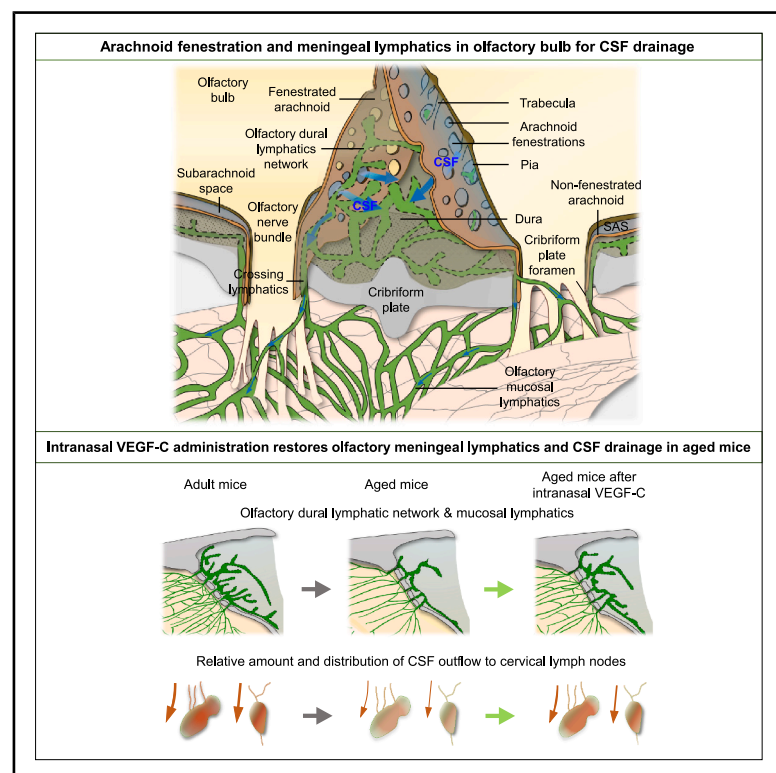


CSF clearance through arachnoid fenestrations to olfactory meningeal lymphatics

Graphical abstract



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In brief

Cerebrospinal fluid (CSF) crosses arachnoid fenestrations near the olfactory bulbs to reach dural lymphatics that traverse the cribriform plate, connect to nasal lymphatics, and drain to cervical lymph nodes. Aging impairs this pathway, but intranasal VEGF-C restores the lymphatics and the CSF outflow.

Highlights

- CSF crosses arachnoid fenestrations and enters dural lymphatics near olfactory bulbs
- Dural lymphatics carrying CSF traverse the cribriform plate and join nasal lymphatics
- Aging reduces arachnoid fenestrations, cribriform plate foramina, olfactory lymphatics, and CSF outflow
- Intranasal VEGF-C normalizes atrophic olfactory lymphatics and impaired CSF outflow

Article

CSF clearance through arachnoid fenestrations to olfactory meningeal lymphatics

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SUMMARY

Meningeal (dural) lymphatics are essential for cerebrospinal fluid (CSF) clearance to cervical lymph nodes, yet the precise pathway is incompletely understood. Using a multifaceted approach in mice, we examined tracer dynamics and the CSF outflow pathway from the subarachnoid space (SAS) to the nasal mucosa and identified a discrete arachnoid region surrounding the olfactory bulbs with abundant fenestrations. Similar arachnoid fenestrations were found in cynomolgus monkeys. Fluorescent tracers in the SAS passed through arachnoid fenestrations into dural lymphatics, which traversed the cribriform plate foramina, joined the nasal lymphatics, and drained to the cervical lymph nodes. In aged mice, the known reduction in CSF outflow was accompanied by lymphatic atrophy in the olfactory dura and nasal mucosa and by fewer arachnoid fenestrations and smaller cribriform plate foramina. Importantly, the lymphatics and CSF clearance were restored to normal by intranasal delivery of vascular endothelial growth factor-C (VEGF-C), thereby documenting the reversibility of the aging-related impairment in CSF clearance.

INTRODUCTION

Cerebrospinal fluid (CSF) plays a pivotal role in maintaining central nervous system (CNS) homeostasis by providing mechanical protection and facilitating the clearance of neurotransmitters and metabolites. As amyloid- β (A β), phosphorylated tau, α -synuclein, and other pathological protein aggregates are also removed by CSF clearance,^{1–3} impaired CSF clearance is thought to contribute to cognitive decline in aging, Alzheimer's disease, and other neurodegenerative disorders.^{4–9} These properties have stimulated interest in the mechanisms that govern CSF outflow, the pathophysiology of dysfunction in CSF drainage, and therapeutic strategies for ameliorating or reversing impairments in CSF clearance.^{10–13}

An estimated 50% of CSF is cleared through lymphatics that drain to the cervical lymph nodes.^{14,15} Through landmark studies,^{12,16–22} many features of the anatomical and functional connections for CSF outflow from the subarachnoid space (SAS) have been identified, including the involvement of dural

lymphatics, extracranial lymphatic networks, and cervical lymph nodes. However, important questions remain about the route CSF takes from the SAS to lymph nodes in the neck. Among these is whether CSF flows through direct connections between the SAS and dural lymphatics^{12,18,22} or first crosses discontinuities in the arachnoid barrier before entering dural lymphatics.²³ Additional proposed routes include CSF flowing through extensions of the SAS that traverse the cribriform plate,^{15,24,25} or through interstitial channels in and around cranial nerves^{14,26,27} to extracranial lymphatics in the nasal mucosa.

There is no dispute that a deeper understanding of the CSF drainage routes and their relative contributions to overall CNS waste clearance will advance the understanding of brain health and neurological diseases. While extensive evidence supports CSF drainage through lymphatics associated with olfactory nerves,^{12,13,16,17,22,23,26–30} other cranial nerves,^{1,18} and spinal nerves,^{31–33} the connections enabling CSF in the SAS to reach extracranial lymphatics have proven challenging to identify with assurance. High-resolution live imaging, strategic use of

tracers, and genetically engineered reporters and overexpression or deletion models in mice have been helpful for assessing the physiological relevance of CSF egress routes. Yet, questions persist on whether CSF reaches extracranial lymphatics in the nasal mucosa by traversing perineurial interstitial spaces or instead through dural lymphatics adjacent to the cribriform plate lymphatics.^{13,15,17,21,23,29,30,34} These questions are still unanswered despite the obvious anatomical proximity of the structures and acceptance that CSF can reach cervical lymph nodes by crossing the cribriform plate.¹³

An interstitial route for CSF drainage from the olfactory cistern³⁵ region of the SAS near the olfactory bulbs was proposed to pass around olfactory nerves through the cribriform plate to the nasal mucosa.^{14,26–28} However, more recent studies using *Prox1*-GFP and *Flt4* (*Vegfr3*)- β -gal reporter mice revealed that CSF tracers enter dural lymphatics near the cribriform plate and are found in the nasal mucosal lymphatics.^{12,22,23,36} These findings implicated the involvement of lymphatics throughout the clearance route but also raised new questions about the mechanism by which CSF in the SAS crosses the arachnoid barrier and enters dural lymphatics.

A plausible route across the arachnoid barrier came with findings of focal discontinuities in the barrier layer near the olfactory bulbs that could serve as entry points for CSF to access the lymphatic system.^{23,36,37} However, most of the evidence was obtained by analyzing histological tissue sections that did not reveal the three-dimensional (3D) architecture of the structures or the functional patency of the discontinuities. This limitation pointed to the need for approaches for real-time visualization of CSF tracer movement and 3D imaging of key structures along the route from the SAS through lymphatics that cross the cribriform plate to the nasal cavity.

With this background, we developed a multifaceted approach to circumvent these limitations by using immunofluorescence staining of whole-mount preparations, scanning electron microscopy (SEM), and functional tracing in *Prox1*-GFP reporter mice³⁸ to map the egress pathway of fluorescent CSF tracers. Using this approach, we sought to (1) identify the 3D architecture of the olfactory dural lymphatic network and compare it to downstream lymphatics in the olfactory mucosa; (2) assess CSF outflow through lymphatics in the olfactory dural network, cribriform plate, and nasal mucosa; (3) identify the size and distribution of fenestrations in the arachnoid near the olfactory bulbs by SEM; (4) compare CSF outflow through the cribriform plate region to arachnoid cuff exit (ACE) sites in the dorsal meninges; (5) identify the effects of aging on the olfactory dural lymphatic network; (6) estimate the contribution of olfactory dural lymphatics to CSF drainage to cervical lymph nodes; and (7) determine whether the dependence of the olfactory dural lymphatic network on vascular endothelial growth factor-C (VEGF-C)/VEGF receptor-3 (VEGFR3) signaling can be used to reverse aging-related changes that impair CSF outflow.

The studies revealed that the olfactory bulb dural lymphatic network in the region between the olfactory bulbs and the cribriform plate bordering the olfactory cistern had a unique 3D architecture and specialized connections to short lymphatics that traversed cribriform plate foramina and joined lymphatics in the olfactory mucosa. Nasal mucosal lymphatics also had a complex 3D

architecture, distinct from the dural lymphatic network. Strikingly, the arachnoid in the olfactory cistern region had abundant fenestrations that CSF tracers in the SAS could traverse to access dural lymphatics. The study also revealed that the known aging-related reduction in CSF outflow was accompanied by regression not only of the olfactory lymphatic network but also of arachnoid fenestrations, cribriform foramina, and nasal lymphatics. Importantly, the aging-related atrophy of dural lymphatics and impairment in CSF outflow could be reversed by intranasal delivery of VEGF-C by an adeno-associated viral vector.

RESULTS

Features of the olfactory dural lymphatic plexus

Lymphatics in the region of the olfactory bulbs and cribriform plate of *Prox1*-GFP mice were examined in sagittal and oblique-coronal tissue sections after immunofluorescence staining (Figure S1A). The 0.3–1.0 mm thickness of the sections, which effectively served as whole mounts, enabled 3D imaging of the architecture and endothelial cell features of the dural lymphatic network located between the olfactory bulbs and on the intracranial surface of the cribriform plate. Lymphatics crossing the cribriform plate and in the olfactory and respiratory regions of the nasal mucosa were similarly visible.

Examination of thick sagittal sections revealed that the olfactory dural lymphatic network extended from the dura between the right and left olfactory bulbs to the midline of the cribriform plate next to the anterior extension of the SAS (olfactory cistern³⁵) (Figures S1B–S1D). This lymphatic network had a single midline location and distinctive architectural features consisting of highly anastomotic vessel segments with rounded protrusions and sparse atypical valves (Figures 1A–1E and S1E; Video S1). The lymphatics were variable in diameter (median diameter 68 μ m, range 23–154 μ m, 10 lymphatics per mouse, $n = 5$ mice) and irregular in shape. Lymphatic endothelial cells (LECs) of the network had features typical of initial lymphatics, including strong *Prox1*-GFP, *VEGFR3*, and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1) immunoreactivities and discontinuous intracellular junctions^{39,40} (Figures 1B–1E). Examination of oblique sections cut in the plane of the cribriform plate and stained for LYVE1 (lymphatics) and β -III-tubulin (axons) confirmed the midline location of the dural lymphatic network and revealed lymphatic branches around olfactory nerve bundles (Figure S2A; Video S2).

Together, findings from both planes of section demonstrated that the olfactory dural lymphatic network consisted of midline initial lymphatics with blunt ends located between the olfactory bulbs that were connected to a midline lymphatic plexus located on the intracranial surface of the cribriform plate (Figures 1 and S2A; Videos S1 and S2). Branches from the midline lymphatic plexus surrounded olfactory nerve bundles and traversed foramina near the midline of the cribriform plate. Lymphatic vessels in cribriform plate foramina were small (median diameter 36 μ m, range 12–99 μ m, 10 lymphatics per mouse, $n = 5$ mice), short (median length 55 μ m, range 31–91 μ m, 10 lymphatics per mouse, $n = 5$ mice), and joined an anastomotic lymphatic plexus in the nasal surface of the cribriform plate (Figures 1, S1B–S1E, and S2A; Videos S1 and S2). Lymphatics in the

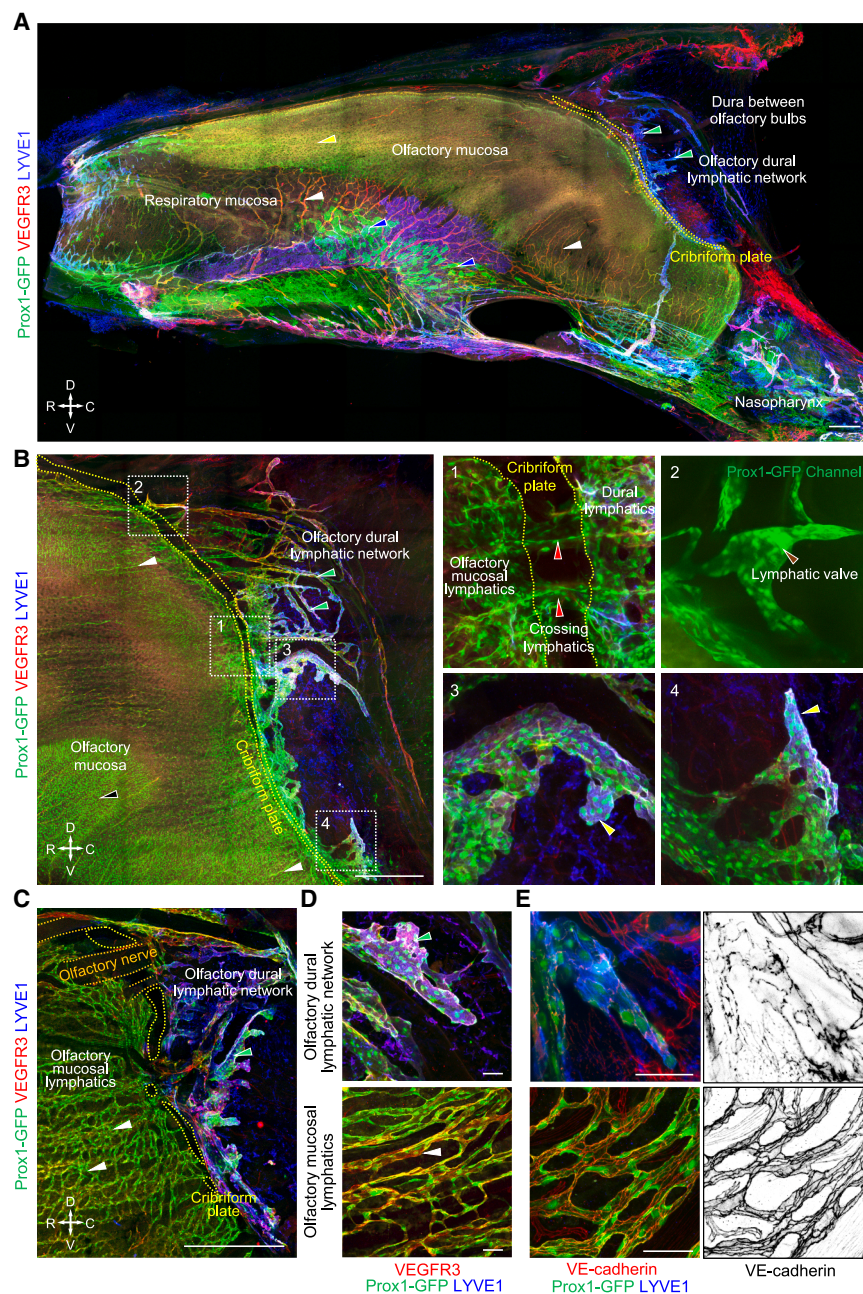


Figure 1. Olfactory dural lymphatic network connections to nasal lymphatics

For a Figure360 author presentation of Figure 1, see <https://doi.org/10.1016/j.cell.2026.06.035>

Figure360

(A) Immunofluorescence images of a mid-sagittal view of lymphatics in the olfactory dural lymphatic network that stain variably for Prox1, VEGFR3, and LYVE1 (green to blue or red) (green arrowheads) in *Prox1-GFP* mice. The dural network consists of initial lymphatics between the olfactory bulbs that join lymphatics on the intracranial surface of the cribriform plate. Also shown are lymphatics in olfactory and respiratory regions of the nasal mucosa that stain for Prox1 and VEGFR3 but not for LYVE1 (green to red) (white arrowheads). Prox1⁺ venous sinusoids (blue arrowheads), which are bright green, are also prominent. Cribriform plate outlined by yellow dotted lines. In the nasal mucosa, olfactory epithelial cells (yellow arrowhead) are also Prox1⁺. Scale bars, 500 μ m. Representative of $n = 6$ mice from three independent experiments.

