Centre Armand Frappier Santé Biotechnologies

Rôle de l'apolipoprotéine D dans la mémoire spatiale et étude de son passage à travers la barrière hémato-encéphalique

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Thèse présenté(e) pour l'obtention
du grade de Philosophiae Doctor (Ph.D.)
en sciences de doctorat en Biologie

Jury d'évaluation

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REMERCIEMENTS

Je tiens tout d'abord à remercier mon directeur de recherche le Pr. Charles Ramassamy, qui m'a accepté dans son laboratoire pour intégrer son équipe de recherche. Grâce à son soutien, les échanges scientifiques, et les conseils qu'il m'a fournis, il a permis de m'accompagner dans ce marathon qu'est le doctorat et d'en arriver là où je suis. Je remercie aussi le Pr. Eric Rassart, qui a accepté de co-diriger ma thèse, grand spécialiste de l'apolipoprotéine D, il m'a permis d'avancer dans mes projets et d'améliorer mes expériences, ainsi qu'un soutien sur la dernière ligne droite.

Je remercie aussi le Pr. Kessen Patten d'avoir accepté la présidence du jury d'évaluation de ma thèse. J'aimerais aussi remercier Pre. Mélanie Plourde et Dre. Sonia Do Carmo pour avoir accepté d'y participer en tant qu'évaluateurs externes. Je remercie aussi tous les jurys qui ont accepté de participer aux précédents examens de mon doctorat (Pre. Catherine Mounier, Pr. Jonathan Brouillette).

Je remercie aussi les organismes subventionnaires qui m'ont accompagné durant mon doctorat : La chaire Louise et André Charron sur la maladie d'Alzheimer, la fondation Armand-Frappier, le CRSNG et le Réseau Québécois de Recherche sur le vieillissement qui m'a permis de voyager pour un congrès international à Las Vegas.

Je tiens aussi à remercier tous les membres du laboratoire, actuels et anciens, avec qui j'ai pu très souvent échanger, nul doute que sans eux le doctorat n'aurait pas été pareil, je garde quelques belles amitiés. Mohamed (avec qui j'ai commencé), Jean-Michel, pour sa disponibilité pour répondre aux questions scientifiques et rugbystiques, Morgane P., qui m'a épaulé durant la première partie du doctorat, Raâfet, Nour, Morgane L., Ahlem, Ghislain, Hermine, Rummy, Sarra, Hai Chao, ainsi que Guylaine. Je remercie aussi tous les stagiaires que j'ai pu encadrer durant mon doctorat : Kévin, Camille D., Laure, Marie, Floriane, Axelle, Chloé. Je suis fier de les avoir encadrés, il est important pour moi de les remercier car ils ont aussi contribué à ce que je devienne un meilleur chercheur en apprenant à transmettre la science et les connaissances.

L'aventure a été belle aussi grâce à une belle gang d'amis rencontrés à l'institut, Mathilde, Etienne, Laura, Guillaume, pour cette première partie de doctorat mais aussi Zoé, Laurie, Clément, Wesley, Anaïs, Alex, Bélinda, Ritou, Richard, Léna, Claudia, Léa, Gab. Je ne les oublie pas et ils font maintenant partie de ma vie, de belles amitiés se sont créés après ça et perdureront autour du monde. Je n'oublie pas toutes les personnes que j'ai croisé avec le comité Socio-Culturel (Guillaume D., Fabien, Hermance, Kévin, Aude) pendant près de 5 ans, ainsi que les

membres du comité organisateur du Congrès Armand Frappier 2017 auquel je suis heureux d'avoir participé et rencontré des gens exceptionnels (Anissa, Sarah M., Arlette, Amélie, Sarah P-C, et d'autres déjà cités plus tôt). Je n'oublie pas de remercier Louise, qui m'a bien montré comment fonctionner l'institut et m'y a vite intégrer, qui fait partie du groupe de M2 avec Laurent, Morgan et Nicolas à s'être expatrié pour la thèse et dont nous avions plaisir à se retrouver de l'autre côté de l'Atlantique.

J'ai aussi vécu plusieurs colocations, et je remercie ceux que j'ai croisé, Stéphanie, Alex, Agnès, Nicolas, Lucie, mais surtout Iza, devenue amie et avec qui je m'entends si bien depuis presque 5 ans et qui m'a soutenu durant toute cette période. Je ne pourrais pas les citer, mais c'était aussi un réel plaisir de découvrir l'improvisation à Montréal et de partager autant de scènes avec des partenaires de jeux incroyables.

Je remercie aussi mes parents, mon frère et ma grand-mère qui m'ont encouragé à partir au Québec pour réaliser ce doctorat, ils ont aussi cru à ce cursus universitaire scientifique, un monde nouveau pour eux. Et je n'oublie pas les amis de France, je pense surtout à Antho et Julie, amis depuis plus de 10 ans, que j'ai comme tout le monde très hâte de retrouver.

RÉSUMÉ

L'apolipoprotéine D (apoD) est une lipoprotéine sécrétée qui est associée à la neuroprotection et au métabolisme lipidique. Chez les rongeurs, son expression est principalement limitée au système nerveux central. La surexpression de l'apoD dans les neurones de souris induit des stéatoses hépatiques non-inflammatoires, ainsi l'apoD cérébrale pourrait avoir une influence périphérique. Nous avons montré que 3h après une injection d'apoD humaine (apoDh) radioactive dans le ventricule cérébral latérale (intracérébroventriculaire (icv)), celle-ci traverse la barrière hémato-encéphalique (BHE) et on retrouve 40% en périphérie, principalement dans le foie et les reins. Nous montrons une forte corrélation entre l'accumulation d'apoDh dans les tissus et les niveaux faibles de glycosylation de la basigine. *In vitro*, nous avons montré que l'apoDh peut traverser la monocouche de cellules endothéliales (bEnd.3) mimant les caractéristiques de la BHE. Cependant, en présence d'un compétiteur pour le récepteur basigin, l'internalisation de l'apoDh dans les cellules de la monocouche n'a pas montré de diminution. Cela suggère qu'un autre récepteur pourrait intervenir dans l'internalisation de l'apoDh. En revanche l'apoDh injectée en périphérie n'a pas été retrouvée dans le cerveau, suggérant que l'apoD cérébrale a pour principal origine le cerveau.

Dans un second temps, nous avons observé l'effet de l'absence d'apoD chez des souris ($apoD^{-/-}$) âgées de 8 mois ayant subi une icv de streptozotocine (icv-STZ), une toxine induisant certaines caractéristiques de la maladie d'Alzheimer (MA). Les souris $apoD^{-/-}$ - STZ ont montré une diminution de leur capacité de mémoire spatiale dans la piscine de Morris. Cette diminution n'ayant pas été vue chez les souris *wild-type* icv-STZ, celle-ci est spécifique à l'absence de l'apoD. Nous avons aussi observé une différence quant au niveau des apolipoprotéines bien connues dans la MA : l'apoE et l'apoJ. L'apoE diminue chez les souris $apoD^{-/-}$ ainsi que dans l'hippocampe chez les souris icv-STZ. L'apoJ est augmentée dans le striatum de toutes les souris icv-STZ ainsi que dans les souris $apoD^{-/-}$. De plus, une augmentation des protéines carbonylées dans l'hippocampe de souris $apoD^{-/-}$, ainsi qu'une diminution du piégeage des radicaux libres dans l'hippocampe, le striatum, le cortex frontal sont observées. Nous avons ainsi montré que le manque d'apoD induit une sensibilité plus importante à la détérioration de la mémoire spatiale, qui pourrait être due à l'augmentation du stress oxydatif, confirmant le rôle bénéfique de l'apoD dans les maladies neurodégénératives.

Mots-clés : Apolipoprotéine D ; Barrière Hémato-Encéphalique ; Stress oxydatif ; Mémoire spatiale ; Streptozotocine ; ICV, Maladie neurodégénératives

ABSTRACT

Apolipoprotein D (apoD) is a secreted lipoprotein that is associated with neuroprotection and lipid metabolism. In rodents, its expression is mainly in the central nervous system. Overexpression of apoD in mouse neurons induces non-inflammatory hepatic steatosis, so brain apoD might have a peripheral influence. We showed that 3h after an injection of radioactive human apoD (apoDh) in the lateral cerebral ventricle (icv), 40% is found in the periphery and crosses the blood-brain barrier (BBB). It is mainly found in the liver and kidneys. We showed a strong correlation between apoDh accumulation in tissues and low levels of basigin glycosylation. *In vitro*, apoDh was shown to cross the bEnd.3 endothelial cell monolayer mimicking the characteristics of the BBB. However, in the presence of a competitor for the basigin receptor, the internalization of apoDh into the monolayer cells did not show a decrease. This suggests that another receptor may be involved in apoDh internalization. On the other hand, apoDh injected in the periphery was not found in the brain, which suggests that the main origin of cerebral apoDh is the brain.

In neurodegenerative diseases, brain apoD would have mainly a neuroprotective role. In a second step, we observed the effect of the absence of apoD in 8-month-old mice ($apoD^{-/-}$) subjected to streptozotocin icv (icv-STZ), a toxin inducing certain characteristics of Alzheimer's disease (AD). The $apoD^{-/-}$ - STZ mice showed a decrease in their spatial memory capacity in the Morris pool. This decrease was not seen in the wild-type icv-STZ mice and is specific to the absence of apoD. We also observed a difference in the level of apolipoproteins well known in AD: apoE and apoJ. ApoE is decreased in $apoD^{-/-}$ mice as well as in icv-STZ mice in the hippocampus. ApoJ is increased in the striatum of all icv-STZ mice as well as in $apoD^{-/-}$ mice. Moreover, an increase in carbonylated proteins in the hippocampus of $apoD^{-/-}$ mice, as well as a decrease in free radical scavenging in the hippocampus, striatum and frontal cortex are observed. We have thus shown that the lack of apoD induces a greater sensitivity to the protection of spatial memory, which could be due to the increase of oxidative stress, confirming the beneficial role of apoD in neurodegenerative diseases.

Keywords: Apolipoprotein D, Blood-Brain Barrier, Oxidative Stress, Spatial Memory, Streptozotocin, ICV, Neurodegenerative Diseases

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LISTE DES ABRÉVIATIONS

4-HNE
: 4-hydroxynonenal

ABCA1
: *ATP-binding cassette*

ADN
: Acide désoxyribonucléique

ADNc
: Acide désoxyribonucléique

APEX
: *Apurici/Apyrimidic Endonuclease-I*

apoD
: Apolipoprotéine D

apoDh
: Apolipoprotéine humaine

apoE
: Apolipoprotéine E

apoJ
: Apolipoprotéine J

APP
: *Amyloid precursor protein*

ARN
: Acide ribonucélique

ARNm
: Acide ribonucléique messager

Aβ
: Amyloid Bêta

BBP, 3
: *Bilin binding protein*

BHE
: Barrière hémato-encéphalique

BSG
: Basigin

Cys
: Cystéine

ERE
: *Estrogen response element*

ERK1/2

extracellular signal-regulated kinase 1/2)

GFAP

Glial fibrillary acidic protein

GRE

Gluc*oticoid response element*

HDL

High density lipoprotein

icv

intracérébroventriculaire

LCAT

lécithine cholestérol acyl-transférase

LCS

Liquide cérébro-spinal

LDL

Low density lipoprotein

LRP1

Low density lipoprotein receptor-related protein

LRP-1

Low density lipoprotein receptor-related protein

LRP2

Low den*sity lipoprotein receptor-related protein*

LRP-2

Low den*sity lipoprotein receptor-related protein*

MA

Maladie d'Alzheimer

Met93

Méthionine de l'apoD en position 93

MetSO

Méthionine sulfoxyde

Myrf

Myelin Regulatory Factor

NFTs

enchevêtrements neurofibrillaires

NF-κB

Nuclear Factor kappa B

PARP-1

Poly ADP-ribose polymerase-1

PLA2

Phospholipase A2

PPARγ

Peroxisome proliferator-activated receptor

PPS1

Préséniline 1

PPS2

Préséniline 2

PRE

Progesterone response element

RAR

Récepteurs nucléaires à l'acide rétinoïques

RBP

Retinol Binding Protein

SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2

siRNA

Small interfering RNA

SNC

Système nerveux central

STZ

Streptozotocine

Tg

Transgénique

VEs

Vésicules extracellulaires

1 INTRODUCTION

1.1 Caractéristiques de l'apolipoprotéine D

L'apolipoprotéine D humaine (apoDh) est une glycoprotéine sécrétée de 29 kDa. Elle est membre de la famille des lipocalines. Il s'agit d'un groupe de protéines capables de transporter de petites molécules hydrophobes (lipides, stéroïdes, etc…) (Drayna *et al.*, 1987; Flower, 1996). Elle a été détectée pour la première fois en 1963 dans le plasma et isolée en 1973 par McConathy et Alaupovic à partir des lipoprotéines à haute densité du plasma (McConathy & Alaupovic, 1973). Elle possède un grand nombre de ligands et plusieurs fonctions lui ont été proposées.

