Acta Neuropathologica

Convective influx/glymphatic system. Tracers injected into the CSF enter and leave the brain along separate periarterial basement membrane pathways

--Manuscript Draft--

Manuscript Number:	ANEU-D-17-00986R2
Full Title:	Convective influx/glymphatic system. Tracers injected into the CSF enter and leave the brain along separate periarterial basement membrane pathways
Article Type:	Original Paper
Keywords:	intramural periarterial drainage, basement membranes, interstitial fluid, cerebrospinal fluid, glymphatic
Corresponding Author:	Roxana Octavia Carare, MD, PhD University of Southampton Southampton, Hampshire UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Southampton
Corresponding Author's Secondary Institution:	
First Author:	Nazira Jamal Albargothy, PhD
First Author Secondary Information:	
Order of Authors:	Nazira Jamal Albargothy, PhD
	David A Johnston, BSc, PhD
	Matthew MacGregor Sharp, BSc
	Ajay Verma, MD, PhD
	Roy Oliver Weller, MD, PhD
	Cheryl Anne Hawkes, BSc, PhD
	Roxana Octavia Carare, MD, PhD
Order of Authors Secondary Information:	
Funding Information:	Biogen (FE-13227) — Prof Roxana Octavia Carare
Abstract:	Tracers injected into CSF pass into the brain alongside arteries and out again. This has been recently termed the "glymphatic system" that proposes tracers enter the brain along periarterial "spaces" and leave the brain along the walls of veins. The object of the present study is to test the hypothesis that 1) tracers from the CSF enter the cerebral cortex along pial-glial basement membranes as there are no perivascular "spaces" around cortical arteries, 2) tracers leave the brain along smooth muscle cell basement membranes that form the Intramural Peri-Arterial Drainage (IPAD) pathways for the elimination of interstitial fluid and solutes from the brain. 2μL of 100μM soluble, fluorescent fixable amyloid β (Aβ) were injected into the CSF of the cisterna magna of 6-10 and 24-30 month-old male mice and their brains were examined 5 and 30 mins later. At 5 mins, immunocytochemistry and confocal microscopy revealed Aβ on the outer aspects of cortical arteries colocalized with α-2 laminin in the pial-glial basement membranes. At 30 mins, Aβ was colocalised with collagen IV in smooth muscle cell basement membranes in the walls of cortical arteries corresponding to the IPAD pathways. No evidence for drainage along the walls of veins was found. Measurements of the depth of penetration of tracer were taken from 11 regions of the brain. Maximum depths of penetration of tracer into the brain were achieved in the pons and caudoputamen. Conclusions drawn from the present study are that tracers injected into the CSF enter and leave the brain along separate periarterial basement membrane pathways. The exit route is along IPAD pathways in which Aβ accumulates

	in cerebral amyloid angiopathy (CAA) in Alzheimer's disease. Results from this study suggest that CSF may be a suitable route for delivery of therapies for neurological diseases, including CAA.

Responses to comments from reviewers for Albargothy et al 05/05/2018

"Convective influx/glymphatic system. Tracers injected into the CSF enter and leave the brain along separate periarterial basement membrane pathways"

Dear Professor Paulus,

Thank you for offering us the opportunity to respond to the comments from reviewers. Our responses are in italics, under each comment.

Reviewer #1:

[1] Abstract line 17 delete the phrase "as there are no perivascular 'spaces' around cortical arteries" That is waving a red flag -- needlessly. Discuss that contention in the discussion, but it does not follow from the results in this paper and should not be in the abstract.

Response

Thank you for this advice. However, we would prefer to retain the phrase "as there are no perivascular 'spaces' around cortical arteries" in the abstract for two reasons: 1] the abstract should be a summary of the important data that are included in the paper. Stating that there are no perivascular "spaces" around cortical arteries is part of our first hypothesis. We feel that this phrase should be included for completeness. 2] From our perspective, this phrase does not "waive a red flag" and is not contentious as we have shown and explained the absence of perivascular "spaces" around cortical arteries in a number of previous publications (see references below).

1. *Morris AW, Sharp MM, Albargothy NJ, Fernandes R, Hawkes CA, Verma A, Weller RO, Carare RO (2016) Vascular basement membranes as pathways for the passage of fluid into and out of the brain. Acta neuropathologica 131:725-736. doi:10.1007/s00401-016-1555-z*
2. *Weller RO, Sharp MM, Christodoulides M, Carare RO, Mollgard K (2018) The meninges as barriers and facilitators for the movement of fluid, cells and pathogens related to the rodent and human CNS. Acta neuropathologica 135:363-385. doi:10.1007/s00401-018-1809-z*
3. *Zhang ET, Inman CB, Weller RO (1990) Interrelationships of the pia mater and the perivascular (Virchow-Robin) spaces in the human cerebrum. J Anat 170:111-123*

2] line 27 Wouldn't "No evidence for the tracers along the walls of veins was found." be a better summary of the results than "No evidence for drainage ....." The separation of hypothesis and observation is much better than in the first version.

Response

Thank you for the suggestion, but we would prefer to retain the current phraseology in the abstract. Tracer is shown in a short segment of vein near the surface of the brain at 30 min in Figure 3a, so technically the phrase "No evidence for the tracers along the walls of veins was found." would be incorrect. "No evidence for drainage ....." states more accurately the findings in the present study.

3] Separate drainage pathways of ISF and CSF. The insertion of the word "largely" on line 181 (and on line 257) helps, but the impression given is still far from that supported by the balance of all the evidence. For at least some solutes there is good evidence for drainage of ISF to CSF and for transfers of material from CSF to blood via arachnoid villi (though its relative importance is very likely to be species and age dependent). The work of people like Davson, Cserr, Bradbury and Szentisvanyi cannot be ignored simply because it is old. The references cited make an excellent case that CSF drains largely via the cribriform plate and that ISF doesn't all drain to CSF, but they do not make the case either that little or no ISF drains to CSF or that CSF drainage via the arachnoid villi is negligible. If you want to make these assertions you should either argue the case or give references to where it is done or both. Nagra 2006 AmJPhysiol 291, 1383 noted that in the rat a significant transfer occurs directly to blood (their early peak). On the other hand Ma et al 2017 Nature Communications 8, 1424 may provide some of the data you need to argue the primacy in mice of CSF drainage via perineural routes. You might also recall that Bedussi and Bakker have quite a different view (see Bedussi et al, 2017, JCBFM 37, 1374). You might want to reread Pollay 2010. You don't need to address questions of how ISF and CSF return to blood in this paper -- so why do so?

Response

We apologise that the relevance of separate routes for drainage of CSF and interstitial fluid (ISF) to the present paper was not made clear in lines 43 and 100. We also realise that the traditional view is that the CSF acts as a "sink" into which interstitial fluid (ISF) drains. However, the mounting evidence for largely separate drainage routes for ISF and CSF began with the work of Helen Cserr and Mike Bradbury. Helen Cserr and her colleagues emphasised the periarterial route for the drainage of radioactive tracers injected into the brain in 1984 (Szentistvanyi I, Patlak CS, Ellis RA, Cserr HF (1984) Drainage of interstitial fluid from different regions of rat brain. American Journal of Physiology 246:F835-844). They noted that there was only 10-15% efflux of radioactive tracer from caudate nucleus or internal capsule into the CSF, although there was more efflux from the mid brain. Szentistvanyi et al also recorded that radioactive tracer injected into the brain was only present in the walls of intracranial arteries and carotid arteries in the neck above the cervical lymph nodes and not below. Our own experiments showed that tracers injected into the brains of mice drain out of the brain along basement membranes in the walls of cerebral capillaries and arteries (Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, Weller RO (2008) Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. Neuropathol Appl Neurobiol 34:131-144. doi:10.1111/j.1365-2990.2007.00926.x). In cerebral amyloid angiopathy, the distribution of a number of amyloids in the basement membranes of cerebral capillaries and arteries exactly mirrors that of the tracers used in experimental studies. Furthermore, amyloid beta is only present in the walls of intracranial arteries and not in the carotid artery in the neck (Shinkai Y, Yoshimura M, Ito Y, Odaka A, Suzuki N, Yanagisawa K, Ihara Y (1995) Amyloid beta - proteins 1-40 and 1-42(43) in the soluble fraction of extra- and intracranial blood vessels. Annals of neurology 38:421-428). This suggests that the distribution of amyloid beta in relation to cerebral arteries in humans is similar to that of radioactive tracers recorded by Szentistvanyi et al. in their experiments in 1984. This chain of observations upon which the hypothesis of largely separate routes for the drainage of ISF and CSF is based is set out in detail in our previous publications and especially in Engelhardt B, Vajkoczy P, Weller RO (2017) The movers and shapers in immune privilege of the CNS. Nature immunology 18:123-131. doi:10.1038/ni.3666 and Engelhardt B, Carare RO, Bechmann I, Flugel A,

Laman JD, Weller RO (2016) Vascular, glial, and lymphatic immune gateways of the central nervous system. Acta neuropathologica 132:317-338. doi:10.1007/s00401-016-1606-5.

Thank you for the reference to Nagra 2006 AmJPhysiol 291, 1383 , who noted that in the rat a significant transfer of CSF occurs directly to blood (their early peak). The early peak in their paper is 10 min after injection into the CSF. Tracers injected into the CSF reach the cervical lymph nodes within 1 min (see Kida S, Pantazis A, Weller RO (1993) CSF drains directly from the subarachnoid space into nasal lymphatics in the rat. Anatomy, histology and immunological significance. Neuropathol Appl Neurobiol 19:480-488). Ten minutes in Nagra's study may be ample time for tracers to transfer from lymph nodes to blood.
Bedussi and Bakker did indeed have quite a different view (see Bedussi et al, 2017, JCBFM 37, 1374), showing that injection of relatively large volumes of tracer into the brain resulted in efflux into the CSF. The differences between the Bakker group and ours were resolved in a subsequent joint review (Bakker EN, Bacskai BJ, Arbel-Ornath M, Aldea R, Bedussi B, Morris AW, Weller RO, Carare RO (2016) Lymphatic Clearance of the Brain: Perivascular, Paravascular and Significance for Neurodegenerative Diseases. Cell Mol Neurobiol. doi:10.1007/s10571-015-0273-8).

Thank you for the suggestion to re-read the article by Pollay 2010. This has been done. But, as can be seen by the following paragraph and the data presented in the present response, concepts and experimental results have moved on since 2010. Quotation from Pollay 2010: "In a rat model, Weller et al also demonstrated the importance of the lymphatic drainage system in the drainage of fluid from the interstitial space of the cortex [53]. It appears that the drainage of cerebral interstitial fluid into the CSF is via the perivasular channels and subsequently entering the lymphatic system through the cribiform plate (figure 9). This supports the view that this pathway serves a primary role in the turnover of the cerebral interstitial fluid." Reference [53] is Weller RO, Kida S, Zhang ET: Pathways of fluid drainage from the brain morphological aspects and immunological significance in rat and man. Brain Pathol 1992, 2:277-284. This work was superseded by experiments by Roxana Carare using soluble fluorescent tracers rather than particulate Indian ink.(Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, Weller RO (2008) Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. Neuropathol Appl Neurobiol 34:131-144. doi:10.1111/j.1365-2990.2007.00926.x) . This work supported more closely the findings of Helen Cserr (Szentistvanyi et al 1984) of drainage of interstitial fluid to lymph nodes.

We have re-read the section of the manuscript between lines 43 and 100 and we feel that it summarises the case put forward in our response to the reviewer's comments. We also consider that it is important to indicate the largely separate routes by which ISF and CSF drain to lymph nodes and we have added two more references that offer a more detailed explanation (Engelhardt B, Carare RO, Bechmann I, Flugel A, Laman JD, Weller RO (2016) Vascular, glial, and lymphatic immune gateways of the central nervous system. Acta neuropathologica 132:317-338. doi:10.1007/s00401-016-1606-5) (Engelhardt B, Vajkoczy P, Weller RO (2017) The movers and shapers in immune privilege of the CNS. Nature immunology 18:123-131. doi:10.1038/ni.3666)

4] pg 2 lines 201-214 These arguments completely ignore the possibility that Aβ is found preferentially in the basement membranes surrounding smooth muscle cells rather than in others because it binds to components present only in those around the smooth muscle cells. Your group has evidence for binding.

Response

We agree that anything is possible. However, the presence of different chemical species of amyloid with the same distribution of basement membranes in artery walls in the different types of CAA (Revesz T, Ghiso J, Lashley T, Plant G, Rostagno A, Frangione B, Holton JL (2003) Cerebral Amyloid Angiopathies: A Pathologic, Biochemical, and Genetic View. Journal of neuropathology and experimental neurology 62:885-898) and the presence of different chemical tracers in the same basement membranes for example dextrans and ovalbumin (Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, Weller RO (2008) Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. Neuropathol Appl Neurobiol 34:131-144. doi:10.1111/j.1365-2990.2007.00926.x) among others (Barua NU, Bienemann AS, Hesketh S, Wyatt MJ, Castrique E, Love S, Gill SS (2012) Intrastriatal convection-enhanced delivery results in widespread perivascular distribution in a pre-clinical model. Fluids Barriers CNS 9:2. doi:10.1186/2045-8118-9-2) suggests that any binding would be for multiple substances rather than specifically for amyloid beta.

