DECIPHERING THE LINK BETWEEN ASTROCYTE REACTIVITY AND NEURONAL DAMAGE IN ALZHEIMER'S DISEASE

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1. Summary

Problem statement. Late-onset Alzheimer's disease (LOAD) is a type of dementia caused by neurodegeneration that affects more than 46 million people worldwide. Main clinical symptoms include loss of memory, language problems, difficulty in doing simple tasks, loss of reasoning capacity, mood changes, and behavioural changes. The cause of LOAD is not clear yet, nor is there a cure. Gross pathological changes include the hallmark amyloid beta plaques and neurofibrillary tangles, as well as loss of synapses, cerebrovascular damage, metabolic changes and activation of non-neuronal cells such as microglia and astrocytes, also called reactive astrocytes. Astrocytes are a type of brain cell that carries out computational and homeostatic roles. Astrocytes are big, bushy cells, encompassing a cell body from which a dense network of primary and secondary processes emerges. Reactive astrocytes present a robust morphological change that consists of the enlargement of primary and secondary processes, as shown by increased immunostaining of the intermediary filament protein GFAP. The functional changes associated with this morphological transformation are unknown. Specifically, to what extent loss of cognition in LOAD is due to astrocyte malfunction, which astrocytic pathways are dysregulated, and how they impact disease pathogenesis, hallmarks, biomarkers, and clinical symptoms, has not been established, despite non-negligible research. A critical problem is that interventional studies performed in mice employing astrocyte-targeted manipulations have produced conflicting evidence with regard to the role of astrocytes in LOAD. In addition, astrocytes produce TNF-alpha, a factor with many roles that include synaptic scaling in the normal brain, and regulation of cell survival and death in many tissues. Critical to this project is that the production of TNF-alpha is dramatically increased in LOAD. It is suspected, but not known, that TNF-alpha could contribute to spine loss and neuronal demise. If this is the case, the mechanism is unclear.

Objectives. The overarching goal was to characterize astrocyte dysfunction in LOAD in order to gain insight into the fundamental question of whether and how alterations of astrocyte-neuron crosstalk may lead to loss of synapses, and ultimately neuronal loss, in order to develop astrocyte-targeted therapies. Four laboratories have collaboratively carried out four Specific Objectives. Specific Objective 1 was the structural and ultrastructural study of astrocytes, using advanced imaging techniques, including electron microscopy. Study materials were postmortem samples from LOAD patients

and animal models. Specific Objective 2 was the identification of the molecular profile of astrocytes, using bioinformatics and omics data from human-brain samples. Specific Objective 3 consisted of functional studies. Specifically, analysis of calcium excitability by calcium imaging, and phagocytosis by subcellular tracking of labelled probes. Study materials were primary astrocyte cultures from mouse brain, and astrocytes overexpressing human alleles of the apolipoprotein type E (ApoE4 and ApoE3). ApoE4 is the most important genetic risk factor in LOAD. In Specific Objective 4 we examined the effect of TNF-alpha in animal models in which the pathways activated by this factor have been genetically manipulated.

2. Results

2.1. Alzheimer's disease causes a decrease in the expression of FAIM-L (long form of Fas apoptotic inhibitory molecule), a protein involved in TNF-alpha signalling that promotes neuronal survival. Unexpectedly, we found that FAIM also regulates neuronal electrical excitability. This suggests that the decreased expression of FAIM-L in Alzheimer's disease may not only contribute to the neurotoxic actions of astrocyte-released TNF-alpha in the disease, but also lead to neural circuit hyperexcitability (refs 1, 3).

• *Amyloid-β reduces the expression of neuronal FAIM-L, thereby shifting the inflammatory response mediated by TNF-alpha from neuronal protection to death*. We detected that FAIM-L was reduced in the hippocampi of patients with LOAD. We also observed that the entorhinal and hippocampal cortex of a mouse model of LOAD ($PS1_{M146L}xAPP_{751sl}$) showed a reduction in this protein before the onset of neurodegeneration. Notably, cultured neurons treated with the cortical soluble fractions of these animals showed a decrease in endogenous FAIM-L, an effect that is mimicked by the treatment with A*β*-derived diffusible ligands (ADDLs). The reduction in the expression of FAIM-L is associated with the progression of the neurodegeneration by changing the inflammatory response mediated by TNF-alpha in neurons. In this sense, we also demonstrate that the protection afforded by TNF-alpha against A*β* toxicity ceases when endogenous FAIM-L is reduced by short hairpin RNA (shRNA) or by treatment with ADDLs. All together, these results support the notion that levels of FAIM-L contribute to determine the protective or deleterious effect of TNF*α* in neuronal cells.

