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Symposium Highlight

BRAIN LYMPHATICS: REDISCOVERY AND NEW INSIGHTS INTO LYMPHATIC INVOLVEMENT IN DISEASES OF HUMAN BRAINS

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ABSTRACT

The brain's lymphatic system is comprised of a glymphatic—meningeal—cervical lymphatic vessel pathway. The study of its mechanism and pathophysiology in neurodegenerative disease has been one of the most exciting topics in basic and translational neuroscience of the last decade. However, while there has been some debate about when the meningeal lymphatics were discovered, it cannot be denied that studies in preclinical models and humans in this century represent a monumental step forward in our understanding of how the brain removes metabolic waste, the role this system plays in neurodegenerative disease, and, most importantly, its potential as a novel therapeutic target. This is a summary of the history, functional anatomy, and role of the brain's lymphatics in neurodegenerative disease.

Keywords: Glymphatics, meningeal lymphatics, cervical lymphatics, CSF, neurodegeneration, Alzheimer's disease, Parkinson's disease.

THE BRAIN'S LYMPHATIC SYSTEM: A BRIEF HISTORICAL PERSPECTIVE

Paolo Mascagni, an Italian physician, published the first textbook on human lymphatics in 1787 titled, "*Vasorum Lymphaticorum Corporis Humani Historia et Ichnographia*." This textbook also contained early descriptions of what have since been confirmed to be functional meningeal lymphatic vessels (MLVs) located within the dura mater layer of the meninges in mice (1), non-human primates (2), and humans (2). However, while Mascagni has largely been credited with this discovery posthumously, it took more than 200 years and repeated evidence to convince the scientific community. As recent as 20 years ago his ideas were still being questioned despite ample evidence. For example, in 2003 The Lancet published a paper criticizing Mascagni's observations, stating that he "*was probably so impressed with the lymphatic system that he saw lymph vessels even where they did not exist—in the brain*." (3) However, this article ignored two centuries of evidence supporting Mascagni's early observations including that from two prominent scientists who came before Mascagni, Frederik Ruysch (1638-1731) and William Cumberland Cruikshank (1745-1800). Both anatomists, Ruysch and Cruikshank described absorbent vessels, now believed to be MLVs. In a similar fashion to Mascagni, their work came under intense scrutiny and was frequently discredited in

their lifetimes (4).

While not a complete review of the works of the 19th century, there are two major contributions to highlight from this period- the experimental work of Gustavo Schwalbe (1869) and Heinrich Quincke (1872). Schwalbe administered Berlin blue into the cranial subarachnoid space of previously exsanguinated rabbits and dogs (5). In both models, the lymphatic vessels and nodes of the neck filled with dye, with the nodes being filled by vessels exiting the jugular foramen and forming a plexus on the anterior cervical muscles. Schwalbe also observed dye in the lymph nodes along the length of the vertebral columns, suggesting that the spinal sub-arachnoid space might be linked to the lymphatic system (6). Quincke's experiments were technically more advanced. In addition to performing his studies in awake and free moving animals (dogs, cats, and rabbits), he was careful to maintain the physiological pressure within the vertebral column by using small bore needles and administering small volumes of cinnabar dye intrathecally. He determined that a subdural space existed in the brain (but not in the spinal cord), that the subarachnoid spaces of the brain and spinal cord communicated, and that there were multiple cerebrospinal fluid (CSF) outflow pathways travelling along structures including, but not limited to, the cervical/submaxillary glands, olfactory/optic/ trigeminal nerves, and intercostal/lumbar/sacral nerves (7).

