1 **TITLE: Post-capillary venules is the locus for transcytosis of**

2 **therapeutic nanoparticles to the brain**

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30 **HIGHLIGHTS:**

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32 • Integration of drug carrier nanotechnology with two-photon microscopy *in vivo*

33 • Real-time nanoscale-resolution imaging of nanoparticle transcytosis to the brain

34 • Distinct trafficking pattern in the endothelium of cerebral venules and capillaries

35 • Venules, not capillaries, is the locus for brain uptake of therapeutic nanoparticles

1 **SUMMARY:**

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3 Treatments of neurodegenerative diseases require biologic drugs to be actively

4 transported across the blood-brain barrier (BBB). To answer outstanding questions

5 regarding transport mechanisms, we determined how and where transcytosis occurs at

6 the BBB. Using two-photon microscopy, we characterized the transport of therapeutic

7 nanoparticles at all steps of delivery to the brain and at the nanoscale resolution *in*

8 *vivo*. Transferrin receptor-targeted nanoparticles were taken up by endothelium at

9 capillaries and venules, but not at arterioles. The nanoparticles moved unobstructed

10 within endothelial cells, but transcytosis across the BBB occurred only at post-

11 capillary venules, where endothelial and glial basement membranes form a

12 perivascular space that can accommodate biologics. In comparison, transcytosis was

13 absent in capillaries with closely apposed basement membranes. Thus, post-capillary

14 venules, not capillaries, provide an entry point for transport of large molecules across

15 the BBB, and targeting therapeutic agents to this locus may be an effective way for

16 treating brain disorders.

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18 **KEYWORDS:**

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20 Blood–brain barrier, transcytosis, two-photon imaging, nanoparticle, transferrin

21 receptor, *in vivo*, awake mice, drug delivery, capillaries, venules.

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24 **INTRODUCTION**

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26 The blood-brain barrier (BBB) is impermeable to most blood-borne substances

27 (Abbott et al., 2010). The paracellular route across the BBB is limited by junctional

28 complexes between brain endothelial cells (BEC). Diffusion across BEC is possible,

29 but restricted to low-molecular weight hydrophobic compounds, and most lipophilic

30 drugs show negligible brain uptake because of the rapid outward transport of

31 xenobiotics by BEC efflux pumps (Mollgard et al., 2017; Pardridge, 2012). Large

32 molecules can enter the brain, but only via vesicular transport, i.e. transcytosis.

33 However, this route is highly selective and strongly suppressed by recently identified

34 homeostatic mechanisms (Ben-Zvi et al., 2014; De Bock et al., 2016; Janiurek et al.,

1   2019; Tietz and Engelhardt, 2015). Consequently, while the low BBB permeability

2   protects the brain against circulating pathogens, it also precludes more than 98% of

3   neuroprotective compounds from reaching the brain, rendering central nervous system

4   (CNS) disorders resistant to most conventional therapies (Banks, 2016; Pardridge,

5   2002, 2012).

6   Drug delivery systems that adapted the receptor-mediated transcytosis (RMT) to

7   shuttle large therapeutic cargo across the BBB are currently at the forefront of the

8   modern pharmacology (Lajoie and Shusta, 2015; Pardridge, 2020; Pulgar, 2018).

9   Coupling carrier molecules to RMT ligands may enhance delivery of therapeutics to

10   the brain, and the flagship RMT ferrying receptor in the brain is the transferrin

11   receptor (TfR) (Johnsen et al., 2019b; Pardridge, 2002, 2020). Nanoparticles, such as

12   liposomes, are versatile drug carriers that can encapsulate a variety of xenobiotics,

13   and functionalized with TfR ligands represent a promising drug delivery approach

14   with diverse therapeutic opportunities (Johnsen et al., 2019b; Pardridge, 2020). The

15   translational potential of TfR-targeted nanoparticles has been tested in preclinical

16   models of Parkinson's, Alzheimer's, Huntington's disease, brain cancer, and stroke,

17   but the level of drug delivery levels rarely meet the thresholds for clinical significance

18   due to failure in nanoparticle transcytosis (Johnsen et al., 2019b).

19   To address this issue, we examined how cerebral vessels handle clinically relevant

20   nanoparticles in the living, intact brain. We employed two-photon microscopy in

21   anesthetized and awake mice to characterize the pharmacokinetics of TfR-targeted

22   nanoparticles at the BBB, intracellular trafficking patterns in BECs, and transcytosis

23   into the brain parenchyma using real-time *in vivo* imaging at nanoscale resolution. We

24   report that TfR-targeted nanoparticles bind efficiently to BECs in venules and

25   capillaries, but are absent in arterioles. Despite the highest uptake by BECs in

26   capillaries, we found that transcytosis occurs only in post-capillary venules that, in

27   contrast to capillaries, are surrounded by a large perivascular space that can

28   accommodate nanoparticle-sized elements (Engelhardt et al., 2017). Our observations

29   indicate that post-capillary venules mediate brain uptake of large drug carriers, which

30   should be considered in the design of next-generation nanoparticles for the treatment

31   of brain disorders.

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1   **RESULTS**

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3   **Two-photon imaging of BBB-targeted nanoparticles *in vivo*.**

4   To investigate how drug nanocarriers interact with the BBB *in vivo*, we used two-

5   photon fluorescence microscopy in mice. Following microsurgery, the brain was

6   imaged via a cranial window over the somatosensory cortex (Figures 1A and 1B). The

7   nanoparticles were designed to resemble clinically approved nanoliposome

8   formulations (Barenholz, 2012), consisting of a

9   distearoylphosphatidylcholine(DSPC)/cholesterol lipid bilayer surrounding an

10   aqueous lumen and with a PEGylated surface to ensure stability in the blood (Figure

11   1C, Table S1). To enable targeting to the brain via TfR, we coupled the nanoparticles

12   to immunoglobulin G (IgG) monoclonal anti-TfR antibody clone RI7217 (Lee et al.,

13   2000). Prior to two-photon imaging experiments, we validated the nanoparticle TfR

14   targeting system by encapsulating BBB-impermeable cisplatin into nanoparticles

15   (Table S1), and injecting the nanoparticles into the bloodstream. The traces of

16   cisplatin were detected in the brain only for nanoparticles functionalized with RI7217,

17   but not stealth (no targeting antibody), or isotype IgG (no TfR recognition) antibodies

18   (Figure 1D).

19   To enable detection in subsequent two-photon imaging experiments, we fluorescently

20   labeled the nanoparticle lipid bilayer with either Atto488 or Atto550 (Figure 1C).

21   Both the Atto550- and Atto488-labeled nanoparticles (RI7-L-A550 and RI7-L-A488)

22   were administered by single bolus injection into the bloodstream (70 nmol$_{lipid}$/g$_{animal}$)

23   and were subsequently imaged in the brain volume containing pial arterioles and

24   venules, penetrating arterioles, ascending venules, pre- and post-capillary vessels, and

25   capillaries (Figure 1E). The blood-circulating nanoparticles exhibited high

26   fluorescence stability over time, indicating a low filtration rate by peripheral organs

27   (Figures 1F and 1G, Movie S1). Nanoparticles retained their single-particle

28   appearance without agglomeration or fusion, as ascertained by laser-extravasation of

29   nanoparticles from the bloodstream into the brain parenchyma after 3 h in circulation

30   (Figures 1H and 1I). A small fraction of nanoparticles was sequestered by leukocytes,

31   but without adverse effects on the brain and systemic physiology that could

32   compromise the BBB (Figure S1, Movie S2).

33   Nanoparticles in the brain *in vivo* exhibited properties of a point source signal with a

34   Gaussian distribution peak denoting the center location of the nanoparticle. The

1   fluorescence distribution standard deviation (σ) was 0.290±0.006 µm and

2   0.296±0.008 µm for RI7-L-A550 and RI7-L-A488, respectively, and did not differ

3   between the two distinctively labeled nanoparticle types (n=20, p=0.5910, t-test)

4   (Figure 1J). In subsequent experiments, the nanoparticles were considered spatially

5   separated when their peak of fluorescence exceeded 2σ from other sources of

6   fluorescence, i.e., vessel lumen, endothelium, or other nanoparticles.

7   Thus, the nanoparticles fulfilled all necessary requirements for *in vivo* imaging:

8   efficient two-photon excitation and emission of fluorescence with the ability to

9   resolve single nanoparticles, high structural stability in the circulation, and no

10   detrimental effects on the brain and systemic physiology.

11

12   **Targeted nanoparticles selectively associate to capillaries and venules.**

13   To delineate vessel lumen, we used FITC-dextran (FITC-dx) injected subsequently to

14   the nanoparticles. The blood-circulating nanoparticles were rapidly recruited to the

15   endothelium, and at 2 h post-injection, appeared as numerous punctae at vessel walls

16   of the capillaries, and of the pial (Figure 2A), ascending, and post-capillary venules

17   (Figures 2B–2E), but were absent from the arterial branches of the brain

18   microvasculature (Figure 2F). The association was driven by interactions of the TfR-

19   Ab with the TfR at the BEC surface and not by stalling of nanoparticles in the vessel

20   lumen, as neither antibody-lacking stealth nanoparticles (Sth-L-A550)(Figures S2A

21   and S2B) nor isotype IgG-functionalized nanoparticles that lack TfR recognition

22   (IgG-L-A550) associated to BECs (Figures S2C and S2D). The nanoparticle

23   association was independent of the type of fluorescent tag, as RI7-L-A488 exhibited

24   the same targeting properties as RI7-L-A550 (Figures S3A–E).

