

Vagus nerve stimulation enhances remyelination and decreases innate neuroinflammation in lysolecithin-induced demyelination

Helen Bachmann^{a,*}, Boris Vandemoortele^{b,c,d}, Vanessa Vermeirssen^{b,c,d}, Evelien Carrette^a, Kristl Vonck^a, Paul Boon^a, Robrecht Raedt^{a,1}, Guy Laureys^{a,1}

^a Ghent University, 4 Brain, Department of Neurology, Ghent University Hospital, Belgium

^b Laboratory for Computational Biology, Integromics and Gene Regulation (CBIGR), Cancer Research Institute Ghent (CRIG), Ghent, Belgium

^c Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium

^d Department of Biomolecular Medicine, Ghent University, Ghent, Belgium

ARTICLE INFO

Keywords:

Vagus nerve stimulation
Multiple sclerosis
Remyelination
Neuroinflammation
Microglia
Astrocytes

ABSTRACT

Background: Current treatments for Multiple Sclerosis (MS) poorly address chronic innate neuroinflammation nor do they offer effective remyelination. The vagus nerve has a strong regulatory role in inflammation and Vagus Nerve Stimulation (VNS) has potential to affect both neuroinflammation and remyelination in MS.

Objective: This study investigated the effects of VNS on demyelination and innate neuroinflammation in a validated MS rodent model.

Methods: Lysolecithin (LPC) was injected in the corpus callosum (CC) of 46 Lewis rats, inducing a demyelinated lesion. 33/46 rats received continuously-cycled VNS (cVNS) or one-minute per day VNS (1minVNS) or sham VNS from 2 days before LPC-injection until perfusion at 3 days post-injection (dpi) (corresponding with a demyelinated lesion with peak inflammation). 13/46 rats received cVNS or sham from 2 days before LPC-injection until perfusion at 11 dpi (corresponding with a partial remyelinated lesion). Immunohistochemistry and proteomics analyses were performed to investigate the extend of demyelination and inflammation.

Results: Immunohistochemistry showed that cVNS significantly reduced microglial and astrocytic activation in the lesion and lesion border, and significantly reduced the Olig2+ cell count at 3 dpi. Furthermore, cVNS significantly improved remyelination with 57.4 % versus sham at 11 dpi. Proteomic gene set enrichment analyses showed increased activation of (glutamatergic) synapse pathways in cVNS versus sham, most pronounced at 3 dpi.

Conclusion: cVNS improved remyelination of an LPC-induced lesion. Possible mechanisms might include modulation of microglia and astrocyte activity, increased (glutamatergic) synapses and enhanced oligodendrocyte clearance after initial injury.

1. Introduction

Multiple Sclerosis (MS), an inflammatory and degenerative central nervous system (CNS) disease, affects around 2.8 million people worldwide [1]. MS pathophysiology has been attributed to immune-mediated demyelination and axonal transection by CNS-infiltrating autoreactive T- and B-lymphocytes [2], along with neurodegenerative mechanisms like mitochondrial deficiency, glutamate excitotoxicity and oxidative stress [2]. The CNS innate immune system, with microglia and astrocytes as main components, also

contributes to MS pathogenesis through chronic inflammation and subsequent disability progression [2–4]. More specifically a close astrocyte-microglia interaction may be critical in chronic-active lesions, characterized by a rim of activated microglia surrounding lesions, and thought to drive disease progression [3].

Microglia play various roles in CNS homeostasis, neuroplasticity and neuroprotection [5,6]. Different microglial morphologies, indicative for their functions, can be distinguished: ramified, transitional/activated, and amoeboid microglia [7]. Ramified microglia are considered homeostatic [8], but under pathological conditions rapidly become activated [9], transforming into an transitional/activated form and

* Corresponding author. Department of Neurology, Ghent University Hospital. Corneel Heymanslaan 10, 9000, Ghent, Belgium.

E-mail address: Helen.Bachmann@UGent.be (H. Bachmann).

¹ shared senior authorship.

Abbreviations			
(B&H)	Benjamin & Hochberg		spectrometry
(CNS)	Central Nervous System	(LFC)	Log fold changes
(cVNS)	Continuously-cycled VNS	(LFB)	Luxol fast blue
(CC)	Corpus callosum	(LPC)	Lysolecithin
(CV)	Cresyl violet	(MWU)	Mann-Whitney <i>U</i> test
(dpi)	Days post-injection	(MGV)	Mean gray value
(GSEA)	Gene set enrichment analyses	(MS)	Multiple sclerosis
(GFAP)	Glial Fibrillary Acidic Protein	(Olig2)	Oligodendrocyte Lineage Transcription Factor 2
(Iba1)	Ionized Calcium-binding Adapter Molecule 1	(OPCs)	Oligodendrocyte precursor cells
(LMEP)	Laryngeal Muscle Evoked Potential	(1minVNS)	One-minute VNS
(LC-MS/MS)	Liquid chromatography coupled to tandem mass	(PBS)	Phosphate-buffered saline
		(Pdgfra)	Platelet-derived Growth Factor Receptor Alpha
		(VNS)	Vagus Nerve Stimulation

ultimately into a highly proinflammatory, amoeboid form [5,8,10–13]. Once activated, microglia can release neurotoxic and inflammatory mediators (cytokines, chemokines, reactive oxygen species), activating other immune cells, increasing inflammation and promoting neuro-axonal damage and neurodegeneration [9].

Similar to microglia, astrocytes have multiple functions, like blood-brain-barrier maintenance and supporting neuroplasticity [14,15], with both pro- and anti-inflammatory properties [2]. In response to inflammatory processes, like activated microglia, astrocytes become reactive, leading to the release of proinflammatory mediators, increased microglia activation and inhibition of mature oligodendrocyte generation, thereby preventing remyelination [2,9,15–18].

In MS, immune-mediated demyelination [19] leads to axonal damage and eventual clinical disability [20,21]. Remyelination, where oligodendrocyte precursor cells (OPCs) generate new oligodendrocytes that remyelinate axons, is one of the most important mechanisms to protect axonal integrity. Remyelination insulates axons from a hostile microenvironment and restores their conductive properties and metabolic interaction with oligodendrocytes [22–24]. Although remyelination is oligodendrocyte-driven, it involves complex regulation by microglia and astrocytes. Microglia promote remyelination by clearing myelin debris and by recruiting and supporting OPCs, while astrocytes secrete factors influencing OPC behavior, impact cholesterol metabolism and recruit microglia [25,26].

Although several disease modifying therapies are currently available for MS, there is still no effective treatment for CNS innate immune cell and neurodegeneration driven disease progression [4,27,28]. Neither is there a therapy that successfully promotes remyelination to restore demyelinated lesions and protect against further axonal damage [29,30]. Based on prior research, demonstrating anti-inflammatory effects of Vagus Nerve Stimulation (VNS) in several inflammatory diseases [31–38], and a recent preclinical stroke study, showing positive effect of transcutaneous auricular VNS on ischemic white matter damage [39], we hypothesized that VNS may have potential as MS treatment. Two VNS protocols were tested: 1) a continuously-cycled VNS (cVNS) protocol, based on studies demonstrating a central modulating effect and norepinephrine increase in the CNS, without inducing hypothermia [40–42]; 2) a one-minute VNS (1minVNS) protocol, based on preliminary work of Hamlin et al. describing increased remyelination induced by a single, per-operative administration of one minute of VNS, aiming to activate the cholinergic anti-inflammatory pathway [43,44].

This study explores the impact of VNS on innate neuroinflammatory responses linked to demyelination [45,46] and remyelination in the rat lysolecithin (LPC)-induced focal demyelination model, a robust model to study remyelinating approaches [47]. LPC acts as a gliotoxin, causing dissolution of oligodendrocyte membranes and cell death, leading to rapid demyelination and secondary inflammation, mainly involving microglia and astrocytes [48–50]. This study focused on two timepoints after injecting LPC in the corpus callosum (CC): 3 days post-injection

(dpi), when demyelination is complete and peak inflammation is found [51,52] (demyelination experiment), and 11 dpi, corresponding with ongoing remyelination [48,51,52] (remyelination experiment).

2. Material and methods

2.1. Animals

Forty-six female Lewis rats (Janvier, France), aged 7 weeks with average body weight of 195.5 ± 13.5 g, were used. Ethical approval was obtained by the animal ethics committee of Ghent University’s Faculty of Medicine and Health Sciences (ECD 20/22). Animals were kept and handled according to European guidelines (Directive 2010/63/EU), housed in controlled environmental conditions (12 h light/dark cycle, temperature 21–24 °C, humidity 55–65 %) with ad libitum access to food and water.

2.2. Experimental timeline

Two subsequent experiments were performed (Fig. 1). In the first performed demyelination experiment, 33 rats were divided into 1minVNS (n = 10/33), cVNS (n = 12/33) and sham (n = 11/33) groups, and perfused at 3 dpi for immunohistochemistry (demyelination and peak inflammation). Second, the remyelination experiment was performed on a new batch of 13 rats. As immunohistochemistry of the demyelination experiment showed efficacy of cVNS, only cVNS was used as treatment group (n = 6/13) compared to sham (n = 7/13), followed by perfusion at 11 dpi (remyelination). Later, five rats were randomly selected from each of the above cVNS and sham groups of the demyelination and remyelination experiments for proteomics (n = 20).

2.3. VNS electrode implantation

Rats were implanted with a custom-made bipolar cuff VNS electrode around the left vagal nerve, as described by El Tahry et al. [53]. Electrode leads were subcutaneously tunneled towards the scalp and rats were transferred into a stereotaxic frame. LPC-injection coordinates were marked (1 mm caudal and 2 mm lateral to bregma) and four anchor screws were placed epidurally in burr holes in the parietal and occipital bone. The 2.54 mm winslow connector pins (RS components, Belgium) of the VNS electrode leads were assembled in a connector block and fixated to the skull with metabond (Super-Bond C&B, Sunmedical, Japan) and dental cement (Simplex Rapid fluid/powder, Kemdent, UK), forming a headcap. Headcap positioning ensured accessibility for later LPC-injection. The skin was closed and meloxicam (1 mg/kg) was administered subcutaneously.