(B) Enlarged view of olfactory dural lymphatic network at four boxes (1–4) in center and right: (1) lymphatics (red arrowheads) crossing cribriform plate; (2) atypical lymphatic valve (brown arrowhead) in the Prox1 (green) channel, which is one optical section from the thick confocal z stack and therefore differs somewhat from the overview image in left; and (3 and 4) initial lymphatics (yellow arrowheads) in the olfactory dural network that stains for Prox1, VEGFR3, and LYVE1. Olfactory mucosal lymphatics (white arrowheads) and olfactory dural lymphatic network (green arrowheads). In the nasal mucosa, olfactory epithelial cells (black arrowhead) are also Prox1⁺. Scale bars, 500 μ m. Representative of $n = 6$ mice from three independent experiments.

(C–E) Immunofluorescence images of mid-sagittal sections showing the olfactory dural lymphatic network (green arrowheads) and olfactory mucosal lymphatics (white arrowheads) in the region of the cribriform plate (C, dotted outline) in *Prox1-GFP* mice. Scale bars, 500 μ m. (C and D) Images comparing staining for VEGFR3 (red) and LYVE1 (blue) in Prox1⁺ lymphatics (green) of the olfactory dura and olfactory mucosa. (C) and (D) show different specimens. (E) 3-color (left) and monochrome (right) confocal images comparing discontinuous VE-cadherin staining of adherens junctions in olfactory dural lymphatics (upper row) and continuous VE-cadherin staining in olfactory mucosal lymphatics (lower row). The left and right

images of each row show the same lymphatics. Scale bars, 50 μ m (D and E). Representative of $n = 5$ mice from three independent experiments.

Anatomical orientation is indicated in the bottom left corners: D, dorsal; V, ventral; R, rostral; C, caudal.

See also Figures S1 and S2 and Videos S1 and S2.

Figure 360: An Author Presentation of Figure 1

sub-cribriform plexus continued in the mucosa of the nasal septum and lateral wall (Figure 1; Videos S1 and S2).

Contrasting features of lymphatics in the olfactory mucosa

Lymphatics in most regions of the olfactory mucosa were structurally very different from the olfactory dural lymphatic network up-

stream (Figures 1C–1E). The mucosal lymphatics lacked LYVE1 immunoreactivity and valves, were smaller (median diameter 22 μ m, range 10–46 μ m, 10 lymphatics per mouse, $n = 5$ mice), were tubular in shape, and had zipper-like intracellular junctions (Figures 1C–1E; Video S1). These lymphatics cascaded through the nasal mucosa along the course of olfactory nerves in the nasal mucosa^{41,42} (Figures 1A–1C; Videos S1 and S2).

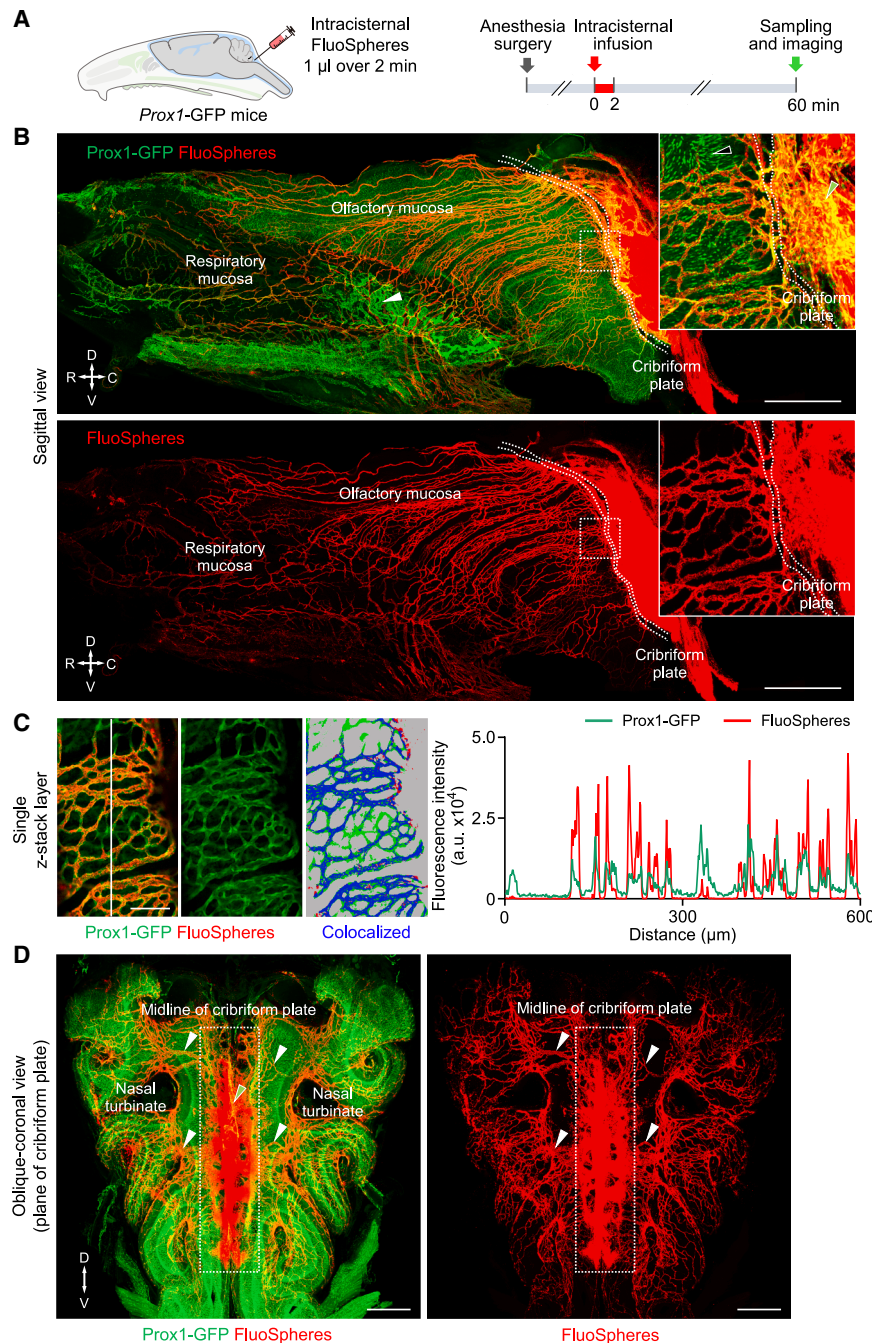


Figure 2. CSF tracer drainage through the olfactory dural lymphatic network to nasal lymphatics

For a Figure360 author presentation of Figure 2, see <https://doi.org/10.1016/j.cell.2026.06.035>

Figure360

(A) Sequence of intracisternal infusion of 1 µL FluoSpheres (diameter 200 nm) over 2 min into *Prox1*-GFP mice followed by tissue processing 60 min later.

(B) Immunofluorescence images of a mid-sagittal section of the nasal septum and dura between olfactory bulbs showing FluoSpheres (red) within lymphatics (green, yellow, or red) bordering the olfactory cistern, crossing the cribriform plate (dashed lines), and in the nasal mucosa of a *Prox1*-GFP mouse. White arrowhead marks *Prox1*⁺ venous sinusoids (green) that lack FluoSpheres. Boxed regions are enlarged in insets to show dural lymphatics (yellow, green arrowhead), olfactory mucosal lymphatics (red), and *Prox1*⁺ olfactory epithelial cells (green, black arrowhead). Scale bars, 1 mm. Representative of *n* = 5 mice from three independent experiments.

(C) Three images of a single optical section from a confocal microscopic z stack of (B) an inset region showing *Prox1*-GFP⁺ lymphatics (green) and FluoSpheres (red), lymphatics alone (green), and colocalization (blue) of FluoSpheres and lymphatics. In the colocalized image, some lymphatics (green) lack FluoSpheres, and small regions show FluoSpheres (red) with a lymphatic pattern where *Prox1* is below the detection threshold. White vertical line marks the region used for the fluorescence intensity profile on the right. Scale bars, 200 µm. Representative of *n* = 5 mice from three independent experiments.

(D) Fluorescence images of an oblique-coronal section in the plane of the cribriform plate showing FluoSpheres (red) in dural lymphatics (green arrowhead) near the midline of the cribriform plate (white box) and in the nasal mucosal lymphatics (white arrowheads) of a *Prox1*-GFP mouse. Strong *Prox1* fluorescence is also widespread in olfactory epithelial cells over the nasal turbinates. Scale bars, 500 µm. Representative of *n* = 5 mice from three independent experiments.

Anatomical orientation is indicated in the bottom left corners: D, dorsal; V, ventral; R, rostral; C, caudal.

See also Figures S2 and S9 and Video S3.

Figure 360: An Author Presentation of Figure 2

CSF outflow through the olfactory dural lymphatic network and nasal lymphatics

To test the contribution of the olfactory lymphatic network to CSF outflow, we infused 1 µL of FluoSpheres (200 nm diameter) over 2 min into the SAS at the cisterna magna of anesthetized *Prox1*-GFP mice (Figure 2A). At 60 min after infusion, FluoSphere fluorescence was strong in the dura between olfactory bulbs and lymphatics of the olfactory dural network (Figures 2B, S2B, and S2C). FluoSphere fluorescence also

highlighted lymphatics that crossed the cribriform plate, formed the plexus on the nasal surface of the cribriform plate, and cascaded through the nasal mucosa over the nasal septum and turbinates (Figures 2B, 2C, S2B, and S2C), consistent with their known involvement in CSF outflow.^{1,12,13,18,21–23,26–30,36,37} Importantly, FluoSphere fluorescence was not detected outside of lymphatics on the nasal surface of the cribriform plate or elsewhere in the nasal mucosa (Figures 2B–2D; Video S3).

Morphological features of arachnoid near olfactory bulbs

The olfactory bulbs, like other regions of the brain, are covered by three layers of meninges: inner pia, middle arachnoid, and outer dura. Immunohistochemical markers have been identified for all layers, with dipeptidyl peptidase-4 (DPP4), epithelial cadherin (E-cadherin), and claudin-11 serving as useful markers of the arachnoid in mice.^{8,43} Staining for these markers over the olfactory bulbs and cribriform plate was surprisingly heterogeneous. DPP4 and E-cadherin staining was discontinuous (discontinuous arachnoid) over the anterior medial surface of the olfactory bulbs and over the midline region of the cribriform plate in the region of the olfactory dural lymphatic network (Figures S3A–S3D and S4A–S4C). The arachnoid in this region had many variably sized (diameter 10–200 μ m), hole-like discontinuities in DPP4 staining (Figures S3A–S3D). By contrast, DPP4 staining was uniform and continuous in the arachnoid (continuous arachnoid) over the superior part of meninges between olfactory bulbs, lateral surfaces of the olfactory bulbs, around olfactory nerve bundles, and distal regions of the cribriform plate (Figures S3A–S3D and S4A–S4C).

Additional differences in the meninges were found to coincide with the discontinuous and continuous regions of arachnoid by immunostaining for selective markers of dural border cells (Slc47a1), arachnoid barrier cells (claudin-11), and inner arachnoid cells (Ppp1r1a).^{8,43} Consistent with the previous findings,^{8,43} regions with uniform staining for DPP4 and E-cadherin had intense staining for claudin-11, Slc47a1, and Ppp1r1a (Figures S4D–S4F). By contrast, regions with discontinuous, weak, and non-uniform staining for DPP4 and E-cadherin had discontinuous and non-uniform staining for claudin-11 and lacked Slc47a1 and Ppp1r1a staining (Figures S4D–S4F).

Continuous and discontinuous regions of arachnoid were accompanied by different types of blood vessels in the adjacent dura. Continuous arachnoid with uniform DPP4 and E-cadherin staining coincided with dural blood vessels that stained for plasmalemma vesicle-associated protein (PLVAP) and CD31 but not for claudin-5 or glucose transporter 1 (GLUT1), whereas regions with discontinuous DPP4 and E-cadherin staining coincided with dural blood vessels that stained for claudin-5, GLUT1, and CD31 but not for PLVAP (Figures S4G and S4H). PLVAP expression is a feature of the fenestrated vasculature of the choroid plexus and pituitary gland and dorsal dura,^{44–46} whereas expression of claudin-5 and GLUT1 without PLVAP is typical of brain regions with the blood-brain barrier.^{47,48} To determine the functional implications of these differences, we compared the permeability of blood vessels in the two regions of dura by injecting OVA-F-555 intravenously as a fluorescent tracer and, 15 min later, examined the distribution of immune cells that had phagocytized the tracer in *Prox1*-GFP mice (Figure S4I). OVA-F-555-containing cells were rare in the region with abundant lymphatics next to discontinuous arachnoid but were abundant in the region with sparse lymphatics next to continuous arachnoid (Figure S4J). These findings are evidence that dural blood vessels that stain for claudin-5 but not PLVAP in regions with discontinuous arachnoid were less permeable to OVA-F-555. Yet to be determined is whether dural blood vessels with less tracer permeability also

have less CSF uptake, a property that could favor greater CSF drainage through dural lymphatics.