25   Lastly, mice co-injected with both nanoparticles exhibited distinct labeling at the

26   vessel walls from both RI7-L-A488 and RI7-L-A550, attesting that the observed

27   punctae represented single nanoparticles (Figure 2G). Scarce presence of merged

28   signals was attributed to overlapping fluorescence of nanoparticles separated by a

29   distance smaller than the diffraction limit of the microscope (Figure 2H).

30

31   **Vessel type determines association density and kinetics.**

32   We isolated BEC-associated from blood-circulating nanoparticles by excluding the

33   signal of circulating nanoparticles that colocalized with FITC-dx in the vessel lumen

34   (Figure 3A). Three-dimensional (3D) reconstruction revealed a spatially

1   heterogeneous association of nanoparticles across the vascular tree (Figure 3B). At 2

2   h post-injection, the highest density, i.e., number of nanoparticles at the vessel

3   surface, was at BECs of capillaries and venules with an exponential decline in

4   ascending and pial venules (Figure 3D). This observation was consistent with the

5   kinetics of association being fastest in capillaries and slowest in pial venules (Figures

6   3E, S3F, and S3G, Movie S3). Of note, nanoparticle binding was ongoing even after 2

7   h post-injection, indicating that the cellular pool of available TfRs was not saturated

8   at this time (Figure 3E).

9

10   **Most of associated nanoparticles are internalized by endothelium.**

11   To determine the type of interaction between nanoparticles and BEC, i.e. the ratio

12   between internalized and adhering nanoparticles, we used transgenic mice expressing

13   cytosolic green fluorescent protein (GFP) in the endothelium (Tie2-GFP; see

14   Methods). This allowed for a comprehensive assessment of endothelium morphology

15   with unprecedented details in the living brain (Figures 4A and S4). We could discern

16   the distinct patterns of nuclei and endothelial cell contact sites (Figure 4B), and the

17   fluorescence signal was compatible with imaging of the nanoparticles (Figure 4C).

18   At 2 h post-injection, TfR-coated nanoparticles exhibited a non-uniform distribution

19   in relation to the vessel wall, appearing either to be internalized or to adhere to the

20   luminal surface of the endothelium (Figure 4D). For each analyzed vessel, we aligned

21   the imaging plane with the center of the vessel radial symmetry (the broadest vessel

22   cross-section), and for each nanoparticle, we extracted the fluorescence profile plot

23   with corresponding endothelium signal (Figure 4E). As a measure of separation, we

24   used the distance between peaks (Δp). The precise spatial localization of nanoparticles

25   is non-trivial because of the diffraction limit, therefore we defined the boundary

26   conditions characterizing nanoparticle location using the previously calculated

27   standard deviation (σ) of Gaussian fluorescence distribution ($\sigma=0.29$ µm; Figure 1J).

28   Using conservative inclusion criteria, the nanoparticles were categorized as

29   *internalized* when separated from endothelium peak signal only by $|\Delta p|<\sigma$ (high

30   fluorescence overlap) and as *adhering* when separated from the endothelium peak

31   signal by at least 2σ, i.e., $|\Delta p|>2\sigma$ (minimal fluorescence overlap). The third group

32   consisted of nanoparticles that could not be categorically classified into adhering or

33   internalized and represented the intermediate group (i.e., *unresolved*, $\sigma \leq |\Delta p| \leq 2\sigma$).

34   Our boundary conditions were further attested by visual inspection of nanoparticles

1   (Figure 4F). Given a wide signal point spread function along the Z-axis (depth), we

2   did not analyze capillaries because of their small diameter and high vessel curvature.

3   At 2 h post-injection, the internalized fractions were 55.9%, 48.0%, and 46.3% of

4   total nanoparticles at the endothelium for main pial venules, pial venules, and

5   ascending venules, respectively (Figure 4G). The corresponding adhering fraction of

6   nanoparticles was 24.0%, 30.9%, and 32.8%, while the unresolved fraction was

7   20.1%, 21.1%, and 20.8%. This indicates that, on average, at least half of the

8   nanoparticles recruited to vessel walls were internalized by endothelium. Noteworthy,

9   within the *unresolved* fraction, we detected nanoparticles with $\Delta p < -\sigma$, with

10   prevalences of 7.1%, 5.2%, and 3.8% for main pial venules, pial venules, and

11   ascending venules, respectively. These nanoparticles were located in proximity to the

12   basolateral membrane of BEC, and their prevalence increased with venule diameter.

13

14   **Directional movement of nanoparticles inside endothelium**

15   Once internalized, the nanoparticles exhibited a relatively high degree of movement

16   over short distances (Figure 5A, Movie S4). The nanoparticles were tracked at 2-3 h

17   post-injection with respect to large vessels, i.e. pial venules, and small vessels, i.e.

18   post-capillary venules with capillaries (Movie S5). We omitted ascending venules

19   because of their varying angled orientation to the imaging plane. From each analyzed

20   vessel, we selected 10 nanoparticles that remained in the imaging plane throughout 30

21   mins of continuous imaging (time interval between frames=30 s) (Figure 5B). The

22   traces were aligned to the point of origin (Figure 5C), and to avoid underestimation of

23   movement in planar *(x,y)* coordinates because of vessel curvature, we projected each

24   trace to a vector *(v)* aligned with a vessel symmetry axis and blood flow direction,

25   both independent from circumference curvature (Figure 5D).

26   The total distance traveled along the vessel axis did not differ between nanoparticles

27   in large venules, and post-capillary venules with capillaries (p=0.1672, Mann–

28   Whitney; Figure 5E). Similarly, the end-position of the nanoparticle relative to the

29   origin was the same for all vessel types (p=0.6694, Mann–Whitney)(Figure 5F). The

30   movement did not occur preferentially along or against the blood flow, as in all vessel

31   types, the median displacement relative to the blood flow direction approximated 0

32   (Figure 5G). Of note, nanoparticles exhibited motility even in capillaries with stalled

33   blood flow (Movie S6). Thus, neither cell morphology, nor differences in blood flow

1   velocities (Santisakultarm et al., 2012), nor blood flow direction influenced the

2   intracellular movement of the nanoparticles.

3   To determine whether intracellular trafficking of nanoparticles occurs by random

4   movement (e.g., diffusion) or is directional, we performed mean squared

5   displacement (MSD) analysis (see Methods). MSD analysis was performed along the

6   vessel axis v (Figure 5H) and MSD(t) significantly departed from linearity (p<0.001,

7   n=673 nanoparticles, Wald–Wolfowitz runs test). This indicates that the movement in

8   the endothelium contains a significant directional component. This suggested that the

9   nanoparticles might be directed towards specific cell regions, which we assessed in

10   the subsequent experiment.

11

12   **Nanoparticles distribute to endothelial cell perinuclear area in venules but not in**

13   **capillaries.**

14   Upon internalization, the nanoparticles localized over time to the perinuclear region

15   of endothelium cells in venules, but not in capillaries (Figures 5I and 5J). To quantify

16   the spatial distribution of nanoparticles in venules, we measured the distances from

17   the geometric center of the nucleus to the nanoparticles (Figure 5K). Our kernel

18   density map shows that the highest probability for finding a nanoparticle in venules

19   was in proximity to the nucleus (Figure 5L), at distances of 0.5–2.5 µm from the

20   nucleus boundary and with numbers decreasing at intermediate (2.5–5 µm) and distal

21   (>5 µm) regions (Figure 5M). Of note, neither vessel type exhibited clustering of

22   nanoparticles at the endothelium perimeter (Figure 5N). This indicated that

23   nanoparticles do not stall in the cytosol areas that contain dense cytoskeleton elements

24   that support cell contact sites, and that nanoparticles do not wedge between adjacent

25   endothelial cells.

26

27   **Nanoparticles are transcytosed to the brain, but only at venules.**

28   The possibility of large nanoparticle transcytosis across the BBB is disputed (Clark

29   and Davis, 2015; Lindqvist et al., 2016; van Rooy et al., 2011; Wiley et al., 2013).

30   Here, we detected nanoparticles present at the basolateral side of the endothelium in

31   both capillaries and venules (Figure 6A). This could result from lateral displacement

32   of nanoparticles, i.e. movement within endothelium along the vessel (Figure 6B).

33   However, in contrast to capillaries, nanoparticles in venules exhibited translocation

34   normal to the vessel lumen (Figure 6C). We observed that nanoparticles that

1 associated with the vessel wall slowed down, but once transcytosed, they rapidly

2 accelerated into the perivascular space (Figure 6D, Movie S7). This occurred only in

3 pial and post-capillary venules and not in capillaries, indicating that the vessel type

4 crucially determines successful transcytosis. Of note, we detected rare events in which

5 blood-borne cells that sequestered nanoparticles crossed the BEC in venules (Movie

6 S8), consistent with previous studies of immune-cell trafficking in the brain

7 (Engelhardt et al., 2017).

