# Neurovascular ageing: transcriptomic readout and implications on therapeutic targeting in Alzheimer's disease

**Zhongqi Li, Bonaventure Ip, Vincent C. T. Mok, Ho Ko***

Ageing is one of the greatest risk factors for neurodegenerative diseases. How the complex biological changes in ageing increase the brain's susceptibility to neurodegeneration remains incompletely understood. Research into neurodegenerative disorders has shifted from a neuron-centric approach, to the contributing roles of age-related neurovascular and glial cell dysfunction. Empowered by single-cell transcriptomic techniques, the molecular diversity of neurovascular and glial cells can now be profiled in very high throughput. Questions arise, on: (i) whether the fine molecular subtypes of cells, such as the brain endothelial cell (EC) subtypes that segregate along the arteriovenous axis (Vanlandewijck et al., 2018), may age differently, and (ii) if the age-related changes are amenable by pharmacological agents. A recent study by our team reported the ageing-associated, genome-wide expression changes in mouse brain EC subtypes (Zhao et al., 2020). Apart from uncovering EC subtype-dependent transcriptomic alterations and their functional implications at the blood-brain barrier (BBB), we provided additional evidence linking vascular changes and Alzheimer's disease (AD). Importantly, we showed that both the transcriptomic and the functional changes in the aged mouse brain ECs are partially reversible by treatment with exenatide, a glucagon-like peptide-1 receptor (GLP-1R) agonist (GLP-1RA). These findings thus provide detailed insights into brain endothelial ageing and imply the therapeutic potential of GLP-1RA in the treatment of age-related vasculopathy.

The role of neurovascular dysfunction in age-related neurodegenerative conditions, such as AD, has been established over the past few decades. On post-mortem neuropathological examinations, as many as 80% of AD patients harbor varying severity of microvascular pathology (Sweeney et al., 2019). In fact, it seems more accurate to consider vascular dementia (VaD) and AD as a continuum rather than distinct diseases. Is neurovascular dysfunction in AD a mere association due to ageing, or could it also causally contribute to AD pathogenesis? Epidemiological and mechanistic studies suggest the latter. In humans, various midlife cardiovascular risk factors, such as diabetes, obesity and hypertension, increase the risk of late-onset AD, and/or are associated with increased amyloid plaque and neurofibrillary tangle depositions (Sweeney et al., 2019). Reduced cerebral blood flow (CBF), impaired neurovascular coupling (NVC), and increased BBB leakage, have been reported in prodromal AD (Montagne et al., 2015; Korte et al., 2020). BBB breakdown, in particular, appears to be a prominent feature whereby its severity correlates with cognitive deficits (Montagne et al., 2015). In animal studies, knockin of human genes causing familial AD (e.g. mutant amyloid precursor protein and/or presenilin) or associated with increased risk of AD (e.g. *APOE4* (apolipoprotein E4)), cause neurovascular dysfunction that precedes neuronal and behavioural deficits (Montagne et al., 2017). Of note, in a human mutant amyloid precursor protein-based model AD model which typically does not develop tauopathy, heterozygous *Pdgfrb* (platelet-derived growth factor receptor β) deletion-induced pericyte loss and vascular deficits led to tau hyperphosphorylation and deposition (Montagne et al., 2017). These evidences suggest that the onset of neurovascular dysfunction is an early event in AD and favours the development of AD neuropathology.