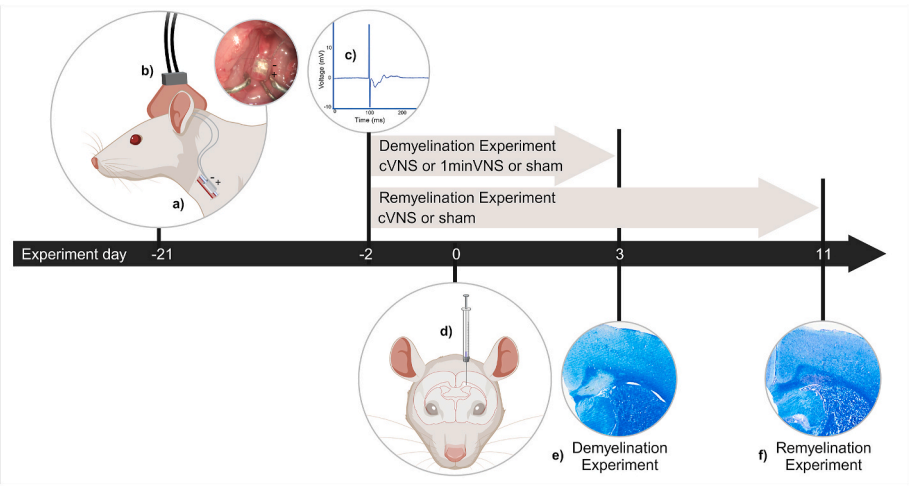


Fig. 1. Experimental time-line. Schematic representation of experimental time line. A custom-made cuff electrode was surgically implanted around the left vagal nerve, with the anode and cathode placed caudally and cranially, respectively (a). The electrode leads were subcutaneously tunneled towards the headcap (b). After LMEP recording (c), animals were divided in VNS (cVNS or 1minVNS) or sham groups, and connected to the stimulation cable. VNS or sham treatment was started two days before the LPC injection in the CC (d). To study the effect on demyelination, histological evaluation was performed 3 dpi, corresponding with complete demyelination and the peak of secondary inflammation (e). For the effect on remyelination histological evaluation was performed 11 dpi, when a partial remyelinated lesion is expected (f). At both timepoints the following outcome parameters were assessed: intensity-corrected demyelination volume (de- and remyelination), Olig2+ cell count and Pdgfra intensity within Olig2+ cells (oligodendrocytes and precursors), Iba1 intensity and Iba1+ cell count (microglia activity and morphotypes, neuroinflammation), and GFAP intensity and GFAP+ cell count (astrocyte activity, neuroinflammation). Proteomics was later performed in a subgroup of the cVNS and sham rats at both timepoints.

2.4. LMEP

Three weeks after VNS implantation, a Laryngeal Muscle Evoked Potential (LMEP) was recorded according to Grimonprez et al. [54], to estimate effective VNS per rat. Recording electrodes were custom-made from 30 Gauge needles and subcutaneously inserted without cutting the skin. One electrode was placed near the left laryngeal muscle (LMEP recording), another one in the pectoralis muscle (detection and differentiation from non-laryngeal muscle contractions). Recordings were referenced to a ground electrode in the right hind paw. Biphasic, square-wave pulses (200 μ s pulse width, 1 Hz frequency) were delivered through the VNS electrode using a constant current stimulator (DS4, Digitimer, UK) with intensity increasing in 0.1 mA steps from 0.1 to 1.0 mA. After tenfold repetition, the average response was used to determine a stimulus-response relationship. LMEP presence indicated correct stimulation of the vagal nerve (Table 1). Rats without clear evidence of an LMEP at 1.0 mA were reallocated to the sham group.

2.5. VNS stimulation

After LMEP recording, rats were connected to custom-designed constant current stimulators, by attaching the stimulation cable to their headcap. For the demyelination experiment (n = 33), VNS was initiated 2 days prior to LPC-injection until 3 dpi (briefly interrupted for LPC-injection). There were three experimental groups based on VNS protocol: 1minVNS, cVNS and sham. In the 1minVNS group, VNS was applied daily for 1 min per day (biphasic pulses, 1.0 mA intensity, 10 Hz frequency, 250 μ s pulse width). In the cVNS group, VNS was applied continuously with a 0.5sON/29.5sOFF duty cycle (biphasic pulses, 1.0

mA intensity, 30 Hz frequency, 250 μ s pulse width). The cathodic first phase was delivered at the cranial electrode contact. In the sham group, rats were connected to the set-up without VNS delivery. For the remyelination experiment (n = 13), cVNS was compared to sham. cVNS and sham were initiated 2 days before LPC-injection until 11 dpi. During cVNS or 1minVNS, the VNS electrode impedance was measured daily to ensure correct stimulation.

2.6. LPC-injection

After isoflurane anesthesia and subcutaneous buprenorphine injection, rats were fixated in a stereotaxic frame. A craniotomy was performed bilaterally above the CC (1 mm caudal and 2 mm lateral to bregma). On each side, 1 μ l of 0.5 % LPC (Sigma-Aldrich, Belgium) in phosphate-buffered saline (PBS) was injected at a flow rate of 0.3 μ l/min in the CC (2.5 mm ventral to the brain surface) using a 10 μ l Hamilton Neuros syringe (point style 4) mounted on a Quintessential Stereotaxic Injector (Stoelting Co., USA). After injection, the needle was left in place for 5 min, before slowly retracting and suturing the skin.

2.7. Transcardiac perfusion

Rats were euthanized 3 or 11 dpi in the demyelination or remyelination experiment respectively, by an intraperitoneally administered overdose of sodium pentobarbital (600 mg/kg, Vétoquinol, Belgium), followed by transcardiac perfusion with room temperature PBS and ice-cold PBS, and rapid brain isolation. Isolated brains were fixated in 4 % paraformaldehyde for 48 h, followed by cryoprotection with increasing sucrose solutions (10%-20%-30 %) at 4 °C. Then, they were frozen in

Table 1
Laryngeal Muscle Evoked Potential (LMEP) recording before stimulation.

	Demyelination experiment			Remyelination experiment	
	Sham (n = 11)	cVNS (n = 12)	1minVNS (n = 10)	Sham (n = 7)	cVNS (n = 6)
Proportion of rats with LMEP present of LMEPs recorded	7/11	12/12	10/10	2/7	6/6
Mean±SD of LMEP threshold (mA)	0.33 ± 0.25	0.38 ± 0.26	0.36 ± 0.31	0.35 ± 0.25	0.50 ± 0.26

isopentane (2-methylbutane, Sigma-Aldrich) and liquid nitrogen (-196°C) and stored in -80°C .

2.8. Immunohistochemistry

For histology, 30 μm thick coronal sections of the CC were made using a cryotome (Leica, Germany). Throughout the complete lesion, including a 630 μm thick margin, every third slice was mounted on a glass slide and stained by luxol fast blue (LFB) with cresyl violet (CV) counterstaining. Slices were scanned with a PANNORAMIC Digital Slide Scanner (Panoramic 250, 3×D Hitech, 40× magnification). For all slices the demyelination surface was determined using ImageJ (Fig. 2a). The mean surface of two subsequent slices (μm^2) was multiplied by 90 μm (30 μm thick slice + 60 μm interslice gap) to calculate the inter-slice volume (μm^3). The lesion volume (mm^3) was then calculated by the total sum of these inter-slice volumes. Using the ImageJ color deconvolution tool, the LFB and CV staining was virtually split for the slice image showing the largest demyelination surface ('mid-lesion slice') after which the level of myelin loss was calculated as the difference in LFB staining intensity (mean gray value (MGV)) between intact and demyelinated CC tissue (Fig. 2c). Lesion volume and level of myelin loss were then combined into the intensity-corrected demyelination volume ($\text{mm}^3 \cdot \text{MGV}$) as the outcome parameter to assess demyelination and remyelination.

On two slices, adjoining the mid-lesion slice, immunohistochemistry was performed as described earlier [55,56]. Here, microglia and astrocytes were stained by an ionized Calcium-binding Adapter Molecule 1 (Iba1) and Glial Fibrillary Acidic Protein (GFAP) double staining, using rabbit anti-Iba1 (1:500, abcam ab178846), mouse anti-GFAP (1:1000, Sigma-Aldrich G3893), goat anti-rabbit Alexafluor 594 (1:1000, abcam ab150088) and goat anti-mouse Alexafluor 488 (1:1000, abcam ab150113). Oligodendrocytes and OPCs were stained by Oligodendrocyte Lineage Transcription Factor 2 (Olig2) and Platelet-derived Growth Factor Receptor Alpha (Pdgfra) double staining, using goat anti-Olig2 (1:100, Biotechne AF2418), rabbit anti-Pdgfra (1:100, Thermo Fisher, PA5-16571), donkey anti-goat Alexafluor 594 (1:1000, Thermo Fisher A32758) and donkey anti-rabbit Alexafluor 488 (1:1000, Thermo Fisher, A32790). Immunostained slices were imaged on a Nikon A1R HD

confocal laser scanning microscope (Nikon Benelux, Belgium) using a 20× Plan APO VC/0.75 Numeric Aperture objective lens. NIS Elements was used as acquisition software, ImageJ for further analysis. After background correction and delineating the lesion area, the mean Iba1-intensity and the mean GFAP-intensity (MGV) were measured within the lesion and within the outer border (approximately 50 μm border surrounding the lesion) as microglial and astrocytic activation markers respectively. For astrocyte and microglia cell counting, a grid of 5000 μm^2 big squares was plotted on the image. Two regions were determined: the lesion (all squares that completely lie within the delineated lesion) and the outer border (complete squares adjacent to the outside border of the lesion) (Fig. 3). For each region, astrocytes and microglia were counted (innate neuroinflammation markers) in five randomly selected squares and the mean cell count per square was calculated. Microglia were morphologically divided into ramified microglia (small cell body, long thin processes with branches [8,10,12]), transitional microglia (activated microglia with enlarged, often irregular, cell body and thick, short processes [11–13]) and amoeboid microglia (round, enlarged cell body without branches [8,10,12]) (Fig. 3). The total microglia count was calculated by the summation of these microglia subcategories.

Similarly, background correction and lesion delineation were performed on the Olig2/Pdgfra stained slices (Fig. 4). Olig2+ cells (oligodendrocyte lineage) were automatically counted within the delineated lesion (cells/ mm^2) by applying the threshold, the watershed, and the analyze particles functions of ImageJ. Within each Olig2+ cell area, the Pdgfra-intensity was measured (MGV), and the average was used for relative OPC abundance within the Olig2+ cell population (OPC marker).

Statistical analysis of immunohistochemistry was performed in SPSS version 27 (Chicago, USA) with statistical significance set at $p \leq 0.05$. Normal distribution was checked by a histogram, QQ-plot and Shapiro-Wilk test. If data were normally distributed, an unpaired t -test was used. Otherwise, a Mann-Whitney U test (MWU) was used. Two-way ANOVA was used on the log-transformed Olig2+ cell count and Pdgfra-intensity to evaluate time and treatment interactions. Unless otherwise stated, data were expressed as mean $\pm$ SD.

