

## RESEARCH ARTICLE

# Peri-arterial pathways for clearance of $\alpha$ -Synuclein and tau from the brain: Implications for the pathogenesis of dementias and for immunotherapy

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## Abstract

**Introduction:** Accumulation of amyloid beta ( $A\beta$ ),  $\alpha$ -synuclein ( $\alpha$ Syn), and tau in dementias indicates their age-related failure of elimination from the brain.  $A\beta$  is eliminated along basement membranes in walls of cerebral arterioles and leptomeningeal arteries (intramural peri-arterial drainage [IPAD]); IPAD is impaired with age. We test the hypothesis that  $\alpha$ Syn and tau are also eliminated from the normal brain along IPAD pathways.

**Methods:** Soluble  $\alpha$ Syn or tau was injected into mouse hippocampus. Animals were perfused 5 minutes to 7 days post-injection. Blood vessels were identified by ROX-SE for light-sheet and immunolabeling for confocal microscopy. IPAD was quantified by measuring the proportion of arterioles with  $\alpha$ Syn/tau.

**Results:**  $\alpha$ Syn and tau are eliminated from the brain by IPAD but with different dynamics.

**Discussion:** Age-related failure of IPAD may play a role in the pathogenesis of synucleinopathies and tauopathies.  $\alpha$ Syn persists within IPAD at 24 hours, which may affect immunotherapy for  $\alpha$ Syn.

## KEYWORDS

Immunotherapy, IPAD, perivascular drainage, tau,  $\alpha$ -synuclein

## 1 | INTRODUCTION

Many dementias are characterized by the accumulation of amyloid beta ( $A\beta$ ),  $\alpha$ -synuclein ( $\alpha$ Syn), tau, or other proteins in the brain, indicating an age-related failure of elimination of these proteins. If preventative measures and therapeutic strategies are to be further developed for the treatment of dementias, a firm understanding is required of how soluble proteins are eliminated from the brain, why such elimination fails with age, and how elimination can be facilitated.

So far, most attention on pathways for elimination and why they fail has centered on Alzheimer's disease (AD). Plaques of insoluble  $A\beta$  are one of the hallmarks of AD but levels of soluble  $A\beta$  in the interstitial fluid (ISF) of the brain are also raised which suggests a failure of elimination of soluble  $A\beta$  and loss of homeostasis of the extracellular environment in the brain.<sup>1,2</sup> Soluble  $A\beta$  in the brain is degraded by tissue enzymes such as neprilysin, absorbed into the blood, and is also eliminated from the brain, with ISF, along peri-arterial lymphatic drainage pathways.<sup>3,4</sup> As there are no conventional lymphatics in the

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brain, ISF and soluble A $\beta$  drain to lymph nodes with other solutes along basement membranes (BMs) in the walls of cerebral capillaries and arteries to cervical lymph nodes; this has been termed intramural peri-arterial drainage (IPAD).<sup>5-7</sup> Experimental studies in mice have shown that IPAD is impaired as arteries age, and such age-related impairment in humans is associated with deposition of insoluble A $\beta$  in IPAD pathways as cerebral amyloid angiopathy (CAA).<sup>8-10</sup> In addition to the accumulation of A $\beta$  in the brain, failure of IPAD appears to contribute to white matter hyperintensities in which fluid accumulates in the white matter in association with increasing age and CAA.<sup>11</sup>

As well as A $\beta$ , insoluble and soluble  $\alpha$ Syn accumulates in the brain in Parkinson disease and Lewy body dementias.<sup>12</sup> Synucleins regulate the kinetics of synaptic vesicle endocytosis,<sup>13</sup> and in the brains of patients with Parkinson disease and Lewy body diseases  $\alpha$ Syn accumulates in neurons as insoluble Lewy bodies.<sup>14,15</sup> In normal human and mouse brains there is a level of 0.5 to 8.0 ng/mL of extracellular  $\alpha$ Syn in the ISF, but this level is higher in mouse models of Parkinson disease,<sup>16</sup> indicating a disease-related failure of elimination of  $\alpha$ Syn from the brain.

In the normal brain, tau is highly enriched in neurons in which it modulates microtubule dynamics and plays a role in mediating axonal transport and synaptic structure and functions together with neuronal signaling pathways.<sup>17</sup> Although Tau accumulates within neurons as neurofibrillary tangles and in other cells in the brain in tauopathies and Lewy body dementias it is also present in the ISF and this level is found to increase in transgenic mice overexpressing tau compared to wild type mice.<sup>18,19</sup>

Mechanisms for intracellular degradation of  $\alpha$ Syn and tau have been extensively investigated.<sup>20,21</sup> It also appears that  $\alpha$ Syn and tau are propagated between cells in the brain and this would involve  $\alpha$ Syn and tau in the ISF in intercellular spaces in the brain.<sup>22,16,23,24</sup> Immunotherapies have been introduced for the removal of  $\alpha$ Syn, tau, and A $\beta$  from the brains of patients with dementia.<sup>25</sup> Most research, however, has concentrated on immunotherapy for AD, entailing the removal of A $\beta$ . Despite the successful removal of insoluble A $\beta$  plaques from the cerebral cortex, immunotherapy for A $\beta$  is associated with a rise in soluble A $\beta$  in the ISF of the brain parenchyma and an increase in the severity of CAA.<sup>26,27</sup> Such complications suggest that impaired IPAD prevents the complete removal of A $\beta$  from the brain and therefore limits the effectiveness of the immunotherapy. In effect, impaired IPAD is a rate-limiting step in immunotherapy for AD.

To ensure maximum effectiveness of immunotherapy for  $\alpha$ Syn and tau, it is crucial to understand the pathways by which these proteins in their soluble forms are eliminated from the brain. The major objective of this study is to determine whether soluble  $\alpha$ Syn and tau drain from the brain along IPAD pathways and how the dynamics of this drainage compare with the elimination of soluble A $\beta$ . Such knowledge would indicate whether therapies to improve the efficiency of IPAD in elderly individuals should be included in protocols for immunotherapy.

