# Demyelination Produces a Shift in the Population of Cortical Neurons That Synapse with Callosal Oligodendrocyte Progenitor Cells

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#### **Visual Abstract**



#### Abstract

Oligodendrocyte progenitor cells (OPCs) receive synaptic input from a diverse range of neurons in the developing and adult brain. Understanding whether the neuronal populations that synapse with OPCs in the healthy brain is altered by demyelination and/or remyelination may support the advancement of

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neuroprotective or myelin repair strategies being developed for demyelinating diseases such as multiple sclerosis. To explore this possibility, we employed cre-lox transgenic technology to facilitate the infection of OPCs by a modified rabies virus, enabling the retrograde monosynaptic tracing of neuron $\rightarrow$ OPC connectivity. In the healthy adult mouse, OPCs in the corpus callosum primarily received synaptic input from ipsilateral cortical neurons. Of the cortical neurons,  $\sim$ 50% were layer V pyramidal cells. Cuprizone demyelination reduced the total number of labeled neurons. However, the frequency/kinetics of mini-excitatory postsynaptic currents recorded from OPCs appeared preserved. Of particular interest, demyelination increased the number of labeled layer II/III pyramidal neurons and also increased at the expense of layer V pyramidal neurons, a change that was largely ameliorated by remyelination. These data suggest that in the healthy adult mouse brain, callosal OPCs primarily receive synaptic input from cortical layer V pyramidal neurons. However, callosal demyelination is associated with a population switch and OPCs equally synapse with layer II/III and V pyramidal neurons to synapse with OPCs, until myelin is restored.

*Key words:* demyelination; NG2 glia; oligodendrocyte progenitor cells; rabies virus; remyelination; synapse

#### Significance Statement

In the CNS, myelination and remyelination involve the differentiation of oligodendrocyte progenitor cells (OPCs) into new oligodendrocytes (OLs), some of which survive to mature and myelinate axons. Throughout this process, neurons communicate with the OPCs and developing OLs. We show that OPCs in the corpus callosum of adult mice predominantly receive synaptic input from layer V cortical pyramidal neurons. However, they synapse equally with layer II/III and layer V neurons following cuprizone demyelination, suggesting that the highly motile OPC processes select alternative presynaptic sites. Five weeks of remyelination sees OPC connectivity bias return to layer V neurons. This provides critical insight into neuron $\rightarrow$ OPC communication and cellular interactions that are impacted in demyelinating diseases such as multiple sclerosis.

#### Introduction

Oligodendrocyte progenitor cells (OPCs) proliferate to sustain their number and differentiate into myelinating oligodendrocytes (OLs) throughout life (Rivers et al., 2008; Hughes et al., 2013; Young et al., 2013; Hill et al., 2014). In the developing or adult central nervous system (CNS), OPCs receive synaptic input from excitatory (Bergles et al., 2000; Kukley et al., 2007, 2008) and inhibitory neurons (Lin and Bergles, 2004; Balia et al., 2017). Neuron→OPC synapses share key structural, molecular, and physiological characteristics with neuronal synapses, including the presence of postsynaptic scaffolding proteins (Li et al., 2024), neurotransmitter receptors, and voltage-gated ion channels (reviewed in Kula et al., 2019; Xiao and Czopka, 2023) that allow OPCs to detect and respond to neuronal input (Bergles et al., 2000; Lin and Bergles, 2004; Gallo et al., 2008; Kukley et al., 2008; Mangin et al., 2008; Li et al., 2024). While the mechanisms that regulate the assembly and maintenance of neuron→OPC synapses remain largely unknown, they appear to be formed with a degree of selectivity. For example, interneuron  $\rightarrow$  OPC synapses preferentially form with fast-spiking cells (Tanaka et al., 2009; Orduz et al., 2015) and the number of inhibitory inputs decline in early postnatal development (Velez-Fort et al., 2010), such that only a small number of inhibitory connections are retained in adulthood (Moura et al., 2023). Instead, OPCs in the adult mouse brain predominantly synapse with excitatory neurons (Mount et al., 2019; Moura et al., 2023).

Neuron→OPC interactions play an important role in CNS health and disease and are implicated in the regulation of OPC proliferation and differentiation, myelination, and the modulation of axonal and synaptic development and plasticity (see Chen et al., 2018; Habermacher et al., 2019; Fletcher et al., 2021; Moura et al., 2022; Buchanan et al., 2023; Xiao and Czopka, 2023; Hill et al., 2024 for comprehensive reviews). In the developing CNS, electrically active axons are preferentially myelinated (Hines et al., 2015) and the chemogenetic activation of CTIP2<sup>+</sup> neurons in the adult mouse hippocampus similarly increases synapse formation between CTIP2<sup>+</sup> neurons and OPCs (Moura et al., 2023). Neuron→OPC synapses also appear to support remyelination, as OPCs rapidly populate demyelinated lesions and synaptically integrate prior to differentiation (Etxeberria et al.,

pSAD∆G-GFP genome plasmid (Addgene 32635) pcDNA-SADB19N (Addgene 32630), pcDNA-SADB19P (Addgene 32631), pcDNA-SADB19L (Addgene 32632), and pcDNA-SADB19G (Addgene 32633) and B7GG, EnvA-BHK and TVA-HEK cell lines were kindly provided by Prof. Edward M Callaway (Salk Institute for Biological Sciences).

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2010; Gautier et al., 2015; Sahel et al., 2015). This process is at least partially activity dependent, as blocking neuronal neurotransmitter release reduces OPC differentiation and lesion remyelination (Gautier et al., 2015). As OPCs form de novo synapses with demyelinated axons (Gautier et al., 2015), it is likely that they receive synaptic input from distinct or at least different neuronal populations in the healthy versus demyelinated brain.

The corpus callosum is the primary commissural region of the brain, composed of myelinated and unmyelinated axons (Sturrock, 1980; Lamantia and Rakic, 1990; Reeves et al., 2012), largely originating from layer II/III and V excitatory cortical pyramidal neurons that span the two hemispheres of the cerebral cortex (Chovsepian et al., 2017; Ku and Torii, 2020). OPCs receive synaptic input from these axons (Kukley et al., 2007; Ziskin et al., 2007; Mount et al., 2019), and the myelination of callosal axons continues throughout life (Young et al., 2013; Tripathi et al., 2017). To determine whether OPCs receive synaptic inputs from discrete subsets of projection neurons, we conditionally infected a small number of OPCs in the subcortical white matter with a monosynaptically restricted, glycoprotein-deleted rabies virus (Wall et al., 2010; Mount et al., 2019; Moura et al., 2023). This approach resulted in the transsynaptic labeling of cortical neurons that project their axons through the corpus callosum, alongside hippocampal neurons that project their axons through the alveus. In healthy adult mice, OPCs received synaptic connections from excitatory neurons in cortical and subcortical regions, largely located within the same hemisphere. Following cuprizone-induced demyelination, the total number of neurons that synapsed onto an OPC was reduced and a higher proportion of the synaptic inputs came from layer II/III cortical pyramidal neurons, at the expense of layer V pyramidal cells. After remyelination, the distribution of virally labeled neurons was partially restored to that observed in the healthy brain. These data suggest that callosal OPCs preferentially receive synaptic input from layer V excitatory cortical neurons, but that demyelination removes this preference, and that layer II/III and layer V neurons are equally likely to synapse with OPCs.

#### **Materials and Methods**

*Pseudotyped glycoprotein-deleted rabies virus*. A glycoprotein-deleted rabies virus encoding EGFP (SADΔG-GFP) was amplified and pseudotyped with the envelope protein of the avian sarcoma leukosis virus subtype A (EnvA) to produce SADΔG-(EnvA)-GFP as previously described (Osakada and Callaway, 2013; Dempsey et al., 2017). Briefly, B7GG cells were cultured in Dulbecco's modified eagle medium plus GlutaMAX (DMEM; Invitrogen)/10% fetal calf serum (FCS; Invitrogen, 10099-141) at 37°C and 5% CO<sub>2</sub>. At ~60% confluency, the B7GG cells were washed with PBS and exposed to DMEM/10% FCS/40% SADΔG-GFP supernatant (from a previous culture) and maintained at 35°C and 3% CO<sub>2</sub> for 5–6 h to enable infection. The medium was replaced with DMEM/10% FCS and the cells cultured at 35°C/3% CO<sub>2</sub> to support SADΔG-GFP replication and release into the culture medium. Supernatant containing the unpseudotyped virus was filtered (0.22 μm; Millipore, SCGP0525) and stored at -80°C.

To pseudotype the virus, BHK-EnvA cells were cultured in DMEM/10% FCS at  $35^{\circ}C/3\%$  CO<sub>2</sub>. When ~60% confluent, the cells were cultured in DMEM/10% FCS/50% unpseudotyped SAD $\Delta$ G-GFP supernatant for 5–6 h. The cells were washed, passaged, and replated at 20% confluency in DMEM/10% FCS and cultured at  $35^{\circ}C/3\%$  CO<sub>2</sub> for ~48 h. The pseudotyped SAD $\Delta$ G-GFP-(EnvA) virus concentrated by ultracentrifugation at 24,000 rpm (~72,000 × *g*) for 2 h at 4°C using a Sorvall WX+ Ultracentrifuge (Thermo Fisher Scientific, 75000100) with a TH-641 Swinging Bucket Rotor (Thermo Fisher Scientific, 54295). The viral pellet was resuspended in 300 µl Hanks' balanced salt solution (HBSS; Invitrogen, 14185052) per tube, then pooled and added to the surface of a 20% sucrose (Sigma)/HBSS cushion (2 ml), and topped with HBSS, before centrifugation at 20,000 rpm (~51,000 × *g*) for 2.5 h at 4°C. The supernatant was aspirated, and the SAD $\Delta$ G-GFP-(EnvA) viral pellet gently resuspended in 100 µl HBSS and placed at ~21°C for 1 h. Aliguots of concentrated SAD $\Delta$ G-GFP-(EnvA) were stored at ~80°C for experimental use.

Effective pseudotyping was confirmed by inoculating HEK293T cells or HEK293T-TVA800 cells (constitutively express the receptor for EnvA), with SAD $\Delta$ G-(EnvA)-GFP. Cells were plated in 24-well plates (Corning, CLS3524) at a density of  $1.5 \times 10^5$  and cultured in DMEM/10% FCS at  $35^{\circ}$ C/3% CO<sub>2</sub> before viral inoculation in serial dilutions ( $10^3-0^9$  in DMEM/ 10% FCS). The number of GFP<sup>+</sup> cells per well was quantified 36 h postinoculation. Viral titer was calculated using the following equations:

Titer of viral stock = (average number of GFP<sup>+</sup> cells per well/ $(0.75 \times 3) \times viral$  preparation dilution factor.

Final SAD $\Delta$ G-(EnvA)-GFP viral titers were  $2.17 \times 10^6$ – $3.06 \times 10^6$  infectious units/ml. The absence of GFP fluorescence in inoculated HEK293T cells confirmed that the pseudotyped SAD $\Delta$ G-GFP-(EnvA) virus could only infect TVA receptor<sup>+</sup> cells.

*Transgenic mice.* Animal experiments were approved by the University of Tasmania Animal Ethics Committee (A0013741 and A0016151) and carried out in accordance with the Australian code of practice for the care and use of animals for scientific purposes. All mice were weaned at postnatal day (P) 35 and group housed (2–5 per cage) in individually ventilated Optimice microisolator cages (Animal Care Systems) at  $21 \pm 2^{\circ}$ C on a 12 h light/dark cycle (lights on 07:00 to 19:00). Mice had *ad libitum* access to standard rodent chow (Barastoc rat and mouse pellets) and water.