1.1.1 Structure de l'apoD

1.1.1.1 Gène de l'apoD

Chez l'Homme, le gène de l'apoD est situé sur le chromosome 3 (Drayna *et al.*, 1987), tandis que chez la souris, il se retrouve sur le chromosome 16 (Warden *et al.*, 1992). Le promoteur de l'apoD humaine (apoDh) dépend de plusieurs éléments régulateurs, comme les éléments de réponse aux stéroïdes. Parmi elles, il y a l'œstrogène, la progestérone, et les glucocorticoïdes (notifiés respectivement dans la littérature comme ERE, PRE, GRE). Le gène de l'apoD est aussi régulé par des facteurs de transcription impliqués dans la réponse inflammatoire, comme NF-κB (Nuclear factor kappa B) (Do Carmo *et al.*, 2007; Do Carmo *et al.*, 2002; Lambert *et al.*, 1993). Dans ce promoteur figurent aussi les éléments de réponse aux acides gras, au sérum et à la phase aigüe de l'inflammation (Do Carmo *et al.*, 2007; Do Carmo *et al.*, 2002; Lambert *et al.*, 1993). La diversité et la multitude des stimuli influençant l'expression de l'apoD démontrent la complexité de cette glycoprotéine et ses rôles multifonctionnels. Sur le plan de l'évolution, le gène de l'apoD a été conservé dans plusieurs espèces de mammifères (rat, souris, homme, etc..). De plus, chez d'autres espèces plus éloignées, des homologues de l'apoD ont été retrouvés, comme chez la bactérie *Escherichia coli* (Bishop et al., 1995), la drosophile (Sanchez *et al.*, 2006) et la plante *Arabidopsis thaliana* (Charron *et al.*, 2002; Charron *et al.*, 2008).

1.1.1.2 La protéine : apoD

De par sa séquence protéique, l'apoD est singulière des autres apolipoproteines, quoiqu'un haut degré d'homologie existe entre elle et la famille des lipocalines. Les lipocalines

sont une famille de protéines qui transportent des petites molécules hydrophobes comme les stéroïdes, rétinoïdes et lipides. Elle dispose de 25% d'homologie de séquence avec *Retinol-Binding Protein* (RBP) ainsi que 30 à 40% d'homologie avec la *Bilin Binding Protein* (BBP), qui est une protéine retrouvée chez les insectes (Drayna *et al.*, 1987; Weech *et al.*, 1991). Il s'agit de protéines de transport capables de se lier à leurs ligands respectifs. La séquence de l'ADN complémentaire (ADNc) de l'apoD permet de déduire une séquence protéique de 189 acides aminés contenant un peptide signal de 20 acides aminés hydrophobes, donnant une protéine mature de 169 acides aminés (Yang *et al.*, 1994). Le poids moléculaire calculé à partir de l'ADNc est de 18,1 kDa, en revanche son poids moléculaire retrouvé physiologiquement varie entre 19 et 32 kDa (Albers *et al.*, 1981; Balbin *et al.*, 1990; Drayna *et al.*, 1986), comme retrouvé sur un gel d'électrophorèse natif. Cette hétérogénéité de poids moléculaire est due à la glycosylation de l'apoD. En effet, celle-ci possède deux sites de glycosylation sur les asparagines en position 45 et 78 (Peitsch & Boguski, 1990; Yang *et al.*, 1994). L'apoD humaine possède 5 cystéines qui peuvent former des ponts disulfures intramoléculaires, entre les cystéines 41 et 165, ainsi qu'entre les cystéines 8 et 114 (Figure 1.1). En revanche, la cystéine en position 116, peut former un pont disulfure avec d'autres protéines associées aux HDL, dont l'apoA-I, l'apoA-II et la lécithine cholestérol acyl-transférase (LCAT), mais aussi avec l'apoD elle-même (Blanco-Vaca *et al.*, 1992; Fielding & Fielding, 1980; Weech *et al.*, 1986; Weech *et al.*, 1991; Yang *et al.*, 1994).

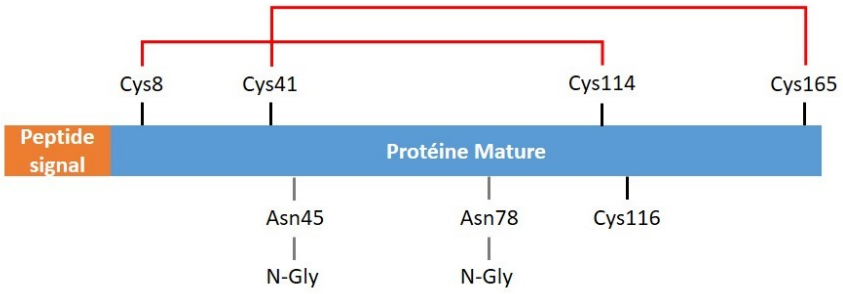

Figure 1.1 : Représentation schématique de la séquence protéique de l'apoD humaine.

Les cystéines (cys) et les ponts disulfures identifiés sont représentés. Les deux asparagines sont notées Asn et représentent les deux sites de glycosylations connus de l'apoD.

Tout comme sa séquence, la structure secondaire de l'apoD est différente des autres apolipoprotéines, elle est prédite avec une petite structure en hélice α et composée principalement de feuillets β (Camato *et al.*, 1989; Drayna *et al.*, 1986; Yang *et al.*, 1994). Il a été proposé un modèle de structure tertiaire de l'apoD basé sur la BBP, qui pour rappel possède 30 à 40 % d'homologie avec l'apoD (Peitsch & Boguski, 1990). Cette protéine est prédite pour être

composée de 8 feuillets β antiparallèles qui sont ouverts sur le haut, et fermés sur la base (Eichinger *et al.*, 2007). Cette structure conique permet à l'apoD de transporter et lier ses ligands en se logeant dans la cavité hydrophobe. De plus, l'apoD est aussi capable de se retrouver sous forme dimérique et tétramérique (Kielkopf *et al.*, 2018).

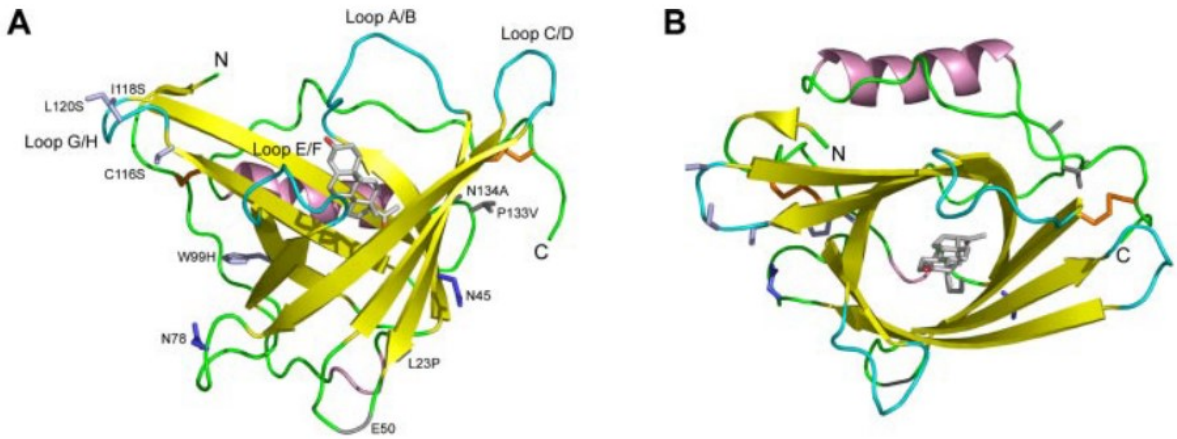

Figure 1.2 : Structure cristallographique de l'apoD humaine avec la progestérone.

A - Représentation transversale – La structure secondaire de l'apoD est constituée de 8 feuillets β antiparallèles (jaune). Les boucles (bleu) A/B, C/D, E/F et G/H font la liaison entre ces 8 feuilles β, le tout formant une cavité hydrophobe. D'autres boucles, en vert, sont aussi présentes à l'opposé de l'entrée de cette cavité. Deux hélices α (en rose), permettent la fermeture de la cavité. L'apoD possède des sites de glycosylations (en bleu) sur l'acide aminé asparagine en position 45 (N45) et 78 (N78). Les ponts disulfures sont indiqués en oranges. B - Représentation de la vue au-dessus de la cavité montrant la liaison avec la progestérone. Figure adaptée de (Eichinger et al., 2007)

Les 8 feuillets β sont reliés par des boucles A/B, C/D, E/F, G/H (Figure 1-2). Les boucles A/B, E/F et G/H sont de nature hydrophobe ce qui permettrait à l'apoD de s'insérer dans les *High-density lipoprotein* (HDL), plus précisément dans la phase lipidique grâce à la présence des cystéines (Eichinger *et al.*, 2007).

1.1.2 L'expression de l'apolipoprotéine D

1.1.2.1 Expression tissulaire

Chez l'humain, l'apoD est très peu exprimée dans le foie et les intestins, contrairement aux autres apolipoprotéines, ce qui, une fois de plus, participe à sa singularité dans la famille des apolipoprotéines (Figure 1-3). Cette protéine est à la fois exprimée durant la période

embryonnaire et au stade adulte, et possède une large distribution tissulaire contribuant à son rôle multifonctionnel (Rassart *et al.*, 2000; Rassart *et al.*, 2020). Elle est très exprimée dans le cerveau, les testicules, les ovaires, les nerfs périphériques, le pancréas, le placenta, les reins (Drayna *et al.*, 1986). Elle est aussi retrouvée dans les fluides, comme le plasma (0,05 à 0,2 mg/ml) (Camato *et al.*, 1989), le liquide cérébro-spinal (LCS) (1,2 µg/ml) (Terrisse *et al.*, 1998) et les fluides kystiques (13,7 à 15,1 mg/ml) (Pearlman *et al.*, 1973; Sanchez *et al.*, 1992).

Chez la souris et le rat, cette expression tissulaire est différente. En effet, chez ces rongeurs, l'ARN de l'apoD est retrouvé majoritairement dans le système nerveux (Boyles *et al.*, 1990b; Seguin *et al.*, 1995). En revanche, chez le singe (Smith *et al.*, 1990), le lapin (Provost *et al.*, 1991b; Provost *et al.*, 1990) et le cochon d'Inde (Provost *et al.*, 1995) la distribution tissulaire est similaire à celle chez l'humain (Drayna *et al.*, 1986). L'apoD aviaire est présente dans l'ovocyte en croissance rapide. Le rôle de transport des lipides durant l'embryogenèse suggère la présence de l'apoD à ce stade de développement chez les ovipares (Vieira *et al.*, 1995; Yao & Vieira, 2002).

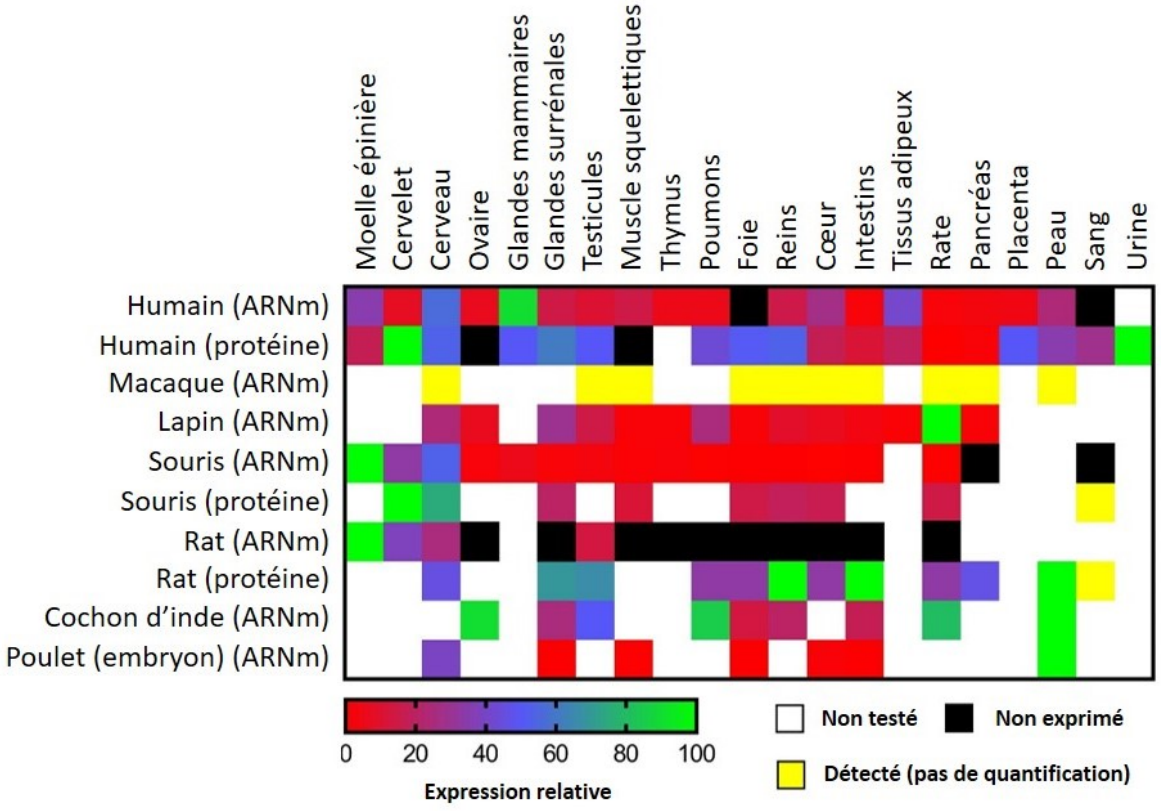

Figure 1.3 : Expression d'ARNm et de protéine relative d'ApoD en fonction des tissus de plusieurs espèces. Figure modifiée de Rassart *et al.*, 2020.