What then, underlies neurovascular changes in ageing that predispose the brain to neurodegeneration? The underlying mechanisms involved are likely multifaceted and necessitate thorough considerations of the roles played by the different cellular components of the neurovascular units (NVUs) – including ECs, mural cells (i.e. smooth muscle cells (SMCs) and pericytes), and astrocytes. The complex interplays between these NVU cells in mediating NVC and maintaining BBB integrity are still under active research. Molecules released by active neurons (e.g. glutamate, adenosine triphosphate) bind to astrocytic receptors whose activation leads to vasodilator (e.g. prostaglandin E2) synthesis and release (Mishra et al., 2016). Capillary ECs can sense extracellular elevation of potassium concentration due to neuronal activity, to initiate retrograde EC hyperpolarization-mediated arteriolar SMC relaxation (Longden et al., 2017). Apart from SMCs, subsets of contractile pre-capillary and capillary pericytes are also mediators of the NVC process (Mishra et al., 2016). While the BBB is primarily formed by tight junction-coupled ECs, the other NVU cells are also essential regulators of BBB integrity. Pericytes are required for the formation and maintenance of an intact BBB, while astrocytes both structurally (by extending their endfeet to ensheath microvessels) and functionally (by ionic and osmolarity regulations, as well as the secretion of regulatory factors) support BBB functions. In theory, an age-related compromise in the structural integrity or functions of any of the NVU cells can lead to BBB breakdown, reduced resting CBF, and impaired NVC, thereby increasing the susceptibility to neurodegenerative changes (**Figure 1A**).

However, given the molecular complexity of the NVU cells, how ageing commonly or differentially impacts on the NVU cell subtypes remains unclear. Based on single-cell RNA sequencing and thorough immunohistochemistry, a landmark paper reported six EC subtypes that are spatially segregated from arteries to veins in the mouse brain (Vanlandewijck et al., 2018). Each of these EC subtypes are distinctly characterized by unique molecular signatures, whereby arterial ECs are enriched with transcription factors while capillary ECs express more transporters, pointing to possible functional specializations. This provided the foundation of our study to investigate how the transcriptomic changes are shared or distinct between the EC subtypes in the aged mouse brain. We similarly classified the ECs into the six subtypes by their marker genes and calculated their differential expressions across age. A couple of observations were made. Firstly, expression changes are the most prominent in capillary ECs. Secondly, while subsequent pathway enrichment analysis revealed altered immune/cytokine pathways in most EC subtypes, clustering of differentially expressed genes (DEGs) potentially impacting on BBB functions and energy metabolism are especially prominent in capillary ECs (**Figure 1B**). The EC subtypes are thus differentially vulnerable in ageing, with those of the capillary bed likely more prone to functional changes.

We then performed two types of analyses to identify AD-associations of the aged brain EC differential expressions. Interestingly, overrepresentation analysis revealed an abundance of AD-associated genes identified from genome-wide association (GWAS) studies, among the human orthologs of the aged capillary EC DEGs which included genes known to be required for BBB integrity or function (e.g. *CD2AP* (CD2-

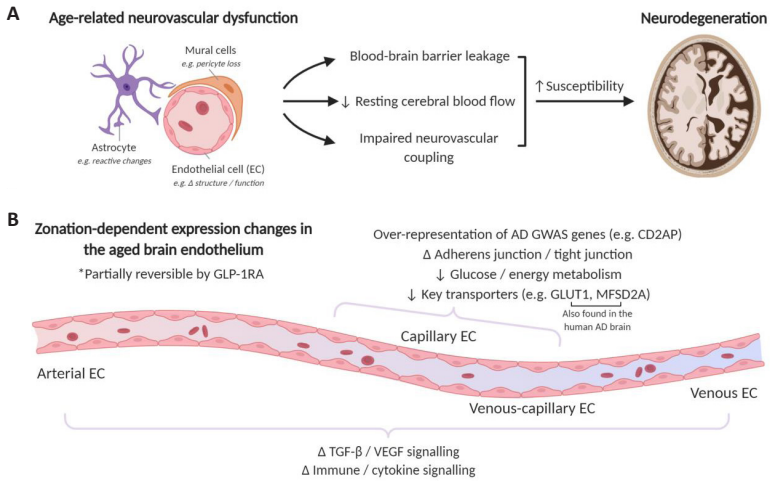


**Figure 1 | Age-related neurovascular dysfunction in neurodegeneration and zonation-dependent endothelial alterations in the aged mouse brain.**
(A) Illustration of the current understandings on the relationship between age-related neurovascular dysfunction and neurodegeneration. (B) Functional implications of the zonation-dependent ageing-associated transcriptomic changes in the endothelial cell subtypes, which can be partially reversed by GLP-1RA treatment. Symbol in A and B: Δ refers to "altered". AD: Alzheimer's disease; EC: endothelial cell; GLP-1RA: glucagon-like peptide-1 receptor agonist; GLUT1: glucose transporter 1; GWAS: genome-wide association; MFSD2A: major facilitator superfamily domain-containing 2A; TGF: transforming growth factor; VEGF: vascular endothelial growth factor.

