Bartha does not have the ability to infect neurons anterogradely, but it can infect them retrogradely (i.e., from post-synaptic to presynaptic neurons) (Enquist, 2002). Therefore, an isogenic version of PRV-Bartha, encoded with a fluorescence protein such as PRV724, could be used to examine the function and survival of RGCs and to label retinorecipient brain regions.

Here, we administered intravitreal injections of PRV724 to ONC mice, and used the tissue optical clearing technique to analyze changes in retinorecipient brain regions.

Methods

Ethics statement

All experiments were carried out in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Pericèd et al., 2020), the Guide for the Care and Use of Laboratory Animals, and the Association for Research in Vision and Ophthalmology (ARVO) statement on animal use. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Center, Zhongshan School of Medicine, Sun Yat-sen University (approval No. 2019-1210; approval date: March 28, 2019).

Experimental animals

We used 3-month-old C57BL/6 or Thy1-Yellow fluorescence protein (Thy1-YFP) transgenic mice (RRID: MGI: 3581649) of both sexes, which had been identified by Y2 and YRH via polymerase chain reaction (PCR) and bred and maintained in pathogen-free conditions, for our experiments. We purchased 3-month-old C57BL/6 mice weighing 20–25 g from the Guangdong Medical Laboratory Animal Center in China (license No. SCXK (Yu) 2022-0002), and obtained 3-month-old Thy1-YFP transgenic mice from the Zhongshan School of Medicine, Sun Yat-sen University. The mice were divided randomly into a control and an ONC group. At least three mice per group were sacrificed in each experiment. The specific numbers are given in Figure 1A-C. We used a total of 74 mice, including 55 C57BL/6 mice and 19 Thy1-YFP mice. RGC survival was analyzed in 8 C57BL/6 mice (four males and four females). The residual RGC fibers and axons in the optic nerves were analyzed in 6 C57BL/6 mice (three males and three females). Retrograde fibers and axons in the optic nerves were analyzed in 7 C57BL/6 mice (three males and four females). An intravitreal injection of PRV724 was administered in 16 Thy1-YFP mice (eight males and eight females). In these mice, an intravitreal injection of phosphate buffer solution (PBS) was administered in three C57BL/6 mice (one male and two females). Two C57BL/6 mice (two males) died prematurely because of the lethality of PRV724. An intravitreal injection of PRV724 was administered in 11 Thy1-YFP mice (eight males and three females), and one mouse (female) died prematurely because of the lethality of PRV724. Counts for RGC staining were performed in 6 Thy1-YFP mice (three males and three females). Among these mice, four brains from the above C57BL/6 mice (two males and two females) and nine brains from the above Thy1-YFP mice (five males and four females) were analyzed after tissue clearing. The anesthesia used for all experiments was an intraperitoneal injection of 1% pentobarbital sodium (80 mg/kg; Merck, Frankfurt, Germany). Generally, mice were euthanized by intraperitoneal injection of 100 mg/kg sodium hydroxide (Sigma-Aldrich, St. Louis, MO, USA).

Identification of Thy1-YFP transgenic mice

A 0.5 cm piece of tail was removed from each mouse using sterilized scissors and used the tissue optical clearing technique to analyze changes in retinorecipient brain regions.

ONC surgery

As previously described (Quan et al., 2020; Wu et al., 2020; Zhan et al., 2020), after ensuring adequate anesthesia, the lower bulbar conjunctiva of each mouse was incised at 180°, and the optic nerve of the left eye was carefully exposed without obvious bleeding under a stereo microscope (Phenix, Shanghai, Jiangxi, China). Then, the optic nerve was crushed for 5 seconds.
Additional Figure 1). To determine Figure 3B), which was similar to eyes intravitreally injected with PBS (n = 9). Imaris version 9.0.2 (Bitplane Statistical analysis to the ONC eye was identified as the contralateral side. The ONC side was identified as the ipsilateral side and the side contralateral PRV724-labeled neurons, and the data were averaged for statistical analysis. Counting was performed for representative images containing each brain tissue.

Microscopy imaging Images of non-cleared samples were acquired using a confocal microscope (LSM 780, Zeiss, Oberkochen, Germany). We used a 10× objective lens (FLUAR, numerical aperture (NA) = 0.5, work distance (WD) = 2 mm), 20× objective lens (PLANAPOCHROMAT, NA = 0.8, WD = 550 μm), and 63× objective lens (PLANAPOCHROMAT, NA = 1.4, WD = 190 μm) for imaging. Image stacks of cleared samples were acquired with a step size of 5 μm using a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany) equipped with an sCMOS camera (Hamamatsu ORCA-flash 4.0 V3). Samples were illuminated from 4 directions horizontally via thin Line Bessel light-sheets, and imaged with a 4× objective lens (Olympus XFLUOR4X/340, NA = 0.28) from the bottom. We used Litscan 3.0 to create light-sheet microscope hyper-stacks as 16-bit grayscale TIFF images for each channel separately.