• *FAIM deficiency promotes seizure susceptibility, locomotor hyperexcitability, cognitive changes and social alterations*. We used FAIM-knockout mice (FAIM-KO) in order to establish the role of the factor in LOAD. Before crossing the mice with LOAD transgenics, we carried out a phenotypical characterization of the FAIM-KO mice in order to gain insight into the function of FAIM in the adult central nervous system (CNS). The study stems from our initial observation of seizure activity in these mice. Seizure incidence, severity and duration in spontaneous conditions or upon manual manipulation were scored in FAIM-KO mice and wild-type (WT) age-matched controls. Electrophysiology studies included input/output curves, paired-pulse facilitation, evoked long-term potentiation (LTP) and seizure generation upon kainic-acid injection (8 mg/kg) in hippocampus. A comprehensive behavioural and functional screening was performed using a battery of 8 tests assessing five dimensions: motor and exploratory functions, emotional status, cognitive functions, social interaction and executive functions. FAIM-KO mice exhibited recurrent age-dependent seizure activity with fast recovery between crises. FAIM-KO also presented slightly larger LTP values in the electrophysiological properties tested, as well as lower propensity to generate hippocampal seizures after injection of kainic acid compared to WT mice. Finally, FAIM-KO mice showed locomotor hyperexcitability, deficits in social interaction, impaired innate nesting behaviour, and deficits in learning and memory abilities. These studies suggest involvement of FAIM in the modulation of brain excitability in vivo. FAIM-KO mice might provide an exceptional genetic model to study downstream mechanisms involved in recurrent epilepsy, and to develop anti-epileptic drugs that might serve to counteract neuronal hyperexcitability in LOAD.

2.2. Alzheimer's disease causes dysfunction of the endolysosomal system in astrocytes. This may have 3 consequences: (i) altered mitophagy leading to bioenergetic failure, (ii) impaired phagocytosis, causing accumulation of dystrophic neurons, and defective clearance of amyloid-beta, thus promoting its aggregation, and (iii) increased calcium responses, which, may, in turn, compromise neuron-astrocyte signalling (refs 2,4,5).

• *Bioinformatic characterization of astrocyte malfunction in Alzheimer's disease using human data.* BACKGROUND. The clustering of GFAP-overexpressing astrocytes around amyloid beta plaques in Alzheimer's disease (AD) points to major phenotypical and hence functional alterations in astrocytes. However, it is not known which astrocytic pathways are dysregulated in human AD brains. Progress on knowledge

about the contribution of astrocytes to AD has been hampered by lack of human data. OBJECTIVE. To characterize pathway dysregulation in astrocytes in AD using human data and bioinformatics in order to help clarify the contribution of astrocytes to the disease, and to inform systems-biology-based therapeutics. METHODS. First, we established the cellular specificity of brain gene clusters by applying the univariate cell-type enrichment score Tau together with hierarchical clustering of RNAseq data from astrocytes, neurons, microglia, endothelial cells and oligodendrocytes isolated from aged human cortices. Second, we defined the functions of such gene clusters using both open access gene ontology platforms and manual curation. Third, we used gene set enrichment analysis (GSEA) to evaluate changes of astrocyte-specific clusters in AD using microarray and RNAseq datasets from postmortem AD tissues and age-matched controls. Neuronal clustering served as a positive control. RESULTS. Neuron-specific clusters related to synaptic structure, synaptic plasticity and neurotransmission were, as expected, downregulated in AD. Salient changes within astrocyte specific are related to growth-related genes, TGF beta pathways, energy-metabolism-related genes and the endolysosomal system. CONCLUSION. Cellular compartmentalization of genes differentially expressed in human AD brains with respect to healthy controls suggests multi-organellar dysfunction in astrocytes.