These findings revealed a previously unrecognized heterogeneity of the meninges near the olfactory bulbs and cribriform plate, which included discontinuous staining of the arachnoid for DPP4, E-cadherin, and claudin-11 in the region of the olfactory dural lymphatic network. This discontinuous staining and presence of scattered hole-like openings in the arachnoid are consistent with previous reports of discontinuities in the arachnoid barrier in this region.²³

Identification of arachnoid fenestrations for CSF exit by SEM

To determine the structural implications of the discontinuous immunohistochemical staining of the arachnoid for DPP4 and other markers, we examined specimens from C57BL/6J mice by SEM. We found that the arachnoid located between the central and inferior regions of the olfactory bulbs and near the midline of the cribriform plate had conspicuous fenestrations of diverse sizes (diameter range 2–12 μ m; mean 4.5 μ m; SD $\pm$ 1.6 μ m; n = 4 mice, 73 fenestrations total) and spacing (Figure 3A). By contrast, no fenestrations were found in the arachnoid over the other regions of the olfactory bulb and lateral cribriform plate (Figures 3A, S5A, and S5B). The presence of abundant fenestrations fits with the hole-like pattern of DPP4 staining of the discontinuous arachnoid. The fibrous meshwork of the dura was visible through arachnoid fenestrations and was obvious after removal of the arachnoid (Figure 3B), consistent with arachnoid fenestrations serving as a direct route for CSF in the SAS to enter the dura.

These findings in mice led us to determine whether non-human primates have these features. SEM studies of *Macaca fascicularis* monkeys prepared by the same methods that were used for mice revealed fenestrations and trabeculae of diverse sizes in the arachnoid covering the medial aspects of the olfactory bulbs, but no fenestrations or trabeculae in the arachnoid outside this region (Figure S5C), as found in mice.

The next step was to confirm that arachnoid fenestrations provide a pathway for substances in the SAS to cross the arachnoid barrier. FluoSpheres (200 nm diameter) were infused into the SAS of anesthetized C57BL/6J mice, and 30 min later, the tissues were fixed by vascular perfusion for SEM examination (Figure 3C). Clusters of FluoSpheres were abundant near and within arachnoid fenestrations bordering the olfactory cistern (Figure 3D) and in the dura beneath the fenestrations (Figure 3E).

This structural evidence for arachnoid fenestrations as a route for CSF drainage was extended by functional studies in which FluoSpheres of two sizes (200 nm or 10 μ m diameter) were infused to determine the impact of blocking fenestrations on drainage of 70 kDa rhodamine-B dextran (RB-dextran) in CSF to cervical lymph nodes (Figure S6A). The larger FluoSpheres could block fenestrations as large as 10 μ m, whereas the smaller FluoSpheres could traverse most fenestrations.

At 1 h after infusion of fluorescent 70 kDa RB-dextran into CSF at the cisterna magna 1 day after FluoSphere infusion, the RB-dextran fluorescence in cervical lymph nodes was similarly strong in the 200-nm FluoSphere group and in the

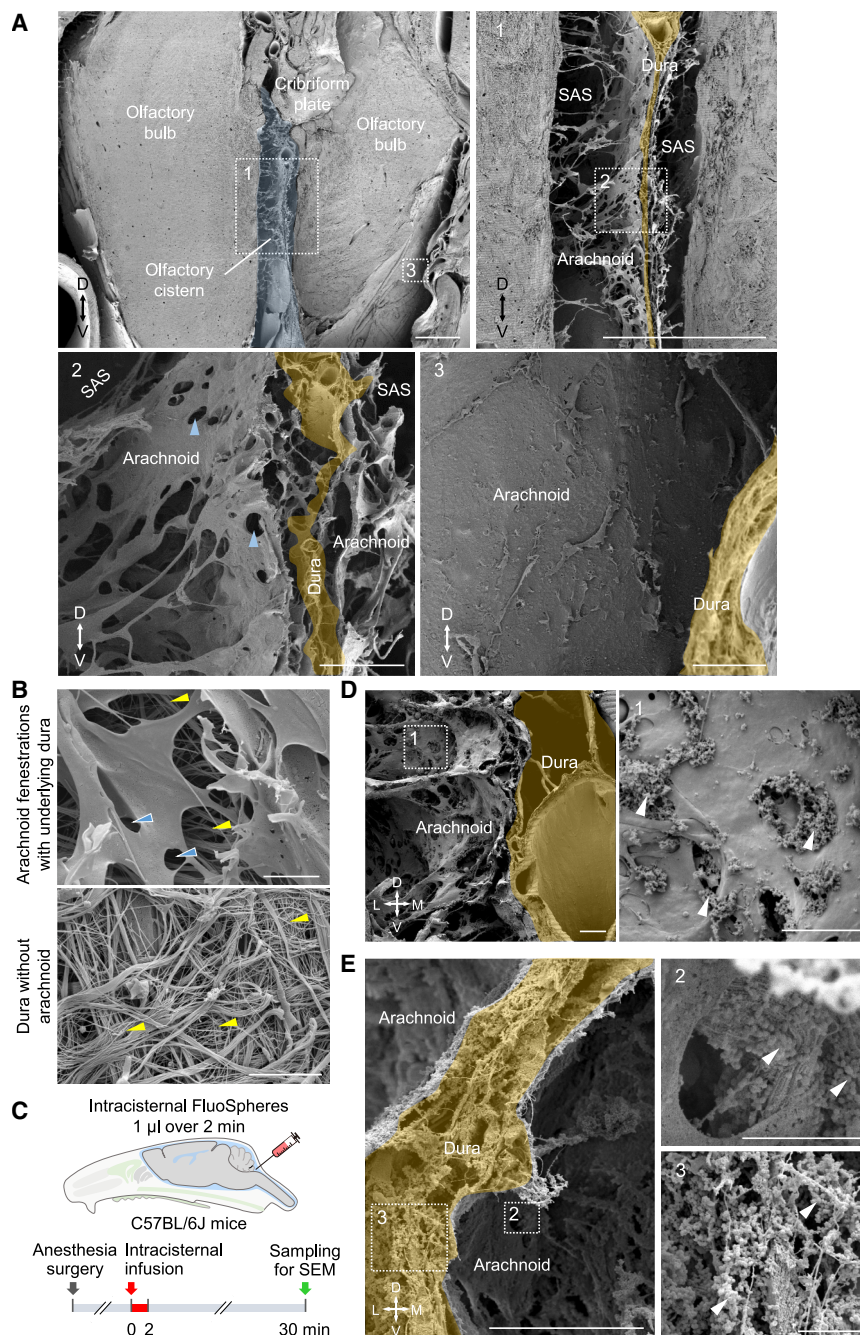


Figure 3. SEM images of fenestrations and FluoSpheres within fenestrations in the arachnoid near olfactory dural lymphatic network

For a Figure360 author presentation of Figure 3, see <https://doi.org/10.1016/j.cell.2026.06.035>

Figure360

(A) SEM images of a 0.4-mm thick oblique-coronal section showing the meninges and SAS (olfactory cistern, light blue shading) in the midline between the left and right olfactory bulbs and near the cribriform plate of a C57BL/6J mouse. Boxed regions 1–3 are enlarged in the other images. Region 1 and 2 at higher magnification show the meninges in the midline next to the olfactory cistern that is lined by arachnoid with abundant fenestrations (arrowheads) that span the SAS. Region 3 is on the lateral aspect of the olfactory bulb, where the arachnoid has no fenestrations. Dura is marked by beige shading. Scale bars: A main image 250 μ m; A inset images (2) and (3) 25 μ m. Representative of $n = 4$ mice from three independent experiments.

(B) SEM images showing arachnoid fenestrations (light blue arrowheads) and the underlying dural fiber network (yellow arrowheads, upper) and dura without the overlying arachnoid (lower) between olfactory bulbs near the cribriform plate. Scale bars, 5 μ m. Representative of $n = 3$ mice from two independent experiments.

(C) Sequence of intracisternal infusion of 1.0 μ l FluoSpheres (diameter 200 nm) over 2 min into C57BL/6J mice followed by tissue preparation 30 min later.

(D and E) SEM images of a 0.4-mm thick oblique-coronal section of the arachnoid bordering the olfactory cistern between the olfactory bulbs showing FluoSphere aggregates within fenestrations and in the adjacent dura (white arrowheads). Dura has beige shading (D and E). Boxed regions 1–3 are enlarged on the right. Scale bars, 20 μ m. Scale bar in boxed region 1, 10 μ m. Scale bars in boxed regions 2 and 3, 3 μ m. Representative images of $n = 4$ mice from three independent experiments.

Anatomical orientation is indicated in the bottom left corners: D, dorsal; V, ventral; L, lateral; M, medial.

See also Figures S3–S6.

PBS control group but was nearly absent in the 10- μ m group (Figures S6B and S6C). Consistent with this observation, RB-dextran-laden phagocytic cells were abundant in the dura overlying the olfactory bulbs adjacent to arachnoid fenestrations in the 200-nm and PBS groups but were rare in the 10- μ m group (Figures S6D and S6E). In support of the functional results, the SAS surface of arachnoid fenestrations was visible by SEM after infusion of 200-nm FluoSpheres or PBS but was covered with 10- μ m spheres after infusion of 10- μ m FluoSpheres (Figure S6F).

CSF outflow from the SAS in the olfactory cistern region, traversing arachnoid fenestrations to dural lymphatics that cross the cribriform plate en route to lymphatics of the nasal mucosa and cervical lymph nodes.

Greater CSF drainage through arachnoid fenestrations near the cribriform plate than through “ACE” sites in the dorsal meninges

The report that CSF can cross the arachnoid barrier at sites where veins that bridge the SAS cross the arachnoid (ACEs⁴⁹)

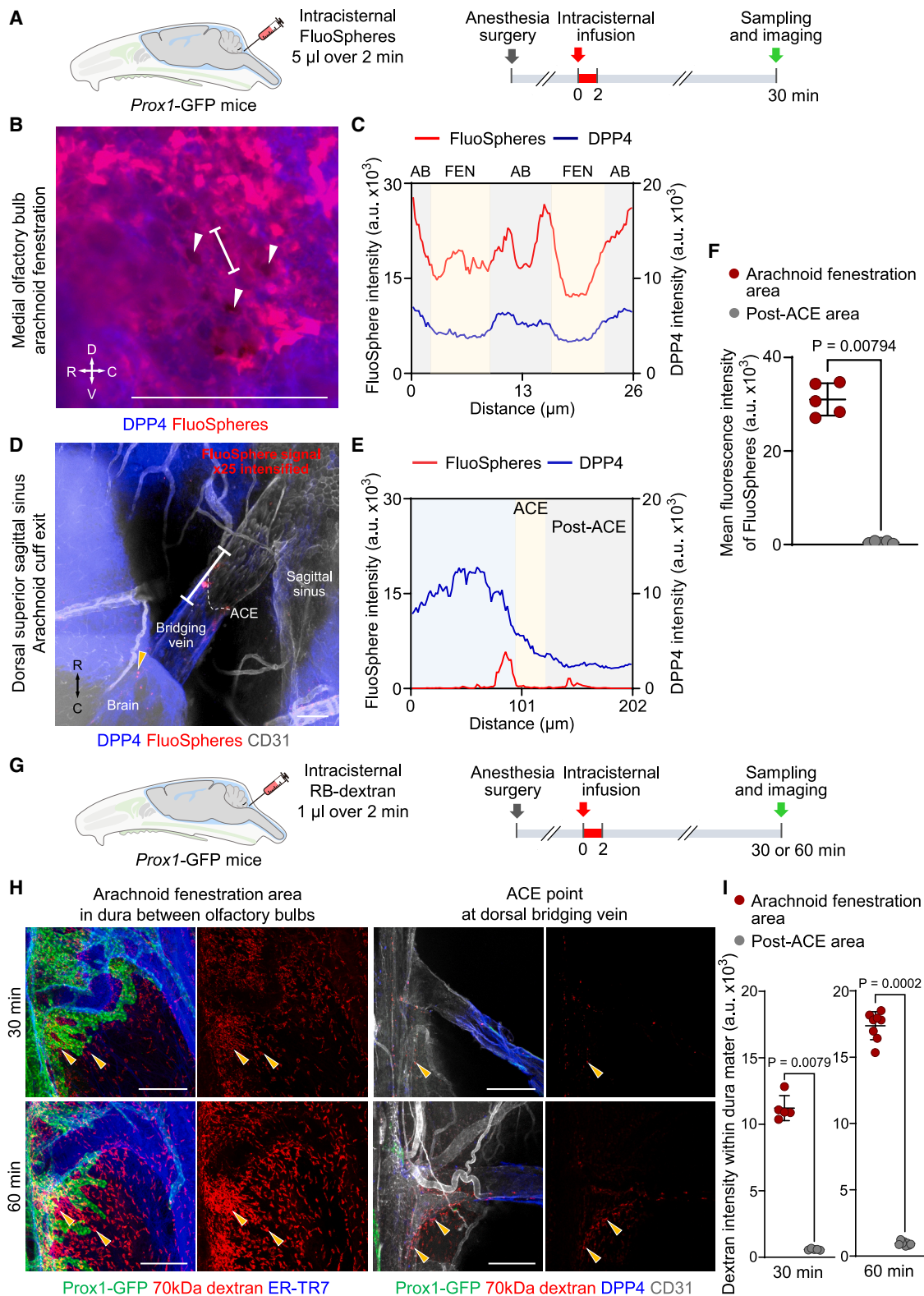


Figure 4. Greater FluoSpheres or RB-dextran drainage through arachnoid fenestrations than at ACE sites

(A) Sequence of intracisternal infusion of 5.0 μ l FluoSpheres (diameter 200 nm) over 2 min into *Prox1*-GFP mice followed by tissue processing 30 min later.

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raised questions of the barrier properties of these sites and the magnitude of their contribution to CSF clearance. To address these questions, we first identified DPP4⁺ regions of the arachnoid around bridging veins at ACE sites. Confocal microscopic imaging revealed that bridging veins had sleeves of DPP4⁺ cells that ended abruptly where the vessels crossed the arachnoid into the dura at the ACE sites near the sagittal and transverse sinuses (Figures S7A–S7C). The abrupt ending of DPP4⁺ cells on veins provided a landmark for determining the contribution of ACE sites versus cribriform region lymphatics to the outflow of CSF tracers in the SAS.

We then infused each of four different CSF tracers into the SAS at the cisterna magna to compare the amount crossing through ACE sites and the cribriform plate region. As the first tracer, 1 μ L of 200-nm FluoSpheres was infused over 2 min, and fluorescence was measured in the two regions 30 min later (Figure S7D). FluoSpheres were rare at post-ACE sites and in dural lymphatics near the bridging veins but were abundant in and around the arachnoid fenestrations and olfactory dural lymphatic network (Figures S7E–S7G and S8A–S8E). FluoSphere fluorescence in regions of dura distal to ACE sites (post-ACE areas) was only 0.85% of the amount in the dura near the discontinuous arachnoid bordering the olfactory cistern (Figures S8B–S8F). To compensate for potentially low values that have been reported for CSF flow from the cisterna magna^{20,50} to ACE sites over the dorsal cortex, we increased the amount of tracer to 5 times the volume of FluoSpheres, which was the maximum tolerated without meningeal injury, and found more FluoSphere fluorescence near bridging veins and post-ACE areas (Figures 4A–4E). However, FluoSphere fluorescence in post-ACE areas was still only 1.45% of the value in olfactory regions of arachnoid fenestrations (Figures 4B–4F).