8 Continuous high-frequency time-lapse imaging required to capture transcytosis events

9 may potentially damage the BBB, leading to extravasation of the blood-borne

10 components to the brain (Choi et al., 2011). Given this possibility, along with the

11 rarity of transcytosis events and limited span of acute imaging experiments (~4 h), we

12 next performed long-term two-photon imaging on awake and mobile mice with

13 chronic cranial window implants (Figures 6E and 6F; see Methods). We imaged the

14 brains of awake Tie2-GFP mice 10-day post-surgery, to identify the cortical areas that

15 contained large venules (Figure 6G). We then reassessed the same loci 2 days later,

16 and subsequently, injected RI7-L-A550 into the bloodstream (50 μL 70

17 $nmol_{lipid}/g_{animal}$). Next, we imaged the animals again (30 min post-injection) to ensure

18 that the endothelium remained structurally intact and examined the same region at 1

19 and 2 days post-injection (Figure 6G). Already at 1 day post-injection, the animals

20 exhibited no significant presence of nanoparticles in the circulation, indicating

21 effective clearance of RI7-L-A550 from the bloodstream. However, at this timepoint

22 we detected substantial amounts of nanoparticles on the abluminal side of the

23 endothelium in the brain parenchyma surrounding venules. This fraction of

24 nanoparticles significantly decreased between 1 and 2 days post-injection but was still

25 abundant, indicating high retention (Figure 6G and H). In contrast to pial, ascending,

26 and post-capillary venules, nanoparticles failed to transcytose to the brain tissue close

27 to capillaries and the arterial branches of the microvasculature (Figure 6G). This

28 confirmed that transcytosis of nanoparticles across the BBB occurs at venules and not

29 at other segments of the brain microvasculature.

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31

32 **DISCUSSION**

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34 Understanding the pharmacokinetics of drug-carrying nanoparticles at the BBB *in*

1   *vivo* is crucial for future drug delivery strategies. For nearly two decades, drug

2   delivery studies have faced methodological challenges to determine how

3   nanoparticles may penetrate the BBB. Our current knowledge on nanoparticle-based

4   drug delivery across the BBB is deduced from chemically processed tissue and

5   biodistribution studies, or whole-brain imaging techniques (Cabezon et al., 2015;

6   Johnsen et al., 2019b; Pardridge, 2012). Moreover, the microanatomy of brain tissue

7   surrounding microvessels vastly differs among arterioles, capillaries, and venules

8   (Engelhardt et al., 2017), but how this heterogeneity of CNS vascular anatomy defines

9   distinct BBB compartments is unclear. Here, using two-photon microscopy, we

10   provide direct insight into nanoparticle delivery through all stages of endothelial

11   vesicular transport: binding, uptake, intracellular trafficking, and exocytosis in the

12   living brain. We report that clinically relevant formulations of TfR-targeted

13   nanoparticles rapidly associate with the BECs, with preferential distribution to

14   capillaries. Upon internalization, nanoparticles exhibit movement kinetics

15   independent of the vessel type, and with subcellular distribution profiles that differ

16   between venules and capillaries. Despite the highest density of nanoparticles in

17   capillaries, transcytosis across the BBB *in vivo* occurs only in venules (Figure 7), the

18   same vascular segment that is involved in immune-cell trafficking (Engelhardt et al.,

19   2017).

20   The association of nanoparticles to the endothelium rapidly declined from capillaries

21   to venules, inversely proportional to the vessel diameter and consistent with the TfR

22   expression profile (Ge et al., 2005; Jefferies et al., 1984). TfR is a recycling receptor

23   with ~10% of the total receptor pool present at the surface of BECs (van Gelder et al.,

24   1995). This amount was sufficient to maintain steady and ongoing recruitment of

25   TfR-targeted nanoparticles to the vessel wall without saturation of the receptor pool,

26   even at the high densities in capillaries. Although anti-TfR antibodies readily cross

27   the BBB (Johnsen et al., 2019b; Niewoehner et al., 2014; Yu et al., 2011), there is a

28   fundamental disagreement as to whether significantly larger TfR-targeted

29   nanoparticles actually enter the brain (Clark and Davis, 2015; Lindqvist et al., 2016;

30   van Rooy et al., 2011; Wiley et al., 2013). Here, we provide direct evidence of TfR-

31   mediated transcytosis of nanoparticles across the endothelial cells of the post-

32   capillary venules, and challenge the concept that nanoparticles undergo transport

33   across the BBB selectively at capillary endothelial cells.

1   The key question is why the highest density of associated nanoparticles in capillaries

2   did not translate to the vascular segment of transcytosis. The lack of nanoparticle

3   passage in capillaries can not be explained solely by impaired dissociation of

4   antibody-functionalized nanoparticle from TfR during exocytosis (Couch et al., 2013;

5   Yu et al., 2011), as transcytosis would also be absent at venules. Instead, we propose

6   that the microanatomy of the tissue surrounding microvessels determines successful

7   nanoparticle entry into the brain, specifically the size of the perivascular space and

8   organization of the basement membrane. Large and post-capillary venules are

9   surrounded by perivascular space located between the endothelium basement

10   membrane and astrocyte glia limitans, but in contrast to venules, capillaries are

11   devoid of perivascular space because both membranes are fused (Engelhardt et al.,

12   2017; Thomsen et al., 2017). This principal anatomical brain feature is present in both

13   murine and human brains (Zhang et al., 1990).

14   We propose that nanoparticles are more likely to transcytose via the route of the least

15   resistance, i.e. into the perivascular space of venules; instead of entering the more

16   restrictive compartment, i.e. the brain parenchyma at the capillary segment (Foley et

17   al., 2012; Wardlaw et al., 2020). By analogy, translocation of blood-borne leukocytes

18   across the BEC into the brain is also possible only at the microvascular segments

19   surrounded by perivascular space capable of accommodating the cells, i.e. at post-

20   capillary venules (Engelhardt et al., 2017). It also is unlikely that nanoparticles were

21   transcytosed at capillaries and subsequently relocated downstream along the capillary

22   wall into the perivascular space of venules due to the interstitial fluid flow. First, the

23   hydraulic resistance around the capillaries is too high for interstitial fluid flow

24   (Faghih and Sharp, 2018). Second, we detected no significant nanoparticle movement

25   following laser extravasation to the brain tissue surrounding capillaries, compared to

26   the rapid progression of nanoparticles extravasated from venules into the perivascular

27   space (Movie S9). Third, if transcytosis occurred in capillaries, a significant fraction

28   of nanoparticles of >100 nm diameter would likely become trapped in the protein

29   meshwork of fused capillary basement membranes (Cabezon et al., 2015; Muldoon et

30   al., 1999), but this we did not observe in chronic imaging experiments.

31   The major concern of the large nanoparticle-based drug delivery systems is the ability

32   of transcytosed nanovehicles to progress within the extracellular space (ECS). *In vivo*

1   diffusion experiments estimate the ECS to be in the range of 38–64 nm (Thorne and

2   Nicholson, 2006), although recent super-resolution imaging revealed ECS clefts of

3   ~100 nm (Tonnesen et al., 2018). These dimensions may not restrict the movement of,

4   e.g., antibodies, but may exclude significantly larger nanoparticles from entry and

5   progression within the ECS (Nance et al., 2012; Thorne and Nicholson, 2006). Here,

6   most nanoparticles were detected in perivascular areas, even after 2 days post-

7   injection, which indicates high perivascular retention. However, we also observed

8   nanoparticles at distances from post-capillary venules corresponding to the location of

9   neuropil, indicating successful passage into the ECS. How ~130-nm nanoparticles

10   might travel within the ECS is unclear but appears to be highly dependent on the level

11   of PEGylation (Nance et al., 2012). In the murine brain, neurons are located on

12   average within short (~15 μm) distances from brain capillaries (Tsai et al., 2009).

13   Although TfR may potentially act as an intraparenchymal target for neurons (Chen

14   and Liu, 2012; Farias et al., 2015), we observed no cellular association patterns of the

15   nanoparticles suggestive of binding to TfRs, which are enriched at the neuronal

16   somata (Farias et al., 2015).

17   In contrast to microvessels embedded in the brain parenchyma, the vasculature in the

18   dura lacks the BBB (Engelhardt et al., 2017; Weller et al., 2018). Contrary to a recent

19   study (Lam et al., 2018), we detected no TfR-targeted nanoparticles in the dura.

20   However, we did observe blood-borne immune cells that infiltrated the brain, carrying

21   along nanoparticles that were hijacked from the bloodstream. Thus, nanoparticles

22   could also enter the CNS in a manner independent of vesicular transcytosis. This

23   phenomenon, in a similar manner to transcytosis, also occurred exclusively in

24   venules. The leakage of large blood-borne macromolecules can reportedly occur via

25   wedging of leukocytes between endothelial cells in venules but not in capillaries

26   (Claudio et al., 1990). However, this phenomenon is associated with severe

27   autoimmune reactions, and we did not detect the clustering of nanoparticles at BEC

28   contact sites.

29   An important consideration is to what extent brain microanatomy might influence

30   nanoparticle transcytosis compared to other BEC features along the vascular

31   segments. First, colloidal glycocalyx at the BEC luminal side may potentially bury

32   macromolecules within its matrix or repel negatively charged nanoconstructs,

33   impairing endocytosis (Cheng et al., 2016; Gromnicova et al., 2016). Indeed, we

1   detected that the internalization of nanoparticles was not an all-or-nothing

2   phenomenon, and ~30-40% total nanoparticles recruited from the blood circulation

3   were restricted to the luminal side of BEC. Although enzymatic shedding of

4   glycocalyx does not improve BEC uptake of positively charged nanoparticles

5   (Gromnicova et al., 2016), we cannot exclude that glycocalyx might affect slightly

6   negatively charged particles (Kutuzov et al., 2018), and in a vessel-type–dependent

7   manner.