associated protein)) (**Figure 1B**, **Additional Table 1**). Caution must be taken when interpreting these results, as GWAS studies are known to exhibit substantial variability and depend on the accuracy of case definition at the first place, which had been challenging for AD. Nonetheless, some of these candidate genes may provide new targets for studying how vascular factors contribute to neurodegeneration. On the other hand, we compared the differential expressions of the mouse brain ECs to that in the human AD brain based on the publicly available bulk RNA sequencing dataset from the Allen Brain Aging, Dementia and TBI study. A couple of genes with concordant expression changes in the human AD brain are worth special highlights. In the human AD brain, we found a downregulation of *GLUT1* (glucose transporter 1, **Additional Table 1**) consistent with previous reports (Sweeney et al., 2019), and reduced *MFSD2A* (major facilitator superfamily domain-containing protein 2A, **Additional Table 1**) expression which had not been reported to be associated with AD (**Figure 1B**). Given the known role of *MFSD2A* in limiting transcytosis at the BBB (Andreone et al., 2017), one may speculate that in a subset of AD patients, reduced *MFSD2A* expression and transport activity could contribute to pathogenesis by increasing nonspecific BBB transport. Additionally, we noted a downregulation of *IFITM3* (interferon-induced transmembrane protein 3, **Additional Table 1**), an interferon-responsive gene, in the AD cohort. Although this seems inconsistent with a recent report on its upregulation in ageing and AD which can promote γ-secretase-mediated amyloid beta production (Hur et al., 2020), differences in human brain regional sampling, sample cell type compositions and age of animal subjects studied could have contributed to the apparent discrepancy.

Given the complexity of the aged brain EC transcriptomic changes, one may wonder if these are potentially reversible, preferably by pharmacological means. We made a heuristic choice of GLP-1RA for testing in the aged mice and showed that 1-month treatment prior to the experimental age (i.e. 18 months old) resulted in expression changes in directions opposite to ageing for nearly 70% of the DEGs (**Figure 1B**). This was also associated with a substantial reduction of nonspecific BBB leakage assayed by 40-kDa dextran-dye conjugate extravasation (Zhao et al., 2020). These effects of exenatide could be related to the metabolic and immunomodulatory effects of GLP-1RA, thereby improving overall neurovascular health in the aged brain. Overall, the results complement recent clinical studies showing that GLP-1RA use reduces the incidence of cognitive impairment and Parkinson's disease in diabetic patients (Brauer et al., 2020; Cukierman-Yaffe et al., 2020), by revealing a neurovascular protective effect which may also generalize to non-diabetic patients with age-related vasculopathy. GLP-1RA may also be applicable to the clinical treatment of established AD. When an GLP-1RA is used in combination with other therapeutic agent(s) that require BBB crossing to attain their therapeutic efficacies (e.g. anti-amyloid beta or tau antibodies), a proper balance between BBB integrity improvement and the brain parenchymal access of the other agent(s) may however need to be attained. Additionally, therapeutic antibodies may be engineered to enhance their BBB-crossing capability.

The precise cellular mechanisms of GLP-1RA in reversing brain endothelial ageing remain unclear. We speculate that there may be a dependence on microglial GLP-1R activation, as our own data also showed reduced microglial expression of activation- and neurodegenerative disease-associated transcripts after exenatide treatment (Zhao et al., 2020). Microglial GLP-1R may serve as an important modulator of neuroinflammatory responses in ageing and neurodegenerative conditions. This is an important question to address, as proving that GLP-1RA acts centrally in the brain would determine the prioritization of existing peptide-based GLP-1R agonists with different BBB-crossing profiles for clinical trials, as well as instruct the future development of small molecule GLP-1RAs. Currently, there are only a few small-molecule GLP-1RAs (e.g. PF-06882961, TTP-273) in clinical development, the BBB-crossing properties of which are unknown. It is also important to test if the aged EC transcriptomic reversal by GLP-1RA is also associated with improvements in other aspects of neurovascular functionality (e.g. resting CBF, NVC). Similar approaches can also be adapted to study the transcriptomic alterations in glial and mural cells in relation to their subtype and regional identities in ageing, and whether GLP-1RAs or other pharmacological agents (**Additional Table 2**) can ameliorate their age-related expression and functional changes.

