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The glymphatic system supports convective exchange of cerebrospinal fluid and brain interstitial fluid that is mediated by perivascular aquaporin-4

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Introduction

The 'glymphatic' system is a brain-wide network of perivascular pathways that supports exchange of cerebrospinal fluid (CSF) and brain interstitial fluid (ISF), contributing to the efflux of interstitial solutes including amyloid β(Iliff *et al.*, 2012; Iliff *et al.*, 2013a; Iliff *et al.*, 2013b). Importantly, this pathway emphasizes a role for astrocytes and the water channel aquaporin-4 (AQP4) in facilitating this exchange, setting it apart from earlier descriptions of perivascular clearance(Rennels *et al.*, 1985). Since its initial characterization in 2012, it has garnered much attention and controversy, including a recent critique(Smith *et al.*, 2017) centered on two discrete elements of the glymphatic system as it was initially described: 1) the occurrence of interstitial bulk flow and 2) the dependence of perivascular CSF-ISF exchange upon the AQP4. Here, we will elaborate the evidence supporting and opposing these points of controversy.

Bulk flow and diffusion in the cerebrum

Solute movement in tissue occurs principally by two processes: diffusion, thermally-driven movement of solutes down their concentration gradients; and bulk flow, solute motion resulting from the pressure-driven movement of its solvent. The rate of diffusion slows with increasing molecular size, while within certain limits the movement by bulk flow is independent of molecular size (Sykova & Nicholson, 2008).

The initial description of the glymphatic system was derived from experiments focused on two exchange processes: CSF solute influx and ISF solute efflux. In vivo 2-photon microscopy and dynamic contrast-enhanced-MRI demonstrated that tracers injected into the

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subarachnoid CSF compartment move rapidly over the brain surface along perivascular spaces surrounding pial arteries and into the brain along perivascular spaces surrounding penetrating arteries (Iliff *et al.*, 2012; Iliff *et al.*, 2013a; Iliff *et al.*, 2013b). In these experiments, the rate of CSF distribution along perivascular pathways did not differ across tracer sizes. Whole-slice imaging following intraparenchymal injection of fluorescent tracers demonstrated that interstitial solute efflux occurs along white matter tracks and large-caliber veins associated with ventricular and cisternal CSF compartments (Iliff *et al.*, 2012; Iliff *et al.*, 2014). Clearance rates of radiolabeled interstitial tracers did not differ between tracers 0.2kD and 10kD in size (Iliff *et al.*, 2012). These findings suggested that brain CSF-ISF exchange involves bulk flow of solutes along perivascular compartments, linked to the efflux of interstitial solutes through interstitial bulk flow.

The description of CSF influx is consistent with widely reported occurrences of bulk-flow dependent CSF movement in low-resistance compartments including the ventricles, cisterns, perivascular spaces and white matter(Rosenberg *et al.*, 1980; Rennels *et al.*, 1985; Ghersi-Egea *et al.*, 1996; Mestre *et al.*, 2018a). A recent study using high-speed particle tracking in perivascular spaces at the brain surface definitively demonstrated rapid, pulsatile bulk flow along these pathways entrained to the cardiac cycle(Mestre *et al.*, 2018b). The extent of bulk flow in distal segments of the vasculature as well as the wider brain interstitium, remains a topic of active debate.

Based on elegant experiments using tracers (Patlak & Fenstermacher, 1975; Pizzo *et al.*, 2018), real-time iontophoresis (RTI) (Nicholson *et al.*, 1979) and fluorescence recovery after photobleaching (FRAP) (Smith *et al.*, 2017), diffusion has been classically thought to dominate solute distribution in the rodent brain, particularly over short distances(Sykova & Nicholson, 2008; Nicholson & Hrabetova, 2017). Though often overlooked, size dependent distribution of CSF tracers away from perivascular compartments was also observed in the initial glymphatic report(Iliff *et al.*, 2012). While these findings were initially ascribed to size-based restriction of solute movement from the perivascular to the wider interstitial compartment by overlapping perivascular astroglial endfeet, these findings are also consistent with a role for diffusion in the local distribution of solutes away from perivascular spaces. However, several experimental studies suggest solute distribution and efflux, particularly over long anatomical distances, cannot be explained by diffusion alone. A seminal study by Cserr and colleagues demonstrated that intraparenchymally-injected solutes spanning 0.9–69kD were cleared from the brain with size-independent kinetics (Cserr *et al.*, 1981), a finding replicated by Iliff et al. (Iliff *et al.*, 2012). Additionally, a comprehensive pharmacokinetic study reported the efflux kinetics of a wide range of solutes from the rat brain following striatal injection (Groothuis *et al.*, 2007). This study reported a complex pattern of efflux, in which both diffusion and bulk flow were observed to contribute to efflux, with their relative contributions dependent upon solute size, chemistry, and interactions with efflux transporters.