Image processing We used Zen 2011 SP2 (Version 8.0.0.273, Carl Zeiss GmbH, Oberkochen, Germany) software to collect non-cleared sample data. The RGC counts and fluorescence intensity values were measured using ImageJ.

Data processing, analysis, 3-dimensional (3D) rendering, and video generation for the cleared samples were performed on an HP workstation Z840 with a 24 core Xeon processor, 256 GB RAM, and an NVIDIA RTX k4000 graphics card, or on an iMac Pro workstation with an 8 core Intel core i9 processor (iRGCs) (Kwang et al., 2010; Mao et al., 2014). Cells co-labeled with RBPM5, opsins, and mRFP were identified as iRGCs infected with PRV724. The axons in the optic nerve were stained with GAP-43.

Tissue optical clearing Optical clearing of the brains was based on the polyethylene glycol-associated solvent system (Jing et al., 2018) and the kits were purchased from Leica Biosystems (Wetzlar, Germany). The brains were immersed in 4% PFA in Carlsbad, CA, USA, Cat# PAI-781, TRIB: AB_2156604) overnight at 4°C. After three washes with PBS-0.1% Tween 20, goat-anti-rabbit Alexa Fluor 488 (1:500, CST, Cat#44125, TRIB: AB_1904025) or goat-anti-mouse Alexa Fluor 488 (1:1000, CST, Cat#6410, TRIB: AB_1904023) was applied. The tissue samples were incubated at 26°C in a dark environment for 2 hours, and then coverslip slides were applied with an antifade reagent with DAPI (F6057, Sigma-Aldrich). The RBPM5 cells were identified as RGCs, and the opsins 4+ cells were identified as the infected RGCs with the ipRGC marker opsin 4 antibody, and found that while some PRV724-injected RGCs were stained with opsin 4, other opsin 4-labeled RGCs were not.

Results

Residual RGCs and axons had survived at 4 weeks after ONC

Previous studies have reported that approximately 10% of RGCs had survived at 4 weeks after ONC (Tran et al., 2019). We wanted to determine whether the axons stretching from the RGCs had survived, so we used a pan-RGC marker NF-L antibody and an axon marker antibody to stain the RGC and RGC nerve fibers, as well as the axons, after ONC administration in 1 eye of 3-month-old mice. As expected, we found that ONC led to a marked reduction in the number of RGCs (n = 4 eyes per group, Figure 2A upper panel). When compared with the number of RGCs in the control (131 ± 23/μm²), the number of RGCs in the central retina 4 weeks after ONC was only 196.0 ± 61.6/μm². Furthermore, 4 weeks after ONC, the numbers of RGCs in the mid-peripheral retina and peripheral retina were 137.0 ± 86.1/μm², respectively. The survival rates were 8.47%, 9.6%, and 13.9% in the central retina, mid-peripheral retina, and peripheral retina, respectively.

To confirm the existence of retrograde nerve fibers, we used the retrograde tracer cholera toxin subunit B (CTB). The results showed that the nerve fibers that remained after ONC included retrograde nerve fibers (control: n = 4 eyes, ONC: n = 3 eyes). Additionally, using an NF-L antibody, we found that 4 weeks after ONC, 74.0%, 48.3%, and 68.3% of the fluorescent signal remained in the central (p = 0.0001), mid-peripheral (p = 0.0013), and peripheral (p = 0.0001) retina, respectively (Figure 2C).

Furthermore, 4 weeks after ONC, the numbers of RGCs in the mid- and peripheral retina were significantly lower in the central retina (p = 0.002) retina, respectively (Figure 2E). CTB-stained nerve fibers were also significantly reduced in the mid- and peripheral (p = 0.002) retina (respectively) (Figure 2F). These data strongly suggest that the nerve fibers in the peripheral retina were less damaged.