Homozygous *RphiGT* transgenic mice [*B6;129P2-Gt*(*ROSA*)*26Sor*<sup>tm1(CAG-RABVgp4,-TVA)Arenk/J, RRID:IMSR\_JAX:024708; Takatoh et al., 2013] that, following Cre-mediated recombination, express the TVA receptor and rabies glycoprotein under the control of the *Rosa26-YFP* promoter, and heterozygous *Pdgfrα-H2BGFP* transgenic mice (RRID:IMSR\_JAX:007669; Hamilton et al., 2003), were purchased from Jackson Laboratories. *Pdgfrα-CreER*<sup>T2</sup> (Rivers et al., 2008) transgenic mice were a kind gift from Prof. William D Richardson (University College London). All mice were backcrossed and maintained on a *C57BL6/J* background. Heterozygous *Pdgfrα-CreER*<sup>T2</sup> mice were crossed with homozygous *RphiGT* mice to produce *Pdgfrα-CreER*<sup>T2</sup>:: *RphiGT* experimental mice. Male and female mice were randomly assigned to treatment groups, but care was taken to ensure that littermates were represented across treatment groups.</sup>

*Pdgfra-H2BGFP* and *Pdgfra-CreER*<sup>T2</sup> mice were genotyped as described (Ferreira et al., 2020; Zhen et al., 2022). In brief, genomic DNA (gDNA) was extracted from ear biopsies by ethanol precipitation and PCR performed using 50–100 ng of gDNA with the following primer combinations: *Cre 5'* CAGGT CTCAG GAGCT ATGTC CAATT TACTG ACCGTA and Cre 3' GGTGT TATAAG CAATCC CCAGAA; *GFP 5'* CCCTG AAGTTC ATCTG CACCAC and *GFP 3'* TTCTC GTTGG GGTCT TTGCTC. *RphiGT* mice were genotyped by PCR detection of TVA using the following primers: *TVA 5'* CGGTT GTTGG AGCTG CTGGT GCTGC TGC and *TVA 3'* TGCAA CGATC AGCAT CCACA TGC. The PCR program (94°C for 4 min, followed by 35 amplification cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 60 s, and 10 min at 72°C) produced DNA products of ~500 bp for Cre, ~242 bp for GFP, and ~300 bp for TVA.

*Tamoxifen administration.* Tamoxifen (Sigma; 10540-29-1) was dissolved to 40 mg/ml in corn oil (Sigma; 8001-30-7) by sonication at 21°C for ~2 h and administered to adult mice by oral gavage as four consecutive daily doses (300 mg/kg). Mice received four consecutive doses of tamoxifen, as we have previously shown that this dosing regimen produces maximum activation of Cre recombinase in OPCs in Pdgfra-CreER<sup>T2</sup> mice (Rivers et al., 2008). We also ensured a minimum of 3 d between the final tamoxifen dose and commencing any intervention, to ensure that OPC recombination was complete and stable prior to viral injection (Rivers et al., 2008). We avoided the simultaneous delivery of tamoxifen and cuprizone, as this can result in weight loss, but primarily because tamoxifen can promote remyelination (Gonzalez et al., 2016). To evaluate neuron-OPC synaptic connectivity in remyelinating mice, tamoxifen delivery was delayed until the end of the remyelinating period, to ensure that essentially all callosal OPCs, including those generated from neural stem cells over the 10 week tracing period (Xing et al., 2014), were targeted by recombination.

*Cuprizone-induced demyelination and remyelination.* To induce demyelination, mice received a diet containing 0.2% (w/w) cuprizone (bis-cyclohexanoneoxaldihydrazone; Sigma, C9012) from P49 for five consecutive weeks. Cuprizone was thoroughly mixed with dry, ground standard rodent chow (Barastoc rat and mouse pellets) and rehydrated with water in a 3:1 (w/v) ratio (Nguyen et al., 2024). Cuprizone chow was prepared as large experimental batches and distributed across cages so that each mouse had *ad libitum* access to ~25 g of hydrated cuprizone-chow, replaced every 2 d.

Stereotaxic viral microinjections. Adult mice received a subcutaneous (s.c.) injection of the nonsteroidal antiinflammatory analgesic meloxicam (Metacam 5 mg/kg at 0.0125%; Ilium, Troy Laboratories) prior to anesthesia induction with 5% isoflurane (Henry Schein) in O<sub>2</sub> delivered at ~0.6 L per minute (Darvall Isoflurane Precision Vapouriser; GVPAD-09175S). Anesthesia was maintained with 1.2-2% isoflurane in O<sub>2</sub> throughout the procedure. Anesthetized mice were secured within a stereotaxic frame (Narishige, SR-5R-HT) and Viscotears liquid eye gel (Novartis Pharmaceuticals) applied to maintain eye moisture. Following sterilization with Microshield chlorhexidine (Schulke), a cocktail of bupivacaine hydrochloride (1 mg/kg at 0.025%; Pfizer) and lignocaine hydrochloride (5 mg/kg at 0.03%; Mavlab) was administered subcutaneously to the scalp to induce local analgesia and 0.9% sterile saline (Livingstone International, DWSC0005) administered to the left or right flank (0.5 ml, s.c.) to prevent dehydration during the procedure. Mice were placed on sterile heating pads to maintain body temperature at 37°C, and the reflex response, breathing rate, mucous membrane color, and capillary refill time (MM/CRT) periodically monitored throughout the surgical procedure. A small cranial incision ( $\leq 1$  cm) was made in the scalp to expose the skull and the periosteum removed. A small burr hole (0.5 mm tip diameter burr) was microdrilled (Fine Science Tools, 19007-05) through the right side of the skull, 1.5 mm posterior and 1 mm lateral to bregma, ensuring the dura remained intact. Then, 800 nl of SADAG GFP-(EnvA) was injected into the corpus callosum (~1 mm below the dura) at a rate of 100nl/min using a UMP3 UltraMicroPump (World Precision Instruments) attached to a 5 µl Hamilton Neuros Syringe (model 75 RN) with 33 gauge beveled needle (Hamilton, 65460-03). The needle was slowly withdrawn to ensure fluid diffusion, and the surgical incision was sutured (Daclon Nylon, GVPI-915 1512). Mice were recovered in their home cage on a 37°C heat pad for ~30 mins until awake, eating and grooming normally.

*Electrophysiology.* Adult *Pdgfra-H2BGFP* mice were killed by cervical dislocation and the brains rapidly dissected into ice-cold sucrose solution (in mM: 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 7 MgCl<sub>2</sub>, and 0.95 CaCl<sub>2</sub>). Then, 250  $\mu$ m acute coronal brain slices were generated using a Leica VT1200s Vibratome and incubated for 30 min at ~32°C in artificial cerebral spinal fluid (300 ± 5 mOsm/kg ACSF; in mM: 119 NaCl, 1.6 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 26.2 NaHCO<sub>3</sub>, 1.4 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, and 11 glucose) saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> and maintained at ~21°C in ACSF saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>.

Whole-cell patch-clamp recordings were made from GFP<sup>+</sup> callosal OPCs in the body of the corpus callosum, underlying the parietal/retrosplenial cortices; the area corresponding to the location of viral injection for the neuroanatomical studies

(~bregma 1.5 mm). Recording pipettes, pulled from glass capillaries had a resistance of 3.6–6.9 MΩ (Harvard Apparatus, W3 30-0057), were filled with a cesium-based internal solution containing the following (in mM): 125 Cs-methanesulfonate, 4 NaCl, 3 KCl, 1 MgCl<sub>2</sub>, 8 HEPES, 9 EGTA, 10 phosphocreatine, 5 MgATP, and 1 Na<sub>2</sub>GTP set to a pH of 7.2 with CsOH, with an osmolarity of 290±5 mOsm/kg (Fulton et al., 2010). Upon breakthrough, access resistance (Ra), capacitance (Cm), and membrane resistance (Rm) were recorded using a HEKA patch-clamp EPC800 amplifier and pClamp 10.5 software (Molecular Devices). As OPCs produce a transient inward current after depolarization beyond -30 mV, the voltagegated inward current was evaluated for each GFP<sup>+</sup> cell by recording their response to 500 ms voltage steps of -70 to -20 mV, -10, 0, +10, and +20 mV. Cells with a capacitance (Cm) of <50 pF and NaV conductance  $\geq100$  pA for the -70 to 0 mV step were classified as OPCs (Pitman et al., 2020). Cells were held at -70 mV and mEPSCs recorded from slices perfused in ACSF containing 1 µM tetrodotoxin (TTX; Abcam), as 2 × 3 min gap-free protocols, sampled at 50 kHz and filtered at 3 Hz (total of 6 min recording per cell). Perfusate was then replaced with ACSF containing 1 µM TTX and 100  $\mu$ M of the secretagogue ruthenium red (Sigma), and after  $\geq$ 3 min perfusion, the recording protocol was repeated. Ra was measured before and after each recording, and a cell was excluded if the Ra exceeded 20 MΩ or deviated >20% from the baseline Ra measurement. As OPCs have a high Rm (>1 G $\Omega$ ), recordings were made without series resistance compensation. Data have not been adjusted for the liquid junction potential. Measurements were made from data files using Clampfit 10.5 (Molecular Devices) and mEPSCs with an amplitude ≥5 pA were identified and analyzed using the MiniAnalysis60 program (Synaptosoft).

*Tissue preparation and immunohistochemistry.* Mice were terminally anesthetized by an intraperitoneal (i.p.) injection of 300 mg/kg sodium pentobarbital (llium) prior to transcardial perfusion with 4% (w/v) paraformaldehyde (PFA; Sigma) in PBS. Brains were dissected and immersion fixed in 4% PFA for 90 min at ~21°C. SAD $\Delta$ G-GFP-(EnvA) transfected brains were transferred to 4°C PBS and the olfactory bulbs and cerebellum removed prior to embedding in 4% (w/v) molecular grade agarose (Bioline Australia) in PBS. Floating serial coronal vibratome sections (40 µm; Leica VT1000S Vibratome) were used for immunohistochemistry (see below), mounted on glass slides (Dako, K8020), and coverslipped with fluorescent mounting medium (Dako). To detect myelin basic protein (MBP), perfusion fixed in 4% PFA for 90 min at ~21°C. The brain slices using a 1 mm brain matrix (Kent Scientific) and further immersion fixed in 4% PFA for 90 min at ~21°C. The brain slices were cryoprotected in 20% (w/v) sucrose (Sigma)/PBS in 4°C overnight and frozen in Shandon Cryomatrix (Thermo Fisher Scientific) using liquid nitrogen. Brain slices were stored at ~80°C until coronal brain cryosections (30 µm, ~bregma -1.5 mm) were floated onto glass slides, allowed to dry, and exposed to -20°C methanol for 10 min prior to commencing immunohistochemistry.