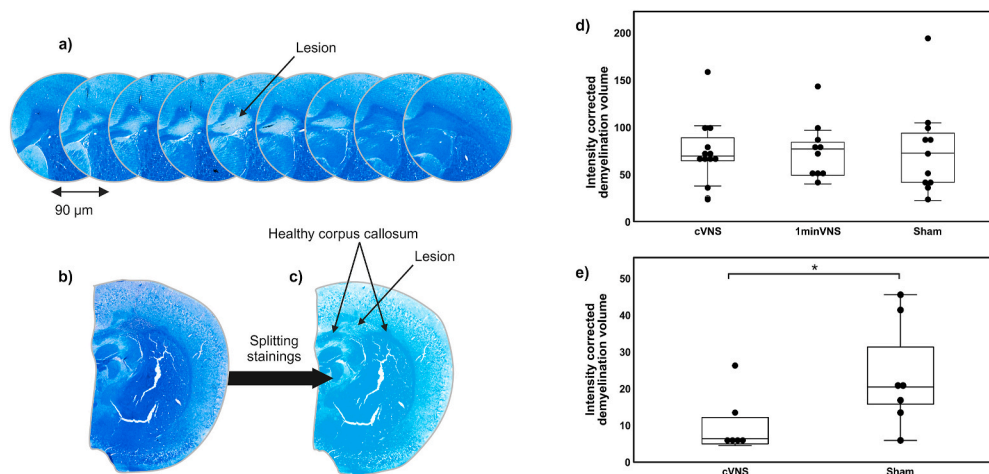


Fig. 2. Demyelination. Demyelination was assessed by the intensity-corrected lesion volume, combining lesion volume and myelin loss within the lesion. An LFB staining and CV counterstaining were performed on every third slice throughout the complete lesion and lesion surface was measured on these slices (a). The sum of the interslice volumes (product of the mean surface of two subsequent slices (μm^2) and 90 μm (space between subsequent slices)) gave the lesion volume. The degree of myelin loss within the lesion was calculated by the difference of the myelin intensity of the healthy CC and the myelin intensity of the lesion. As the purple CV counterstaining could interfere with the LFB-stained blue-colored myelin, the LFB-CV staining (b) were virtually split in ImageJ, before measuring the myelin intensity on the LFB staining alone (c). On 3 dpi, there was no significant reduction of the intensity-corrected lesion volume, indicating that there was no prophylactic effect against lesion induction by cVNS or 1minVNS (d). On 11 dpi, there was a significant effect of cVNS on remyelination, as the intensity-corrected demyelination volume was 57.4 % reduced compared to sham (MWU: $p = 0.046$) (e). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

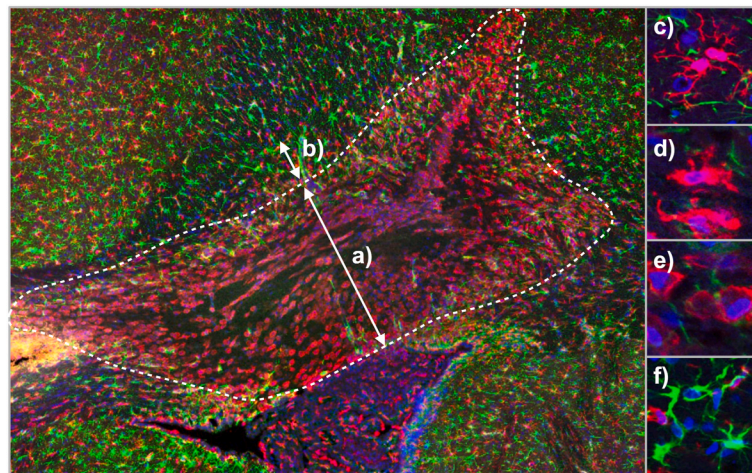


Fig. 3. Iba1/GFAP staining. An Iba1/GFAP double staining was used to analyze microglia and astrocytes respectively (Iba1 red, GFAP green, DAPI blue). The lesion border was delineated (dashed line), and Iba1- and GFAP-intensity were measured within the lesion (a). Additionally, microglia and astrocytes were counted in 5 random selected squares within the lesion (a), and the outer border surrounding the lesion (b). Based on morphology, microglia were subdivided in ramified (c), transitional (d), and amoeboid microglia (e). Astrocytes are shown in (f). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.9. Proteomics

Proteomics was performed in five cVNS and sham rats of both timepoints ($n = 20$). For each rat, one slice, adjoining the Iba1/GFAP-stained slice, was washed with PBS, and the lesion was cut out and prepared for high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (described in Supplementary material). LC-MS/MS runs of all samples were searched using the DiaNN algorithm, version 1.8.1. Spectra were searched against the *Rattus norvegicus* (TaxID: 10,116) protein sequences in the Uniprot database (06_2023) [57]. Shotgun results were further analyzed in the R programming language version 4.2.2. Protein expression matrices were prepared by filtering the DIA-NN main report output table at a precursor and protein library, q-value cut-off of 1 %, and retaining only proteins identified by at least one proteotypic peptide. iBAQ intensity columns were added to the matrix using DIAgui's R package get_IBAQ function [58]. PG.MaxLFQ intensities were log2-transformed and replicate samples were grouped. Proteins with less than three valid values in at least one group were removed and missing values were imputed from a normal distribution centered around the detection limit (package DEP) leading to a list of 8172 quantified proteins in the experiment.

Statistical analyses were performed on log2-transformed, imputed PG.MaxLFQ intensities. Differentially expressed proteins were identified using limma [59] with empirical Bayes procedure and p-values corrected using Benjamini & Hochberg's (B&H) procedure (adjusted $p \leq 0.05$ and $|\log \text{ fold changes (LFC)}| \geq 1$). Samples were grouped based on treatment and condition and contrasts were defined as follows: DE_cVNS_vs_DE_sham = DE.cVNS - DE.sham, and RE_cVNS_vs_RE_sham = RE.cVNS - RE.sham (Fig. 5).

For gene set enrichment analyses (GSEA), proteins were sorted based on decreasing limma moderated t-statistics. GSEA were performed against a combination of gene sets from databases and literature (Table 2) using R packages clusterProfiler [60] and enrichplot [61] with $n\text{Perm} = 1000$, $\text{minGSSize} = 3$, $\text{maxGSSize} = 800$ and B&H correction (adjusted $p \leq 0.05$). For each gene set analysis, the top 10 up- and downregulated pathways were visualized, and running enrichment score plots were plotted only for literature gene sets with significant enrichment. Protein IDs were mapped to gene symbols using UniProt's mapping service if needed [62]. Human gene symbols in the Zhong microglia gene set were mapped to mouse gene symbols using the mapping table provided by the mouse GEM [63,64].

3. Results

3.1. Demyelination experiment

3.1.1. VNS effect on demyelination

At 3 dpi, the intensity-corrected demyelination volume did not differ between cVNS ($75.6 \pm 33.8 \text{ MGv} \times \text{mm}^3$) and sham ($76.1 \pm 48.0 \text{ MGv} \times \text{mm}^3$) (MWU: $p = 0.951$), or 1minVNS ($74.8 \pm 30.6 \text{ MGv} \times \text{mm}^3$) and sham (Fig. 2d. MWU: $p = 0.944$). The number of Olig2+ cells in the demyelinating lesions was significantly lower for cVNS ($1191 \pm 224 \times 10^{-6} \text{ cells/mm}^2$), but not for 1minVNS ($1519 \pm 645 \times 10^{-6} \text{ cells/mm}^2$) compared to sham ($1643 \pm 482 \times 10^{-6} \text{ cells/mm}^2$) (MWU: resp. $p = 0.008$ and $p = 0.324$). Pdgfra-intensity of Olig2+ cells was not significantly different for cVNS ($237.3 \pm 100.4 \text{ MGv}$) or 1minVNS ($223.6 \pm 43.4 \text{ MGv}$) relative to sham ($278.8 \pm 112.2 \text{ MGv}$) (MWU: resp. $p = 0.196$ and $p = 0.231$).

3.1.2. VNS effect on microglia during demyelination

Lesion Iba1-intensity was significantly lower for cVNS ($96.8 \pm 28.4 \text{ MGv}$) but not for 1minVNS ($110.3 \pm 36.4 \text{ MGv}$) compared to sham ($129.6 \pm 43.1 \text{ MGv}$) (Fig. 6a. T-test resp. $p = 0.042$ and 0.284). The total microglia count was significantly reduced in cVNS ($6.93 \pm 1.36 \text{ cells/square}$) compared to sham lesions ($8.85 \pm 2.25 \text{ cells/square}$) (Table 3. Fig. 6b. T-test: $p = 0.021$). Further analysis of the microglia subtypes, showed that this resulted from the combined reduction of amoeboid and transitional microglia in cVNS ($6.67 \pm 1.46 \text{ cells/square}$) compared to sham ($8.59 \pm 2.37 \text{ cells/square}$) (Fig. 6c. T-test: $p = 0.028$). For ramified microglia, similar counts were found in cVNS and sham lesions (MWU: $p = 0.925$). Conversely, total, amoeboid, transitional and ramified microglia, were not significantly different between 1minVNS and sham (Table 3). At the outer border, the Iba1-intensity was significantly reduced in cVNS ($37.4 \pm 16.0 \text{ MGv}$) but not in 1minVNS ($42.3 \pm 19.9 \text{ MGv}$) compared to sham ($55.2 \pm 24.0 \text{ MGv}$) (Fig. 6d. T-test resp. $p = 0.047$ and 0.198). Transitional microglia were reduced in cVNS ($0.51 \pm 0.23 \text{ cells/square}$) and 1minVNS ($0.44 \pm 0.35 \text{ cells/square}$) compared to sham ($1.02 \pm 0.73 \text{ cells/square}$) (Fig. 6f. T-test resp. $p = 0.049$ and 0.048). Total, amoeboid and ramified microglia at the outer border were not significantly changed (Table 3).

3.1.3. VNS effect on astrocytes during demyelination

Lesion GFAP-intensity was significantly reduced for cVNS ($50.0 \pm 22.9 \text{ MGv}$) and showed a trend towards reduction for 1minVNS ($52.6 \pm$

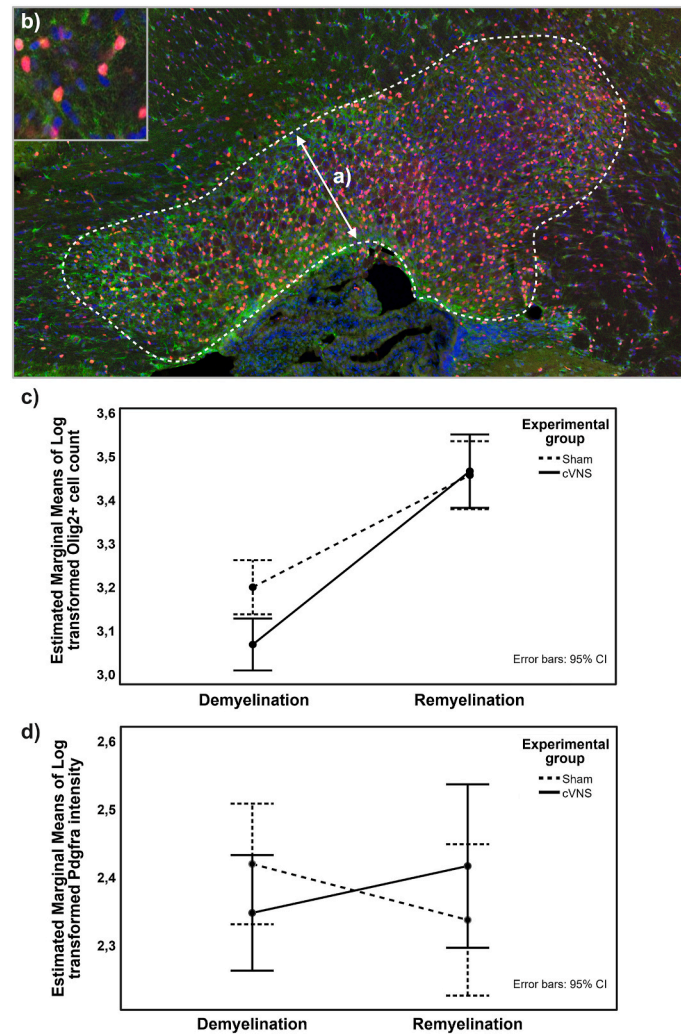


Fig. 4. Olig2/Pdgfra staining. An Olig2/Pdgfra double immunostaining was used to analyze oligodendrocytes and OPCs. Within the lesion (a), the Olig2+ cells (red) were selected and counted by the automatic cell count function in ImageJ. Within the area of each Olig2+ cell, the mean Pdgfra-intensity (green) was measured for that Olig2+ cell (b). Next, the mean of the Pdgfra-intensities of all Olig2+ cells within the lesion was calculated, indicating the relative OPC abundance within the Olig2+ cell population (OPC marker). (c) Olig2+ cell counts were significantly increased from 3 dpi to 11 dpi in both cVNS and sham (MWU: $p = 0.001$ and $p < 0.001$ resp.). While at 3 dpi, Olig2+ cells were significantly lower in cVNS compared to sham (MWU: $p = 0.008$), cVNS reached similar Olig2+ cell counts to sham at 11 dpi (MWU: $p = 1.000$). Two-way ANOVA showed a trend of significant interaction between timing and treatment concerning Olig2+ cells ($p = 0.054$). (d) Pdgfra-intensity of Olig2+ cells was not significantly different between 3dpi and 11dpi in cVNS or sham (MWU: $p = 0.261$ and $p = 0.258$ resp.), or between cVNS and sham compared at 3dpi (MWU: $p = 0.196$) or 11 dpi (T-test: $p = 0.245$). Two-way ANOVA did not show significant interaction between timing and treatment for Pdgfra-intensity ($p = 0.142$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

28.4 MGv) compared to sham (84.9 ± 46.6 MGv) (Fig. 7a. MWU: resp. $p = 0.027$ and $p = 0.091$). GFAP-intensity at the outer border was also significantly reduced for cVNS (55.8 ± 21.5 MGv), but not for 1minVNS (65.6 ± 33.7 MGv), compared to sham (85.0 ± 35.4 MGv) (Fig. 7b. T-test: resp. $p = 0.039$ and $p = 0.238$). Regarding astrocyte counts, there were no significant differences between groups (Table 3).