Residually labeled RGCs in ONC eyes

Retrogradely labeled RGCs in ONC eyes

To investigate the retinoreceptor connections related to RGCs, we used retrograde trans-synaptic virus PRV274 constructed with mRFP, an isogenic version of PRV-Bartha. Three weeks after ONC, PRV274 was intravitreally injected into the ONC eyes of the experimental group or the normal eyes of the control group (D21). After 6–7 days (D27–28), we collected whole-mount retinas or frozen retina sections (Figure 1B). Because 1 × 10⁴ pfu/mL PRV274 did not obviously infect RGCs in the control group (n = 3 eyes, Figure 3A), we used 1 × 10⁵ pfu/mL PRV274 to stain the RGCs and found that there were no fluorescent signals in the retinas of the contralateral eye (n = 4 eyes; Figure 3C), which was similar to eyes intravitreally injected with PBS (n = 3 eyes; Figure 3D).

To verify the infection specificity of PRV274 in RGCs, we used the pan-RGC marker RBPM5 antibody to stain the retina. The results showed that PRV274-labeled RGCs were present in the processed samples (iRGCs) (Kwang et al., 2010; Mao et al., 2014), but the retinas of PRV274-injected eyes were distorted and swollen because of PRV toxicity (n = 3 eyes per group; Figure 3G). To determine whether PRV274 reduced the number of RGCs, we counted the number of RGCs in the control and ONC groups using whole-mount retina staining with RBPM5 antibody, which is a pan-RGC marker. When compared with the data in Figure 1D, there was no significant loss of RGCs after intravitreal injection of PRV274 over 5–6 days in the results shown in Figure 3D. However, the number of PRV274-injected eyes was significantly lower than the control group (p < 0.005; Figure 3H, I). The results showed that the PRV274 infection was mostly limited in RGCs regardless of ONC administration. Although there were fewer RGCs in ONC eyes, the proportion of infected RGCs appeared to be higher. By counting the number of RGCs with red fluorescence (mRFP+ RGCs) and the total number of RGCs in the infected regions of retinas, we found that the percentage of mRFP+ RGCs/total RGCs in control eyes showing a PRV274 intravitreal injection was 5.8 ± 1.2% (n = 16 eyes), whereas the percentage of RBPM5+ RGCs/total RGCs in ONC eyes was 10.9 ± 2.1% (n = 11 eyes). There was a small but significant difference between these 2 groups (p = 0.04, Figure 3J).

A previous study showed that PRV-Bartha exclusively infects iRGCs (Vinay et al., 2007), and among the 46 types of RGCs, 2 types of iRGCs and all 5 types of ipRGCs were infected with PRV-Bartha (Jing et al., 2018; Tran et al., 2019; Yang et al., 2020). iRGCs accounted for over 82% of the ONC RGC 60 days after the ONC procedure (Pérez de Sevilla Müller et al., 2014). Hence, we next confirmed whether PRV274 exclusively infected iRGCs, as reported in a previous study (Vinay et al., 2007). We stained the retinas with the ipRGC marker opsin 4 antibody, and found that while some PRV274-infected RGCs were stained with opsin 4, other opsin 4-labeled RGCs were not.
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Overview of PRV724-traced retinorecipient regions

After confirming our labeling strategy, we sought to better identify the whole brain neurons innervated by the RGCs. Accordingly, 6–7 days after the intravitreal injection of PRV724 in the left eye, we removed the whole brain with or without the eyeballs and optic nerve (Thy1–YFP control mice n = 4, wild-type control mice n = 4). The mouse brains were analyzed using whole brain optical tissue clearing, with a transparency time of 7 days (Figure 1C). As expected, the brains were fully transparent with no visible shrinkage (Figure 4A), and they could be imaged via a light-sheet microscope (Figure 4B and Additional Video 1). The locations of different brain areas were determined following standard procedures, as described in the Methods section (Additional Figure 1A–H). To better locate the positions, we used Thy1–YFP mice. We found that PRV724 was able to label the RGCs in C57BL/6 (wild-type) mice as well as in Thy1–YFP mice, regardless of ONC administration (Additional Figure 3A). There was no significant difference in the number of RGCs in the C57/BL6 mice (wild-type) versus Thy1–YFP mice, regardless of ONC administration (n = 3 eyes per group; Additional Figure 3B). Also, the percentage of RFP+RGCs/total RGCs in the C57/BL6 (wild-type) and Thy1–YFP mice did not differ significantly, regardless of ONC administration (n = 5 eyes per group; Additional Figure 3C).