As the second tracer, anti-LYVE1 antibody was infused and measured as immunofluorescence at 30 min in the same regions as for FluoSpheres. LYVE1 antibody fluorescence associated with Prox1⁺ olfactory dural lymphatics was 22.6-fold the amount in dural lymphatics around the transverse sinus (Figures S8G and S8H).

As the third tracer, ovalbumin-Alexa Fluor 555 (OVA-F-555) was infused and examined in the same regions 15 min later (Figure S8I). Fluorescence was strong in presumptive phagocytic immune cells in the dura bordering fenestrated regions of the arachnoid and in olfactory dural lymphatics and olfactory mucosal lymphatics, but little or none was found in the dura

bordering regions of continuous arachnoid (Figure S8J). OVA-F-555 was also readily detected along bridging veins in the SAS but stopped abruptly at ACE sites, and little or none was detected beyond these sites or in dural lymphatics near the sagittal or transverse sinuses (Figure S8K).

As the fourth tracer, 70 kDa RB-dextran was infused and examined between the olfactory bulbs and near ACE sites 30 or 60 min after infusion (Figure 4G). At both time points, fluorescence in RB-dextran taken up by immune cells in the dura near olfactory arachnoid fenestrations was 19.2- and 18.3-fold the amount in the dura of the post-ACE region at 30 and 60 min (Figures 4H and 4I).

Together, these tracer data align with a recent report³⁶ and provide evidence that CSF tracers ranging from 45 kDa ovalbumin and 70 kDa dextran to antibodies and 200-nm particles can exit the SAS through arachnoid fenestrations near the olfactory cistern and cribriform plate and can also exit at ACE sites over the dorsal cortical surface, but the amount is much greater near the cribriform plate than at dorsal ACE sites under the conditions of these experiments.

Olfactory dural lymphatic contribution to CSF drainage to cervical lymph nodes

CSF outflow has been reported to be impaired after olfactory nerves and nasal lymphatics are destroyed by intranasal infusion of ZnSO₄.³⁰ We used this approach to estimate the proportional contribution of olfactory and nasal lymphatics to total CSF drainage to cervical lymph nodes (Figure S9A). The area density of Prox1⁺ lymphatics near the olfactory bulb and cribriform plate was 36% less in the ZnSO₄ group than in the controls. The value for lymphatics in the olfactory mucosa was 31% less in the ZnSO₄ group but was not different for lymphatics in the respiratory region of the nasal mucosa (Figures S9B–S9E).

Consistent with these findings, 70 kDa RB-dextran fluorescence at 60 min after intracisternal infusion was 60% less in submandibular lymph nodes and 40% less in deep cervical lymph nodes in the ZnSO₄ group than in the controls (Figures S9F and S9G). Corresponding values in the ZnSO₄ group after infusion of A β (1–42) Hi-Lyte Fluor 647 were 49% less in submandibular lymph nodes and 26% less in deep cervical lymph nodes (Figures S9H and S9I).

These changes after intranasal ZnSO₄ were not accompanied by changes in ventricular volume measured by magnetic resonance imaging (MRI) (Figures S9J and S9K), intracranial

(B–F) Immunofluorescence images and corresponding fluorescence intensity profiles for FluoSpheres and DPP4 along the line indicated by white bars, and (F) comparison of mean fluorescence intensity of FluoSpheres near arachnoid fenestrations or post-ACE sites in each region of interest (see STAR Methods). The confocal microscope laser of FluoSphere channel had the same settings for fluorescence profiles and measurements in (B), (C), (E), and (F) to ensure comparability. The FluoSpheres fluorescence intensity scale is 25-fold higher in (D) than in (B). In (C), AB, arachnoid barrier; FEN, fenestration. The white arrowheads in (B) mark fenestrations with FluoSpheres. Yellow arrowhead in (D) marks FluoSphere fluorescence within DPP4⁺ region on the brain. Scale bars, 100 μ m. Each dot is the value for one mouse. Error bars indicate mean $\pm$ SD. $n = 5$ per group from three independent experiments. a.u., arbitrary unit. p values calculated by two-tailed Mann-Whitney U test.

(G) Sequence of intracisternal infusion of 1.0 μ L 70 kDa RB-dextran over 2 min into Prox1-GFP mice followed by tissue processing 30 min or 60 min later.

(H and I) Immunofluorescence images of lymphatics in the dura between the olfactory bulbs and a bridging vein at an ACE site in the dorsal dura showing regions of RB-dextran (yellow arrowheads) in adult Prox1-GFP mice 30 or 60 min after intracisternal infusion. Graphs of the area density of RB-dextran fluorescence in the dura mater near arachnoid fenestrations and ACE sites. Scale bars, 200 μ m. Each dot represents the average value for three regions in one mouse. Error bars indicate mean $\pm$ SD. $n = 5$ –8 mice per group from three independent experiments. a.u., arbitrary unit. p values calculated by two-tailed Mann-Whitney U test.

Anatomical orientation is indicated in the bottom left corners: D, dorsal; V, ventral; R, rostral; C, caudal.

See also Figures S7 and S8.

Figure 360: An Author Presentation of Figure 4

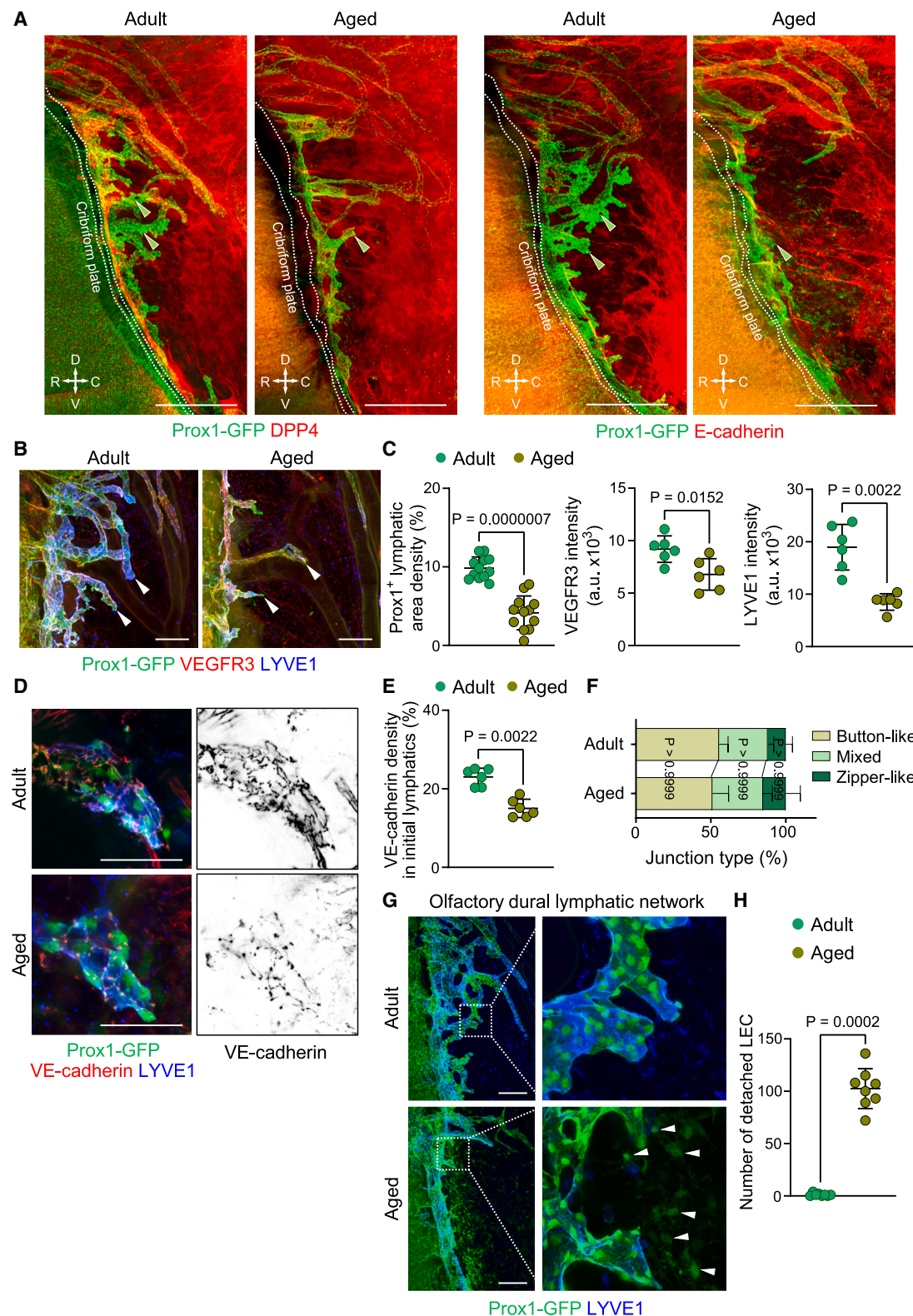


Figure 5. Aging-related atrophy of olfactory dural lymphatic network

(A) Immunofluorescence images of mid-sagittal sections comparing the Prox1⁺ olfactory dural lymphatic network (green) (green arrowheads) on the intracranial side (right) of the cribriform plate (dotted white lines) of adult (8–10 weeks) and aged (85–100 weeks) Prox1-GFP mice. Arachnoid between the olfactory bulbs and

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pressure measured with a transducer in the brain parenchyma (Figure S9L), or the extent of dural lymphatics in basolateral or dorsal regions around the brain (Figures S9M and S9N), consistent with previous reports.³⁰

These findings are evidence that partial destruction of olfactory neurons and neighboring lymphatics was accompanied by a corresponding reduction in CSF outflow of A β to cervical lymph nodes, without changes in ventricular volume, intracranial pressure, or lymphatics around dorsal or basolateral regions of the brain. Although the reduction in CSF outflow with aging is anticipated to have a similar decrease in A β clearance, new approaches are needed to determine whether CSF outflow to cervical lymph nodes is abolished by selective and complete destruction of olfactory lymphatics. The question of whether CSF absorption by cerebral blood vessels undergoes compensatory increases under these conditions also warrants further work.

Atrophy of the olfactory dural lymphatic network during aging

Multiple studies have shown that CSF outflow decreases during aging.^{22,51–53} The evidence includes less CSF drainage to superficial and deep cervical lymph nodes in aged mice,^{5,12,18,20,22,53} but it is unknown whether lymphatics in the olfactory dural network near the cribriform plate undergo similar changes during aging. To address this question, we compared olfactory dural lymphatics in aged mice (85–100 weeks old) and young adults (8–10 weeks old) and found they were less numerous (–58% Prox1⁺ area) and had weaker staining for VEGFR3 (–26%) and LYVE1 (–55%) in aged mice (Figures 5A–5C and S10A). Vascular endothelial cadherin (VE-cadherin) immunofluorescence was also less (–35%) in aged mice, but the relative proportions of button-like, zipper-like, and mixed intercellular junctions were similar in the two groups (Figures 5D–5F). Although the relationship between VE-cadherin expression and junction morphology is still to be determined, the findings indicate that aging attenuates VE-cadherin expression at intercellular junctions of olfactory dural lymphatics without altering the proportions of button- or zipper-like junctions. Another change was the appearance of seemingly detached and fragmented Prox1⁺ LECs in the aged mice (Figures 5G and 5H), similar to those found in the nasopharyngeal lymphatic plexus of aged mice.²²

Further differences in aged mice included fewer Prox1⁺ lymphatics in the olfactory mucosa (–48%) and respiratory mucosa (–50%) (Figures S10B and S10C). In addition, SEM imag-

ing of the SAS surface of the arachnoid in this region revealed adherent cells (Figure 6A) that were identified as CD45⁺ by immunohistochemical staining (Figure S10D), and arachnoid fenestrations near the midline of the cribriform plate of aged mice were smaller (–51%) and less numerous (–59%) (Figures 6B and 6C).

The aging-related reduction of olfactory dural lymphatics and CSF outflow in mice led us to determine whether similar changes occurred in the bony foramina of the central part of the cribriform plate through which most branches of the olfactory dural lymphatic network crossed to the nasal mucosa. Measurements revealed that in young adults, foramina within 350 μ m of the midline were larger (median diameter 99 μ m) than those located more laterally (median diameter 42 μ m) (Figures S11A–S11D). In aged adults, medial foramina of the cribriform plate were 24% smaller, 49% less numerous, and occupied 64% less overall area (area density) than in young adults, but lateral foramina were unchanged (Figures 6D–6F). As reflected by the area density values, linear regression analysis of foramen size and number gave values of $R^2 = 0.2749$, $p = 0.3644$ in young adults; $R^2 = 0.6069$, $p = 0.1204$ in aged mice; and $R^2 = 0.7712$, $p = 0.0008$ in both groups analyzed together, consistent with a link between the two features in aged mice.

To identify which structural changes appeared first during aging, we compared the dural lymphatic density, total cribriform plate foramen area, and number of arachnoid fenestrations in three age groups: young adult (8–12 weeks old), early middle-aged (30–35 weeks old), and late middle-aged (45–50 weeks old) mice. The three parameters were similar in adult and early middle-aged mice, but all values were smaller in late middle-aged mice. Olfactory dural lymphatic density was 22% less, the medial cribriform plate foramen area was –24% smaller, and arachnoid fenestrations were 55% less numerous in late middle-aged mice than in young adults (Figures S11E–S11G).

The coincidence of these changes during aging argues against causal relationships and is evidence that they occurred in parallel with other changes in the cribriform plate region that reduced CSF efflux through olfactory lymphatics to cervical lymph nodes. Aging-related atrophy also occurs in the nasopharyngeal and hard palatal lymphatic plexus and in dural lymphatics near dorsal venous sinuses in mice.^{5,12,18,20,22} Changes in the cribriform plate during aging have also been reported in humans.^{30,54} Additional research is needed to identify the causal mechanisms for these changes.

near the cribriform plate is stained for DPP4 (left) or E-cadherin (right). Scale bars, 500 μ m. Representative of $n = 8$ mice per age group from four independent experiments.

(B and C) Images and measurements showing a less extensive olfactory dural lymphatic network (arrowheads) in aged mice after staining for Prox1-GFP, VEGFR3, and LYVE1 immunoreactivity in adult and aged Prox1-GFP mice. Scale bars, 200 μ m. Each dot represents one mouse. $n = 6$ –12 mice per group from four independent experiments. Error bars indicate mean $\pm$ SD. a.u., arbitrary unit. p values calculated by two-tailed Mann-Whitney U test.

