nature neuroscience

Matters arising

https://doi.org/10.1038/s41593-025-01897-3

A curious concept of CNS clearance

Received: 9 June 2024

Accepted: 28 January 2025

Published online: 11 March 2025

Check for updates

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ARISING FROM A. Miao et al. *Nature Neuroscience* https://doi.org/10.1038/s41593-024-01638-y (2024)

Over the past decade, multiple lines of research have shown that sleep decreases amyloid-β and tau burden compared with wakefulness, and glymphatic clearance is increased during sleep in both human and mouse brains[1–6]. A recent study by Miao et al.[7] has questioned these findings. We here raise concerns regarding experimental methodology, analytical rigor, and theoretical and mathematical assumptions in the Miao et al.[7] study. The conclusion of that study—brain clearance is reduced during sleep and anesthesia—is not supported by the data presented.

Conceptual misunderstanding of brain clearance

Metabolic waste clearance from the brain is a process whereby proteins are degraded in situ (by ubiquitination or autophagy) or exported from the brain to the periphery[8]. Brain clearance is experimentally defined as a decline in reference molecules in brain homogenates or as their evacuation to peripheral tissues[8]. Miao et al.[7] instead measure dye displacement between the caudate–putamen and an optical probe placed in the prefrontal cortex, thus defining clearance as redistribution of the tracer within the brain. This is fundamentally flawed. Moving garbage around your home will not eliminate your waste problem. Waste must be flushed out of the brain to be cleared.

Misrepresenting modeling of brain clearance mechanisms

Clearance of the tracer occurs via a combination of advection (clearance by bulk fluid motion) and diffusion in brain. Miao et al.[7] estimate the tracer diffusivity by fitting experimental measurements to equation 8, which is not a solution of a diffusion or advection–diffusion equation and is at odds with centuries of established physics[9]. Miao et al.[7] give no justification for the mathematical form of equation 8, which attempts to model advective transport as a reaction term. The authors argue that their measurements are inconsistent with a naive approximation of transport by pure diffusion, thus an unspecified mechanism that is neither diffusion nor advection must be responsible. They define this mechanism as 'clearance', contradicting accepted definitions, ignoring the possibility that diffusivity and advection may change between wake and anesthesia/sleep, dismissing established research, and introducing confusion into the field.

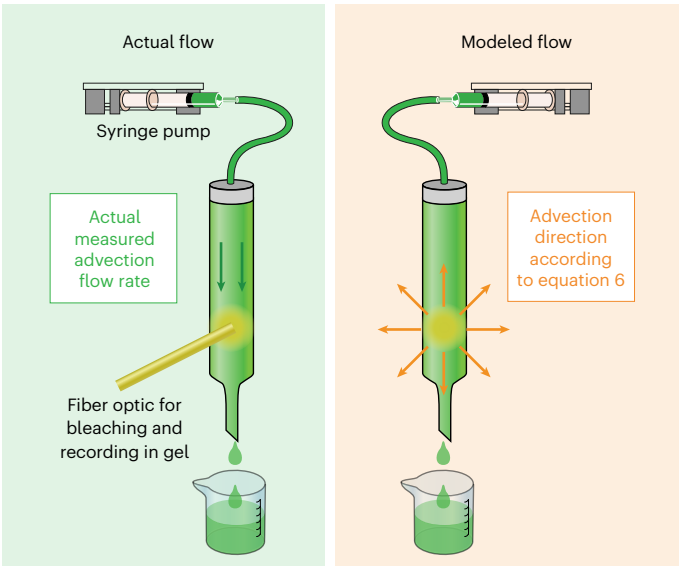

Fig. 1 | Discrepancy between flow velocity model and actual velocity. Equation 6 models velocity as purely radial, emanating from the origin, whose location is not stated but presumably is the center of the observation region (right). However, the experiment being modeled involved no such outward flow; rather, the velocity pointed downward (left). Depending on the location, downward flow is aligned with, orthogonal to or in direct opposition to outward flow. Additionally, the modeled flow fails to conserve fluid mass, making it impossible. Equation 6 cannot be used to model the experiment shown in Extended Data Fig. 5 of Miao et al.[7] or any others.

