Whole-brain fluorescence-MRI coregistration for precise anatomical mapping of virus infection

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Abstract:

Neurotropic virus infections cause tremendous disease burden. Methods visualizing infection in the whole brain remain unavailable which greatly impedes understanding of viral neurotropism and pathogenesis. We devised an approach to visualize the distribution of neurotropic virus infection in whole mouse brain *ex vivo*. Optical projection tomography (OPT) signal was coregistered with a unique magnetic resonance imaging (MRI) brain template, enabling precise anatomical mapping of viral distribution, and the effect of type I interferon on distribution of infection was analyzed. Guided by OPT-MR, we show that Langat virus specifically targets sensory brain systems and the lack of type I interferon response results in an anatomical shift in infection patterns in the brain. We confirm this regional tropism, observed with whole brain OPT-MRI, by confocal and electron microscopy to provide unprecedented insight into viral neurotropism. This approach can be applied to any fluorescently labeled target in the brain.

Introduction:

Virus species from multiple virus families are neuroinvasive and neurotropic^{1,2}. These viruses not only cross the physical brain barriers, but also infect the central nervous system, potentially leading to lethal encephalitis or long-term neurological sequelae among survivors³⁻⁵. While global disease burden remains high, better understanding of viral pathogenesis is required for the development of effective diagnosis, therapy, and prevention measures^{6,7}. One of the major obstacles for studying viral pathogenesis within the entire brain is the lack of feasible imaging approaches to visualize virus infection at cellular to subcellular levels, while still providing a global view within a preserved anatomical tissue context.

While confocal microscopy provides invaluable information at the subcellular level, any three-dimensional (3D) information provided by the approach is limited to the μm – mm range. Since neurons consist of cell bodies with long axons that can project to distant brain areas, it is extremely challenging to obtain an overall view of viral distribution within the context of the entire brain based solely on tissue section analyses. Mesoscopic techniques such as optical projection tomography (OPT) and light sheet fluorescence microscopy (LSFM) are 3D imaging techniques for transparent mesoscopic sized (mm - a few cm) tissue samples; and therefore, are suitable for whole rodent brain imaging. These methods can provide information at cellular resolution while still capturing the entire tissue in 3D and have been used in various applications for targeted imaging, such as, whole organ distribution of specific protein expression via immunolabeling⁸⁻¹⁰. However, anatomical information obtained from OPT, based on tissue autofluorescence, is limited. Magnetic resonance imaging (MRI), conversely, is widely used for anatomical brain imaging since it has exquisite contrast and resolution¹¹. In its turn, structural MRI is not suitable for imaging pathogens.

For a holistic view of viral brain infection, here, we devised an imaging approach that, for the first time, provides spatial distribution of virus infection in the brain, with precise anatomical mapping. Specifically, we generated an MRI template for *ex vivo* optically cleared adult mouse brains and coregistered it with whole brain viral signal acquired from 3D OPT images (~0.5 cm³). We used this approach to evaluate, the detailed distribution of Langkat virus (LGTV), a model neurotropic flavivirus, in wild type (WT) mice and mice lacking type I interferon (IFN) response, *Ifnar*^{-/-}. Type I IFN response is the first line of antiviral defense in the brain and can, therefore, influence viral distribution¹². In conjunction with confocal and electron microscopy, which confirmed the presence of virus infection in the identified areas, we can also identify the cellular tropism of virus infection in the brain. The developed MRI template and MRI-OPT coregistration approach enable precise anatomical mapping of any signal obtained from *ex vivo* optical brain images. It is an invaluable tool to study whole brain viral distribution with precise anatomical context and is applicable for any pathogen.

Results:

Whole brain 3D imaging of virus infection

Previous studies have confirmed LGTV infection in rodent brains using immunohistochemistry or quantitative PCR¹³⁻¹⁵. However, the complete overview of viral distribution throughout the whole brain is still unknown. Therefore, we first established an approach for imaging virus infection in the whole brain and tested its biological value using WT and *Ifnar*^{-/-} mice. We infected the mice with LGTV, immunolabeled the brains against LGTV nonstructural protein 5 (NS5), optically cleared and scanned the brains with OPT. This novel approach allowed visualization of virus