We focused on retinorecipient nuclei known to receive robust RGC innervation including the suprachiasmatic nucleus (SCN), ventral part of the lateral geniculate complex (LGV), intergeniculate leaflet (IGL), olivary pretectal nucleus (OPN), and superior colliculus (SC) (Hattar et al., 2002; Beier et al., 2021). However, PRV724 labeling of neurons was inconsistent in the SCN, OPN, and SC (data not shown), apart from the LGV and IGL. Hence, data from the SCN, OPN, and SC were not included in this study. Consistent with previous studies using PRV-Bantha (Card et al., 1991; Smith et al., 2000; Pickard et al., 2002; Smeraski et al., 2004), we observed trans-sympathetically labeled neurons in the paraventricular hypothalamic nucleus (PVH), Edinger-Westphal nucleus (EWN), oculomotor nucleus, IGL, LGv, and superior vestibular nucleus (SUV) in both hemispheres (Figure 5A–C). Unexpectedly, PRV724 labeled neurons were also observed in the baxilateral amygdala (BLA), central amygdalar nucleus (CeA), red nucleus (RN), VII nucleus, and gigantocellular reticular nucleus (GRN) in both hemispheres (Figures 5A, C, and D).

Common properties of PRV724-traced retinorecipient regions

As shown in Figure 6A, the signals of PRV724-labeled neurons were distributed equally across the ipsilateral and contralateral BLA (P = 0.498), while the signals of PRV724-labeled neurons were distributed equally across the ipsilateral and contralateral CeA (P = 0.0073) and contralateral PVH (P = 0.0164) (n = 4; Figure 6B–D). As shown in Figure 6E, PRV724-labeled neurons in the IGL and LGv were distributed equally across both hemispheres (P > 0.05) and P = 0.0992), while the signals of PRV724-labeled neurons were mostly located in the contralateral oculomotor nucleus (P = 0.0305) (n = 4; Figure 6F–H). The EWN is located at the midline, so there was no comparison between the ipsilateral and contralateral sides (Figure 6I).

As shown in Figure 7A, the signals of PRV724-labeled neurons were distributed equally across the ipsilateral and contralateral LC (P = 0.9875), SUV (P = 0.8996), and RN (P = 0.8424) (n = 4; Figure 7B–D). As shown in Figure 7E, PRV724-labeled neurons were distributed equally across the ipsilateral and contralateral VII nucleus (P = 0.9418), as well as the GRN (P = 0.7549) (n = 4; Figure 7F and G). Our results confirmed that PRV724-labeled RGCs send more projections to the contralateral PVH, oculomotor nucleus, and CeA.

ONC administration selectively alters PRV724-traced retinorecipient regions

Given that the ONC process decreases the survival of RGCs but also on the function and structure of the brain (Zhan et al., 2020; Zhang et al., 2020), we next examined changes in projections from PRV724-labeled RGCs after ONC using optical tissue clearing (Thy1–YFP control mice = 3 eyes; Figure 3K upper row). PRV724-infected RGCs were also co-stained with opsin 4 in the ONC group (n = 3 eyes; Figure 3K bottom row). However, not all PRV724-labeled cells were co-stained with opsin 4 (Figure 3K middle row). Hence, PRV724 might not exclusively infect ipRGCs. Additionally, when GAP-43 antibody was used to stain the intact optic nerve crossover at the optic chiasm, no red fluorescent signals were observed (n = 3; Additional Figure 2A–D). This suggests that PRV724 did not reveal the axons of RGCs. Taken together, these findings confirm that PRV724 can label RGCs in ONC eyes.
Figure 3 | Retrograde labeling of RGCs in ONC eyes.
(A, B) Comparison of different dose of PRV724 intravitreal injection. 1 × 10⁶ pfu/mL PRV724 infected fewer RGCs than 3 × 10⁶ pfu/mL PRV724. Confocal images of the retina in the control group after intravitreal injection of 1 × 10⁶ pfu/mL (n = 3 eyes) or 3 × 10⁶ pfu/mL PRV724 (red) (n = 3 eyes), co-stained with RBPMS (Alexa Fluor 488, green). (C) The contralateral retinas were not infected with PRV724. Confocal images of the retina contralateral to the PRV724-injected eye (red) (n = 3 eyes), co-stained with RBPMS (Alexa Fluor 488, green). (D) An intravitreal injection of PBS produced no mRFP signals compared with an intravitreal injection of PRV724. Confocal images of retinas from PBS-injected eyes (n = 3 eyes) co-stained with RBPMS (Alexa Fluor 488, green). (E) PRV724 did not infect fewer RGCs in the ONC mice than in the control mice. Confocal images of retinas from ONC mice with PRV724-injected eyes (n = 3 eyes) co-stained with RBPMS (Alexa Fluor 488, green). (F) PRV724 infected a higher ratio of RGCs in the ONC group than in the control group. Co-labeling of RGCs and PRV724 was observed from flat-mounted retinas. Figure shows magnified confocal images of flat-mounted retinas from both control and ONC mice after an intravitreal injection of PRV724 (red) (n = 3 eyes), with RGCs stained with RBPMS (Alexa Fluor 488, green). Yellow triangles show the co-labeled RGCs (mRFP+ RGCs). (G) PRV724 infected a greater ratio of RGCs in the ONC group than in the control group. Co-labeling of RGCs and PRV724 was observed from cross-sectioned retinas. Figure shows retinal cross-sections from both control and ONC mice after an intravitreal injection of PRV724 (red) (n = 3 eyes), with RGCs stained with RBPMS (Alexa Fluor 488, green) and nuclei stained with DAPI (blue). Red arrows show the co-labeled RGCs. (H) Quantification of the RGCs in the control group and the PRV724-injected control group (n = 3 eyes per group; the number of RGCs in the control group is shown in Figure 1C). (I) Quantification of the RGCs in the ONC group and the PRV724-injected ONC group (n = 3 eyes per group; the number of RGCs in the ONC group is shown in Figure 1C). (J) Quantification of the ratio of mRFP+ RGCs/total RGCs in the control (n = 16 eyes) and PRV724-injected ONC groups (n = 11 eyes). (K) PRV724 did not infect fewer ipRGCs in the ONC group than in the control group. Confocal immunostaining images of cross-sections from wild-type mice in the control group and ONC group after intravitreal injection of PRV724 (red). The ipRGCs were stained with opsin 4 (Alexa Fluor 488, green) and the nuclei were stained with DAPI (blue) (n = 3 eyes per group). Green triangles indicate ipRGCs. Red triangles indicate PRV724-labeled cells. Yellow triangles show the PRV724-infected ipRGCs. Scale bars: 50 µm. Data from 3 independent experiments are presented as the mean ± SEM. *P < 0.05 (two-way analysis of variance followed by Sidak’s multiple comparisons). DAPI: Diamidino-phenyl-indole; ipRGCs: intrinsically photosensitive retinal ganglion cells; mRFP: monomeric red fluorescent protein; ONC: optic nerve crush; PRV: pseudorabies virus; RBPMS: RNA binding protein with multiple splicing; RGCs: retinal ganglion cells.