*We thank Hei-Ming Lai, Junzhe Huang and Leo Yan for helpful comments on the manuscript.*
*The present work was supported by a Croucher Innovation Award from the Croucher Foundation (to HK); a Faculty Innovation Award (FIA2017/B/01) from the Faculty of Medicine, the Chinese University of Hong Kong (CUHK) (to HK); the Gerald Choa Neuroscience Centre, the Margaret K. L. Cheung Research Centre for Parkinsonism Management, Faculty of Medicine, CUHK (to VCTM and HK); the Collaborative Research Fund (C6027-19GF) and the Area of Excellence Scheme (AoE/M-604/16) of the University Grants Committee of Hong Kong (to HK).*

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**Date of submission:** November 16, 2020
**Date of decision:** January 6, 2021
**Date of acceptance:** February 6, 2021
**Date of web publication:** April 23, 2021

**https://doi.org/10.4103/1673-5374.313042**
**How to cite this article:** *Li Z, Ip B, Mok VCT, Ko H (2021) Neurovascular ageing: transcriptomic readout and implications on therapeutic targeting in Alzheimer's diseas. Neural Regen Res 16(12):2411-2412.*

**Open peer reviewer:** *Melissa Schepers, Hasselt University, Belgium.*
**Additional files:**
***Additional file 1:*** *Open peer review report 1.*
***Additional Table 1:*** *Highlights of AD GWAS genes or genes with concordant expression changes in aged mouse brain endothelium and in the human AD brain.*
***Additional Table 2:*** *Highlights of experimental therapeutics with vascular protective effects for neurovascular ageing and/or AD in pre-clinical development or clinical trials.*

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*P-Reviewer: Schepers M; C-Editors: Zhao M, Liu WJ, Qiu Y; T-Editor: Jia Y*

**Table 1 Highlights of AD GWAS genes or genes with concordant expression changes in aged mouse brain endothelium and in the human AD brain**

| Gene name | AD GWAS gene? | Δ Exp (aged mouse brain EC by scRNA-seq) | Δ Exp (human AD brain by bulk RNA-seq) | Vascular-related and/or (potential) AD-related functional roles (reference) |
|---|---|---|---|---|
| *CD2AP* | ✓ | ↑ | No reported change | • Required for the establishment or maintenance of intact BBB (Hum Mol Genet. 2015;24(23):6667-6674)<br>• May modulate amyloidogenesis and tau-mediate neurotoxicity (Aging Dis. 2019;10(4):901-907) |
| *DLC1* | ✓ | ↑ | $\uparrow^{AB}$ *Also in EC by snRNA-seq (Nat Neurosci. 2019;22(12):2087-2097) | • Required for ICAM-1 adhesome stabilization, leukocyte spreading and diapedesis (Cell Rep. 2018;24(12):3115-3124)<br>• Negative regulator of angiogenesis (Cancer Lett. 2017; 398: 46-51) |
| *GLUT1* | ✗ | ↓ | $\downarrow^{AB}$ | • Main glucose transporter across the BBB<br>• Reduced vascular expression in the human AD brain (Ann Neurol. 1994;35(5):546-51; Virchows Arch. Int. J. Pathol. 1994;425:69-72)<br>• Haploinsufficiency in the *APP*$^{Sw/0}$ mouse AD model exacerbates neuropathology (Nat Neurosci. 2015; 18(4): 521-530) |
| *MFSD2A* | ✗ | ↓ | $\downarrow^{AB}$ | • Transport of docosahexaenoic acid (DHA) into the brain (Nature. 2014;509(7501):503-6)<br>• Required for the formation of intact BBB in development (Nature. 2014;509(7501):507-511)<br>• Negative regulator of transcytosis across the BBB (Neuron. 2017;94(3):581-594.e5) |
| *IFITM3* | ✗ | ↓ | $\downarrow^{AB}$ / $\uparrow^{Mayo}$ | • Part of the γ-secretase complex and modulates its activity (Nature. 2020;586(7831):735-740)<br>• Constitutive expression by EC and inhibits viral invasion (J Virol. 2016;90(24):11157-11167; Nat Chem Biol. 2019;15(3):259-268) |
| *IGF1R* | ✗ | ↑ | $\uparrow^{Mayo}$ | • Aberrant signaling reported in the human AD brain (J Alzheimers Dis. 2005;7(1):63-80)<br>• Genetic ablation in neuron or inhibition by small molecule ameliorates neuroinflammation and neuropathology in the APP/PS1 mouse AD model (J Neurosci. 2015;35(33):11500-11513; Front Cell Neurosci. 2020;14:200) |