5] pg 3 line 241 To continue playing devils advocate: Surely CAA is a process which requires focii or nucleation. If those foci are present only in the basement membranes of the smooth muscle cells, then regardless of the route of efflux, those basement membranes will be where you see the deposits.

Response

The exact mechanisms by which Abeta deposits in its fibrillar form in the lamina densa of smooth muscle cell basement membranes are not completely clear. Deposition of insoluble Abeta occurs in extracellular spaces of the brain as plaques and in capillary and artery walls. Electron microscopy has shown that, non-fibrillar and fibrillar Abeta are present in smooth muscle cell basement membranes, mainly in the lamina densa (Wisniewski HM, Wegiel J (1994) Beta-amyloid formation by myocytes of leptomeningeal vessels. Acta Neuropathol (Berl) 87:233-241) and gradually increase in amount separating the two elements of the adjacent smooth muscle cell basement membranes. (Keable A, Fenna K, Yuen HM, Johnston DA, Smyth NR, Smith C, Al-Shahi Salman R, Samarasekera N, Nicoll JA, Attems J, Kalaria RN, Weller RO, Carare RO (2016) Deposition of amyloid beta in the walls of human leptomeningeal arteries in relation to perivascular drainage pathways in cerebral amyloid angiopathy. Biochimica et biophysica acta 1862:1037-1046. oi:10.1016/j.bbadis.2015.08.024).

The presence of fibrillary amyloid within the brain parenchyma and in vessel walls suggest that there may be multiple factors involved in the nucleation and precipitation of fibrillar Abeta. Drainage of tracers along perivascular (IPAD) pathways is impaired with age (Hawkes CA, Hartig W, Kacza J, Schliebs R, Weller RO, Nicoll JA, Carare RO (2011) Perivascular drainage of solutes is impaired in the ageing mouse brain and in the presence of cerebral amyloid angiopathy. Acta neuropathologica 121:431-443. doi:10.1007/s00401-011-0801-7) and Abeta tends to progress from soluble to fibrillar Abeta when there is stasis. A possible explanation for the deposition of fibrillary Abeta in the IPAD pathways is the age-related slowing of the perivascular clearance mechanisms from the brain-thus precipitation in both brain and the clearance pathways. Although CAA develops predominantly in the basement membranes of leptomeninges and large cortical arteries (Type 2 CAA), it is also observed in the basement membrane of capillaries (Type 1 CAA) in advanced stages of AD (Thal et al. (2008) Cerebral amyloid angiopathy and its relationship to Alzheimer's disease Acta Neuropathol. 115:599–609). Thus, the multiple sites of fibrillary Abeta the brain

parenchyma and in the capillary and artery basement membranes suggest that it is not just basement membranes of the smooth muscle cells that are foci for nucleation of Abeta.

6] pg 3 line 260 glympathic system not systems

Response: *Thank you; this has been corrected*

7] pg 3 line 260-162 This is an excellent example of where you need to separate results and interpretation. This should be worded "In electron microscope studies there have not been any perivascular spaces around arteries in the cerebral cortex." The cynic would continue -- of course not, you can't fix a space.

Response

Although in well-fixed tissue electron microscopy does not demonstrate spaces around cortical arteries either in humans or mice, there are numerous accounts of artifactual spaces (see Weller RO, Sharp MM, Christodoulides M, Carare RO, Mollgard K (2018) The meninges as barriers and facilitators for the movement of fluid, cells and pathogens related to the rodent and human CNS. Acta neuropathologica 135:363-385. doi:10.1007/s00401-018-1809-z). Dilated perivascular spaces in the cerebral white matter and basal ganglia can be visualised by MRI (Shams S, Martola J, Charidimou A, Larvie M, Granberg T, Shams M, Kristoffersen-Wiberg M, Wahlund LO (2017) Topography and Determinants of Magnetic Resonance Imaging (MRI)-Visible Perivascular Spaces in a Large Memory Clinic Cohort. J Am Heart Assoc 6. doi:10.1161/JAHA.117.006279). Perivascular spaces in the basal ganglia can be demonstrated by electron microscopy (Pollock H, Hutchings M, Weller RO, Zhang ET (1997) Perivascular spaces in the basal ganglia of the human brain: their relationship to lacunes. J Anat 191 (Pt 3):337-346), as can perivascular spaces in the white matter (Carare in press).

The following has been inserted into the manuscript on ***Page 3 Lines 105-108*** *".....although dilated perivascular spaces do develop in other parts of the brain such as cerebral white matter and the basal ganglia (Shams S, Martola J, Charidimou A, Larvie M, Granberg T, Shams M, Kristoffersen-Wiberg M, Wahlund LO (2017) Topography and Determinants of Magnetic Resonance Imaging (MRI)-Visible Perivascular Spaces in a Large Memory Clinic Cohort. J Am Heart Assoc 6. doi:10.1161/JAHA.117.006279). Perivascular spaces in the basal ganglia can be demonstrated by electron microscopy (Pollock H, Hutchings M, Weller RO, Zhang ET (1997) Perivascular spaces in the basal ganglia of the human brain: their relationship to lacunes. J Anat 191 (Pt 3):337-346)"*

8] The nanoparticles are found in the basement membranes, they are not (or at least have not been) seen to pass along them. You can of course, assert the interpretation that "the observation of probes penetrating via perivascular spaces" was in fact "the observation of probes that had penetrated via basement membranes" -- but you don't have evidence for one or the other. You cannot infer in vivo from fixed tissue without careful argument and that is still missing. (Please remember that when Davson took on the electron microscopists on extracellular spaces, Davson won!)

Response

The problem here is that we have been unable to demonstrate the perivascular "spaces" around cortical arteries in well-fixed tissue in electron microscope preparations whereas we have been able to demonstrate perivascular spaces around arteries in the basal ganglia. We

agree that flow of nanoparticles into the brain along pial-glial basement membranes has not been directly visualised. However, the most logical explanation for the presence of nanoparticles in pial glial basement membranes following injection of the nanoparticles into the CSF is that they entered by this route. Page 3, line 116 has been modified by the insertion of the words "most probable".

9] pg 3 lines 271-277. I support the second referee's opinion on the status of these objectives. You have not achieved these objectives. For the first, entry could still be via free spaces. For the second see the comments below. What you have done is well worth doing, but it has not (yet) answered these questions. What you have done is show the locations of the tracers at two time points. There are arguments you can give which support the existence of two separate pathways (itself an important result) but they don't yet prove it as you don't yet have evidence of outward flow along the inner pathway. At the late time point there would only be a little tracer in the outward pathway regardless of whether or not it is the major route of efflux. If it is, it will be flushed into CSF quickly and hence will have only a low concentration.

Response

Thank you for your interesting interpretation of our results. We have presented our current observations i.e. at 5 min after injection of tracer into the CSF it is present in the pial glial basement membrane on the outer aspect of artery walls. The tracer co-localises with proteins within the pial glial basement membrane. At 30 min after injection, tracer is present in the basement membranes of capillaries in the old mice and in smooth muscle cells in the tunica media of arteries in old and young mice in a similar distribution to the IPAD pathway identified by intracerebral injection studies and by CAA. We have put forward what we feel is a reasonable interpretation of these findings and a working hypothesis that tracer enters the brain from the CSF along pial-glial basement membranes and leaves the brain along IPAD pathways.

[10] lines 278-281 The third objective is much more objective -- and it has been achieved.

[11] lines 280-281 You do yourselves down. This is an important study of the basic physiology of the process. Don't justify it with just an application. Perhaps "An important practical application of this study relates to drug delivery from the CSF which will be ....."

Response

Our results are mostly relevant to intrathecal drug delivery, so we have expanded the discussion to reflect this. Page 11 lines 445-454

[12] pg 11 lines 863 to 870 There is a more conventional explanation for the decrease in Aβ reaching CSF once plaques have formed. The soluble Aβ concentrations in the parenchyma are thought (see Cirrito, J. R. et al (2003) J. Neurosci. 23: 8844-8853) to be lower once plaques are formed, because the plaques take up a significant fraction of the Aβ that is being produced. Before plaques form ISF is super-saturated in the "soluble" forms of Aβ -- the plaques need to be seeded.

Response

Thank you for pointing out the paper by Cirrito, J. R. et al (2003) from Holtzman's group. There are other papers recording that in transgenic APP mice soluble A beta levels in the brain are continually elevated, increasing 4-fold at 2 months and 33-fold in the APP23 Tg mice at 20 months of age when compared to the control mice (Kuo YM, Beach TG, Sue LI, Scott S, Layne KJ, Kokjohn TA, Kalback WM, Luehrs DC, Vishnivetskaya TA, Abramowski D, Sturchler-Pierrat C, Staufenbiel M, Weller RO, Roher AE (2001) The evolution of A beta peptide burden in the APP23 transgenic mice: implications for A beta deposition in Alzheimer disease. Mol Med 7:609-618). In mice there is overexpression of APP, but elevation of soluble Abeta levels has been recorded at post-mortem in brains from demented patients with Alzheimer's disease (McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, Masters CL (1999) Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Annals of neurology 46:860-86; Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, Beach T, Kurth JH, Rydel RE, Rogers J (1999) Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. The American journal of pathology 155:853-862).

It is possible that age-related impairment of IPAD results in loss of homoeostasis in the brain parenchyma and that the rise in the level of soluble Abeta in Alzheimer's disease reflects such loss of homoeostasis (Weller RO, Hawkes CA, Carare RO, Hardy J (2015) Does the difference between PART and Alzheimer's disease lie in the age-related changes in cerebral arteries that trigger the accumulation of Abeta and propagation of tau? Acta neuropathologica 129:763-766. doi:10.1007/s00401-015-1416-1)

[13] These results can be interpreted in a number of ways, one of which you have chosen to present as the inevitable conclusion of your observations. If, as you say, the tracer is entering the tissue along the external basement membrane at the time of the 5 min snapshot and leaving the tissue along the smooth muscle cells at the time of the 30 min snapshot, then there should have been an intermediate time and place for the tracer to have been in transit from one to the other. Where and when? If the transit from one to the other occurs along the length of the arteries/arterioles there is one interpretation, if it occurs only at the capillaries one has the interpretation you've given. Your present discussion is leaping ahead of the results until you know which. Careful measurements of relative concentrations along the two pathways as a function of time should be able to resolve this issue. It sounds like a lot of work!

Response

We agree that, ideally, measurements should be taken at every conceivable time point and at every conceivable point in the anatomy of the inflow and outflow system for tracers in the CSF entering and leaving the brain. At the moment we have snapshots at 5 min and 30 min after injection of tracer into the CSF and it is from these snapshots that we have formulated our working hypothesis.

Drainage of tracers out of the brain following intracerebral injection is impaired with age (Hawkes CA, Hartig W, Kacza J, Schliebs R, Weller RO, Nicoll JA, Carare RO (2011) Perivascular drainage of solutes is impaired in the ageing mouse brain and in the presence of cerebral amyloid angiopathy. Acta neuropathologica 121:431-443. doi:10.1007/s00401-011-0801-7 , such that a greater number of capillaries show tracer in their basement membranes at a set time following intracerebral injection. In the present study, tracer is present in the basement membranes of capillaries at 30 min following injection of tracer into the CSF in the older age group of mice. This gives some indication of the possible pathway

for drainage of tracers out of the brain. We have used this information to support our working hypothesis that the tracers are draining along basement membranes of capillaries and arteries as they pass out of the brain along the IPAD pathway. Furthermore, the presence of tracer in astrocyte processes within the brain parenchyma (as reported in the present paper) suggests that tracer has entered the interstitial fluid and been taken up by these cells.

Reviewer #2:

In their revision, the authors have substantially revised the presentation of their findings, but have conducted no additional experiments to address the comments of either Reviewer 1 or Reviewer 2. Thus, while the self-confirmatory tone of the prior submission is largely muted, the most important criticisms of these two reviewers from the prior submission remain largely unresolved.

1] Both reviewers noted that that the technical approaches and the two time points utilized in this study prevent any sense of the direction of tracer movement from being defined. Given that this is one of the central controversies in the field, addressing this issue of key importance. In response, the authors have now cited a prior study from their group which suffers from the same technical limitations, and a 2-photon microscopy study using a very different injection paradigm than either their own or those from Nedergaard and colleagues. This latter study uses a pressure injection approach (5-20 PSI for 10-30 s) to introduce tracer for intravital imaging. While this approach may be relevant to the field of convection-enhanced delivery, it is unclear if the results are relevant to the physiology movement of fluid and solutes out of brain tissues, which the authors of the present study implicitly note by using a 'small' volume of 0.5ul. In their response to Reviewer 2, the authors note the technical difficulties in conducting these studies. But that is the very reason that this question is important to answer. A study that moved the field forward would devise a clever way to do this.

2] Related to this issue, is the inability to distinguish the route of tracer movement between the 5 and 30 min time points, including resolving between organized 'flow' and simple distribution by diffusion. This is a second key element of controversy in the field that the authors do not address.