Misalignment between modeled and actual flow

Advection effects are quantified by fitting equation 6 to measurements from experiments akin to the one outlined in Extended Data Fig. 5 in Miao et al.[7] However, equation 6 presumes a flow emanating radially from the origin, which is physically impossible because it fails to conserve fluid mass. Radial flow also contradicts the actual flow direction

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https://doi.org/10.1038/s41593-025-01897-3

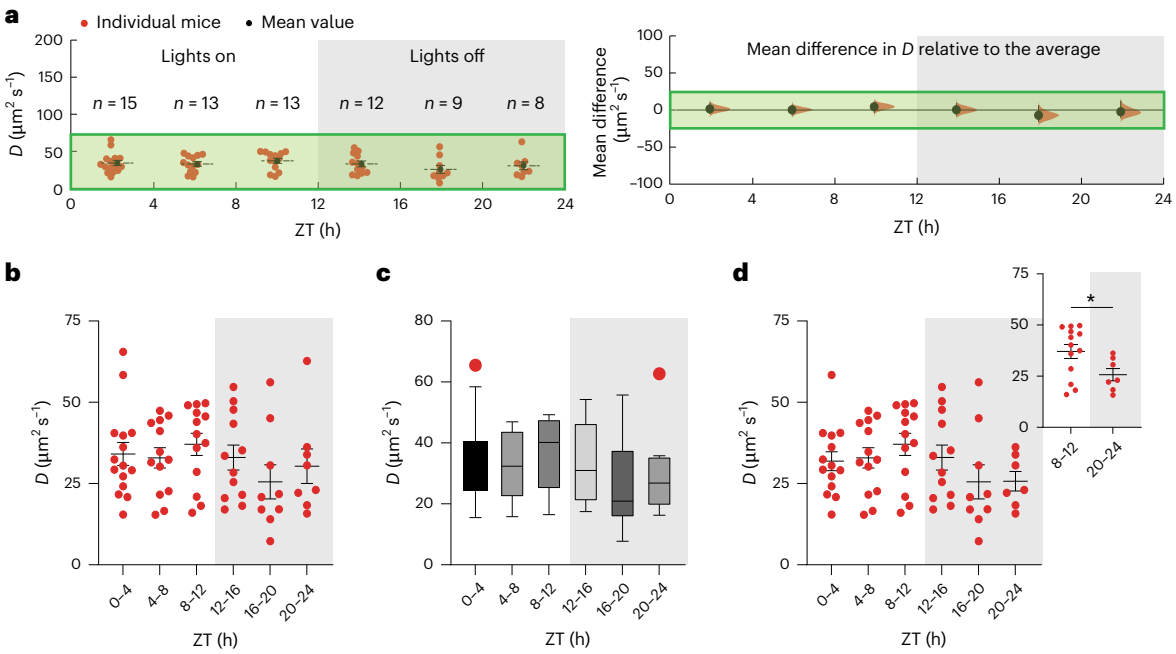

Fig. 2 | Data visualization, unequal sampling and improper statistical analysis may cause interpretation errors in diurnal variation of diffusion. a, Fig. 1e in the original manuscript (green shaded boxes indicate data ranges). **b**, Data re-graphed to show data variability and 4-h intervals (red dots represent individual animals; error bars indicate the mean ± s.e.m.; gray box denotes the dark phase). **c**, Tukey box plots showing statistical outliers in the ZT0–4 and ZT20–24 bins (red dots). **d**, Data re-plotted without outliers in **c**, with the significant ZT8–12 and ZT2–24 comparison (insert; red dots represnt individual animals; error bars indicate the mean ± s.e.m.; gray box denotes the dark phase; $^*P = 0.0413$).

(Fig. 1), entering from the top and exiting at the bottom. Because the modeled flow is impossible and differs essentially from the experimental one, fitting equation 6 will not quantify transport parameters.

Misrepresented brain states

The brain states presented in this study are incorrectly described, and figure design and labels are misleading. Figure 2d–f in Miao et al.[7] indicates cohorts under different anesthesia conditions for 12 h; however, the mice were anesthetized only once with a single bolus injection at the start of the experiment, which is not mentioned in the legend, or the Results and Methods sections. Pentobarbital induces an anesthetic plane for 10–300 min, ketamine–xylazine for 30–45 min and dexmedetomidine (with ketamine) for 20–30 min, meaning that these cohorts were in a state of post-anesthesia recovery and not anesthetized for 92% (11 h of 12 h) of the experiments[10]. This is important, because our published data restricted the studies of glymphatic influx and clearance to deeply anesthetized mice and can, therefore, not be compared to the data of Miao et al.[7]. The data in Fig. 2 must be represented as post-anesthesia data rather than anesthesia data to avoid confusion in the field. Additionally, the recordings in Fig. 2g of Miao et al.[7] were done during sleep deprivation and rebound sleep. Post-anesthesia arousal is associated with hyperactivity in mice[11], and rebound sleep differs from normal sleep and requires several days for complete recovery[12].