infection throughout the whole brain. We observed that *Ifnar*^{-/-} mice were more susceptible for infection (Supplementary Fig. 1) and that viral distribution in these mice was strikingly different when compared to WT mice (Fig. 1a, Supplementary Fig. 2, Supplementary Movie 1). In *Ifnar*^{-/-} brains, we observed pronounced infection, spanning from the interior wall of the lateral ventricles to the anterior cerebrum and olfactory bulbs, resembling the rostral migratory stream, in addition to third ventricle and fourth ventricle choroid plexus (ChP). To complement OPT data, we used LSFM to visualize fourth ventricle ChP infection at high resolution (Supplementary Movie 2). We also observed weak infection in the cerebral cortex compared to other areas. By contrast, in WT mice, infection was mainly localized in cerebral cortex, with no detectable infection in rostral migratory stream, lateral ventricle, and fourth ventricle (Fig. 1a, Supplementary Fig. 2, and Supplementary Movie 1). Of note, we also detected some low-level unspecific signal in uninfected brains (mock group) (Fig. 1a and Supplementary Fig. 2), most likely due to ventricular antibody trapping or unspecific antibody absorption in meninges during whole brain immunolabeling.

In theory, OPT or LSFM imaging allows for the study of any immunolabeled pathogen or protein of choice. However, it greatly depends on the availability of specific antibodies that can withstand harsh chemical treatment and their ability to penetrate the brain. Detection of an endogenously expressed fluorescent protein could bypass the need for these specific antibodies. Several GFP-expressing pathogens already exist; however, several fluorescent proteins, especially green fluorescent protein (GFP), are severely quenched during the clearing process¹⁰. Therefore, we investigated whether an anti-GFP antibody could be used as a general OPT probe. Accordingly, we infected *Ifnar*^{-/-} mice with GFP-expressing LGTV (LGTV-GFP) and immunolabeled the brain *ex vivo* with anti-NS5 and anti-GFP antibodies. We observed a clear overlap between OPT signals from viral NS5 and GFP-expressing virus, indicating the possibility to visualize any GFP-

expressing pathogen in the brain using OPT without the need of specific antigens (Fig. 1b, Supplementary Movie 3). Taken together, we were able to visualize viral infection and viral distribution in the whole brain using 3D imaging. Furthermore, our 3D OPT approach can be translated to study whole brain distribution of any GFP- labeled pathogen.

OPT-MRI coregistration for anatomical mapping of virus infection

We have now shown that OPT allows visualization of viral brain infection with great sensitivity and high spatial resolution. However, the anatomical information obtained from tissue autofluorescence is insufficient to provide a detailed anatomical reference frame for the multitude of structures the brain can be divided in ¹⁶ and which are involved in different physiological functions. Therefore, we are in need of an improved anatomical reference frame, allowing precise identification of the infected brain areas.

To address this, we coregistered our viral OPT images with structural MR images. Because our OPT images are acquired *ex vivo* after extensive chemical tissue processing, we acquired *ex vivo* MR images after chemical preprocessing and OPT acquisition (Fig. 2a). We then coregistered these MR images with viral OPT signal, which resulted in fusion images with good spatial alignment (Fig. 2b). To obtain an anatomical reference with improved anatomical detail and tissue contrast and to overcome high MR-scanning costs of individual brains in the future, we created a high-resolution *ex vivo* MRI brain template, acquired after tissue bleaching and clearing for optical imaging. We designed a novel study- and sequence specific brain template by serial longitudinal registration of ten optically cleared brains and generated corresponding tissue probability maps (TPMs) (Fig. 2 c-e), required for image segmentation and image normalization (warping) to the

template, as described in the Materials and methods. Afterwards, we created a study-specific animal preset in the toolbox SPMMouse (<http://spmmouse.org>)¹⁷, which resulted in improved warping and normalization of viral OPT signal to the template. Finally, we normalized individual differences in tissue deformation to compensate for natural variation in brain size, which allowed for improved coregistration of viral signal, mainly in the olfactory bulbs. In optimized OPT-MRI template fusion images, the viral signal was displayed within a detailed anatomical context provided by MRI (Fig. 2d,f), allowing for precise identification of infected brain regions.