Les expressions d'apoD indiquées sur la figure s'appuient sur plusieurs articles. L'expression relative est indiquée en pourcentage d'expression d'apoD pour chaque tissu et espèces. Chez certaines espèces et

tissus, l'apoD a été détectée mais n'a pas été quantifiée (indiqué en jaune). Les données proviennent des articles suivants : pour l'humain (Uhlen *et al.*, 2015) (https://www.proteinatlas.org/ENSG00000189058-APOD/tissue), (Ben-Ari Fuchs *et al.*, 2016; Fishilevich *et al.*, 2016) données disponibles : https://www.genecards.org/cgi-bin/carddisp.pl?gene=APOD, (Drayna *et al.*, 1986; Seguin *et al.*, 1995) ; chez le macaque (Smith *et al.*, 1990), le lapin (Provost *et al.*, 1990) ; la souris (Cofer & Ross, 1996; Do Carmo *et al.*, 2009b; Li *et al.*, 2016b; Seguin *et al.*, 1995) ; le rat (Boyles *et al.*, 1990b; Seguin *et al.*, 1995) ; le cochon d'Inde (Provost *et al.*, 1995) ; le poulet (Ganfornina *et al.*, 2005) . Figure modifiée de Rassart *et al.*, 2020.

1.1.2.2 Expression cellulaire

L'ARNm de l'apoD est principalement exprimé par les fibroblastes, plus précisément ceux à proximité des vaisseaux sanguins (Provost *et al.*, 1991b; Smith *et al.*, 1990). Les oligodendrocytes et astrocytes sont les deux types cellulaires qui synthétisent majoritairement l'apoD dans le système nerveux central (SNC). En revanche, dans le système nerveux périphérique, ce sont les fibroblastes endoneuraux qui produisent l'apoD (Boyles *et al.*, 1990a; Boyles *et al.*, 1990b; Boyles *et al.*, 1989). Il a été montré que dans des conditions normales *in vitro*, que l'apoD pouvait être sécrétée et réinternalisée par des cellules NIH/3T3, des fibroblastes de souris. Après cette internalisation, l'apoD était retrouvée dans la zone périnucléaire des fibroblastes. Cette localisation est différente dans des conditions de stress (arrêt de croissance ou stress inflammatoire) et l'apoD est alors retrouvée dans le cytoplasme et le noyau. L'apoD nucléaire aurait pour origine le milieu extracellulaire, et les auteurs suggèrent qu'elle pourrait jouer un rôle de régulateur transcriptionnel (Do Carmo *et al.*, 2007).

Récemment, quelques études ont mentionné la présence de l'apoD dans les vésicules extracellulaires (VEs) dans le sérum mais aussi du LCS (Ben Khedher *et al.*, 2021; Cheow *et al.*, 2016; Jiang *et al.*, 2019; Pascua-Maestro *et al.*, 2018; Przybycien-Szymanska *et al.*, 2016).

1.1.3 Les ligands de l'apoD

Comme vu précédemment, la structure cristallographique de l'apoD permet de mettre en évidence une cavité hydrophobe où les ligands de l'apoD pourraient se loger (Figure 1.2). Plusieurs ligands ont déjà été documentés dans la littérature.

1.1.3.1 L'acide arachidonique

L'acide arachidonique est le ligand physiologique qui dispose de la plus grande affinité avec l'apoD (Morais Cabral *et al.*, 1995). Elle se lie avec une affinité 100 fois supérieure à celle de la progestérone. En revanche, l'apoD recombinante produite chez la bactérie *Escherichia coli*, ne montre pas de différence d'affinité de liaison de l'apoD avec la progestérone ou l'acide

arachidonique (Vogt & Skerra, 2001). L'absence de glycosylation chez les bactéries peut expliquer cette différence d'affinité, par conséquent la protéine recombinante sans glycosylation aurait la même affinité pour la progestérone et l'acide arachidonique. Ce cas démontre l'importance de la glycosylation pour déterminer l'affinité de l'apoD avec ses ligands. Les analyses sur les dynamiques moléculaires suggèrent que la liaison entre l'acide arachidonique et ses dérivés, avec l'apoD se produit de manière flexible pour l'ouverture de la cavité hydrophobe. L'acide arachidonique est le précurseur de la synthèse des eicosanoïdes (prostaglandines et leucotriènes) (Kuehl & Egan, 1980). Ces agents sont produits localement dans l'organisme et répondent à différents stimuli. Ils sont impliqués dans l'inflammation, l'agrégation de plaquettes et la régulation cellulaire (Kuehl & Egan, 1980). L'acide arachidonique possède plusieurs fonctions, et intervient notamment dans la signalisation cellulaire (Piomelli, 1993). Dans ce contexte l'apoD pourrait jouer un rôle de transporteur de l'acide arachidonique, ce dernier étant impliqué dans un rôle de régulation et protection cellulaire.

1.1.3.2 **Hormones stéroïdiennes**

L'apoD a été caractérisée comme une protéine de liaison avec la progestérone dans le fluide kystique mammaire (Pearlman *et al.*, 1973). Des études de co-cristallisation ont confirmé cette interaction (Eichinger *et al.*, 2007). Cependant, tous les stéroïdes ne montrent pas une affinité avec l'apoD, c'est le cas de l'oestradiolβ, ceci suggérant que l'oestrogène n'est possiblement pas un ligand naturel de l'apoD (Ruiz *et al.*, 2013). Le promoteur du gène de l'apoD possède plusieurs éléments régulateurs des stéroïdes, ainsi les stéroïdes influenceraient l'expression de l'apoD (Lambert *et al.*, 1993). L'apoD aurait donc un lien direct entre la protéine et la progestérone, mais les hormones stéroïdes pourraient aussi influer sur son expression génique.

1.1.3.3 **Le cholestérol**

Initialement, l'apoD a été identifiée comme un composé des HDL. Les HDL sont des lipoprotéines permettant le transport de lipides aux cellules. L'apoD a aussi été retrouvée pour être associée à LCAT, apoA-I ou CETP (protéine de transfert d'ester cholesteryl), où elle ferait partie d'un complexe responsable du transport du cholestérol des tissus périphériques vers le foie pour qu'il soit métabolisé (Blanco-Vaca *et al.*, 1992; Spreyer *et al.*, 1990; Weech *et al.*, 1991; Yang *et al.*, 1994). En présence de l'apoD, l'activité d'estérification de la LCAT est augmentée (Fielding & Fielding, 1980; Steyrer & Kostner, 1988). L'association de LCAT avec l'apoD

permettrait de mieux stabiliser cette enzyme. De plus, cette association a permis de mettre en évidence le fait que l'apoD pouvait se lier au cholestérol et ses dérivés estérifiés (Drayna *et al.*, 1986). Cependant, bien qu'une liaison entre l'apoD et le cholestérol a été mise en évidence, il apparait que cette liaison est de faible affinité (Patel *et al.*, 1997). À l'heure actuelle, cette liaison est encore controversée, car d'autres études ne l'ont pas détectée (Morais Cabral *et al.*, 1995; Ruiz *et al.*, 2013). L'explication de cette controverse pourrait s'expliquer car la liaison se ferait de manière indirecte, *via* la sphingomyéline qui fait partie des ligands de l'apoD (Ruiz *et al.*, 2013).

1.1.3.4 **Autres ligands**

D'autres ligands de l'apoD ont été identifiés dans la littérature, notamment des dérivés de l'hème, tels que la bilirubine (Peitsch & Boguski, 1990). Cette interaction permettrait à la bilirubine d'être associée aux HDL (Goessling & Zucker, 2000). De plus le niveau de glycosylation de l'apoD interférerait son affinité de liaison avec la bilirubine (Vogt & Skerra, 2001). L'acide rétinoïque qui est impliqué dans la différenciation cellulaire, la sphingomyéline et les sphingolipides qui sont des constituants importants des particules des HDL et de la membrane plasmique sont des ligands de l'apoD identifiés (Breustedt *et al.*, 2006; Rhinn & Dolle, 2012; Ruiz *et al.*, 2013; Vance, 2012). Bien que leurs interactions aient été démontrées, elles demeurent faibles et la pertinence de celle-ci est controversée. En revanche, la large variété des ligands pouvant s'associer démontre la multiplicité des activités de l'apoD.

1.1.4 **Récepteurs de l'apoD**

Après avoir été sécrétée par les cellules, l'apoD peut ensuite être internalisée par ces mêmes cellules ou par d'autres (Do Carmo *et al.*, 2007; Najyb *et al.*, 2016). Son internalisation est dépendante de récepteurs, notamment de la basigine (aussi nommé CD147), une glycoprotéine transmembranaire (Najyb *et al.*, 2015). La basigine joue un rôle dans plusieurs activités cellulaires comme la croissance, la différentiation, la survie et l'adhésion cellulaire (Muramatsu & Miyauchi, 2003). Elle est exprimée dans de nombreux tissus et est aussi impliquée dans plusieurs pathologies comme le cancer ou la maladie d'Alzheimer (MA) (Iacono *et al.*, 2007). Dans un premier temps, l'interaction entre l'apoD et la basigine a été mise en évidence dans les cellules 293T, une lignée immortalisée de cellules rénales humaines. Cette interaction a ensuite été confirmée par la visualisation d'une co-localisation de la basigine et de l'apoD à la surface des cellules 293T. Dans cette même étude, la diminution transitoire de l'expression de basigine induit une diminution de l'internalisation de l'apoD dans ces cellules. Dans des conditions

normales, l'apoD est peu internalisée dans les SH-5YSY, une lignée cellulaire immortalisée de neurones humains. Au contraire, la surexpression de basigine dans les cellules SH-5YSY induit une augmentation d'internalisation de l'apoD. Enfin l'internalisation de l'apoD est diminuée en présence d'un ligand bien connu de la basigine, la cyclophylline A, confirmant que l'internalisation de l'apoD est en partie médiée par la basigine. Cette étude a permis de mettre en évidence un lien étroit entre l'internalisation de l'apoD et le récepteur basigine.

Outre la basigine, l'apoD semble aussi se lier aux récepteurs aux apolipoprotéines. En effet, lorsque des cellules de la moelle épinière sont traitées avec de l'apoD (5 µg/ml) pendant 2 à 6 jours, cela induit une augmentation de la quantité des protéines PSD-95, la synaptophysine ainsi que la synaptotagmine, qui sont des protéines synaptiques (Kosacka *et al.*, 2009). Lorsque qu'un inhibiteur des récepteurs aux apolipoprotéines est ajouté avant le traitement à l'apoD, cette augmentation n'a pas lieu, mettant en évidence l'importance de ce récepteur dans cette signalisation. Il est aussi important de noter que l'apoD n'est pas retrouvée sous une seule forme dans le corps. Elle peut être retrouvée liées aux HDLs, dans les VEs ou sous forme dimérisée ou tétramérisée, ainsi l'apoD peut entrer dans les cellules par de multiples voies d'internalisation.

1.2 Rôle dans la périphérie

1.2.1 Rôle dans le processus développemental

Une modification de l'expression de l'apoD est observée durant la grossesse et la période de développement embryonnaire. Chez les embryons de cochons d'Inde, il a été observé de faibles niveaux de transcrits d'apoD (Provost *et al.*, 1991a). Chez la souris, l'expression embryonnaire d'apoD commence entre 8 et 9 jours et est sélectivement contrôlée jusqu'à la naissance (Sanchez *et al.*, 2002). Chez les rats l'induction d'apoD dans la période embryonnaire coïncide avec la myélination et la synaptogénèse (Ong *et al.*, 1999) . Enfin chez le poulet, l'apoD est rapidement présente durant la croissance des oocytes et est associée avec la mobilisation et le transport des lipides (Vieira *et al.*, 1995).