For immunohistochemistry, vibratome sections or cryosections were incubated in blocking solution [0.1% (v/v) Triton X-100 (Sigma) and 10% FCS in PBS] for 1 h at ~21°C. All antibodies were diluted in blocking solution, applied to sections, and incubated overnight at 4°C. Primary antibodies included the following: goat anti-PDGFRα (1:200; R&D Systems, RRID: AB\_2236897), rat anti-GFP (1:2,000; Nacalai Tesque, RRID: AB\_10013361), rat anti-MBP (1:200; Abcam, RRID: AB\_305869), rabbit anti-ASPA (1:200; Millipore, RRID: AB\_2827931), mouse anti-NeuN (1:500; Millipore, RRID: AB\_2298772), rabbit anti-parvalbumin (1:2,000; Abcam, RRID: AB\_2924658), and rabbit anti-GFAP (1:1,000; Dako, RRID: AB\_10013382). Secondary antibodies were conjugated to AlexaFluor-488, –568 or –647 (Invitrogen) and included the following: donkey anti-mouse (1:1,000), donkey anti-goat (1:1,000), donkey anti-rabbit (1:1,000), and donkey anti-rat (1:500). Nuclei were labeled using Hoechst 33342 nuclear stain (1:1,000; Invitrogen, 62249).

*Microscopy and image analysis.* Low (20× air objective) and high (40× water objective) magnification confocal images were collected using an UltraView Nikon Ti Confocal Microscope with Volocity Software (PerkinElmer), or Ultraview Eclipse Nikon Ti Microscope with a differential spinning disk attachment (ANDOR) running NIS-Elements Advanced Research software (Nikon). Standard excitation and emission filters for DAPI (Hoechst 33342), FITC (Alexa Fluor-488), TRITC (Alexa Fluor-568), and CY5 (Alexa Fluor-647) were used. Images were captured with a 1–3 µm *z*-spacing and stitched to form a composite image of a defined region of interest.

To quantify myelin content, a 20× single *z*-plane image was collected for a single field of view of the medial corpus callosum for each coronal cryosection immunolabeled to detect MBP. Images were analyzed in ImageJ (NIH) by a researcher blind to the experimental conditions. Images were changed to 16 bit grayscale and manual thresholding performed to identify all MBP<sup>+</sup> pixels within the dorsal fornix – a white matter tract largely unaffected by cuprizone feeding (Yang et al., 2009; Hertanu et al., 2023). MBP coverage was then quantified in the corpus callosum, using the same thresholding parameter. The proportion of the *x*-*y* area covered by MBP labeling was quantified in three randomly placed 100  $\mu$ m<sup>2</sup> boxes in the medial corpus callosum for each of three sections per mouse, and data are expressed as mean area covered by MBP<sup>+</sup> pixels (%).

To quantify the number and regional distribution of RABV-GFP<sup>+</sup> cells, 20× confocal image stacks (3 μm *z*-spacing) were captured from serial coronal sections at and surrounding the injection site and were stitched to form composite images. Manual cell counts were performed using Photoshop CS6 (Adobe) by an experimenter blind to treatment. Each brain region was defined with reference to the Hoechst nuclear staining pattern and anatomical features cross-referenced against the Mouse Brain Atlas (Franklin and Paxinos, 2007). RABV-GFP<sup>+</sup> cells were included in the analysis if they contained a Hoechst<sup>+</sup> nucleus and a cellular identity assigned if the cell coexpressed cell-specific markers and/or could be morphologically categorized. More specifically, RABV-GFP<sup>+</sup> cells that expressed PDGFRα were identified as OPCs, while

those that expressed ASPA had a small spherical nucleus and supported multiple straight fluorescent segments characteristic of myelin sheaths (Osanai et al., 2017) were identified as OLs. Astrocytes were identified by their expression of glial fibrillary acidic protein (GFAP) or morphologically distinct star-shaped cell bodies and highly ramified processes (Matyash and Kettenmann, 2010; Vasile et al., 2017; Zhou et al., 2019). Neuronal nuclear protein (NeuN) or parvalbumin (PV) expression were used to help classify RABV-GFP<sup>+</sup> neurons. Excitatory pyramidal neurons were further classified by their distinct pyramidal shaped cell bodies and apical and basal dendritic trees including their large apical dendrites with a high density of dendritic spines (De Felipe and Fariñas, 1992). Interneurons colabeled for parvalbumin and/or elaborated a smaller dendritic tree that lacked spines and could be identified with reference to previously defined interneuron classifications (Porter et al., 2001; Dumitriu et al., 2007; Feldmeyer et al., 2018; Laturnus et al., 2020).

The number and anatomical distribution of RABV-GFP<sup>+</sup> neurons in each brain was quantified by performing highthroughput imaging of serial coronal vibratome brain sections using an Olympus VS120 Slide Scanner (Olympus) fluorescent microscope, running VS200 ASW V3.3 software, with standard excitation and emission filters for DAPI (Hoechst 33342), FITC (Alexa Fluor-488), and TRITC (Alexa Fluor-568). Low magnification (10× air objective) images were captured at 5  $\mu$ m *z*-spacing across the central 35  $\mu$ m of each section and stitched to form a single composite maximum projection image of each coronal brain section. Images were converted to 16 bit RGB TIFF files before blinded cell counts were performed manually in Photoshop CS6. Brain regions and cortical laminar were defined using Hoechst nuclear staining and anatomical landmarks defined in the Mouse Brain Atlas (Franklin and Paxinos, 2007).

RABV-GFP<sup>+</sup> cells that possessed a clear Hoechst-labeled nucleus were quantified across ~120 sequential serial sections per brain between ~bregma 2.0 and -4.0, with an estimated  $\leq 2\%$  of tissue lost during tissue sectioning. Sections containing the remaining rostral portion of the cortex and cerebellum were analyzed in a small number of mice, but as no RABV-GFP<sup>+</sup> neurons were identified within these regions, they were excluded from future analyses. To account for variability in the number of RABV-GFP<sup>+</sup> neurons/glia labeled within each brain, data were normalized by expressing the number of RABV-GFP<sup>+</sup> cells in each region as a percentage of the total RABV-GFP<sup>+</sup> cells detected in each brain or within a defined anatomical region.

To quantify cFos expression by NeuN<sup>+</sup> neurons in the cortex, previously generated confocal image stacks that were stitched to encompass the visual cortex of n=3 control and 5 week 0.2% (w/w) cuprizone demyelinated mice (Nguyen et al., 2024) were downloaded for analysis. Each image captured Hoescht33342, NeuN, and c-fos labeling. Cortical laminar were defined based from nuclei distributions using Hoescht33342. Cell quantification was performed manually in layers II/III and V. Each NeuN<sup>+</sup> cell was identified and counted in layer II/III or V of the visual cortex using the cell counter function in Image J (NIH). These data were used to determine NeuN<sup>+</sup> cell density in each layer, and each NeuN<sup>+</sup> cell was evaluated for cFos expression. Within the same tissue sections, we also evaluated the relative intensity of cFos expression in cortical layer II/III and V of demyelinated mice. Individual regions of interest were drawn around NeuN<sup>+</sup> cFos<sup>+</sup> cells within each layer. Background fluorescence was evaluated in five regions of interest drawn pseudorandomly in each layer, to encompass areas without cFos<sup>+</sup> labeling. Mean gray value and integrated pixel density were measured using Image J (NIH). Corrected total cell fluorescence (CTCF) was calculated for cells within layer II/III or V of demyelinated mice using the following formula: [CTCF = Integrated Density – (Area of selected cell × Mean fluorescence of background readings)].

Statistical analyses. Statistical comparisons were performed using Prism 9.5.1 (GraphPad). Data were evaluated using the Kolmogorov–Smirnov (KS) or Shapiro–Wilk (SW) normality tests. Normally distributed data were analyzed using an unpaired or paired *t* test or one- or two-way ANOVA followed by a Tukey's multiple-comparison test. A test for linear trend was performed following a one-way ANOVA where indicated. When unequal variance was observed between groups, determined by the Brown–Forsythe test or *F* test to compare variance, data were analyzed using a *t* test with a Welch's correction, or a Welch's ANOVA followed by a Dunnett's T3 multiple-comparisons post hoc test. Data that were not normally distributed were square root transformed to satisfy assumptions of normality and homoscedasticity of the variances followed by analysis by parametric tests where indicated. mEPSC data were analyzed in R (v4.4.2) using Ime4, emmeans, and easystats packages for restriction maximum likelihood (REML) linear mixed models to determine the effect of cuprizone feeding with individual mouse included as a random effect term. *p* values < 0.05 were considered statistically significant. Statistical information is presented in the corresponding figure legend. The summary of the statistical analysis is shown in Tables 1 and 2. Data are presented as mean ± standard deviation unless otherwise specified.

Data were included/excluded from analysis based on a set of predetermined criteria. Specifically, injection of the SAD $\Delta$ G-GFP-(EnvA) virus into the subcortical white matter of *RphiGT* control mice resulted in the nonspecific labeling of 15 ± 7 neurons, and previous studies have estimated the minimum number of synaptic inputs received by individual callosal OPCs to be between ~11 and 24 (Kukley et al., 2007; Mount et al., 2019). Therefore, if the number of RABV-GFP<sup>+</sup> neurons labeled in *Pdgfra-CreER<sup>T2</sup> :: RphiGT* experimental mice was <2-fold greater than 15±7, it was assumed that insufficient viral labeling of OPCs or transsynaptic labeling of connected neurons occurred in that mouse. Based on this assumption, n = 1 *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mouse from the cuprizone demyelinated cohort, and n = 2 *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice from the remyelination cohort were excluded and not used to analyze the regional distribution of RABV-GFP<sup>+</sup> neurons. Additionally, n = 1 *Pdgfra-CreER<sup>T2</sup> :: RphiGT* mouse from the remyelination cohort was not used to analyze the regional distribution of RABV-GFP<sup>+</sup> neurons. Additionally, n = 1 *Pdgfra-CreER<sup>T2</sup> :: RphiGT* mouse from the remyelination cohort was not used to analyze the regional distribution of RABV-GFP<sup>+</sup> neurons. Additionally, n = 1 *Pdgfra-CreER<sup>T2</sup> :: RphiGT* mouse from the remyelination cohort was not used to analyze the regional distribution of RABV-GFP<sup>+</sup> neurons.

