

Reply to: " A curious concept of CNS clearance "

Matters Arising: Nature Neuroscience 2025

Nicholas P. Franks^{1,2} & William Wisden^{1,2}

¹Department of Life Sciences, Imperial College London, London SW7 2AZ, UK

²UK Dementia Research Institute, Imperial College London, London SW7 2AZ, UK.

Summary

All of the technical objections raised by Plá *et al.* regarding our recent paper¹ are either misunderstandings, misleading, or simply incorrect. It seems to us that the aim of their comments is to discredit the conclusions of our work, rather than to seriously question the methodology.

Despite the assertion of Plá *et al.* that a decade of work has supported the idea that "glymphatic" clearance is increased during sleep², this is not correct. In fact, there is virtually no data showing directly that this is the case. Most studies claiming enhanced clearance are based on the unproven³, and in our opinion fallacious, belief that *entry into* the brain from the CSF is a reliable surrogate of brain clearance, even though only a small percentage³ of tracer enters the brain after cisterna magna injection. Our results¹ show unambiguously that there is greater retention of tracer injected into the brain during sleep and anesthesia compared to waking. Studies⁴ that show increased fluctuations of CSF movement during sleep are no more evidence of increased CSF bulk flow and clearance, than increased fluctuations in the EEG during sleep are evidence of increased brain activity. Both simply reflect coordinated neuronal activity.

In a recent paper⁵, the Nedergaard group, in a remarkable reversal of their previous position, no longer claim that clearance depends on the length of sleep or NREM delta power⁶, but rather that clearance correlates with short bursts of microarousals - short periods of *waking* - that occur *during* sleep⁵. Moreover, the vasomotion they claim is driving clearance during these microarousals has been shown to operate continuously in the waking state⁷. Finally, they concede in that paper⁵, which has also been shown experimentally⁷, that clearance would be greatly inhibited by anesthetics, exactly the result we report¹, and which they seek to discredit.

Responses to the comments of Plá *et al.*

1. Conceptual misunderstanding of brain clearance. We do not think that there is any misunderstanding about the meaning of brain clearance - it is simply removal of substances from the brain. If there is any misunderstanding, it is in the minds of those who believe that this can be measured by influx *into* the brain. (See also point 2 below.)

2. Misrepresenting modeling of brain clearance mechanisms. We don't agree. The basic equation we used (equation [2]) describes a purely diffusive redistribution of the injected tracer, which is then modified by a clearance term (see equation [8]) that accounted accurately for the reduced tracer observed *in vivo*. The form of the clearance term was empirical and this fitted the *in vivo* data well and hence allowed us to quantify clearance. The statement that this approach is "at odds with centuries of established physics", referencing a student textbook on diffusion, cannot be taken seriously.

No matter what equations are used, the increased concentrations of tracer arriving at the frontal cortex during sleep and anesthesia compared to waking are most simply interpreted as reduced clearance along the path that the tracer has taken between the CPu and the cortex. This is consistent with the histology experiments (Fig. 3 in Miao *et al*).

3. Misalignment between modeled and actual flow. This is a misunderstanding. The *in vitro* experiment described in Extended Data Fig. 5 was designed to get an estimate of the maximum bulk flow that, statistically, we could have excluded. These *in vitro* experimental data alone were all we used for this estimate. (The equation that best fitted the data - equation 6, which *did* assume radial flow - was not used for this estimate).

4. Misrepresented brain states. We did not. It is clear from the Methods that we used bolus injections, as used by the Nedergaard lab⁶ (and adopted by us). These doses cause long-lasting effects for many hours, as is evident from the EMG and EEG; importantly, however, most clearance occurs in the initial 2-4 hours. It has yet to be determined exactly which facets of the anesthetic state inhibit clearance.