3.2. Remyelination experiment

3.2.1. VNS effect on remyelination

At 11dpi, intensity-corrected demyelination volume was significantly reduced with 57.4 % in the cVNS group (10.1 ± 8.4 MGv $\times$ mm³) compared to sham (23.7 ± 14.4 MGv $\times$ mm³) (Fig. 2e. MWU: $p = 0.046$). Compared to 3 dpi, Olig2+ cells were significantly increased at 11 dpi for cVNS and sham (MWU: $p = 0.001$ and $p < 0.001$ resp.), with cVNS reaching similar Olig2+ cell count ($2993 \pm 760 \times 10^{-6}$ cells/mm²) as sham ($2909 \pm 563 \times 10^{-6}$ cells/mm²) (MWU: $p = 1.000$).

Pdgfra-intensity of Olig2+ cells was not significantly different between 3dpi and 11dpi for cVNS or sham (MWU: $p = 0.261$ and $p = 0.258$ resp.), nor between cVNS (273.5 ± 88.9 MGv) and sham (223.3 ± 57.8 MGv) (T-test: $p = 0.245$) at 11dpi. Two-way ANOVA showed a trend of significant interaction between timepoint and treatment for Olig2+ cell count, but not for Pdgfra-intensity (Fig. 4 $p = 0.054$ and $p = 0.142$ resp.).

3.2.2. VNS effect on microglia during remyelination

Lesion Iba1-intensity showed a trend towards reduction in cVNS (185.0 ± 57.7 MGv) compared to sham (268.0 ± 80.5 MGv) (T-test: $p = 0.060$). Iba1-intensity at the lesion border was significantly reduced (T-test: $p = 0.027$) in cVNS (59.3 ± 20.9 MGv) compared to sham (97.3 ± 30.9 MGv). There was no significant difference of amoeboid, transitional, ramified or total microglia counts in the lesion or outer border (Table 3).

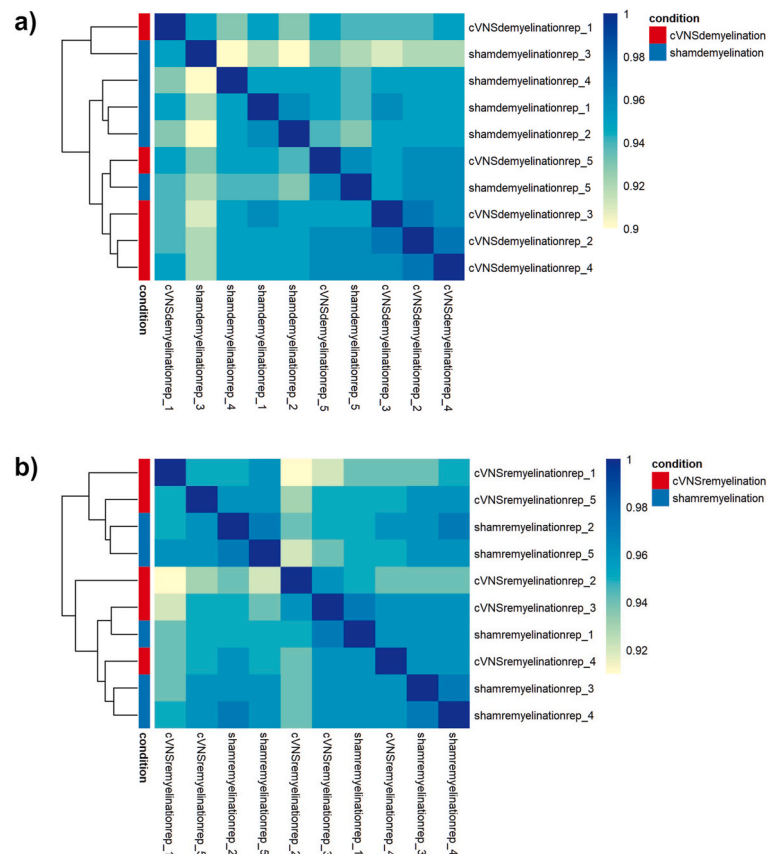


Fig. 5. Proteomics. Between-sample correlation heatmaps were constructed using hierarchical clustering of Spearman correlation coefficients between protein expression values of all pairs of samples in demyelination (a) and remyelination (b). There was no significant effect of cVNS on differential protein expression on either demyelination or remyelination (p-adjusted ≤ 0.05 & $|\text{LFC}| \geq 1$) (not shown).

Table 2

Databases and Gene sets used for Gene set enrichment analysis.

Databases	Accessed through
Gene Ontology (GO) Biological Processes	ClusterProfiler [55]
GO Cellular Component	ClusterProfiler [55]
GO Molecular Function	ClusterProfiler [55]
Reactome [60]	ReactomePA [61]
Gene sets from literature ^a	Reference
Dowell 2009 (astrocyte-secreted proteins)	Dowel et al., 2009 [62]
Dumont 2007 (mature rat oligodendrocytes)	Dumont et al., 2007 [63]
Zhong microglia (gene set downloaded from https://www.gsea-msigdb.org/gsea/msigdb/human/geneset/ZHONG_PFC_MAJOR_TYPES_MICROGLIA.html (2023/12/12))	Zhong et al., 2018 [64]
Santiago microglia (three separate protein lists were created for protective microglia (IL10-Control), homeostatic microglia (TGFB-Control) and inflammatory microglia (LPS-Control) using the following cut-offs: adjusted $p \leq 0.05$ & diff >0 (upregulated) or diff <0 (downregulated))	Santiago et al., 2023 [65]

^a Lists given in supplementary material.

3.2.3. VNS effect on astrocytes during remyelination

Lesion GFAP-intensity showed a trend towards reduction in cVNS (98.0 ± 27.0 MGv) compared to sham (147.4 ± 55.7 MGv) (T-test: $p = 0.068$). At the lesion border, GFAP-intensity was significantly reduced (T-test: $p = 0.027$) in cVNS (56.0 ± 24.5 MGv) compared to sham (103.9 ± 39.7 MGv). Astrocyte counts were not significantly different in the lesion or outer border (Table 3).

3.3. Proteomics analysis

No differentially expressed proteins were demonstrated in cVNS versus sham during demyelination (3 dpi), or between cVNS or sham during remyelination (11 dpi). Since zero differentially expressed proteins were identified at both timepoints, GSEA was performed, avoiding hard LFC or adjusted p-value cut-off when biological differences are too modest to meet significance [65]. GSEA using GO showed significant activation of synapse-related pathways (like post-synapse organization) in cVNS compared to sham, and more specifically activation of the glutamatergic synapse pathway, at 3 dpi (Fig. 8a). Similarly, GSEA using Reactome showed significant activation of synapse-related pathways (most interestingly: neurexins and neuroligins, playing a role in synapse formation and neuroplasticity), and of glutamate-related pathways (like unblocking of NMDA receptors, glutamate binding and activation) in cVNS at 3 dpi (Fig. 8b). GSEA using GO and Reactome showed significant activation of membrane- and synapse-associated pathways (neurexins and neuroligins) in cVNS at 11 dpi (Fig. 8c and d). GSEA using literature based gene sets (Fig. 8e and f), showed a significant reduction of astrocyte secreted proteins, as reported by Dowell et al. [66], in cVNS at 3 dpi. GSEA using proteins of mature rat oligodendrocytes reported by Dumont et al. [67], showed a significant downregulation of mature oligodendrocytes proteins in cVNS at both timepoints. Based on the gene set of Santiago et al. [68], no significant changes in inflammatory, anti-inflammatory or protective state of microglia were found. However, GSEA using the microglia gene set of Zhong et al. showed upregulation of microglia proteins of developing prefrontal cortex, thought to modulate neuronal apoptosis, neurogenesis and synaptic pruning, in cVNS at 3 dpi [69].

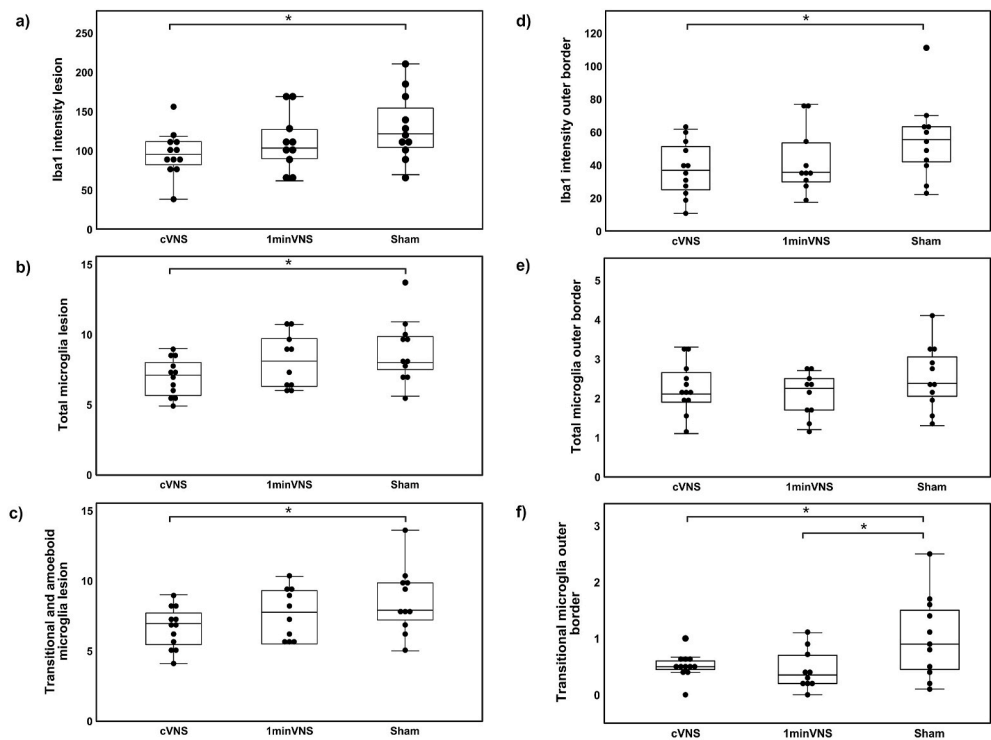


Fig. 6. Effect on microglia during demyelination. At 3 dpi, cVNS significantly reduced the Iba1-intensity in the lesion (a) and the lesion’s total microglia count (b), resulting from a combined amoeboid and transitional microglia reduction (c), indicating reduced activated microglia by cVNS. At the outer border, cVNS significantly reduced the Iba1-intensity (d). Total microglia count at the outer border was not significantly different (e). However, both cVNS and 1minVNS significantly reduced the transitional microglia count at the outer border (f). Homeostatic ramified microglia were not reduced by VNS at the lesion or outer border.