Highly accurate brain map of viral infection

The OPT images displaying viral signal in the whole brain together with the study-specific brain template, provide a detailed anatomical reference frame after coregistration. This allowed us to proceed to our ultimate goal, namely, to evaluate the differential viral distribution patterns in WT and *Ifnar*^{-/-} mice (Fig. 3). The high-resolution viral signal displayed within the detailed anatomy, afforded by OPT-MRI coregistration offered unprecedented detail and insights into viral brain distribution. In *Ifnar*^{-/-} mice, we detected specific infection in grey matter (GM), white matter (WM), and the ventricular system (Fig. 3a-c, left panels). For GM, we observed viral signal in the granular cell layer of the olfactory bulb, entorhinal cortex, dorsal endopiriform nucleus, piriform area, agranular insula, primary visual field, and primary somatosensory cortex (upper limb and barrel field). In WM, we detected viral signal in the olfactory limb of the anterior commissure, anterior forceps of corpus callosum, and supra-callosal WM. The identified regions are part of distinct sensory systems, such as the olfactory system, visual system, and somatosensory system. We also observed viral signal in ChP and the interior wall of lateral ventricles, third ventricle, and fourth ventricle. Of note, ChP of the lateral ventricle, subventricular zone, and the olfactory limb of the anterior commissure are all parts of the rostral migratory stream, suggesting specific

infection of the ventriculo–olfactory neurogenic system by LGTV. In WT mice, however, the viral signal was limited to entorhinal cortex, piriform area, primary visual field and the third ventricle (Fig. 3a-c, right panels), suggesting a major influence of the type I IFN response on virus infectivity and distribution in the mouse brain.

Validation of OPT-MRI viral signals by confocal and electron microscopy

Guided by OPT-MRI fusion images, we confirmed LGTV infection in the brain regions identified as infected in whole brain imaging on sections using confocal and electron microscopy. Apart from confirming the presence of viral infection in these areas, it also allowed us to investigate viral cellular tropism, leading to new biological insights. In rostral migratory stream, we observed viral infection in immature neurons in both mouse models (Fig. 4a). However, we only observed viral infection in leptomeninges, endothelial cells lining the blood vessels in the subarachnoid space, ChP epithelial cells and ependymal cells lining the ventricular wall in *Ifnar*^{-/-} but not WT brains (Fig. 4b-c and Supplementary Fig. 3a,b). This result confirms that the signal observed from these areas in the OPT images of LGTV infected and mock WT brains was due to non-specific antibody absorption (Fig. 1a and Supplementary Fig. 2). To further confirm the presence of virus, we imaged fourth ventricle ChP of *Ifnar*^{-/-} brain, identified as highly infected on OPT-MRI, using electron microscopy. Transmission electron microscopy (TEM) imaging of ChP epithelial cells, marked by the presence of cilia, revealed extreme distortion of endoplasmic reticulum (ER) membranes in the infected compared to mock tissue (Fig. 4d and Supplementary Fig. S3c,d). High-magnification analysis revealed virus particles in the ER lumen (Fig. 4d, inset a) and the formation of viral replication complexes, as bud-like invaginations of the ER membrane (Fig. 4d, inset ii). Segmentation of volume images taken by focused ion beam milling-scanning electron microscopy (FIB-SEM) showed the 3D architecture of replication complexes within ER of infected cells (Fig.

4e-f, Supplementary Fig. 3e and Supplementary Movie 4). Lastly, we investigated infection in cerebral cortex, focusing on the piriform area and entorhinal cortex. In both mouse models, we did not observe infection in astrocytes (Supplementary Fig. 3f). However, we observed infection in mature neurons in WT brain, while microglia remained uninfected (Fig. 4g). Surprisingly, we observed the opposite cellular tropism in *Ifnar*^{-/-} brains, namely infected microglia and refractory neurons, (Fig. 4g), indicating that the type I IFN response shapes cellular tropism of viral infection in the brain. Taken together, our OPT-MR fusion technique allowed us to specifically identify infected regions, which could then be used to further investigate viral behavior in more detail, leading to the discovery of this remarkable tropism shift under influence of the type 1 IFN response.

Discussion:

Imaging techniques provide pivotal information on biological processes in cells and tissues. Each technique has its own advantages and limitations, such as spatial resolution and specimen size, which makes it challenging to observe detailed biological phenomena while maintaining an overview of the whole organ and its anatomical reference frame. Here, we report a whole brain imaging approach, which allows visualization of fluorescent targeted signals from OPT and precise anatomical brain mapping when applied in combination with our developed MR template for optically cleared brains. This allowed subsequent targeting of brain regions for higher-resolution imaging using confocal and 3D electron microscopy. The novel approach can be utilized as a tool to improve our understanding of viral pathogenesis or, in fact, of any immunolabeled target, in the whole mouse brain.