AD: Alzheimer's disease; GWAS: genome-wide association study; DLC1: deleted in liver cancer 1; EC: endothelial cell; IGF1R: insulin-like growth factor 1 receptor; scRNA-seq: single-cell RNA sequencing; RNA-seq: RNA sequencing; ΔExp: expression change. For the expression changes in the human AD brain, superscript $^{AB}$ denotes that found in the Allen Brain Aging, Dementia and TBI study dataset, while $^{Mayo}$ denotes that found in the Mayo Clinic cohort.

**Table 2 Highlights of experimental therapeutics with vascular protective effects for neurovascular ageing and/or AD in pre-clinical development or clinical trials**

| **Proposed therapeutic agent/approach** | **Example(s)** | **Rationale/proposed mechanism(s) of action** (reference) |
|---|---|---|
| Angiotensin receptor blocker | Candesartan, Telmisartan | • Treatment of hypertension if any<br>• Inhibition of dysregulated renin-angiotensin signalling in AD (J Alzheimers Dis. 2018;62(3):1443-1466) |
| Glucagon-like peptide-1 receptor agonist | Exenatide, Liraglutide, Semaglutide | • Multiple mechanisms including improvement in brain metabolism (Front Aging Neurosci. 2016;8:108), amelioration of neuroinflammation (Nat Med. 2018;24(7):931-938) and reversal of vascular ageing (Nat Commun. 2020;11(1):4413) |
| HMG-CoA reductase inhibitor | Simvastatin | • Lowering lipids and thereby improving general and neurovascular health |
| Mechanistic target of rapamycin (mTOR) inhibitor | Rapamycin | • Inhibition of mTOR which is involved in driving age-related cerebrovascular deficits (Aging Cell. 2020;19(1):e13057) |
| Nicotinamide adenine dinucleotide ($NAD^+$) precursor | Nicotinamide mononucleotide | • Increasing cellular $NAD^+$ to improve oxidative metabolism and reverse age-related endothelial dysfunction (Aging Cell. 2016;15(3):522-530) |
| Poly(ADP-ribose] polymerase 1 (PARP1) inhibitor | PJ34 | • Inhibition of PARP1 which is involved in amyloid beta-induced endothelial vasomotor dysfunction (Stem Cell Res Ther. 2018;9(1):224; Nat Commun. 2014;5:5318) |
| Phosphodiesterase inhibitor | Cilostazol, Tadalafil | • Improving endothelial function and cerebral blood flow |
| Gene therapy | Endothelium-targeted sirtuin 7 (*Sirt7*) expression | • *Sirt7*: improvement of neovascularization and amelioration of ageing features (Sci Adv. 2020;6(8):eaay5556) |
| Factors in young blood | Young plasma transfusion | • Multiple mechanisms including ameliorating neuroinflammation and endothelial aging phenotype (Cell Rep. 2020;30(13):4418-4432.e4) |