Figure 4 | Overview of tissue-cleared brain.
(A) Representative images of a brain before clearing (left), after clearing (middle), and after RI matching (right). (B) View from different angles of the 3D cleared brain from a Thy1-YFP mouse. Scale bar: 5 mm in A, 100 µm in B. 3D: Three-dimensional; RI: refractive index; YFP: yellow fluorescent protein.
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ONC administration selectively eliminates projections from RGCs in the BLA, CeA, PVH, IGL, LGv, oculomotor nucleus, and EWN.

**Figure 5** | Overview of PRV724-traced retinorecipient regions in control and ONC mice. (A) There were fewer PRV724-labeled neurons in the BLA, CeA, and PVH of ONC mice versus control mice. The locations of the BLA, CeA, and PVH in the control (left) and ONC (right) mice. (B) There were fewer PRV724-labeled neurons in the IGL, LGv, and EWN in the control mice than in the ONC mice. The locations of the IGL, LGv, oculomotor nucleus, and EWN in the control (left) and ONC (right) mice. (C) The number of PRV724-labeled neurons was unchanged in the LC, SUV, and RN in ONC mice. The locations of the LC, SUV, and RN in the control (left) and ONC (right) mice. (D) The number of PRV724-labeled neurons was unchanged in the VII nucleus and GRN in the ONC mice. The locations of the VII nucleus and GRN in the control (left) and ONC (right) mice. Scale bars: 500 μm. BLA: Basolateral amygdala nucleus; CeA: central amygdalar nucleus; EWN: Edinger-Westphal nucleus; IGL: intergeniculate leaflet of the lateral geniculate complex; LGV: ventral part of the lateral geniculate complex; ONC: optic nerve crush; PRV: pseudorabies virus; PVH: paraventricular hypothalamic nucleus; RN: red nucleus; SUV: superior vestibular nucleus.

**Discussion**

The main goals of this study were to identify the retinorecipient regions of RGCs in mice brains, to map their changes after ONC, and to offer a practicable approach for evaluating neural regeneration in future. To ensure that signals continued to be sent from the RGCs after ONC, we first confirmed that ~ 13.9% of the RGCs survived the procedure, along with retrograde nerve fibers and axons in the optic nerves. Next, to determine whether PRV724, a new isogenic version of PRV-Bartha, had the same neurotropic effect on RGCs, we performed unilateral intravitreal injection of PRV724. The retinal immunofluorescence data showed that PRV724 primarily infected RGCs in both control and ONC mice. We then performed entire brain imaging using a tissue clearing technique to fully scan the retinorecipient regions that received projections from PRV724-labeled RGCs, and investigated the changes in these retinorecipient regions after ONC. Together, our results demonstrate that PRV724-traced retinorecipient regions changed in specific areas after ONC, including the BLA, CeA, PVH, IGL, LGv, oculomotor nucleus, and EWN. Thus, reconnection between RGCs and these areas might be a good measure of optic nerve regeneration.