Table 1. Statistical comparison of RABV-GFP<sup>+</sup> neuron number and distribution per mouse in untreated versus cuprizone "control" cohorts

Comparison	Test	Statistics	Power (95% CI of difference)
RABV-GFP <sup>+</sup> neurons (#)	Unpaired <i>t</i> test	T=0.4342, p=0.6772	-1,358 to 1,968
RABV-GFP <sup>+</sup> cortical neurons (#)	Unpaired t test	T=0.5216, p=0.6181	-621.6 to 973.4
RABV-GFP <sup>+</sup> cortical neurons (%)	Unpaired <i>t</i> test on sqrt transformed data	$T = 0.003 \ p = 0.99$	-2.122 to 2.116
RABV-GFP <sup>+</sup> contralateral cortical neurons (#)	Unpaired <i>t</i> test	T=0.2560, p=0.8053	-128.9 to 160.2
RABV-GFP <sup>+</sup> contralateral cortical neurons (%)	Unpaired <i>t</i> test	T=0.5307, p=0.6120	-6.843 to 4.334
RABV-GFP <sup>+</sup> hippocampal neurons (#)	Unpaired <i>t</i> test	<i>T</i> =0.2509, <i>p</i> =0.8091	-890.0 to 1,101
RABV-GFP <sup>+</sup> hippocampal neurons (%)	Unpaired <i>t</i> test on sqrt transformed data	T = 0.03 p = 0.97	-1.484 to 1.447
RABV-GFP <sup>+</sup> contralateral hippocampal neurons (#)	Unpaired t test	<i>T</i> = 0.5837, <i>p</i> = 0.5777	-171.0 to 103.3
RABV-GFP <sup>+</sup> contralateral hippocampal neurons (%)	Unpaired <i>t</i> test on sqrt transformed data	T = 1.74 p = 0.12	-2.551 to 0.3836
RABV-GFP <sup>+</sup> contralateral	Unpaired <i>t</i> test	<i>T</i> = 0.1.338, <i>p</i> = 0.2228	-12.94 to 3.587
RABV-GFP <sup>+</sup> thalamic/ hypothalamic neurons (%)	Unpaired <i>t</i> test	<i>T</i> = 1.397, <i>p</i> = 0.2052	-0.7646 to 2.970
RABV-GFP <sup>+</sup> neurons per cortical region (%)	Two-way ANOVA	interaction $F_{(5,42)} = 0.4727$ , $p = 0.7944$ ; region $F_{(5,42)} = 6.090$ , $p = 0.0003$ ; treatment $F_{(1,42)} = 0.0180$ , $p = 0.8941$	Difference between treatments; –6.309 to 7.206
RABV-GFP <sup>+</sup> neurons per hippocampal region (%)	Two-way ANOVA	interaction $F_{(3,28)} = 1.835$ , $p = 0.1638$ ; region $F_{(3,28)} = 2,962$ , $p < 0.001$ ; treatment $F_{(1,28)} = 3,585e-009$ , $p < 0.9999$	Difference between treatments; –1.711 to 1.711
RABV-GFP <sup>+</sup> cortical layer distribution (%)	Two-way ANOVA	Interaction $F_{(3,28)} = 0.353$ , $p = 0.7836$ , cortical layer $F_{(3,28)} = 69.46$ , $p < 0.0001$ , treatment $F_{(1,28)} = 1.164 \times e-005$ , $p = 0.9973$	Difference between treatments; -4.944 to 4.961

#### **Results**

#### SADAG-GFP-(EnvA) infected OPCs decline in number within 7 d

To characterize the population of neurons that synapse onto OPCs in the healthy adult mouse corpus callosum, we labeled OPCs with the glycoprotein-deleted SAD $\Delta$ G-GFP-(EnvA) rabies virus. This virus has been modified to restrict infection to cells that express TVA, the cognate receptor for EnvA. Furthermore, deletion of the gene encoding the rabies virus glycoprotein (RVG) has rendered the virus unable to cross the synapse, unless RVG is expressed by the infected cell. Delivery of tamoxifen to *Pdgfra-CreER*<sup>T2</sup> :: *RphiGT* transgenic mice induces the expression of TVA and RVG in PDGFRa<sup>+</sup> OPCs, renders OPCs susceptible to infection and labeling by the SAD $\Delta$ G-GFP-(EnvA) virus, and allows the monosynaptic transfer of the virus to label neurons that directly synapse with the labeled OPCs in vivo (Mount et al., 2019).

Previous studies have demonstrated that the in vivo injection of SAD $\Delta$ G-GFP-(EnvA) virus can label some neurons and glia around the injection site, even in the absence of Cre-induced TVA expression (Marshel et al., 2010; Rancz et al., 2011; Takatoh et al., 2013). Therefore, we first delivered tamoxifen to P42 C57BL/6 (*n* = 3) and *RphiGT* control mice (*n* = 4) and injected SAD $\Delta$ G-GFP-(EnvA) into the corpus callosum at P49. After 7 d, RABV-GFP<sup>+</sup> cells were not detected in C57BL/6 injected brains. The small number of RABV-GFP<sup>+</sup> cells (46 ± 14) detected in the brains of *RphiGT* control mice were proximal to the injection site, but spanned the corpus callosum, hippocampus, and cortex (Extended Data Fig. 1-1*A*). A subset of these cells were identified as RABV-GFP<sup>+</sup> NeuN<sup>+</sup> neurons (15 ± 7 cells) and had the distinct morphology of excitatory cortical pyramidal neurons (Extended Data Fig. 1-1*B–D*), and fewer still (~1–2 per brain) were interneurons. The remaining RABV-GFP<sup>+</sup> cells were glia, including OLs (18 ± 8 cells; Extended Data Fig. 1-1*E–G*) and astrocytes (7 ± 6 cells; Extended Data Fig. 1-1*H–J*). Of note, RABV-GFP<sup>+</sup> cells did not express the OPC marker PDGFR $\alpha$ , indicating that OPCs lacking the TVA receptor are not prone to nonspecific labeling by SAD $\Delta$ G-GFP-(EnvA) (Extended Data Fig. 1-1*K*).

In contrast, when SAD $\Delta$ G-GFP-(EnvA) was injected into the corpus callosum of P49 *Pdgfra*-*CreER*<sup>T2</sup> :: *RphiGT* transgenic mice (Fig. 1*A*), PDGFRa<sup>+</sup> OPCs within the cortex (Fig. 1*B–D*), corpus callosum (Fig. 1*E–G*), and hippocampus (Fig. 1*H–J*) became labeled with RABV-GFP. In total, 207 RABV-GFP<sup>+</sup> OPCs were identified across n = 24*Pdgfra*-*CreER*<sup>T2</sup> :: *RphiGT* mice analyzed 1–7 d postinjection. Approximately 82% of these were located within the corpus

	Figure	Graph	Data structure	Type of test	<i>p</i> value	Power (95% CI of difference)
а	1	К	Sqrt transformed	One-way ANOVA: Tukey's post hoc test	<0.0001 CTX versus CC <0.0001 CTX versus HIP 0.0528 CC versus HIP <0.0001	
b		L	Sqrt transformed	One-way ANOVA; Tukey's post hoc test	0.012 1 DPI versus 7DPI 0.01 2DPI versus 7 DPI 0.058 0.0004	
	_	_		trend	0.0004	
c d	2	В I	Sqrt transformed Normally distributed	Welch's <i>t</i> test Welch's ANOVA	0.012 0.0045	10.21 to 43.62
е		J	Normally distributed	Dunnett's post hoc test Two-way ANOVA	HIP versus TH/HYTH; 0.0136 Hemisphere <0.0001	23.91 to 104.2 –78.02 to –68.59
				Tukey's post hoc tests	lpsi versus contra all regions; 0.0001 lpsi versus CTX: <0.0001	-82.86 to -52.60
f		к	Normally distributed	One-way ANOVA	Ipsi versus contra HIP; <0.0001	-85.34 to -55.09
			nonnany alothoatoa	Tukey's post hoc tests	CA1 versus CA2; <0.0001	78.62 to 92.17
					CA1 versus CA3; <0.0001	82.92 to 96.47
g		L	Normally distributed	One-way ANOVA	CA1 versus subiculum; <0.0001 0.0012	84.56 to 98.11
•				Tukey's post hoc tests	Somatosensory versus auditory; 0.0066	6.726 to 60.08
					Somatosensory versus cingulate; 0.0014	11.46 to 64.82
					Somatosensory versus insular; 0.0015	11.40 to 64.76
h		Б	Normally distributed		Somatosensory versus rhinal; 0.0016	11.06 to 64.42
		Г	Normally distributed	Tukey's post hoc test	II/III versus V; 0.0008 IV versus V; 0.0003 V versus V! <0.0001	-47.23 to -13.64 -50.38 to -16.78 26.68 to 60.28
I	2-1	D	Normally distributed	Paired <i>t</i> test	<0.0001	98.93 to 100.1
J	3	Е	Normally distributed	One-way ANOVA	<0.0001	10 50 to 22 72
				Tukey's post floc tests	<0.0001	19.59 10 52.75
					<0.0001	-33.92 10 -20.79
k		J	Normally distributed	Two-way ANOVA Tukey's post hoc tests	Region <0.0001 Control: CTX versus HIP; 0.0036 Demyelinated: CTX versus CC; 0.0053	-0.1558 to 0.2438 0.1906 to 1.028 -1.002 to -0.1650
					Demyelinated: CC versus HIP; 0.0001	0.4077 to 1.245
Ι		К	Normally distributed/ nonparametric	Kolmogorov–Smirnov test	0.0021	
m		L	Normally distributed	Welch's <i>t</i> test; <i>F</i> test to compare variance	0.0203	
n		М	Normally distributed/ nonparametric	Kolmogorov-Smirnov test	0.0002	
0	4	A	Sqrt transformed	One-way ANOVA Tukey's post hoc test	0.0019 Control versus demyelinating: 0.0309	
				Tukey's post hoc test	Control versus remyelinating; 0.0023	
р	5	F	Sqrt transformed	One-way ANOVA	0.0163	
				Tukey's post hoc test	Control versus demyelinated; 0.0232	
						(Table continues.)

#### Table 2. Continued

	Figure	Graph	Data structure	Type of test	p value	Power (95% CI of difference)
q		G	Sqrt transformed	One-way ANOVA Tukey's post hoc tests	0.0003 Control versus demyelinated;	1.051 to 10.97
					0.0167 Control versus remyelinating; 0.0003	3.895 to 12.21
r		Н	Sqrt Transformed	One-way ANOVA Tukey's post hoc tests	0.0116 Control versus demyelinated;	0.2532 to 6.662
					0.0336 Control versus remyelinating; 0.0270	0.3414 to 5.962
S		I	Sqrt transformed	One-way ANOVA Tukey's post hoc test	0.0026 Control versus remyelinating;	2.558 to 11.17
t		J	Sqrt transformed	One-way ANOVA	0.0050	
			·	Tukey's post hoc test	Control versus demyelinating; 0.0744	-0.1149 to 2.709
		ĸ	Sart transformed		Control versus remyelinating 0.0052 0.0003	0.5368 to 3.014
u		ĸ	Sqrt transformed	Tukey's post hoc test	Control versus demyelinating; 0.0167	1.051 to 10.97
					Control versus remyelinating 0.0003	3.895 to 12.21
v		М	Normally distributed	Two-way ANOVA Tukey's post hoc tests	Region; 0.0108 Retrosplenial control versus	-43.38 to -13.03
		_			Somatosensory control versus remyelinating; 0.0333	1.037 to 31.39
W	6	D	Normally distributed	Two-way ANOVA	0.0006	
				Tukey's post hoc tests	Control versus demyelinated layer V; 0.0129	18.61 to 187.4
					Control versus remyelinating layer V; >0.0001	66.82 to 214.9
					Control layer II/III versus V; 0.0015 Control layer IV versus V: 0.0005	-1/9.3 to -33.77 -187.6 to -42.00
x		E	Normally distributed	Two-way ANOVA	Control layer V versus VI; 0.0005 Cortical layer <0.0001; interaction	67.22 to 212.8
				Tukey's post hoc tests	0.0062 Control versus demyelinated layer	1.716 to 26.59
					Control versus remyelinating layer V; 0.0376	-27.38 to -0.6611
					Control layer II/III versus V; <0.0001	-42.10 to -20.65
					Control layer II/III versus VI; 0.0203	1.420 to 22.87
					Control layer V versus VI: <0.0001	-45.22 to -25.77 32.79 to 54.24
					Demyelinated layer II/III versus IV; 0.013	3.105 to 35.28
					Demyelinated layer II/III versus VI; 0.0119	3.305 to 35.48
					0.0012	-40.08 to -7.900
					Demyelinated layer V versus VI; 0.0011	8.100 to 40.28
					Remyelinating layer II/III versus V; <0.0001	-42.74 to -16.46
					Remyelinating layer IV versus V; <0.0001 Remyelinating layer V versus VI	-55.79 to -29.52
					<0.0001	
						(Table continuês.)