Enfin chez l'humain, l'apoD est fortement exprimée dans l'endomètre durant l'implantation et dans le placenta (Drayna *et al.*, 1986; Kao *et al.*, 2002). Les niveaux d'apoD plasmatique diminuent durant la gestation, diminution qui est accentuée chez les femmes ayant une prise de poids excessive durant cette période (Do Carmo *et al.*, 2009a).

1.2.2 **Influence dans les voies métaboliques**

L'apoD et les lipides ont des liens étroits, tout particulièrement dans les tissus adipeux où il y a une corrélation positive entre la masse adipeuse, les niveaux de leptines et l'expression de l'apoD hypothalamique (Liu *et al.*, 2001). L'apoD plasmatique diminue significativement pendant une grossesse sans complication (Do Carmo *et al.*, 2009a). Comme cité précédemment, l'apoD diminue aussi chez les femmes gravides ayant une prise de poids excessive durant cette période. Chez ces femmes, la concentration d'apoD est étroitement et négativement corrélée avec les paramètres lipidiques tels que l'indice de masse corporel, le cholestérol total, les concentrations en HDL et LDL, la concentration en triglycérides, acide gras libres, apoA-I et apoB-100 (Do Carmo *et al.*, 2009a).

Le traitement d'adipocytes 3T3-L1 en présence d'acide arachidonique provoque l'augmentation du niveau d'incorporation des transporteurs du glucose, GLUT1 et GLUT4, dans les membranes plasmatiques. De ce fait, cela induit l'augmentation de l'absorption du glucose (Nugent *et al.*, 2001). Une partie de l'effet de l'apoD sur l'homéostasie du glucose dans le tissu adipeux pourrait être dû à la modulation de la production et de la libération d'eicosanoïdes.

Le rôle de l'apoD dans le métabolisme lipidique a été montré dans une étude sur des souris surexprimant l'apoD humaine dans le système nerveux central (Do Carmo *et al.*, 2009b). Ces souris ont montré une accumulation d'apoD dans le foie et lentement développé une stéatose hépatique et musculaire, accompagnée d'une résistance à l'insuline (Do Carmo *et al.*, 2009b). L'apoD cérébrale jouerait un rôle important sur la périphérie dans ce modèle de souris génétique. Cependant ces souris ne sont pas obèses, ni diabétiques et ne développent pas d'inflammation. Le développement de cette pathologie est déclenché au moins partiellement par des évènements signalisés précis impliquant des prostanoïdes dérivés de l'activation de l'acide arachidonique et activation de PPARγ (Desmarais *et al.*, 2019; Labrie *et al.*, 2015). L'apoD induit une accumulation lipidique qui n'est pas dûe à une lipogenèse plus active mais par une augmentation de la capture des lipides. Une caractéristique importante de la stéatose hépatique induite par l'apoD est que les acides gras omega-3 et oméga-6 (incluant l'acide arachidonique) sont accumulés préférentiellement sous forme d'acides gras sursaturés ou mono-insaturés. De plus, les acides gras oméga 3 sont accumulés de manière plus rapide que les acides gras oméga 6, montrant un équilibre omega3/omega6 plus favorable dans les souris transgéniques que dans les souris contrôles.

1.2.3 **Implication dans les maladies cardio-vasculaires et métaboliques**

L'expression d'apoD varie dans plusieurs pathologies métaboliques, telles que le déficit en HDL ou la maladie de Tangier (Alaupovic *et al.*, 1981), le déficit familial en LCAT (Albers *et al.*, 1985) ainsi que dans le diabète de type 2 (Baker *et al.*, 1994; Hansen *et al.*, 2004). Ces maladies et l'apoD seraient reliées par un problème de métabolisme lipidique.

La maladie de Tangier, est une maladie génétique causée par la mutation du gène transporteur *ATP-binding casette* (ABCA1). Il s'agit d'une protéine responsable du transport du cholestérol intracellulaire vers la surface cellulaire, permettant son transfert vers les HDL (Bodzioch *et al.*, 1999). Cette perte de fonction favorise l'apparition d'athérosclérose due à l'accumulation de cholestérol en périphérie (Bodzioch *et al.*, 1999). Chez les patients atteints d'athérosclérose, l'apoD est retrouvée enrichie dans les HDL. Ceci pourrait être un mécanisme compensatoire pour maintenir une homéostasie du cholestérol, où l'apoD prendrait la fonction de ABCA1 (Vaisar *et al.*, 2007). Dans les athéromes, qui sont des plaques composées de lipides et glucides retrouvés dans les artères, l'apoD est retrouvée et est associée à l'accumulation de cholestérol dans les athéromes de patients atteints d'athérosclérose ou chez des modèles murins de cette maladie (Perdomo & Henry Dong, 2009; Sarjeant *et al.*, 2003). L'apoD permettrait d'éliminer le cholestérol en excès dans les cellules, pour les associer au HDL, permettant ensuite l'élimination de celui-ci par le foie (Pfrieger, 2003). Ce mécanisme permet aux HDL de protéger l'organisme vis-à-vis du développement de l'athérosclérose en éliminant le cholestérol (Tall, 2008).

Dans un modèle d'artérosclérose létal de souris, le gène de l'apoD est surexprimé (Tsukamoto *et al.*, 2013). De plus, l'apoD protège partiellement les souris contre un blocage temporaire de l'artère coronaire, mais elle protège aussi des cellules isolées de rat du muscle cardiaque contre la privation temporaire d'oxygène (Tsukamoto *et al.*, 2013). L'apoD aurait un rôle cardioprotecteur dans les cas d'artères obstruées notamment grâce son activité antioxydante.

1.2.4 **Fonctions de différenciation et de croissance cellulaire**

Des cultures primaires de fibroblastes humains ont montré que suite à un arrêt de la croissance cellulaire, l'apoD était induite (Provost *et al.*, 1991a). Cette induction a aussi été rapportée dans des cultures de fibroblastes quiescents et sénescents et dans d'autres types cellulaires (Do Carmo *et al.*, 2002).

L'apoD peut être sécrétée puis réinternalisée dans des conditions de culture cellulaires dites normales (Do Carmo *et al.*, 2007; Leung *et al.*, 2004; Sarjeant *et al.*, 2003). Elle est capable de moduler sélectivement la réponse de prolifération aux facteurs de croissance dans le muscle lisse en inhibant la translocation nucléaire de la forme active de ERK1/2 (*extracellular signal-regulated kinase 1/2)* (Sarjeant *et al.*, 2003).

Son expression peut aussi être induite suite à différentes conditions de stress, telles que le stress oxydatif et inflammatoire, ou un traitement aux UV (Do Carmo *et al.*, 2007). Ces observations ont été faites à des concentrations de H_2O_2 ou des doses spécifiques d'UV qui induisent un arrêt de croissance mais pas d'apoptose. L'augmentation de l'expression de l'apoD peut se faire par liaison d'un facteur nucléaire dans la région promotrice de l'apoD, comme les facteurs nucléaires PARP-1 (*Poly ADP-ribose polymerase-1*) et APEX (*Apurinic/Apyrimidic Endonuclease-I*), qui sont des facteurs hautement exprimés dans des conditions d'arrêt de croissance cellulaire (Levros *et al.*, 2010).

D'autre part, l'apoD est aussi impliquée dans la différenciation cellulaire. L'acide rétinoïque et le 1,25-dihydroxyvitamine D3, qui sont deux médiateurs cellulaires, participent à l'induction de l'expression d'apoD dans les cellules de cancers mammaires (Lopez-Boado *et al.*, 1997; Lopez-Boado *et al.*, 1994). Les récepteurs nucléaires à l'acide rétinoïques (RAR) induisent une inhibition significative de la prolifération cellulaire, ceci favorisant l'induction de l'expression d'apoD. De cette observation, il a été suggéré que l'apoD pourrait être un marqueur biochimique de la différentiation et de l'arrêt de la prolifération cellulaire contrôlée par les RAR dans les cellules de cancer mammaires (Lopez-Boado *et al.*, 1996). Une observation similaire a été effectuée aussi dans les cancers de la prostate (Aspinall *et al.*, 1995), où l'apoD est retrouvée principalement dans l'épithélium glandulaire prostatique. Sa présence est alors associée à un état non-prolifératif ou de différentiation. Pour résumer, l'apoD est stimulée lors d'un arrêt de croissance, dans les cellules sénescentes et durant les stress oxydatifs et inflammatoires.

D'après toutes ces études, l'expression de l'apoD est inversement corrélée avec la croissance cellulaire, et cette expression est plutôt retrouvée dans le phénomène de différenciation cellulaire. Ces observations ont permis d'étudier plus en profondeur le rôle étroit que pourrait avoir l'apoD dans plusieurs cancers.

1.2.5 **Rôle dans le cancer**

L'apoD est exprimée et contrôlée dans plusieurs cancers. Elle est surexprimée dans de nombreux types de carcinomes, comme ceux du sein, des ovaires, de la prostate, de la peau et

du système nerveux central. Dans la prostate, le pancréas et la peau, un haut niveau d'expression d'apoD est associé avec une invasion du cancer (Ashida *et al.*, 2004; Aspinall *et al.*, 1995; Hall *et al.*, 2004; Zhang *et al.*, 1998). De plus, dans le cadre des cancers du poumon, une haute expression des transcrits d'apoD est aussi associée avec une survie plus courte et sans récidive (Cury *et al.*, 2019). Des recherches sur les cancers précoces du sein et du système nerveux central ont montré qu'ils sont fortement associés avec une augmentation de l'expression de l'apoD. Celle-ci est retrouvée principalement dans les carcinomes hautement différenciés, non invasifs et non métastatiques (Hunter *et al.*, 2002; Porter *et al.*, 2003; Serra Diaz *et al.*, 1999). La surexpression de l'apoD est souvent liée à une diminution des rechutes de cancer et, contrairement aux cancers du poumon, est associée à une meilleure survie des patients atteints de carcinomes (Diez-Itza *et al.*, 1994). Comme évoqué précédemment, l'expression de l'apoD augmente lors de la différenciation, ce qui a été constaté dans le cadre de la progression des cancers, (Diez-Itza *et al.*, 1994; Porter *et al.*, 2003; Serra Diaz *et al.*, 1999). En revanche, l'augmentation d'expression de l'apoD peut être associée à un caractère invasif des cancers, notamment dans les cancers de la prostate, de la peau et du pancréas (Ashida *et al.*, 2004; Aspinall *et al.*, 1995; Hall *et al.*, 2004; Zhang *et al.*, 1998). Dans ce contexte, l'apoD semble être augmentée, favorisant ainsi la progression de la maladie (Iacobuzio-Donahue *et al.*, 2002; Miranda *et al.*, 2003; Ryu *et al.*, 2001; West *et al.*, 2004). Cependant, à l'heure actuelle, il reste à déterminer si l'augmentation d'apoD dans ces cancers est une cause ou une conséquence de la maladie, et donc des changements d'état cellulaires.

1.3 Rôle de l'apoD dans le système nerveux

1.3.1 L'apoD dans le système nerveux périphérique (SNP)

Les cellules fibroblastiques du système nerveux périphérique (SNP) sécrètent de l'apoD. Cette sécrétion est accentuée dans les nerfs périphériques pendant une régénération faisant suite à une lésion (Boyles *et al.*, 1990a; Spreyer *et al.*, 1990). Dans les faits, la forme protéique de l'apoD et son ARN augmentent, les niveaux sont respectivement 500 et 40 fois plus importants dans les nerfs périphériques pendant la régénération. Les niveaux d'autres apolipoprotéines comme l'apoA-I, A-IV, et E, sont aussi multipliés respectivement par 26, 14 et 250 comparés à leurs concentrations normales. L'apoD et l'apoE semblent impliquées dans ce processus de régénération et sont synthétisées localement (Boyles *et al.*, 1990a). La dégénération des nerfs périphériques entraîne une libération du cholestérol provenant de la dégradation des gaines de myéline qui est principalement absorbée et stockée par les macrophages (Boyles *et al.*, 1990a;

Boyles *et al.*, 1990b). Les lipoprotéines contenant de l'apoA-I et de l'apoE sont principalement impliquées dans la régénération du nerf, en transportant le cholestérol des macrophages vers les cellules de Schwann intratubulaires qui myélinisent les axones en cours de régénération. En effet, les cellules de Schwann réagissent en augmentant l'expression des récepteurs aux LDL pendant la remyélination, qui probablement va aider à capturer les lipoprotéines (Boyles *et al.*, 1989).