(D–F) Images and measurements showing less VE-cadherin staining of intercellular junctions in initial lymphatics of the olfactory dural lymphatic network in aged Prox1-GFP mice. Scale bars, 50 μ m. Each dot represents one mouse. $n = 6$ mice per group from three independent experiments. Error bars indicate mean $\pm$ SD. p values calculated by two-tailed Mann-Whitney U test or by two-way ANOVA test followed by two-tailed Holm-Sidak's multiple comparison post-hoc test.

(G and H) Confocal microscopic images comparing Prox1⁺ initial lymphatics of the olfactory dural lymphatic network in adult and aged Prox1-GFP mice. In the aged mouse, some lymphatic endothelial cells (LECs, white arrowheads) have weak Prox1 staining and could be detached. Scale bars, 200 μ m. Each dot represents one mouse. $n = 8$ mice per group from four independent experiments. Error bars indicate mean $\pm$ SD. p values calculated by two-tailed Mann-Whitney U test.

Anatomical orientation is indicated in the bottom left corners: D, dorsal; V, ventral; R, rostral; C, caudal.

See also Figures S10 and S11.

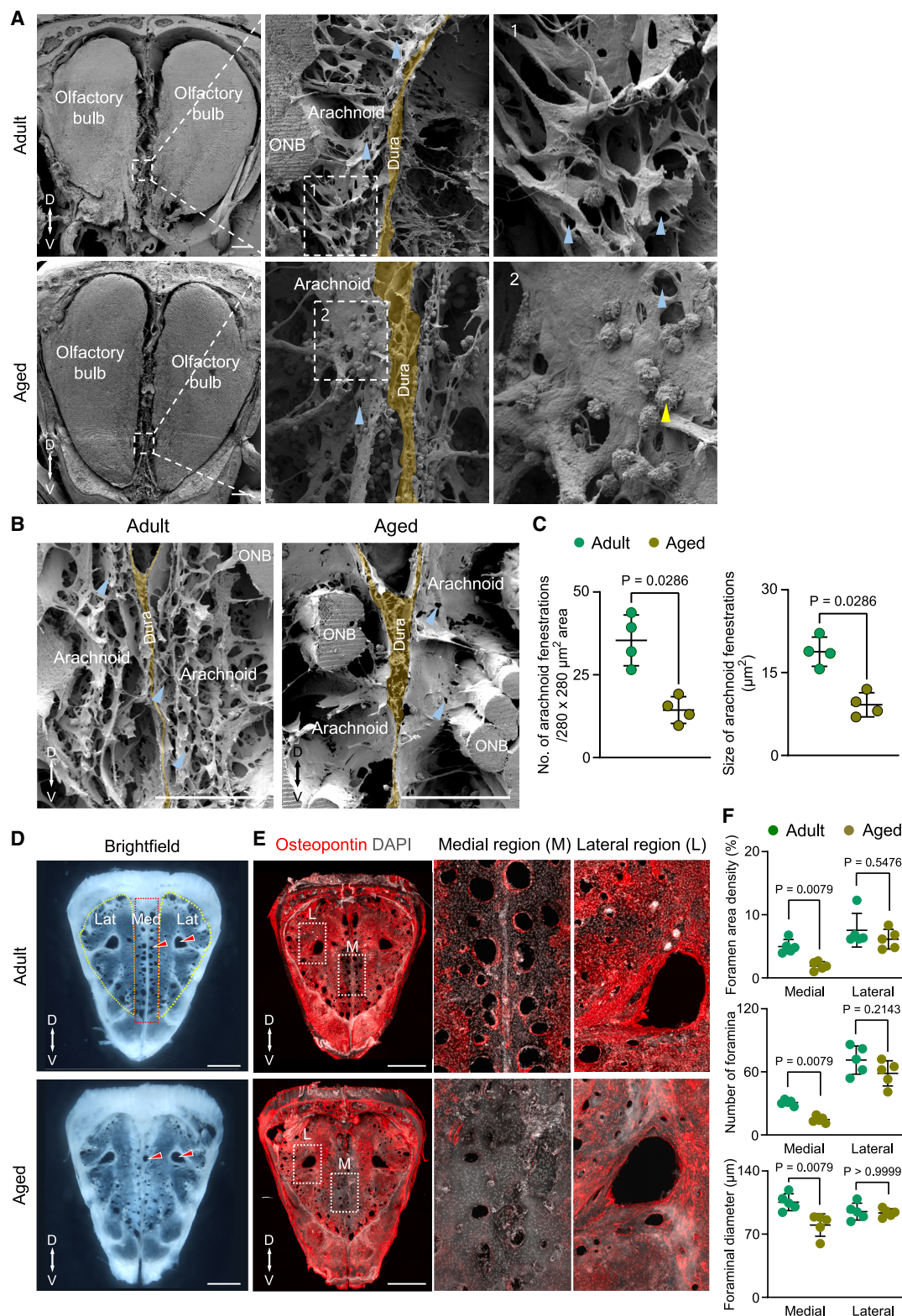


Figure 6. Aging-related alterations in arachnoid fenestrations next to the olfactory cistern and cribriform plate foramina

(A) SEM images of 0.4-mm oblique-coronal sections through the olfactory bulbs in the plane of the cribriform plate comparing the abundance of arachnoid fenestrations next to the olfactory cistern in adult (8–10 weeks) and aged (85–100 weeks) C57BL/6J mice. Boxed regions 1 and 2 in the overview images are

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Atrophy or expansion of olfactory dural lymphatics and CSF outflow by manipulating VEGFR3 signaling

As reported previously, dural lymphatic networks can be selectively reduced by blocking VEGFR3 signaling or expanded by exposure to VEGF-C.^{5,10,16,17,19,20,22,33,55} We sought to determine whether lymphatics in the region of the olfactory bulb, cribriform plate, and nasal mucosa can be manipulated in similar loss- and gain-of-function approaches.

To test the dependency of these lymphatics on VEGF-C/VEGFR3 signaling, soluble mouse VEGFR3 (VEGF-C trap) was overexpressed in *Prox1*-GFP mice by i.p. delivery of 1×10^{12} gene copies of adeno-associated virus (AAV) serotype-9 encoding mouse VEGFR-3 (1–4) (AAV-VR3 [1–4]), which expresses VEGF-C trap or control AAV9-VEGFR3 (4–7) (AAV-VR3-control), as previously described^{10,17,19} (Figure S12A).

At 6 weeks, lymphatics in the AAV-VR3 (1–4) group were significantly less numerous in the olfactory bulb dura (–58%), olfactory mucosa (–65%), and respiratory nasal mucosa (–73%) than in the corresponding regions after AAV-VR3-control (Figures S12B–S12F). Large reductions after AAV-VR3 (1–4) were also found in the dorsal dura (–97%) and basolateral dura (–84%) (Figures S12G and S12H). By comparison, no differences were found in the overall area of medial foramina in the cribriform plate or the number of arachnoid fenestrations after VEGF-C trap (Figures S12I and S12J).

The potential for expansion of these lymphatics was explored by overexpressing mouse VEGF-C (mVEGF-C) in *Prox1*-GFP mice by intracisternal delivery of 5×10^{10} gene copies of AAV serotype-9 encoding mVEGF-C (AAV-VEGF-C) or AAV9-empty control vector (AAV-control) (Figure S13A).¹⁰ At 6 weeks, lymphatics in the AAV-VEGF-C group were more abundant in the olfactory bulb dura (+97%), nasal olfactory mucosa (+115%), and nasal respiratory mucosa (+75%) compared with the corresponding regions in the AAV-control group (Figures S13B–S13F). Increases were even greater in the dorsal dura (+445%) and basolateral dura (+133%) (Figures S13G and S13H).

Consistent with earlier findings,^{5,10,16,19,22,33} these results show variable responses of diverse regions of dural lymphatics but support the feasibility of manipulating dural lymphatics around the cribriform plate and the use of AAV-VEGF-C to determine the reversibility of lymphatic atrophy and impaired CSF outflow in aged mice.

Restoration of dural lymphatics and CSF outflow in aged mice by nasal AAV-VEGF-C

To build on evidence that intranasal viral delivery of a VEGF-C-expressing viral vector can expand the nasal lymphatic network,⁵⁶ we asked whether the same approach could be used to expand the intracranial network of dural lymphatics near the olfactory bulbs. To address this question, we delivered 10^{11} gene copies of the AAV-VEGF-C or AAV-control vectors intranasally to *Prox1*-GFP mice at ages 8–12-weeks; 6 weeks later, CSF drainage of 70 kDa RB-dextran to cervical lymph nodes was measured, and dural lymphatics between the olfactory bulbs were examined and quantified (Figure S14A). In mice that received the AAV-VEGF-C vector, the olfactory dural lymphatic network was larger (+21%), and RB-dextran fluorescence in submandibular and deep cervical lymph nodes was greater (+16%) than after the AAV-control vector (Figures S14B and S14C). However, the number of arachnoid fenestrations and the combined area of medial foramina of the cribriform plate were similar after intranasal viral delivery of AAV-VEGF-C or AAV-control (Figures S14D and S14E).

Based on the finding that the lymphatic network near the cribriform plate can be expanded by AAV-VEGF-C delivered intranasally, we asked whether lymphatic atrophy and impaired CSF clearance in aged mice can be reversed by the same approach (Figure 7A). We found that 6 weeks after intranasal delivery of AAV-VEGF-C to *Prox1*-GFP mice at ages 85–100 weeks, lymphatics in the dura between the olfactory bulbs were doubled (+101%) and were also increased in the nasal olfactory mucosa (+72%) and nasal respiratory mucosa (+126%) (Figures 7B and 7C) but not in the dorsal dura, basolateral dura, or nasopharynx (Figures S14F–S14I). Arachnoid fenestrations and medial cribriform plate foramina were unchanged (Figures S14J and S14K).

The effect of intranasal delivery of AAV-VEGF-C on CSF outflow through lymphatics near the cribriform plate of aged *Prox1*-GFP mice was assessed by measuring RB-dextran fluorescence in cervical lymph nodes 60 min after intracisternal infusion of 1 μ l of the tracer into the SAS at the cisterna magna (Figure 7A). Outflow values in young adults were compared with aged mice with or without AAV-VEGF-C. Without AAV-VEGF-C, RB-dextran fluorescence values were less in submandibular (–50%) and deep cervical (–40%) lymph nodes of aged mice than in young adults. In comparison, RB-dextran

enlarged in the center and right images. Fewer fenestrations (light blue arrowheads) and more attached cells resembling leukocytes (yellow arrowheads) are present in the arachnoid of the aged mouse. Dura (beige shading). ONB, olfactory nerve bundle. Scale bars, 250 μ m. Representative images are from $n = 4$ mice from three independent experiments.

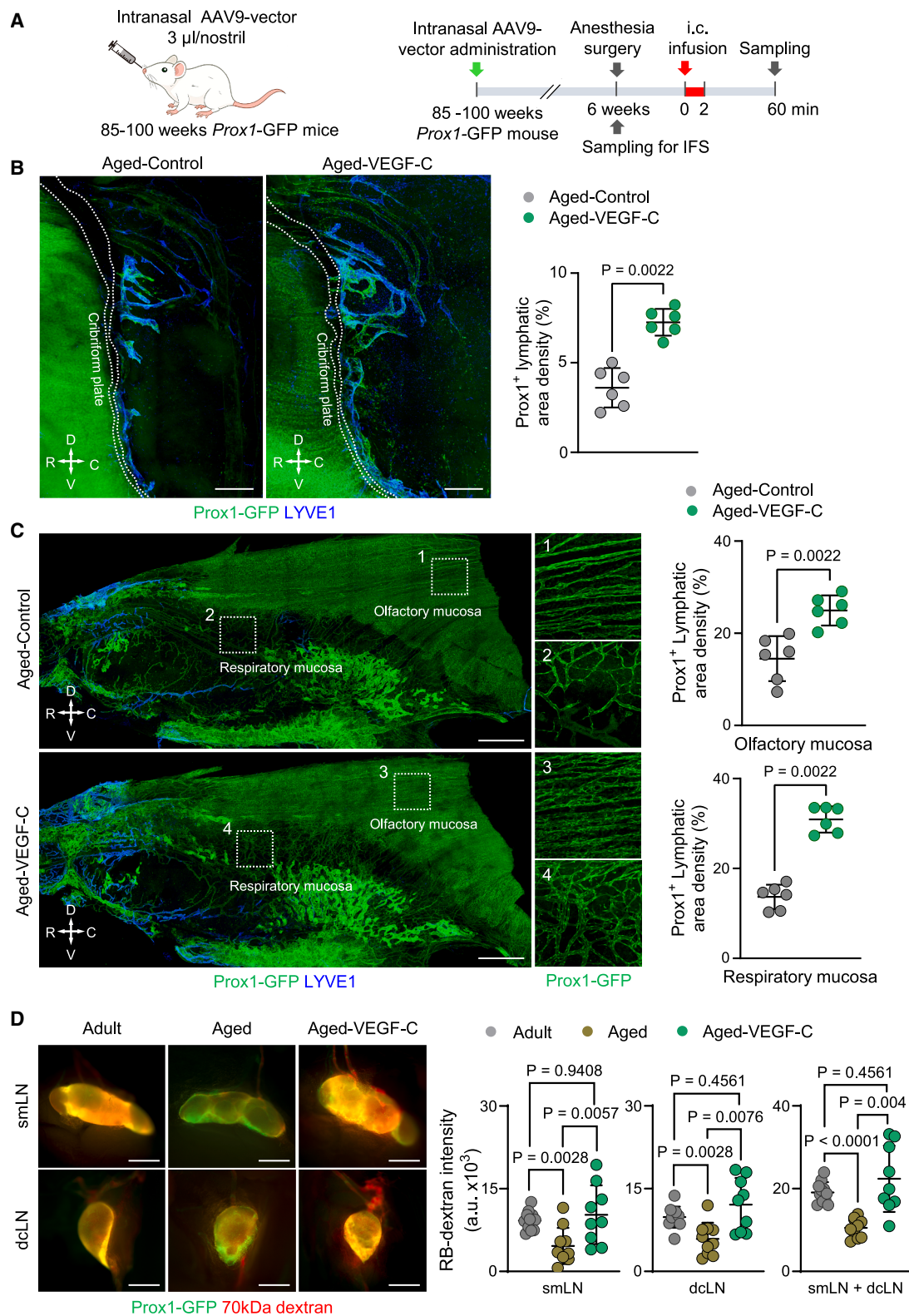
(B and C) SEM images similar to (A) comparing the arachnoid fenestrations (blue arrowheads) bordering the olfactory cistern near the cribriform plate of adult (8–10 weeks) and aged (85–100 weeks) C57BL/6J mice. (C) Comparison of the number and size of arachnoid fenestrations between adult and aged mice. Dura (beige shading). Scale bars, 100 μ m. Each dot represents one mouse. $n = 4$ mice per group from three independent experiments. Error bars indicate mean $\pm$ SD. p values calculated by two-tailed Mann-Whitney U test.