Only a few RGCs survive after ONC, and approximately 1% of their axons pass through the crush site at the optic nerve (Duan et al., 2015). Our data were consistent with previous findings in that few RGCs survived, and almost half of their nerve fibers had been lost. This was accompanied by a striking decrease in the number of axons in the optic nerves. When studying retinorecipient regions using a retrograde trans-synaptic virus, the integrity...
of the retrograde nerve fibers should first be confirmed. Previous studies have shown that retrograde tracer CTB can be used to label axons after ONC, and while most retrograde axons are expected to be lost, a few retrograde axons generally survive and pass through the injury site (Duan et al., 2015; Patel et al., 2020; Hilla et al., 2021). Our findings from retinal tissue indicate that retrograde nerve fibers were present after ONC. Together, these data suggest that although ONC seriously damages the optic nerve, a retrograde physiological structure exists. Thus, this model provides an opportunity to study retinorecipient regions using a retrograde trans-synaptic virus.

Recent studies have shown that PRV724 can be used to trace neurons via injection into the anterior chamber of the eyes or brain area (Yang et al., 2021; Zhai et al., 2021). We confirmed that PRV724 could label RGCs, as well as some ipRGCs. Moreover, PRV724 labeled other cells in the retina. Viney et al. observed that during the first wave of infection (0.5–4 days), almost 99% of PRV152 (an isogenic version of PRV constructed with green fluorescence protein)-labeled cells were ipRGCs (Viney et al., 2007). One possible explanation is that PRV724 infected Müller cells near the cell bodies of infected RGCs through gap junctions, as described in a previous study (Viney et al., 2007). The extra labeling of other cells in the retina is related to the local circuit of ipRGCs (Viney et al., 2007). However, in the present study we could not confirm that the PRV724-labeled cells were exclusively ipRGCs. Additionally, we found that the number of RGCs in the PRV724-infected retinas did not significantly differ from that in non-infected retinas, despite the fact that PRV724 caused retrograde disorder and distortion. This enabled us to rule out the possibility that PRV724 affected RGC survival.

Via intravitreal or intracerebral injection of PRV, a previous report found PRV-labeled neurons to be distributed in the SCN, IGL, LGv, and PVH in rodent brains (Smith et al., 2000). Another study in golden hamsters found that PRV-labeled neurons were not only present in the SCN, LGv, IGL, and PVH, but also in the EWN, lateral terminal nucleus, and OPN (Pickard et al., 2002). Furthermore, Smersaki et al. (2004) observed that PRV-labeled neurons were distributed in multiple regions in rat brains, including the nucleus of the tractus solitarius, the superior salivatory nucleus, and ventral mammillary nucleus were labeled. Previous studies have indicated that the seemingly disparate distributions of PRV-labeled retinorecipient nuclei might be caused by differences in the susceptibility of neurons in different animals (i.e., golden hamster, rat, and mouse) as well as limitations associated with slice analysis. For instance, large datasets such as those containing hundreds or even thousands of rodent brain sections may be susceptible to missing information. Hence, methods that enable viewing of the entire brain are preferable for visualizing retinorecipient regions connected to RGCs. However, deep regions in opaque tissue are impossible to image without slicing. In tissue clearing, the refractive index of the medium is matched to that of scattering particles to produce optical transparency in the tissue (Ueda et al., 2020), and light-sheet microscopes have an extended range of fast imaging options for large samples. Our light-sheet imaging method for the entire brain enabled a more comprehensive assessment of retinorecipient regions connected to RGCs via PRV labeling. Although our results have similar characteristics to previous studies, there are some distinct findings to discuss.

Previous studies on ipRGCs have shown that they send projections to the SCH to control circadian rhythms (Hattar et al., 2002) and to the OPN to participate in the pupil light-reflex (Hattar et al., 2008; Sonoda et al., 2020). However, although there were some PRV724 retinal cells co-labeled with opsin 4, we did not consistently observe PRV724-labeled neurons in these areas in all 9 brains (control: n = 4, ONC: n = 5, data not shown). One study found that the SCH was labeled with PRV only if the OPN was labeled in some mice (Smersaki et al., 2004). This might depend on the number of PRV724-infected ipRGCs in different animals, and so this issue requires further investigation.