Considering the mesoscopic sample size (mm-cm) and the reasonable scan times (~ 90 min per brain) to obtain high-resolution (13.2 μm for our setting), OPT seems ideally suited to visualize viral distribution in the whole mouse brain. However, since OPT systems are not commercially available, LSFM is often applied. Our approach could also be applied to LSFM to visualize viral distribution in the whole brain with even higher resolutions as compared to OPT but with consequent elongated scanning times. One should also keep in mind that, in contrast to OPT and MRI, LSFM results in images with non-isotropic voxels, leading to slight mismatch alignment of LSFM-MR fusion images.

To date, various mouse brain templates that can be used as an anatomical reference are available. However, they are either based on histology^{16,18} or, *in vivo* or *ex vivo in situ* MRI¹⁹⁻²¹. However, OPT images are acquired *ex vivo* after bleaching and optical clearing. These chemical treatments result in a certain level of tissue alteration, which lead to misalignment when coregistering brain OPT signals to these existing templates. Therefore, we designed a study-specific brain template consisting of *ex vivo* MR scans, acquired after OPT pre-processing, and coregistered it with viral OPT signal, which yielded exact alignment and allowed precise identification of infected brain regions. It should be noted here, however, that the template is based on brains that underwent particular chemical permeabilization and bleaching and, that were cleared with BABB (see Materials and methods). The application of distinct pre-processing and clearing protocols, such as iDisco²², may affect the brain differently and lead to divergent brain deformations.

In this study, we also showed that OPT-MRI coregistration set the base for novel insights in infection biology. The fusion images revealed distinct infection patterns driven by type I IFN response. In *Ifnar*^{-/-} mice, infection mainly localized in regions belonging to the visual, somatosensory, and, most extensively, olfactory system, while in WT infection was mainly

restricted to regions processing olfactory information, which have been previously reported for other flaviviruses^{23,24}. The susceptibility of the olfactory system to LGTV infection in both WT and *Ifnar*^{-/-} brains implies that the type I IFN response is, in itself, not sufficient to protect this neuronal circuit from viral infection. In contrast, the susceptibility of the ChP and leptomeninges, which were only permissive to viral infection in *Ifnar*^{-/-} brains, indicates that the antiviral properties of these tissues greatly depend on type I IFN response. Lastly, while infection in *Ifnar*^{-/-} brains could be found in both WM and GM, infection in WT brains was restricted to the latter. This restriction might, at least in part, relate to the surprising cellular tropism shift from mature neurons in WT to microglia in *Ifnar*^{-/-} brains (Fig. 4g). In WT, the virus infected the cell body of mature neurons, residing in GM, while in *Ifnar*^{-/-}, the virus infected microglia, which reside both in GM and WM. To the best of our knowledge, such striking cellular tropism shift has not been reported to date *in vitro* or *in vivo*.

Collectively, we demonstrate that OPT-MRI fusion images enable precise whole brain mapping of viral 3D fluorescence signal and, that the creation of such images opens the door to novel insights in infection biology. Since the approach is not only applicable to image viral infection but can be translated to any fluorescently labeled target in the brain, visualized by OPT or LSM, it holds the power to improve our knowledge and understanding of any neurological condition with anatomical precision.

Materials and Methods:

Animals

C57BL/6 WT mice and interferon alpha receptor knockout (*Ifnar*^{-/-}) mice in C57BL/6 background were kindly provided by N.O. Gekara (Department of Molecular Biology, Umeå University;

current address: Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Sweden)¹². Mice were bred as homozygotes and maintained under specific pathogen-free conditions. All animal experiments were conducted at the Umeå Centre for Comparative Biology (UCCB). All animal experiments were approved by the regional Animal Research Ethics Committee of Northern Norrland and by the Swedish Board of Agriculture (ethical permits: A9-2018 and A41-2019), and all procedures were performed according to their guidelines.

Viruses

LGTV strain TP21 was a kind gift from G. Dobler (Bundeswehr Institute of Microbiology, Munich, Germany) and GFP-expressing LGTV (LGTV-GFP) infectious clone was a kind gift from A. Pletnev (NIAID, NIH)²⁵. LGTV stock was produced in VeroB4 cells, a kind gift from G. Dobler,²⁶ and harvested on day 3 post infection when cytopathic effect was apparent. A 1:1 mixture of VeroB4 and HEK 293T cells, a kind gift from F. Weber (Biomedical Research Center Seltersberg (BFS), Justus Liebig University Giessen, Germany) was transfected with the LGTV-GFP plasmid using GeneJuice® Transfection Reagent (#70967, Sigma Aldrich, USA), following manufacturer's protocol. Virus supernatant was harvested on day 7 post infection. Both virus stocks were titrated on VeroB4 cells using focus-forming assay²⁶.