#### Table 2. Continued

	Figure	Graph	Data structure	Type of test	p value	Power (95% CI of difference)
aa	6-1	А	Normally distributed	Two-way ANOVA	Treatment 0.0006 Cortical layer <0.0001 Interaction 0.046	
				Tukey's posttest	Layer II/III: control versus demyelinated 0.0025	287.9 to 1,080
					Control: Layer II/III versus V < 0.0001	761.1 to 1,553
					Demyelinated: Layer II/III versus 5 < 0.0014	350.5 to 1,143
		В	Normally distributed	Two-way ANOVA	Treatment 0.0004	
				Tukey's posttest	Layer II/III: control versus demyelinated 0.008	-1,203 to -227.2
					Layer V: control versus demyelinated 0.023	-1,168 to -92.64
		С	Normally distributed	Two-way ANOVA	Treatment 0.0002	
				Tukey's posttest	Layer II/III: control versus demyelinated 0.012	-48.84 to -6.23
					Layer V: control versus demyelinated 0.006	-51.61 to -9.007
		D	Normally distributed	Paired t test	0.0629	-1,675 to 104.7

callosum; ~15% within the cortex, typically along the injection tract; and ~4% within the hippocampal CA1 or alveus, in close proximity to the corpus callosum (Fig. 1K).

The number of RABV-GFP<sup>+</sup> PDGFRa<sup>+</sup> OPCs declined over time:  $19 \pm 11$  RABV-GFP<sup>+</sup> OPCs were detected per mouse at 1 d;  $12 \pm 9$  at 2 d;  $10 \pm 9$  at 3 d;  $9 \pm 9$  at 4 d; and  $4 \pm 5$  at 5 d and by 7 d postinjection, RABV-GFP<sup>+</sup> OPCs could no longer be detected (Fig. 1*L*). While the SAD $\Delta$ G-GFP virus has been reported to induce neuronal cell death after ~16 d (Wickersham et al., 2007), the loss of RABV-GFP<sup>+</sup> OPC within 7 d of infection suggests that OPCs are less tolerant of viral amplification than neurons. The loss of RABV-GFP<sup>+</sup> OPCs by 7 d postinfection prevents the direct association of single RABV-GFP<sup>+</sup> OPCs with specific neuronal subset at 7 d but does not preclude a population level characterization of the synaptically connected neurons.

# Callosal OPCs preferentially synapse with from ipsilateral pyramidal neurons projecting from the CA1 region of the hippocampus and layer V of the cortex

In contrast to RABV-GFP<sup>+</sup> OPCs, RABV-GFP<sup>+</sup> neurons were not detected in *Pdgfra-CreER<sup>T2</sup>* :: *RphiGT* mice until 3 d postinjection and increased in number ~13-fold between 3 (111 ± 119 cells) and 7 days (1,413 ± 687 cells; Fig. 2A,B). This was significantly higher than the number of RABV-GFP<sup>+</sup> neurons in *RphiGT* mice (compare Extended Data Fig. 1-1K and Fig. 2B; Welch's t test: t = 4.070, p = 0.027), consistent with the successful transsynaptic trafficking of SADAG-GFP from infected OPCs to presynaptic neurons in *Pdgfra-CreER<sup>T2</sup>* :: *RphiGT* mice. Based on the location of RABV-GFP<sup>+</sup> neuronal soma at 7 d, we deduced that the infected population of callosal and CA1/alveus OPCs primarily receive synaptic input from cortical (478 ± 397 RABV-GFP<sup>+</sup> neurons per mouse, mean ± SD) or hippocampal neurons (929 ± 558 RABV-GFP<sup>+</sup> neurons per mouse, mean ± SD; Fig. 2*C*–*H*), respectively. A total of 35 ± 18% of the RABV-GFP<sup>+</sup> neurons identified in each brain were in the cortex, 65 ± 18% in the hippocampus, and <1% in the thalamic and hypothalamic nuclei (Fig. 2*I*). Then, 84 ± 7% of all labeled neurons were ipsilateral to the injection site, including ~78% of the RABV-GFP<sup>+</sup> cortical and ~85% of the hippocampal neurons (Fig. 2*C*,*J*). Thalamic and hypothalamic connectivity was exclusively ipsilateral (Fig. 2*J*).

To examine the regional distribution of RABV-GFP<sup>+</sup> neurons within the cortex and hippocampus, data from both hemispheres were combined. Within the hippocampus,  $92 \pm 4\%$  of RABV-GFP<sup>+</sup> neurons were found to reside in the CA1 region, with a smaller proportion in CA2 ( $6 \pm 4\%$ ) or CA3 ( $2 \pm 3\%$ ; Fig. 2*K*). Of the cortical neurons that synapsed onto callosal OPCs,  $39 \pm 18\%$  were located in the somatosensory cortex;  $17 \pm 23\%$  in the visual cortex, and  $14 \pm 10\%$  in the parietal cortex, with RABV-GFP<sup>+</sup> neurons also being identified in the retrosplenial ( $12 \pm 9\%$ ), motor ( $13 \pm 11\%$ ), and auditory cortices ( $39 \pm 18\%$ ), but rarely (<1% per region) within the cingulate, insular, or rhinal cortices (Fig. 2*L*). A small proportion of RABV-GFP<sup>+</sup> neurons were parvalbumin<sup>+</sup> interneurons ( $0.24 \pm 0.19\%$ , ~ 1–4 cells per brain; Extended Data Fig. 1-2*A*–*D*), but the majority (>99%) had the morphology of glutamatergic projection neurons.

Most axons that traverse the mouse corpus callosum project from neurons in cortical layers II/III and V, and a small minority project from neurons in layers IV and VI (Wise, 1975; Fame et al., 2011; Chovsepian et al., 2017). A laminar distribution analysis was possible for RABV-GFP<sup>+</sup> cortical neurons spanning the parietal, somatosensory, auditory, and visual cortices (74 ± 15% of all RABV-GFP<sup>+</sup> cortical neurons per mouse), as their laminar organization allowed identification of layers I, II/III, IV, V, and VI, using Hoechst nuclear staining (Fig. 2*M*). RABV-GFP<sup>+</sup> cortical neurons were distributed



**Figure 1.** RABV-GFP<sup>+</sup> OPCs are located in the corpus callosum, cortex, and hippocampus of *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice. *A*, Experimental time-course schematic. *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice received tamoxifen from P42–45 and were injected with the SADΔG-GFP-(EnvA) virus at P49. Labeled RABV-GFP<sup>+</sup> OPCs were visualized 1, 2, 3, 4, 5, and 7 d postinjection. *B–J*, Representative compressed confocal images of RABV-GFP<sup>+</sup> (green) PDGFRa<sup>+</sup> (red) OPCs, with Hoechst 33342 labeled nuclei (HST; blue), in the cortex (CTX; *A–C*), corpus callosum (CC; *D–F*), and hippocampus (HIP; *G–I*) of adult *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice injected with the SADΔG-GFP-(EnvA) virus. Scale bars represent 20 µm. *K*, The proportion of RABV-GFP<sup>+</sup> OPCs within the CTX, CC, and HIP of individual *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice (*n* = 19). One-way ANOVA *F*<sub>(2,54)</sub> = 79.93, *p*<0.0001; data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. *K*, Quantification of the total number of RABV-GFP<sup>+</sup> OPCs in *Pdgfra-CreER<sup>T2</sup> :: RphiGT* nice (*n* = 24) at 1, 2, 3, 4, 5, and 7 days postinjection. One-way ANOVA *F*<sub>(5,18)</sub> = 19.20, *p* = 0.0004, *R*<sup>2</sup> = 0.94. Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. Error bars display mean ± SD. \**p* ≤ 0.05, \*\*\*\**p* ≤ 0.001 by Tukey's multiple-comparison posttest. Extended data Figure 1-1 supports Figure 1.



**Figure 2.** Callosal OPCs primarily receive synaptic input from ipsilateral pyramidal neurons in cortical layer V. **A**, Experimental time-course schematic and representative compressed confocal image of serial coronal brain sections from *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice 3 and 7 d postinjection (**D**) of the SADAG-GFP-(EnvA) virus into the corpus callosum. Images encompass the injection site and display RABV-GFP (white). **B**, Quantification of the total number of RABV-GFP<sup>+</sup> neurons 3 and 7 d after the SADAG-GFP-(EnvA) virus was delivered into the corpus callosum of P42 + 7 *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mose, **C** and **D** and **C** and **B** and

across layers II/III, IV, V, and VI in all mice (Fig. 2*N*,*O*). Unsurprisingly, no RABV-GFP<sup>+</sup> neurons were located in layer I, a region predominantly composed of interneurons (Larkum, 2013) that do not extend their axons into the corpus callosum (Chovsepian et al., 2017). The majority of RABV-GFP<sup>+</sup> cortical neurons were located within layer V and were morphologically classified as pyramidal neurons ( $52 \pm 9\%$ ; 198 ± 185 neurons per brain). The remaining RABV-GFP<sup>+</sup> cortical neurons were equally distributed across layers II/III, IV, and VI (Fig. 2*N*–*P*). While fewer RABV-GFP<sup>+</sup> neurons were in the contralateral cortex, their distribution across cortical layers was the same (Fig. 2*Q*). As neurons originating in layer II/III comprise the largest population of transcallosal axons in the mouse brain (Fame et al., 2011; Chovsepian et al., 2017), these data suggest that in the adult mouse corpus callosum, OPCs preferentially form synaptic connections with layer V pyramidal neurons.

#### Cuprizone-mediated demyelination reduces the number of neurons that synapse with OPCs

To investigate the impact of demyelination on neuron $\rightarrow$ OPC synaptic connectivity, P49 *Pdgfra-CreER*<sup>72</sup> :: *RphiGT* transgenic mice were maintained on a control diet or transferred to 0.2% (w/w) cuprizone for 5 weeks to induce demyelination (Nguyen et al., 2024) prior to SADAG-GFP-(EnvA) delivery. As cuprizone withdrawal allows significant spontaneous remyelination to occur over a 4 week period (Skripuletz et al., 2011; Gingele et al., 2020; Palavra et al., 2022), a separate cohort of demyelinated *Pdgfra-CreER*<sup>72</sup> :: *RphiGT* mice were returned to a control diet, to support remyelination prior to SADAG-GFP-(EnvA) virus delivery. In C57BL/6 mice, 5 weeks of cuprizone feeding induces a ~31% decrease in MBP<sup>+</sup> pixel coverage in the corpus callosum at ~bregma –1.5 mm (Fig. 3*A*–*C*,*E*) and a 5 week remyelination period restores MBP<sup>+</sup> pixel coverage in the corpus callosum to control levels (Fig. 3*D*,*E*).

When SAD $\Delta$ G-GFP-(EnvA) virus was injected into the corpus callosum of control or demyelinated P84 *Pdgfra-CreER*<sup>T2</sup> :: *RphiGT* transgenic mice (Fig. 3*F*–*H*), a similar number of PDGFR $\alpha^+$  OPCs became RABV-GFP<sup>+</sup> in control (10 ± 4 cells) and demyelinated (7 ± 9 cells) mice (Fig. 3*I*), and most of the RABV-GFP<sup>+</sup> OPCs were located within the corpus callosum (control 63 ± 26% vs demyelinated 77 ± 20%; Fig. 3*J*). Perhaps unsurprisingly, demyelination was associated with lateral extension of the OPC labeling along the corpus callosum (*F* test to compare variance *p* = 0.02; Fig. 3*K*,*L*) and a greater rostrocaudal distribution (Fig. 3*M*,*N*). This is likely the result of increased viral diffusion in the absence of myelin or the increased migration of infected OPCs.