In this study we test the hypothesis that  $\alpha$ Syn and tau are eliminated from the brain along IPAD pathways but possibly with dynamics that vary from those of A $\beta$ .  $\alpha$ Syn and tau were injected separately into the mouse hippocampus, and the distribution of these two proteins over

## RESEARCH IN CONTEXT

1. Systematic review: The accumulation of  $\alpha$ -synuclein ( $\alpha$ Syn), tau, and amyloid beta (A $\beta$ ) in the brain is a hallmark of dementias, indicating the failure of elimination of these proteins with advancing age. Soluble A $\beta$  drains from the brain along basement membranes in the walls of arteries; such drainage is impaired with age and after immunization against A $\beta$ . The dynamics for the drainage of  $\alpha$ Syn and tau from the brain are unclear and are the focus of this article.
2. Interpretation: Results show that both  $\alpha$ Syn and tau are eliminated from the brain along artery walls with dynamics that are similar, but not identical, to those of A $\beta$ . Age-related impairment of peri-arterial drainage for  $\alpha$ Syn and tau is likely to be an important factor in the accumulation of these proteins in aging and demented brains.
3. Future directions: Therapeutic strategies to enhance peri-arterial drainage from the aging brain will improve the elimination of  $\alpha$ Syn, tau, and A $\beta$  in the prevention of dementias, and these strategies should be considered also in the context of immunotherapy.

space and time in the brain parenchyma and in the walls of blood vessels was recorded by light-sheet and confocal microscopy at 5 minutes, 30 minutes, 24 hours, or 7 days post-injection. The results allow the dynamics of IPAD for  $\alpha$ Syn and tau to be assessed and to be compared with the IPAD dynamics for A $\beta$ .

## 2 | METHODS

### 2.1 | Animals

Male C57BL/6 10 to 11-week-old mice were housed in groups of 5-10, kept under a standard 12-hour light/dark cycle, and fed a standard RM1 chow diet (SDS, UK) and water *ad libitum*. All procedures were carried out in accordance with animal care guidelines stipulated by the United Kingdom Animals (Scientific Procedures) Act 1986, Home Office license.

### 2.2 | Intracerebral injections

#### 2.2.1 | Anesthesia

To maintain a sufficient heart rate and oxygen saturation, 2% Isoflurane was mixed with concentrated O<sub>2</sub> (1.7 L/min) and used throughout the procedure<sup>28</sup> with a standard system of anesthesia. Internal body

temperature was regulated at 37°C using a rectal probe, a homeothermic blanket, and a temperature control system (BASi).

## 2.2.2 | Labeling of tau protein

Recombinant human 2N4R-tau (Abcam, ab87400) was prepared in sterile filtered phosphate-buffered saline (PBS) (pH 8.7) using a 7K MWCO Zeba spin desalting column (Thermo scientific, 89889) following the manufacturer's instructions. The resulting tau was concentrated to 0.2 mg/mL using VivaSpin concentrator (10000 MWCO, VivaScience). The tau was added to Alexa-Fluor-488-TFP-ester (Thermo Scientific, A37570) and mixed for 1 hour on a Cleaver vortex and incubated at 4°C overnight. Unconjugated dye was removed using Zeba columns. Tau-Alexa488 conjugates contained two moles of dye per mole protein.

## 2.2.3 | Intracerebral injections

Surgery was performed using a stereotaxic frame (KOPH instruments, Model 900) with a digital manipulator (World Precision Instruments). A burr hole was created in the skull using a Tech2000 Micromotor drill (RAM Products, INC) with 0.7 mm burr. One-half microliter of either 60  $\mu$ M recombinant human  $\alpha$ Syn(1-140)-HiLyteFluor-488 (AnaSpec) or Tau-Alexa488 was injected into the right hippocampus (anterior-posterior 2 mm; medial-lateral 1.5 mm; dorsal-ventral 1.7 mm) using a 33 gauge Hamilton needle (Essex Scientific Laboratory Supplies Ltd.) and Microinjection syringe pump (UMP3T-1; World Precision Instruments) at a rate of 0.25  $\mu$ L/minute (supplementary figure 1a) The syringe was left in situ for 2 minutes to prevent reflux. Mice were killed by intracardiac perfusion of fixative after a further 5 minutes, 30 minutes, 24 hours, or 1 week ( $n = 5$  per group). Buprenorphine was administered for analgesia.

## 2.2.4 | Perfusion fixation

For confocal analysis, at the end of each time point mice were terminally anesthetized with pentobarbitone (200 mg/kg) and intracardially perfused with PBS (0.01 M) followed by 4% paraformaldehyde (PFA) (in 0.01 M PBS, pH 7.4) at a rate of 5 mL/minute. For light-sheet analysis, mice were perfused at 5 minutes or 24 hours with PBS followed by 20 mL of 40  $\mu$ M 5-Carboxy-X-Rhodamine Succinimidyl Ester (ROXSE) (to visualize blood vessels) at 3 mL/min, and then 4% PFA at 5 mL/min. Brains were immersed in 4% PFA for 3 hours at 4°C and then cryoprotected in 30% sucrose.

## 2.3 | Tissue processing for light-sheet microscopy

Brains were dehydrated in 50% to 100% methanol concentrations (12 hours each at RT). Brains were delipidated in 30% dichloromethane

(DCM) 60% methanol for 1 hour, followed by 100% DCM for 4 hours, and then cleared in Dibenzyl ether (DBE) for over 12 hours. Brains were imaged using light-sheet microscope (Ultramicroscope II, LaVision Biotec).

## 2.4 | Immunohistochemistry for confocal microscopy

Twenty micrometer coronal slices were prepared on a Leica Cryostat. Immunolabeling of  $\alpha$ -smooth-muscle-actin-Cy3 (SMA) (1:200, Sigma-Aldrich, Dorset, UK) and collagen IV (1:400, ABCAM, Cambridge, UK) was performed on sections 200  $\mu$ m anterior to the injection site (supplementary Figure S1b). Immunolabeling of perivascular macrophages using CD206 (1:200, Serotec), which is uniquely expressed on perivascular macrophages<sup>29</sup> or astrocytes (GFAP, 1:200, Dako), was performed on adjacent sections. Sections were scanned using an SP8 confocal-laser scanning microscope (Milton Keys, UK).