8   Second, our data show that after internalization, the nanoparticles exhibited similar

9   movement dynamics regardless of vessel type and consistent with cytoskeleton-

10   assisted transcytosis (Soldati and Schliwa, 2006). However, in contrast to capillaries,

11   post-capillary and pial venules exhibited the preferential distribution of nanoparticles

12   to perinuclear areas, which corresponds to the position of late endosomes in the

13   transcytotic pathway that contain components directed for degradation (De Bock et

14   al., 2016; Grant and Donaldson, 2009). Although this suggests a more restrictive

15   intracellular barrier for the trafficking of nanoparticles in venules than in capillaries, it

16   supports the notion that despite segmental differences in RMT, it is ultimately the

17   microanatomy of the brain surrounding microvessels that plays a decisive role for

18   successful transcytosis.

19

20   Lastly, the vessel-dependent manner in which the brain handles TfR-targeted

21   nanoparticles may potentially explain conflicting reports on the delivery of other TfR-

22   targeted entities, such as antibodies, where the robust ability to penetrate the BBB is

23   often opposed by evidence for the same type of entity indefinitely trapped within the

24   capillaries (Friden et al., 1991; Moos and Morgan, 2001). Our findings may be of

25   relevance for other than TfR-targeting approaches and potentially explain why other

26   targeting ligands e.g. COG133, angiopep-2, or CRM197 that performed successfully

27   in capillaries *in vitro*, failed to enhance nanoliposome uptake *in vivo* (van Rooy et al.,

28   2011). Our methodological platform is first to describe the nanoparticle fate in the

29   living brain at nanoscale resolution in live, and also, awake animals. It is suitable for

30   examining the potential effects of disease states on large molecule therapeutics

31   delivery, e.g. during stroke-induced imbalance in transcytosis (Knowland et al.,

32   2014), reduction of perivascular spaces (Mestre et al., 2018), or ECS changes in

33   edema or during brain activity (Tonnesen et al., 2018). Our findings may also help to

34   avoid pitfalls in design of drug delivery systems, e.g. in Alzheimer disease, where Aβ

1   is deposited along pericapillary and periarteriolar spaces (Tarasoff-Conway et al.,

2   2015), rather than in venular perivascular space, which is the locus of nanoparticle

3   transcytosis.

4   In summary, we reveal the transcytosis pathway across the BBB of targeted clinically

5   relevant nanoparticles. Our identification of post-capillary venules as the key site for

6   transcytosis may aid efforts to develop efficient therapeutic approaches for drug

7   delivery to the CNS. As an accessible part of the vascular tree, post-capillary venules

8   may be poised to serve as a preferred route for macromolecule and nanoparticle

9   delivery across the BBB.

10

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12

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20

21   **AUTHOR CONTRIBUTIONS (CRediT taxonomy).**

22   Conceptualization, Kr. Ku., Ka. Kr. and M. J. L.; Methodology, Kr. Ku. and Ka. Kr.;

23   Formal Analysis, Kr. Ku.; Investigation, Kr. Ku., Ka. Kr., K.B.J., M. A. L., and M.

24   L.; Resources - T.M., T. L. A. and M. J. L.; Writing – Original Draft, Kr. Ku.; and M.

25   J. L.; Writing – Review & Editing, Kr. Ku., Ka. Kr., K.B.J., T.M., and M.J.L.;

26   Visualization, Kr. Ku.; Funding Acquisition, T.M., T. L. A. and M. J. L.; Supervision

27   M. J. L.

28

29

30   **DECLARATION OF INTERESTS**

31

32   All authors declare no conflict of interest.

33

1

Methods

3

Animals

All animal experiments were approved by the Danish National Ethics Committee and followed ARRIVE guidelines. We used wild-type C57Bl/6 mice, age 5–7 months (23–31 g) and age-matched (25–32 g) homozygous Tg(TIE2GFP)287Sato/J transgenic reporter mice (Tie2-GFP mice, #003658, The Jackson Laboratory) expressing GFP under the endothelial-specific receptor tyrosine kinase (Tie2) promoter (Motoike et al., 2000).

11

Animal preparation for acute imaging

Surgery was performed as previously described, with minor modifications (Kucharz and Lauritzen, 2018). Briefly, animals were anesthetized by intraperitoneal (i.p.) injection of xylazine (10 µg/g_{animal}) and ketamine (60 µg/g, then 30 µg/g_{animal}, at 20–25 min intervals). A tracheotomy was performed for mechanical respiration (180–220 µL volume at 190–240 strokes/min; MiniVent Type 845, Harvard Apparatus) with O_2-supplemented air (1.5–2 mL/min). Two catheters were inserted, one into the left femoral artery for injection of compounds and nanoparticles, and for monitoring mean arterial blood pressure (MABP; Pressure Monitor BP-1, World Precision Instruments), and the other into the femoral vein for anesthesia infusion during imaging. The animal was turned to the prone position, the scalp was removed, the periosteum was removed with a $FeCl_3$-soaked cotton bud, and the exposed skull was glued (Loctite Adhesives) to a custom-made metal head plate. A craniotomy was performed over the right somatosensory cortex (3 mm lateral, 0.5 mm posterior to bregma; Ø=4 mm; 4500 rpm dental drill). The bone flap was carefully lifted, the dura removed, and 1% agarose (type III-A, Sigma-Aldrich) in artificial cerebrospinal fluid (aCSF; in mM: NaCl 120, KCl 2.8, Na_2HPO_4 1, $MgCl_2$ 0.876, $NaHCO_3$ 22, $CaCl_2$ 1.45, glucose 2.55, pH=7.4) was applied on the brain surface. An imaging coverslip (~4×4 mm, 0.08-mm thick; Menzel-Gläser) was positioned onto the craniotomy, leaving a ~0.5-mm gap for glass microelectrode insertion. The animal was transferred to the imaging stage, and the anesthesia was changed to continuous infusion of α-chloralose (50 mg/kg BW per hour) via an intravenous catheter.

1   Mice were allowed to rest for 25 min before the imaging procedures. Prior to

2   imaging, a ~50 µL blood sample was collected via the arterial catheter for blood gas

3   evaluation (ABL, Radiometer), and the respiration rate and volume were adjusted if

4   necessary. To ensure physiological conditions, we monitored end-tidal CO_2 levels and

5   MABP, and body temperature was maintained at 37°C using a rectal thermistor-

6   regulated heating pad.

7

8   **Animal preparation for awake/chronic awake imaging**

9   The surgery was performed as previously described, with minor modifications

10   (Holtmaat et al., 2009). Briefly, 4 h prior to the surgery, the animals were injected

11   with dexamethasone (4.8 mg/g BW; Dexavit, Vital Pharma Nordic). The anesthesia

12   was induced with 5% isoflurane (ScanVet) in O_2-supplemented air (10%). Eyes were

13   lubricated with eye ointment (Viscotears, Novartis), and the animal's head was

14   shaved and mounted onto a stereotactic frame. The body temperature was maintained

15   during all steps of the surgery at 37°C using a rectal thermistor-regulated heating pad.

16   Surgery was performed in an aseptic environment with heat-sterilized surgical tools.

17   The shaved skin was disinfected with chlorhexidine/alcohol (0.5%/74%; Kruuse).

18   Next, carprofen (5 mg/kg BW; Norodyl, Norbrook), buprenorphine (0.05 mg/kg BW;

19   Temgesic, Indivior), and lidocaine (100 µL 0.5%) were subcutaneously injected under

20   the scalp. The anesthesia was reduced to 1.8%–2.0% isoflurane, the scalp was

21   removed, and the bone surface was cleaned from the periosteum with an ultrasonic

22   drill (Piezosurgery, Mectron). A craniotomy was performed over the right

23   somatosensory cortex (2 mm lateral, 0.5 mm posterior to bregma; Ø=3 mm), the bone

24   flap was carefully lifted, and the exposed brain temporarily covered with a hemostatic

25   absorbable gelatin sponge (Spongostan®, Ferrosan, Denmark) pre-wetted with ice-

26   cold aCSF. The cranial opening was filled with aCSF, then sealed with an autoclave-

27   sterilized round imaging coverslip (Ø=4 mm, 0.17-mm thick; Laser Micromachining

28   LTD). The rim of the coverslip was secured with a thin layer of Vetbond (3M), and a

29   lightweight stainless steel head plate (Neurotar) was positioned on the top of the skull

30   in alignment with the cranial window. The skull was coated with adhesive resin

31   cement (RelyX Ultimate, 3M) to secure the exposed bone, including skin incision rim,

32   and to firmly attach the metal plate to the head. Next, the animals were transferred

33   onto a pre-warmed heating pad to wake from anesthesia (~5 min) in a cage

34   supplemented with pre-wetted food pellets for easy chow and hydration.

1   Post-operation care consisted of subcutaneous injections of Temgesic (3 h) and

2   Rimadyl (24 and 48 h post-surgery, doses as before). Animal welfare was closely

3   monitored during the 7 days of post-surgical recovery and subsequent imaging

4   training procedures. All animals underwent recurrent 30-min/day training sessions

5   before the imaging to gradually habituate to the mobile cage system (Neurotar) with

6   sugar-supplemented water as a reward (~14 days training). Given that no catheters

7   were mounted in chronically imaged animals, the nanoparticles were injected

8   retroorbitally 10 days post-surgery during brief (~2 min) isoflurane anesthesia (5%).

9   This administration route was preferential to, e.g., tail vein injections, because it

10 provided better control over the injectant volume. The imaging sessions never

11 exceeded 45 min.