- *Phagocytic clearance of presynaptic dystrophies by reactive astrocytes in Alzheimer's disease*. Reactive astrogliosis, a complex process characterized by cell hypertrophy and upregulation of components of intermediate filaments, is a common feature in brains of LOAD patients. Reactive astrocytes are found in close association with neuritic plaques; however, the precise role of these glial cells in disease pathogenesis is unknown. In this study, using immunohistochemical techniques and light and electron microscopy, we report that plaque-associated reactive astrocytes enwrap, engulf and may digest presynaptic dystrophies in the hippocampus of amyloid precursor protein/presenilin-1 (APP/PS1) mice. Microglia, the brain phagocytic population, was apparently not engaged in this clearance. Phagocytic reactive astrocytes were present in 35% and 67% of amyloid plaques at 6 and 12 months of age, respectively. The proportion of engulfed dystrophic neurites was low, around 7% of total dystrophies around plaques at both ages. This fact, along with the accumulation of dystrophic neurites during disease course, suggests that the efficiency of the astrocyte phagocytic process might be limited or impaired. Reactive astrocytes surrounding and engulfing dystrophic neurites were also detected in the hippocampus

of LOAD patients by confocal and ultrastructural analysis. We posit that the phagocytic activity of reactive astrocytes might contribute to clear dysfunctional synapses or synaptic debris, thereby restoring impaired neural circuits and reducing the inflammatory impact of damaged neuronal parts and/or limiting the amyloid pathology. Therefore, potentiation of the phagocytic properties of reactive astrocytes may represent a potential therapy in LOAD.

- *ApoE4-elicited lysosomal and mitochondrial dysfunction in astrocytes.*

Apolipoproteins (ApoE) are cholesterol carriers that exist in three alleles in humans: E2, E3 and E4. ApoE4 is the most important genetic risk factor in LOAD, but its pathogenic mechanism, which is probably multifactorial, remains unclear. In our studies we seek to understand the effect of intracellular ApoE4 in astrocytes, the cells that produce most ApoE in the brain. We posit that ApoE4 disrupts normal lipid trafficking in astrocytes thereby causing organelle dysfunction. As study materials we used immortalized astrocytes with targeted replacement of endogenous ApoE with human ApoE isoforms, generated by the Holtzman group. As readouts, we examined calcium concentrations using calcium imaging, and a battery of pharmacological approaches, as well as mitochondrial dynamics, by tracking mitochondrial fusion and fission with fluorescence microscopy and computerized morphometry. We found three main differences between ApoE4 and ApoE3 astrocytes. First, the canonical calcium transients elicited by ATP, noradrenaline and acetylcholine in astrocytes (a marker of astrocyte excitability) were regulated by extracellular lipids in ApoE3 astrocytes but not in ApoE4 astrocytes. By contrast, ApoE4 astrocytes present larger concentration of calcium inside acidic organelles related to lysosomes, which leads to greater and longer calcium transients upon stimulation with ATP and other agonists. Second, mitochondria in ApoE4 astrocytes showed defective fission, as evidenced by the increased contents in mitochondrial networks in ApoE4 cells challenged by stimuli that fragment mitochondria, such as oligomycin. They also showed greater motility, as shown by measurements of squared displacement (D2, Imaris), decreased expression of PARKIN, a protein key to mitophagy, decreased production of ATP and increased production of lactate. Third, untargeted lipidomics of whole astrocytes revealed a decreased ratio of lysophosphatidylcholine/phosphatidylcholine in ApoE4 astrocytes, as compared to ApoE3 astrocytes. We conclude that ApoE4 astrocytes present altered calcium fluxes due to increased calcium accumulation in lysosomes, associated with impaired mitochondrial dynamics, and a switch of energy metabolism to glycolysis. Current

experiments are dedicated to identify the calcium channels that account for differences between ApoE3 and ApoE4 astrocytes, as well as to examine cause-effect relationships between lysosomal and mitochondrial dysfunction in ApoE4 astrocytes and the contribution of changes in phospholipid composition.

3. Relevance and future implications

- The finding that FAIM-L is decreased in patient postmortem samples and mouse models of LOAD provides a target for neuroprotective strategies to preserve neuron integrity, as well as a biomarker of neuronal damage.

- The finding that FAIM depletion causes epileptic status (which is an overlooked hallmark of LOAD in patients and animal models) and alteration of electrophysiological parameters in mouse brain cortex suggests that FAIM regulates circuit activity in the brain and paves the way to therapies that correct neuronal hyperexcitability in LOAD by restoring FAIM expression.

- The multi-organellar and multifunctional failure caused by dysfunction of the endolysosomal system in astrocytes, as discovered in several LOAD-related models, suggests that recovery of this organelle will be key to achieving global improvement of astrocyte-specific homeostatic and computational functions. Systems biology will be necessary to identify druggable nodes with multi-functional effects from protein-to-protein interaction maps generated from astrocytic molecular data.

4. Generated literature

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