L'apoD et l'apoE sont exprimées dans les nerfs à partir de 3 à 6 jours après la lésion, le pic d'expression est observé 3-4 semaines après, ensuite on observe une diminution jusqu'à 10 semaines pour revenir à un niveau normal (Goodrum, 1993). Ainsi, un rôle de liaison et de transport du cholestérol est attribué à ces apolipoprotéines, assurant un maintien de l'homéostasie et la réutilisation du cholestérol pendant la régénération (Boyles *et al.*, 1989; Goodrum, 1993; Goodrum *et al.*, 1995). Par conséquent, il apparait ici qu'il s'agit d'un mécanisme de transfert et de recyclage du cholestérol plutôt que de son remplacement. Des résultats similaires ont été observés chez le lapin et le singe (Boyles *et al.*, 1990a). Des résultats *in vivo* vont dans le même sens que ces observations. En effet, une co-culture de neurones de la moelle épinière de rat avec les adipocytes 3T3-L1 a montré une amélioration de la neuritogénèse et de la synaptogénèse (Kosacka *et al.*, 2009). Il a été observé que lors de cette co-culture, les gènes de l'apoD et l'apoE étaient hautement activés. De plus, dans cette même étude, quand les neurones de rats seuls étaient traités avec de l'apoD, une augmentation de synaptophysine et synaptotagmine était observée. Ces protéines sont des protéines pré et post-synaptiques qui contribuent à la synaptogénèse. Par ailleurs, ce traitement induit un accroissement plus important des neurites. En parallèle, les récepteurs aux apolipoprotéines sont aussi hautement exprimés dans ces conditions. Une inhibition de ces récepteurs lors du traitement aux apolipoprotéines n'induit pas d'augmentation pour les protéines synaptiques, montrant que l'apoD et l'apoE seraient impliquées dans l'augmentation de cette expression par le biais direct ou indirect des récepteurs aux apolipoprotéines.

Dans un modèle génétique de souris déficiente en apoD (apoD$^{-/-}$), la vitesse de transmission au nerf moteur est diminuée ainsi que l'épaisseur des gaines de myélines dans les nerfs (Ganfornina *et al.*, 2010). Inversement, lorsqu'une rupture du nerf sciatique est effectuée chez des souris surexprimant l'apoD dans les neurones, une amélioration plus efficace et rapide des fonctions locomotrices est observée. D'autre part, le manque d'apoD retarde la régénération et la remyélination axonale, ces phénomènes sont stimulés en excès d'apoD (Ganfornina *et al.*, 2010).

1.3.2 **L'apoD dans le système nerveux central (SNC)**

L'apoD est sécrétée dans le SNC par les oligodendrocytes et les astrocytes et peut participer au processus de réinnervation (Boyles *et al.*, 1990a; Navarro *et al.*, 1998; Patel *et al.*, 1995; Provost *et al.*, 1990). Deux ligands potentiels de l'apoD, la progestérone et la prégnénolone, sont synthétisés aussi par les astrocytes et les oligodendrocytes dans le SNC (Hu *et al.*, 1987). La prégnénolone a été montrée pour être accumulée sous sa forme sulfatée dans les cerveaux et nerfs sciatiques des humains et rats (Hu *et al.*, 1987; Morfin *et al.*, 1992). Dans ce cas, l'apoD pourrait être impliquée dans le transport local de ces hormones stéroïdiennes. La formation et la régulation de la connectivité synaptique sont connues pour être régulées par les récepteurs aux stéroïdes (McEwen & Woolley, 1994)**.** En particulier, les récepteurs aux œstrogènes et à la progestérone sont retrouvés dans l'hippocampe, qui est l'aire de formation et de stockage de la mémoire. L'expression de la progestérone est par ailleurs induite par les œstrogènes (McEwen & Woolley, 1994). Comme l'expression de l'apoD a été montrée pour être modulée par les œstrogènes dans plusieurs situations et que l'apoD lie très probablement la progestérone, il pourrait exister une relation indirecte entre ces deux différents éléments (McEwen & Woolley, 1994). De plus, dans le cerveau, il a été montré que l'expression d'apoD était influencée par l'âge et le genre (Ordonez *et al.*, 2012).

Le manque d'apoD chez la souris induit un vieillissement prématuré chez des souris jeunes, cependant sans affecter l'espérance de vie de l'animal (Sanchez *et al.*, 2015). Ce vieillissement prématuré est notamment observé par l'intermédiaire de l'activation de plusieurs gènes comme GFAP et celui de la protéine liant le Ca^{2+} : S100a6 connues pour être régulé positivement avec l'âge (Sanchez *et al.*, 2015). Outre les effets de l'âge comme l'hyperkinésie ou des déficits de mémoire, des différences moléculaires sont observées dans le cortex et l'hippocampe. La GFAP, qui est un marqueur de l'activité gliale, est retrouvée de manière plus importante dans les cerveaux de souris âgées déficientes en apoD. De plus, des dommages oxydatifs et inflammatoires augmentés sont aussi retrouvés. Ces observations traduisent des signes précoces du vieillissement et favorisent les dommages cérébraux dans les souris déficientes en apoD. De plus une diminution du nombre de neurones est aussi observée dans le cortex de ces souris âgées (diminution d'environ 1/3). Pour les auteurs (Sanchez *et al.*, 2015), l'augmentation de l'apoD dans les cerveaux âgés est un mécanisme d'antivieillissement, car cette augmentation est aussi retrouvée dans de nombreuses espèces.

1.3.3 **L'apoD entre le cerveau et la périphérie**

La barrière hémato-encéphalique (BHE) sépare le sang du cerveau et permet un passage filtré des molécules entre ces deux compartiments. Cette BHE a pour fonction principale d'isoler le cerveau de la circulation sanguine. Les cellules endothéliales sont les cellules principales de la BHE et sont liées par les jonctions serrées permettant à cette barrière d'être imperméable. Avec les péricytes et les astrocytes, elles représentent la BHE (Iadecola, 2017). Plusieurs composés peuvent traverser cette barrière notamment des nutriments, ions, peptides, médicaments et hormones (Banks, 2016; Galea & Perry, 2018; Zlokovic, 2011). Cette capacité de barrière fortement restrictive permet aux cellules endothéliales de la BHE de réguler étroitement l'homéostasie du SNC, ce qui est essentiel pour permettre une fonction neuronale et pour protéger le SNC des toxines, agents pathogènes, de l'inflammation et des maladies. Dans plusieurs maladies comme les traumatismes crâniens, les accidents vasculaires cérébraux, la sclérose en plaques ou les maladies neurodégénératives, on retrouve une perte totale ou partielle de cette restriction (Daneman, 2012; Zlokovic, 2008).

À l'heure actuelle, aucune étude n'a permis de mettre en évidence le passage de l'apoD à travers la BHE. Contrairement à l'Homme, chez la souris, l'expression de l'ARNm de l'apoD est exclusivement retrouvée dans la SNC (Cofer & Ross, 1996; Do Carmo *et al.*, 2009b; Li *et al.*, 2016b; Rassart *et al.*, 2020; Seguin *et al.*, 1995). Cependant, un haut niveau de protéine est retrouvé dans les tissus périphériques dont le foie, ce qui suggère que l'apoD est capable de traverser la BHE (Boyles *et al.*, 1990b; Rassart *et al.*, 2020). Cette hypothèse a été appuyée par une autre étude, chez des souris surexprimant l'apoDh dans les neurones, un niveau détectable d'apoDh était retrouvé dans le foie (Do Carmo *et al.*, 2009b). De plus, ces souris développent des stéatoses hépatiques et musculaires (Desmarais *et al.*, 2019; Do Carmo *et al.*, 2009b; Labrie *et al.*, 2015), causées très probablement car l'apoDh modulerait le métabolisme dans ces tissus. Ainsi cela suggère que l'apoD produite dans le cerveau peut aussi influencer la périphérie comme le montre l'apparition de ces stéatoses. De plus, l'internalisation de l'apoD est dépendante de la basigine (Najyb *et al.*, 2015). Ce récepteur est retrouvé à la surface des cellules endothéliales de la BHE, tout comme LDLR (Wagner *et al.*, 2012; Zuchero *et al.*, 2016).

Bien que la BHE représente une surface 10 fois supérieure au plexus choroïde (Takeshita & Ransohoff, 2012), l'apoD pourrait aussi traverser cette barrière. En effet un haut niveau expression d'ARNm a été retrouvé dans l'épithélium du plexus choroïde, tout comme l'apoE qui régule son expression (Jansen *et al.*, 2009; Janssen *et al.*, 2013). Ainsi, cette structure pourrait

participer aussi au passage de l'apoD vers la périphérie en induisant une augmentation de son expression.

1.3.4 L'apoD et son rôle dans le cerveau

L'apoD dans le cerveau pourrait jouer un rôle de protection ou de prévention contre le stress oxydatif. En effet dans des conditions normales, des souris sans apoD montrent une augmentation des lipides peroxydés dans le cerveau, qui n'est pas observée dans les poumons, sans induire une modification sur les niveaux de protéines carbonylés (Ganfornina *et al.*, 2008). Cependant chez des souris surexprimant l'apoDh dans les neurones, le niveau de peroxydation lipidique est similaire aux souris sauvages, montrant qu'un excès d'apoD ne réduit pas le niveau basal des produits d'oxydation. Dans cette même étude, suite à un traitement de paraquat, qui est connu pour générer la peroxydation des lipides (Beal, 2002), les souris apoD$^{-/-}$ montrent une augmentation de la peroxydation lipidique plus importante que les souris sauvages. En revanche, lorsque les souris surexpriment l'apoDh dans les neurones, après un traitement de 2 semaines de paraquat, les niveaux de peroxydation lipidique ne sont pas modifiés, contrairement aux souris sauvages qui ont eu ce même traitement (Ganfornina *et al.*, 2008). Ainsi, l'excès d'apoD pourrait contrer l'induction exogène de stress oxydatif mais pas dans des conditions normales.

De plus, chez ces souris, les capacités locomotrices et d'apprentissage sont altérées. Au contraire, la surexpression neuronale de l'apoDh produit l'effet opposé après un traitement induisant un stress oxydatif, c'est-à-dire une augmentation de la survie ainsi que la prévention de la peroxydation lipidique (Ganfornina *et al.*, 2008). Parallèlement, chez une autre espèce étudiée, la drosophile, la perte de l'homologue de l'apoD montre également une augmentation de la sensibilité au stress oxydatif et à la peroxydation lipidique (Sanchez *et al.*, 2000). De plus, ces drosophiles montrent une espérance de vie réduite (Sanchez *et al.*, 2006). Au contraire, l'effet inverse est observé chez les drosophiles surexprimant cet homologue, c'est-à-dire une résistance plus importante au stress oxydatif et une augmentation de la longévité (Hull-Thompson *et al.*, 2009; Ruiz *et al.*, 2013; Walker *et al.*, 2006).

Il y a une évidence grandissante que la fonction de l'apoD s'étend plus largement au simple transport de molécules lipophiliques, et qu'elle a un lien direct avec le bon fonctionnement cérébral. En effet, il a été démontré que l'apoD pouvait catalyser la réaction de réduction des hydroperoxydes de l'acide arachidonique en hydroxydes *via* sa méthionine 93 (Met93) (Bhatia *et al.*, 2012). Cette étude montre que l'apoD jouerait un rôle prépondérant dans la prévention de l'apparition de sous-produits toxiques de la peroxydation lipidique comme le 4-hydroxynonenal

(4-HNE) et l'acroléine, et ainsi limiter les effets du stress oxydatif en inhibant la peroxydation lipidique. Dans une lignée de cellules hippocampiques d'origine de souris (HT22), l'apoD est localisée dans le cytoplasme sous conditions physiologiques. En revanche, lors d'un traitement avec du H_2O_2 (inducteur de stress oxydatif) à une concentration induisant l'apoptose, l'apoD est retrouvée dans le noyau (Martinez-Pinilla *et al.*, 2015). Cependant, dans les maladies neurodégénératives, l'apoD s'accumule dans la membrane nucléaire de neurones et cellules gliales dans plusieurs aires cérébrales. Dans ces cas-là, l'apoD n'est jamais retrouvée dans le noyau, ce qui suggère que, l'apoD n'y exercerait pas un rôle spécifique dans le noyau, mais que cette localisation est due à un changement structural et fonctionnel de la cellule suite à une induction de stress oxydatif.

Plusieurs études suggèrent que l'apoD serait exprimée en réponse aux altérations cérébrales, notamment au stress oxydatif, et ainsi son importante expression aurait pour rôle de protéger les neurones (Belloir *et al.*, 2001; Kalman *et al.*, 2000; Thomas *et al.*, 2003c).

L'apoD est une protéine pouvant être transportée dans des vésicules extracellulaires (VEs), qui jouent un rôle important dans les communications intercellulaires en transférant leur contenu à des cellules réceptrices (Maas *et al.*, 2017). Dans le SNC, sa présence dans les VEs produites par les cellules gliales à destination des neurones a été mise en évidence (Pascua-Maestro *et al.*, 2018). Dans cette étude *in vitro,* les auteurs montrent que l'apoD est exclusivement transportée des cellules gliales vers les neurones par les VEs. De plus, ces vésicules contenant l'apoD participent à la protection neuronale lors d'un traitement au paraquat, un herbicide puissant induisant un stress oxydatif. Chez les astrocytes, l'apoD extracellulaire induit une réaction de protection autocrine suite à un traitement de paraquat. Cette réaction est moins importante lorsque ce traitement est effectué chez des astrocytes déficientes en apoD.