12

13 **Nanoparticle preparation**

14 1,2-Distearoyl-*sn*-glycero-3-phosphocholine   (DSPC),   ovine   cholesterol,   1,2-

15 distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene   glycol)-

16 2000]   ammonium   salt   (DSPE-PEG2k),   and   1,2-distearoyl-*sn*-glycero-3-

17 phosphoethanolamine-N-[maleimide(polyethylene   glycol)-2000]   ammonium   salt

18 (DSPE-PEG2k-maleimide) were purchased from Avanti Polar Lipids (Alabaster, AL,

19 USA). The stealth nanoparticles were prepared to consist of DSPC/cholesterol/DSPE-

20 PEG2k   (molar   composition: 56.3:38.2:5.5),   and   the   antibody-functionalized

21 nanoparticles to consist of DSPC/cholesterol/DSPE-PEG2k/DSPE-PEG2k-maleimide

22 (molar   composition: 56.3:38.2:5:0.5).   The   fluorescent   nanoparticles   were

23 supplemented with 0.5 mol% of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine

24 labeled with Atto488 (Atto488 DPPE) or Atto550 (Atto550 DPPE; Atto-Tec, Siegen,

25 Germany). To obtain lipid powder mixtures of the above compositions, the

26 constituent lipids were dissolved in tert-butanol (Sigma-Aldrich, St. Louis, MO,

27 USA)/Milli-Q water solution (9:1) and heated to 40–50°C to ensure complete

28 dissolution. The lipid solutions were then plunge-frozen in liquid N_2 and lyophilized

29 overnight to remove the solvent (ScanVac CoolSafe lyophilizer, LaboGene, Allerød,

30 Denmark).

31

32 **Nanoparticle fluorescent tagging**

33

1   To obtain fluorescently labeled nanoparticles, the lyophilized lipids were hydrated in

2   70°C phosphate-buffered saline (PBS; 10 mM phosphate, 137 mM sodium chloride,

3   2.7 mM KCl, pH 7.4; tablets from Sigma-Aldrich) to a 50 mM lipid concentration.

4   The lipid suspensions were vortexed seven times in 5-min intervals, then subjected to

5   five freeze-thaw cycles by alternate placement in a liquid N_2 and a 70°C water bath.

6   Next, the lipid suspensions were extruded 21 times through a 100-nm polycarbonate

7   filter (Whatman, GE Healthcare) at 70°C using a mini-extruder (Avanti Polar Lipids)

8   to form nanoparticles.

9

10   **Nanoparticle targeting**

11   We used a high-affinity (K_D=6 nM) monoclonal anti-TfR antibody clone RI7217 to

12   functionalize nanoparticles (Johnsen et al., 2018). The antibody was produced in-

13   house using the hybridoma technique at Laboratory for Neurobiology, Aalborg

14   University, Denmark. The antibody specificity was previously determined using

15   surface plasmon resonance (Johnsen et al., 2018). To functionalize the nanoparticles

16   with either RI7217 or a rat isotype IgG control (Thermo Fisher Scientific, Waltham,

17   MA, USA), we prepared solutions of 8 mg/mL antibody in borate buffer (100 mM

18   borate, 2 mM EDTA, pH 8.0; all Sigma-Aldrich). The antibody concentrations were

19   determined from the absorbance at 280 nm (NanoDrop 2000c spectrophotometer,

20   NanoDrop Products, Thermo Fisher Scientific) using mass extinction coefficients of

21   1.3 $(mg/mL)^{-1}$ cm^{-1} and 1.5 $(mg/mL)^{-1}$ cm^{-1} for RI7217 and IgG isotype, respectively,

22   determined in a separate micro-BCA experiment. Traut's reagent (Thermo Fisher

23   Scientific) was added to a reagent-to-antibody molar ratio 10:1 in Protein LoBind

24   tubes (Eppendorf, Hamburg, Germany), and the solutions were incubated for 1 h at

25   room temperature under constant shaking at 500 rpm. Using Amicon Ultra-4 30 kDa

26   centrifugal filter units (Merck, Darmstadt, Germany), we transferred the thiolated

27   antibodies to PBS and determined their concentration using the NanoDrop 2000c as

28   described above. Next, we added 1.05 mg of newly prepared thiolated antibodies to

29   700 µL of newly prepared nanoparticles (lipid concentration ~35–40 mM) in Protein

30   LoBind tubes and replaced the air phase in the tubes with N_2. The samples were then

31   incubated for 24 h at room temperature under constant shaking at 500 rpm, allowing

32   the thiolated antibodies to couple to the maleimide groups the surface of the

33   nanoparticles. The antibody-functionalized nanoparticles were separated from

34   unbound antibodies using a Sepharose CL-4B (GE Healthcare, Little Chalfont, UK)

1   size-exclusion chromatography column eluted with PBS (dimensions, 1.5×20 cm;

2   flow rate, 1 mL/min). The recovered nanoparticles were pooled in Amicon Ultra-4

3   100 kDa centrifugal filter units (Merck) and concentrated by centrifuging at 2000 ×g

4   until the lipid concentration was increased to 30–40 mM.

5

6   **Nanoparticle cisplatin loading, targeting and detection.**

7   To prepare cisplatin-loaded nanoparticles, *cis*-diammineplatinum(II) dichloride

8   (cisplatin; Sigma-Aldrich) was dissolved in PBS to a nominal concentration of 8.5

9   mg/mL (Peleg-Shulman et al., 2001). The solutions were magnetically stirred for 1 h

10   at 70°C and subsequently left at room temperature for 15 min, allowing undissolved

11   cisplatin crystals to precipitate. The supernatants were transferred to new vials and

12   magnetically stirred while being heated to 70°C. The solutions were then added to

13   lyophilized lipids, resulting in 50 mM lipid suspensions that were magnetically stirred

14   for 1 h at 70°C and extruded as described above for the fluorescently labeled

15   nanoparticles. The samples were cooled to room temperature to allow any residual

16   cisplatin crystals to precipitate, and the supernatants were run on a Sepharose CL-4B

17   size-exclusion chromatography column eluted with PBS (dimensions 1.5×20 cm, flow

18   rate 1 mL/min) to remove free cisplatin. The recovered nanoparticles were

19   concentrated using Amicon Ultra-4 100 kDa centrifugal filter units by centrifuging at

20   2000 ×g.

21   To prepare antibody-functionalized cisplatin-loaded nanoparticles, the antibodies

22   were thiolated as described above. Then, 0.5 mg of newly prepared thiolated antibody

23   was added to 700 µL of newly prepared cisplatin-loaded nanoparticles (lipid

24   concentration ~13 mM) in a Protein LoBind tube. The nanoparticles were finally

25   incubated, recovered, and concentrated as described above for the fluorescently

26   labeled antibody-functionalized nanoparticles.

27   Brain uptake of cisplatin was measured using ICP-MS as recently described (Johnsen

28   et al., 2019a).

29

30   **Nanoparticle properties *in vitro***

31   The phosphorus concentrations of the liposome stock solutions were determined using

32   inductively coupled plasma mass spectrometry (iCAP Q ICP-MS, Thermo Fisher

33   Scientific). We estimated phospholipid concentrations by subtracting the phosphorus

34   background of the PBS buffer and estimated total lipid concentrations by dividing the

1   phospholipid concentrations by 0.618, taking into account that cholesterol does not

2   contain phosphorus. For the cisplatin-loaded nanoparticles, we also used ICP-MS to

3   determine the platinum concentrations. The size of the nanoparticles (dissolved in

4   PBS) was measured using dynamic light scattering, and the zeta potential of the

5   nanoparticles in phosphate-glucose buffer (10 mM phosphate, 280 mM glucose, pH

6   7.4; reagents from Sigma-Aldrich) was measured using mixed measurement mode

7   phase analysis light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern,

8   UK). The antibody conjugation level on the functionalized nanoparticle was

9   determined using the micro-BCA assay (reagents purchased from Thermo Fisher

10 Scientific), performed by incubating samples (including bovine serum albumin [BSA]

11 standard samples) for 1 h in a 60°C water bath and then transferring them to a 96-well

12 plate to measure their absorbance at 562 nm using a Spark multimode microplate

13 reader (Tecan, Männedorf, Switzerland). To account for the small contribution of

14 lipids in the micro-BCA assay (Kessler and Fanestil, 1986; Kristensen et al., 2019),

15 we also performed the micro-BCA on non-functionalized nanoparticles, which

16 allowed for the subtraction of the lipid contribution to determine the amount of

17 antibody conjugated to the nanoparticles. The hydrodynamic diameter (D_h) of RI7-

18 functionalized Atto 550-tagged (RI7-L-A550) and Atto 488-tagged (RI7-L-A488)

19 nanoparticles was in the range of d_h=~135–140 nm (Table S1) and comparable to

20 other TfR-targeted clinically relevant formulations (Johnsen and Moos, 2016; Lam et

21 al., 2018). Both RI7-L-A550 and RI7-L-A488 had a low polydispersity index (≤0.13),

22 indicating high size homogeneity. Assuming the nanoparticles contained on average

23 ~2.5×10^5 lipids, the conjugation level of 30 g/mol$_{lipid}$ corresponded to ~50 antibodies

24 per nanoparticle (Kristensen et al., 2019).