Responses to points [1-2]

We thank the reviewer #2 for the insightful comments and we will certainly bear them in mind for the design of future experiments.

The objectives of the experiments reported in the present paper were 1] to demonstrate in young and aged mice that the pial-glial basement membranes are the entry route into the brain for soluble tracers from the CSF and 2] to test the hypothesis that tracers entering the brain from the CSF drain out of the brain parenchyma along the IPAD pathways. 3] to determine the range of depths of penetration of tracer alongside arteries in different regions of the brain and for the time points of 5 and 30 min in young and aged mice. We consider that we have achieved these objectives.

Furthermore we have tried to answer to points raised in by reviewers #1 and #2 in their first reviews. We are sorry if we had not been able to answer all the criticisms but we have

answered questions to the best of our ability. Some of the questions raised in the second reviews by reviewer #2 have been answered in our responses to reviewer #1.

3] In response to Reviewer 1's critiques (#13) concerning the possibility of artifactual vascular labeling resulting from pressure injection ascribed to Iliff et al. (2012) being a possible confound to their own data, the authors simply cite yet more defects from the Iliff study. It is the veracity of the present study's findings that are the subject of the review. Pointing to the defects in another study doesn't nullify the impact those defects for the study in question.

Response

With regard to our mention of Iliff's study, we are putting forward possible reasons for the discrepancies between Iliff's findings and our findings in the present paper. We feel that this is a valid insertion.

[4] In response to this reviewer's questions regarding the strikingly low variance observed in these studies, the authors have changed the presentation so that the data are not shown with log-transformation. This doesn't address the general surprise at observing such large effects with such small (n=3) sample sizes. A post-hoc power analysis of this data does not ameliorate these concerns, given that if the data were flawed in some way systematic way, so too would be the power analysis.

Response

As described in the manuscript, "the distance of the Aβ-positive blood arteries from the surface or base of the brain was measured by drawing two perpendicular lines (one travelling in the direction of the vessel and a tangent of the surface/base of the brain; see supplementary Figure 1) and determining the distance of the vessel from the point at which the two lines meet." These measurements were applied consistently across all brain regions that were analysed in young and aged animals. Because the quantification data in some brain areas were not normally distributed, log transformation was applied to all groups and analysed using one- or two-way ANOVA with Sidak's posthoc test to control for multiple comparisons. These log transformed data were presented in the original manuscript not to obscure variations between groups but to accurately reflect the data as analysed. Based on the recommendation from Reviewer 1, the raw values (mean $\pm$ S.E.M from individual animals) were presented in the figures of the revised manuscript. As previously indicated, post-hoc power calculations indicate that the n=3 used in the study was sufficient to detect a significant difference between brain regions and age groups (86-97% power, $\alpha = 0.05$) due to large effect sizes (Cohen's f = 4.06-5.76) that are underpinned by biological variations in regional and age-related clearance of solutes, as previously reported (Szentistvanyi I, Patlak CS, Ellis RA, Cserr HF (1984) Drainage of interstitial fluid from different regions of rat brain. American Journal of Physiology 246:F835-844; Hawkes CA, Hartig W, Kacza J, Schliebs R, Weller RO, Nicoll JA, Carare RO (2011) Perivascular drainage of solutes is impaired in the ageing mouse brain and in the presence of cerebral amyloid angiopathy. Acta neuropathologica 121:431-443). The method of quantification, statistical analyses and

presentation of the data in the Figures are all described in full in the revised manuscript (Page 5-6 Lines 202-226).

[5] In response to reviewer comments concerning both the effect of anesthesia, and of mode of injection, particularly when seeking to make a comparison with other parallel studies, the authors essentially reply that that they will 'take note' of these differences and will 'bear [them] in mind' in future experiments. However, these are issues that confound the present findings and their interpretation, and as such, they should be addressed in the present study.

Response

We recognise that we have not measured changes in CSF pressure in the cisterna magna during injections or estimated the changes in pressure over the superior surfaces of the cerebral hemispheres at a distance from the cisterna magna. Nevertheless, we consider that our observations are valid and that our findings support our working hypothesis that tracer enters the brain from the CSF along pial-glial basement membranes and leaves the brain along the IPAD pathway.

With regard to anaesthesia - we have undertaken experiments testing the effects on heart rate, pulse, distension, breath rate, breath distension and arterial oxygen saturation for isoflurane and for another common anaesthetic used in rodent studies: 10mg/ml ketamine with 1mg/ml xylazine. The result is displayed in online resource 1 and demonstrates that isoflurane does not reduce the heart rate or oxygen saturation as does ketamine. As all groups were anaesthetised with isoflurane, we conclude that the effects observed are not due to an effect of anaesthesia. (see page 4 lines 148-151: page 14 lines 569-575)

Convective influx/glymphatic system. Tracers injected into the CSF enter and leave the brain along separate periarterial basement membrane pathways

Nazira J Albargothy[1], David A Johnston[1], Matthew MacGregor-Sharp[1], Roy O Weller[1], Ajay Verma[2], Cheryl A Hawkes[3]*, Roxana O Carare[1]*

[1]Faculty of Medicine, University of Southampton, U.K

[2]Biogen, U.S.A

[3]Open University, U.K

*Contributed equally to the study

Corresponding Author and email address

Key words: intramural periarterial drainage, basement membranes, interstitial fluid, cerebrospinal fluid, glymphatic

Abstract

Tracers injected into CSF pass into the brain alongside arteries and out again. This has been recently termed the “glymphatic system” that proposes tracers enter the brain along periarterial “spaces” and leave the brain along the walls of veins. The object of the present study is to test the hypothesis that 1) tracers from the CSF enter the cerebral cortex along pial-glial basement membranes as there are no perivascular “spaces” around cortical arteries, 2) tracers leave the brain along smooth muscle cell basement membranes that form the Intramural Peri-Arterial Drainage (IPAD) pathways for the elimination of interstitial fluid and solutes from the brain. 2μL of 100μM soluble, fluorescent fixable amyloid β (Aβ) were injected into the CSF of the cisterna magna of 6-10 and 24-30 month-old male mice and their brains were examined 5 and 30 mins later. At 5 mins, immunocytochemistry and confocal microscopy revealed Aβ on the outer aspects of cortical arteries colocalized with α-2 laminin in the pial-glial basement membranes. At 30 mins, Aβ was colocalised with collagen IV in smooth muscle cell basement membranes in the walls of cortical arteries corresponding to the IPAD pathways. No evidence for drainage along the walls of veins was found. Measurements of the depth of penetration of tracer were taken from 11 regions of the brain. Maximum depths of penetration of tracer into the brain were achieved in the pons and caudoputamen. Conclusions drawn from the present study are that tracers injected into the CSF enter and leave the brain along separate periarterial basement membrane pathways. The exit route is along IPAD pathways in which Aβ accumulates in cerebral amyloid angiopathy (CAA) in Alzheimer’s disease. Results from this study suggest that CSF may be a suitable route for delivery of therapies for neurological diseases, including CAA.

Introduction:

Apart from blood, there are two major extracellular fluids associated with the brain and spinal cord, namely CSF in the ventricles and subarachnoid spaces, and interstitial fluid (ISF) in the extracellular spaces of the brain and spinal cord. CSF and ISF appear to drain from the brain along largely separate pathways to regional lymph nodes [11]. Tracers injected into the CSF drain from the subarachnoid space to cervical lymph nodes by channels that pass through the cribriform plate and join lymphatic channels in the nasal submucosa [18, 20, 27]. Similarly, there is lymphatic drainage of CSF alongside cranial and spinal nerves and into lymphatics in the dura mater [3, 8, 11, 22].

There are no conventional lymphatic vessels in the brain parenchyma, but there is lymphatic drainage of ISF to cervical lymph nodes [11, 39]. When minute amounts of radioactive tracer are injected into deep grey matter of the rat brain, they drain to cervical lymph nodes along the walls of cerebral and intracranial arteries [39]. High-resolution studies using formalin-fixable fluorescent tracers suggest that the drainage pathways for ISF and solutes from the brain are the basement membranes in the walls of cerebral capillaries and arteries [4]. Injection of fluorescent tracer in small amounts of 0.5 µL through fine 50 µm glass cannulae ensures that no tracer leaks into the CSF [4]. At 5 min following these injections, tracers are within the basement membranes of cerebral capillaries and in basement membranes around smooth muscle cells in the tunica media of cerebral arteries. By 30 mins post-injection, no tracer remains in basement membranes in the walls of capillaries or arteries but the course of each artery involved is outlined by perivascular macrophages containing tracer [4].

Flow of tracer along smooth muscle cell basement membranes in the walls of arteries has been demonstrated by *in vivo* multiphoton imaging [2]. Ultrastructural studies have shown that, following injection of soluble amyloid-β (Aβ) into the mouse brain as a tracer, Aβ pervades the extracellular spaces of the brain and is in continuity with Aβ within capillary basement membranes [24]. In artery walls, however, tracer injected into the brain parenchyma is confined to basement membranes surronding smooth muscle cells in the tunica media and is not observed in basement membranes associated with either endothelial cells or with pial-glial basement membranes on the external aspects of the tunica media [4]. It appears, therefore, that tracer is unlikely to enter artery walls either via the endothelium or radially from the brain parenchyma through the glia limitans. From these observations it appears that tracers in the ISF pass from the brain parenchyma into basement membranes of capillaries and flow out of the brain along smooth muscle cell basement membranes in the walls of arteries [2, 4]. This pathway has been termed the Intramural Peri-Arterial Drainage (IPAD) pathway. The relevance of IPAD to neurodegenerative disease in humans is emphasised by the pattern of deposition of Aβ in the walls of cerebral capillaries and arteries as cerebral amyloid angiopathy (CAA). In the early stages of CAA, the pattern of deposition of Aβ corresponds, exactly, with the IPAD pathways identified by fluorescent tracer studies in the mouse [14, 19, 29].

Although tracers and Aβ in CAA outline the endothelial/glial basement membranes

around capillaries, tracers and Aβ are limited to basement membranes around smooth muscle cells in the tunica media of arteries within the brain and leptomeninges. Neither the endothelial basement membranes nor the outer pial-glial basement membranes are outlined by tracer or by the deposition of Aβ [4].

There is, therefore, evidence from experimental studies and from CAA that IPAD is a physiological pathway for drainage of ISF and solutes from the brain. Evidence from radioactive tracer studies [36] and from the drainage of Aβ from the brain [26] strongly support the concept that the IPAD route is the lymphatic drainage pathway for ISF and solutes from rodent and human brain.Radioactive tracers injected into the brain are present in the walls of intacranial arteries but not in the carotid artery in the neck [36]. Furthermore, biochemical analyses of Aβ in the walls of human arteries from 20 years of age onwards detected increasing amounts of Aβ in the walls of intracranial arteries with age but very little Aβ was detected at any age in the extracranial carotid arteries in the neck [33]. These results would corelate with Aβ draining from the brain along artery walls and passing to lymph nodes closely applied to the carotid artery below the base of the skull [7].

Despite the largely separate drainage pathways for CSF and ISF, the two fluids do mix within the brain. Tracers injected into the CSF pass into the brain and spinal cord tissue along the outer aspects of arteries and this system has been variously named as convective tracer influx and the glymphatic system [17, 30]. Electron microscope studies have shown that there are no perivascular spaces around arteries in the cerebral cortex [23, 37, 40] although dilated perivascular spaces do develop in other parts of the brain such as cerebral white matter and the basal ganglia [32]. Perivascular spaces in the basal ganglia have been demonstrated by electron microscopy [28].

When nanoparticles are injected as tracers into the CSF of mice, they appear to pass into the brain along the basement membrane between the coatings of pia mater on the outer aspect of cortical arteries and the glia limitans on the surface of the brain, the pial-glial basement membrane [24]. Previous studies have suggested that tracers injected into the CSF pass into the brain along assumed periarterial “spaces” and drain out of the brain along the walls of veins [17]. Having defined by electron microscopy the most probable entry pathway for particulate tracers passing into the brain from the CSF as the pial-glial basement membrane, we used injections of tracer into the CSF of the cisterna magna of mice to achieve three objectives. Our first two major objectives in the present study are: (1) to demonstrate by immunocytochemistry that the pial-glial basement membranes are the entry route into the brain for soluble tracers from the CSF and (2) to test the hypothesis that tracers entering the brain from the CSF drain out of the brain parenchyma along the IPAD pathways. Groups of mice were examined 5 min and 30 min after injection of fluorescent Aβ tracer into the cisterna magna of young 2-10 week and aged 24-30 month old mice.

The third major objective in this study is to determine the range of depths of penetration of tracer alongside arteries in different regions of the brain and for the time points of 5 and 30 min in young and aged mice. The importance of this study relates to drug delivery from the CSF and will be dealt with further in the discussion.

Materials and Methods:

Animals

Male C57BL/6 mice 6–10 weeks (young) and 24–30 months (old) were housed at the Biomedical Research Facility (BRF) at Southampton General Hospital (Southampton, UK). All mice were housed in groups of 4–10. They were kept under standard 12-hr light/dark cycle and fed a standard RM1 chow diet (SDS, UK) and water ad libitum. Environmental enrichment consisted of wooden sticks, red plastic tunnels and other bedding. All procedures were carried out in accordance with animal care guidelines stipulated by the United Kingdom Animals (Scientific Procedures) Act 1986, Home Office licence (PPL 30/3095).