Virus infection in mouse model

For the experiments, the animals (7- to 11-week-old, mixed gender) were randomly divided into the following groups: WT, mock-infected; WT, LGTV-infected; *Ifnar*^{-/-}, LGTV-infected. After sedation with ketamine (100 µg/g body weight) and xylazine (5 µg/g body weight), the animals were intracranially inoculated with 1000 pfu of LGTV or with 10,000 pfu of LGTV-GFP

suspended in 20 μ L of PBS. Infected mice were euthanized using O₂ deprivation when they developed any one severe symptom, such as: >20% weight loss, bilateral eye infection, diarrhea, or hind-limb paralysis; or when they developed three milder symptoms, such as: >15% weight loss, unilateral eye infection, facial edema, ruffled fur, or overt bradykinesia, and/or development of stereotypies. Following euthanasia, cardiac perfusion was performed using 20 mL of PBS, followed by 20 mL of 4% w/v paraformaldehyde (PFA) in PBS, after which the perfused brain was removed for *ex vivo* analysis.

Whole-mount immunohistochemistry (IHC) and OPT

PFA-fixed brain was fluorescently immunolabeled with an antibody against viral NS5 (Supplementary Table 1) and processed for OPT imaging, as previously described^{27,28}. Briefly, the brain was dehydrated in a stepwise gradient of methanol (MeOH), permeabilized by repeated freeze–thawing in MeOH at –80 °C and a bleaching solution (MeOH:H₂O₂:DMSO at 2:3:1) to quench tissue autofluorescence. For immunolabeling, specimens were rehydrated in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% v/v TritonX-100), blocked with 10% v/v normal goat serum (NGS) (#CL1200-100, Nordic Biosite, Sweden), 5% v/v DMSO, and 0.01% w/v sodium azide in TBST at 37 °C for 48 h, and labeled with primary (chicken anti-NS5 and/or rabbit anti-GFP, Supplementary Table 1) and secondary (goat anti-chicken Alexa Fluor 680 and/or donkey anti-rabbit Alexa Fluor 594, Supplementary Table 1) antibodies diluted in blocking buffer. They were incubated at 37 °C for 4 d at each staining step. The stained tissue was mounted in 1.5% w/v low melting point SeaPlaque™ agarose (#50101, Lonza, Switzerland) and optically cleared using a BABB solution (benzyl alcohol (#1.09626.1000, Supelco, USA) : benzyl benzoate (#10654752, Thermo Fisher Scientific, USA) at 1:2).

OPT imaging was performed with an in-house developed near-infrared OPT (NIR-OPT) system described in detail in Eriksson et al.²⁸, with a zoom factor of 1.6 or 2.0, that resulted in an isotropic voxel dimension of 16.5 μm^3 and 13.2 μm^3 , respectively. To obtain specific fluorescent viral (NS5) signal coupled with Alexa Fluor 680, anti-GFP antibody coupled with Alexa Fluor 594, and tissue autofluorescence signals, OPT images were acquired at the following filter settings: Ex: 665/45 nm, Em: 725/50 nm (exposure time: 7000 ms); Ex: HQ565/30, Em: 620/60 (exposure time: 5000 ms); and Ex: 425/60 nm, Em: 480 nm (exposure time: 500 ms), respectively.

To increase the signal-to-noise ratio for NS5, the pixel intensity range of all images was adjusted to display the minimum and maximum, and a contrast-limited adaptive histogram equalization (CLAHE) algorithm with a tile size of 64×64 was applied to projection images acquired in the NS5 channel²⁹. Tomographic reconstruction was performed using NRecon software (v.1.7.0.4, Skyscan microCT, Bruker, Belgium) with additional misalignment compensation and ring artifact reduction. Image files were converted to Imaris files (.ims) using the Imaris file converter (v9.5.1, Bitplane, UK). NS5 signal from all imaged brains was adjusted to display at min = 0, max = 200, and gamma = 1.2, and colored using red glow color scheme. The signal was superimposed onto the corresponding tissue autofluorescence image using 3D iso-surface rendering in Imaris software (v9.5.1, Bitplane).