25

26 **Nanoparticle systemic interactions *in vivo***

27 In contrast to other TfR ligands, RI7217 does not compete with endogenous

28 transferrin, and TfR vascular expression is highly specific to the brain (van Rooy et

29 al., 2011). We observed no pathological changes in exhaled CO_2 levels, mean arterial

30 blood pressure MABP, or brain activity after nanoparticle administration in acute

31 experiments (Figure S1). Furthermore, we detected no atypical behavior, weight loss,

32 or signs of neuroinflammation in animals monitored up to 48 h post-injection in

33 chronic imaging experiments.

1

Other fluorescent probes

FITC-dextran (MW 10 kDa, 0.5%, Sigma-Aldrich), TRITC-dextran (MW 65 kDa, 1%, Sigma-Aldrich), or bovine serum albumin Alexa Fluor 488-conjugate (BSA-Alexa 488, 1%, Invitrogen) was administered as a single bolus injection (50 µL) via a femoral arterial catheter. All were dissolved in sterile saline and administered subsequently to nanoparticles. In addition to delineating a vessel lumen, lack of extravascular leak of dyes indicated preserved BBB structural integrity after the microsurgery.

Imaging setup

In vivo two-photon imaging was performed with a SP5 upright laser scanning microscope (Leica Microsystems) coupled to MaiTai Ti:Sapphire laser (Spectra-Physics). The images were collected using a 20× 1.0 water-immersion objective. The fluorescence signal was split by FITC/TRITC filter and collected by two separate multi-alkali photomultipliers after 525–560 nm and 560–625 nm bandpass filter (Leica Microsystems). The fluorophores were excited at 870 nm with the 14 mWatt output power at the sample. The images were collected using LAS AF v. 4.4 (Leica Microsystems) in 16-bit color depth and exported to ImageJ for further analysis (v. 1.52a; NIH). 3D reconstructions were performed via volume rendering in Amira v. 6 (FEI Visualization Sciences Group).

Surface density calculation

To assess the spatio-temporal properties of nanoparticles association to the endothelium, we monitored the association of nanoparticles for 2 h after injection with respect to all cerebral vessel types using hyperstack (4-dimensional) imaging. Data were recorded as a series of Z-stacks in bidirectional scanning mode with triple frame averaging, from 387.5 µm×387.5 µm area (2048×2048 pixel resolution) and 144 µm depth span (Z-step size 2.50 µm) with 7.5-min intervals between consecutive Z-stacks. The cerebral microvessels were classified as pial arterioles or venules, penetrating arterioles, post-capillary venules, ascending venules, or capillaries based on their morphology and second harmonics generation (Grubb et al., 2020; Janiurek et al., 2019). The nanoparticles were counted from all vessels in the field of view,

1   with each individual vessel followed over time. The vessel surface area was

2   calculated from vessel diameter delineated by FITC-dx or TRITC-dx signal and the

3   length of the vessel measured in 3D. The association density was obtained from a

4   nanoparticle count per corresponding vessel surface area [nanoparticles/μm^2].

5

6   **Subcellular distribution mapping**

7   We imaged the surface of the vessels, i.e., a ~5 μm planar optical section aligned

8   with the vessel circumference. When measuring distances from the nucleus

9   geometric center to nanoparticles, we set the distance cut-off point to 11 μm to

10   exclude the nanoparticles that belonged to neighboring endothelial cells and to avoid

11   distribution bias due to the non-concentric spindle-like geometry of endothelial cells.

12   In addition, we excluded nanoparticles located in line from the geometric center

13   towards the vessel wall, where the cut-off distance exceeded the vessel boundary.

14   We took this step to minimize the effect of the vessel curvature on the estimation of

15   the distance.

16

17   **MSD analysis**

18   To characterize nanoparticle movement dynamics, we used the mean square

19   displacement (MSD) analysis (Weimann et al., 2013). Time-lapse recordings were

20   collected in bidirectional scanning mode from 387.5 μm×387.5 μm area (2048×2048

21   pixel resolution) for 30 min at 30-s intervals (60 data timepoints). Each nanoparticle

22   trajectory was manually traced, treating the fluorescence radial symmetry center as a

23   nanoparticle location coordinate. The planar (x,y) trajectories were projected to a

24   vector *(v)* aligned with a vessel symmetry axis and with the direction of the blood

25   flow. For every trajectory, the displacements in *v* coordinate in time *t* were extracted

26   for each multiplier of the smallest resolved time interval *d* (i.e., *t=d*, *t=2d*,

27   *t=3d…t=i*d*), where *i*=60 timepoints and *d*=30 s. In simple model systems, the

28   significant deviation of MSD(t) from linearity with the increase of *t* indicates a non-

29   stochastic (directional) movement component (Weimann et al., 2013). We assessed

30   MSDv(*t*) linearity with the least squares linear regression fit weighted by the inverse

31   of data point variance (Michalet, 2010), followed by Wald–Wolfowitz runs test.

32

33   **Electrophysiological recordings**

1 Electrocortical brain activity (ECoG) was recorded via a heat-pulled borosilicate glass
2 electrode containing an Ag/AgCl filament and filled with aCSF (electrode tip Ø, 2–3
3 μm; inner Ø, 0.86 mm; outer Ø, 1.5 mm; Sutter Instrument; resistance 1.5–2.0 MΩ).
4 The electrode was inserted under the glass coverslip ~50 μm into the cerebral cortex,
5 and the reference electrode was positioned in the neck muscle. The total electrical
6 signal was filtered (3000 Hz low-pass filter), then amplified 10× (AP311 analog
7 amplifier; Warner Instruments), and the alternate current-ECoG component (i.e.,
8 spontaneous brain activity) was obtained after further 100× amplification and 0.5 Hz
9 high-pass filter (NL 106 analog amplifier and NL 125/126 analog filter, NeuroLog).
10 Analog data were digitized (Power 1401, CED) at 20 kHz. For the exhaled CO_2,
11 MABP (the raw readout) was collected. All data were recorded in Spike2 software (v.
12 7.02a; CED).
13

14 **Statistical analysis**
15 Following Pearson's normality test, either an unpaired two-tailed Student's t-test
16 with Welch's correction (normally distributed data) or Mann–Whitney test (non-
17 normally distributed data) was used. All statistical analyses were performed in Prism
18 v.8.2 (GraphPad). The sample size was selected based on our previous two-photon *in*
19 *vivo* experiments (Kucharz and Lauritzen, 2018). Data was plotted in Prism v.8.2. or
20 in OriginPro 2018 (OriginLab Corporation).
21

22 **Exclusion criteria**
23 Prior to injection of nanoparticles, all animals with abnormal blood pressure (<50
24 mmHg), abnormal brain ECoG activity, or significant (>2 μm/min) brain movement
25 in *x*, *y*, or *z* coordinates were excluded from analysis (3 of 48 animals).

26
27
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15

16

17   **FIGURE LEGENDS**

18

19   **Figure 1. Two-photon imaging of liposome nanoparticles *in vivo*.**

20   **A)** Mouse after the preparative surgery for acute imaging.

21   **B)** Features of an acute cranial window.

22   **C)** Principal components of targeted nanoparticles used in the study.

23   **D)** RI7217-functionalized nanoparticles drive accumulation of cisplatin payload

24   in the brain, in contrast to nanoparticles functionalized with isotype IgGs or

25   devoid of antibody functionalization (stealth liposomes). %ID/g is percentage

26   of injected dose of encapsulated cisplatin per gram tissue; *n*=4 mice.

27   **E)** 3D reconstruction of the brain microvasculature delineated by circulating

28   nanoparticles.

29   **F)** Time-lapse images of blood-circulating nanoparticles following injection into

30   the bloodstream. Long panels are kymographs of fluorescence at demarked

31   lines. See also Movie S1.

32   **G)** Upper panel: nanoparticle fluorescence signal over time. Lower panel:

33   fluorescence signal from RI7-L-A550 and RI7-L-A488 nanoparticles after 2 h

34   of continuous imaging matches the stability of FITC-dx and outperforms

1   TRITC-dx, commonly used in imaging studies (ANOVA). *(n)*=number of

2   mice

3   **H)** Laser-extravasated nanoparticles into brain parenchyma after 3 h after

4   injection into the circulation. Nanoparticles retained their discrete and

5   homogeneous appearance.

6   **I)** Laser-extravasated RI7-L-A488 and RI7-LA550 into the brain parenchyma 3

7   h after injection into the circulatiion. No liposome fusion or aggregation

8   occured when present in circulation or in the brain.

9   **J)** Average of fluorescence profile plots of nanoparticles laser-extravasated to the

10   brain 3 h after injection into the circulation. The average standard deviation

11   (σ) of signal distribution did not differ between distinctively labeled

12   nanoparticles. *(n)*=number of nanoparticles.

13 *p<0.05; ***p<0.001, *ns*=non-significant. Data are means±SEM.

14

15 **Figure 2. Robust association of targeted nanoparticles to capillaries and post-**

16 **capillary venules.**

17   **A–E)** *In vivo* images of RI7-L-A550 nanoparticles (red) *in vivo* 3 h after injection

18   to the circulation. Co-injected circulating FITC-dx delineates vessel lumen

19   (green). Liposomes associate with vessel walls at pial, ascending venules,

20   post-capillary venules, and capillaries.

21   **F)** No association of liposomes to an arterial branch of the brain

22   microvasculature.

23   **G)** Co-injection of both RI7-L-A550 and RI7-L-A488 revealed discrete punctae

24   of both nanoparticles at the vessel walls.