Michael Földi, a pioneer in the field of modern lymphology, expanded on this work in the mid-20th century. He observed drainage in lymphatic vessels within the dura mater at the jugular foramen. Földi described these as a "prelymphatic-lymphatic" pathways that ran perivascular until the jugular foramen at which point, they merged with lymphatic vessels and travelled along the internal carotid artery to the deep cervical lymph nodes (dCLNs) (8). In this same paper he also described lymphatics connecting the subarachnoid space and the cervical lymphatics in the nasal cavity and orbita. In addition, Földi discovered and named the clinical condition of "lymphostatic encephalopathy," which results in individuals with cervical lymphatic obstruction (9-11). He would later further characterize this in animal models, demonstrating that cervical lymphatic obstruction leads to cerebral, retina, and optic nerve edema (12,13).

All of this laid the groundwork for this century's two most influential findings related to the brain's lymphatic system. The first of these was the discovery of the glymphatic system by the Nedergaard group (14,15). By imaging with fluorescent CSF tracers and *in vivo* 2-photon and *ex vivo* confocal microscopy they discovered that more than 40% of subarachnoid CSF enters the brain parenchyma along paravascular spaces that surround the penetrating arteries in the brain (14). Using these same techniques, they also identified a critical role for aquaporin-4 (AQP4) water channels in regulating the convective bulk flow of fluid from the para-arterial CSF influx pathway through the interstitium. This was shown using transgenic *Aqp4*-null mice, which demonstrated a significant reduction in parenchymal CSF influx compared to wildtype controls. As a follow-up, using a radiotracer clearance assay, these mice also showed a significant reduction in their interstitial solute clearance. These findings nicely complemented a discovery by Li et al., demonstrating that the cerebral meningeal stomata were likely a component of the cerebral prelymphatic capillary system and involved in regulating lymphatic egress from the perivascular space (16).

While Nedergaard's work has received much fanfare, and rightfully so, the preceding work of her contemporaries, including Roxanne Carare and the late Roy O. Weller, should not be overlooked. Combined, they provided important descriptions of the role of vasomotion in fluid clearance from the brain in addition to identifying CSF/ISF drainage pathways. In 1993, Weller's group injected India ink into the cisterna magna and found that CSF drained into the dCLNs and lumbar para-aortic nodes. Additionally, his work showed that CSF drains from the subarachnoid space along lymphatic pathways through the cribiform plate into the nasal submucosa (17). He also hypothesized that under non-pathological conditions the brain eliminates amyloid-beta (Aβ) within the ISF that drains