Table 3
Overview of microglia and astrocyte cell counts (cells/square) at the lesion and the outer border.

		Demyelination experiment			Remyelination experiment	
		Sham (n = 11)	cVNS (n = 12)	1minVNS (n = 10)	Sham (n = 7)	cVNS (n = 6)
Lesion	Microglia	8.85 ± 2.25	6.93 ± 1.36 ^{c b}	8.12 ± 1.88 ^b	25.74 ± 3.64	26.47 ± 6.41 ^a
	Transitional microglia	4.25 ± 1.21	3.61 ± 1.41 ^b	3.94 ± 1.28 ^b	12.24 ± 1.59	13.12 ± 2.11 ^b
	Amoeboid microglia	4.35 ± 2.24	3.06 ± 1.42 ^a	3.67 ± 1.50 ^b	12.63 ± 4.48	12.50 ± 5.80 ^a
	Ramified microglia	0.26 ± 0.23	0.27 ± 0.21 ^a	0.51 ± 0.33 ^a	0.87 ± 0.59	0.85 ± 0.68 ^a
	Astrocytes	0.25 ± 0.17	0.25 ± 0.19 ^b	0.32 ± 0.22 ^a	4.41 ± 0.89	4.40 ± 1.29 ^a
Outer border	Microglia	2.54 ± 0.81	2.24 ± 0.65 ^b	2.08 ± 0.54 ^b	4.66 ± 0.49	6.17 ± 2.63 ^a
	Transitional microglia	1.02 ± 0.73	0.51 ± 0.23 ^{c b}	0.35 ± 0.60 ^{c a}	0.54 ± 0.44	0.30 ± 0.28 ^b
	Amoeboid microglia	0.26 ± 0.26	0.53 ± 0.50 ^a	0.33 ± 0.47 ^a	0.00 ± 0.00	0.00 ± 0.00
	Ramified microglia	1.26 ± 0.43	1.20 ± 0.34 ^a	1.31 ± 0.45 ^b	4.11 ± 0.40	5.87 ± 2.76 ^a
	Astrocytes	1.35 ± 0.74	1.38 ± 0.57 ^b	1.52 ± 0.48 ^b	4.20 ± 0.75	4.40 ± 0.36 ^b

Mean ± SD of the cell counts.

^a Non-continuous data, MWU was used.
^b Continuous data, unpaired *t*-test was used.
^c Significant difference (*p* ≤ 0.05) of cVNS or 1minVNS compared to sham.

4. Discussion

In this study, VNS therapy was tested in an LPC-induced demyelination rat model, demonstrating reduced microglial and astrocytic inflammation, as well as improved remyelination. In the first performed demyelination experiment, the clearest effect was found in cVNS, and only minimal changes were found in 1minVNS.

We found that cVNS reduced demyelination with 57.4 % versus sham at the remyelination timepoint (11 dpi), indicating improved remyelination. At the demyelination timepoint (3 dpi), there was no difference in level of demyelination, indicating no (preventive) effect of VNS against the direct, membrane-disrupting effect of LPC causing initial demyelination [50]. Investigation of the oligodendrocyte markers

showed significantly lower Olig2+ cell counts in cVNS at demyelination, that significantly increased and reached similar levels between cVNS and sham during remyelination. Relative OPC abundance within the Olig2+ cells did not differ between cVNS and sham, nor between timepoints. In the LPC model, remyelination generally starts around 7 dpi [48,51], with Olig2+/Pdgfra + OPC recruitment starting from 3 dpi, followed by proliferation and differentiation of these OPCs into remyelinating Olig2+ oligodendrocytes [70–72]. The early Olig2+ cells (3 dpi) thus probably represent mature oligodendrocytes that survived initial injury. Proteomics GSEA further supported our oligodendrocyte effect, showing downregulated mature oligodendrocyte proteins in cVNS at both timepoints [67]. Remyelination is mainly driven by newly differentiated oligodendrocytes, generated from infiltrating OPCs [73].

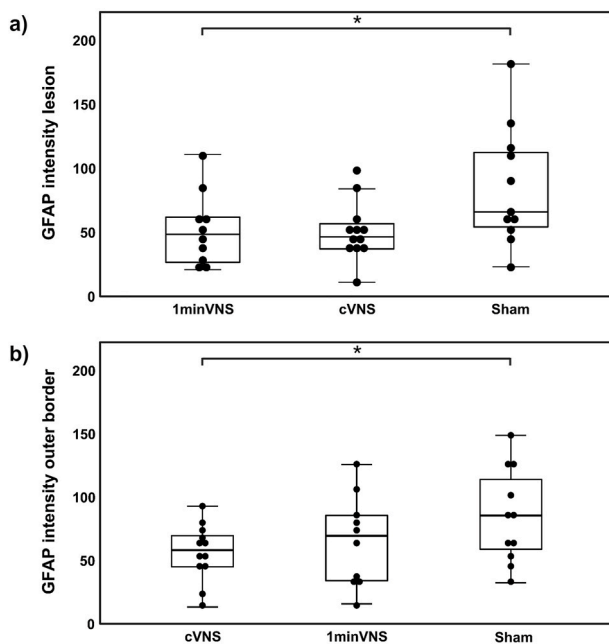


Fig. 7. Effect on astrocytes during demyelination. At 3 dpi, cVNS significantly reduced the GFAP-intensity at the lesion (a) and at the outer border (b).

Surviving mature oligodendrocytes that stay in place after initial injury are unable to efficiently remyelinate axons [73,74], and can even counteract repair and OPC-driven remyelination [75–78]. It is possible that VNS enhances microglial clearance of injured oligodendrocytes and myelin debris, which favors remyelination [79]. In addition, GSEA showed significant upregulation of synapse- and glutamate-related pathways in cVNS at demyelination. Stimulation of neuronal activity and release of glutamate can improve remyelination, by stimulating OPCs to differentiate into new myelinating oligodendrocytes [80–82]. Indeed, Etxeberria et al. found that demyelinated axons form functional glutamatergic synapses onto migrating OPCs at 2–3 days post-LPC-injection [83]. One week after lesioning, this glutamatergic input was significantly reduced in the differentiated oligodendrocytes, indicating that it is lost upon oligodendrocyte differentiation and this axon-oligodendrocyte synapse formation is a transient, regulated step during axonal remyelination [83]. Newly-generated OPCs do not receive synaptic inputs during active proliferation after demyelination, but gain synapses during remyelination, meaning that glutamatergic synaptic inputs may contribute to inhibit OPC proliferation and instruct OPCs to differentiate into new myelinating oligodendrocytes [81,82]. The remyelination effect of cVNS might thus also be exerted through increasing neuronal activity and glutamatergic synapses, improving OPC-driven remyelination. Possibly, VNS influences transient OPC changes between 3dpi and 11dpi, such as enhanced infiltration, proliferation, or differentiation, potentially overlooked at our timepoints.

Our study showed that cVNS reduced microglia and astrocyte activation, indicating a possibly novel treatment strategy to tackle CNS innate neuroinflammation. Microglia and astrocytes contribute to MS pathogenesis through chronic inflammation, impeding myelin repair. They are involved in ‘smoldering’ chronic-active lesions known to be strong predictors of MS progression [2–4,84–86]. The effects of cVNS on microglia were most pronounced at 3 dpi, when inflammation peaks [51,52], with reduced Iba1-expression and microglia cell count, both sensitive markers of microglia activation [87], in the demyelinated lesion. The reduced microglia number was due to a decrease in amoeboid and transitional microglia, both morphologies that appear upon stress-induced microglial activation and linked to pro-inflammatory functions [5,11–13]. cVNS also reduced Iba1-expression and transitional microglia count at the outer border. Conversely, the homeostatic,

ramified microglia were not reduced by cVNS, suggesting that cVNS reduced activated microglia, without reducing microglia with possible neuroprotective and restorative roles [79]. Proteomics GSEA showed increased proteins linked to microglia of developing prefrontal cortex, associated with homeostatic functions in neurogenesis and synaptic pruning [69]. Furthermore, at 3 dpi, cVNS significantly reduced GFAP-expression at the lesion and outer border, indicating reduced astroglial activation [16], and proteomics GSEA showed reduced astrocyte-secreted proteins [66]. At 11 dpi, the effects of cVNS on microglia and astrocytes were less pronounced: showing only a significant reduction of Iba1-and GFAP-expression in the outer border and a trend towards less Iba1-and GFAP-expression in the lesions [16,87].

The regulatory role of the vagus nerve in inflammation and the anti-inflammatory effects of VNS have been demonstrated in several inflammatory diseases [31–38]. For CNS inflammation specifically, it was already shown that VNS reduced lipopolysaccharide induced neuroinflammation and ischemic induced inflammation [39,88]. Regarding its central effects, the locus coeruleus, the brain’s main source of norepinephrine, is thought to be crucial [34,54,89]. Through activation of β 2-adrenoreceptors of microglia, astrocytes and neurons, VNS-induced NE release reduces neuroinflammatory and neurodegenerative processes [34,89–92]. Another potential effect of increased NE signaling involves modulation of excitatory synapses and synaptic plasticity [93–100]. Alexander et al. found that VNS modulates excitatory synapses through upregulation of postsynaptic density proteins like neuroligin 1, and pathway analysis suggested that glutamate receptor signaling is a primary target [93], which is consistent with our GSEA findings.

Future research should assess clinical impact by neurobehavioral testing, probably more applicable at other injection sites (e.g. internal capsule) [101–103]. Oligodendrocytes, microglia and astrocytes, should be further investigated, using single-cell techniques and additional timepoints (e.g. 7 dpi to assess transient OPC changes during early remyelination).

5. Conclusion

Our study showed that VNS improved remyelination and modulated microglia and astrocyte activation, indicating its potential to treat dysfunctional remyelination and innate immune cell activation in MS. GSEA on proteomics showed upregulation of (glutamatergic) synapse-related pathways in VNS, possibly involved in activating synaptic signaling between neurons and OPCs, supporting OPC-driven remyelination. Other working mechanisms might include reduced microglia and astrocyte inflammation, and enhanced oligodendrocyte clearance after initial injury. The effect was protocol dependent, showing optimal results by cVNS, indicating that parameters should be chosen carefully.

Funding

Helen Bachmann is a fellow of the Research Foundation Flanders (FWO) (1S25620N), funding was provided by the Charcot Research Fund (<https://www.fondation-charcot.org/>). These funding agencies were not involved in the study design, data collection, analysis, writing, and submission of the paper.

Data availability

Proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE [104] partner repository with the dataset identifier PXD050858, see also Supplementary Material.