L'injection d'acide kaïnique en périphérie chez la souris est connue pour induire une neurotoxicité, particulièrement dans l'hippocampe qui est très sensible dû à la grande quantité de récepteur kaïnate dans cette région. Les souris surexprimant l'apoDh au niveau neuronal montrent une résistance à l'acide kaïnique injecté en périphérie. Cette résistance est expliquée par une diminution de la réponse inflammatoire induisant une protection contre l'apoptose dans l'hippocampe, physiologiquement induite chez des souris sauvages (Najyb *et al.*, 2016).

Dans d'autres contextes de stress, l'apoD peut avoir un rôle bénéfique. En effet, lors d'une infection cérébrale du coronavirus humain (HCoV-OC43) provoquant une encéphalite aigüe chez la souris ; une augmentation des transcrits et de la protéine d'apoD dans le cerveau a été observée (Do Carmo *et al.*, 2008). Celle-ci coïncide avec l'activation gliale et une fois le virus

éliminé, l'expression d'apoD revient à un niveau normal. Les souris surexprimant l'apoDh dans les neurones montrent un taux de survie 3 fois plus important à cette infection. Ceci est corrélé avec l'activation gliale et démontre le rôle important de l'apoD dans la régulation de l'inflammation. Cependant l'inflammation peut aussi être causée par les espèces réactives oxygénées induites par le stress oxydatif (Naik & Dixit, 2011).

Cet état de la littérature suggère le rôle bénéfique de l'apoD de neuroprotection suite à une induction de stress oxydatif ou inflammatoire, ainsi que son rôle dans réparation neuronale, notamment par le biais de rôle de transporteur lipidique.

1.3.4.1 **L'apoD et la mémoire**

A ce jour, peu d'études ont établi un lien entre la mémoire et l'apoD. Il a été vu chez les souris apoD$^{-/-}$, une diminution de l'épaisseur des gaines de myéline. La myéline forme une gaine permettant d'isoler les axones des neurones et ainsi d'accélérer la vitesse de conduction des potentiels d'actions. Des souris déplétées de la protéine Myrf, qui est un facteur de régulation de la myéline, montrent une apparition de trouble de la mémoire spatiale (Steadman et al., 2020). Les cellules responsables de la production de myéline dans le SNC sont les oligodendrocytes. Un manque de production de myéline chez ces cellules chez les souris induit des déficits de mémoire spatiale (Xin & Chan, 2020). L'apoD pourrait être impliquée dans la mémoire spatiale en transportant la myéline vers les axones, sa déplétion impliquerait une fragilité de la mémoire spatiale. L'apoD a déjà été montrée pour être impliquée dans ce processus de régénération de la myéline dans le SNP (Boyles et al., 1990a).

Ces tests *open-field* ont montré une diminution des capacités locomotrices chez les souris apoD$^{-/-}$ (Ganfornina *et al.*, 2008). Ces résultats confirment ceux précédemment vus chez la drosophile avec la protéine homologue de l'apoD (Sanchez *et al.*, 2006). De plus les auteurs montrent que les souris Tg-apoDh montrent une activité plus importante. En contrepartie, les souris sans apoD montrent une capacité d'apprentissage moindre que des souris sauvages.

1.3.4.2 **L'apoD et la maladie d'Alzheimer**

Une modification de l'expression de l'apoD a été observée dans le cadre de plusieurs maladies neurodégénératives. Dans la maladie d'Alzheimer (MA), son expression est augmentée au niveau du liquide cérébro-spinal (LCS) ainsi que dans l'hippocampe et le cortex cérébral

(Desai *et al.*, 2005; Kalman *et al.*, 2000; Terrisse *et al.*, 1998). Cependant, cette augmentation n'a pas été observée dans le plasma de patients diagnostiqués pour la MA (Perrotte *et al.*, 2019).

La MA est une maladie dégénérative, progressive et irréversible. C'est la démence la plus commune, caractérisée par une perte de mémoire et des troubles cognitifs. C'est en 1906, que le Dr Aloïs Alzheimer identifie trois lésions caractéristiques de cette maladie : les plaques amyloïdes, les dégénérescences neurofibrillaires (NFTs) (Figure 1-4) ainsi que la présence de gouttelettes lipidiques dans les cellules gliales du cortex (caractéristique moins étudiée que les deux premières) (Alzheimer, 1907; Alzheimer *et al.*, 1995). La première est due à l'accumulation du peptide amyloïde β qui va former ces plaques qui sont retrouvées au niveau extracellulaire. Les NFTs ont principalement pour cause l'hyperphosphorylation de la protéine Tau, qui va induire une déstructuration des microtubules. Le peptide Aβ peut se retrouver sous différentes formes, dont les deux plus représentées dans la forme 1-40 et 1-42, de respectivement 40 et 42 acides aminés.

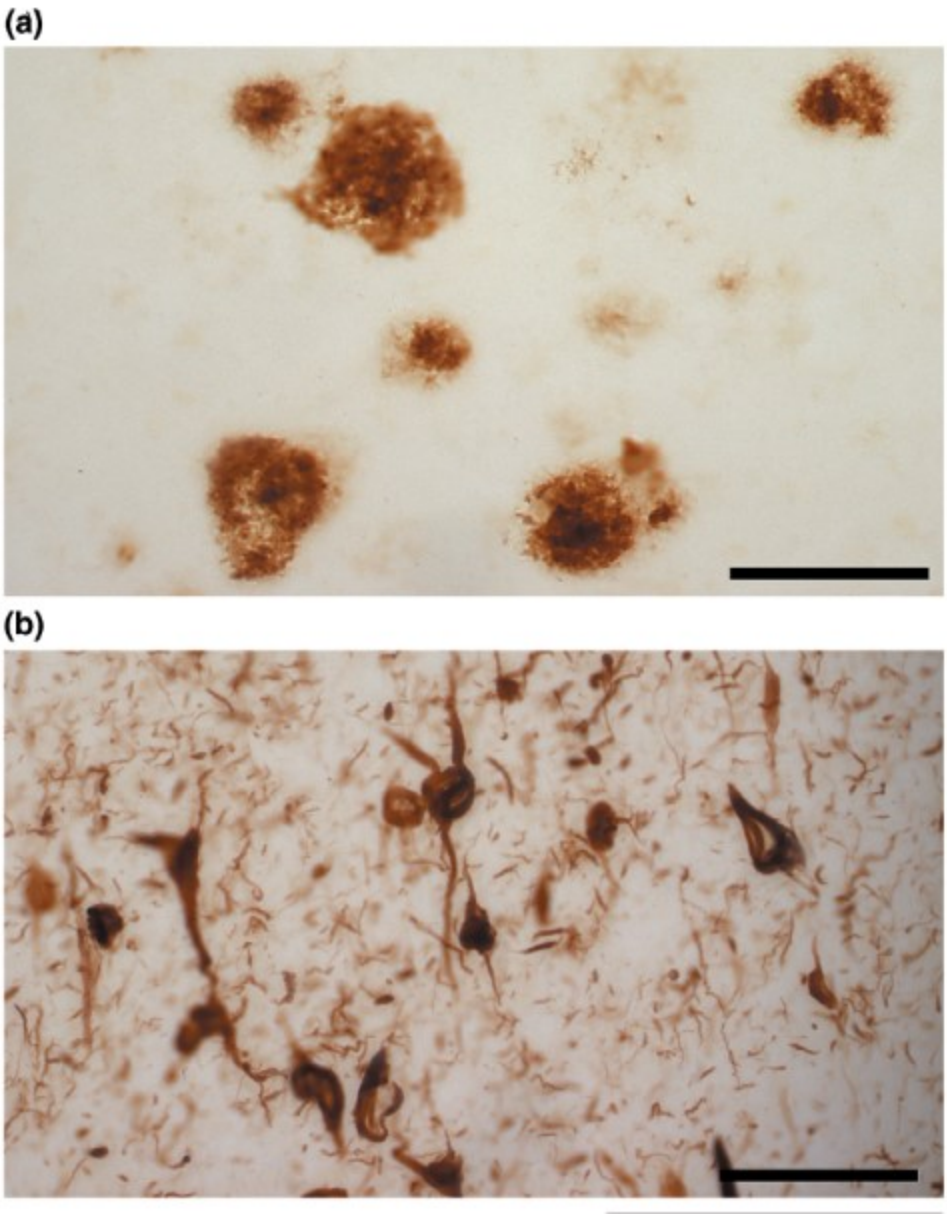

Figure 1.4 : Plaques séniles et enchevêtrements neurofibrillaires dans le cerveau de patients atteints de la maladie d'Alzheimer.

(a) Microphotographie représentant les plaques amyloïdes dans le cerveau Alzheimer. Les plaques Amyloïdes ont été visualisées par immunomarquage avec un anticorps spécifique anti-Aβ42. Barre d'échelle : 125 µm. (b) Microphotographie représentant les enchevêtrements neurofibrillaires. Les enchevêtrements ont été visualisés par immunomarquage avec un anticorps spécifique anti-PHF1. Barre d'échelle : 62.5 µm. (LaFerla & Oddo, 2005)

Deux formes de la maladie co-existent :

- La première et la plus répandue dite sporadique représentant 90 à 95% des malades d'Alzheimer. Celle-ci possède plusieurs facteurs de risques dont les principaux sont le vieillissement et la présence de l'allèle ε4 de l'apoE (Henderson & Finch, 1989; Ling *et al.*, 2003).

- La seconde est dite familiale, et où 150 mutations identifiées sur 3 gènes sont responsables de l'apparition de la MA. Ces 3 gènes sont ceux codant pour la protéine APP (*Amyoid Precursor Protein*), qui est comme son nom l'indique, le précurseur du peptide Aβ, et deux gènes codant pour PPS1 et PPS2 qui sont les protéines présénilines 1 et 2 (protéines associées aux γ-sécrétases qui participe au clivage de l'APP pour la génération de Aβ) (Selkoe, 2001).

Voie non-amyloidogenique : Clivage de APP par l'α-sécrétase, libérant sAPPα et C83, ce dernier étant

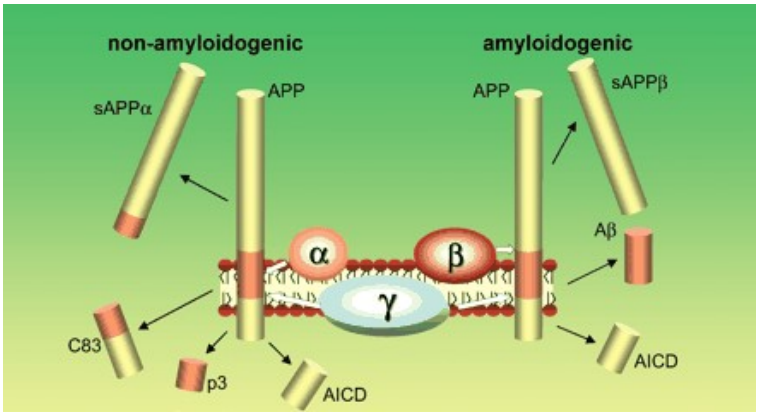

Figure 1.5 : Clivage de l'APP par les deux voies protéolytiques.

ensuite clivé par la γ-sécrétase, libérant p3 et AICD. Voie amyloidogenique : la β-sécrétase va cliver l'APP en sAPPβ et un fragment restant ancré à la membrane, ce dernier étant clivé en Aβ et AICD (Pearson & Peers, 2006).

Les niveaux élevés d'apoD trouvés dans le cerveau sont corrélés avec les enchevêtrements neurofibrillaires (NFTs) mais pas avec les plaques séniles (Belloir *et al.*, 2001; Glockner & Ohm, 2003). La transcription de l'apoD est déjà altérée quand les NFTs sont formés, ce qui suggère que l'augmentation d'apoD survient durant les premiers stades de la maladie. Une étroite relation a été établie avec l'apoD, dans l'hippocampe, dont le niveau corrèle avec la propagation des NFTs (Glockner & Ohm, 2003). Les stades de propagation des NFTs ont été décrit par Braak en 1991 (Braak & Braak, 1991) et sont couramment appelés stades de Braak. Les niveaux d'apoD sont corrélés avec ces stades de Braak.

Chez des souris PDAPP âgées, cette expression augmentée a aussi été observée (Thomas *et al.*, 2001c). Les souris PDAPP développent plusieurs lésions caractéristiques de la MA et expriment la forme mutée de la protéine précurseur de l'amyloïde : l'APP impliquée dans la forme familiale de la MA, représentant 5 à 10 % des personnes diagnostiquées (Games *et al.*, 1995). Une microgliose, astrogliose, perte synaptique et de nombreux dépôts amyloïdes sont observés chez ces souris (Chen *et al.*, 1998; Masliah *et al.*, 1996). Face à l'accumulation de la Aβ, les cellules gliales produiraient de l'apoD pour faire face à cette accumulation.