25   **H)** Time-lapse images of laser-extravasated nanoparticles in brain parenchyma.

26   The proximity of nanoparticles causes overlapping fluorescence signal.

27 *pV*=pial venule; *ascV*=ascending venule*; pcV*=post-capillary venule; *cap*=capillaries;

28 *penA*=penetrating arteriole; *pA*=pial arteriole.

29

30 **Figure 3. Nanoparticle association density and kinetics are determined by vessel**

31 **type.**

32   **A)** The difference between RI7-L-A550 (red) and FITC-dx (green, vessel lumen)

33   fluorescence signal used to show the fraction of non-circulating nanoparticles

34   that contour vessel boundaries (RI7-L-A550nc, cyan).

B) 3D reconstruction of the nanoparticles associatiod to endothelium 2 h post-injection. The recuruited (non-circulating) nanoparticles (RI7-L-A550nc, cyan) are superimposed on the signal from liposomes circulating in the bloodstream (gray).

C) Upper panel: brain microvasculature following co-injection of RI7-L-A550 nanoparticles (red) and FITC-dx (vessel lumen, green) into circulation. Squares indicate areas magnified in lower panels. Lower panels: association of nanoparticles over time. See also Movie S3.

D) Nanoparticle distribution 2 h post-injection. Each data point is a single vessel. Blue areas indicate vessel segments. Dashed line demarks lognormal distribution trendline. Inset illustrates vessel classification.

E) Nanoparticles associated most rapidly with capillaries and most slowly with large venules. Surface density=# of nanoparticles per μm^2 vessel wall area.

pV=pial venule; *ascV*=ascending venule*; pcV*=post-capillary venule; *cap*=capillaries; *pcA=pre-capillary arteriole; penA*=penetrating arteriole; *pA*=pial arteriole. Data are means±SEM.

Figure 4. Prevalence of internalized nanoparticles in the brain vasculature.

A) Brain endothelium *in vivo* imaged in Tie2-GFP mice.

B) Detailed morphology of endothelial cells *in vivo* with noticeable cell boundaries and bright nuclei.

C) Simultaneous imaging of the brain endothelium and nanoparticles 2 h after RI7-L-A550 injection into circulation.

D) Pial venule with a clear presence of nanoparticles. Boxes 1–4 represent magnified areas on the right panels. #5 is the magnified area presented in E).

E) Upper panel: nanoparticles (red) at two distinct locations (*I., II.*) in relation to the endothelium (Tie2-GFP, green). Dashed lines indicate the axes of fluorescence profiles in the lower panel. Lower panel: fluorescence profiles of a nanoparticles with low (*I.)* and high signal overlap (*II*). Δp demarks signal peaks separation.

F) Examples of internalized (|Δp|<σ), unresolved (σ<|Δp|<2σ), and adhering (|Δp|>2σ) nanoparticles. σ=0.29 µm. Scale bars are provided in both σ and µm. Numbers at arrowheads show Δp value in σ units.

1   **G)** Histogram of percentage distribution of internalized (*int.*)*,* unresolved (*unr.*),

2   and adhering (*adh.*) liposomes. Inset illustrates vessel type division. Values

3   over horizontal lines are the sum of underneath bins. *n*=number of liposomes,

4   values in brackets are the number of mice. Histogram bin=σ=0.29 µm.

5 Image insets denote the position of imaging plane relative to a vessel perimeter.

6 *mV=main pial venule; pV=*pial venule; *ascV=*ascending venule*; pcV=post-capillary*

7 *venule; cap=*capillaries; *penA=*penetrating arteriole; *pA=*pial arteriole.

8

9 **Figure 5. Nanoparticles exhibit directional movement in all vessel types, but**

10 **distinct subcellular distribution.**

11   **A)** Time-lapse images of nanoparticle movement in large venules. *Left panel:*

12   vessel surface scan. *Right panel:* vessel cross-section scan. Arrowheads

13   indicate the movement of a nanoparticle. See also Movie S4

14   **B)** Tracking of nanoparticles. *Left panel:* circles outline traced nanoparticles.

15   *Middle panel:* movement traces during 30 min of continuous time-lapse

16   imaging. *Right panel:* isolated movement traces (black) with contours

17   delineating the microvessel (gray). See also Movie S5

18   **C)** Movement traces in *x,y* (Cartesian) coordinate system with respect to a vessel

19   type. (0,0) corresponds to the initial position of a nanoparticle.

20   **D)** Translation of the movement in *x,y* coordinates into the movement relative to

21   the blood flow direction *v*. *v>*0 indicates the movement along the blood flow,

22   and *v<*0 is against the blood flow direction.

23   **E-F)** Vessel type does not influence the total distance traveled and the

24   displacement (end-position) of a nanoparticle. Time span=30 min. *n*=number

25   of nanoparticles, values in brackets are the number of mice.

26   **G)** Blood flow direction does not affect the direction of movement of

27   nanoparticles in large (pial venules) and small microvessels (post-capillary

28   venules and capillaries).

29   **H)** MSD analysis showing significant deviation of MSD(t) from linearity (Wald–

30   Wolfowitz runs test). Solid line shows least squares linear regression fit.

31   **I)** Nanoparticles preferentially distributed to perinuclear areas of venules.

32   **J)** No preferential distribution in capillaries.

K) Calculation of nanoparticles location (bold solid lines) in relation to nucleus perimeter (solid edges). Distance lines meet at the geometric center of a nucleus (dashed lines).

L) Density map of nanoparticle distribution in relation to the geometric center of the nucleus. Kernel=2σ. The heat-map represents the probability of liposome presence at a given coordinate.

M) Nanoparticle distribution in relation to nucleus boundary (bin=0.5 µm). Numbers over horizontal lines are the sum of underneath bins. Empty bars are liposomes overlapping with nucleus signal *(non-classified)*. *n*=number of liposomes; number in brackets indicates number of animals.

N) Nanoparticles do not distribute to the endothelial cells perimeter and contact sites. Dashed lines indicate cell boundaries.

Insets in images denote the position of imaging plane relative to a vessel perimeter. *pV*=pial venule; *pcV*=post-capillary venule; *cap*=capillary; *nuc*=nucleus; *MSDv*=mean squared displacement in *v* coordinate. E–G) Data are medians with interquartile ranges (IQR). H) Data are means±SEM.

Figure 6. Post-capillary venules is the locus for TfR-mediated transcytosis.

A) Internalized nanoparticles located at the basolateral side of endothelium in a capillary and a pial venule.

B) Lateral nanoparticle movement in capillaries.

C) Movement of a nanoparticle perpendicular to the vessel lumen.

D) Nanoparticle crossing the endothelium into the perivascular space in venules. The images show a nanoparticle accelerating in the perivascular space. See also Movie S7.

E) Schematic drawing of a mouse head and craniotomy for chronic two-photon imaging.

F) Microscope stage with movement-unrestricted awake animal. The objective is stationary, and the air-pressurized pad reacts reciprocally to the mouse movement.

G) Time course of nanoparticle penetration to brain parenchyma. Images are Z-stack maximum intensity projection. *Upper rows:* Nanoparticles enter the brain at post-capillary venular segments; *Lower row:* No nanoparticle

1   transcytosis to the brain at capillaries. *null*=fluorescence signal prior to

2   nanoparticles injection.

3   **H)** High-resolution images of nanoparticles upon transcytosis in pial, ascending

4   and precapillary venules.

5   *pA*=pial artery; *pV*=pial venule; *pcV*=post-capillary venule; *cap*=capillary.

6

7 **Figure 7. Results summary.**

8

9

10 **SUPPLEMENTAL FIGURES LEGENDS**

11

12 **Figure S1. No adverse systemic effects on physiology.**

13   **A)** Blood-circulating leukocytes with sequestered nanoparticles (arrowheads). See

14   also Movie S2.

15   **B)** Leukocytes with sequestered nanoparticles preserve their rolling and

16   endothelium adherence properties.

17   **C)** The uptake of nanoparticles is unlikely driven by the RI7217 moiety, as it is

18   also present e.g. for fluorescently labeled albumin (BSA-Alexa488).

19   **D)** No significant effect of nanoparticles on brain activity (electrocorticogram,

20   *ECoG*), exhaled CO_2 levels (exCO_2), and mean arterial blood pressure

21   (*MABP*), all measured simultaneously with imaging.

22

23 **Figure S2. No nanoparticle association without TfR-targeting moiety.**

24   **A-B)** No association of stealth Atto 550-tagged nanoparticles (Sth-L-A550) to

25   pial vessels and capillaries.

26   **C-D)** No association of isotype IgG Atto 550-tagged nanoparticles (IgG-L-A550)

27   to pial vessels and capillaries.

28

29 **Figure S3. RI7-L-A488 follows RI7-L-A550 spatio-temporal distribution.**

30   **A–D)** High resolution *in vivo* images of RI7-L-A488 nanoparticles (green) 2 h

31   post-injection. Co-injected circulating TRITC-dx delineates vessel lumen

32   (red). Liposomes are readily present at pial, ascending venules, post-capillary

33   venules, and capillaries *in vivo*.

E) No association of isotype IgG A488-tagged nanoparticles with pial vessels and capillaries.

F-G) Association of RI7-L-A488 over time *in vivo*. Nanoparticles associated most rapidly to endothelium of capillaries and most slowly to large venules. Surface density=# of nanoparticles per μm^2 vessel wall area. Inset illustrates vessel type classification.

mV=main pial venule; *pV*=pial venule; *ascV*=ascending venule*; pcV*=post-capillary venule; *cap*=capillaries; *pA*=pial arteriole.