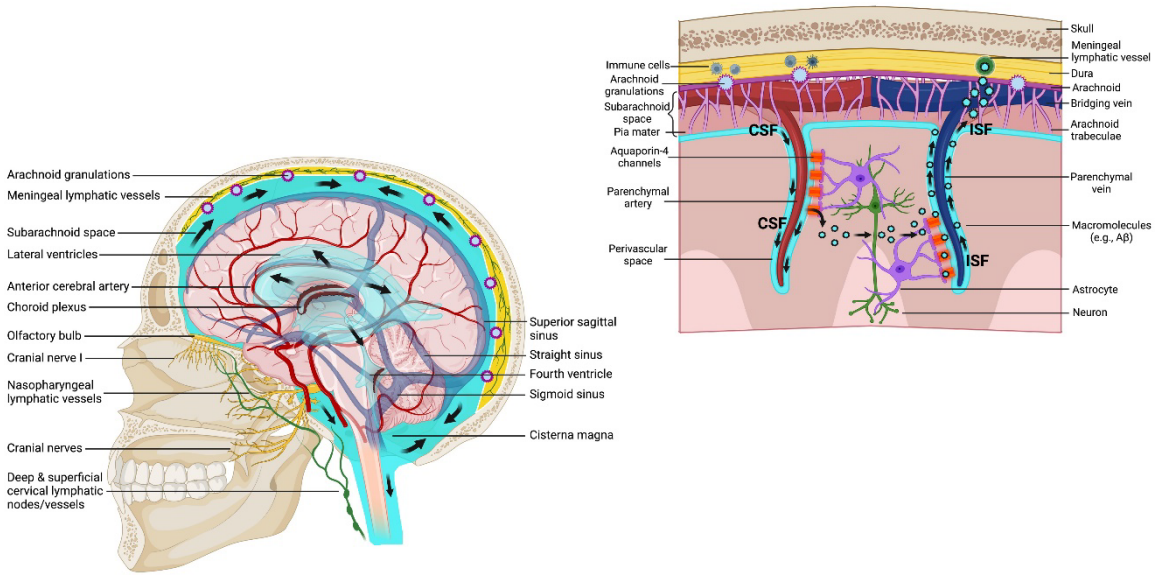

Fig. 1. Production of cerebrospinal fluid (CSF) and its movement within the central nervous system (CNS) along the glymphatic-meningeal-cervical lymphatic pathway. *CSF (black arrows) is produced in the choroid plexus of the lateral and third ventricles, before exiting via the fourth ventricle into the subarachnoid space and its perivascular extensions, known as Virchow-Robin spaces. Low frequency arteriolar oscillations then drive CSF through the narrowing Virchow-Robin spaces and into the brain via Aquaporin-4 (AQP4) channels, where it moves throughout the parenchyma via convective flow. CSF, now combined with interstitial fluid containing dissolved macromolecules (e.g. amyloid beta), exits via AQP4 channels into the paravenous space, where it is then transported to noncontractile meningeal lymphatic vessels (MLVs) in the dura mater. CSF may also exit the brain along cranial (e.g. olfactory) and spinal nerves before, in addition to CSF collected in the MLVs, ultimately draining into the cervical lymph nodes (CLNs) via their respective cervical lymphatic vessels (CLVs). Adapted from Li et al, 2022 (22). Created in BioRender. Bartlett, M. (2024) BioRender.com/b77t632 and BioRender.com/b44w228.*

via periarterial pathways and that the accumulation of these peptides contributes to the development of cerebral amyloid angiopathy (18). A decade later, Carare and Weller confirmed that the brain drains solutes to the peripheral lymph nodes through a perivascular route following injections of both dextran and ovalbumin in the striatum of mice (19). Using mathematical modeling, they also demonstrated how pulsatile arterial flow could reverse solute transport out of the brain via the perivascular pathways they had previously identified (20).

The second major discovery in this century was the identification and confirmation of MLVs in mice by the Kipnis group (1). In his group's seminal paper using molecular lymphatic biomarkers such as *Lyve-1* and *Prox1*, they were able to identify functional lymphatic vessels along the dural sinuses (1). Moreover, they were able to confirm that these vessels transport fluid and immune cells from the CSF to the dCLNs. In this same year, Alitalo's group also identified the presence of a lymphatic network in the dura mater of mice. Using transgenic mice with a VEGF-C/D trap, which lacked lymphatic vessels in the dura, they showed a decrease in macromolecule clearance from the brain and a blockage of the normal transport from the subarachnoid space to the dCLNs (21). Combined, the work of both Kipnis and Alitalo provided evidence for the clearance of macromolecules and solutes via the dural lymphatics following their absorption of CSF from the subarachnoid space and glymphatic system. While there has been

criticism that these publications (and others) did not account for the experimental work that preceded, it cannot be denied that this work has advanced the field and reinvigorated the neuroscience communities' interest in the lymphatic system.