Anaesthesia

C57BL/6 mice were anaesthetised using a standard isoflurane anaesthesia system. An anaesthesia induction chamber was precharged with 5% isoflurane for 5 minutes and the oxygen flow meter was set to approximately 1.7 $lmin^{-1}$ with the indicator ball floating between the 1 and 2 hash marks. The isoflurane vaporizer was then reduced to 1–2 % and the mouse was left in the chamber for 3–5 minutes. We chose isoflurane after our pilot experiments in n=3 wild type mice determined that isoflurane did not reduce the heart rate or oxygen saturation in C57BL/6 mice to the same degree as our previous anaesthetic (ketamine and xylazine), see Online Resource 1. The level of anaesthesia was monitored using the pedal withdrawal reflex response. Once the mouse reached a surgical plane of anaesthesia (no reflex) it was transferred from the induction chamber onto a surgical bed with a breathing device and mask that supplied a constant flow of 1 to 2 % isoflurane and 1.7 $lmin^{-1}$ oxygen to keep the animal safely anesthetised during the surgical procedure. The core body temperature was monitored using a rectal probe and maintained at 37°C using an electronic heating pad. The cornea was protected using Lacri-Lube eye ointment.

Intracisternal injections

The mouse was placed prone on a stereotaxic frame and head-restrained with ear bars after the area between the head and shoulders was shaved. Under a dissection microscope, a 10 mm midsagittal incision of the skin was made inferior to the occipital crest. To expose the dura covering the cisterna magna, the subcutaneous tissue and occipital muscles (biventer cervicis and rectus capitis dorsalis) were separated using blunt-ended forceps and the posterior atlanto-occipital membrane was incised. Using a cotton swab, the area was treated with a cotton bud impregnated with viscous glycerol to help prevent CSF reflux. A glass capillary micropipette (Sigma, UK) with an adjusted diameter (of < 50 µm, made using a Sutter P97 Flaming Brown Pipette puller) was positioned perpendicular to the ear bars and advanced gently to penetrate the dura until resistance was overcome indicating entry into the cisterna magna. The capillary had been loaded with 2 µl of 100 µM Aβ1-40 Hylite Fluor 555 (Bioscience), which was injected over 2.5 minutes at a rate of 0.8 $\mu lmin^{-1}$. The microcapillary was left in situ for 2 minutes to prevent reflux and mice were culled 5 minutes or 30 minutes after withdrawal of the glass capillary through overdose with pentobarbital (200 $mgkg^{-1}$). Mice were intracardially perfused with 0.01M phosphate buffer saline (PBS) followed by 4% paraformaldehyde (PFA)

in 0.01M PBS at a rate of 5 $mlmin^{-1}$. Brains were dissected from the skull, post-fixed in 4 % PFA for 6-7 hours and cryoprotected with 30% sucrose in 0.01 M PBS for 48 hours. The brains were sectioned into 20 µm thick coronal slices using a cryostat and stored at -20 °C.

Double-labeling immunofluorescence

For double labelling immunohistochemistry, tissue sections were incubated overnight with anti-α-smooth muscle actin (SMA) FITC (1:200, Sigma-Aldrich, Dorset, UK) for the identification of arteries. Anti-collagen IV (1:400, ABCAM, Cambridge, UK) was used to mark the basement membranes of blood vessels (Thermo Fisher Scientific, Paisley, UK). Brain sections were also incubated with rat monoclonal anti-α-2 laminin (1:200) to identify the astrocyte basement membranes of the glia limitans abutting the outer aspect of cortical arteries [13] or mouse monoclonal anti-GFAP (1:100) or polyclonal rabbit anti-CD163 (1:100) at 4 °C for 24 hr in a moist chamber. All primary antibodies were diluted in 0.1% PBST. Subsequently sections were incubated with the appropriate secondary antibodies conjugated to Alexafluor fluorophores. Photomicrographs of triple labelling immunohistochemistry were captured across the brain starting anterior from the olfactory bulbs moving posteriorly towards the cerebellum. Sections were analyzed using an SP8 confocal laser-scanning microscope (Milton Keys, UK) and exported to Image J software. Montages were created with Photoshop CS6.

Cerebral blood vessel classification

Arteries were differentiated from veins using an SMA marker, which only stains the smooth muscle cells that are present in arteries and arterioles. Vessels that were negative for SMA and had a diameter < 10 µm were classified as capillaries.

Quantitative assessment of Aβ-positive blood vessels

To compare the depth of tracer 5 minutes and 30 minutes post-intracerebral injection in young and old mice, the distance of the Aβ-positive blood arteries from the surface or base of the brain was measured by drawing two perpendicular lines (one travelling in the direction of the vessel and a tangent of the surface/base of the brain; see Online Resource 2) and determining the distance of the vessel from the point at which the two lines meet. A total of 108 full coronal sections were analyzed in 6 – 10 week old mice (n = 3 per time point) and 56 sections in 24–30 month old mice (n = 3 per time point) and all the Aβ-positive blood SMA-positive arteries were quantified in this way. The sections quantified were taken at the level of the olfactory bulbs, cortex, ventricles, hippocampus, midbrain and cerebellum. The total length of each region (e.g. olfactory bulbs) was 180 µm.

Statistical analysis

The average of all distances in each brain region per mouse were used for statistics (n=3/group). Data were checked for normality using the D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests. As the data in some groups were not normally distributed, all values were Log_{10} transformed to obtain a normal distribution. One-way analysis of variance (ANOVA) with Sidak's post-hoc test was

used to compare the distance at which Aβ was seen from the reference point at the surface of the brain within the same brain region at 5 and 30 minutes after intracisternal injections. Two-way ANOVA with Sidak's post-hoc test was used to compare the distance at which Aβ was seen from the reference point at the surface of the brain between young and old mice at 5 and 30 minutes after intracisternal injections. Statistical analyses were performed using log-transformed data, with corresponding p-values indicated on graphs that were generated using non-transformed data.

Results:

Anatomical distribution of β-amyloid after its injection into cisternal CSF

First, the possible route of entry of Aβ1-40 (referred to from here as Aβ) contained within the CSF into the brain of 6–10 week old mice was evaluated by double-labelling immunohistochemistry and confocal microscopy. The anatomical distribution of Aβ in the brain and the type of Aβ-positive blood vessels were determined by staining the sections with an SMA marker to differentiate arteries from veins, a general basement membrane marker (collagen IV) and a specific astrocytic basement membrane marker (α-2 laminin). The anatomical compartment in which the tracer was contained was defined and compared after 5 and 30 minutes of injection of tracer into the cisterna magna.

6–10 week old mice, 5 minutes post-injection,

Aβ was detected within the walls of leptomeningeal and cortical arteries (Figure 1). Immunocytochemistry with multiple labelling of collagen IV, α-2 laminin and Aβ tracer confirms the presence of Aβ tracer in the brain disposed along pial-glial basement membranes on the outer aspects of arteries. Figure 1a shows a blood vessel penetrating the cerebral cortex with Aβ tracer, stained red, in the vessel wall. Co-localisation of Aβ tracer and α-2 laminin in the pial-glial basement membrane is shown as yellow. Co-localisation of Aβ tracer and collagen IV in the artery wall is depicted as fuchsia-pink. At higher magnification in figure 1b, α-2 laminin in the astrocyte basement membranes of the glia limitans on the surface of the brain is stained green and co-localisation of Aβ and α-2 laminin wall of the artery is shown as yellow. Confirmation that the Aβ tracer is in basement membranes on the outer aspect of arteries 5 min after injection into the CSF is seen in figure 1c. The blood vessel in longitudinal view is identified as an artery by the presence of smooth muscle cells stained green for smooth muscle actin in its wall. Aβ tracer colocalizes with collagen IV (blue) as a fuchsia pink colour in a single fine layer of basement membrane on the outer aspects of the artery. This corresponds to the position on the outer aspect of the artery to the pial-glial basement membrane [24].

6–10 week old mice, 30 minutes post-injection,

Aβ was detected in the basement membranes surrounding smooth muscle cells within the walls of leptomeningeal and cortical arteries but not in the walls of capillaries and veins. The vessel in longitudinal view in figure 1d is an artery

identified by the smooth muscle cells in its wall stained green by α smooth muscle cell actin (Figures 1d, 1f, 1j). At 30 min after injection into the cisterna magna, Aβ tracer (Fig 1e, 1k) 1 is seen in a spiral configuration that represents the smooth muscle cell basement membranes within the artery wall; the fuchsia-pink staining (Fig 1d, 1i) represents co-localisation of Aβ tracer in the walls of arteries in figure 1e, 1k and collagen IV in the smooth muscle cell basement membranes (Fig 1g, 1j, 1l). In a single optical section from an arteriole, fuchsia pink co-localisation of collagen IV and Aβ is seen running between green-stained smooth muscle cells in the tunica media of the arteriole (Fig 1h).

24–30 month old mice, 5 minutes post-injection

At 5 min following injection of Aβ tracer into the cisterna magna, the distribution of tracer is similar in the 24-30 month old mice to that found in the young 6-10 week old mice. Aβ tracer was observed in the walls of arterioles, as depicted by immunostaining for green smooth muscle actin in Figures 2 a, b and in the walls of capillaries, as shown in Figure 2c. In Figure 2d, a blood vessel penetrating the surface of the cerebral cortex shows Aβ tracer within the vessel wall and co-localizing with α-2 laminin indicating its presence in the pial glial basement membrane on the abluminal aspect of the vessel. At higher magnification, in a single channel image in Figure 2e-f, α-2 laminin in the glia limitans on the surface of the brain is stained green. Co-localisation of α-2 laminin and Aβ tracer in the pial-glial basement membrane on the outer aspect of the vessel wall is depicted in yellow.

24–30 month old mice, 30 minutes post-injection

The pattern of distribution of Aβ tracer in the walls of blood vessels in the older mice at 30 min after intracisternal injection is slightly different from that in the young mice. With regard to the arteries, the distribution of Aβ tracer is the same in the two age groups. Figure 3a shows an artery and vein at the surface of the cerebral cortex. The artery is identified by the staining of smooth muscle cells in its wall; Aβ is present in the same spiral pattern as smooth muscle cell basement membranes in the tunica media. A leptomeningeal artery in Figure 3 a,b shows the corresponding spiral pattern of smooth muscle cells in the tunica media. Aβ tracer is present in a short region of the wall of a vein near the subarachnoid space in Figure 3a. Aβ tracer, red, colocalizes with cells stained green for the macrophage marker CD 163 (Figure 3c). In addition, Aβ tracer co-localizes with glial fibrillary acidic protein (GFAP) in perivascular astrocyte processes in Figure 3d.

Periarterial penetration of β-amyloid tracer into the brain parenchyma

Next, the periarterial distances of Aβ from the surface of the brain at 5 and 30 minutes after intracisternal injection were determined using the quantification method illustrated in Online resource 1. Normally distributed data presented in the form of histograms show the Log_{10} values.

Regional differences in distance of periarterial penetration of Aβ into the parenchyma after 5 minutes in 6–10 week old mice

Soluble Aβ injected into the CSF was only observed along the walls of

leptomeningeal and cortical arteries after 5 and 30 minutes of injection. No Aβ was observed in the walls of capillaries or veins. The depth of penetration of Aβ along the walls of cortical arteries was calculated in eleven different brain regions. At 5 min post-injection, there was no statistically significant difference between the periarterial Aβ distance in the olfactory bulbs and the frontal, parietal, and entorhinal cortices. However, the distance at which Aβ was detected in the walls of arteries was significantly greater in the visual cortex, caudoputamen, thalamus, pons and cerebellar molecular layer compared to the olfactory bulbs ($P < 0.0001$). The greatest distance at which Aβ was detected from the surface of the brain was 253.18μm and this was observed in the pons. No Aβ was detected in the corpus callosum.

Regional differences in the distance of periarterial penetration of Aβ after 30 minutes in 6–10 week old mice.

Regional differences in the depth from the brain surface at which Aβ within the walls of arteries was located in the parenchyma at 30 min from the intracisternal injection, were largely similar to those detected after 5 min. The main differences were in greater depths of penetration in the olfactory bulbs and the cortical, subcortical and posterior brain regions. There was a statistically significant difference in the distance at which periarterial Aβ was detected within the parenchymal arteries in all eleven regions compared to the olfactory bulbs ($P \leq 0.0001$), Fig 4.

Regional differences in the periarterial distance of Aβ penetration into the parenchyma in 24 – 30 month old mice

After 5 minutes of injection into the cisterna magna of 24 – 30 month old mice, the greatest periarterial distance along which Aβ was detected was in the pons and the least was detected in the thalamus. The periarterial Aβ distance in the olfactory bulbs was significantly greater than the frontal cortex and the thalamus, but significantly less than the caudoputamen, tectum/tegmentum and pons ($P<0.0001$). The periarterial Aβ distance in the caudoputamen was significantly higher than in the frontal cortex, thalamus and tectum/tegmentum.