(D and E) Bright-field and osteopontin immunofluorescence images of the cribriform plate in *Prox1*-GFP mice. (D) Medial (Med, red dashed line) and lateral (Lat, yellow dashed lines) regions of interest (ROIs). (E) More abundant medial foramina (red arrowheads) in adult (8–10 weeks, upper row) than in aged mice (85–100 weeks, lower row). Boxes mark medial (M) and lateral (L) regions enlarged in adjacent images. Scale bars, 1 mm. Representative of $n = 5$ mice from three independent experiments.

(F) Graphs showing larger area density, total number, and diameter of medial foramina of the cribriform plate in adult than in aged mice. Each dot represents one mouse. $n = 5$ mice per group from three independent experiments. Error bars indicate mean $\pm$ SD. p values calculated by two-tailed Mann-Whitney U test.

Anatomical orientation is indicated in the bottom left corner: D, dorsal; V, ventral.

See also Figures S10 and S11.



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fluorescence in aged mice that received AAV-VEGF-C was more than doubled in submandibular (+122%) and deep cervical (+105%) lymph nodes compared with aged controls and overlapped the range of values in untreated young adults (Figure 7D). The lack of difference between AAV-VEGF-C-treated aged mice and untreated young adults was evidence that the aging-related reduction in CSF clearance can be reversed.

DISCUSSION

The CSF outflow and immune functions of dural lymphatics are receiving greater attention as the contributions of brain waste clearance to multiple neurological disorders are being elucidated.^{1,3,8,9,13,57} This project was designed to address questions about CSF clearance pathways that have proven difficult to answer. By examining the 3D structure and function of key components of the route CSF takes from the SAS to nasal lymphatics, the study characterized the architecture of the olfactory dural lymphatic plexus bordering the olfactory cistern over the midline of the cribriform plate and between the olfactory bulbs. This lymphatic network was found to receive CSF from the SAS through fenestrations in the arachnoid and have branches that traverse foramina in the cribriform plate to join lymphatics in the olfactory mucosa. Atrophy of the olfactory dural lymphatic network, arachnoid fenestrations, and cribriform plate foramina accompanied the known reduction in CSF outflow during aging. Importantly, intranasal delivery of AAV-VEGF-C viral vector reversed the aging-related atrophy of lymphatics in the olfactory dura and nasal mucosa and restored CSF outflow to the levels found in untreated young adults.

Dural lymphatics contribute to the clearance of CSF that contains brain metabolites, A β , phosphorylated tau, α -synuclein, and other neurotoxic substances that can impair neuronal function.^{1,3,8,9,13,57} Dural lymphatics also serve as exit pathways for immune cells and cytokines/chemokines to cervical and paravertebral lymph nodes.^{1,3,8,9,13,55,57} The vital role of dural lymphatics in maintaining brain homeostasis is highlighted by the consequences of manipulating VEGF-C/VEGFR3 signaling^{5,10,16,17,19,20,22,33,55} or blocking CSF outflow by ligating cervical lymphatics in mice.^{58,59} Lymphatics that drain to lymph nodes in the neck are estimated to account for about half of the total CSF outflow.^{14,15}

Building on findings of landmark studies,^{12,15–22} we addressed unresolved questions of the anatomical and functional connections for CSF clearance from the SAS through dural lymphatics across the cribriform plate to cervical lymph nodes. Among them were whether CSF enters dural lymphatics through direct connections with the SAS^{12,18,22} or through discontinuities in the arachnoid barrier,²³ and whether non-lymphatic pathways contribute to transit across the cribriform plate en route to nasal lymphatics and cervical lymph nodes.^{14,15,26–28} The challenges in resolving these questions are reflected by contrasting interpretations of contemporary immunofluorescence and other imaging data, where some describe lymphatics that cross the cribriform plate, and others argue against direct lymphatic connections in favor of non-lymphatic CSF flow across the cribriform plate to lymphatics in the nasal mucosa beneath the cribriform plate.^{15,17}

Studies with 200-nm FluoSpheres in *Prox1*-GFP mice and 3D confocal microscopic and SEM imaging revealed that the CSF tracer in the SAS crossed arachnoid fenestrations near the olfactory bulbs and entered dural lymphatics with multiple unusual features. These dural lymphatics formed a midline network of vessels with blind-ended protrusions that joined lymphatics around olfactory nerve bundles on the cranial surface of the cribriform plate. Branches of these lymphatics traversed cribriform plate foramina and joined the mucosal lymphatic plexus on the nasal surface of the cribriform plate. From the sub-cribriform plexus, relatively straight branches cascaded through the nasal mucosa and joined cervical lymphatics that drained to cervical lymph nodes along previously identified pathways.¹²

Dural lymphatics near the olfactory bulbs had LECs that expressed VEGFR3 and LYVE1, were joined by discontinuous intracellular junctions typical of initial lymphatics, had few valves, and lacked smooth muscle coverage. By contrast, lymphatics in the olfactory mucosa were narrower, had LECs without LYVE1 staining, and were interconnected by continuous, zipper-like intracellular junctions typical of collecting lymphatics.⁶⁰ As LYVE1 is a commonly used lymphatic marker, the absence of LYVE1 staining of lymphatics in the nasal mucosa contributed to the debate over whether lymphatics cross the cribriform plate.^{21,23}

In the context of the route of CSF drainage, FluoSpheres were clearly within lymphatics but were not detected outside lymphatics anywhere on the nasal surface of the cribriform plate

Figure 7. Reversal of aging-related atrophy of lymphatics in the olfactory dural network and nasal mucosa and restoration of CSF outflow after intranasal VEGF-C

(A) Sequence of intranasal administration of AAV9-mVEGF-C (aged-VEGF-C) or control vector AAV9-empty (aged-control) into aged (85–100 weeks) *Prox1*-GFP mice at a dose of 1×10^{11} virus particles. 6 weeks later, tissues were prepared for immunofluorescence staining or measurement of CSF outflow to lymph nodes 60 min after an intracisternal infusion of 1.0 μ l of 70 kDa RB-dextran over 2 min.

(B and C) Immunofluorescence images and measurements of mid-sagittal sections showing more extensive *Prox1*⁺ lymphatics in the olfactory dural lymphatic network, olfactory mucosa, and respiratory mucosa of aged mice after intranasal AAV-VEGF-C vector. Boxed regions 1–4 show nasal mucosa enlarged in four images at the right. Scale bars, 500 μ m. Each dot represents one mouse. $n = 6$ mice per group from three independent experiments. Error bars indicate mean $\pm$ SD. p values calculated by two-tailed Mann-Whitney U test.

(D) Images and measurements of RB-dextran fluorescence in submandibular (smLN) and deep cervical (dcLN) lymph nodes of adult (8–12 weeks) and aged (85–100 weeks) *Prox1*-GFP mice. Values in aged mice after empty vector (aged) are lower than in adult mice but in aged mice after AAV9-mVEGF-C vector (aged-VEGF-C) are similar to adult mice. Scale bars, 1 mm. Each dot represents one mouse. $n = 9–11$ mice per group from three independent experiments. Error bars indicate mean $\pm$ SD. a.u., arbitrary unit. p values calculated by two-tailed Mann-Whitney U test.

Anatomical orientation is indicated in the bottom left corners: D, dorsal; V, ventral; R, rostral; C, caudal.

See also Figures S12–S14.

or elsewhere in the nasal mucosa. This finding provides important support for CSF flowing through the lymphatic route but not detectably through a non-lymphatic route. The intranasal location exposed CSF-carrying lymphatics to oscillating forces from respiration, cardiac pulsations, and mastication that could influence transport effectiveness.

SEM imaging revealed that arachnoid fenestrations were blocked by 10- μ m FluoSpheres, which substantially reduced CSF drainage to cervical lymph nodes and provided functional confirmation of the fenestration route in CSF transit. Confocal imaging of DPP4 staining revealed corresponding discontinuities in the arachnoid barrier, consistent with the SEM evidence that fenestrations were restricted to regions near the olfactory bulbs and cribriform plate. The 2–12- μ m size and spatial distribution of arachnoid fenestrations support their function as a gateway for CSF and brain waste to access dural lymphatics en route to cervical lymph nodes. Arachnoid fenestrations have also been described at spinal nerve exit sites,⁶¹ but whether similar fenestrations exist around other cranial nerve exits and whether these fenestrations are the only routes for CSF flow through the arachnoid barrier remains to be determined. Although the images of arachnoid fenestrations reflect the highest resolution currently achievable by SEM and confocal microscopy of whole mounts, future experiments using cellular and subcellular approaches should reveal additional details relevant to CSF leakage.

An important unresolved question concerns the relative amounts of CSF cleared from the brain and spinal cord through different regional meningeal lymphatics identified to date. We approached this issue by comparing CSF tracer clearance through arachnoid fenestrations near the cribriform plate and ACE sites in the dorsal dura by using tracers ranging in size from 45 kDa ovalbumin and 70 kDa RB-dextran to $\sim$ 150 kDa anti-LYVE1 antibody and 200-nm FluoSpheres. Much greater clearance of all CSF tracers was found through the cribriform plate route than near dorsal ACE sites around bridging veins. Although body position, the reportedly low CSF flow to the region over the dorsal brain,^{20,50} and other factors⁴⁹ could contribute to the observed difference, further experiments are needed to determine the relative contribution of each outflow route to overall CSF clearance when suitable methods become available.

CSF outflow through lymphatics decreases with age.^{22,51–53} The reduction in CSF drainage to superficial and deep cervical lymph nodes has been documented in multiple studies.^{5,12,18,20,22,53} We previously described aging-related regression and structural alteration of lymphatics in the basolateral dura, nasopharyngeal plexus, olfactory mucosa, and hard palate plexus that reduce CSF outflow to cervical lymph nodes in mice.^{12,20,22} Added to these changes, the present studies of aged mice revealed atrophy of dural lymphatics near the olfactory bulbs and cribriform plate and lymphatics in the olfactory mucosa and respiratory mucosa.⁶⁰ Atrophy of these lymphatics was accompanied by fewer arachnoid fenestrations and fewer cribriform plate foramina, all of which could contribute to the reduction in CSF outflow to cervical lymph nodes.

The size and number of cribriform plate foramina have long been linked to olfaction and the dimensions of olfactory nerve bun-

dles.^{54,62–66} Similarly, aging-related changes in the cribriform plate have been associated with impaired olfaction^{54,66} and are increasingly linked to reduced CSF outflow and neurological disorders.^{30,54,67} The cribriform plate,⁶⁸ like other parts of the skull,^{69–71} contains bone marrow and osseous channels that connect to the immune cell environment around olfactory nerves. Sustained inflammation or other processes that lead to the regression of cribriform plate foramina could exaggerate the reduction of CSF outflow and brain waste clearance in aging. Although the reversibility of changes in bony foramina has not been examined, age-related remodeling of the cribriform plate could represent a structural bottleneck in restoring CSF outflow through this route.

A recently developed method for noninvasive mechanical massage was found to increase CSF outflow through the superficial lymphatic pathway to cervical lymph nodes of aged mice,¹² although the efficacy of the approach in producing a sustained increase has not been tested in mice or humans. As shown in other regions of dural lymphatics,^{10,12,16,20,22,55,72} lymphatics in the dura bordering the olfactory cistern and in the nasal mucosa were expanded by sustained activation of VEGF-C/VEGFR3 signaling by intracisternal delivery of AAV-VEGF-C, while inhibition of this signaling by systemic delivery of AAV-soluble VEGFR3 had the opposite effect. Both approaches had the drawback of widespread effects on lymphatics. Intranasal delivery of AAV-VEGF-C overcame this limitation by selectively expanding lymphatics of the olfactory dural network and nasal mucosa of aged mice without inducing similar changes in lymphatics elsewhere.

Importantly, CSF outflow to cervical lymph nodes was restored in aged mice by intranasal delivery of AAV-VEGF-C despite the absence of recovery in arachnoid fenestrations or cribriform plate foramina. One interpretation is that arachnoid fenestrations and cribriform plate foramina are not rate-limiting in CSF outflow under these conditions but could become limiting as the atrophy progresses. Alternatively, VEGF-C-driven expansion of the lymphatic network could overcome any limitation in CSF outflow resulting from aging-related atrophy of arachnoid fenestrations and cribriform plate foramina under the conditions of these experiments. Regardless, additional work is needed to safely image and manipulate dural lymphatics, arachnoid fenestrations, and cribriform plate foramina in mice and primates and to compare the morphological and functional similarities and differences in CSF outflow in these species.

Overall, this study uncovered multiple previously unknown features of the CSF clearance pathway from the SAS through the cribriform plate to cervical lymph nodes, including the size, abundance, and restricted distribution of arachnoid fenestrations and the 3D architecture of the olfactory dural lymphatic network. By extending the work to aged mice, the studies revealed that dural lymphatics near the olfactory bulbs and cribriform plate atrophied during aging and that the loss of lymphatics was accompanied by reductions in arachnoid fenestrations, medial cribriform plate foramina, and CSF outflow to cervical lymph nodes. Intranasal delivery of AAV-VEGF-C reversed the lymphatic atrophy and restored CSF outflow in aged mice to the levels of young adults, despite not reversing the aging changes in arachnoid fenestrations or cribriform plate foramina.

Limitations of the study

Several limitations influence the interpretation of these results. The use of 200-nm FluoSpheres, SEM imaging, and confocal imaging of DPP4 staining did not exclude additional CSF outflow pathways for small solutes to cross the arachnoid and cribriform plate for uptake by nasal lymphatics. New approaches are needed to image the routes for small CSF solutes to move from the SAS to lymphatics and to elucidate the ultrastructure of these pathways in mice and humans. Real-time two-photon microscopic imaging was unsuccessful due to the inability to create an optically stable surgical window in this region without disrupting tissue architecture. Transmission electron microscopy (TEM) imaging of electron-dense tracers, serial block-face SEM with 3D reconstructions, functional MRI, and synchrotron imaging could be useful, but multiple technical challenges and anatomical constraints of the cribriform plate region limit what can currently be learned. Further studies are also warranted to identify the effects of anesthesia, body position, and other factors on CSF tracer distribution and the observed greater drainage through arachnoid fenestrations and cribriform plate lymphatics than through dorsal ACE sites. The aging studies leave multiple unanswered questions, including the duration of the AAV-VEGF-C-induced reversal, the lack of recovery of cribriform plate foramina, and the extent to which the approach can ameliorate neurological deficits in aging. These limitations provide a strong rationale for further studies of the clearance routes for CSF solutes through dural lymphatics, crossing the cribriform plate, and other exit pathways from the skull and vertebral column of mice and primates.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gou Young Koh (gykoh@kaist.ac.kr).

Materials availability

All unique/stable materials generated in this study are available from the [lead contact](#) with a completed materials transfer agreement.