To determine if the population of neurons that synapse with callosal OPCs was altered by demyelination, P85 control and demyelinated Pdgfra- $CreER^{T2}$  :: RphiGT mice were injected with SAD $\Delta$ G-GFP-(EnvA) and maintained on standard chow for 7 d to ensure robust neuronal RABV-GFP expression (P85 + 7D). We first compared the number and location of RABV-GFP<sup>+</sup> neurons in P85 + 7D control mice (n = 5) with that of our previous P49 + 7D controls (n = 4; Fig. 2). While highly variable, we found that the number and distribution of RABV-GFP<sup>+</sup> neurons was equivalent across cohorts (statistical comparison in Table 1), and data from the n = 9 control mice were pooled prior to comparison with the demyelinated mice. As the number of RABV-GFP<sup>+</sup> neurons in control mice was highly variable (F test to compare variance, p = 0.0083), we performed a square root data transformation prior to data analysis. We determined that fewer RABV-GFP<sup>+</sup> neurons were labeled in demyelinated mice compared with controls (Fig. 4A, Table 2). These data are consistent with OPCs forming fewer synapses with demyelinated axons.

The reduced number of RABV-GFP<sup>+</sup> neurons in cuprizone-demyelinated mice is consistent with a previous report that white matter OPCs within demyelinated focal lesions receive fewer synaptic inputs (Etxeberria et al., 2010; Gautier et al., 2015; Sahel et al., 2015). To explore the possibility that OPCs receive fewer synaptic inputs in the demyelinated corpus callosum, we performed whole-cell patch-clamp electrophysiology of callosal OPCs in young adult *Pdgfra-H2BGFP* mice that received a standard or cuprizone diet for 5 weeks. Mice were returned to normal chow for 1 day prior to whole-cell patch-clamp recordings from GFP<sup>+</sup> OPCs in the body of the corpus callosum (~bregma -1.5 mm) obtained in acute brain slices. The identity of GFP<sup>+</sup> OPCs was confirmed by a membrane capacitance (Cm) <50 pF and the presence of a TTX-sensitive voltage-gated sodium currents >100 pA, evoked by a voltage step from -70 to 0 mV (Fig. 4*B*; Pitman et al., 2020). Cuprizone demyelination did not alter the membrane resistance or capacitance of OPCs (Table 3), and mEPSC frequency was also unchanged (recordings made in the presence of TTX; Fig. 4*C*–*E*). We would not expect a change in the composition of presynaptic neurons to impact mEPSC properties. This was confirmed by measurement of mEPSC amplitude and decay time (Fig. 4*F*,*G*). However, the low frequency of mEPSCs in OPCs restricted the number of events available for analysis. To overcome this limitation, OPCs were exposed to the secretagogue, ruthenium red, to increase the frequency of glutamate release in the presence of TTX. This increased mEPSC frequency significantly for

nuclear stain (HST; white) was used to identify each cortical layer (I, II/III, IV, V, and VI). *N*, Compressed confocal image of the somatosensory cortex of a P42 + 14 *Pdgfra-CreER*<sup>T2</sup> :: *RphiGT* transgenic mouse 7 d postinjection of the SAD $\Delta$ G-GFP-(EnvA) virus into the corpus callosum, showing RABV-GFP<sup>+</sup> (green) cortical neurons distributed across the cortical laminar. *O*, Quantification of the total number of RABV-GFP<sup>+</sup> cortical neurons in cortical layers II/III, IV, V, and VI of individual *Pdgfra-CreER*<sup>T2</sup> :: *RphiGT* transgenic mice (*n* = 4). One-way ANOVA: *F*<sub>(3,12)</sub> = 1.904, *p* = 0.183. *P*, The proportion of RABV-GFP<sup>+</sup> cortical neurons in cortical layers II/III, IV, V, and VI of individual *Pdgfra-CreER*<sup>T2</sup> :: *RphiGT* transgenic mice (*n* = 4). One-way ANOVA: *F*<sub>(3,12)</sub> = 1.904, *p* = 0.183. *P*, The proportion of RABV-GFP<sup>+</sup> cortical neurons in cortical layers II/III, IV, V, and VI of individual *Pdgfra-CreER*<sup>T2</sup> :: *RphiGT* transgenic mice (*n* = 4). One-way ANOVA: *F*<sub>(3,12)</sub> = 1.909, *p* < 0.0001. *Q*, The proportion of ipsilateral and contralateral RABV-GFP<sup>+</sup> cortical neurons within cortical layers II/III, IV, V, and VI, in individual *Pdgfra-CreER*<sup>T2</sup> :: *RphiGT* transgenic mice (*n* = 4). Two-way ANOVA: interaction *F*<sub>(3,24)</sub> = 0.772, *p* = 0.521; laminar *F*<sub>(3,24)</sub> = 33.13, *p* < 0.0001; and hemisphere *F*<sub>(1,24)</sub> = 2.839 × 10<sup>-5</sup>, *p* = 0.996. Data are expressed as mean ± SD. Closed circles represent individual animals. Scale bars represent 100 µm (*D*-*G* and *M*, *N*). \**p* ≤ 0.05, \**rp* ≤ 0.001, \*\*\*\**p* ≤ 0.001 by Dunnett's T3 (*I*) or Tukey's (*J*-*L* and *P*) multiple-comparison posttest. Extended Data Figure 2-1 supports Figure 2.



**Figure 3.** Cuprizone induces reversible demyelination of the corpus callosum and alters the distribution but not the number of RABV-GFP<sup>+</sup> OPCs. **A**, Experimental time-course schematic. C57BL/6 control mice were retained on normal chow and tissue collected at P84. Demyelinated mice received 0.2% (w/w) cuprizone chow from P49 and tissue was collected at P84. Remyelinating mice received 0.2% (w/w) cuprizone chow P49–P84 and were returned to normal chow until tissue was collected at P119. **B–D**, Confocal images of the medial corpus callosum (CC) in coronal brain sections immunolabeled to detect MBP (white), taken from a control (**A**), demyelinated (**B**), and remyelinating (**C**) adult C57bL/6 mice. **E**, Quantification of the area of the medial CC labeled for MBP in individual control, demyelinated, and remyelinating C57bL/6 mice (n = 5 per group; mean ± SD). One-way ANOVA:  $F_{(2,12)} = 78.92$ , p < 0.0001. **F**, Experimental time-course schematic. *Pdgfra*-CreER<sup>T2</sup> :: *RphiGT* mice received tamoxifen from P42–45. Control mice were retained on control diet for the duration of the experiment. Demyelinated mice were transferred to 0.2% (w/w) cuprizone chow F84. All mice were on a normal diet and received the SADAG-GFP-(EnvA) virus at P85. RABV-GFP<sup>+</sup> OPCs were analyzed at P87 (3D). **G–H**, Compressed confocal

control and demyelinated OPCs (Fig. 4*H*) and increased the number of mEPSCs that could be evaluated to confirm that mEPSC amplitude (Fig. 4*I*) and decay time (Fig. 4*J*) were unchanged by demyelination.

# Callosal OPCs primarily receive synaptic input from ipsilateral cortical neurons in the demyelinated and remyelinating brain

To determine whether demyelination or myelin repair alters the type of neuron that synapses with callosal OPCs, we analyzed the location of RABV-GFP<sup>+</sup> neurons within the brains of control, demyelinated, or remyelinating mice. To induce demyelination, *Pdgfra-CreER<sup>T2</sup>* :: *RphiGT* mice received 5 weeks of cuprizone chow (0.2% w/w), and to induce remyelination, mice were returned to normal chow for a further 5 weeks (Fig. 5A). While demyelinated and remyelinating mice had significantly fewer RABV-GFP<sup>+</sup> neurons per brain compared with control mice (Fig. 4A), RABV-GFP<sup>+</sup> neurons were still primarily located in the ipsilateral hemisphere of control (86 ± 6%), demyelinated (88 ± 3%), and remyelinating (95 ± 4%) mice (Fig. 5*B*,*C*).

Though infected OPCs remained synaptically connected to cortical, hippocampal, and thalamic/hypothalamic neurons following demyelination and remyelination, the relative distribution of the labeled neurons was different in the demyelinated brain. The total number of total cortical RABV-GFP<sup>+</sup> neurons was unchanged (Fig. *5E*) but they comprised a greater proportion of the RABV-GFP<sup>+</sup> neurons in the demyelinated mice (Fig. *5F*). In the hippocampus, the total number of RABV-GFP<sup>+</sup> neurons was decreased in demyelinated and remyelinating mice compared with controls, and hippocampal neurons comprised a smaller proportion of total RABV-GFP<sup>+</sup> neurons (Fig. *5G*,*H*). When examining the contralateral side of the brain, we determined that the number of RABV-GFP<sup>+</sup> neurons located within the cortex was decreased in remyelinating mice and represented a smaller proportion of the RABV-GFP<sup>+</sup> neurons (Fig. *5I*,*J*). The number of contralateral hippocampal RABV-GFP<sup>+</sup> neurons was similarly reduced in the remyelinating mice (Fig. *5K*) but represented a similar proportion of total RABV-GFP<sup>+</sup> neurons (Fig. *5L*). The proportion of RABV-GFP<sup>+</sup> neurons located in the thalamic and hypothalamic nuclei remained low in all conditions, typically accounting for <5% of RABV-GFP<sup>+</sup> neurons (Fig. *5M*).

RABV-GFP<sup>+</sup> neurons were primarily located in the retrosplenial, motor, parietal, somatosensory, visual, auditory, and cingulate cortices of all mice, with a small number (<1% of cortical RABV-GFP<sup>+</sup> neurons) in the insular, rhinal, limbic, and temporal association cortices (Fig. 5*N*). In remyelinating mice, a higher proportion of RABV-GFP<sup>+</sup> neurons were found in the retrosplenial cortex and a decreased proportion in the somatosensory cortex compared with controls (Fig. 5*N*), which may suggest that retrosplenial connections are prioritized by OPCs. In contrast, there was no change in the proportion of RABV-GFP<sup>+</sup> hippocampal neurons located within CA1-3 (Fig. 5*O*).