## 2.5 | Statistical analysis and quantification of $\alpha$ Syn-positive blood vessels

The area selected for analysis encompassed the dentate gyrus extending to the CA1 region of the hippocampus proper. Blood vessels within the hippocampal sulcus were not quantified. The number of arterioles/venules/capillaries with  $\alpha$ Syn or tau in their BMs was counted within this area and normalized to the total number of arterioles/venules/capillaries. Blood vessels were classified as capillaries (diameter <10  $\mu$ m), arterioles (SMA-positive blood vessels with diameter larger than 10  $\mu$ m), or veins (SMA-negative blood vessels with diameter larger than 10  $\mu$ m).<sup>3,9</sup> One-way analysis of variance was conducted with Bonferroni corrections for post hoc analysis.

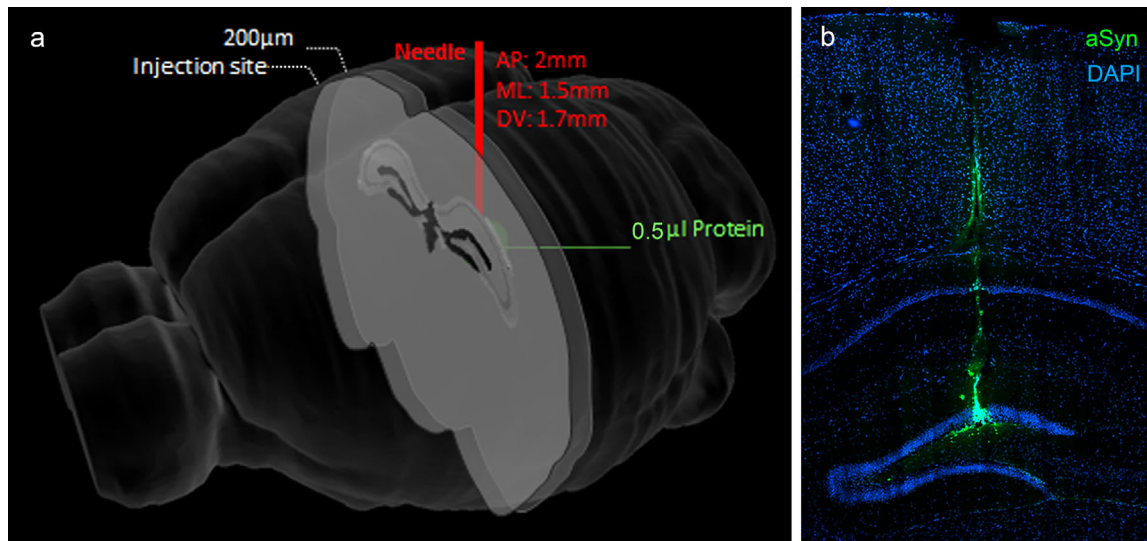
## 3 | RESULTS

### 3.1 | Distribution of $\alpha$ Syn and tau

To establish the change in overall distribution of  $\alpha$ Syn or tau tracers after their injection into the hippocampus, whole brains were imaged using light-sheet microscopy at 5 minutes or 24 hours post-injection (PI).

Figure 1A is a diagram showing the site of injection of  $\alpha$ Syn and tau, and Figure 1B illustrates the injection site of  $\alpha$ Syn into the dentate gyrus of the mouse hippocampus.

At 5 minutes PI,  $\alpha$ Syn and tau diffused within the hippocampus (Figures 2A and 3A), and did not diffuse to surrounding brain regions even by 24 hours (Figures 2B and 3B). At 5 minutes PI,  $\alpha$ Syn surrounded the transverse hippocampal arteries (THAs) and longitudinal hippocampal arteries (LHAs) (Figure 2A, Supplementary Movie 1), whereas minimal tau was localized to hippocampal blood vessels, mainly the posterior cerebral artery (PCA) (Figure 3A, Supplementary Movie 2). By 24 hours, diffuse  $\alpha$ Syn had largely disappeared and perivascular  $\alpha$ Syn



**FIGURE 1** Site of injections of both  $\alpha$ -synuclein or tau into the mouse hippocampus. A, Diagrammatic representation of a mouse brain showing the site (in red) of 0.5  $\mu$ L injections of either  $\alpha$ -synuclein or tau into the hippocampus. Brain sections for confocal microscopy and quantitative measurements were taken 200  $\mu$ m posterior to the injection site. B, Site of injection of  $\alpha$ -synuclein (green). The needle track through the cortex is seen at the top of the figure leading to the injection site in the dentate gyrus of the hippocampus (DGH). No reflux was observed and no spread of  $\alpha$ -synuclein into the subarachnoid space, cerebral ventricles, or contralateral cerebral hemisphere was detected. A similar pattern of injection was achieved with tau. The section is stained with DAPI to show nuclei (blue) in the brain parenchyma.

was observed around the THAs and LHA toward the PCA (Figure 2C,D, Supplementary Movie 3). At 24 hours PI, tau was localized to more vessels and continued along branches of the PCA toward the surface of the brain (Figure 3B, Supplementary Movie 4). At 24 hours PI, tau appeared as small puncta in the parenchyma of the hippocampus as well as around blood vessels, contrasting with the diffuse appearance at 5 minutes PI (Figure 3C,D).

### 3.2 | $\alpha$ Syn and tau enter IPAD

To investigate whether extracellular  $\alpha$ Syn and tau enter IPAD pathways, the distribution of  $\alpha$ Syn and tau was analyzed by confocal microscopy at 5 minutes, 30 minutes, 24 hours, and 7 days PI.