At 30 minutes, the greatest periarterial distance at which Aβ was observed in the parenchyma was no longer in the pons but in the caudoputamen, followed by the pons. Similar to the young mice, the periarterial distance of Aβ was significantly greater after 30 min compared to 5 min in all of the brain regions analysed including the olfactory bulbs, frontal cortex, caudoputamen, thalamus, tectum/tegmentum and pons (one-way ANOVA, $P < 0.0001$), Fig 5.

The comparison between the patterns of the periarterial distance of Aβ entry from the CSF into the parenchyma at 5 and 30 minutes after intracisternal injection for both young and old mice is presented in Online Resource 3.

Discussion:

The first two major objectives in the present study were: (1) to demonstrate by immunocytochemistry that the pial-glial basement membranes are the entry route into the brain for soluble tracers from the CSF and (2) to test the hypothesis that tracers entering the brain from the CSF drain out of the brain parenchyma along the

IPAD pathways that are lymphatic drainage pathways from the brain for the elimination of fluid and solutes.

To fulfil these objectives, we performed multi-labelling immunocytochemistry and confocal microscope studies on young 6-10 week old mice and on aged 24-30 month old mice, both at two separate time points of 5 and 30 mins after injection of Aβ tracer into the CSF of the cisterna magna.

Our study showed similar but not exactly identical results in mice in the two age groups. We confirmed by immunocytochemistry and confocal microscopy our previous electron microscope studies that, within 5 minutes of injection into the CSF, tracer were present in the brain within basement membranes on the outer aspects of cortical artery walls. More exactly, the electron microscope study [24] and the present results using immunocytochemistry for α-2 laminin [9] identified tracer in pial-glial basement membranes. These results suggest that one pathway for entry of tracers into the brain from the CSF is along the pial-glial basement membranes between the layer of pia mater and astrocytes in the perivascular glia limitans (figure 6). This entry pathway was observed in the young and in aged mice.

Basement membranes and cell components surrounding cortical arteries are compacted so that there is no perivascular “space” for transport of tracers [23, 37]; transport appears to be along basement membranes as shown in the present study. This contradicts the statement in the glymphatic literature that there is transport of tracers into the brain along perivascular ”spaces” [17]. These authors used low resolution two-photon microscopy which did not resolve the microanatomy of the perivascular compartment. Furthermore, the subarachnoid space appears to have been mistaken for perivascular space in the electron micrographs [17].

At 30 mins following injection into the CSF of the cisterna magna, in the present study, Aβ tracer co-localised with collagen IV in basement membranes within the walls of arteries. These basement membranes are in the position of smooth muscle cell basement membranes in the tunica media of arteries [4, 14, 27, 29] and correspond to the Intramural Peri-Arterial Drainage (IPAD) pathways that are the proposed route of drainage of interstitial fluid and solutes from the brain.

The pattern of distribution of Aβ tracer in the artery wall at 30 minutes after injection into cisternal CSF was similar in the young 6-10 week and the old 24-30 month old mice. Aβ tracer was located in the spirally arranged smooth muscle cell basement membranes within the tunica media of arteries within the cerebral cortex. This is the same IPAD pathway identified previously by intracerebral injections of tracer in mice and the same IPAD pathway in which the Aβ is deposited in CAA [5, 19, 29]. Electron microscope studies of CAA have shown that fibrillary Aβ is initially deposited in the lamina densa that forms the junction zone between basement membranes of adjacent smooth muscle cells in the tunica media [12, 38].

With the increase in volume of deposited Aβ in CAA, the mass of Aβ separates the basement membranes of adjacent smooth muscle cells [5, 19]. Although adjacent basement membranes are fused at the lamina densa in a normal artery, it appears that the two parts of the basement membrane can be separated by the deposition of amyloid [12]. Nevertheless, co-localisation of tracer with laminin in previous studies

[4] and collagen IV in the present study suggest that bulk flow of tracers, therefore of ISF and other solutes, out of the brain is along basement membranes that form the IPAD pathways.

The results of the present investigation suggest a possible route for connection between the inflow of tracers from the CSF along pial-glial basement membranes and the outflow along smooth muscle cell basement membranes in the IPAD pathway. In the older 24-30 month old mice, Aβ tracer injected into the CSF was located within astrocytes and in macrophages in the brain parenchyma at 30 minutes. This suggests that tracer had entered the extracellular, ISF compartment of the brain and been taken up by these cells. Futhermore, Aβ, tracer was located in the walls of brain capillaries associated with collagen IV in the capillary basement membranes. It seems most probable that the Aβ,tracer drains out of the brain initially along basement membranes in the walls of cerebral capillaries and then along smooth muscle cell basement membranes in the walls of cerebral arterioles and arteries. This is effectively the same IPAD pathway identified for the outflow of tracers injected directly into the brain parenchyma [4]

Previous studies have suggested that tracers entering the brain from the CSF drain out of the brain along the walls of veins [17]. This suggestion is derived not from injection of tracers into the CSF but from intracerebral injections of tracer. Veins were not conclusively identified in these studies, and it is unclear whether the tracer was even present the walls of blood vessels or in the surrounding brain tissue. In Iliff et al [17] the veins were identified by the absence of NG2Ds-Red rather than immunocytochemistry for the absence of smooth muscle actin [17]. Recent studies demonstrate that NG2Ds-Red is not specific for identification of arteries, so it is possible that some of the vessels that were NG2-DsRed negative may indeed have been smooth muscle actin positive arterioles [15].

It is also likely that in this study [17] tracer leaked from the brain into the CSF during the intracerebral injections due to the use of a large 33G needle for injecting a large 1µl volume of tracer. This would account for the appearance of tracer as oval patches resembling macrophages surrounding veins in the subarachnoid space. Furthermore, the time of examination of the brain was one hour in ref 20 rather than the much earlier time of 5min at which it has been shown that tracers, injected intracerebrally, drain out of the brain along IPAD pathways [4]. In the present study, tracer was only rarely detected in the walls of veins for short distances very near the surface of the brain and this probably represented incursion of tracer from the subarachnoid space. For example, the vein in Fig 3a shows a small amount of Aβ (red) in its wall near the surface of the brain but no Aβ in the wall further into the brain. This short ingress of tracer into the brain from the SAS along veins has been recorded in [17] and recent studies [1, 13, 27].

Other recent studies cast doubt upon the circulation of Aβ from the periarterial convective influx compartment to the perivenous compartment for clearance into the CSF [34, 35]. Our results support the convective influx of Aβ along arterial perivascular compartments in the pial-glial basement membranes, but we did not observe perivenous clearance into the CSF. This field of work is significant not only for the pathogenesis of CAA, but also for the distribution of drugs into the

parenchyma after intrathecal delivery, as well as for the interpretation of biomarkers in the CSF.

The third objective of the present study was to determine the range of depths of penetration of tracer alongside arteries in different regions of the brain at the time points of 5 and 30 min in young and aged mice. We demonstrated that the pons is the region with the highest penetration of Aβ at both ages at 5 minutes after intracisternal injection. Recently we showed in vivo that entry of contrast agent is most efficient in the midbrain of dogs and the pial-glial basement membranes were also thickest in the midbrain [10]. This suggests that the brainstem is an area most efficiently reached by therapeutic agents from the CSF, most likely also explaining the success of intracisternally administered antisense oligonucleotides in the treatment of spinal muscular atrophy (SMA) [6, 21]. SPINRAZA is an anti-sense oligonucleotide that alters the splicing of Survival Motor Neuron (SMN) pre-messenger RNA in order to increase production of full-length normal SMN protein. Following a randomized, double-blind, sham-controlled study in infantile-onset SMA where SPINRAZA (www.spinraza.com) was administered intrathecally, the motor milestones improved significantly and the numbers of deaths decreased. Our results suggest that it is possible for solutes to reach the neurons in the brainstem within 5 minutes increasing thus the understanding of how anti-sense oligonucleotides that are in development for intrathecal administration in the treatment of neurological diseases may reach their targets [31].

Although in the present study the depth at which Aβ was observed in the brain increased after 30 minutes, most was observed in the pial glial basement membranes of arteries. This suggests that therapeutic agents would need to then diffuse from the pial-glial basement membranes towards their target, reducing the concentration and therapeutic efficiency. Only 10–15% of the intrathecally administered dose of proteins in cynomolgus monkeys was observed within the parenchyma at 2.5h after injection of tracer into the CSF and by 24h this decreased to 2% [25].

The exact dynamics of biomarkers in the CSF associated with neuropathological conditions remain unclear. Although with advancement of Alzheimer's disease, there is a decrease in the amount of both Aβ1-40 and 1-42 in the CSF, there is a corresponding increase in total and phosphorylated forms of tau [16]. It is probable that the Aβ becomes entrapped in the IPAD pathways as CAA and therefore less Aβ is able to reach the CSF; this is in contrast to the more soluble tau that may be able to filter more readily along the chains of glycoproteins and proteoglycan components of the basement membranes surrounding smooth muscle cells.

In conclusion, we suggest that the present observations support the working hypothesis that tracers injected into the CSF pass into the brain along pial-glial basement membranes on the outer aspects of cortical arteries, enter the brain parenchyma and drain out of the brain along pericapillary and periarterial basement membranes that form the IPAD pathway for the drainage of ISF and solutes from the brain. In older mice, this pathway is impaired, as by 30 minutes after intracisternal injection, the tracer is observed in the capillary basement membranes, as well as in astrocytes and macrophages.

Figure legends:

Figure 1 - Anatomical entry route of Aβ into the young adult brain from the CSF at 5 and 30 minutes post-injection. Young 6-10 week old mice: *Entry of amyloid-β (Aβ) tracer into the brain from the CSF at 5 mins (a-c). Drainage of Aβ tracer from the brain along the walls of arteries at 30 mins (d-l).* (a) *5 mins*: The surface of the cerebral cortex (blue line) and a cortical artery passing into the brain with Aβ tracer red in its walls. Aβ tracer colocalized with α2-laminin in the pial-glial basement membrane (yellow) indicated by the white arrow. (b) *5 mins:* A single optical section of the artery enclosed in the box in (a) showing α2-laminin in the astrocyte basement membrane of the glia limitans on the surface of the brain (green) and co-localisation of Aβ and α2-laminin in the pial-glial basement membrane on the outer aspect of the wall of the artery (yellow) (white arrow); (c) 5mins: the profile of a cortical artery with smooth muscle cells (green) in its wall shows Aβ tracer (red) colocalized (pink) with collagen IV (blue) in the pial basement membrane on the outer surface of the artery (white arrows). *At 30 min after injection as shown in figures (d-l), the distribution of the Aβ in the artery walls is very different from that seen at 5 mins in figures (a-c).* (d) An artery identified by green smooth muscle actin in its wall (f, j) shows Aβ tracer (red, e, k) colocalized (pink) with collagen IV (blue, g, l) within the wall of the artery in a spiral or ladder-type distribution (arrows in d), closely resembling the pattern of deposition of Aβ in the walls of arteries as cerebral amyloid angiopathy (CAA); h) a single optical section from the artery in d) showing the Aβ tracer (red) between the smooth muscle actin staining (green).

Figure 2 - Anatomical entry route of Aβ into old 24–30 month old mouse brain from the CSF at 5 minutes post-injection: (a-c): Arteries showing Aβ (red) along the walls of arterioles of 10-20μm diameter labelled with collagen IV (blue) and smooth muscle actin (green); (d) An artery entering the cerebral cortex (arrow) showing Aβ (red) extending into the brain. (e): Enlargement of the artery in (d) showing laminin α-2 staining in the glia limitans on the surface of the brain (green) and co-localisation of the amyloid with laminin α-2 in the vessel wall (yellow) as indicated by the arrow. (f) single optical section of artery in e); g) single channel image of laminin α-2 from d); h) single channel image of collagen IV from d) ; i) single channel image of Aβ from d)

Figure 3 - Anatomical route for drainage of Aβ out of the brain following entry from the CSF at 30 minutes post-injection in old 24-30 month mice: **(a)** An artery and vein at the surface of the brain. The artery is identified by smooth muscle cells in its wall (green). Aβ tracer (red) is present in a ladder-like pattern in the wall of the artery. Aβ (red) in the wall of a vein is only seen at the surface suggesting that the Aβ here has entered from the CSF and not drained from the brain. Only occasional veins had Aβ in their walls. (b) Enlargement of the artery in (a) showing the ladder-like distribution of Aβ that resembles the distribution of amyloid in CAA [29]. At the surface of the brain is a branch of a leptomeningeal artery showing the ladder-like distribution of smooth muscle cells in the tunica media. The Aβ is in the intramural periarterial drainage (IPAD) pathway; c) Macrophages take up Aβ tracer at 30 min after injection. The arrows indicate Aβ (red) within macrophages stained for the macrophage marker CD163 (green); d) Astrocytes are stained for GFAP (green). Co-localisation (yellow) of Aβ and GFAP indicates uptake of Aβ tracer by perivascular astrocytes.