Light sheet fluorescent imaging

High-resolution images of ChP in the fourth ventricle of individual *Ifnar*^{-/-} brains, previously scanned using OPT, were acquired by LSFM. To compensate for any phototoxic effects on immunofluorescence from homogeneous sample illumination during OPT acquisition, the sample was relabeled using both primary and secondary antibodies (see above) and cleared in BABB

without agarose mounting. The brain was then scanned using an UltraMicroscope II (Miltényi Biotec, Germany) with a 1× Olympus objective (PLAPO 2XC, Olympus, Japan), coupled to an Olympus zoom body providing 0.63–6.3× magnification with a lens-corrected dipping cap MVPLAPO 2× DC DBE objective (Olympus). For image acquisition, left and right light sheets were merged with a 0.14 numerical aperture, which resulted in a light sheet *z*-thickness of 3.87 μm and 60% width, while using a 10–15 step blending dynamic focus across the field of view. Image sections were generated by Inspector Pro software (v7.0124.0, LaVision Biotec GmbH, Germany) and stitched together using the TeraStitcher script (v9), implemented in Inspector Pro. Stitched images were then converted into Imaris files (*.ims files) using the Imaris file converter (v9.5.1, Bitplane).

MRI acquisition

After OPT or LSFM imaging, 10 brains were rehydrated in TBST. T1-weighted images were then acquired at 9.4 Tesla using a preclinical MR system (Bruker BioSpec 94/20, Bruker, Germany) equipped with a cryogenic RF coil (MRI CryoProbe, Bruker) running Paravision 7.0 software (Bruker). Specifically, Modified Driven Equilibrium Fourier Transform (MDEFT) sequence with 5 repetitions (TR: 3000 ms; TE: 3 ms; TI: 950 ms; voxel dimension: 40 × 40 × 40mm³) was performed. Postprocessing of images involved the realignment and averaging of individual repetitions using statistical parametric mapping (SPM8) (the Wellcome Trust Centre for Neuroimaging, UCL, UK; www.fil.ion.ucl.ac.uk/spm) implemented in Matlab (R2014a, The MathWorks Inc., USA).

MRI template creation

A study-specific MRI template was created using bias-corrected (SPM8) MR images (n = 10), which were realigned and averaged using serial longitudinal registration in SPM12 implemented in Matlab (R2015b, The MathWorks Inc.). Then, all individual MR scans were coregistered with the template image. Study- and sequence-specific segments and TPMs were created using a 2-step segmentation and DARTEL pipeline initially based on in-house generated tissue priors. Briefly, a primary segmentation and DARTEL algorithm was applied to individual T1-weighted images to generate preliminary tissue priors for the study, using the toolbox SPMmouse. The entire process was then repeated (segmentation + DARTEL) using the tissue priors generated at the first step to produce study- and sequence-specific TPMs.

Creation of OPT-MRI fusion images

OPT images with viral signal and autofluorescence signal were reconstructed in DICOM format using NRecon software (v.1.7.0.4, Bruker) followed by their conversion into NifTi using PMOD VIEW tool (v.4.2, PMOD Technologies Inc., Switzerland) or the dcm2nii tool in MRICron software for OPT and MR images, respectively. Coregistration of OPT with the study-specific MRI template was performed using the toolbox SPMmouse in SPM8. Voxel-to-voxel affine transformation matrices were calculated using individual tissue autofluorescence from OPT images and applied to the corresponding viral OPT images. Fusion images of viral OPT signal were created for each individual brain using its own MRI and with the study-specific MRI template using the PMOD VIEW tool or Amira-Avizo software (v6.3.0, Thermo Fisher Scientific) for 3D renderings. Finally, brain areas with viral signal were identified according to the Turone Mouse Brain Template and Atlas (TMBTA) ²¹.

IHC for brain slice

PFA-fixed brain was washed in PBS, dehydrated overnight in a 30% w/v sucrose solution, snap-frozen on dry ice in Optimal Cutting Temperature medium (#361603E, VWR, USA), and stored at -80 °C until cryosectioning. The organ was sectioned along the sagittal plane at 10 µm thickness using a rotatory microtome cryostat (Microm Microtome Cryostat HM 500M, Microm, USA). Brain sections were permeabilized and blocked in 10% v/v NGS, 0.2% v/v TritonX-100, an 1% w/v bovine serum albumin in PBS for 1 h at room temperature (RT), immunolabelled with primary antibodies overnight at 4 °C, and then labeled with fluorescent secondary antibody for 1 h at RT in the dark. For immunodetection, the primary and secondary antibodies (Supplementary Table 1) were diluted in 2% v/v NGS and 0.5% v/v TritonX-100 in PBS. Confocal fluorescence microscopy was performed using a Zeiss 710 confocal microscope (Zeiss, Germany) controlled by Zeiss Zen interface (v.14.0.19.201) with Axio Observer inverted microscope equipped with Plan Apochromat 20×/0.8, C-Apochromat 40×/1.2, and Plan Apochromat 63×/1.4 objective lens. Large-area imaging was performed using a Nikon Eclipse Ti-E inverted microscope (Nikon, Japan) with an DU897 ANDOR EMCCD camera controlled by Nikon NIS Elements interface, equipped with Nikon CFI Plan Apochromat 10× (N.A. 0.45) DIC objective. To portray the large area, composite images were created by stitching together individual images using NIS-Elements AR software (v5.21.03, Nikon).