$\alpha$ Syn and tau were located in BMs of arterioles at 5 minutes PI, observed as regions of co-localization between the green tracer and blue collagen IV producing a cyan color (Figure 4, arrows). At 24 hours PI, there were traces of tau in arteriolar BMs (Figure 4D); however, the majority appears intracellular, possibly within perivascular macrophages (Figure 6).  $\alpha$ Syn persists in arteriolar BMs at 24 hours PI (Figure 4C) and 7 days, but only the leptomeningeal arteries of the superior hippocampal fissure contain  $\alpha$ Syn (Figure 4E). Characteristic features of IPAD are observed in Figure 4D in which the  $\alpha$ Syn was distinctly located within BMs surrounding smooth muscle cells, (Figure 4C, arrowhead).

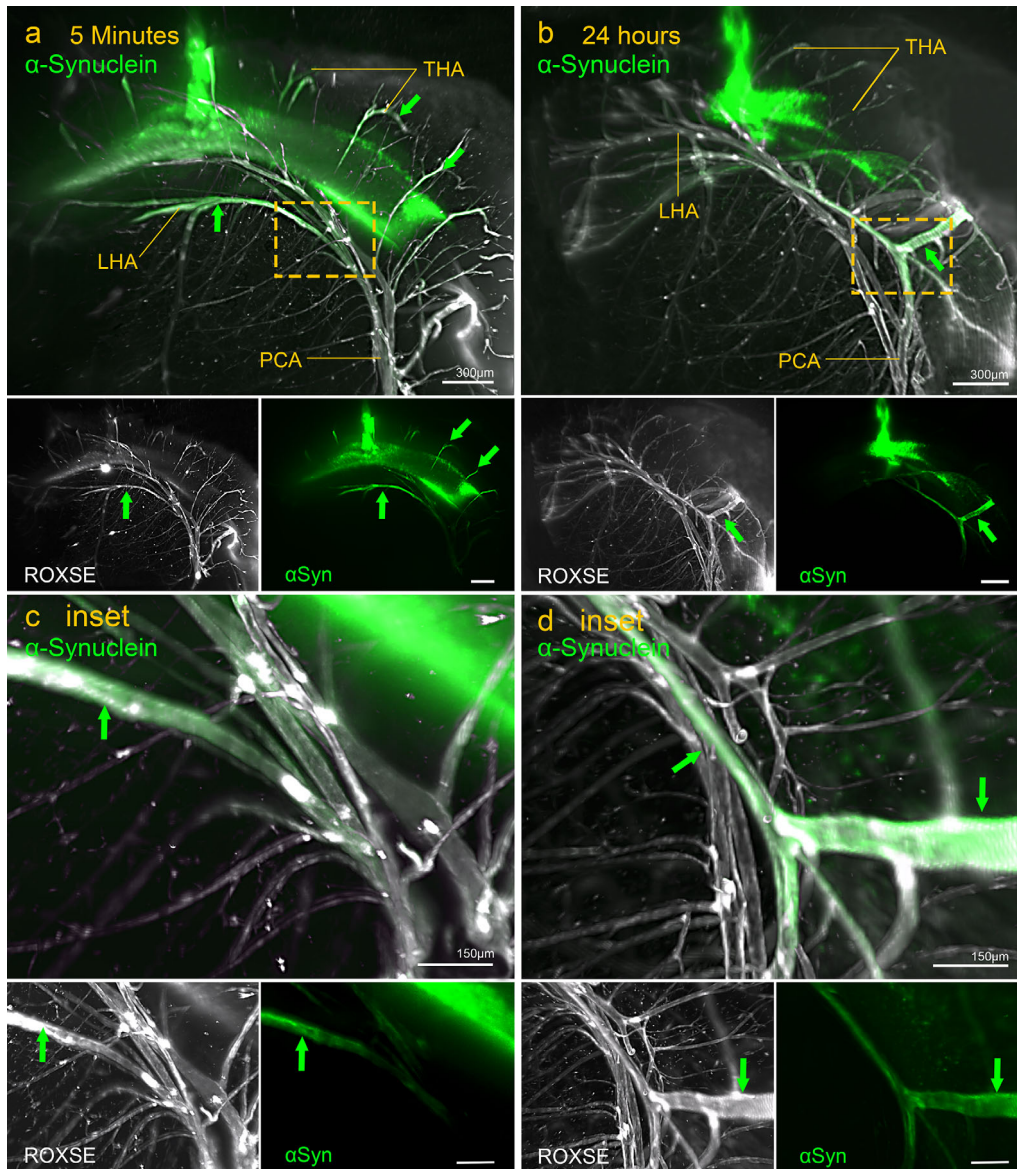
Quantification of the proportion of each blood vessel type that contained  $\alpha$ Syn or tau over time is shown in Figure 5. The majority of vessels with tracer in their BMs were arterioles (5 minutes:  $\alpha$ Syn: 60%; Tau: 40%), with <15% of venular and capillary BMs containing tracer at each

time point. By 24 hours,  $\alpha$ Syn- or tau-positive venules and capillaries decreased to 0% and 6%, respectively. No difference between the proportion of arterioles, venules, or capillaries with tau in their walls was found between 30 minutes and 24 hours PI (Figure 5B). There was a significant decline in the proportion of  $\alpha$ Syn-positive vessels over 7 days, with the highest proportion occurring between 5 and 30 minutes (Figure 5A). Although there is a slight increase in  $\alpha$ Syn-positive arterioles at 7 days compared to 24 hours, this is not significant.

To confirm that the tracer was in the BMs of vessels and not within abutting astrocyte end feet, brain sections were immunolabeled with GFAP (Supplementary Figure S1). Slight co-localization of perivascular GFAP with  $\alpha$ Syn was observed at 30 minutes but not at 5 minutes or 24 hours. Tau was occasionally taken up by perivascular astrocytes at 5 minutes PI, but not at 30 minutes or 24 hours (Supplementary Figure S1b, d, f).