Plá *et al* further argue that sleep deprivation causes stress. However, our method of sleep deprivation (novel objects presented each hour for 5 hours, with no handling) causes minimal stress, and more importantly, clearance during this state (Fig. 2g) is identical to that during normal waking (Fig. 2d-f in Miao *et al*). We note that in Nedergaard's most recent paper⁵ sleep deprivation is also used, but there the mice are handled, which is significantly more stressful (assayed by corticosterone levels).

5. No confirmation of consistent injection volumes. There was no reason to believe that different volumes were injected - the injection syringe is a positive-displacement device, and the same volumes were injected into the brain. Subsequent measurements of tracer levels 10 minutes after injection confirmed this ($p=0.72$). The removal of the dummy cannula after injection in our photometry experiments is the exactly the same procedure used by the Nedergaard laboratory⁸. Also, for our histology experiments, the indwelling cannula was never removed, and we obtained the same results. Indeed, a recent *bioRxiv* deposition from the Nedergaard lab⁹, reports experiments essentially identical to ours. Although they did not study the effects of sleep *per se*, they did compare injections at different circadian times and under KET-XYL anesthesia. Their results are entirely consistent with our findings (see their Fig. 1c) and show that shortly after injection, the tracer levels close to the cannula appear to be, within error, the same in the KET-XYL and awake groups (meaning the injection volumes of tracer must have been the same), but the tracer concentrations diverge with time due to the greatly increased clearance during the waking state. The authors⁹ ignore this obvious explanation.

Finally, Plá *et al.* criticize our histology experiments for using only 3-hour and 5-hour assessments "missing earlier time points". Yet in Nedergaard's latest paper⁵, clearance is only assessed at 5 hours.

6. Invalid tracer compromises diffusion measurements. This is misleading. There is nothing unusual or unique about the tracer we used. AF488, together with countless other small molecules and tracers, may cross the blood brain barrier to some extent¹⁰. However, the balance between this clearance route and clearance through the CSF is unknown, as are the mechanisms by which sleep and anesthesia reduce clearance. The possibility that some AF488 is cleared through the blood does not invalidate its use for studying parenchymal diffusion; rather, in our view, this strengthens the generality of our findings.

7. Injected volume far exceeds physiologically available space. We disagree. These comments completely ignore the critical parameter of *rate* of injection. To be clear, for our clearance experiments

we injected only 0.5 μ l (injected at 0.05 μ l/min into the CPu). The Nedergaard lab uses⁸ twice this volume and four times this rate. For the diffusion experiments, we used 10 μ l (injected at 0.1 μ l/min into the CPu) where the aim was to achieve a uniform distribution of the tracer in the frontal cortex. Even at the low rate we used, there might be some local damage at the site of injection, but the diffusion measurements were made in the cortex, some 3 mm distant, so damage here is unlikely.

8. No assessment of brain damage and inflammation. The Nedergaard lab has shown⁸ with identical cannulation to ours, that brain clearance returns to baseline levels 24 hours after cannulation. We waited 7 days. Moreover, there is no reason to believe that any damage would preferentially affect the tracer redistribution during waking compared with the sleeping or anesthetized states.

Tissue damage in the bleaching experiments is unlikely - we used levels of light exposure during bleaching that were 10% of those routinely using in optogenetics experiments¹¹.

9. Unclear data visualization and improper analysis. Our diffusion data using one-way ANOVA showed no significant change over 24 hours. The reanalysis by Plá *et al.* is flawed at several levels. A one-way ANOVA can certainly be used with unequal sample sizes provided Levene's test of homogeneity shows no significance (for our data $p=0.96$). One disadvantage is that if significance is found, other methods are needed to assess frequency, amplitude and phase. We found no significance so no further steps were warranted. What Plá *et al.* have done to find significance is to take our publicly deposited data and first ignore 70% of it; they then perform a *t*-test on two chosen bins, completely ignoring the problem of multiple comparisons. Moreover, even then, "significance" is only achieved (just, $p = 0.0413$) by eliminating a data point that was "a potential outlier". Their whole approach is flawed. It is also worth noting that even if significance were to be found, this is no direct relevance to sleep, since multiple changes occur over the circadian cycle, and such a change could be circadian.