Fixation, resin embedding, and staining of tissue for electron microscopy

ChP from the brain of LGTV-infected *Ifnar*^{-/-} mice were prepared for electron microscopy by cardiac perfusion with 20 mL of 0.1 M phosphate buffer, followed by 20 mL of 2.5% w/v glutaraldehyde and 4% w/v PFA in 0.1 M phosphate fixative solution. Fourth ventricle ChP was

dissected and further immersed in the same fixative solution for an additional 24 h. The tissue was stained and subsequently embedded in resin using the rOTO (reduced osmium tetroxide, thiocarbohydrazide, osmium tetroxide) method³⁰. The tissue samples were placed in a solution of 1.5% w/v potassium ferrocyanide and 2% w/v osmium tetroxide (OsO₄), and then incubated in Pelco Biowave Pro+ (Pelco, Fresno, USA), a microwave tissue processor (“the processor”), under vacuum for 14 min. After two rinses with MilliQ water on the bench, the samples were washed twice with MilliQ water in the processor without vacuum pressurization. Then, the samples were incubated in 1% w/v thiocarbohydrazide solution for 20 min. After another MilliQ water rinse on the bench, the samples were again washed twice in the processor (no vacuum). Next, the samples were placed in 2% w/v OsO₄ solution and run in the processor under vacuum for 14 min, followed by an additional water and processor wash. The samples were placed in 1% w/v uranyl acetate solution and run in the processor under vacuum for 12 min, followed by another water and processor wash. The samples were then dehydrated in an ethanol gradient series, by removing and adding ethanol solutions of increasing concentrations (each step was performed in processor, without vacuum pressurization): 30%, 50%, 70%, 80%, 90%, 95%, and 100% (twice). The dehydrated samples were infiltrated with an increasing concentration of Durcupan ACM resin (Sigma-Aldrich) using the following stepwise ratios of ethanol to Durcupan resin: 1:3, 1:1, and 3:1. All steps were performed in the processor for 3 min under vacuum. The two final infiltration steps were performed in 100% resin. Finally, the samples were transferred to tissue molds, and placed at 60 °C for 48 h for complete polymerization of the resin.

TEM

Using a Reichert UltraCut S ultramicrotome (Leica, Germany), resin-embedded samples were trimmed, and 50 nm sections were cut using a diamond knife and placed onto copper slot grids. Resin-embedded sections were imaged using a 120 kV Talos L120C transmission electron microscope (Thermo Fischer Scientific) fitted with a LaB6 filament and Ceta 4k × 4k CMOS camera sensor. Images were acquired at 2600×, 8500×, and 36,000× magnification corresponding to a pixel size of 54.3, 17.0, and 4.1 Å/px, respectively, at the specimen level. TEM images were analyzed by ImageJ software (NIH).

Volume imaging using FIB-SEM

Resin-embedded tissue blocks were trimmed, mounted on SEM stubs, and then coated with a 5 nm platinum layer using a Q150T-ES sputter coater (Quorum Technologies, UK) before FIB-SEM volume imaging. Data was acquired using Scios Dual beam microscope (FIB-SEM) (Thermo Fischer Scientific). Electron beam imaging was acquired at 2 kV, 0.1 nA current, 1.9×1.9 nm pixel spacing, 7 mm working distance, 10 μs acquisition time, and 3072×2048 resolution using a T1 detector. SEM images were acquired every 20 nm. The working voltage of gallium ion beam was set at 30 kV, and 0.5 nA current was used for FIB milling. The specimens were imaged at 5×5 μm block face and 5 μm depth. FIB milling and SEM imaging were automated using the Auto Slice and View software (Thermo Fischer Scientific). SEM volume images were aligned and reconstructed using ImageJ (NIH) with linear stack alignment, with SIFT and MultiStackRegistration plugins^{31,32}. Analysis and segmentation of SEM volume images was done using Amira-Avizo software (v2020.3.1, Thermo Fisher Scientific).