La perte d'apoD dans des souris APP-PS1, un modèle murin présentant des plaques amyloïdes, induit une augmentation du nombre de plaques amyloïdes dans l'hippocampe (Li *et al.*, 2015). Au contraire, chez ce modèle animal APP-PS1, une surexpression d'apoD humaine dans les neurones entraîne une diminution du nombre de plaques. Cette observation était associée avec une réduction du peptide Aβ 1-40 et 1-42. Dans une lignée cellulaire hippocampique de souris, l'expression d'apoD était induite après un traitement d'Aβ 25-35 : forme tronquée du peptide mais toxique (Martinez *et al.*, 2012). Cet étroit lien entre l'apoD et l'Aβ a aussi été mis en valeur par la détection de l'apoD dans les plaques amyloïdes de patients atteints de la MA (Del Valle *et al.*, 2016). En 2005, une colocalisation entre l'apoD et les plaques amyloïdes a été observée dans le cortex temporal de patients atteints de la MA (Desai *et al.*, 2005). Celle-ci était plus importante dans les plaques compactes fibrillaires amyloïdes.

Le rôle étroit entre l'apoD et l'amyloïde a aussi été retrouvé dans l'angiopathie cérébrale amyloïde (Kuiperij *et al.*, 2020). Cette étude a montré une corrélation spécifique entre l'apoD et cette pathologie. Celle-ci est caractérisée par une accumulation d'Aβ dans le système vasculaire cérébral et constitue une cause majeure d'hémorragies intracérébrales et de déclin cognitif chez les personnes âgées (Viswanathan & Greenberg, 2011).

Le facteur de risque le plus important dans la MA sporadique est la présence de l'isoforme ε4 de l'apoE (Henderson & Finch, 1989). C'est aussi un important transporteur de cholestérol et il favorise la réparation des dommages cérébraux (Liu *et al.*, 2013). Le génotype de l'apoE influence aussi les niveaux d'apoD (Glockner & Ohm, 2003), mais sont indépendants de la concentration d'apoE (Terrisse *et al.*, 1998). Ce lien entre le génotype de l'apoE et les niveaux d'apoD a été confirmé chez des modèles de souris exprimant les différents allèles de l'apoE. En effet, les souris exprimant les allèles ε3 et ε4 de l'apoE exprime faiblement l'apoD (Jansen *et al.*, 2009). Ceci s'explique par la capacité à ces deux allèles, contrairement à l'allèle ε2, de se lier au promoteur de l'apoD pour réprimer son activité et diminuer l'expression de l'apoD (Levros *et al.*,

2013). Les souris déficientes en apoE (apoE$^{-/-}$) expriment des niveaux plus importants d'apoD, ce qui pourrait être un mécanisme compensatoire de l'absence d'apoE (Jansen *et al.*, 2009).

L'apoJ est aussi impliquée dans la MA, son expression est notamment augmentée dans le cerveau et le plasma de patients atteints de la MA (May *et al.*, 1990; Perrotte *et al.*, 2019). L'apoJ est aussi connue pour être impliquée dans la maladie, notamment dans l'élimination du peptide Aβ vers la périphérie (Zlokovic, 1996; Zlokovic *et al.*, 1994). En effet, l'apoJ est capable de lier le peptide et le transporter à la barrière hémato-encéphalique (BHE) pour l'amener en périphérie *via* les récepteurs RAGE et LRP2. Tout comme l'apoD, l'apoJ montre une corrélation positive avec la progression de la maladie ainsi que les stades de Braak (Del Valle *et al.*, 2016). D'autre part, l'apoJ est capable d'inhiber l'agrégation du peptide Aβ (Oda *et al.*, 1995). L'apoE et l'apoJ, ensemble, coopèrent pour diminuer les niveaux d'Aβ et les plaques (DeMattos *et al.*, 2004).

L'apoD possède plusieurs variants génétiques. Certaines de ces variations ont été documentées pour représenter des facteurs de risques de la forme sporadique de la MA. Ces variations génétiques de l'apoD ont été retrouvées chez trois populations géographiquement éloignées : finlandaise, chinoise et afro-américaine (Chen *et al.*, 2008; Desai *et al.*, 2003; Helisalmi *et al.*, 2004). Ces observations génétiques indiquent le rôle étroit de l'apoD dans la maladie, et son importance.

Pour reproduire des similitudes de la MA, la plupart des études utilisent des modèles de souris génétiques arrivant à reproduire des marqueurs histologiques comme l'hyperphosphorylation de tau ou la présence de plaques amyloïdes. Bien que ces modèles soient utiles, ils reposent sur des modifications génétiques et ressembleraient plutôt à des symptômes de la MA familiale qui regroupent 5 à 10 % des malades. De plus, la plupart des études cliniques réalisées pour la MA ont échoué, 99,6% des médicaments montrant des effets positifs chez les modèles génétiques d'animaux ne fonctionnent pas chez l'Homme (Drummond & Wisniewski, 2017). Pour plusieurs scientifiques, le terme de diabète de type 3 est utilisé pour parler de la MA (de la Monte & Wands, 2008). Un autre modèle mimant des caractéristiques de la MA existe. Il s'agit de l'injection intracérébroventriculaire de streptozotocine (icv-STZ) (Lannert & Hoyer, 1998). Il s'agit d'une molécule diabétogène qui initialement, était utilisée pour induire le diabète chez les animaux quand cette molécule était injectée en périphérie. Ce composé sélectionne spécifiquement les cellules bêta du pancréas qui produisent de l'insuline. La STZ affecte les organes exprimant le transporteur de glucose 2 (GLUT2), ce récepteur permettant à la STZ de traverser la membrane cellulaire (Wang & Gleichmann, 1998). Lorsqu'elle est injectée en

périphérie, elle n'affecte pas le cerveau, le composé ne traversant pas la BHE, en absence de GLUT2 (Park, 2011). Le traitement de la STZ agit notamment en détruisant les récepteurs à l'insuline, induisant une résistance à celle-ci, comme retrouvée dans le cerveau de patients atteints de la MA et chez le diabète de type 2 (Salkovic-Petrisic *et al.*, 2013; Steen *et al.*, 2005). Cependant lorsque l'injection se fait dans le ventricule cérébral latéral, les dommages se font directement dans le cerveau induisant plusieurs comportements communs à la MA. En entrant dans les cellules de mammifère, la STZ va inhiber la synthèse de l'ADN par alkylation des brins d'ADN, ceci affectant toutes les étapes du cycle cellulaire des cellules mammifères. En effet, l'icv-STZ est bien connue pour induire une neuroinflammation, du stress oxydatif (en créant des dysfonctions mitochondriales) et des altérations biochimiques. Chez des rats traités avec une icv-STZ, une accumulation d'Aβ, une hyperphosphorylation tau, un stress oxydatif, une neuroinflammation plus accrues ont été retrouvés (Zhang *et al.*, 2018). Bien que le mode d'action ne soit pas encore bien connu, plusieurs effets sont répertoriés. A certaines concentrations, la STZ induit des déficits de mémoires spatiales (Li *et al.*, 2016a), tout comme une hyperphosphorylation de tau, des dysfonctions synaptiques et une apoptose neuronale dans les cerveaux de rongeurs, plusieurs dysfonctions retrouvées dans la MA sporadique (Zhang *et al.*, 2020). À ce jour, l'icv-STZ n'a pas été utilisée pour étudier le rôle de l'apoD.

1.3.4.3 **La schizophrénie**

La schizophrénie est un trouble mental sévère causé par un mauvais fonctionnement de l'activité des neurotransmetteurs qui affectent les systèmes glutamatergique, dopaminergique et sérotoninergique (Jones & Pilowsky, 2002; Konradi & Heckers, 2003). Dans cette maladie, une carence d'acide arachidonique est observée (Thomas *et al.*, 2003b; Yao *et al.*, 2005). Ce lipide est pour rappel le ligand avec la meilleure affinité de liaison pour l'apoD (Morais Cabral *et al.*, 1995). Dans le plasma et le cerveau de patients atteints de schizophrénie, une modulation de l'expression de l'apoD a été montrée (Mahadik *et al.*, 2002; Thomas *et al.*, 2003a; Thomas *et al.*, 2001b). Dans le cerveau de patients traités avec de la clozapine, un médicament antipsychotique atypique efficace contre la schizophrénie, une augmentation des niveaux d'apoD a été retrouvée (Ciapparelli *et al.*, 2003; Mahadik *et al.*, 2002; Thomas *et al.*, 2001b). Dans les membranes des érythrocytes de patients traités avec la clozapine, une augmentation d'acide arachidonique a été relevée (Vaddadi, 1992; Walker *et al.*, 1999). L'augmentation de l'expression de l'apoD pourrait faciliter l'incorporation d'acide arachidonique dans la membrane, comme le démontre une étude chez la souris (Thomas & Yao, 2007).

1.3.4.4 **Autres maladies neurodégénératives**

Tout comme dans la MA, l'expression d'apoD est également augmentée chez les patients atteints de la maladie de Parkinson. Cette augmentation a été constatée en particulier dans les cellules gliales de la *substantia nigra,* structure particulièrement touchée dans la maladie de Parkinson, dont les neurones dopaminergiques se projettent vers le striatum (Ordonez *et al.*, 2006). Cette augmentation est aussi retrouvée dans le plasma de patients (Waldner *et al.*, 2018).

La maladie de Niemann-Pick de type C est une maladie neurodégénérative héréditaire très grave due à une dérégulation de l'homéostasie des lipides. Le transport du cholestérol est particulièrement affecté dans cette maladie, entraînant une accumulation de cholestérol non estérifié dans les endosomes tardifs et les lysosomes (Kolodny, 2000). Dans un modèle murin de la maladie (souris NPC), l'apoD est augmentée dans le plasma ainsi que dans le cerveau. Cette augmentation est aussi présente dans le thymus, le cœur et le tissu adipeux (Suresh *et al.*, 1998; Yoshida *et al.*, 1996). Cette maladie est associée avec une neurodégénérescence progressive, ceci étant expliqué par une plus grande sensibilité des cellules nerveuses vis-à-vis de la perturbation du trafic intracellulaire du cholestérol (Sevin *et al.*, 2007; Suresh *et al.*, 1998). Le cerveau étant un organe particulièrement riche en cholestérol notamment dans les gaines de myéline entourant les axones, la concentration de cholestérol dans la membrane influence l'activité nerveuse (Dietschy & Turley, 2004). Pour pallier la dérégulation du cholestérol intracellulaire chez la souris, l'apoD pourrait être augmentée.

La sclérose en plaques est une maladie auto-immune qui affecte le SNC. Dans cette maladie, le système immunitaire ne fonctionne pas normalement et provoque des dysfonctions induisant des problèmes moteurs, sensitifs ou encore cognitifs. Dans le LCS de ces patients, l'apoD est également augmentée (Reindl *et al.*, 2001).

En revanche, l'augmentation observée n'est pas générale aux maladies neurodégénératives car elle n'est pas retrouvée dans la démence fronto-temporale (Bhatia *et al.*, 2019). L'augmentation de l'apoD est donc une observation retrouvée dans plusieurs neuropathologies, ce qui pourrait s'expliquer par le fait que l'apoD serait synthétisée pour limiter les effets de ces agressions.

1.4 Hypothèse et objectifs de recherche

A ce jour, l'apoD possède des propriétés neuroprotectrices et semble principalement avoir une activité au sein du cerveau. Cependant, chez des souris surexprimant l'apoDh dans les

neurones, on remarque un développement de stéatose hépatique et musculaire, ainsi l'apoD cérébrale pourrait avoir un effet sur la périphérie (Do Carmo *et al.*, 2009b). Le passage de l'apoD dans le sens du cerveau au sang à travers la BHE n'a pas été étudié. D'autre part, étudier le passage de l'apoD dans le sens opposé, du sang vers le cerveau, serait aussi pertinent. En effet, certaines maladies neurodégénératives ont des facteurs de risques périphériques (diabètes, maladie cardiovasculaire pour la MA), l'apoD qui est augmentée dans le cerveau dans la MA pourrait entre autres avoir une origine périphérique, l'étude de son passage du sang vers le cerveau serait donc pertinente. Récemment, l'internalisation de l'apoD a été montrée pour se faire de manière dépendante avec le récepteur basigine, celui-ci pourrait donc être impliqué dans le passage de l'apoD à travers la BHE (Najyb *et al.*, 2015).