Conclusions

Our diffusion experiments suggest that, for the tracers we studied, diffusion is the predominant mechanism that moves metabolites through the brain parenchyma, rather than bulk flow¹². Indeed, this has been well established by others¹³⁻¹⁵, and this element of the "glymphatic hypothesis" is, therefore, almost certainly wrong. Our data go one important step further in showing that vigilance states (sleep, wake, anesthesia) have no effect on tracer movement.

Our clearance measurements show that more tracer is retained in the brain during sleep and anesthesia compared to during waking. We are confident that the claim of Xie *et al.*² that sleep and anesthesia speed clearance 20-fold, the other key element of the "glymphatic hypothesis", is also wrong. Indeed, based on the recent findings from ourselves¹ and the Nedergaard group⁵, there is little left of the original glymphatic hypothesis other than the name.

Acknowledgements: This work was supported by the Wellcome Trust, grant 220759/Z/20/Z (N.P.F., WW); and the UK Dementia Research Institute (award number UK DRI-5004) through UK DRI Ltd, principally funded by the Medical Research Council (W.W., N.P.F.). For the purpose of open access, the author has applied a Creative Commons Attribution (CC BY) license to any Author Accepted Manuscript version arising.

1. Miao, A., *et al.* Brain clearance is reduced during sleep and anesthesia. *Nat Neurosci* **27**, 1046-1050 (2024).
2. Xie, L., *et al.* Sleep drives metabolite clearance from the adult brain. *Science* **342**, 373-377 (2013).
3. Hladky, S.B. & Barrand, M.A. The glymphatic hypothesis: the theory and the evidence. *Fluids Barriers CNS* **19**, 9 (2022).
4. Fultz, N.E., *et al.* Coupled electrophysiological, hemodynamic, and cerebrospinal fluid oscillations in human sleep. *Science* **366**, 628-631 (2019).
5. Hauglund, N.L., *et al.* Norepinephrine-mediated slow vasomotion drives glymphatic clearance during sleep. *Cell* **Jan 8th Online ahead of print**(2025).
6. Hablitz, L.M., *et al.* Increased glymphatic influx is correlated with high EEG delta power and low heart rate in mice under anesthesia. *Sci Adv* **5**, eaav5447 (2019).
7. van Veluw, S.J., *et al.* Vasomotion as a Driving Force for Paravascular Clearance in the Awake Mouse Brain. *Neuron* **105**, 549-561 e545 (2020).
8. Pla, V., *et al.* A real-time in vivo clearance assay for quantification of glymphatic efflux. *Cell Rep* **40**, 111320 (2022).
9. Kroesbergen, E., *et al.* Glymphatic clearance is enhanced during sleep. *bioRxiv*, 2024.2008.2024.609514 (2024).
10. Hladky, S.B. & Barrand, M.A. Elimination of substances from the brain parenchyma: efflux via perivascular pathways and via the blood-brain barrier. *Fluids Barriers CNS* **15**, 30 (2018).
11. Zhang, F., *et al.* Optogenetic interrogation of neural circuits: technology for probing mammalian brain structures. *Nat Protoc* **5**, 439-456 (2010).
12. Iliff, J.J., *et al.* A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Sci Transl Med* **4**, 147ra111 (2012).

13. Asgari, M., de Zelicourt, D. & Kurtcuoglu, V. Glymphatic solute transport does not require bulk flow. *Sci Rep* **6**, 38635 (2016).
14. Jin, B.J., Smith, A.J. & Verkman, A.S. Spatial model of convective solute transport in brain extracellular space does not support a "glymphatic" mechanism. *J Gen Physiol* **148**, 489-501 (2016).
15. Holter, K.E., *et al.* Interstitial solute transport in 3D reconstructed neuropil occurs by diffusion rather than bulk flow. *Proc Natl Acad Sci U S A* **114**, 9894-9899 (2017).