Data and code availability

The data underlying this article are available in the article and in its online [supplemental information](#). This paper does not report original code. Additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

S.P.H., C.J., and G.Y.K. conceived and designed the study. S.P.H. and C.J. performed most of the experiments with contributions from D.R.C. S.P.H., C.J., and G.Y.K. wrote the manuscript under the supervision of D.M.M. and G.Y.K. S.P.H., C.J., M.J.Y., H.J., J.-H.Y., C.M.Y., L.A.A., and G.Y.K. analyzed

and interpreted the data and generated the figures. S.P.H., C.J., M.J.Y., H.J., J.-H.Y., C.M.Y., and L.A.A. actively discussed the experiments and the results under the supervision of W.-S.C., K.A., D.M.M., and G.Y.K. J.S. and K.S.L. prepared the primate tissue samples. S.J.S. performed genotyping analysis for mouse strain maintenance. J.J. oversaw the care of mice. S.A. and K.A. provided AAV9-mVEGF-C and AAV9-mVEGFR3 and reviewed the manuscript. S.P.H., D.M.M., and G.Y.K. supervised and directed the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-mouse LYVE1	AngioBio	Cat#11-034; RRID: AB_2813732
Rat monoclonal anti-mouse LYVE1 (clone 223322)	R&D Systems	Cat# MAB2125; RRID: AB_2138528
Goat polyclonal anti-mouse VEGFR3	R&D Systems	Cat# AF743; RRID: AB_355563
Goat polyclonal anti-mouse VE-cadherin	R&D Systems	Cat# AF1002; RRID: AB_2077789
Rabbit polyclonal anti- β -III-tubulin	Biologend	Cat# 802001; RRID: AB_2564645
Rabbit polyclonal anti-mouse osterix	Abcam	Cat# ab22552; RRID: AB_2194492
Goat polyclonal anti-mouse DPP4	R&D Systems	Cat# AF954; RRID: AB_355739
Rat monoclonal anti-ER-TR7 (clone ER-TR7)	SantaCruz Biotechnology	Cat# sc-73355; RRID: N/A
Goat polyclonal anti-E-cadherin	R&D Systems	Cat# AF748; RRID: AB_355568
Rabbit monoclonal anti-Col1a1 (clone E8F4L)	Cell Signaling Technology	Cat# 72026; RRID: AB_2904565
Rabbit polyclonal anti-Slc47a1	Alomone Labs	Cat# ANT-131; RRID: AB_2756729
Rabbit polyclonal anti-claudin-11	BiCell	Cat# 00211; RRID: N/A
Rabbit monoclonal anti-Ppp1r1a (clone SN0756)	Invitrogen	Cat# MA5-32310; RRID: N/A
Rat monoclonal anti-mouse PLVAP (clone MECA-32)	BD Biosciences	Cat# 550563; RRID: AB_393754
Hamster monoclonal anti-mouse CD31 (clone 2H8)	Merck	Cat# MAB1398Z; RRID: N/A
Rat monoclonal anti-mouse CD31 (clone MEC13.3)	BD Biosciences	Cat# 557355; RRID: AB_396660
Rabbit polyclonal anti-GLUT1	Merck	Cat# 07-1401; RRID: N/A
Rabbit polyclonal anti-claudin-5	Invitrogen	Cat# 34-1600; RRID: N/A
Goat polyclonal anti-mouse Osteopontin	R&D Systems	Cat# AF808; RRID: AB_2194992
Bacterial and virus strains		
AAV9-CAG-mVEGF-C	Antila et al. ¹⁰	N/A
AAV9-CAG-empty	Antila et al. ¹⁰	N/A
AAV9-mVEGFR3 ₁₋₄ -lg	Antila et al. ¹⁹	N/A
AAV9-mVEGFR3 ₄₋₇ -lg	Antila et al. ¹⁹	N/A
Chemicals, peptides, and recombinant proteins		
Zinc sulfate monohydrate, 99%	Thermo Fisher Scientific	Cat# 389802500
Formaldehyde	Junsei	Cat# 69360-0380
Tissue-clearing reagent CUBIC-L [for animals]	TCI	Cat# T3740
2,2'-Thiodiethanol	Sigma Aldrich	Cat# 88561
Histodenz™	Sigma Aldrich	Cat# D2158
Glutaraldehyde, 25% solution	Junsei	Cat# 111-30-8
3-methylbutyl acetate (Isoamyl acetat)	Junsei	Cat# 123-92-2
Osmium tetroxide (OsO ₄)	EMS	Cat# 20816-12-0
Beta-Amyloid (1-42), HiLyte™ Fluor647-labeled	AnaSpec	Cat# AS-64161
FluoSpheres™ Carboxylate-Modified Microspheres (200 nm, 660/680 nm)	Thermo Fisher Scientific	Cat# F8807
FluoSpheres™ Carboxylate-Modified Microspheres (200 nm, 365/415 nm)	Invitrogen	Cat# F8805

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FluoSpheres™ Polystyrene Microspheres (10 μm, 365/415 nm)	Invitrogen	Cat# F8829
Rhodamine B-conjugated 70 kDa dextran	Invitrogen	Cat# D1841
Alexa-555 conjugated ovalbumin	Invitrogen	Cat# O34782

Experimental models: Organisms/strains

Mouse: Prox1-GFP, FVB	Choi et al. ³⁸	N/A
Mouse: C57BL/6J	Animal Center of Aging Science of Korea Basic Science Institute	N/A
Cynomolgus macaque monkeys: <i>Macaca fascicularis</i>	National Primate Research Center of the Korea Research Institute of Bioscience and Biotechnology	N/A

Software and algorithms

ZEN blue 2.3	Carl Zeiss	v2.3.69.1010
ZEN Black 2.1 SP3	Carl Zeiss	v14.0.4.201
Fiji/ImageJ	ImageJ	https://imagej.net/ij/
GraphPad Prism 10 (GraphPad Software, version 10.5.0).	GraphPad Software	v10.5.0
Imaris (10.2.0)	Oxford instruments	v10.2.0
DaVinci Resolve 20	Blackmagic Design	v20.3.2

Study approval

All animal care and experimental procedures were approved for mice by the Institutional Animal Care and Use Committees of the Institute for Basic Science (IBS-2025-017) and for *Macaca fascicularis* monkeys by the Korea Research Institute of Bioscience and Biotechnology (KRIBB-AEC-24079).

EXPERIMENTAL MODELS

Animals

Prox1-GFP mice³⁸ (FVB background, 8 to 12-week-old for adult, 30 to 35-weeks-old for early middle-aged, 45 to 50-weeks-old for late middle-aged, and 85 to 100-weeks-old for aged mice) were transferred, established, bred, and maintained in specific pathogen-free (SPF) animal facilities at the Korea Advanced Institute of Science and Technology (KAIST). Adult (8 to 12-weeks-old), early middle-aged (30 to 35-weeks-old), late middle-aged (45 to 50-weeks-old), and aged (85 to 100-weeks-old) C57BL/6J mice were purchased from the Animal Center of Aging Science of Korea Basic Science Institute. All mice were bred under a 12 h light-dark cycle at 23–24°C and 40–60% humidity in the SPF animal facility and received *ad libitum* access to a standard chow diet (PMI LabDiet) with water. Mice of both sexes were used for all of the experiments. Mice were anesthetized by i.p. injection of a combination of ketamine (80 mg/kg) and xylazine (12 mg/kg) for all experimental procedures except for mice used in the CSF drainage assay to the cervical lymph nodes, which were anesthetized by i.p. injection of urethane (1.5 g/kg) to minimize the cardiorespiratory effects.¹² Body temperature was maintained at 36.5–37.5°C during surgical and imaging procedures.

Cynomolgus macaque monkeys, *Macaca fascicularis* (7–18 years old), maintained at the National Primate Research Center of the Korea Research Institute of Bioscience and Biotechnology, were housed individually to prevent physical contact and minimize potential harm during the experiment, but had visual and auditory interactions with neighbouring monkeys. Cage dimensions (60 cm × 80 cm × 80 cm) followed the guidelines of the National Institutes of Health (USA). Monkeys were housed with a 12 h light/dark cycles at 22–26°C and 45–55% humidity and fed a commercial monkey diet (Teklad 2050™, Envigo), assorted fruits, and *ad libitum* water, and given rubber and plastic toys for environmental enrichment. Monkeys were anesthetized by intramuscular injection of ketamine (5 mg/kg) and atropine (0.02 mg/kg) before the procedures and supplemented as necessary during procedures. Veterinary monitoring adhered to the nonhuman primates research guidelines.⁷³

METHOD DETAILS

Anatomical planes of sections used to examine cribriform plate region

Structures in the region of the cribriform plate were examined in sections cut in sagittal, oblique-coronal, and axial planes. Sagittal sections were cut near the midline or parallel to the midline to view the rostral-caudal and superior-inferior extents of structures (*sagittal view*). In mice, oblique-coronal sections were cut in a plane parallel to the surface of the cribriform plate (Figure S1A).

This plane was tilted forward (anterior) $\sim 49^\circ$ from the standard coronal plane (*oblique-coronal view*).⁷⁴ This plane enabled examination of medial-lateral extent of dural lymphatics, olfactory nerve bundles, cribriform plate foramina, and the nasal sub-cribriform lymphatic plexus of mice by confocal microscopy and SEM. For SEM studies of monkeys, transverse (axial/cross) sections were cut at the level of the cribriform plate.

Specimen preparations for histological analysis

Mice were anesthetized and perfused through the left cardiac ventricle with ice-cold phosphate buffered saline (PBS) followed by 2% paraformaldehyde (PFA). Blood and fixative drained from a puncture in the right atrium. Immediately after cardiac perfusion, the mandible was removed to expose the hard and soft palates. Both palates were excised using fine forceps, and the zygomatic arch was removed with curved fine scissors. The orbital contents and skin covering the head were removed, and the head and neck were separated at the C2 vertebral level. The head was post-fixed in 2% PFA overnight at 4°C and thoroughly washed in PBS. Samples were then incubated in 10% EDTA and 10 mM Tris-HCl in PBS at 4°C for 7 to 10 days, followed by a 1-day PBS wash to minimize tissue shrinkage. With the dorsal surface of the head facing upward, a midline incision was made from the inferior cerebral vein to the skull base using a blade, separating the anterior cranial region containing the olfactory bulb from the posterior cranial region. A second incision was made to separate the nasal region from the anterior cranial region, and tissues from both regions were examined.

For imaging of tissues near the olfactory bulbs, the anterior cranial region was embedded in 3% low-melting agarose (Invitrogen) using a 12-well plate. After solidification, the embedded specimen was removed from the well, and excess agarose was trimmed with a blade. In preparation for making sagittal sections, trimmed samples were glued to the vibratome stage with the lateral side of the head facing downward and the superior sagittal sinus aligned parallel to the cutting planes. For oblique-coronal sections, samples were mounted with the olfactory bulb facing downward and tilted forward approximately 49° from the coronal plane (Figure S1A). The nasal septum, nasal mucosa, and nasopharyngeal tissues were removed from head specimens decalcified with 10% EDTA and 10 mM Tris-HCl in PBS at 4°C for 7 to 10 days as described previously.^{22,60}

Sections 0.3–1 mm in thickness were cut with a vibratome (VT1200S, Leica) from mounted samples in the PBS-filled vibratome chamber. Sections were incubated in CUBIC-L (TCI chemicals) for 30 to 360 min, depending on specimen size, followed by a 1-day PBS wash to remove residual clearing reagents.

To visualize the dorsal and basal meninges, the posterior cranial region was first separated from the brain as described previously.²⁰ Under the microscope, the dorsal skull was removed with fine curved scissors, cutting the bone in an oval shape. The brain was then carefully removed to expose the basolateral meninges. For imaging of the dorsal meninges over the intact brain, specimens were embedded in 3% low-melting agarose (Invitrogen) in a 12-well plate. After solidification, the embedded tissue was removed from the well, and excess agarose was trimmed with a blade.

Sagittal sections 1.5–2 mm in thickness were cut with a vibratome on samples glued to the vibratome stage with the dorsal skull facing upward and the superior sagittal sinus aligned parallel to the cutting plane for sagittal sectioning. Sections were incubated in CUBIC-L for 300 to 600 min, depending on specimen size, followed by a 1-day PBS wash to remove residual clearing reagents.

Infusion of fluorescent tracers into the subarachnoid space

Prox1-GFP mice anesthetized with urethane (1.5 g/kg, i.p.) in the prone position were placed in a stereotaxic frame under a surgical microscope. By using a mouthpiece, the head was tilted to a 90° angle in relation to the body axis to facilitate access to the cisterna magna. After the skin was incised in the midline of the posterior neck, the muscle layers were carefully separated with microscissors. The atlanto-occipital membrane overlying the cisterna magna was superficially penetrated by a 33-gauge NanoFil needle (World Precision Instruments). One or 5 μl of tracer was infused into the SAS over 2 min using a microsyringe (88000, Hamilton) and a micro-infusion machine (Fusion 100, Chemyx Inc). Tracers included: (i) FluoSpheres (diameter, 200 nm, polystyrene microbeads, carboxylated-modified surface, dark red fluorescent (660/680 nm), F8807, Thermo Fisher Scientific); (ii) Alexa-555 conjugated ovalbumin (O34782, Invitrogen); (iii) Rhodamine B-conjugated dextran (70 kDa, 25 mg/ml) (Rhodamine B-dextran, D1841, Invitrogen), (iv) anti-LYVE1 (1 mg/ml, rat monoclonal, clone #223322, MAB2125, R&D Systems) antibody, and (v) A β (1–42), HiLyte™ Fluor 647-labeled (1 mg/ml, AS-64161, AnaSpec). The needle was left in position for 8 min and then slowly removed to prevent CSF leakage. The muscle layer and skin were sutured with 6-0 black silk (SK617, Ailee). At 15 to 60 min after the infusion, mice were euthanized, and tissues were prepared for imaging the nasal septum, cribriform plate, and dorsal meninges.

Scanning electron microscopy

Specimens from anesthetized C57BL/6J mice were prepared for SEM by vascular perfusion of fixative containing 4% PFA and 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4). The head was removed and immersed in the same fixative overnight at 4°C . Samples were decalcified in 10% EDTA and 10 mM Tris-HCl in PBS at 4°C for 7–10 days. The solution was replaced every other day. After decalcification, the nasal cavity and brain were removed from the head, and the tissue, including olfactory mucosa, cribriform plate, and olfactory bulb, was embedded in 3% low-melting agarose (Invitrogen).

Specimens from anesthetized cynomolgus monkeys were prepared for SEM by carotid artery perfusion of ice-cold PBS followed by 4% PFA. After removing the dorsal skull with a hand drill, the brain was carefully removed to expose the skull base. While lifting the brain, an incision was made near the olfactory bulb tracts to prevent detachment of the olfactory bulbs from the dura. The head and

neck were separated at the C2 vertebral level, the mandible and zygomatic bone were removed, orbital contents were excised, and then the specimens were further trimmed under the microscope to expose the olfactory bulbs and cribriform plate. Specimens were further fixed by immersion in 4% PFA and 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) overnight at 4°C and then decalcified in Calci-Clear Rapid (HS-105, National Diagnostics) over 2 days. The cribriform plate and adjacent tissues were isolated and decalcified further for 14 days in 10% EDTA and 10 mM Tris-HCl in PBS at 4°C. After the decalcification, the tissue was embedded in 3% low-melting agarose (Invitrogen).