#### OPCs form fewer synaptic connections with layer V pyramidal neuron axons in the demyelinated corpus callosum

To compare the laminar distribution of RABV-GFP<sup>+</sup> cortical neurons under control, demyelinated, and remyelinating conditions, RABV-GFP<sup>+</sup> neurons in the parietal, somatosensory, auditory, and visual cortices were classified as belonging to layer I, II/III, IV, V, or VI (Fig. 6*A*–*C*). Demyelination was associated with a significant decrease in the number (Fig. 6*D*) and proportion (Fig. 6*E*) of RABV-GFP<sup>+</sup> cortical neurons located in layer V. Conversely, the proportion of RABV-GFP<sup>+</sup> cortical neurons located in layer II/III increased in demyelinated mice (*p* = 0.050). In the remyelinating mice, layer V RABV-GFP<sup>+</sup> cortical neurons located mice (*p* = 0.050). In the remyelinating mice, layer V RABV-GFP<sup>+</sup> cortical neuron number remained low compared with controls (Fig. 6*D*) but comprised a higher proportion of cortical RABV-GFP<sup>+</sup> neurons than for demyelinated mice (Fig. 6*E*). Indeed, under control conditions, 52 ± 8% of the synapses that OPC formed with cortical neurons were with layer V pyramidal cells and a significantly smaller proportion with layers II/III (21 ± 9%), IV (18 ± 5%), or VI (9 ± 5%; Fig. 6*F*) pyramidal neurons. Following demyelination, the distribution of RABV-GFP<sup>+</sup> cortical neurons changed, and the proportion of RABV-GFP<sup>+</sup> neurons was more evenly distributed between layers II/III (33 ± 4%) and V (38 ± 3%; Fig. 6*G*). Following remyelination, the population of cortical neurons that synapsed with callosal OPCs resembled that of control mice, as 52 ± 17% RABV-GFP<sup>+</sup> neurons were layer V neurons (Fig. 6*H*). These

image of coronal brain section from a control (A) or cuprizone demyelinated (B) adult Pdgfra-CreER<sup>T2</sup> :: RphiGT transgenic mice 3D after the SADAG-GFP-(EnvA) virus (white) was injected into the corpus callosum (CC). The cortex (CTX), CC, hippocampus (HIP), and the location of viral microinjection (marked "X") are indicated on the images. I, Quantification of the total number of RABV-GFP<sup>+</sup> PDGFRa<sup>+</sup> OPCs per control or demyelinated Pdgfra-CreER<sup>T2</sup> :: RphiGT SAD $\Delta$ G-GFP-(EnvA) injected transgenic mouse (n = 5 per group). Unpaired t test: t = 0.678, p = 0.517. J, The proportion of RABV-GFP<sup>+</sup> OPCs located in the CTX, CC, and HIP in control or demyelinated *Pdgfrα-CreER<sup>T2</sup> :: RphiGT* transgenic mice (*n* = 5 per group). Two-way ANOVA: region × treatment  $F_{(2,24)} = 0.715$ , p = 0.499; region  $F_{(2,24)} = 18.63$ , p < 0.0001; treatment  $F_{(1,24)} = 0.286$ , p = 0.597. Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. K, The distance (mm) of individual callosal and hippocampal RABV-GFP<sup>+</sup> OPCs from the site of viral microinjection on the medial-lateral axis within control (n = 35 RABV-GFP<sup>+</sup> OPCs) or demyelinated (n = 25 RABV-GFP<sup>+</sup> OPCs) Pdgfra-CreER<sup>72</sup> :: RphiGT transgenic mice (n = 5 mice per group). KS test, p = 0.0021. L, The mean distance (mm) of each RABV-GFP<sup>+</sup> OPC from the site of viral microinjection along the medial-lateral axis, per control or demyelinated Pdgfrα-CreER<sup>T2</sup> :: RphiGT transgenic mouse (n = 5 per group). Welch's t test: t = 2.052, df = 4.503, p = 0.102. M. The distance (mm) of individual callosal and hippocampal RABV-GFP<sup>+</sup> OPCs from the site of viral microinjection on the rostral-caudal axis within control (n = 35 RABV-GFP<sup>+</sup> OPCs) or demyelinated (n = 25 RABV-GFP<sup>+</sup> OPCs) Pdgfrα-CreER<sup>T2</sup> :: RphiGT transgenic mice cohorts (n = 5 mice per group). KS test, p = 0.0002. N, The mean distance (mm) of each RABV-GFP<sup>+</sup> OPC from the site of viral microinjection along the rostral-caudal axis, in control or demyelinated Pdgfra-CreER<sup>72</sup> :: RphiGT transgenic mouse (n = 5 per group). Welch's t test: t = 1.22, df = 7.617, p = 0.25. Data are expressed as mean ± SD. Closed circles represent individual animals (E, I-L, N), or individual OPCs (K,M). Scale bars represent 100 μm (**B–D**) or 500 μm (**G–H**). \*\**p* ≤ 0.01, \*\*\**p* ≤ 0.001, \*\*\*\**p* ≤ 0.0001 by Tukey's multiple-comparison posttest (**E**, **J**).



Figure 4. The number of RABV-GFP<sup>+</sup> neurons detected in Pdgfra-CreER<sup>T2</sup>:: RphiGT transgenic mice decreases following cuprizone demyelination. A, Quantification of the total number of RABV-GFP<sup>+</sup> neurons in *Pdgfra-CreER<sup>72</sup> :: RphiGT* transgenic mice 7D after callosal injection with the SAD $\Delta$ G-GFP-(EnvA) virus in control, demyelinated or remyelinating mice ( $n \ge 5$  per group). One-way ANOVA:  $F_{(2,16)} = 9.486$ , p = 0.0019; with Tukey's posttest. Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. B, Representative traces of voltage-gated sodium currents evoked by a depolarizing step from -70 to 0 mV recorded from GFP<sup>+</sup> OPCs (in the absence and presence of tetrodotoxin; TTX), newly born OLs (pre-OLs), and mature OLs, in the corpus callosum of acute brain slices generated from P85 Pdgfra-H2BGFP transgenic mice. C, D, Example traces showing mEPSCs recorded from GFP<sup>+</sup> callosal OPCs in acute brain slices generated from P85 Pdgfrα-H2BGFP transgenic mice following 5 weeks of control (C) or cuprizone (0.2% w/w) (D) diet, in the presence of TTX or TTX and the secretagogue ruthenium red (RR). Representative examples of individual ePSCs show the fast rise and decay times. E, H, The frequency of mEPSCs recorded from individual GFP<sup>+</sup> callosal OPC of control or demyelinated mice. Recordings were made in the presence of TTX (E; REML, linear mixed effect ANOVA: F(1,9.784) = 0.1211, p = 0.7352) or TTX and RR (H; REML linear mixed effect model: F<sub>(1,16)</sub> = 0.795, p = 0.3857). F, I, The mean amplitude of mEPSCs recorded in individual callosal OPC of control or demyelinated mice. Recordings were made in the presence of TTX (F: REML, linear mixed effect ANOVA: F<sub>(1,7.054)</sub> = 0.594, p = 0.465) or TTX and RR (J: REML, linear mixed effect ANOVA: F(1,6.973) = 2.574, p = 0.152). G, J, The mean decay time for mEPSCs recorded in individual callosal OPC of control or demyelinated mice. Recordings were made in the presence of TTX (G: REML, linear mixed effect ANOVA: F<sub>(1.8.602)</sub> = 3.299, p = 0.2042) or TTX and RR (REML, linear mixed effect ANOVA:  $F_{(1,16)} = 1.6083$ , p = 0.222). Data are expressed as mean ± SD. Closed circles represent individual animals (A = 9 control, 4 demyelinated, 6 remyelinating) or individual cells (*E–J*:6 control and 12 demyelinated OPCs).  $*p \le 0.05$ ,  $**p \le 0.01$ .

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Mean ± SD	Pipette resistance	Ra (Ω)	Rm (Ω)	Cm (pF)	I <sub>Na</sub> (pA) –70 to 0 mV step
Control ( $n = 6$ OPCs) Demyelinated ( $n = 12$ OPCs)	$4.5 \pm 0.9$ $5.0 \pm 0.9$	$4.7 \pm 1.9$ $8.9 \pm 2.7$	$828 \pm 600 \\ 835 \pm 707$	$26.3 \pm 6.0$ $25.3 \pm 3.9$	-229±89 -231±90

Mean ± SD.



**Figure 5.** In the demyelinated brain callosal OPCs more frequently synapse with ipsilateral cortical neurons. **A**, Experimental time-course schematic. *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice received tamoxifen from P42–45 and were split into three groups. Control mice received normal chow while demyelinated and remyelinating mice received 0.2% (w/w) cuprizone chow from P49 to P84. Control and demyelinated mice were injected with SADΔG-GFP-(EnvA) virus at P85 and analyzed at P92. Remyelinating mice were injected at P119 and analyzed at P126. **B–D**, Representative coronal brain sections from control (**A**), demyelinated (**B**), and remyelinating (**C**) adult *Pdgfra-CreER<sup>T2</sup> :: RphiGT* transgenic mice 7 d postinjection of the SADΔG-GFP-(EnvA) virus (white) into the corpus callosum. **E**, **F**, Quantification of the total number of RABV-GFP<sup>+</sup> neurons within the cortex of *Pdgfra-CreER<sup>T2</sup> :: RphiGT* control, demyelinated, and remyelinating mice (**E**; one-way ANOVA:  $F_{(2,16)} = 2.283$ , p = 0.134) and the proportion of RABV-GFP<sup>+</sup> neurons that were found within the cortex (**F**; one-way



**Figure 6.** In the demyelinated brain, OPCs receive fewer synapses from layer V and more from layer II/III. *A*–*C*, Compressed confocal images of the cortex of control (*A*), demyelinated (*B*), and remyelinating (*C*) adult *Pdgfra-CreER*<sup>72</sup> :: *RphiGT* transgenic mice, 7 d after the SADΔG-GFP-(EnvA) virus was injected into the corpus callosum, showing RABV-GFP<sup>+</sup> (green) cortical neurons distributed across cortical layers I, II/III, IV, V, and VI. Scale bars represent 100 µm. *D*, *E*, The total number (2-way ANOVA: interaction  $F_{(6,64)} = 1.945$ , p = 0.087; laminar  $F_{(3,64)} = 4.196$ , p = 0.009; treatment  $F_{(2,64)} = 8.276$ , p = 0.001) of RABV-GFP<sup>+</sup> cortical neurons positioned in layers II/III, IV, V, and VI of *Pdgfra-CreER*<sup>72</sup> :: *RphiGT* control, demyelinated, or remyelinating mice (9 controls, 4 demyelinated, and 6 remyelinating mice) and the proportion of RABV-GFP<sup>+</sup> cortical neurons in each laminar (2-way ANOVA: interaction  $F_{(6,64)} = 3.354$ , p = 0.006; laminar  $F_{(3,64)} = 59.97$ , p < 0.0001; treatment  $F_{(2,64)} = 0.005$ , p = 0.9995). *F*–*H*, Data from *E*, displaying post hoc analysis of the proportion of RABV-GFP<sup>+</sup> cortical neurons in cortical laminar II/III, IV, V, and VI of *Pdgfra-CreER*<sup>72</sup> :: *RphiGT* control (*F*), demyelinated (*G*), or remyelinating (*H*) mice. Data are expressed as mean ± SD. \* $p \le 0.05$ , \*\* $p \le 0.01$ , \*\*\*\* $p \le 0.001$ , \*\*\*\* $p \le 0.001$  by Tukey's multiple-comparisons posttest. Extended Data Figure 6-1 supports Figure 6.