Figure 7  The brain regions that receive projections from RGCs are unchanged after ONC. Light-sheet images of Thy1-YFP mouse brains from the control mice and those subjected to ONC with PRV724 intravitreal injection. (A) The number of PRV724-labeled neurons did not significantly decrease after ONC in the LC, SUV, and RN. Magnified images of the LC, SUV, and RN. Top panel: control. Bottom panel: ONC. The band at the top of the figure indicates the ipsilateral and contralateral sides of the brain. (F, G) Quantification of PRV724-labeled neurons in the LC, SUV, and RN. Black dots: control (n = 4), red squares: ONC (n = 5). E) The number of PRV724-labeled neurons did not significantly decrease after ONC in the LC nucleus and GRN. Magnified images of the LC nucleus and GRN are shown. Top panel: control. Bottom panel: ONC. Scale bars: 100 μm in A and E. The band at the top of the figure indicates the ipsilateral and contralateral sides of the brain. (F, G) Quantification of PRV724-labeled neurons in the VII nucleus and GRN. Black dots: control (n = 4), red squares: ONC (n = 5). Data from three independent experiments are presented as the mean ± SEM and analyzed by a two-way analysis of variance followed by Sidak’s multiple comparisons.

GRN: Gigantocellular reticular nucleus; LC: locus ceruleus; ONC: optic nerve crush; PRV: pseudorabies virus; RN: red nucleus; SUV: superior vestibular nucleus; YFP: yellow fluorescent protein.
The death of most RGCs explained our results in the LGV, IGL, CeA, BLA, PVH, and EWN after ONC. The LGV processes not only visual-related information but also non-visual information (Monavarfeshani et al., 2017). The IGL is another non-visual processor that receives projections mainly from ipRGCs, although it also receives inputs from other types of RGCs (Hattar et al., 2005). The amygdala is a limbic structure that is involved in a variety of functions, including emotion and memory. The CeA is associated with conditioned fear, anxiety, and the secretion of corticotrophin-releasing factor (Kalin et al., 2004). The CeA participates in mediating eyelid conditioning (Lee and Kim, 2004) and receives information about visually conditioned stimuli and projections from the LGV (Halverson and Freeman, 2010; Farley et al., 2018). Hence, the CeA may be strongly related to the processing of the related information from RGCs. The BLA has complex connections with multiple areas of the brain (Hirniry et al., 2021). The most well-studied function of the BLA is fear control (Adhikan et al., 2015). Recent evidence has shown that the release of glutamate is playing a crucial role in the circuitries of the BLA. Thereafter, the projection from the LGV (Smith et al., 2000; Viney et al., 2007; Yang et al., 2021), whereas studies on ipRGCs have not shown that they project to the PVH (Hattar et al., 2002; Ecker et al., 2010; Cui et al., 2015; Sonoda et al., 2020; Beier et al., 2021). The PVH has recently been identified as a center of the brain that mediates the responses of the HPA axis and the sympathetic nervous system. It receives inputs from the CeA, SUV, and GRN (Chen et al., 2021). In addition, although ipRGCs play an important role in regulating circadian rhythms, little is known about their function in the context of the GRN. Hence, the PVH might mainly receive visual-related projections from the CeA, and the loss of these projections after ONC could have resulted in a decrease in the number of PRV724-labeled neurons in the PVH. Light stimulation has been found to activate the noradrenergic pathway, of which the LC is a central component (Li et al., 2017; Szabadi, 2018), and then project to the EWN to control pupil diameter (Nobukawa et al., 2021). Other studies have found that ipRGCs participate in the control of the pupil light-reflex (Hattar et al., 2006; Sonoda et al., 2020). These results suggest that the LC might receive non-visual projections from ipRGCs, and then project these signals to the EWN. Taken together, these data indicate that brain areas including the LGV, IGL, CeA, BLA, PVH, and EWN might receive projections from specific RGCs that died after ONC, whereas the LC receives projections from specific ipRGCs that had survived at 4 weeks after ONC.

We also examined the distribution of PRV724-labeled neurons in regions related to motor function including the SUV, oculomotor nucleus, RN, VII nucleus, and GRN, and found that apart from that in the oculomotor nucleus, these distributions remained unchanged after ONC. The GRN is the main output center for the regulation of locomotor patterns and rhythms (Lemieux and Briel, 2000). The LGv also participates in the regulation of locomotor patterns and rhythms (Lemieux et al., 2010). The LGv output center for the regulation of locomotor patterns and rhythms (Lemieux and Briel, 2000).