### 3.3 | Tau, but not $\alpha$ Syn, is taken up by perivascular macrophages (PVMs)

At 24 hours PI, perivascular tau is mainly within cells rather than in BMs (Figure 4D). To establish the perivascular location of tau at 24 hours, brain sections were immunolabeled with the PVM marker, CD206.  $\alpha$ Syn did not co-localize with CD206 at 24 hours (Figure 6A), or at any other time point. Tau, however, was found within CD206-positive PVM at 24 hours (Figure 6B), but not at 5 to 30 minutes. The tau-positive perivascular macrophages (PVMs) were mainly surrounding cortical arterioles, leptomeningeal arteries, a few veins, and some capillaries.



**FIGURE 2** Light-sheet images showing the distribution of  $\alpha$ -synuclein ( $\alpha$ Syn) at 5 minutes (left-hand images) and 24 hours (right-hand images) post injection (PI). Green arrows indicate arteries with  $\alpha$ -synuclein in their walls. A, At 5 minutes PI,  $\alpha$ Syn (green) is diffusely distributed within the dentate gyrus and in the walls of arteries (white) in the hippocampus. The transverse hippocampal arteries (THAs) and longitudinal hippocampal arteries (LHAs) that arise from the posterior cerebral artery (PCA) have  $\alpha$ Syn in their walls (green arrows). Smaller images (below), separately stained white for blood vessels by ROXSE and green for  $\alpha$ Syn show that branches of THAs (arrows) have  $\alpha$ Syn in their walls. The boxed area in (A) is enlarged in (C) inset. B, At 24 hours PI, the diffuse  $\alpha$ Syn in the dentate gyrus is notably reduced as is the  $\alpha$ Syn surrounding the THAs and LHAs. Periarterial  $\alpha$ Syn (green arrows) at 24 hours PI is more prominent in the walls of larger arteries like the PCA (see images separately stain for ROXSE and  $\alpha$ Syn below). The boxed area is enlarged in (D) inset. Comparing 5 minutes, (C) inset, with 24 hours, (D) inset. Perivascular  $\alpha$ Syn (green) is more prominent in the walls of larger arteries (green arrows) at 24 hours (D) inset than at 5 minutes (C) inset.

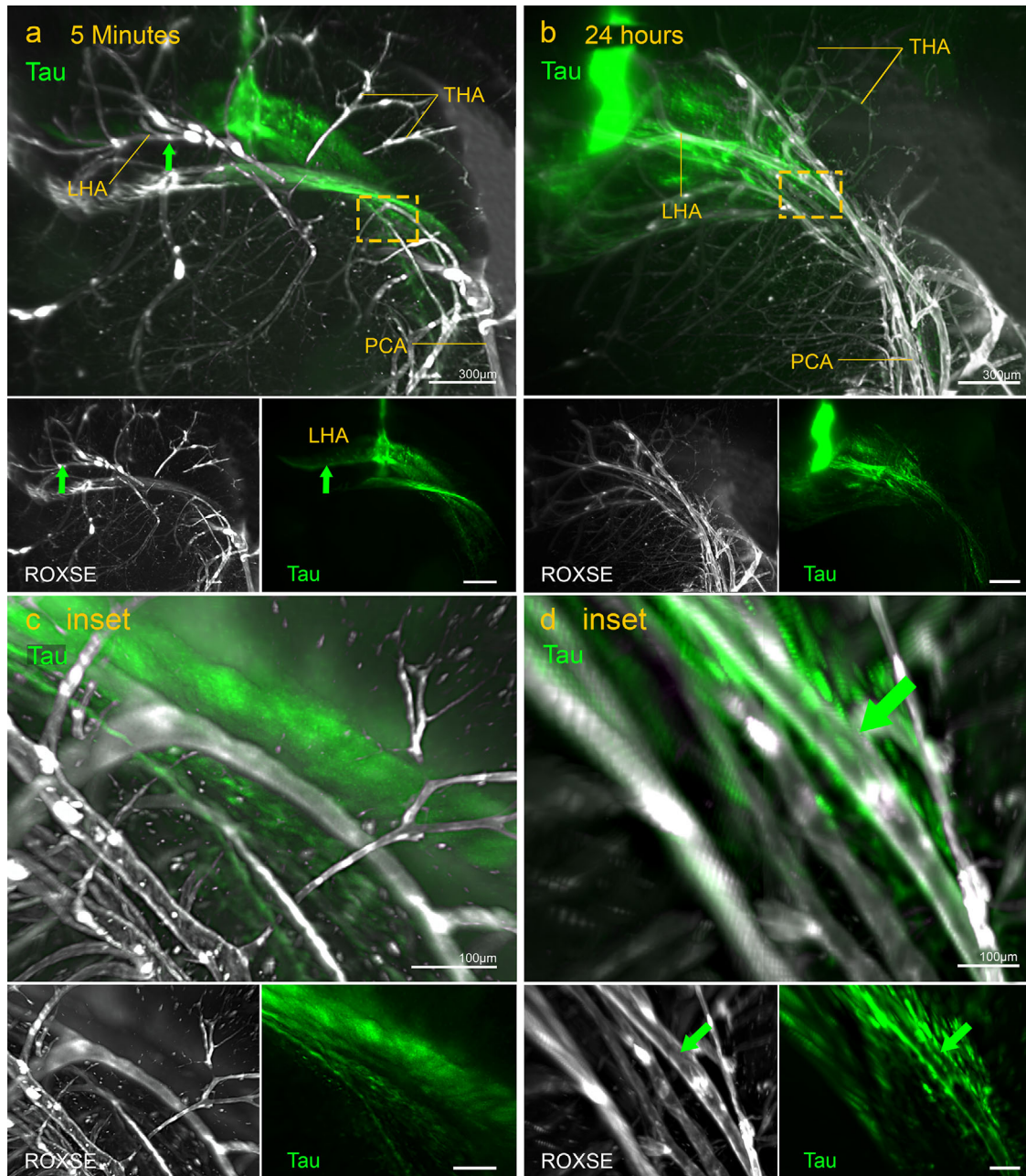
## 4 | DISCUSSION

### 4.1 | $\alpha$ -Synuclein and tau are eliminated from the normal brain along periarterial pathways (IPAD) but with different dynamics

In the present study, light-sheet microscopy and confocal microscopy have shown that  $\alpha$ Syn and tau are eliminated from the brain along

intramural peri-arterial drainage (or IPAD) pathways in the walls of cerebral arterioles in a manner similar to the elimination of  $A\beta$  and other solutes.<sup>3,9</sup> This has important implications for the pathogenesis and management of dementias in which  $\alpha$ Syn and tau accumulate in the brain.