CRedit authorship contribution statement

Helen Bachmann: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis,

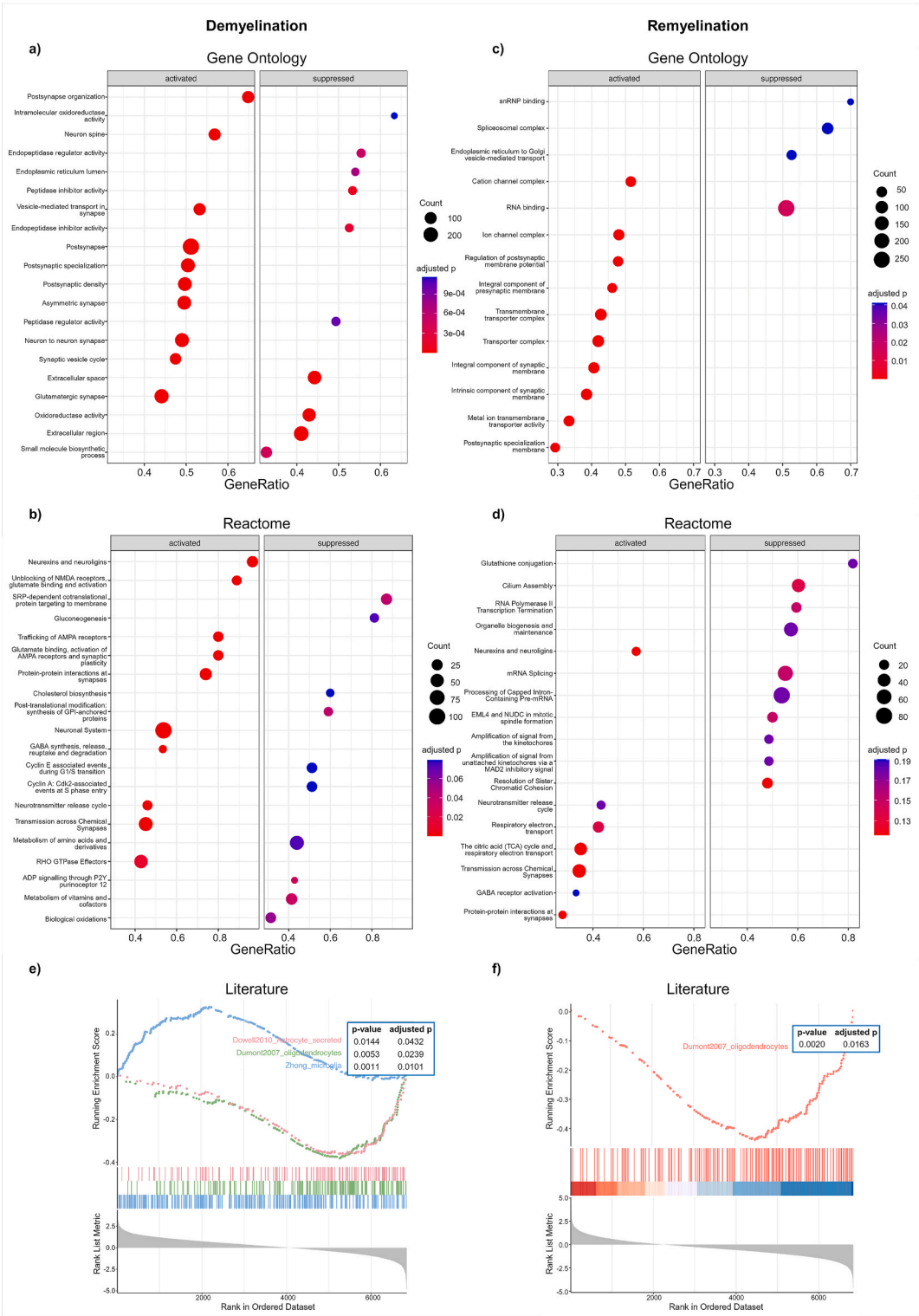


Fig. 8. Rank-based gene set enrichment analysis (GSEA) on proteomics analyses. Shown are adjusted p-values, count (number of genes overlapping with the gene set), and ratio (count/number of genes in the pathway). Enrichments are more significant if the overlap with the gene set is mostly located at the beginning (high t-statistics) or end (low t-statistics) of the gene set. A significant effect on synapse-related pathways and glutamatergic synapse pathways was found in cVNS during demyelination (3 dpi) when using the Gene Ontology (GO) (categories: Biological Processes, Cellular Components, Molecular Function) (a) and Reactome databases (b), and to a lesser extend also an effect on synapse related pathways during remyelination (11 dpi) was observed using the GO (c) and Reactome databases (d). Running enrichment plots for GSEA using literature-based gene sets are shown in (e) for demyelination and (f) for remyelination. Ranked metric represents descending t-statistics of the gene list, colored vertical lines represent an overlapping gene with a given gene set and the dotted line represents the running enrichment score of a given gene set. At demyelination, increased microglia proteins correlated to developmental prefrontal cortex, reduced mature oligodendrocyte proteins and reduced astrocyte-secreted proteins were found in cVNS (e). At remyelination, reduced mature oligodendrocyte proteins were found in cVNS (f).

Conceptualization. **Boris Vandemoortele**: Writing – review & editing, Visualization, Formal analysis. **Vanessa Vermeirssen**: Writing – review & editing, Supervision, Conceptualization. **Evelien Carrette**: Writing – review & editing, Conceptualization. **Kristl Vonck**: Writing – review & editing, Conceptualization. **Paul Boon**: Writing – review & editing, Conceptualization. **Robrecht Raedt**: Writing – review & editing, Supervision, Methodology, Conceptualization. **Guy Laureys**: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Helen Bachmann, Boris Vandemoortele, Vanessa Vermeirssen, Evelien Carrette, Robrecht Raedt, Guy Laureys have nothing to disclose.

Kristl Vonck received consultancy fees from LivaNova Europe and Synergia Medical.

Paul Boon received consultancy fees from Livanova Europe.

Acknowledgement

The authors thank the Ghent Light Microscopy CORE, the VIB Proteomics CORE, the Biobank of the University Ghent Hospital and in particular Anne-Sophie Bultinck and Eline Van Severen in supporting this paper. Figures were partially created with Biorender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.brs.2024.04.012>.

References

- [1] Walton C, King R, Rechtman L, Kaye W, Leray E, Marrie RA, et al. Rising prevalence of multiple sclerosis worldwide: insights from the Atlas of MS, third edition. *Mult Scler j* 2020;26:1816–21. <https://doi.org/10.1177/1352458520970841>.
- [2] Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. *Nat Rev Immunol* 2015;15:545–58. <https://doi.org/10.1038/nri3871>.
- [3] Absinta M, Maric D, Gharagozloo M, Garton T, Smith MD, Jin J, et al. A lymphocyte–microglia–astrocyte axis in chronic active multiple sclerosis. *Nature* 2021;597:709–14. <https://doi.org/10.1038/s41586-021-03892-7>.
- [4] Giovannoni G, Popescu V, Wuerfel J, Hellwig K, Iacobaeus E, Jensen MB, et al. Smouldering multiple sclerosis: the ‘real MS’. *Ther Adv Neurol Disord* 2022;15:175628642110667. <https://doi.org/10.1177/17562864211066751>.
- [5] Leyh J, Paeschke S, Mages B, Michalski D, Nowicki M, Bechmann I, et al. Classification of microglial morphological phenotypes using machine learning. *Front Cell Neurosci* 2021;15. <https://doi.org/10.3389/fncel.2021.701673>.
- [6] Tremblay ME, Stevens B, Sierra A, Wake H, Bessis A, Nimmerjahn A. The role of microglia in the healthy brain. *J Neurosci* 2011;31:16064–9. <https://doi.org/10.1523/JNEUROSCI.4158-11.2011>.
- [7] Lier J, Streit WJ, Bechmann I. Beyond activation: characterizing microglial functional phenotypes. *Cells* 2021;10:2236. <https://doi.org/10.3390/cells10092236>.
- [8] Choi S, Hill D, Guo L, Nicholas R, Papadopoulos D, Cordeiro MF. Automated characterisation of microglia in ageing mice using image processing and supervised machine learning algorithms. *Sci Rep* 2022;12. <https://doi.org/10.1038/s41598-022-05815-6>.
- [9] Heneka MT, Kummer MP, Latz E. Innate immune activation in neurodegenerative disease. *Nat Rev Immunol* 2014;14:463–77. <https://doi.org/10.1038/nri3705>.
- [10] Choi S, Guo L, Cordeiro MF. Retinal and brain microglia in multiple sclerosis and neurodegeneration. *Cells* 2021;10. <https://doi.org/10.3390/cells10061507>.
- [11] Hinwood M, Morandini J, Day TA, Walker FR. Evidence that Microglia Mediate the Neurobiological Effects of Chronic Psychological Stress on the Medial Prefrontal Cortex n.d. <https://doi.org/10.1093/cercor/bhr229>.
- [12] Karperien A, Ahammer H, Jelinek HF. Quantitating the subtleties of microglial morphology with fractal analysis. *Front Cell Neurosci* 2013;1–34. <https://doi.org/10.3389/fncel.2013.00003>.
- [13] Gómez-González B, Escobar A. Prenatal stress alters microglial development and distribution in postnatal rat brain. *Acta Neuropathol* 2010;119:303–15. <https://doi.org/10.1007/s00401-009-0590-4>.
- [14] Dong Y, Benveniste EN. Immune function of astrocytes. *Glia* 2001;36:180–90. <https://doi.org/10.1002/glia.1107>.
- [15] Mayo L, Trauger SA, Blain M, Nadeau M, Patel B, Alvarez JI, et al. B4GALT6 regulates astrocyte activation during CNS inflammation. *Nat Med* 2014;20:1147–56. <https://doi.org/10.1038/nm.3681>.
- [16] Zhou B, Zuo YX, Jiang RT. Astrocyte morphology: diversity, plasticity, and role in neurological diseases. *CNS Neurosci Ther* 2019;25:665–73. <https://doi.org/10.1111/cns.13123>.
- [17] Voskuhl RR, Peterson RS, Song B, Ao Y, Beth L, Morales J, et al. Neurobiology of disease reactive astrocytes form scar-like perivascular barriers to leukocytes during adaptive immune inflammation of the CNS. <https://doi.org/10.1523/JNEUROSCI.1514-09.2009>.
- [18] Bush TG, Puvanachandra N, Horner CH, Polito A, Ostensfeld T, Svendsen CN, et al. Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron* 1999;23:297–308. [https://doi.org/10.1016/S0896-6273\(00\)80781-3](https://doi.org/10.1016/S0896-6273(00)80781-3).
- [19] Lubetzki C, Stankoff B. Demyelination in multiple sclerosis. 89–99. <https://doi.org/10.1016/B978-0-444-52001-2.00004-2>; 2014.
- [20] Clark KC, Josephson A, Benusa SD, Hartley RK, Baer M, Thummala S, et al. Compromised axon initial segment integrity in EAE is preceded by microglial reactivity and contact. *Glia* 2016;64:1190–209. <https://doi.org/10.1002/glia.22991>.
- [21] Criste G, Trapp B, Dutta R. Axonal loss in multiple sclerosis. 101–113. <https://doi.org/10.1016/B978-0-444-52001-2.00005-4>; 2014.
- [22] Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, et al. Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* 2012;487:443–8. <https://doi.org/10.1038/NATURE11314>.
- [23] Morrison BM, Lee Y, Rothstein JD. Oligodendroglia: metabolic supporters of axons. *Trends Cell Biol* 2013;23:644–51. <https://doi.org/10.1016/j.TCB.2013.07.007>.
- [24] Franklin RJM, Goldman SA. Glia disease and repair—remyelination. *Cold Spring Harbor Perspect Biol* 2015;7:1–28. <https://doi.org/10.1101/cshperspect.a020594>.
- [25] Nemes-Baran AD, White DR, DeSilva TM. Fractalkine-dependent microglial pruning of viable oligodendrocyte progenitor cells regulates myelination. *Cell Rep* 2020;32:108047. <https://doi.org/10.1016/j.CELREP.2020.108047>.
- [26] Rawji KS, Gonzalez Martinez GA, Sharma A, Franklin RJM. The role of astrocytes in remyelination. *Trends Neurosci* 2020;43:596–607. <https://doi.org/10.1016/j.TINS.2020.05.006>.
- [27] Feinstein A, Freeman J, Lo AC. Treatment of progressive multiple sclerosis: what works, what does not, and what is needed. *Lancet Neurol* 2015;14:194–207. [https://doi.org/10.1016/S1474-4422\(14\)70231-5](https://doi.org/10.1016/S1474-4422(14)70231-5).
- [28] Absinta M, Maric D, Gharagozloo M, Garton T, Smith MD, Jin J, et al. A lymphocyte–microglia–astrocyte axis in chronic active multiple sclerosis. *Nature* 2021;597:709–14. <https://doi.org/10.1038/s41586-021-03892-7>.
- [29] Ranger A, Ray S, Szak S, Dearth A, Allaire N, Murray R, et al. Anti-LINGO-1 has no detectable immunomodulatory effects in preclinical and phase 1 studies. *Neurol Neuroimmunol Neuroinflamm* 2018;5:e417. <https://doi.org/10.1212/NXI.0000000000000417>.
- [30] Cree BAC, Cutter G, Wolinsky JS, Freedman MS, Comi G, Giovannoni G, et al. Safety and efficacy of MD1003 (high-dose biotin) in patients with progressive multiple sclerosis (SPI2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Neurol* 2020;19:988–97. [https://doi.org/10.1016/S1474-4422\(20\)30347-1](https://doi.org/10.1016/S1474-4422(20)30347-1).
- [31] Kwan H, Garzoni L, Liu HL, Cao M, Desrochers A, Fecteau G, et al. Vagus nerve stimulation for treatment of inflammation: systematic review of animal models and clinical studies. *Bioelectron Med* 2016;3:1–6.
- [32] Sun P, Zhou K, Wang S, Li P, Chen S, Lin G, et al. Involvement of MAPK/NF-κB signaling in the activation of the cholinergic anti-inflammatory pathway. In: Experimental colitis by chronic vagus nerve stimulation; 2013. <https://doi.org/10.1371/journal.pone.0069424>.
- [33] Meregnani J, Clarençon D, Vivier M, Peinnequin A, Mouret C, Sinniger V, et al. Anti-inflammatory effect of vagus nerve stimulation in a rat model of inflammatory bowel disease. *Auton Neurosci* 2011;160:82–9. <https://doi.org/10.1016/j.autneu.2010.10.007>.
- [34] Bassi GS, Dias DPM, Franchin M, Talbot J, Reis DG, Menezes GB, et al. Modulation of experimental arthritis by vagal sensory and central brain stimulation. *Brain Behav Immun* 2017;64:330–43. <https://doi.org/10.1016/j.bbi.2017.04.003>.
- [35] Levine YA, Koopman FA, Faltys M, Caravaca A, Bendele A, Zitnik R, et al. Neurostimulation of the cholinergic anti-inflammatory pathway ameliorates disease in rat collagen-induced arthritis. *PLoS One* 2014;9:e104530. <https://doi.org/10.1371/journal.pone.0104530>.
- [36] Werner MFP, Fraga D, Melo MCC, Souza GEP, Zampronio AR. Importance of the vagus nerve for fever and neutrophil migration induced by intraperitoneal LPS injection. *Inflamm Res* 2003;52:291–6. <https://doi.org/10.1007/s00011-003-1174-8>.
- [37] Zhang P, Han D, Tang T, Zhang X, Dai K. Inhibition of the development of collagen-induced arthritis in Wistar rats through vagus nerve suspension: a 3-month observation. *Inflamm Res* 2008;57:322–8. <https://doi.org/10.1007/s00011-008-8070-1>.
- [38] Pavlov VA, Tracey KJ. The cholinergic anti-inflammatory pathway. *Brain Behav Immun* 2005;19:493–9. <https://doi.org/10.1016/j.bbi.2005.03.015>.
- [39] Long L, Zang Q, Jia G, Fan M, Zhang L, Qi Y, et al. Transcutaneous auricular vagus nerve stimulation promotes white matter repair and improves dysphagia symptoms in cerebral ischemia model rats. *Front Behav Neurosci* 2022;16:811419. <https://doi.org/10.3389/FNBEH.2022.811419>.