ANOVA: F<sub>(2,16)</sub> = 5.38, p = 0.0163; 9 controls, 4 demyelinated, and 6 remyelinating mice). Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. G, H, Quantification of the total number of RABV-GFP<sup>+</sup> neurons in the hippocampus of Pdgfra-CreER<sup>72</sup> :: RphiGT control, demyelinated, and remyelinating mice (G; one-way ANOVA: F<sub>(2,16)</sub> = 13.30, p = 0.0003) and the proportion of neurons located within the hippocampus (H; one-way ANOVA: F(2,16) = 5.971, p = 0.016; 9 controls, 4 demyelinated and 6 remyelinating mice). Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. I, J, Quantification of the number of RABV-GFP+ neurons within the contralateral cortex of Pdgfrα-CreER<sup>T2</sup> :: RphiGT control, demyelinated, and remyelinating mice (I; one-way ANOVA: F<sub>(2,16)</sub>=8.864, p = 0.0026) and the proportion of RABV-GFP<sup>+</sup> cortical neurons located within the contralateral hemisphere (*J*; one-way ANOVA: F<sub>(2,16)</sub> = 7.49, p = 0.005; 9 controls, 4 demyelinated, and 6 remyelinating mice). Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. K, L, Quantification of the number of RABV-GFP<sup>+</sup> neurons within the contralateral hippocampus of Pdgfra-CreEA<sup>T2</sup> :: RphiGT control, demyelinated, and remyelinating mice (K; one-way ANOVA: F(2,16) = 13.30, p = 0.0003) and the proportion of RABV-GFP<sup>+</sup> hippocampal neurons located within the contralateral hemisphere (L; one-way ANOVA:  $F_{(2,16)} = 1.824$ , p = 0.19; 9 controls, 4 demyelinated, and 6 remyelinating mice). Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. M, The proportion of RABV-GFP<sup>+</sup> neurons that were located within the thalamic or hypothalamic nuclei of Pdgfra-CreER<sup>T2</sup> :: RphiGT control, demyelinated, or remyelinating mice (one-way ANOVA: F<sub>(2,16)</sub> = 0.656, p = 0.53). Data were square root transformed for analysis to satisfy the assumptions of normality and homoscedasticity of the variances. N, The proportion of cortical RABV-GFP<sup>+</sup> neurons located in each cortical region of Pdgfra-CreER<sup>T2</sup> :: RphiGT control, demyelinated, or remyelinating mice. Note that cortical regions containing >1% of RABV-GFP<sup>+</sup> neurons are shown. Two-way ANOVA: interaction  $F_{(12,112)}$ =2.325, p = 0.0108; region  $F_{(6,112)} = 13.75$ , p < 0.0001; treatment  $F_{(2,112)} = 0.00187$ , p = 0.9981. **0**, The proportion of hippocampal RABV-GFP<sup>+</sup> neurons within each hippocampal subregion of Pdgfra-CreER<sup>T2</sup> :: RphiGT control, demyelinated, or remyelinating mice. Two-way ANOVA: interaction F<sub>(6,60)</sub> = 1.042, p = 0.4072; region  $F_{(3,60)} = 3,289$ , p < 0.0001, treatment  $F_{(2,60)} = 0.0677$ , p = 0.93047. Data are expressed as mean ± SD. \* $p \le 0.05$ , \*\* $p \le 0.01$ , \*\*\*\* $p \le 0.0001$  by Tukey's multiple-comparison posttest.

data suggest that following a demyelinating injury, OPCs increasingly synapse with layer II/III and have a similar likelihood of synapsing with layer II/III or layer V pyramidal neurons until myelin is restored, after which they again favor layer V pyramidal cells.

To explore the possibility that the shift in the population of cortical neurons synapsing with OPCs following demyelination relates to a change in cortical neuron activity, we quantified the expression cFos in neurons within the visual cortex of control and demyelinated mice. Intriguingly, in the visual cortex, cuprizone demyelination reduces the density of NeuN<sup>+</sup> cells in layer II/III but not layer V (Extended Data Fig. 6-1*A*). The density and proportion of NeuN<sup>+</sup> cells that express cFos is increased in both layer II/III and layer V in demyelinated mice (Extended Data Fig. 6-1*B*,*C*) but the relative intensity of cFos expression tends to be greater in cells from layer II/III compared with layer V (Extended Data Fig. 6-1*D*). These data may indicate differential activity changes in cortical neuron populations following demyelination.

#### Discussion

OPCs in the developing and adult mouse brain receive synaptic input from neurons (Bergles et al., 2000; Lin and Bergles, 2004; Ziskin et al., 2007). However, it is unclear whether OPCs form synapses with any active axon in their territory or target specific neuron populations. By labeling a subset of OPCs in the adult mouse corpus callosum with a modified rabies virus, which is limited to monosynaptic retrograde tracing, it was possible to visualize neurons that directly synapse onto the labeled OPCs. Infected OPCs received input, almost exclusively, from excitatory cortical and subcortical neurons that project their axons within the vicinity of the infected OPCs. The vast majority of cells labeled by the retrograde virus were ipsilateral CA1 hippocampal neurons and ipsilateral layer V cortical neurons that presumably synapsed with labeled OPCs but was also associated with callosal OPCs forming synapses equally with layer II/III neurons and layer V cortical neurons. Remyelination was associated with OPCs reverting to the patterns of synaptic communication seen in control mice.

The body of the corpus callosum, at ~bregma –1.5 mm, primarily contains the axons of neurons from the motor, somatosensory, parietal, and, to a lesser extent, visual, auditory, and retrosplenial cortices (Ku and Torii, 2020). Our data indicate that callosal OPCs synapse with the axons of neurons projecting from each of these regions. As the majority of RABV-GFP<sup>+</sup> neurons were ipsilateral excitatory pyramidal neurons, OPCs may preferentially synapse with more proximal regions of axons or extend processes to synapse with axons in the external capsule, which contains corticofugal and corticocortical projecting axons, many of which have ipsilateral targets (Khibnik et al., 2014; Allen Mouse Brain Atlas, mouse.brain-map.org/experiment/show/657041814). The large number of ipsilateral RABV-GFP<sup>+</sup> hippocampal neurons also suggests that OPCs within the CA1 and alveus are synaptically integrated. The alveus is a major efferent tract that allows hippocampal neurons within CA1-3 and the subiculum to send caudal projections into the ipsilateral fimbria-fornix (Daitz and Powell, 1954; Cenquizca and Swanson, 2007; Lingford-Hughes and Kalk, 2012; Duvernoy et al., 2013). RABV-GFP<sup>+</sup> labeled OPCs may also contact the subset of CA1 hippocampal neurons that project dorsally around the corpus callosum to the retrosplenial, somatosensory, auditory, and visual cortices (Cenquizca and Swanson, 2007) and hippocampal CA1-3 neurons projecting to homotopic and heterotopic contralateral hippocampal regions though the hippocampal commissure (Voneida et al., 1981; Tamamaki et al., 1988; Leung et al., 1992; Cenquizca and Swanson, 2007; Duvernoy et al., 2013). Contact with these hippocampal commissural axons could account for the small number of RABV-GFP<sup>+</sup> neurons in the contralateral hippocampus.

It was unexpected that ~52% of RABV-GFP<sup>+</sup> cortical neurons were layer V neurons and ~21% were layer II/III neurons as some estimates indicate that layer II/III neurons account for up to 80% of the callosal axon population (Wise, 1975; Fame et al., 2011; Gaspard and Vanderhaeghen, 2011; Chovsepian et al., 2017). These data suggest that in adulthood, OPCs preferentially synapse with layer V axons, an idea that is further supported by OPCs synapsing equally with layer V and layer II/III neurons following demyelination. Layer V can be further subdivided into Va and Vb, and the location of most RABV-GFP<sup>+</sup> layer V neurons immediately under layer IV (Figs. 2, 6) suggests that they are Va "slender tufted" pyramidal neurons that primarily comprise corticocortical association neurons and callosal commissural neurons (Veinante et al., 2000; Larsen et al., 2007; Groh et al., 2010; Gaspard and Vanderhaeghen, 2011; Grant et al., 2012; Malmierca et al., 2014; Wang et al., 2018).

Layer V pyramidal neurons are among the first population myelinated, with myelin added from ~P10 in the developing mouse brain (Battefeld et al., 2019) and while less is known about the detailed timing of myelination of layer II/III pyramidal cells, MBP localization suggests that it begins at ~P21 (Vincze et al., 2008). In the motor cortex, layer V pyramidal neurons receive new myelin internodes throughout life and their myelination can be enhanced by increasing their activity (Gibson et al., 2014). The axons of layer V somatosensory and visual cortical neurons can have continuous stretches of myelination along axons projecting toward the corpus callosum, while layer II/III neurons are intermittently myelinated in adult mice (Tomassy et al., 2014). These observations suggest that layer V neurons are more heavily myelinated than layer II/III neurons. However, the level of myelination can also vary with axon region (Tomassy et al., 2014; Call and Bergles, 2021) and only ~8% of the somatosensory layer V axon collaterals that project to layer I are myelinated, and of those, most are sparsely myelinated (Call and Bergles, 2021). It is unclear whether layer V axons that traverse the corpus callosum are heavily or sparsely myelinated. However, as OPCs form synapses with unmyelinated axons segments (Kukley et al.,

2007; Ziskin et al., 2007; Gautier et al., 2015), our data suggest that layer V axons are intermittently myelinated in the corpus callosum.

Intrinsic neuronal properties impact neuronal activity and influence the formation of  $axon \rightarrow OPC$  synapses. Under normal conditions, layers II/III and Va pyramidal neurons have similar firing patterns, both generating trains of regularly spaced, or intermittent but repetitive action potential bursts upon depolarization (Dégenètais et al., 2002; Moyer et al., 2002). As OPCs preferentially synapse with electrically active neurons (Moura et al., 2023), it is possible that OPCs target layer II/III neurons in the demyelinated corpus callosum because of their altered firing pattern. Whole-cell patch-clamp recordings from layer II/III neurons in the demyelinated anterior cingulate cortex indicate that more current is required to elicit an equivalent action potential firing frequency (Kawabata et al., 2025). However, this may be a homeostatic plasticity response to reduce firing, as cuprizone demyelination is associated with a significant increase in the proportion of layers II/III and V neurons that express cFos in the visual cortex (Extended Data Fig. 6-1). A detailed study of spontaneous activity across the cortical laminar would be required to understand whether the relative activity of these populations might explain the change in OPC preference following demyelination.

We predict that OPC synapse formation precedes differentiation and remyelination. High-frequency action potential trains can increase the amount of free residual calcium within axons and facilitate glutamate release at axon $\rightarrow$ OPC synapses (Nagy et al., 2017). In vivo, the high-frequency stimulation of axons in the corpus callosum of adult mice is also associated with increased OPC differentiation and an increase in the number of newborn EdU<sup>+</sup> oligodendrocytes (Nagy et al., 2017). Similarly, the optogenetic or chemogenetic activation of a subset of cortical neurons promotes the remyelination of these neurons in the corpus callosum of healthy (Gibson et al., 2014; Mitew et al., 2018) and demyelinated mice (Ortiz et al., 2019). Given the high proportion of callosal axons that are remyelinated after cuprizone withdrawal, this is likely to include a substantial number of layer II/III neurons. It would be interesting to determine the increase in layer II/III neuron $\rightarrow$ OPCs synapse formation indicate that layer II/III neurons are prioritized for remyelination.

Our study provides further evidence that OPCs located in the corpus callosum preferentially form synaptic connections with neuronal subpopulations predominantly in the cortex and hippocampus. By showing that OPCs can synapse with different neurons in health and following demyelination, we provide further insight into neuron-OPC communication and the cellular interactions that are impacted in demyelinating diseases such as multiple sclerosis.

#### **Data Availability**

All individual data points are provided in the data figures or in the extended data of the manuscript. Requests for any other data files should be directed to Dr. Carlie Cullen (carlie.cullen@mater.uq.edu.au) or Prof. Kaylene Young (kaylene.young@utas.edu.au).

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