Figure 4 - Periarterial penetration of Aβ is of greater distance after 30 minutes than after 5 minutes in cortical, subcortical and posterior brain regions of young mice. Bar charts of periarterial Aβ distance against time after injection into cisternal CSF (5 and 30 minutes) in the olfactory bulbs (a), somatomotor area (b), somatosensory area and caudoputamen (c), thalamus and hypothalamus (d), superior colliculus, pontine reticular nucleus, entorhinal and visual areas (e) and cerebellar molecular layer (f). Values are presented as mean ± SEM of untransformed data, with p values indicated for log10-transformed data (one-way ANOVA with Sidak's post-hoc).

Figure 5 - Regional differences in periarterial distance of penetration of Aβ at 5 and 30 minutes post-injection in old mice. (a) Schematic showing a sagittal mouse brain section with five brain levels (I – V) from which distance measurements were taken in 6 brain regions at 5 minutes after injection of Aβ into cisterna magna. (Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: http://mouse.brain-map.org/static/atlas.) (b) Bar charts of periarterial Aβ distance against time after injection into cisternal CSF (5 and 30 minutes) in the olfactory bulbs (teal), somatomotor area (green), caudoputamen (blue), thalamus (salmon), superior colliculus (pink) and pontine reticular nucleus (orange). Values are presented as mean ± SEM of untransformed data, with p values indicated for log10-transformed data (one-way ANOVA with Sidak's post-hoc).

Figure 6 - Pathways for influx of CSF into the brain and drainage of CSF/ISF out of the brain along capillary and periarterial basement membranes. (1) Entry of tracers from the CSF into the brain along pial-glial basement membranes on the outer aspects of artery walls. The sites of entry of tracer into the brain extracellular compartment are unclear and could be multiple. (2) CSF enters the brain parenchyma and (3) mixes with interstitial fluid (ISF). (4) The mixture of CSF/ISF diffuses through the narrow extracellular spaces of the brain to enter (5) basement membranes in the walls of capillaries to drain out of the brain along (6) basement membranes of smooth muscle cells in the tunica media in the walls of arterioles and arteries (IPAD pathway).

Key to the Microanatomy of the capillary and artery walls: **BM1**: endothelial basement membrane. **BM2**: smooth muscle cell basement membrane identified in this study by the presence of collagen IV. **BM3**: basement membrane between pia mater (pink) and smooth muscle cell. **BM4**: pia-glial basement membrane between the pia mater and the astrocytes of the glia limitans. In this study BM4 is identified by the presence of α2-laminin [9, 13]. **Proposed route of IPAD:** Tracer is located in the capillary endothelial basement membrane (5) apparently entering from the brain parenchyma. In the artery wall, tracer is observed in the basement membranes between smooth muscle cells (light purple) but not in the basement membranes on the outer aspect of the artery wall or in the endothelial basement membrane of the artery wall (both dull blue) [4, 27]. The light green arrow indicates the proposed Intramural Peri-Arterial Drainage (IPAD) pathway for fluid and solutes out of the brain.

Online Resource legends:

Online Resource 1- Male C57BL/6 mice 10 weeks old (n=3) were anaesthesised either with isoflurane (1% with 0.8 litre/min O^2) or with 10mg/ml ketamine & 1mg/ml xylazine. The oxygen saturation, heart and breathing rates were recorded for 10 minutes using a non-invasive infrared thigh sensor attached to a MouseOx Plus Oximeter running premium software (STARR Life Sciences, Holliston, Ma, USA). The isoflurane maintained the heart rate and oxygen pressure at physiological levels when compared to ketamine&xylazine.

Online Resource 2 - Method for measuring distance of periarterial Aβ penetration into the brain. (a) Sagittal view of mouse brain illustrating the locations (I – VI) at which representative slices were taken to analyse the penetration of Aβ in the brain from the CSF. (© 2018 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: http://mouse.brain-map.org/static/atlas.) (A, I-VI) Sections were taken from the level of the olfactory bulbs, cerebral cortex, lateral ventricles, anterior hippocampus, midbrain/hindbrain and cerebellum. The total length of each level analysed per mouse (n = 3/group) was 180 μm (double headed arrow). (b) Coronal view of the brain levels selected for analysis; mouse brains for this study were sectioned coronally. (c) Confocal micrographs corresponding with the boxes in B, III (1-3). (c, 1) Micrograph illustrating two perpendicular lines, a tangent to the midline fissure in the cerebral and another line in the direction of the Aβ-positive vessel. (c, 2) Same quantification method applied to the Aβ-positive vessel in the caudoputamen in relation to the lateral ventricle (asterisk). (c, 3) Distance of Aβ-positive vessel measured in the striatum in relation to the base of the brain. Scale bar: 150 μm.

Online Resource 3 - Distance of periarterial Aβ penetration into the brain in young and old mice at 5 and 30 mins post-injection. Bar charts of periarterial Aβ distance against time after injection into cisternal CSF (5 and 30 minutes) in the olfactory bulbs (a), somatomotor area (b), caudoputamen (c), thalamus (d), superior colliculus (e) and pontine reticular nucleus (f) of young and old mice. Values are presented as mean ± SEM of untransformed data, with p values indicated for log10-transformed data (two-way ANOVA with Sidak's posthoc).

References:

1 Abbott NJ, Pizzo ME, Preston JE, Janigro D, Thorne RG (2018) The role of brain barriers in fluid movement in the CNS: is there a 'glymphatic' system? Acta neuropathologica 135: 387-407 Doi 10.1007/s00401-018-1812-4

2 Arbel-Ornath M, Hudry E, Eikermann-Haerter K, Hou S, Gregory JL, Zhao L, Betensky RA, Frosch MP, Greenberg SM, Bacskai BJ (2013) Interstitial fluid drainage is impaired in ischemic stroke and Alzheimer's disease mouse models. Acta neuropathologica: Doi 10.1007/s00401-013-1145-2

3 Aspelund A, Antila S, Proulx ST, Karlsen TV, Karaman S, Detmar M, Wiig H, Alitalo K (2015) A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. The Journal of experimental medicine 212: 991-999 Doi 10.1084/jem.20142290

4 Carare RO, Bernardes-Silva M, Newman TA, Page AM, Nicoll JA, Perry VH, Weller RO (2008) Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology. NeuropatholApplNeurobiol 34: 131-144

5 Carare RO, Hawkes CA, Jeffrey M, Kalaria RN, Weller RO (2013) Review: cerebral amyloid angiopathy, prion angiopathy, CADASIL and the spectrum of protein elimination failure angiopathies (PEFA) in neurodegenerative disease with a focus on therapy. Neuropathology and applied neurobiology 39: 593-611 Doi 10.1111/nan.12042

6 Chiriboga CA, Swoboda KJ, Darras BT, Iannaccone ST, Montes J, De Vivo DC, Norris DA, Bennett CF, Bishop KM (2016) Results from a phase 1 study of nusinersen (ISIS-SMN(Rx)) in children with spinal muscular atrophy. Neurology 86: 890-897 Doi 10.1212/WNL.0000000000002445

7 Clapham R, O'Sullivan E, Weller RO, Carare RO (2010) Cervical lymph nodes are found in direct relationship with the internal carotid artery: significance for the lymphatic drainage of the brain. Clin Anat 23: 43-47 Doi 10.1002/ca.20887

8 Cserr HF, Knopf PM (1992) Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. ImmunolToday 13: 507-512

9 Di Russo J, Hannocks MJ, Luik AL, Song J, Zhang X, Yousif L, Aspite G, Hallmann R, Sorokin L (2017) Vascular laminins in physiology and pathology. Matrix biology : journal of the International Society for Matrix Biology 57-58: 140-148 Doi 10.1016/j.matbio.2016.06.008

10 Dobson H, MacGregor Sharp M, Cumpsty R, Criswell TP, Wellman T, Finucane C, Sullivan JM, Weller RO, Verma A, Carare RO (2017) The perivascular pathways for influx of cerebrospinal fluid are most efficient in the midbrain. Clin Sci (Lond): Doi 10.1042/CS20171265

11 Engelhardt B, Carare RO, Bechmann I, Flugel A, Laman JD, Weller RO (2016) Vascular, glial, and lymphatic immune gateways of the central nervous system. Acta neuropathologica 132: 317-338 Doi 10.1007/s00401-016-1606-5

12 Frackowiak J, Zoltowska A, Wisniewski HM (1994) Non-fibrillar beta-amyloid protein is associated with smooth muscle cells of vessel walls in Alzheimer disease. Journal of neuropathology and experimental neurology 53 637-645

13 Hannocks MJ, Pizzo ME, Huppert J, Despande T, Abbott NJ, Thorne RG, Sorokin L (2017) Molecular characterization of perivascular drainage pathways in the murine brain. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism: 271678X17749689 Doi 10.1177/0271678X17749689

14 Hawkes CA, Jayakody N, Johnston DA, Bechmann I, Carare RO (2014) Failure of Perivascular Drainage of beta-amyloid in Cerebral Amyloid Angiopathy. Brain Pathol 24: 396-403 Doi 10.1111/bpa.12159

15 He L, Vanlandewijck M, Raschperger E, Andaloussi Mae M, Jung B, Lebouvier T, Ando K, Hofmann J, Keller A, Betsholtz C (2016) Analysis of the

brain mural cell transcriptome. Scientific reports 6: 35108 Doi 10.1038/srep35108

16 Hoglund K, Kern S, Zettergren A, Borjesson-Hansson A, Zetterberg H, Skoog I, Blennow K (2017) Preclinical amyloid pathology biomarker positivity: effects on tau pathology and neurodegeneration. Translational psychiatry 7: e995 Doi 10.1038/tp.2016.252

17 Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, Benveniste H, Vates GE, Deane R, Goldman SAet al (2012) A Paravascular Pathway Facilitates CSF Flow Through the Brain Parenchyma and the Clearance of Interstitial Solutes, Including Amyloid beta. Science translational medicine 4: 147ra111 Doi 10.1126/scitranslmed.3003748

18 Johnston M, Zakharov A, Papaiconomou C, Salmasi G, Armstrong D (2004) Evidence of connections between cerebrospinal fluid and nasal lymphatic vessels in humans, non-human primates and other mammalian species. Cerebrospinal fluid research 1: 2-15

19 Keable A, Fenna K, Yuen HM, Johnston DA, Smyth NR, Smith C, Al-Shahi Salman R, Samarasekera N, Nicoll JA, Attems Jet al (2016) Deposition of amyloid beta in the walls of human leptomeningeal arteries in relation to perivascular drainage pathways in cerebral amyloid angiopathy. Biochimica et biophysica acta 1862: 1037-1046 Doi 10.1016/j.bbadis.2015.08.024

20 Kida S, Pantazis A, Weller RO (1993) CSF drains directly from the subarachnoid space into nasal lymphatics in the rat. Anatomy, histology and immunological significance. NeuropatholAppl Neurobiol 19: 480-488

21 Kolb SJ, Coffey CS, Yankey JW, Krosschell K, Arnold WD, Rutkove SB, Swoboda KJ, Reyna SP, Sakonju A, Darras BTet al (2016) Baseline results of the NeuroNEXT spinal muscular atrophy infant biomarker study. Annals of clinical and translational neurology 3: 132-145 Doi 10.1002/acn3.283

22 Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, Derecki NC, Castle D, Mandell JW, Lee KSet al (2015) Structural and functional features of central nervous system lymphatic vessels. Nature: Doi 10.1038/nature14432

23 MacGregor Sharp M, Bulters D, Brandner S, Holton J, Verma A, Werring DJ, Carare RO (2018) The fine anatomy of the perivascular compartment in the human brain: relevance to dilated perivascular spaces in cerebral amyloid angiopathy. Neuropathology and applied neurobiology: Doi 10.1111/nan.12480

24 Morris AW, Sharp MM, Albargothy NJ, Fernandes R, Hawkes CA, Verma A, Weller RO, Carare RO (2016) Vascular basement membranes as pathways for the passage of fluid into and out of the brain. Acta neuropathologica: Doi 10.1007/s00401-016-1555-z

25 Papisov MI, Belov VV, Gannon KS (2013) Physiology of the intrathecal bolus: the leptomeningeal route for macromolecule and particle delivery to CNS. Molecular pharmaceutics 10: 1522-1532 Doi 10.1021/mp300474m

26 Pappolla M, Sambamurti K, Vidal R, Pacheco-Quinto J, Poeggeler B, Matsubara E (2014) Evidence for lymphatic Abeta clearance in Alzheimer's transgenic mice. Neurobiology of disease 71: 215-219 Doi 10.1016/j.nbd.2014.07.012

27 Pizzo ME, Wolak DJ, Kumar NN, Brunette E, Brunnquell CL, Hannocks MJ, Abbott NJ, Meyerand ME, Sorokin L, Stanimirovic DBet al (2017) Intrathecal antibody distribution in the rat brain: surface diffusion, perivascular transport, and osmotic enhancement of delivery. The Journal of physiology: Doi 10.1113/JP275105

28 Pollock H, Hutchings M, Weller RO, Zhang ET (1997) Perivascular spaces in the basal ganglia of the human brain: their relationship to lacunes. J Anat 191 (Pt 3): 337-346

29 Preston SD, Steart PV, Wilkinson A, Nicoll JA, Weller RO (2003) Capillary and arterial cerebral amyloid angiopathy in Alzheimer's disease: defining the

perivascular route for the elimination of amyloid beta from the human brain. NeuropatholAppl Neurobiol 29: 106-117