- [40] Lysebettens W Van, Vonck K, Larsen LE, Stevens L, Bouckaert C, Germonpr C, et al. Identification of vagus nerve stimulation parameters affecting rat hippocampal electrophysiology without temperature effects. *Brain Stimul* 2020; 13:1198–206. <https://doi.org/10.1016/j.brs.2020.05.011>.
- [41] Van Lysebettens W, Vonck K, Larsen LE, Sprengers M, Carrette E, Bouckaert C, et al. Hypothermia masks most of the effects of rapid cycling VNS on rat hippocampal electrophysiology. 2019. p. 29. <https://doi.org/10.1142/S0129065719500084>. <https://doi.org/10.1142/S0129065719500084>.
- [42] Raedt R, Clinckers R, Mollet L, Vonck K, El Tahry R, Wyckhuys T, et al. Increased hippocampal noradrenaline is a biomarker for efficacy of vagus nerve stimulation in a limbic seizure model. *J Neurochem* 2011;117:461–9. <https://doi.org/10.1111/j.1471-4159.2011.07214.x>.
- [43] Hamlin N, Levine Y, Chernoff D. Bioelectronic therapy attenuates demyelination in a lysolecithin-induced multiple sclerosis model. *ECTRIMS Online Library*; 2018.
- [44] Hamlin N. Vagus nerve stimulation attenuates demyelination and accelerates remyelination in a lysolecithin-induced multiple sclerosis model. *ECTRIMS Online Library*; 2017.
- [45] Kalakh S, Mouihate A. The promyelinating properties of androstenediol in gliotoxin-induced demyelination in rat corpus callosum. <https://doi.org/10.1111/nan.12237>; 2015.
- [46] Kalakh S, Mouihate A. Demyelination-induced inflammation attracts newly born neurons to the white matter. *Mol Neurobiol* 2017;54:5905–18. <https://doi.org/10.1007/s12035-016-0127-5>.
- [47] Keough MB, Jensen SK, Wee Yong V. Experimental demyelination and remyelination of murine spinal cord by focal injection of lysolecithin. *JoVE* 2015; 2015. <https://doi.org/10.3791/52679>.
- [48] Merrill JE. In vitro and in vivo pharmacological models to assess demyelination and remyelination. *Neuropsychopharmacology* 2009;34:55–73. <https://doi.org/10.1038/npp.2008.145>.
- [49] McMurran CE, Zhao C, Franklin RJM. Toxin-based models to investigate demyelination and remyelination. 377–396, https://doi.org/10.1007/978-1-4939-9072-6_21; 2019.
- [50] Plemel JR, Michaels NJ, Weishaupt N, Capriariello AV, Keough MB, Rogers JA, et al. Mechanisms of lysophosphatidylcholine-induced demyelination: a primary lipid disrupting myelinopathy. *Glia* 2018;66:327–47. <https://doi.org/10.1002/glia.23245>.
- [51] Mouihate A, Kalakh S. Ganaxolone enhances microglial clearance activity and promotes remyelination in focal demyelination in the corpus callosum of ovariectomized rats. *CNS Neurosci Ther* 2020;26:240–50. <https://doi.org/10.1111/cns.13195>.
- [52] Schultz V, van der Meer F, Wrzoz C, Scheidt U, Bahn E, Stadelmann C, et al. Acutely damaged axons are remyelinated in multiple sclerosis and experimental models of demyelination. *Glia* 2017;65:1350–60. <https://doi.org/10.1002/glia.23167>.
- [53] El Tahry R, Mollet L, Raedt R, Delbeke J, De Herdt V, Wyckhuys T, et al. Repeated assessment of larynx compound muscle action potentials using a self-sizing cuff electrode around the vagus nerve in experimental rats. *J Neurosci Methods* 2011; 198:287–93. <https://doi.org/10.1016/j.jneumeth.2011.04.007>.
- [54] Grimonprez A, Raedt R, De Taeye L, Larsen LE, Delbeke J, Boon P, et al. A preclinical study of laryngeal motor-evoked potentials as a marker vagus nerve activation. *Int J Neural Syst* 2015;25. <https://doi.org/10.1142/S0129065715500343>.
- [55] Desloovere J, Boon P, Larsen LE, Goossens MG, Delbeke J, Carrette E, et al. Chemogenetic seizure control with clozapine and the novel ligand JHU37160 outperforms the effects of levetiracetam in the intrahippocampal kainic acid mouse model. *Neurotherapeutics* 2022;19:342–51. <https://doi.org/10.1007/s13311-021-01160-0>.
- [56] Stevens L, Larsen LE, Van Lysebettens W, Carrette E, Boon P, Raedt R, et al. Optimized parameters for transducing the locus coeruleus using canine adenovirus type 2 (CAV2) vector in rats for chemogenetic modulation research. *Front Neurosci* 2021;15. <https://doi.org/10.3389/fnins.2021.663337>.
- [57] Uniprot n.d. www.uniprot.org (accessed December 19, 2023).
- [58] Gerault M-A. DIAGui: DIAGui package n.d. <https://rdrr.io/github/mgerault/DIAGui/man/DIAGui.html> (accessed December 13, 2023).
- [59] Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;43. <https://doi.org/10.1093/nar/gkv007>. e47–e47.
- [60] Yu G, Wang L-G, Hu E, Xiao L, Meijun C, Giovanni D, et al. ClusterProfiler: a universal enrichment tool for interpreting omics data. <https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>. [Accessed 18 December 2023].
- [61] Yu G, Hu E, Goa C. Enrichplot: visualization of functional enrichment result. 1.22.0, <https://bioconductor.org/packages/enrichplot>. [Accessed 18 December 2023].
- [62] Bateman A, Martin M-J, Orchard S, Magrane M, Ahmad S, Alpi E, et al. UniProt: the universal protein knowledgebase in 2023. *Nucleic Acids Res* 2023;51: D523–31. <https://doi.org/10.1093/nar/gkac1052>.
- [63] Wang H, Robinson JL, Kocabas P, Gustafsson J, Anton M, Cholley P-E, et al. Genome-scale metabolic network reconstruction of model animals as a platform for translational research. *Proc Natl Acad Sci USA* 2021;118. <https://doi.org/10.1073/pnas.2102344118>.
- [64] Wang H, Robinson JL, Gustafsson J. Mouse-GEM: the generic genome-scale metabolic model of *Mus musculus*. 118, Version 1.8.0; 2023. <https://github.com/SysBioChalmers/Mouse-GEM/>. [Accessed 19 December 2023].
- [65] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 2005;102:15545–50. <https://doi.org/10.1073/pnas.0506580102>.
- [66] Dowell JA, Johnson JA, Li L. Identification of astrocyte secreted proteins with a combination of shotgun proteomics and bioinformatics. *J Proteome Res* 2009;8: 4135–43. <https://doi.org/10.1021/pr900248y>.
- [67] Dumont D, Noben J, Moreels M, Vanderlocht J, Hellings N, Vandenabeele F, et al. Characterization of mature rat oligodendrocytes: a proteomic approach. *J Neurochem* 2007;102:562–76. <https://doi.org/10.1111/j.1471-4159.2007.04575.x>.
- [68] Santiago JV, Natu A, Ramelow CC, Rayaprolu S, Xiao H, Kumar V, et al. PREPRINT: identification of state-specific proteomic and transcriptomic signatures of microglia-derived extracellular vesicles. *bioRxiv* 2023. <https://doi.org/10.1101/2023.07.28.551012>.
- [69] Zhong S, Zhang S, Fan X, Wu Q, Yan L, Dong J, et al. A single-cell RNA-seq survey of the developmental landscape of the human prefrontal cortex. *Nature* 2018;555: 524–8. <https://doi.org/10.1038/nature25980>.
- [70] Keough MB, Jensen SK, Yong VW. Experimental demyelination and remyelination of murine spinal cord by focal injection of lysolecithin. *JoVE* 2015. <https://doi.org/10.3791/52679>.
- [71] Kalakh S, Mouihate A. Enhanced remyelination during late pregnancy: involvement of the GABAergic system. *Sci Rep* 2019;9:7728. <https://doi.org/10.1038/s41598-019-44050-4>.
- [72] Chu T, Zhang YP, Tian X, Ye C, Zhu M, Shields LBE, et al. Dynamic response of microglia/macrophage polarization following demyelination in mice. *J Neuroinflammation* 2019;16:188. <https://doi.org/10.1186/s12974-019-1586-1>.
- [73] Mezydło A, Treiber N, Ullrich Gavalanes EM, Eichenseer K, Ancău M, Wens A, et al. Remyelination by surviving oligodendrocytes is inefficient in the inflamed mammalian cortex. *Neuron* 2023;111:1748–59. <https://doi.org/10.1016/j.neuron.2023.03.031>. e8.
- [74] Franklin RJM, Frisén J, Lyons DA. Revisiting remyelination: towards a consensus on the regeneration of CNS myelin. *Semin Cell Dev Biol* 2021;116:3–9. <https://doi.org/10.1016/j.semcdb.2020.09.009>.
- [75] Manitt C, Colicos MA, Thompson KM, Rousselle E, Peterson AC, Kennedy TE. Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord. *J Neurosci* 2001;21:3911–22. <https://doi.org/10.1523/JNEUROSCI.21-11-03911.2001>.
- [76] Bin JM, Rajasekharan S, Kuhlmann T, Hanes I, Marcal N, Han D, et al. Full-length and fragmented netrin-1 in multiple sclerosis plaques are inhibitors of oligodendrocyte precursor cell migration. *Am J Pathol* 2013;183:673–80. <https://doi.org/10.1016/j.ajpath.2013.06.004>.
- [77] Tepavčević V, Kerninon C, Aigrot MS, Meppiel E, Mozafari S, Arnould-Laurent R, et al. Early netrin-1 expression impairs central nervous system remyelination. *Ann Neurol* 2014;76:252–68. <https://doi.org/10.1002/ANA.24201>.
- [78] Tepavčević V, Lubetzki C. Oligodendrocyte progenitor cell recruitment and remyelination in multiple sclerosis: the more, the merrier?. <https://doi.org/10.1093/brain/awac307>; 2022.
- [79] Kotter MR, Setzu A, Sim FJ, Van Rooijen N, Franklin RJM. Macrophage depletion impairs oligodendrocyte remyelination following lysolecithin-induced demyelination. *Glia* 2001;35:204–12. <https://doi.org/10.1002/glia.1085>.
- [80] Maas DA, Angulo MC. Can enhancing neuronal activity improve myelin repair in multiple sclerosis? *Front Cell Neurosci* 2021;15. <https://doi.org/10.3389/fncel.2021.645240>.
- [81] Gautier HOB, Evans KA, Volbracht K, James R, Sitnikov S, Lundgaard I, et al. Neuronal activity regulates remyelination via glutamate signalling to oligodendrocyte progenitors. *Nat Commun* 2015;6:8518. <https://doi.org/10.1038/ncomms9518>.
- [82] Sahel A, Ortiz FC, Kerninon C, Maldonado PP, Angulo MC, Nait-Oumesmar B. Alteration of synaptic connectivity of oligodendrocyte precursor cells following demyelination. *Front Cell Neurosci* 2015;9. <https://doi.org/10.3389/fncel.2015.00077>.
- [83] Etcheberria A, Mangin J-M, Aguirre A, Gallo V. Adult-born SVZ progenitors receive transient synapses during remyelination in corpus callosum. *Nat Neurosci* 2010; 13:287–9. <https://doi.org/10.1038/nn.2500>.
- [84] Polvinen E, Matilainen M, Nylund M, Sucksdorf M, Airas LM. TSPO-detectable chronic active lesions predict disease progression in multiple sclerosis. <https://doi.org/10.1212/NXI.0000000000200133>; 2023.
- [85] Kamma E, Lasisi W, Libner C, Ng HS, Plemel JR. Central nervous system macrophages in progressive multiple sclerosis: relationship to neurodegeneration and therapeutics. *J Neuroinflammation* 2022;19. <https://doi.org/10.1186/s12974-022-02408-y>.
- [86] Frischer JM, Weigand SD, Guo Y, Kale N, Parisi JE, Pirkio I, et al. Clinical and pathological insights into the dynamic nature of the white matter multiple sclerosis plaque. *Ann Neurol* 2015;78:710–21. <https://doi.org/10.1002/ana.24497>.
- [87] Wittekindt M, Kaddatz H, Joost S, Staffeld A, Bitar Y, Kipp M, et al. Different methods for evaluating microglial activation using anti-ionized calcium-binding adaptor protein-1 immunohistochemistry in the cuprizone model. *Cells* 2022;11: 1723. <https://doi.org/10.3390/cells11111723>.
- [88] Meneses G, Bautista M, Florentino A, Díaz G, Acero G, Besedovsky H, et al. Electric stimulation of the vagus nerve reduced mouse neuroinflammation induced by lipopolysaccharide. *J Inflamm* 2016;13:1–11. <https://doi.org/10.1186/s12950-016-0140-5>.