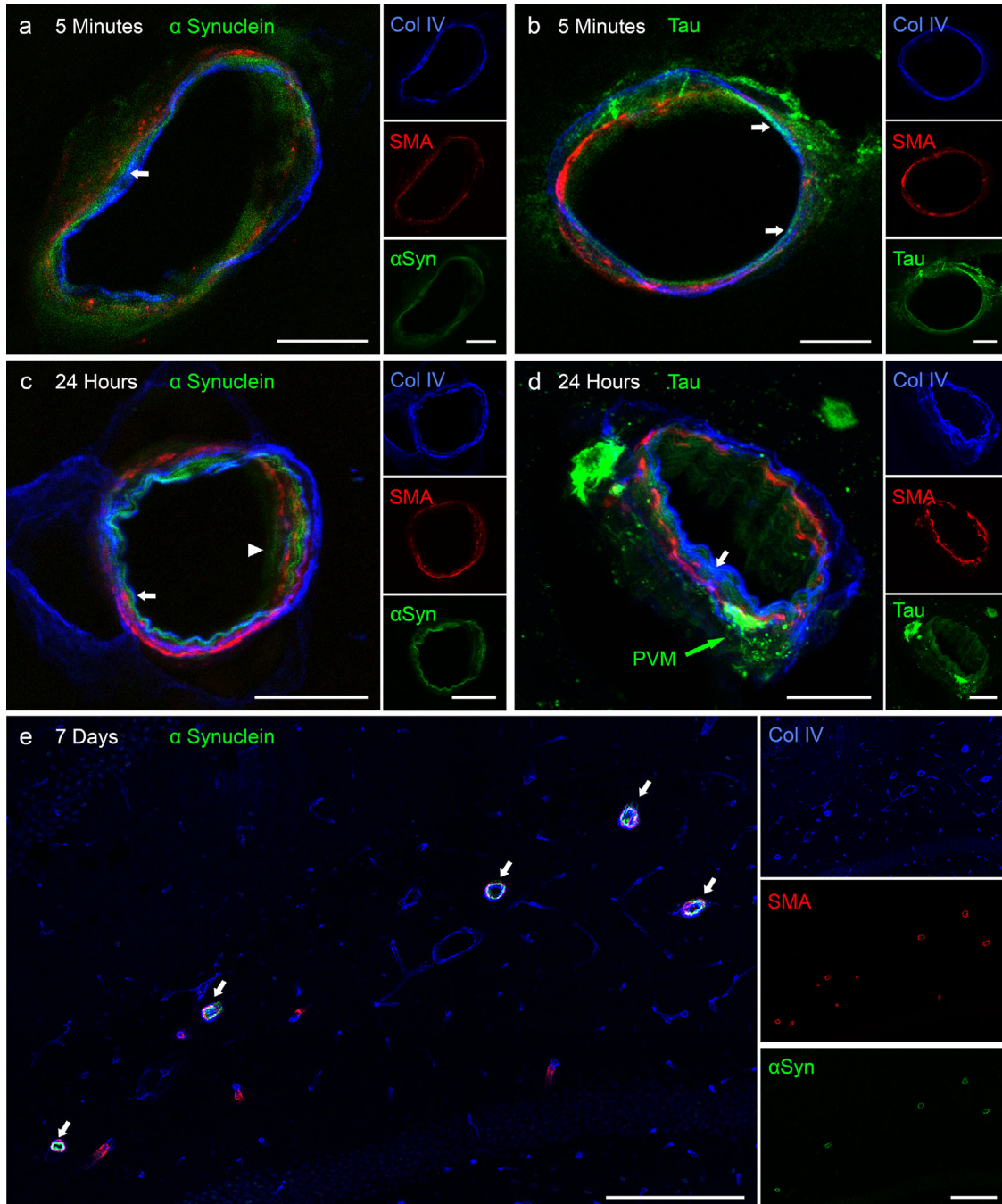
There are some differences in the dynamics of IPAD between  $A\beta$ ,  $\alpha$ Syn, and tau. Examination of the drainage pattern of tau and  $\alpha$ Syn through the whole hippocampus by light sheet microscopy showed



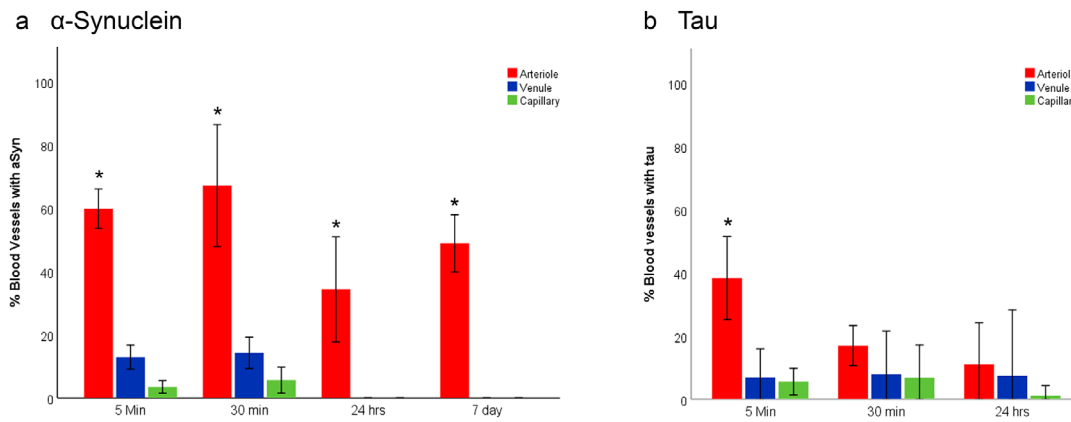
**FIGURE 3** Light-sheet images showing the distribution of tau (green) at 5 minutes (left-hand images) and 24 hours (right-hand images) post-injection (PI). Green arrows indicate arteries with tau in their walls. A, tau (green) at 5 minutes PI is diffusely distributed within the dentate gyrus and in the walls of the hippocampal vasculature (white), in particular, branches of the longitudinal hippocampal arteries (LHAs) (see green arrows in (A) and in images stained for ROXSE and tau below). The boxed area is reproduced at higher magnification in (C) inset. B, At 24 hours PI, there is still some diffuse tau that is faintly detectable in the walls of the transverse hippocampal arteries (THA) or longitudinal hippocampal arteries (LHA), which branch from the PCA (see (D) inset). However, tau is present in oval profiles (see green arrow in (D) inset) that could represent perivascular macrophages (see Figure 6).