30 Rennels ML, Gregory TF, Blaumanis OR, Fujimoto K, Grady PA (1985) Evidence for a 'paravascular' fluid circulation in the mammalian central nervous system, provided by the rapid distribution of tracer protein throughout the brain from the subarachnoid space. Brain research 326 47-63

31 Rinaldi C, Wood MJA (2018) Antisense oligonucleotides: the next frontier for treatment of neurological disorders. Nature reviews Neurology 14: 9-21 Doi 10.1038/nrneurol.2017.148

32 Shams S, Martola J, Charidimou A, Larvie M, Granberg T, Shams M, Kristoffersen-Wiberg M, Wahlund LO (2017) Topography and Determinants of Magnetic Resonance Imaging (MRI)-Visible Perivascular Spaces in a Large Memory Clinic Cohort. J Am Heart Assoc 6: Doi 10.1161/JAHA.117.006279

33 Shinkai Y, Yoshimura M, Ito Y, Odaka A, Suzuki N, Yanagisawa K, Ihara Y (1995) Amyloid beta-proteins 1-40 and 1-42(43) in the soluble fraction of extra- and intracranial blood vessels. Annals of neurology 38 421-428

34 Smith AJ, Verkman AS (2017) The "glymphatic" mechanism for solute clearance in Alzheimer's disease: game changer or unproven speculation? The FASEB journal : official publication of the Federation of American Societies for Experimental Biology: Doi 10.1096/fj.201700999

35 Smith AJ, Yao X, Dix JA, Jin BJ, Verkman AS (2017) Test of the 'glymphatic' hypothesis demonstrates diffusive and aquaporin-4-independent solute transport in rodent brain parenchyma. Elife 6: Doi 10.7554/eLife.27679

36 Szentistvanyi I, Patlak CS, Ellis RA, Cserr HF (1984) Drainage of interstitial fluid from different regions of rat brain AmJ Physiol 246: F835-F844

37 Weller RO, Sharp MM, Christodoulides M, Carare RO, Mollgard K (2018) The meninges as barriers and facilitators for the movement of fluid, cells and pathogens related to the rodent and human CNS. Acta neuropathologica 135: 363-385 Doi 10.1007/s00401-018-1809-z

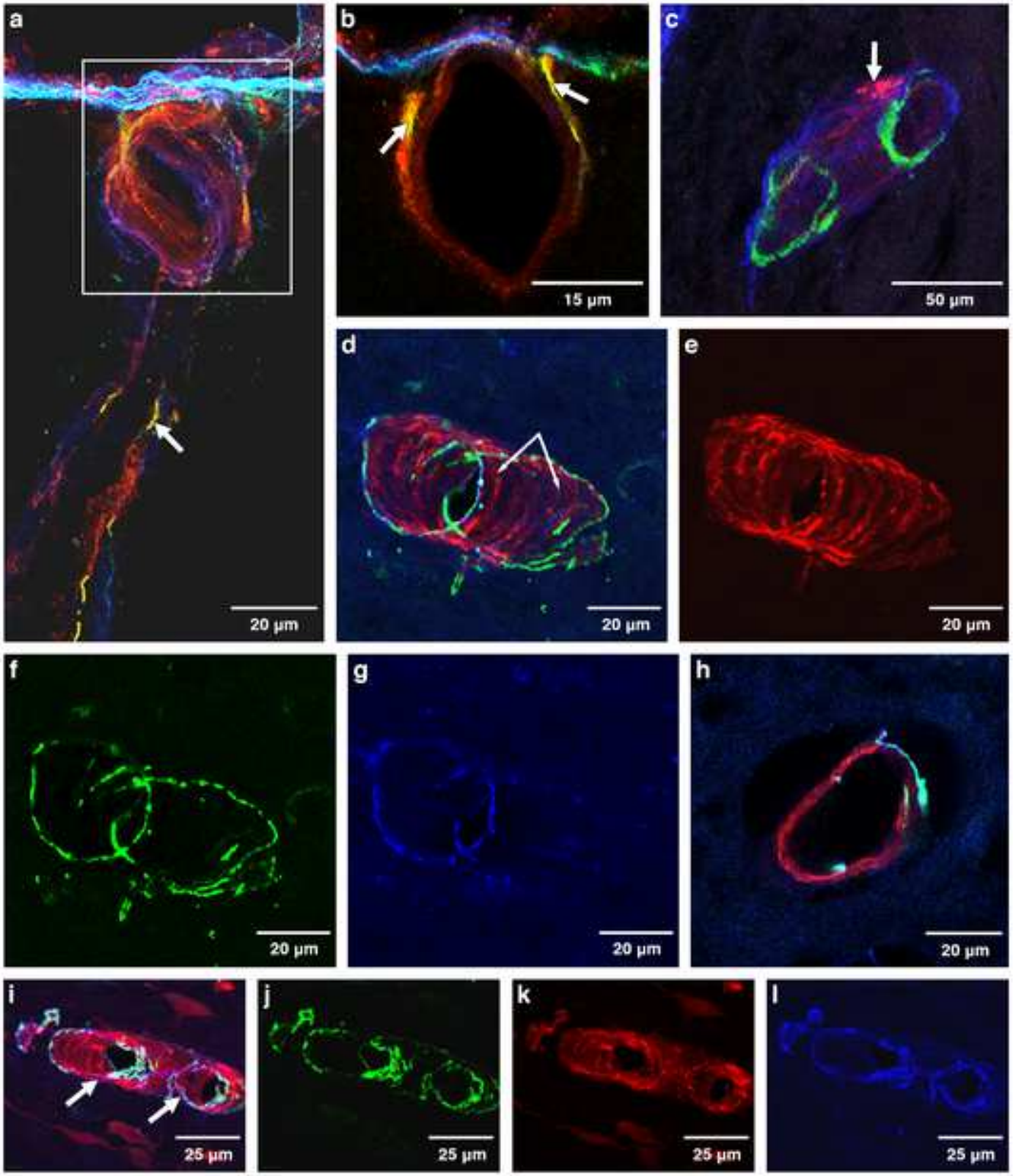
38 Wisniewski HM, Wegiel J (1994) Beta-amyloid formation by myocytes of leptomeningeal vessels. Acta neuropathologica 87: 233-241

39 Yamada S, dePasquale M, Patlak CS, Cserr HF (1991) Albumin outflow into deep cervical lymph from different regions of rabbit brain. American journal of physiology 261: H1197-H1204

40 Zhang ET, Inman CB, Weller RO (1990) Interrelationships of the pia mater and the perivascular (Virchow-Robin) spaces in the human cerebrum. J Anat 170: 111-123