- [89] Krahl SE, Clark KB, Smith DC, Browning RA. Locus coeruleus lesions suppress the seizure-attenuating effects of vagus nerve stimulation. *Epilepsia* 1998;39:709–14. <https://doi.org/10.1111/J.1528-1157.1998.TB01155.X>.
- [90] De Keyser J, Laureys G, Demol F, Wilczak N, Mostert J, Clinckens R. Astrocytes as potential targets to suppress inflammatory demyelinating lesions in multiple sclerosis. *Neurochem Int* 2010;57:446–50. <https://doi.org/10.1016/j.neuint.2010.02.012>.
- [91] Laureys G, Gerlo S, Spooren A, Demol F, De Keyser J, Aerts JL. β 2-adrenergic agonists modulate TNF- α induced astrocytic inflammatory gene expression and brain inflammatory cell populations. *J Neuroinflammation* 2014;11. <https://doi.org/10.1186/1742-2094-11-21>.
- [92] Laureys G, Clinckens R, Gerlo S, Spooren A, Wilczak N, Kooijman R, et al. Astrocytic β 2-adrenergic receptors: from physiology to pathology. *Prog Neurobiol* 2010;91:189–99. <https://doi.org/10.1016/j.pneurobio.2010.01.011>.
- [93] Alexander GM, Huang YZ, Soderblom EJ, He XP, Moseley MA, McNamara JO. Vagal nerve stimulation modifies neuronal activity and the proteome of excitatory synapses of amygdala/piriform cortex. *J Neurochem* 2017;140: 629–44. <https://doi.org/10.1111/jnc.13931>.
- [94] Olsen LK, Solis E, McIntire LK, Hatcher-Solis CN. Vagus nerve stimulation: mechanisms and factors involved in memory enhancement. *Front Hum Neurosci* 2023;17. <https://doi.org/10.3389/fnhum.2023.1152064>.
- [95] Roosevelt RW, Smith DC, Clough RW, Jensen RA, Browning RA. Increased extracellular concentrations of norepinephrine in cortex and hippocampus following vagus nerve stimulation in the rat. *Brain Res* 2006;1119:124–32. <https://doi.org/10.1016/j.brainres.2006.08.048>.
- [96] Follesa P, Biggio F, Gorini G, Caria S, Talani G, Dazzi L, et al. Vagus nerve stimulation increases norepinephrine concentration and the gene expression of BDNF and bFGF in the rat brain. *Brain Res* 2007;1179:28–34. <https://doi.org/10.1016/j.brainres.2007.08.045>.
- [97] Shen H, Fuchino Y, Miyamoto D, Nomura H, Matsuki N. Vagus nerve stimulation enhances perforant path-CA3 synaptic transmission via the activation of β -adrenergic receptors and the locus coeruleus. *Int J Neuropsychopharmacol* 2012;15:523–30. <https://doi.org/10.1017/S1461145711000708>.
- [98] Olsen LK, Moore RJ, Bechmann NA, Ethridge VT, Gargas NM, Cunningham SD, et al. Vagus nerve stimulation-induced International Journal of Neuropsychopharmacology cognitive enhancement: hippocampal neuroplasticity in healthy male rats. *Brain Stimul* 2022;15:1101–10. <https://doi.org/10.1016/j.brs.2022.08.001>.
- [99] Boucard AA, Chubykin AA, Comoletti D, Taylor P, Südhof TC. A splice code for trans-synaptic cell adhesion mediated by binding of neuroligin 1 to α - and β -neurexins. *Neuron* 2005;48:229–36. <https://doi.org/10.1016/j.neuron.2005.08.026>.
- [100] Singh SK, Stogsdill JA, Pulimood NS, Dingsdale H, Kim YH, Pilaz LJ, et al. Astrocytes assemble thalamocortical synapses by bridging NRX1 α and NL1 via hevin. *Cell* 2016;164:183–96. <https://doi.org/10.1016/j.cell.2015.11.034>.
- [101] Yamazaki R, Ohno N, Huang JK. Acute motor deficit and subsequent remyelination-associated recovery following internal capsule demyelination in mice. *J Neurochem* 2021;156:917–28. <https://doi.org/10.1111/jnc.15142>.
- [102] Luo Q, Ding L, Zhang N, Jiang Z, Gao C, Xue L, et al. A stable and easily reproducible model of focal white matter demyelination. *J Neurosci Methods* 2018;307:230–9. <https://doi.org/10.1016/j.jneumeth.2018.05.024>.
- [103] Aykan SA, Xie H, Lai JH, Zheng Y, Chung DY, Kura S, et al. Focal subcortical white matter lesions disrupt resting state cortical interhemispheric functional connectivity in mice. *Cerebr Cortex* 2021;31:4958–69. <https://doi.org/10.1093/cercor/bhab134>.
- [104] Perez-Riverol Y, Bai J, Bandla C, García-Seisdedos D, Hewapathirana S, Kamatchinathan S, et al. The PRIDE database resources in 2022: a hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res* 2022;50:D543–52. <https://doi.org/10.1093/nar/gkab1038>.