Figure S4. Two-photon imaging of brain microvasculature endothelium *in vivo*.

A) Projected Z-stacks from Tie2-GFP mouse cortex showing brain microvasculature endothelium *in vivo*. The high-resolution scan details the subcellular morphology of arterioles and venules.

pV=pial venule; *ascV*=ascending venule*; pcV*=post-capillary venule; *cap*=capillaries; *penA*= penetrating arteriole; *pA*=pial arteriole; *nuc*=nucleus; cs=cell boundaries/endothelium contact sites.

SUPPLEMENTAL MOVIES LEGENDS:

Movie S1. Blood-circulating nanoparticles show high fluorescence stability over time. Time is relative to the time of nanoparticle injection.

Movie S2. A small fraction of nanoparticles is sequestered by circulating leukocytes.

Movie S3. Nanoparticle association to the brain microvasculature over time. Concurrent imaging of RI7-L-A550 nanoparticles (red) with FITC-dextran (FITC-dx, green). Time is relative to the time of nanoparticle injection.

Movie S4. Time-lapse recording of nanoparticle (RI7-L-A550) movement in the brain endothelial cells. The endothelium is visible in green (Tie2-GFP). *Left panels*: pial venule; *right panel*: post-capillary venule.

1   **Movie S5.** Time-lapse recording of nanoparticle (RI7-L-A550) motility at the
2   capillary segment with examples of nanoparticle tracing. Circles denote individual
3   nanoparticles.

4

5   **Movie S6.** Nanoparticles exhibit movement, even at stalled capillaries.

6

7   **Movie S7.** Right panel: nanoparticle (RI7-L-A550, red) transcytosis into the
8   perivascular space at the level of post-capillary venules. Left panel: no nanoparticle
9   transcytosis in capillaries. The vessel lumen is delineated by circulating FITC-dextran
10   (FITC-dx, green).

11

12   **Movie S8.** Leukocyte entry into the brain with previously sequestered nanoparticles
13   (RI7-L-A550, red) in the blood circulation. Vessel lumen is delineated by circulating
14   FITC-dextran (FITC-dx, green).

15

16   **Movie S9.** No significant nanoparticle movement following laser-extravasation from
17   the blood to the brain at capillaries. In contrast, rapid progression in the brain of
18   nanoparticles extravasated from venules. Lines denote vessel lumen boundaries.

A RI7-L-A550 | RI7-L-A550
100 μm | penA ▲ ▲pA
| ▲pV
| cap ▲ascV
| 20 μm

FITC-dx | FITC-dx
| penA ▲ ▲pA
| ▲pV
| cap

RI7-L-A550nc | RI7-L-A550 nc
| ▲pV ▲ascV
| cap

B RI7-L-A550 nc
750 μm
750 μm
▲pV
▲pA
200 μm
cap

C RI7-L-A550 FITCdx 7.5 min | RI7-L-A550 FITCdx 120 min
ascV ▲
pcV ▲
cap ▲
10 μm
▲pV
10 μm

7.5 min | 30 min | 60 min | 90 min | 120 min
pcV ▲
cap ▲
10 μm

7.5 min | 30 min | 60 min | 90 min | 120 min
▲pV
10 μm

D
- main pial venule
- pial venule
- ascending venule
- post-cap. venules and capillaries

venule/capillary | ascending venule | pial venule | main pial venule

surface density [liposomes / μm²]
0.20
0.15
0.10
0.05
0.00
2   4   8   16   32   64   128
vessel diameter [μm]

E
- main pial venule
- pial venule
- ascending venule
- post-cap. venules and capillaries

surface density [liposomes / μm²]
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
15   30   45   60   75   90   105   120
time [min]

A Tie2-GFP — labels: *pA*, *ascV*, *pV*, *penA*, *cap*. Scale bar 100 μm.

B Tie2-GFP panels: *pA*, *pV*, *pA*, *pV*, *cap*. Scale bars 20 μm, 20 μm, 10 μm.

C RI7-L-A550 / Tie2-GFP. Labels: *pV*, *pV*, *pV*, *pV*, *ascV*, *pcV*, *mV*, *cap*, *cap*. Scale bars 50 μm, 5 μm, 10 μm, 5 μm.

D RI7-L-A550 + Tie2-GFP. Insets #1, #2, #3, #4, #5. Scale bars 20 μm, 5 μm.

E RI7-L-A550 / Tie2-GFP. Scale bar 2 μm. Plot: norm. fluorescence vs distance [μm], traces RI7-L-A550 (red), Tie2-GFP (green), regions I. and II., Δρ, brain / blood.

F |Δρ|<σ internalized 0.42 σ; σ<Δρ<2σ unresolved 1.30 σ; Δρ>2σ adhering 3.72 σ. Scale bars 2σ, 2 μm.

G main pial venule (black), pial venule (red), ascending venule (orange).
n=256 (5) — int. 55.9%, adh. 24.0%
n=430 (5) — int. 48.0%, adh. 30.9%
n=341 (5) — int. 46.3%, adh. 32.8%
int., unr., adh.
distribution [%] vs Δρ [σ=0.29 μm]

A

RI7-L-A550 / Tie2-GFP — Δt = 0 min, Δt = 2 min, Δt = 4 min (left panels, 20 μm and 2 μm scale bars)

RI7-L-A550 / Tie2-GFP — Δt = 0 min, Δt = 2 min, Δt = 4 min (right panels, 10 μm and 2 μm scale bars)

B t = 0 min: RI7-L-A550; t = 0→30 min: RI7-L-A550 + traces; t = 0→30 min: isolated traces (10 μm scale bars)

C pial venules; post cap. venules + capillaries. Plot of y [μm] vs x [μm], pV (blue) / pcV+cap (yellow)

D movement in (x,y) coordinates; movement rel. to the blood flow (v). Plot of v vs time [min], blood flow direction

E total distance traveled [μm] — 190 (5), 480 (5), pV / pcV+cap, ns

F displacement [μm] — 190 (5), 480 (5), pV / pcV+cap, ns

G displacement rel. to blood flow direction [μm] — 190 (5), 480 (5), pV / pcV+cap

H MSDv [μm²] vs time [min], linear fit. P (runs test) <0.0001; deviation from linearity Significant; slope 0.0007706. fit residuals [μm²] vs time [min]

I venules — RI7-L-A550 / Tie2-GFP (10 μm scale bars), nuc

J capillaries — RI7-L-A550 / Tie2-GFP, nuc (scale bars)

K RI7-L-A550 / Tie2-GFP, cut-off, nucl. boundary, nucl. (5 μm scale bar)

L density map, probability distribution [%], min–max, cut-off (2 μm scale bars)

M distribution [%] vs distance from nucleus perimeter [μm]; proximal 0-2.5 μm, intermediate 2.5-5.0 μm, distal 5.0-8.5 μm, non-classified; n=3534 (5)

N RI7-L-A550+Tie2-GFP; RI7-L-A550; Tie2-GFP (10 μm scale bars)

A

cap — RI7-L-A550 / Tie2-GFP — brain, blood

ven — RI7-L-A550 / Tie2-GFP — brain, blood

2 µm | 5 µm

B

RI7-L-A550 / TRITC-dx — cap

RI7-L-A550 | Δt = 8 min | Δt = 16 min

5 µm

C

RI7-L-A550 / TRITC-dx — ven

RI7-L-A550 | Δt = 1 min | Δt = 2 min

5 µm

D

RI7-L-A550 / FITC-dx

0 s | 20 s | 40 s | 60 s | 80 s | 100 s

5 µm | 2 µm

E

objective, water, coverglass, head plate, dental cement, acrylic glue, skull bone, dura, brain, aCSF

head plate, midline axis, coverglass, dental cement, craniotomy

F

objective, head plate, static support, mouse momentum, reciprocal pad movement, mobile pad

G

| | -2 days | -30 min | RI7-L-A550 | 30 min | 1 day | 2 days |

venules

Tie2-GFP — null, pV | Tie2-GFP — null, pV | Tie2-GFP / RI7-L-A550 — pV | Tie2-GFP / RI7-L-A550 — pV | Tie2-GFP / RI7-L-A550 — pV

50 µm

null | null | RI7-L-A550 | RI7-L-A550 | RI7-L-A550

50 µm

capillaries

Tie2-GFP — null, cap, pA | Tie2-GFP — null, cap, pA | Tie2-GFP / RI7-L-A550 — cap, pA | Tie2-GFP / RI7-L-A550 — cap, pA | Tie2-GFP / RI7-L-A550 — cap, pA

50 µm

H

2 days — Tie2-GFP / RI7-L-A550

post-capillary venules

#2, #1, #3, pcV, pcV

50 µm | #1 10 µm | #2 10 µm | #3 10 µm

post-capillary venules
TRANSCYTOSIS

brain
• nanoparticle

perivascular space
endothelium
basement membrane
endothelium

blood

capillaries
NO TRANSCYTOSIS

brain

glia limitans
basement membrane
endothelium
basement membrane
endothelium

blood

glia limitans
basement membrane

transcytosed
nanoparticles

perinuclear
clustering

perivascular
space

dense
association

no
association

perivascular
space

ascending
venules

post-capillary
venules

capillaries

pre-capillary
arterioles

penetrating
arterioles

nanoparticle
transcytosis

vessel
diameter

association
density