that tau is not present within as many blood vessel walls as  $\alpha$ Syn or  $A\beta$ . The number of arterioles with tau in their BMs (IPAD pathways) at 5 minutes PI is less than half of what has been reported for  $A\beta$ ,<sup>9</sup> suggesting that tau is not as readily cleared by IPAD as  $A\beta$  or  $\alpha$ Syn. However, when tau enters IPAD pathways it exhibits dynamics similar to those of  $A\beta$ .<sup>3,9</sup> In young mice, both  $A\beta$  and tau enter IPAD pathways in the walls of arterioles within 5 minutes of injection into the brain,

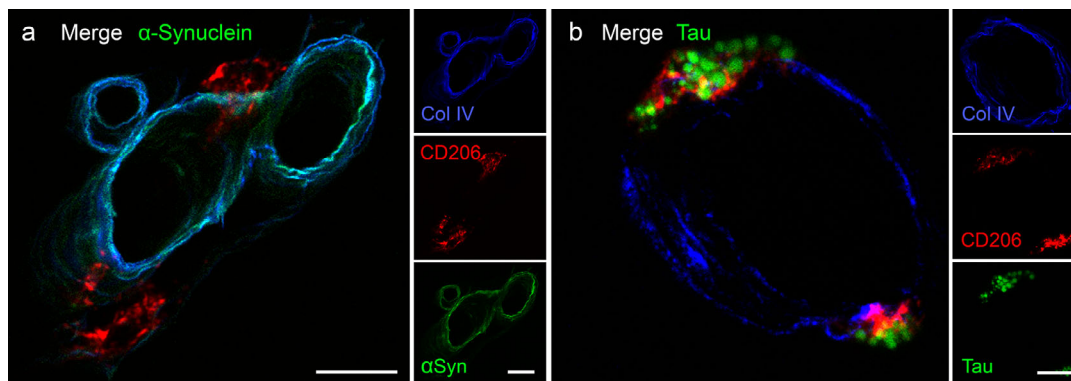
and by 24 hours, most of the  $A\beta$  and tau have disappeared from the drainage pathways but remain in perivascular macrophages.  $\alpha$ Syn exhibits slightly different dynamics for IPAD; at 5 minutes after injection,  $\alpha$ Syn is in IPAD pathways of hippocampal arterioles but in contrast to  $A\beta$  and tau,  $\alpha$ Syn is still present and easily detectable in IPAD pathways at 24 hours and 7 days PI. It is unlikely that this is due to  $\alpha$ Syn becoming "stuck" within the arteriole walls, as light-sheet



**FIGURE 4** Distribution of  $\alpha$ -synuclein and tau within the walls of hippocampal arteries of adult mice. A,  $\alpha$ -synuclein ( $\alpha$ Syn - green) is diffusely distributed within the collagen IV of basement membranes (BMs - blue) of arterioles at 5 minutes (arrow) and (C) at 24 hours (arrows). B, At 5 minutes PI, tau (green) is also diffusely distributed within collagen IV in BMs (blue) of arterioles (arrows). D, At 24 hours PI, very little tau (green) remains within the BMs of arterioles (arrow). Tau is also present in cells of the brain parenchyma surrounding the vessel. The cell closely applied to the vessel wall may be a perivascular macrophage (PVM). E, In contrast to tau,  $\alpha$ Syn remains within BMs of arterioles in the superior hippocampal fissure (arrows) at 7 days. Co-localization of  $\alpha$ Syn with collagen IV can be seen as cyan regions in the arterioles (arrows). Characteristic pattern of IPAD is seen in (c) (arrowhead) as  $\alpha$ Syn (green) spirals around smooth muscle cells (red) within the collagen IV basement membrane (blue). Scale bar = 10  $\mu$ m



**FIGURE 5** Quantification of the change in proportion of blood vessels containing  $\alpha$ -synuclein (A) or tau (B) over time. A, There is a significant reduction in the proportion of arterioles, venules, and capillaries containing  $\alpha$ -synuclein or tau at 24 hours. In the first 24 hours, there is a 25% to 33% decrease in the number of arterioles with  $\alpha$ -Synuclein within their walls. This decreased significantly to 20% to 40% by 24 hours ( $P < .014$ ). The proportion of venules and capillaries containing  $\alpha$ -synuclein was significantly lower than arterioles at all time points ( $* = P < .0001$ ). By 24 hours there were no venules or capillaries with  $\alpha$ Syn in their basement membranes (BMs) ( $P \leq .001$ ). B, At 5 minutes PI of tau, there is a significantly higher proportion of arterioles (31% higher) compared to venules and capillaries with tau in their BMs ( $* = P < .0001$ ). The proportion of arterioles with tau in their walls decreases significantly by 21% in the first 30 minutes PI ( $P = 0.01$ ). This decreases significantly by a further 6% by 24 hours ( $P = 0.002$ ). There is no difference in the proportion of venules and capillaries with tau in their walls over 24 hours. (one-way ANOVA with Bonferroni corrections for multiple comparisons). Error bars represent  $\pm 2SD$