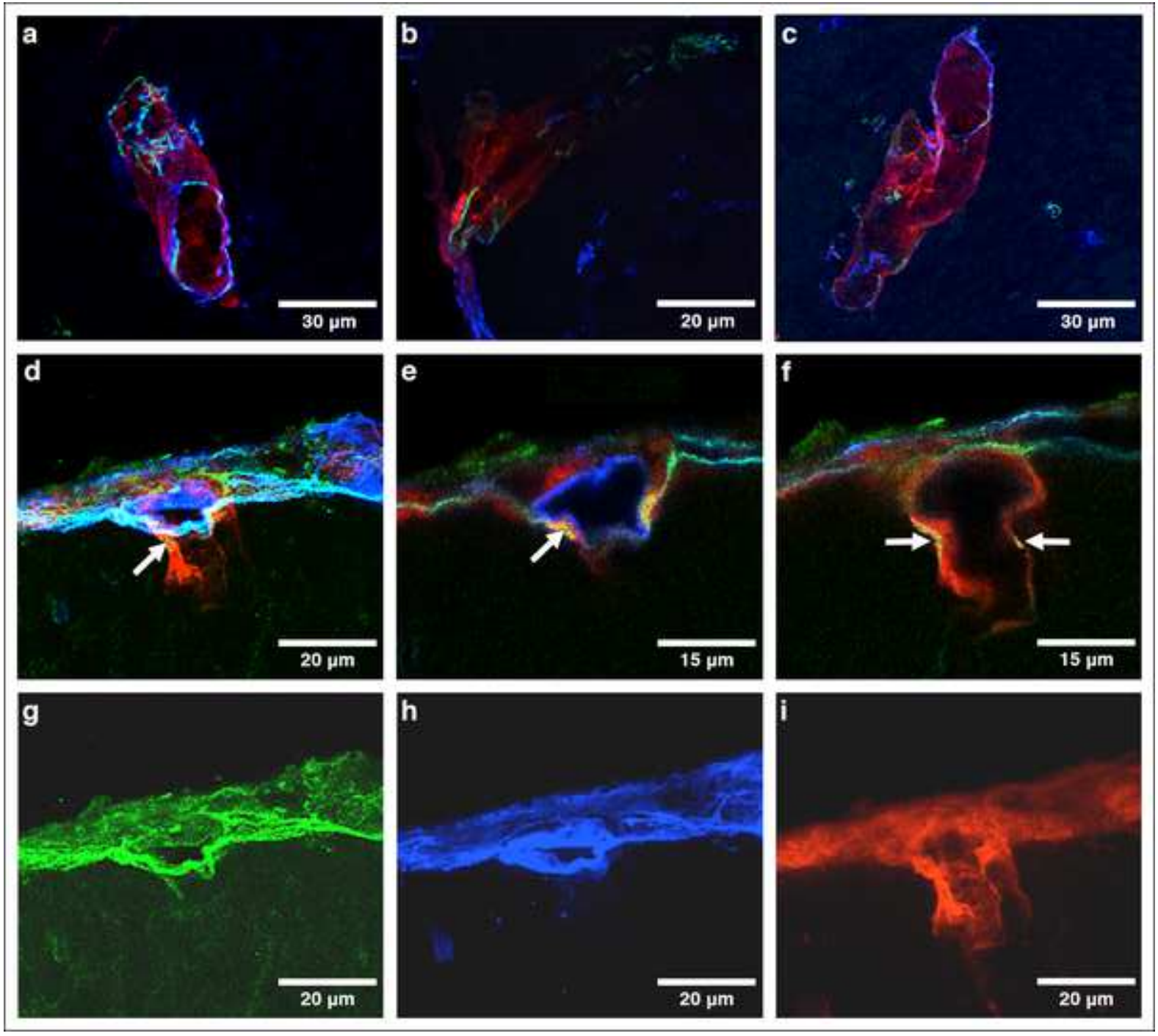
figure

Click here to download figure Figure 1 130318.tiff



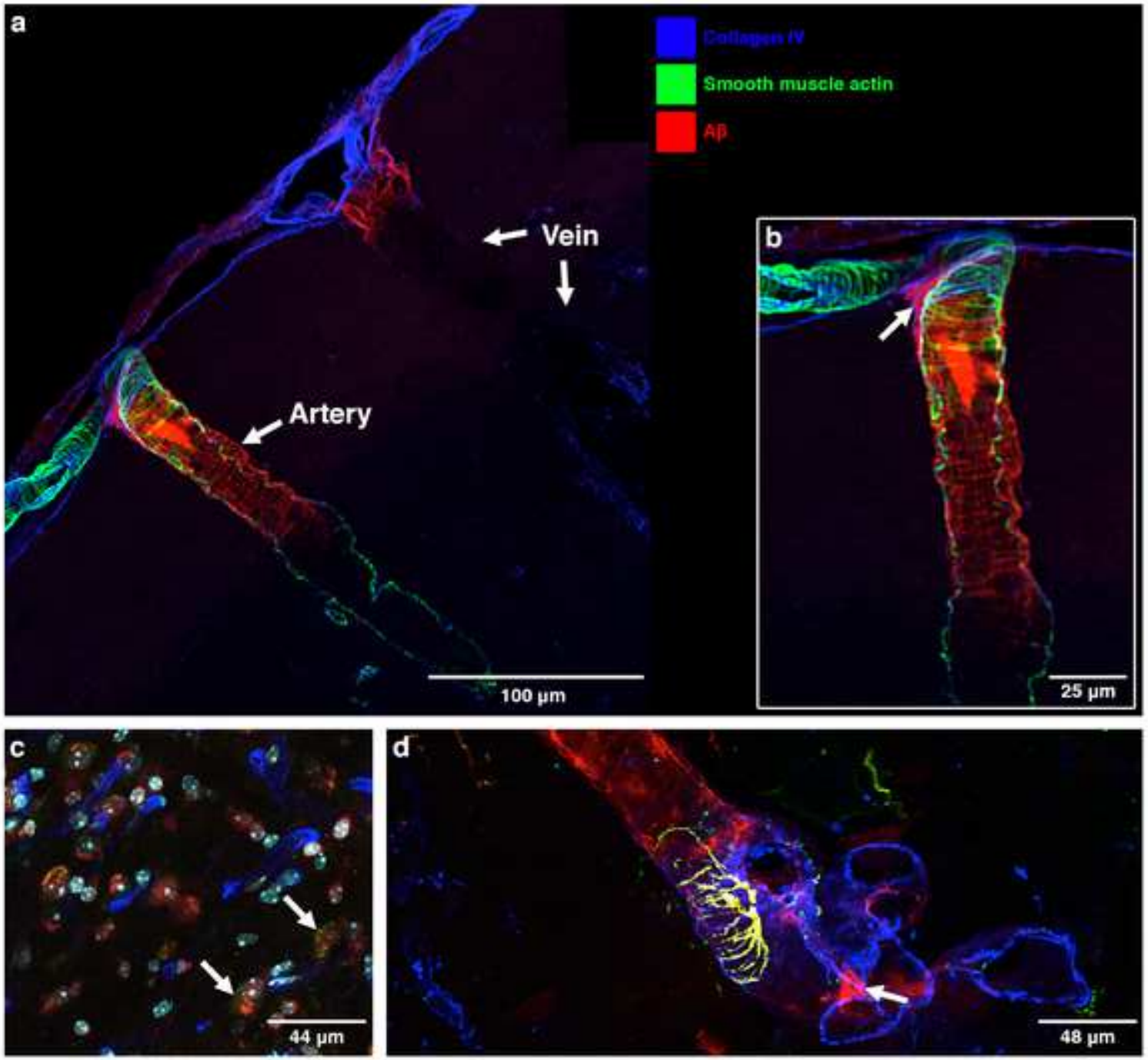
figure

Click here to download figure Figure 2 130318.tiff



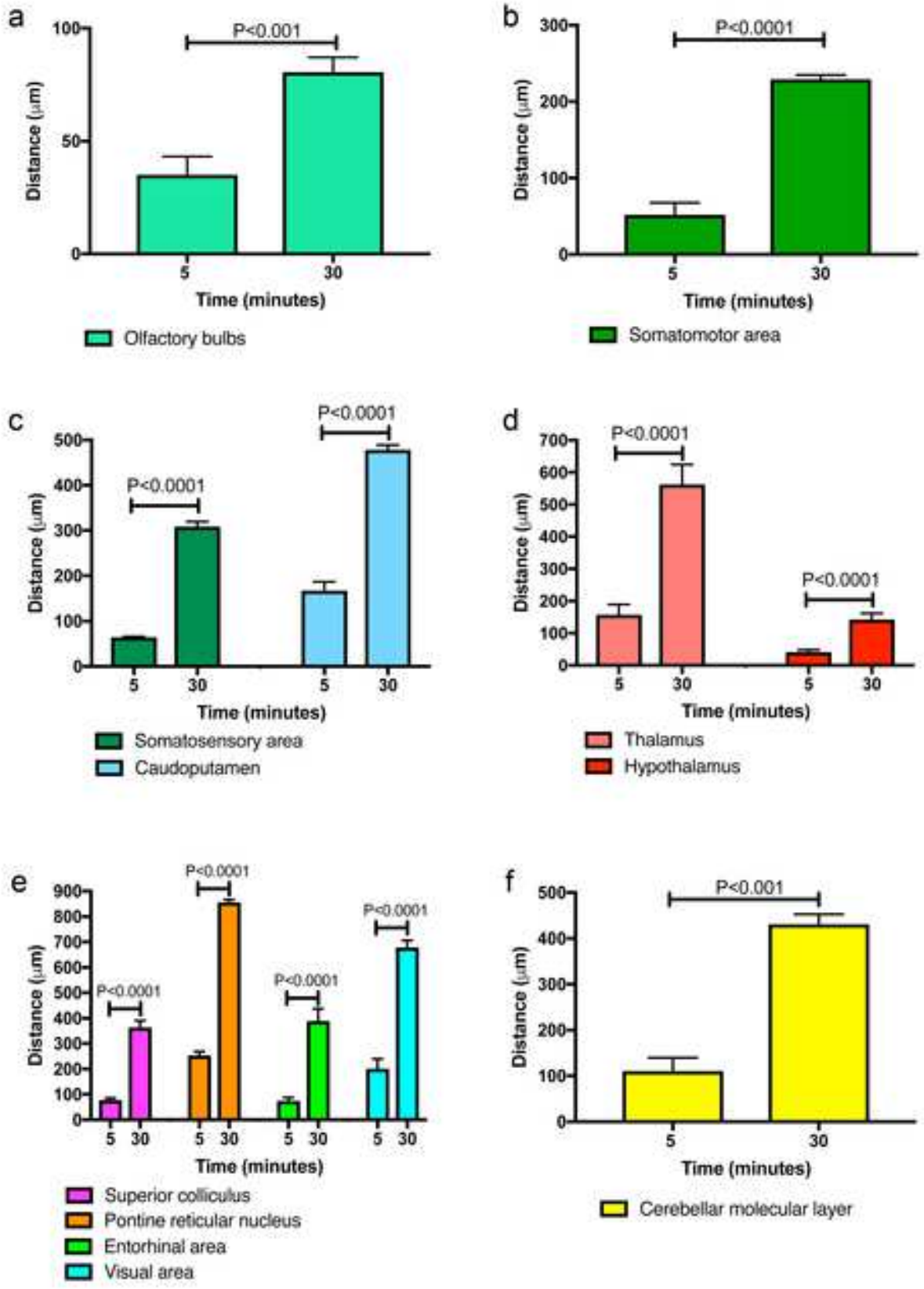
figure

Click here to download figure Figure 3 130318.tiff



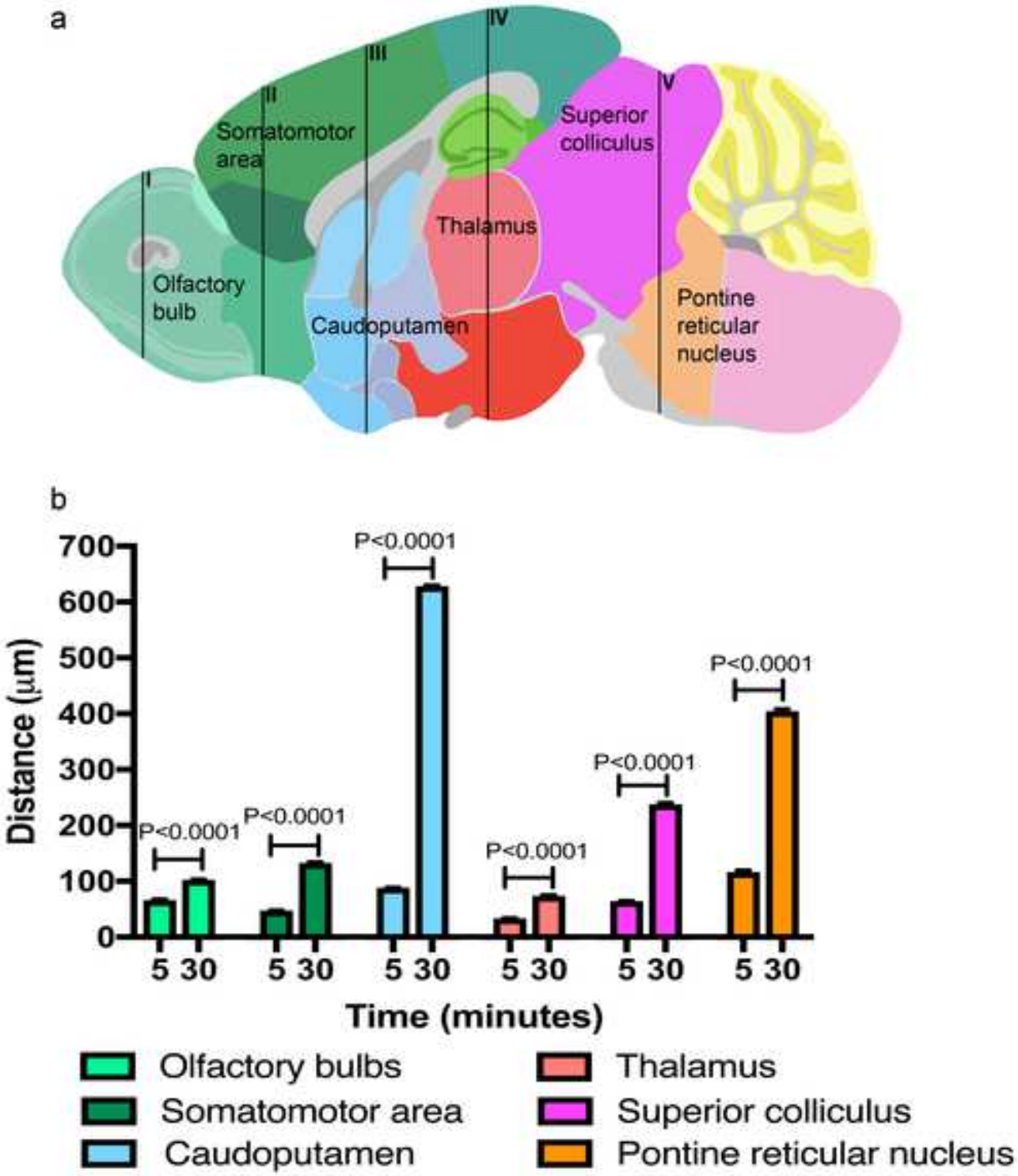
figure

Click here to download figure Figure 4 130318.tiff



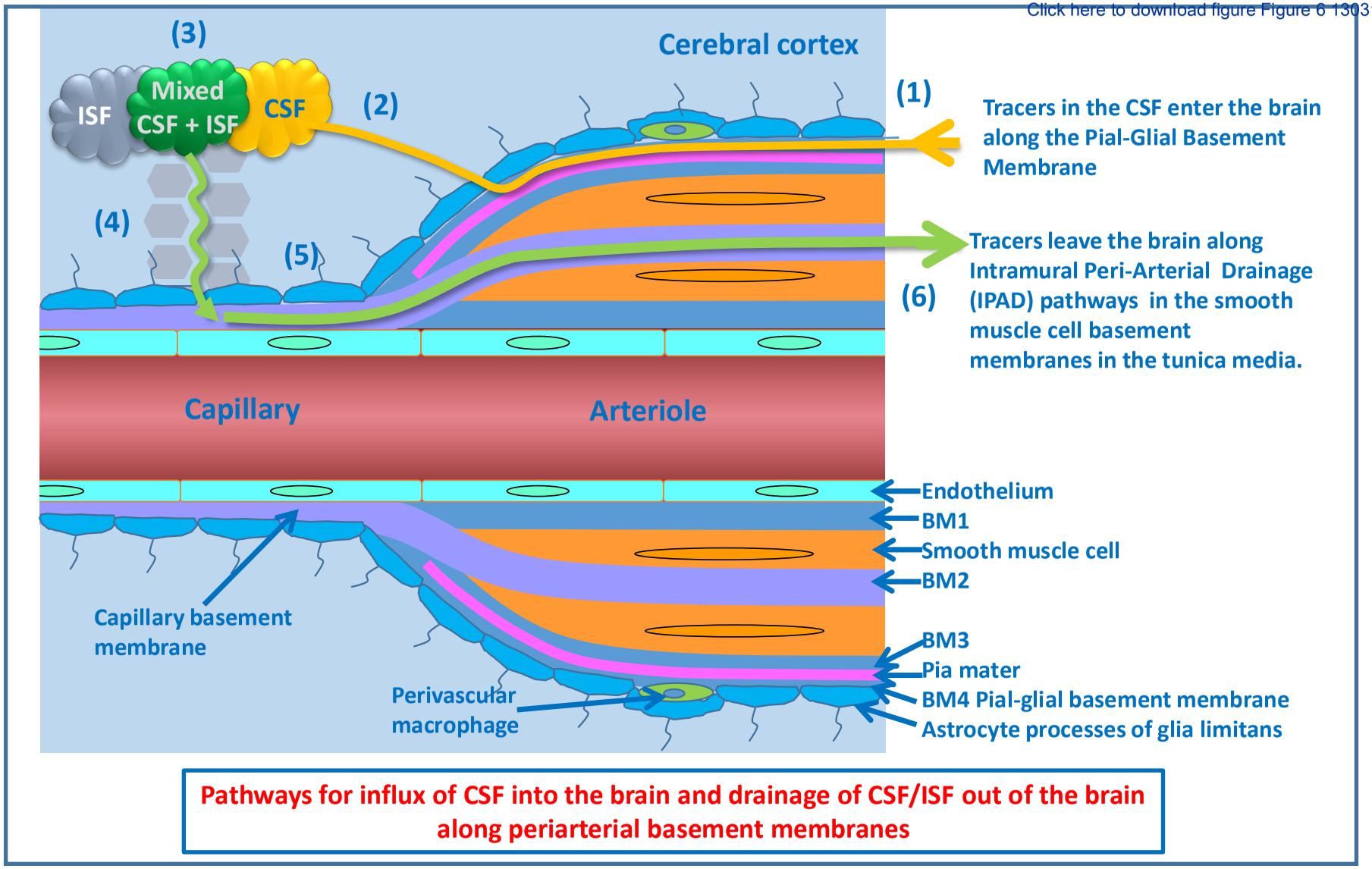
figure

Click here to download figure Fig 5 130318.tiff



figure

Click here to download figure Figure 6 130318.pptx



Pathways for influx of CSF into the brain and drainage of CSF/ISF out of the brain along periarterial basement membranes

electronic supplementary material

electronic supplementary material

electronic supplementary material