THE GLYMPHATIC-MENINGEAL-CERVICAL LYMPHATIC PATHWAY

Historically, it has been accepted that a key role of CSF was to aid in waste removal from the central nervous system (CNS). However, the last decade has shed much light onto the physiological mechanisms by which this occurs and its importance in maintaining brain health. Following its production in the choroid plexus, CSF (along with dissolved macromolecules) follows a complex route before being cleared from the CNS (*Fig. 1*) (22). First, CSF is propelled through perivascular spaces by the cerebral microcirculation's vasomotion, which is the result of low frequency arteriolar oscillations (23). CSF then accesses bidirectional AQP4 channels, located on the end-feet of astrocytes encasing the brain's micro vessels. These AQP4 channels regulate water exchange and allow for CSF to enter the brain parenchyma. Once in the parenchyma, CSF and solutes follow a convective (24) and diffusive (25) trajectory, where they are collected in the paravenous space, before being transported to noncontractile MLVs located in the dura mater at the base of the skull (26) and parasagittal areas (1,21). Additionally, CSF enters lymphatic vessels that travel parallel to olfactory nerves, before crossing the cribriform plate, where it is then absorbed by the nasopharyngeal lymphatic plexus and drained into the dCLNs (27,28). This olfactory pathway has been identified in multiple mammalian species (29). Regardless, both paths follow a unidirectional flow through contractile deep and superficial cervical lymphatic vessels, before emptying into their respective CLNs (1,26,30,31).

TARGETING THE LYMPHATIC SYSTEM IN THE TREATMENT OF NEURODEGENERATIVE DISEASE

The discovery of the brain's glymphatics (14) and the "*re-rediscovery*" (32) of functional MLVs (1) has changed our previously misconceived view of the brain as being immune privileged and lacking lymphatic drainage. In fact, these critical and groundbreaking studies have laid a foundation for the brain's lymphatic system to be a novel therapeutic target for multiple CNS disorders. This is supported by studies demonstrating that aging reduces CSF flow through the lymphatic vessels (33). Moreover, disruptions along this pathway impair drainage and lead to the accumulation of toxins and more specifically peptides and proteins associated with neurodegenerative disease.

At the glymphatic level, AQP4 has been identified to be directly involved in the clearance of Aβ in wild-type mice (34) and inhibition of this channel leads to increased Aβ pathology in mouse models of Alzheimer's disease (AD) (35,36). Glymphatic impairment has also been found to play a role in the pathophysiology of Huntington's disease (37), frontotemporal dementia (38), and amyotrophic lateral sclerosis (ALS) (39,40). Whereas AQP4 redistribution into the parenchymal processes may trigger the formation of a neuroprotective "glial net" that shields neurons from Aβ aggregates (41), ablation of the MLVs with the photoconvertible drug Visudyne, has also been shown to worsen pathology in both models of AD (30) and traumatic brain injury (42). Whereas enhancing MLV function improves cognition (30) and the efficacy of monoclonal antibodies in 5xFAD mice (43). Lastly, at the cervical lymphatic level, ligation of the dCLNs has been shown to increase the accumulation of Aβ and alpha-synuclein in both Parkinson's (44) and AD (45) models, respectively. Conversely, blocking the cerebral lymphatic system by removing the dCLNs prior to a middle cerebral artery occlusion significantly reduces infarct size and edema, while also improving motor function, post-stroke (45, 46). This likely results from a reduction in the influx of immune cells into the brain (45,46).

TRANSLATION TO HUMANS AND FUTURE THERAPIES

While the discussion above is focused on preclinical models, the same functional structures throughout the CNS have been identified in humans (2,47) and appear to undergo significant impairment with age (48) and neurodegenerative disease (49,50). Accordingly, as the brains' lymphatic system ages, toxic peptides, like Aβ, can accumulate resulting in the development of cognitive impairment and eventually AD. Attempts to reduce Aβ deposits or block its aggregation have had limited success and FDA approved immunotherapies are costly with significant side-effects. However, there are no approved therapies that address the pathophysiology seen in the brain's lymphatics. This presents an opportunity for novel therapies that target this system for enhancement at any point along this glymphatic—meningeal—cervical lymphatic pathway to increased CSF and ISF flow carrying excess toxic metabolites, peptides, and proteins out of the CNS. The discovery of such therapies would be groundbreaking in their application, ability to address numerous neurological diseases, and their potential to improve health outcomes for millions of individuals.

CONFLICT OF INTEREST

All authors declare that no financial conflict of interest exists.

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