Data availability:

All data are available in the main text or the supplementary materials. MR template will be deposited at NeuroImaging Tools and Resources Collaboratory (NITRC) and publicly available upon publication of the manuscript.

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NC, SMAW, ES, JZ, LAC, UA, DM and AKÖ conceptualized the manuscript. NC, SMAW, ES, MH, FM, JZ, EN and DW developed methodology and carried out the experiments. LAC, UA, DM and AKÖ provided supervision and critical evaluation with the manuscript. NC, SMAW, ES, LAC, UA, DM and AKÖ wrote the original draft of the manuscript and all authors reviewed and edited the manuscript.

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Figures:

Fig. 1. OPT reveals the distribution of LGTV infection in the adult mouse brain shaped by type I IFN response. **a** Volumetric 3D-render of the brain of mock and LGTV infected mouse immunolabeled with anti-NS5 (virus marker; red glow) antibody. The signal intensity was normalized within an individual brain and adjusted to identical minimum and maximum. Five mice per group were analyzed and representative images are shown. The brain images for the remaining animals ($n = 4$ per group) are shown in Supplementary Fig. 2. For each image pair, the top and lateral views of the same specimen are shown. **b** Volumetric 3D-render of a half of brain from *Ifnar^{-/-}* mouse infected with LGTV-GFP. The brain was immunolabeled with anti-NS5 (red) and anti-GFP antibodies (green). Signal overlap is shown in the merge image. Anatomical outlines were created by iso-surface rendering of autofluorescence signals. All scale bars = 2000 μ m.

Fig. 2. OPT-MRI coregistration and template creation. **a** Sectional image of a single-subject MRI with insets showing zoomed image at (i) olfactory bulb and (ii) anterior commissure. **b** Fusion image created using viral OPT signal (red) with own MR scan. **c** Sectional image of the developed MRI brain template with insets showing zoomed image at (i) olfactory bulb and (ii) anterior commissure, showing improved anatomical details compared to own MR (a). **d** Fusion images created using viral OPT signal (red) with the template. **e** Study-specific tissue probability maps, perfectly fitting the template. GM, gray matter; WM, white matter; CSF, cerebrospinal fluid. **f** Detailed anatomical mapping of viral OPT signal with the template in 3D.

Fig. 3. Brain mapping of viral signal using the developed OPT-MR fusion approach. **a-c** Anatomical mapping of brain regions after coregistration of viral OPT signal (red) with the study-specific MR brain template (gray). **d** Schematic illustration of slices of the brain template shown in A–C, viewed from the sagittal plane.

Fig. 4. Validation of LGTV infection in mouse brain using confocal and electron microscopy. **a** Maximum-intensity projection of confocal z-stack captured at olfactory bulb from sagittal brain sections (10 μ m). The sections were immunolabeled using antibodies against viral NS5, immature neuron from rostral migratory stream (DCX) and the nucleus (DAPI). Scale bars = 20 μ m. **b** Confocal microscopy images of infected cells in the fourth ventricle ChP (indicated by asterisk), immunolabeled with antibodies against NS5 and mature neuron (TUBB3). Scale bars = 100 μ m. **c**

594 Fourth ventricle ChP were immunolabeled with antibodies against NS5 and ChP epithelial cell
595 (AQPI). Scale bars = 20 μ m. **d** TEM images of brain sections fixed in resin. The image captured
596 a heavily infected cell indicated by dilated ER membrane, as compared to ER membrane of
597 uninfected ChP shown in Supplementary Fig. 3D. Scale bar = 1 μ m. Insets show high
598 magnification images of (i) virus particles and (ii) replication complexes within dilated ER. Scale
599 bar = 200 nm. **e** FIB-SEM volume imaging shows viral replication complexes within dilated ER.
600 Scale bar = 500 nm. **f** Segmentation image created from the 3D volume images in (e). Scale bar =
601 500 nm. **g** Maximum-intensity projection of confocal *z*-stack captured at cerebral cortex from
602 sagittal brain sections (10 μ m). The sections were immunolabeled using antibodies against viral
603 NS5, mature neuron (NeuN) and microglia (Iba1). The images are representative of at least 3
604 biological replicates for confocal images and 2 biological replicates with 3-5 technical replicates
605 for TEM and FIB-SEM. Scale bars = 20 μ m.