The size-independent distribution of CSF tracers along perivascular spaces and efflux of interstitial solutes from the brain over long distances led to the initial description of the glymphatic pathway to include a component of interstitial bulk flow. Since then, computational modeling studies in line with experimental studies on interstitial diffusion

suggest that bulk flow in the brain interstitium over short distances is unlikely under physiological conditions due to the hydraulic resistance of this compartment (Asgari *et al.*, 2016; Jin *et al.*, 2016; Holter *et al.*, 2017; Faghih & Sharp, 2018). Interestingly, a recent computational study used primary RTI data from several published studies estimated a theoretical interstitial bulk flow velocity of 50 μm/min (Ray *et al.*, 2019). At such a low rate, it was predicted that diffusion dominates the distribution of efflux of small molecular weight interstitial solutes, while convective transport was predicted to be important in the distribution of solutes more than 3 kD in size, including peptides, proteins and oligomers important in the setting of neurodegeneration. Together, these results suggest that brain solute efflux is likely driven by both bulk flow and diffusion, although their relative contributions remain undefined. An additional possibility is that bulk flow may be restricted to permissive low-resistance pathways including perivascular spaces and white matter tracks, while local diffusion accounts for solute movement over the short distances between such pathways.

Based on these studies, we suggest that the 'glymphatic' hypothesis be reframed to emphasize the contribution of both interstitial diffusion and perivascular bulk flow to the long-distance distribution of tracers in the brain. The precise mechanisms of movement over short distances requires further investigation. While modeling studies to date have supported a role for diffusion, as of yet no experimental evidence precludes the contribution of interstitial bulk flow to these processes. As more precise characterizations of microanatomy are derived with technological advances in ultrastructural studies (Korogod *et al.*, 2015; Tonnesen *et al.*, 2018), and greater spatiotemporal resolution is obtained when measuring solute movement, the details of this pathway will likely become clearer.

The role of AQP4 in perivascular CSF-ISF exchange

One novel element of the glymphatic model is the dependence of perivascular CSF-ISF exchange on the astroglial water channel AQP4 (Iliff *et al.*, 2012). Iliff et al. reported that CSF tracer influx and interstitial tracer efflux were both dramatically slowed in *Aqp4* knockout mice, a finding which Smith et al. could not replicate in a different *Aqp4* knockout mouse line (Smith *et al.*, 2017). Subsequently, however, a study from a consortium of five labs confirmed *Aqp4* gene deletion impairs glymphatic CSF tracer influx and interstitial solute distribution and clearance using four different *Aqp4* knockout lines (Mestre *et al.*, 2018a). Furthermore, this study reported that *Snta1* knockout mice, which express normal AQP4 levels but lack perivascular AQP4 localization, also exhibit impaired CSF tracer influx. Several additional studies report that deletion of *Aqp4* slows distribution and clearance of other interstitial solutes, including lactate (Lundgaard *et al.*, 2017), tau (Iliff *et al.*, 2014), ApoE (Achariyar *et al.*, 2016) and adeno-associated viruses (Murlidharan *et al.*, 2016). These studies demonstrate that AQP4 plays a key role in both the influx of CSF solutes into the brain parenchyma and in the clearance of interstitial solutes from brain tissue. Marked variability in the magnitude of the effect of *Aqp4* gene deletion, including between those of Smith et al. and other groups, suggests that other factors may be influencing CSF-ISF exchange.

One possibility is that differences in anesthesia and other technical details underlie these discrepant findings. Groothius et al., as well as a recent study suggest a significant role for anesthetics in modulating both CSF tracer influx and ISF solute efflux (Groothuis *et al.*, 2007; Hablitz *et al.*, 2019). Importantly, Hablitz et al. demonstrated that tribromoethanol (Avertin), the drug used in the study by Smith et al., reduced CSF influx to approximately half the magnitude seen when using ketamine-xylazine. Additional technical differences including injection rate and volume may contribute to the inconsistent results. One important criticism of the publications generated involving the glymphatic pathway is the variability reported in tracer exchange. This further articulates the need for greater standardization between experimental protocols, not including anesthesia, but also injection paradigms and the monitoring of physiological state.

Although these experimental studies confirm the role of AQP4 is supporting perivascular CSF-ISF exchange, the biophysical basis of this role remains unresolved. Impairment of perivascular exchange in *Snta1* knockout mice that parallels that of *Aqp4* knockout mice(Mestre *et al.*, 2017) suggests that localization of AQP4 to the perivascular endfoot is critical to this process. It remains unclear how water conductance through AQP4 relates to proposed driving forces of perivascular fluid movement, including arterial pulsation, and whether other active or passive astroglial solute transporters participate in this process. Recent insights regarding the density of endfoot ensheathment of the vasculature (Korogod *et al.*, 2015) and the identification of novel transporters at the endfoot domain (Boulay *et al.*, 2017; Simon *et al.*, 2018) may provide important new leads in beginning to define the role of AQP4 and astroglial endfoot solute transport in macroscopic exchange between the CSF and interstitial compartments.

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