**FIGURE 6** Perivascular macrophages (PVMs) are involved in the clearance of tau but not of  $\alpha$ -synuclein at 24 hours following hippocampal injection in 10-week-old mice. A, No co-localization is seen between CD206-positive PVMs (red) and  $\alpha$ -synuclein (green) at 24 hours PI or any other time point (data not shown). B, At 24 hours PI of tau, CD206-positive PVMs (red) have engulfed punctate aggregates of tau (green). Scale bar = 10  $\mu$ m

imaging clearly showed that  $\alpha$ Syn moved away from the THAs and LHAs toward the PCA over time (Figure 2).  $\alpha$ Syn does not appear to be taken up by PVMs as is the case with tau and  $A\beta$ .<sup>30</sup> This may be due to the mechanism of uptake in which tau is more readily taken up by macropinocytosis,<sup>31</sup> but internalization of  $\alpha$ Syn has been found to occur in a receptor-dependent mechanism.<sup>32,33</sup> The differences in dynamics between  $\alpha$ Syn and tau may be due to interactions between  $\alpha$ Syn and BMs in the IPAD pathways or, in the present experiments, it may be due to aggregates forming in the  $\alpha$ Syn solution injected into the mice as shown in supplementary Figure S2. Previous studies have shown that particulate nanospheres of diameters  $>15$  nm do not enter IPAD pathways but remain within the brain parenchyma,<sup>34</sup> suggesting that larger aggregates may not drain as efficiently as soluble monomers. In addition to arterioles,  $\alpha$ Syn and tau were located around

capillaries and veins. Capillary walls are the initial pathways for IPAD, but there is no firm evidence that veins are involved in perivascular drainage of solutes from the brain. Some studies have suggested that veins are pathways for elimination of solutes from the brain into the lymphatic system.<sup>35</sup> There are, however, major problems with these studies: (1) brains were examined 1 hour after the injection of tau or  $A\beta$ , so the time of maximum drainage along IPAD at 5 to 30 minutes had passed<sup>3</sup>; (2) IPAD appears to be driven by waves of smooth muscle cell contraction in the walls of arteries (vasomotion)<sup>36</sup>; (3) veins have few if any smooth muscle cells in their walls, so the motive force for perivascular drainage appears to be absent, whereas there is a continuous route along the walls of arteries in the brain to cervical lymph nodes no such route along veins has been characterized.<sup>37</sup> In CAA, insoluble  $A\beta$  is deposited in IPAD pathways in the walls of cerebral arteries,



whereas only minimal amounts of A $\beta$  are associated with veins.<sup>6</sup> It seems, therefore, that IPAD is the major route for drainage of ISF, A $\beta$ ,  $\alpha$ Syn, tau, and other solutes from the brain to cervical lymph nodes and that there is no firm evidence for a perivenous route of drainage.

Impairment of IPAD associated with aging of cerebral arteries also plays a major role in the accumulation of A $\beta$  in the brain in AD and in the deposition of A $\beta$  in the IPAD pathways in CAA.<sup>9</sup> CAA with the accumulation of insoluble A $\beta$  and other proteins in IPAD pathways is major evidence that IPAD is involved in the drainage of solutes from the brain in humans.<sup>9,38</sup> The presence of tau in the walls of arteries in human brains has been reported,<sup>39</sup> but tau does not accumulate as CAA in the same way as A $\beta$ . Although A $\beta$  shows a firm propensity to form fibrillary amyloid in IPAD pathways in CAA, tau does not appear to exhibit such properties of aggregation. There are no reports of  $\alpha$ Syn in the walls of arteries in the human brain; however, it may be that angiopathy occurs only when a threshold of concentration of  $\alpha$ Syn is reached. The results of the present study suggest that IPAD is a route for elimination of soluble  $\alpha$ Syn from the brain, and therefore any intervention that may increase  $\alpha$ Syn in the extracellular spaces may overcome the capacity of the clearance pathways for  $\alpha$ Syn and may result in disruption of homeostasis and possibly also angiopathy, with other proteins building up as the normal capacity for IPAD is impaired.

#### 4.2 | Facilitating removal of $\alpha$ -synuclein, tau, and A $\beta$ by IPAD in the prevention and treatment of dementias and as an adjunct to immunotherapy

The finding that  $\alpha$ Syn and tau drain from the brain along IPAD pathways in a manner similar to A $\beta$  has implications for both treatment and immunotherapy for  $\alpha$ Syn- and tau-related dementias. Clinical trials to identify drugs that will facilitate the drainage of A $\beta$  along the impaired IPAD pathways by stimulating the vascular smooth muscle cells as the motive force for IPAD are underway.<sup>40,41</sup> Immunization trials for A $\beta$  are showing promise as reported very recently, with reductions in amyloid, phospho-tau, and neurofilament light.<sup>42</sup>

Anti-tau immunotherapy attempts are ongoing<sup>43</sup> and two more recent active immunization trials are demonstrating modest improvements in neurodegeneration.<sup>44</sup> Although the exact mechanisms for the action of the vaccines are not known, there is evidence for the uptake of tau by glial cells.<sup>45</sup> Our present work shows the involvement in perivascular macrophages in the clearance of tau. Tau oligomeric specific monoclonal antibodies clear the brain of synuclein deposits as well as toxic forms of oligomeric synuclein,<sup>46</sup> but the mechanism requires clarification.

Different immunotherapy compounds against  $\alpha$ Syn are under development<sup>47,48,43</sup> and one aspect to consider is that if immune complexes do form as in anti-A $\beta$  immunotherapies, in high doses they may block the IPAD pathways, as we show here that  $\alpha$ Syn drains along the BMs of arterioles and remains there for 24 hours after its intraparenchymal injection.<sup>49,50</sup> Our results support the importance of IPAD for the clearance of  $\alpha$ Syn and tau from the brain, and this should

be considered in the context of immunotherapy trials against  $\alpha$ Syn or tau.

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#### DECLARATION OF INTERESTS

The authors have no competing interests to declare.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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