The cribriform plate midline was used as an anatomical reference for consistency of tissue orientation in SEM studies. Sections 0.4 mm in thickness were cut in a coronal-oblique (mice) or coronal (monkeys) plane with a vibratome, post-fixed in 2% OsO₄ containing 2.5% PFA-2.5% glutaraldehyde 0.1M phosphate buffer (pH 7.2) for 5 h, and then dehydrated in graded concentrations of ethanol followed by critical-point drying in an E3000 Critical Point Dryer (Polaron). Critical-point-dried samples were placed on a piece of carbon tape and sputter-coated with gold in a SC502 Sputter Coater (Polaron). Specimens were imaged with a Quanta 250 Field Emission Scanning Electron microscope (FEI Quanta 250 FEG, FEI, Hillsboro, OR) at the Korea Research Institute of Bioscience and Biotechnology.

Chemical ablation of olfactory sensory neurons and lymphatics

Anesthetized mice were exposed to ZnSO₄ to induce regression of olfactory sensory neurons and nasal lymphatics as previously described.³⁰ A catheter (24 Ga. 0.75 in., Becton Dickinson) was inserted through each nostril to target olfactory mucosa. 2.5 μl of ZnSO₄ (10% ZnSO₄ in sterile distilled water) or sterile distilled water was then delivered to each nostril. Immediately thereafter, mice were inverted to minimize inhalation of ZnSO₄ and unintended exposure of other regions. Mice were kept on a heating pad for a few hours until they awakened.

Magnetic resonance imaging

Magnetic resonance imaging (MRI) experiments were performed using a 9.4 T MR system (Bruker BioSpin, Germany). An 86 mm inner diameter volume coil was used for radiofrequency transmission, and a 10 mm inner diameter surface coil for reception. A three-dimensional inversion recovery T₂-weighted (IR-T₂) sequence was used to visualize the ventricles. Acquisition parameters were repetition time (TR) = 2100 ms, effective echo time (TE) = 42 ms, field of view was 14 x 16 x 10 mm³, and matrix size of 140 x 160 x 100 μm³, yielding an isotropic resolution of 100 x 100 x 100 μm³. The total acquisition time was less than 45 min. Respiration and body temperature were continuously monitored and maintained using a physiological monitoring system. Anesthesia was induced with 5% isoflurane and maintained with 2% isoflurane during imaging.

Intracranial pressure measurement

Intracranial pressure (ICP) was measured following a craniotomy over the left somatosensory cortex (anterior-posterior: -1.7 mm, medial-lateral: 2.45 mm). An intracranial pressure transducer (SPR-671NR, Millar) was inserted 2.2 mm into the brain parenchyma. The transducer was connected to a pressure control unit (PCU-2000, Millar), and signals were recorded for 5 min using a data recorder (IWX214, iWorkx Systems). Mean ICP was calculated from the recorded data.

FluoSphere blockade of arachnoid fenestrations near the olfactory bulb

Prox1-GFP mice were anesthetized with i.p. injection of ketamine (80 mg/kg) and xylazine (12 mg/kg), and the cisterna magna was surgically exposed. A total of 4.0 μl FluoSpheres or PBS was injected as described for the infusion of fluorescent tracers into the SAS. FluoSpheres of two diameters were used at a concentration of 2 x 10⁸ particles per milliliter: (i) 200-nm diameter FluoSpheres (Carboxylate-Modified Microspheres, Blue fluorescent 365/415 nm, F8805, Invitrogen), or (ii) 10-μm diameter FluoSpheres (Polystyrene Microspheres, blue fluorescent (365/415), F8829, Invitrogen). Following injection, the surgical site was sealed using Vetbond (084-1469SB, 3M). 24 hours later, mice were re-anesthetized with urethane (1.5 g/kg, i.p.), and the cisterna magna was re-exposed. Rhodamine B-conjugated dextran (70 kDa, 25 mg/ml; D1841, Invitrogen) was infused (1 μl over 2 min) into the SAS using a micro-syringe (88000, Hamilton) connected to a micro-infusion pump (Fusion 100, Chemyx Inc.). The second injection site was positioned to avoid the initial surgical region.

Modulation of VEGF-C/VEGFR3 signaling by AAV9 transduction

Anesthetized mice received an infusion of VEGF-C (mVEGF-C), 3 μl (5 × 10¹⁰ viral particles) of AAV9-empty or AAV9-mVEGF-C at 0.5 μl per 1 min into the SAS at the cisterna magna of *Prox1*-GFP mice, as previously described.¹⁰ For systemic inhibition of VEGFR3 signaling, *Prox1*-GFP mice received a single i.p. dose (10¹² viral particles in 200 μl) of recombinant AAV encoding the ligand-binding domain 1-4 of mVEGFR3 fused to the IgG Fc domain (AAV9-mVEGFR3₁₋₄-Ig). Control mice received the same dose of AAV encoding the domains 4-7 of mVEGFR3, which do not bind VEGF-C or VEGF-D (AAV9-mVEGFR3₄₋₇-Ig), fused to the IgG Fc domain, as previously described.¹⁹ For intranasal delivery of VEGF-C, 3.5 μl (1 × 10¹¹ viral particles) of AAV9-empty or AAV9-mVEGF-C was infused intranasally into anesthetized aged mice, as previously described.⁵⁶ Six weeks after viral administration, tissues around the cribriform plate, nasal septum, dorsal and basal meninges, and nasopharynx were removed for histological analysis as for other experimental protocols.

Immunofluorescence staining

For immunofluorescence staining, sections were permeabilized, incubated in blocking buffer containing 5% donkey serum in 1% Triton-X 100 in PBS with or without 5% DMSO for 1 h at room temperature, and then incubated in a mixture of primary antibodies diluted 1:400 in the blocking buffer overnight or 3 days at 4°C.

The primary antibodies included: anti-mouse LYVE1 (rabbit polyclonal, 11-034, AngioBio); anti-mouse LYVE1 (rat monoclonal, clone #223322, MAB2125, R&D Systems); anti-mouse VEGFR3 (goat polyclonal, AF743, R&D Systems); anti-mouse VE-cadherin (goat polyclonal, AF1002, R&D Systems); anti- β -III-tubulin (rabbit polyclonal, 802001, Biolegend); anti-mouse Osterix (rabbit polyclonal, ab22552, Abcam); anti-mouse DPP4 (goat polyclonal, AF954, R&D Systems); anti-ER-TR7 (rat monoclonal, clone ER-TR7, sc-73355, Santa Cruz Biotechnology); anti-E-cadherin (goat polyclonal, AF748, R&D Systems); anti-Col1a1 (rabbit monoclonal, clone E8F4L, 72026, Cell Signaling Technology); anti-Slc47a1 (rabbit polyclonal, ANT-131, Alomone labs); anti-claudin-11 (rabbit polyclonal, 00211, BiCell); anti-Ppp1r1a (rabbit monoclonal, clone SN0756, MA5-32310, Invitrogen); anti-mouse PLVAP (rat monoclonal, clone MECA-32, 550563, BD Biosciences); anti-mouse CD31 (hamster monoclonal, clone 2H8, MAB1398Z, Merck); anti-mouse CD31 (rat monoclonal, clone MEC13.3, 557355, BD Biosciences); anti-GLUT1 (rabbit polyclonal, 07-1401, Merck); anti-claudin-5 (rabbit polyclonal, 34-1600, Invitrogen); anti-mouse Osteopontin (goat polyclonal, AF808, R&D Systems); anti-human PDPN (rat monoclonal, clone NZ1.2, MABC117, Merck); anti-human CD31 (sheep polyclonal, AF806, R&D Systems); anti-human LYVE1 (rabbit polyclonal, ab36993, Abcam).

After several washes with PBS, sections were incubated with secondary antibodies diluted 1:1000 in the blocking buffer for 4–6 h at room temperature or overnight or 2 days at 4°C. Secondary antibodies included: Alexa Fluor 488, Cy3, 594, or 647-conjugated anti-rabbit (711-545-152, 711-585-152, 711-605-152), anti-rat (712-585-153, 712-605-153), anti-goat (705-585-147, 705-605-147), anti-hamster (127-475-160, 127-605-160), anti-syrian hamster (107-165-142), or anti-sheep (713-605-147) (Jackson ImmunoResearch). Other secondary antibodies included Alexa Fluor 405-conjugated anti-rabbit (A48258), anti-rat (A48268), or anti-goat (A48259), all from Thermo Fisher Scientific, and DyLight 405-conjugated anti-hamster (127-475-160, Jackson ImmunoResearch).

Nuclei were stained with DAPI (62248, Thermo Fisher Scientific) at a 1:1000 dilution. After several washes with PBS, sections were mounted with Vectashield (Vector Laboratories) or refractive index matching solution (D-PROTOSS).⁷⁵

Imaging and morphometric analysis

Immunofluorescent images were obtained using either a LSM800, LSM880, or LSM 980 confocal microscope (Carl Zeiss). Image acquisition and processing were performed with a ZEN blue 2.3 (v2.3.69.1010) and ZEN Black 2.1 SP3 (v14.0.4.201) software (Carl Zeiss). For both whole mounts and sections tissues, tiled or single z-stack images covering the full depth and width were captured and rendered as maximum intensity projections. All images were acquired at resolutions of 512×512 or $1,024 \times 1,024$ pixels using an air objectives Plan-Apochromat $\times 10/0.45$ numerical aperture (NA) M27, Plan-Apochromat $20\times/0.8$ NA M27 or water immersion objective LD C-Apochromat $\times 40/1.1$ NA Corr M27 with multichannel acquisition performed per z-stack.

Morphometric analysis was performed on orthogonal projections of confocal images using Zen software (Carl Zeiss) or Fiji (ImageJ, <https://imagej.net/ij/>). For fluorescence profile analysis in the arachnoid fenestration, arachnoid cuff exit, olfactory dural lymphatic network, and dorsal dural lymphatics after intracisternal infusion of FluoSpheres, profile lines were drawn with a stroke thickness of 5 using Zen software. Mean fluorescence intensity of FluoSpheres was measured in six randomly selected regions of arachnoid fenestrations ($20 \mu\text{m} \times 20 \mu\text{m}$ each) and six post-ACE areas ($20 \mu\text{m} \times 20 \mu\text{m}$ each) using ZEN software in each mouse. Mean fluorescence intensity of RB-dextran was measured in three randomly selected regions ($200 \mu\text{m} \times 100 \mu\text{m}$ each) in the olfactory bulb dura adjacent to the fenestrated arachnoid and three randomly selected post-ACE areas ($200 \mu\text{m} \times 100 \mu\text{m}$ each) in each mouse. LYVE1 fluorescence was measured within a defined region-of-interest (ROI) ($945 \mu\text{m} \times 945 \mu\text{m}$) in images of dorsal dura and olfactory bulb cistern dura after Prox1⁺ fluorescence was masked and removed using ImageJ.

Imaging was performed at the following field sizes in specimens from mice that received AAV9-empty, AAV9-mVEGF-C, AAV9-mVEGFR3₁₋₄-Ig, AAV9-mVEGFR3₄₋₇-Ig, ZnSO₄ or distilled water: olfactory bulb/cirbriform plate region meninges (ROI, 2.69 mm $\times$ 3.97 mm), nasal septum (ROI, 709 $\mu\text{m} \times$ 709 μm), dorsal meninges (ROI, 3.9 mm $\times$ 3.95 mm), basal meninges (ROI, 3.97 mm $\times$ 3.95 mm), and nasopharyngeal lymphatic plexus (ROI, 3.97 mm $\times$ 3.97 mm).

Immunofluorescence images were analyzed using Fiji/ImageJ software to quantify and compare the Prox1⁺ area of control and experimental groups. Prox1⁺ area, VEGFR3 intensity, LYVE1 intensity, VE-cadherin⁺ intensity, and detached LECs in aged and adult mice were compared in ROIs defined in immunofluorescence images analyzed by Zen or Fiji/ImageJ software. Prox1⁺ lymphatic areas were measured in dorsal meninges (ROIs, 2600 $\mu\text{m} \times$ 2100 μm) and basolateral meninges (ROIs, 3200 $\mu\text{m} \times$ 2100 μm) after intracisternal injection of AAV9-empty, AAV9-mVEGF-C, AAV9-mVEGFR3₁₋₄-Ig, or AAV9-mVEGFR3₄₋₇-Ig. VE-cadherin⁺ junctional patterns in olfactory bulb dural lymphatics were analyzed in 40 $\mu\text{m} \times$ 40 μm regions as previously described.⁷⁶ Arachnoid fenestrations were quantified by counting all fenestrations visible in 280 $\mu\text{m} \times$ 280 μm fields of confocal images of DPP4-stained arachnoid.

For all cirbriform plate analysis, foramina located within 350 μm of the midline were considered 'medial', and those further from the midline were considered 'lateral'. Analysis of the number, size, and distribution of cirbriform plate foramina in adult mice was characterized on binary images, ROI size of 1000 $\mu\text{m} \times$ 1500 μm using Fiji/ImageJ. Foramina were analyzed with the Analyze Particle tool. Foramen diameter was calculated from the area assuming circularity. The distance of each foramen from

the midline was measured. The number, size, and area density of cribriform plate foramina in adult and aged mice were quantified separately within medial and lateral regions, and each value was normalized to the total cribriform plate area of the same specimen. Binary images were generated from immunofluorescence images in Fiji/ImageJ, and analyses were performed using Zen software and Fiji/ImageJ.

3-D renderings of lymphatic networks and other structures were prepared from confocal microscopic image z-stacks obtained from 1-mm thick tissue sections as previously described.^{10,12,16,20,22,55,72} Fluorescence images were acquired with an LSM 980 microscope (Carl Zeiss) and then reconstructed using volume rendering and orthogonal slicer in Imaris (10.2.0) software.

To compare the intensity of dextran fluorescence in lymph nodes after intracisternal infusion of dextran, superficial, accessory, and deep cervical lymph nodes were exposed in mice after perfusion fixation, as described in the tissue preparation section, and identified by using a fluorescence surgical microscope (SZX16, Olympus). Images were acquired using a fluorescence stereo zoom microscope (AxioZoom V16).²² Dextran signal intensity was measured by drawing ROIs around Prox1⁺ regions of lymph nodes with Zen software. For each type of lymph node, the signals from the left and right sides were combined as one data point.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Sample size was determined empirically without prior statistical estimation. Data from experiments were randomized, and investigators were blinded to allocation during experiments and outcome assessment. The statistical significance of differences between groups was determined by the two-tailed Mann-Whitney U test or two-tailed Tukey's multiple comparison test using GraphPad Prism 10 (GraphPad Software, version 10.5.0). Frequency distributions of size of foramina in medial and lateral regions of the cribriform plate were compared by the Kolmogorov-Smirnov two-sample test. Values are presented as means $\pm$ standard deviation (s.d.). Statistical significance was set at $P < 0.05$.