No confirmation of consistent injection volumes

Real-time three-dimensional magnetic resonance imaging and SPECT analyses from our group reveal considerably lower tracer injection into the brains of awake mice compared to sleeping or anesthetized mice[13]. Miao et al.[7] fail to validate the injected tracer volumes, thus not capturing this difference. In Fig. 2 of Miao et al.[7], an optical fiber detects tracer at a distant pinhole, while Fig. 3 measures tracer content in fixated brains at 3 h or 5 h after injection only, missing earlier time points. This leads to the wrong conclusion of greater clearance when, in reality, less tracer was injected in the awake group. The authors also replace the infusion cannula with a dummy after injection, which relieves pressure and likely allows tracer to escape. Furthermore, the injection site's peak fluorescence along the anteroposterior axis varies between mice (Fig. 3b in Miao et al.[7]). Without consistent injectate volume and position, comparisons of brain clearance between groups cannot be trusted.

Invalid tracer compromises diffusion measurements

Unconjugated Alexa Fluor crosses the blood–brain barrier, invalidating its use for studying parenchymal diffusion[14]. The authors ignore efflux to the vascular compartment. The signal that Miao et al.[7] detect with an optical fiber in prefrontal cortex is a mixture of Alexa Fluor in both the vascular compartment and the tissue. As such, it cannot be used to calculate dispersion in brain, which is the fundamental basis for all the equations utilized.

Injected volume far exceeds physiologically available space

Volumetric analysis shows that caudate–putamen averages 20.5 µl ± 0.5 µl in mice[15], with the extracellular space comprising 3.1 µl. Miao et al.[7] inject 10 µl in Fig. 1 (FRAP experiments), nearly half the regional volume and threefold larger than the extracellular space. Such extreme fluid volumes will impose mechanical and osmotic stress, which will have brain-wide impacts and alter neural activity. The injury induced by a large injection volume in Fig. 1 in Miao et al.[7] likely varies between awake and anesthetized mice, as anesthetics are potent neuroprotective agents.

No assessment of brain damage and inflammation

Miao et al.[7] do not include histological analysis of injury and reactive gliosis from multiple cannulations. Miao et al.[7] also fail to show the cannula placement, a standard practice in the field. This omission is

critical, as their design involves repeated large-volume tracer injections and 30 s of ultraviolet exposure to the cortex, likely inducing immune responses and artifacts. The authors need to document these effects to clarify their potential impact on the findings.

Unclear data visualization and improper analysis

The over-scaled *y* axis in Fig. 1d,e downplays data variability (Fig. 2a,b), and interquartile range analysis reveals statistical outliers (Fig. 2c). Reanalysis shows a nonsignificant one-way analysis of variance ($F(5, 62) = 1.361$, $P = 0.2513$). However, the statistics in Miao et al.[7] are inappropriate for testing diurnal variations in brain clearance:

For time-series analysis of individual mice, the appropriate parametric test is a cosinor analysis (a modified general linear model). This requires exact Zeitgeber time (ZT) and equal sample sizes. Miao et al.[7] does not report exact ZT and has less data points at night. Appropriate non-parametric tests would also need ZT reported. To utilize the current dataset, we ran two-sided *t*-tests on diffusion coefficients at time points that were 12 h apart. Comparing early day and night or midday and midnight was not significant (ZT0–4 versus ZT12–16: $t(24) = 0.2382$, $P = 0.8137$; ZT4–8 versus ZT16–20: $t(20) = 1.281$, $P = 0.2148$). There were significant differences in diffusion coefficients between late day and night (ZT8–12 versus ZT20–24: $t(18) = 2.197$, $P = 0.0413$; Fig. 2d) indicating an underlying diurnal variation.

In sum, several important flaws have been identified in Miao et al.[7]. Its design is fundamentally uninformative, and the results are confounded by experimental artifacts. Consequently, the data fail to support meaningful conclusions, whether regarding glymphatic clearance or other aspects of brain fluid transport.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-025-01897-3.

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Acknowledgements
This work was supported by National Institutes of Health grant R01AT012312 (to M.N.); NINDS R01AT011439 (to M.N.); U19 NS128613 (to M.N.); the Simons Foundation (to M.N.); Novo Nordisk Foundation NNF20OC0066419 (to M.N.); the Lundbeck Foundation R386-2021-165 (M.N.); The Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (to M.N.); JPND/HBCI 1098-00030B (to M.N.); JPND/Good Vibes 2092-00006B (to M.N.); DOD W911NF2110006 (to M.N.); Independent Research Fund Denmark 3101-00282B (to M.N.); US Army Research Office grants MURI W911NF1910280 (to M.N.); and American Heart Association Career Development Award 941177 (L.M.H.).

Author contributions
M.N., V.P. and E.K.: Writing—original draft preparation. V.P., E.K., S.D., M.J.G., L.M.H., E.N., A.L.G., T.E., R.S.G., D.H.K., J.H.T. and M.N.: Writing—reviewing and editing.

Competing interests
The authors declare no competing interests. M.N. is a paid consultant for CNS2 for unrelated work.

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Peer review information *Nature Neuroscience* thanks the anonymous reviewers for their contribution to the peer review of this work.