The location procedures of the brain area.

The video of tissue-cleared Thy1-YFP mouse brain under light-sheet microscope.

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Additional Video 1: The video of tissue-cleared Thy1-YFP mouse brain under light-sheet microscope.

Additional files:

Additional Figure 2: Location procedures of the brain area.

Additional Video 2: PRV724 did not label the axons.

Additional Figure 3: Wild-type mice and Thy1-YFP mice shared the same characteristics.

We thank Professor Lian-Yan Huang from Department of Pathophysiology, Zhongshan School of Medicine, Sun Yat-sen University for offering the Thy1-YFP transgenic mice, Guo-Guang Qiu from Laboratory Animal Center and Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-sen University for assistance with breeding animals, De-Ling Li, Wei-Ting Zeng, Xin-Yi Zhang and Li-Ling Liu from State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University for assistance with histology, and Professor Wei Sun and Cui Q from Ministry of Education Key Laboratory of Information Technology, Sun Yat-Sen University for assistance with 3D reconstruction.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (81870086, 81972134), and the National Key Research and Development Program of China (2016YFA0501200). The authors declare no conflicts of interest.

Author contributions: Study design: ZY, MBY, YQL, YDQ, YLW; experiment implementation: ZYH, YRW, ZL, DFS, HYW; manuscript draft: ZY, MBY, YQL; KLW. All authors revised it critically and approved the final version of the manuscript.

Conflicts of interest: LWZ and HYW are employed by Light Innovation Technology Ltd., Hong Kong Special Administrative Region, China. All authors declare no conflict of interest.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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C-Editor: Zhao M; S-Editors: Yu J, Li CH; L-Editors: Yu J, Song LP; T-Editor: Jia Y
Additional Figure 1 The location procedures of the brain area.

Light-sheet images from cleared brains were obtained from the Y-axis to the X-axis (transverse). Take the CeA region as an example. (A) Locate the transverse section of the PRV724 (red dots) labeled areas. (B) Compare with the 3D Allen Brain Atlas. (C) Locate the sagittal section of the same PRV724 labeled areas. (D, E) Compare the sagittal section with the 2D and 3D Allen Brain Atlas. (F) Locate the coronal section of the same PRV724 labeled areas. (G, H) Compare the coronal section with the 2D and 3D Allen Brain Atlas. 2D: Two-dimensional; 3D: three-dimensional; CeA: central amygdalar nucleus; PRV: pseudorabies virus.
Additional Figure 2 PRV724 did not label the axons.

(A) Confocal immunostaining images of the ON and OC of control mice 6 days after PRV724 intravitreal injection, showing axons stained with GAP-43 (Alexa Fluor 488, green) and nuclei stained with DAPI (blue). (B) The regions of OC and left ON of a control mouse are further magnified. (C) Confocal immunostaining images of the ON and OC of an ONC mouse after intravitreal injection of PRV724, showing axons stained with GAP-43 (Alexa Fluor 488, green) and nuclei stained with DAPI (blue). (D) The regions of control OC and left ON of an ONC mice are further magnified. Scale bars: 50 µm. DAPI: Diamidino-phenyl-indole; GAP-43: growth-associated protein-43; OC: optic chiasm; ON: optic nerve; ONC: optic nerve crush; PRV: pseudorabies virus.
Additional Figure 3 Wild-type mice and Thy1-YFP mice shared the same characteristics.

(A) The number of total RGCs or PRV724-infected RGCs do not differ in WT mice or Thy1-YFP mice before and after ONC. Immunostaining images of the central, mid-peripheral and peripheral retinas with RBPMS (Alexa Fluro 647, gray, labeling RGCs), Thy1-YFP RGCs (green), and PRV724-labeled RGCs (red). In the control and ONC groups of Thy1-YFP mice with PRV724 intravitreal injection. Scale bars: 50 µm. (B) Quantification of the number of RGCs in the WT and Thy1-YFP mice in control and ONC groups (n = 3 eyes per group). The WT data are from Figure 2D. (C) Quantification of the mRFP+ RGC ratio in Thy1-YFP mice compared with that in WT mice in the control and ONC groups. The WT mice data are from Figure 3J (n = 5 eyes per group). Data from 3 independent experiments are presented as the mean ± SEM, and were analyzed by two-way analysis of variance followed by Sidiki’s multiple comparisons. mRFP: Monomeric red fluorescent protein; ns: not significant; ONC: optic nerve crush; PRV: pseudorabies virus; RBPMS: RNA binding protein with multiple splicing; RGCs: retinal ganglion cells; WT: wild-type; YFP: yellow fluorescent protein.