Dans le cerveau, l'apoD démontre des propriétés neuroprotectrices en plus d'être augmentée dans la MA. Plus spécifiquement son rôle n'a pas encore été totalement démontré bien qu'on lui donne une implication dans la lutte contre la peroxydation lipidique ainsi que dans le recyclage de la myéline, la déficience de celle-ci retardant la régénération des axones chez les souris (Ganfornina *et al.*, 2008; Ganfornina *et al.*, 2010). De plus, aucune étude à ce jour, n'a montré un rôle de l'apoD dans la préservation de mémoire spatiale lors de l'initiation d'agressions neuropathologiques. Les apoE et apoJ sont déjà bien documentées dans la MA, leur étude serait pertinente dans le cadre d'une déficience d'apoD pour montrer l'importance de cette dernière sur l'expression des autres. En effet, chez des souris apoE$^{-/-}$ une augmentation de l'expression de l'apoD est observée, montrant l'existence d'un mécanisme de compensation quand l'apoE est déficiente (Jansen *et al.*, 2009). Chez des rats déficients en apoE, cette augmentation a aussi été observée, où les niveaux d'apoD ont été retrouvé à des niveaux 50 fois plus élevés que chez les souris sauvages (Terrisse *et al.*, 1999). De plus, l'apoE peut se lier sur la région promotrice de l'apoD avec différentes efficacités en fonction de l'allèle de l'apoE (Levros *et al.*, 2013). Enfin, l'apoJ et l'apoD montrent toutes les deux une corrélation positive avec la progression de la MA ainsi qu'avec les stades de Braak (Del Valle *et al.*, 2016). L'apoD a été suggéré pour limiter la peroxydation lipidique, mais à ce jour, aucune étude n'a montré les effets d'une déficience d'apoD sur la capacité antioxydante ainsi que les protéines carbonylées. Ainsi, l'étude de marqueurs du stress oxydatif chez des souris apoD$^{-/-}$ apporterait une confirmation du rôle étroit entre l'apoD et la protection contre le stress oxydatif.

L'hypothèse comporte ainsi deux parties. Tout d'abord, l'apoD retrouvée dans le cerveau n'a que pour seule origine celui-ci et ne proviendrait pas de la périphérie, cependant l'apoD cérébrale pourrait se retrouver dans des organes périphériques. Enfin, l'apoD cérébrale joue un

rôle majeur dans le retard de l'apparition des symptômes dans la MA, et sa déficience induirait des déficits de mémoire spatiale ainsi qu'une augmentation des marqueurs oxydatifs.

Objectifs :

Les objectifs de travail sont les suivants :

- ✓ Etude du passage de l'apoD à travers la BHE dans les sens de la périphérie au cerveau et inversement chez des souris et établir si ce passage est basigine dépendant chez une lignée de cellules endothéliales connue pour mimer la BHE.
- ✓ Montrer les effets sur la mémoire spatiale d'une déficience d'apoD lors d'une icv-STZ ainsi qu'observer des effets sur les capacités antioxydantes ainsi que sur l'expression l'apoE et l'apoJ dans plusieurs structures cérébrales.

Ces résultats appuieraient l'intérêt d'étudier cette protéine dans la protection des capacités cérébrales et plus spécifiquement la mémoire. Ils contribueraient aussi à mieux comprendre le trafic de l'apoD dans le corps et plus spécifiquement les échanges de l'apoD entre la périphérie et le cerveau. Ce projet pourrait amener de nouvelles perspectives d'expériences sur les plans fondamentaux, cliniques et curatifs concernant l'apoD et les maladies où elle pourrait être impliquée.

2 CEREBRAL APOLIPOPROTEIN D EXITS THE BRAIN AND ACCUMULATES IN PERIPHERAL TISSUES

L'apolipoprotéine D cérébrale quitte le cerveau et s'accumule dans les tissus périphériques

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Titre de la revue ou de l'ouvrage :

International Journal of Molecular Sciences.Titre de la revue ou de l'ouvrage où l'article a été soumis, accepté ou publié

Publié le 16 avril 2021

DOI : https://doi.org/10.3390/ijms22084118

Résumé en Français

L'Apolipoprotéine D (ApoD) est une lipocaline sécrétée, associée à la neuroprotection et au métabolisme des lipides. Chez les rongeurs, la majeure partie de son expression se produit dans le système nerveux central. Malgré cela, l'ApoD a des effets importants dans les tissus périphériques, ce qui indique que l'ApoD neuronale peut atteindre les organes périphériques. Nous avons cherché à déterminer si l'ApoD cérébrale pouvait atteindre la circulation et s'accumuler dans les tissus périphériques. Trois heures ont été nécessaire pour que plus de 40% de l'ApoD humaine radiomarquée (ApoDh), injectée bilatéralement, sorte du système nerveux central (SNC). Une fois dans la circulation, l'ApoDh s'accumule principalement dans les reins/urines, le foie et les muscles. L'accumulation spécifique de l'ApoDh dans ces tissus a été retrouvée pour être fortement corrélée à l'expression de la basigine faiblement glycosylée (BSG, CD147). Nous avons observé que l'ApoDh traversait les monocouches de cellules endothéliales b.End.3 de la barrière hémato-encéphalique. Cependant la Cyclophilline A, un ligand de la basigine, n'a pas eu d'impact sur les taux d'internalisation de l'ApoDh dans les cellules b.End.3, ce qui indique que l'externalisation de l'apoD du cerveau est soit indépendante de la BSG, soit dépendante d'autres types cellulaires. Dans l'ensemble, nos données ont montré que l'ApoD peut quitter rapidement et efficacement le SNC et atteindre le foie et les reins/urine, organes liés au recyclage et à la sécrétion des lipides et des toxines. Cela indique que la surexpression cérébrale au cours d'épisodes neurodégénératifs peut servir à évacuer du SNC, les ligands neurotoxiques de l'apoD.

Contribution des auteurs :

Dans cet article j'ai participé à toutes les expériences *in vivo,* plus précisément, nous avons apporté notre expertise en réalisant les icv d'apoDh radioactive. L'optimisation de cette injection s'est faite avec Morgane Perrotte. L'analyse des résultats *in vivo* a été réalisée par Frédérik Desmarais avec l'appui de Gaétan Ravaut et Guillaume Fyfe-Desmarais. J'ai exclusivement réalisé et analysé les expériences sur les cellules endothéliales b.End.3. J'ai avec Frédérik Desmarais et Karl-F Bergeron écrit le manuscrit ; Charles Ramassamy, Catherine Mounier et Eric Rassart ont corrigé ce manuscrit et supervisé l'étude.

2.1 Abstract

Apolipoprotein D (ApoD) is a secreted lipocalin associated with neuroprotection and lipid metabolism. In rodent, the bulk of its expression occurs in the central nervous system. Despite this, ApoD has profound effects in peripheral tissues, indicating that neural ApoD may reach peripheral organs. We endeavor to determine if cerebral ApoD can reach the circulation and accumulate in peripheral tissues. Three hours was necessary for over 40% of all the radiolabeled human ApoD (hApoD), injected bilaterally, to exit the central nervous system (CNS). Once in circulation, hApoD accumulates mostly in the kidneys/urine, liver, and muscles. Accumulation specificity of hApoD in these tissues was strongly correlated with the expression of lowly glycosylated basigin (BSG, CD147). hApoD was observed to pass through bEnd.3 blood brain barrier endothelial cells monolayers. However, cyclophilin A did not impact hApoD internalization rates in bEnd.3, indicating that ApoD exit from the brain is either independent of BSG or relies on additional cell types. Overall, our data showed that ApoD can quickly and efficiently exit the CNS and reach the liver and kidneys/urine, organs linked to the recycling and excretion of lipids and toxins. This indicated that cerebral overexpression during neurodegenerative episodes may serve to evacuate neurotoxic ApoD ligands from the CNS.

Keywords: apolipoprotein D; basigin; protein accumulation; blood-brain barrier

2.2 Introduction

Apolipoprotein D (ApoD) is a 25–30 kDa glycosylated protein belonging to the lipocalin superfamily of hydrophobic molecule carriers [1–4]. ApoD is known for its ability to bind various ligands, including arachidonic acid (ARA) and progesterone [1,5,6]. ApoD can bind ARA and mediate its release from cell membranes. Furthermore, since ARA is the precursor of the eicosanoid class of lipid inflammation mediators, ApoD's capacity to bind ARA allows it to modulate the production of eicosanoids and attenuate inflammation [7,8]. ApoD can also limit inflammation and oxidative stress by reducing oxidized lipids [1,9–12]. Because it is overexpressed during many neurodegenerative diseases and stresses, ApoD is considered an important factor in brain protection and repair [9,11,13–15]. ApoD is also a member of the apolipoprotein family as it associates with lipoproteins (mainly high-density lipoproteins) in the blood. In humans, contrary to most apolipoproteins, ApoD is minimally produced in the liver and intestines [1,5]. Rather, mRNA expression is found, although in varying quantities, in several tissues including the central nervous system (CNS), mammary glands, spleen, adipose tissues,

adrenals, and skin [1,16,17]. In contrast to humans, in mice and rats, ApoD mRNA expression is mainly restricted to the CNS [1,2,18–20]. However, moderate to high protein levels are found in peripheral tissues including the liver, suggesting that the protein could cross the blood brain barrier (BBB) [1,21].

In support to the hypothesis that apoD can exit the CNS, the overexpression of human ApoD (hApoD) in the CNS of transgenic mice leads to an elevated level of apoD in the livers, likely originating from expression in the CNS [19]. These transgenic mice develop hepatic and muscular steatosis [8,19,22] probably because apoD modulates the metabolism of these tissues.

Beside its ability to circulate as part of lipoproteins, up to 10% of plasma ApoD is found in the protein phase, indicating that ApoD may also circulate freely in its soluble form [23]. Three recent studies also highlighted the presence of ApoD in extracellular vesicles [24–26]. ApoD has been shown to be transported to neurons by extracellular vesicles originating from astrocytes [26]. ApoD can also be found associated with cerebrospinal fluid lipoproteins [27–29]. ApoD was also observed in urine [30]. Together, the results show that ApoD transport is polyvalent and complex.

The Basigin (BSG) receptor, also known as cluster of differentiation 147 (CD147), mediates ApoD internalization in neurons [14]. BSG is also responsible for the internalization of other proteins such as caveolin with its affinity being dependent on BSG glycosylation level [31]. BSG is expressed in a wide range of tissues and cell types [32] (data available at https://www.proteinatlas.org/ENSG00000172270-BSG/tissue) (Access on: 25, November, 2020). Circulating ApoD, through BSG-mediated internalization, could therefore accumulate in many different structures. BSG is expressed in mouse brain endothelial cells, the main component of the blood-brain barrier (BBB) [33]. The BBB is the largest barrier separating the peripheral circulation from the CNS [34]. It is a highly selective barrier, composed of tightly linked endothelial cells that cover the bloodstream capillaries. Astrocytes and pericytes also participate in the architecture and function of the BBB. In addition to BSG, apolipoprotein receptors such as Low-density lipoprotein receptor (LDLR), LDLR-related protein (LRP1) and Megalin are also expressed in BBB endothelial cells and are involved in transporting ligands across the barrier [35].

The mouse brain microvascular endothelial cell line bEnd.3 is the most characterized in terms of tight junction proteins [36,37] and is thus often used to study the endothelial component of the BBB [38]. These cells express the tight junction proteins ZO-1, occludin, and claudin-5 [39] and possess fluorescein permeability similar to those of primary mouse endothelial cells [40]. These mouse brain capillary endothelial cells contain several receptors present on the surface of endogenous BBB endothelial cells, including apolipoprotein receptors such as LDLR and LRP1.

These cells also possess receptors that participate in the transcytosis process of proteins such as transferrin and lactoferrin [35].

In this study, we endeavor to determine if cerebral ApoD can reach the bloodstream from the brain and if BSG plays a role in this process. We also set out to determine in which peripheral organs ApoD preferentially accumulates once in circulation.

2.3 Results

2.3.1 hApoD Exits the Central Nervous System and Reaches Peripheral Tissues

We endeavored to establish whether hApoD can exit the brain and reach the bloodstream and accumulate in peripheral organs. For this, the cerebral ventricles of mice were injected with radiolabeled hApoD or albumin as a control. Injected proteins were allowed to diffuse from the ventricles and tissue/fluid for 3 or 6 h. Samples were subsequently taken throughout the mouse body. During this procedure, mice were perfused with a saline solution to avoid contamination in tissues stemming from potential radiolabeled proteins in the blood. Also, BBB integrity was confirmed by circulating an Evans blue solution in perfused mice (Figure S3). As expected, 3 h after ICV injection of radiolabeled proteins, most of the recovered albumin (93%; Figure 1A), remained sequestered in the brain. Within this time frame, 41% of the recovered hApoD was found in the periphery, showing that hApoD can exit the brain compartment. Interestingly, a significant proportion of hApoD accumulated in the liver (6.06%) but the major fraction was found in urine (28.87%). Radioactivity levels in the bloodstream were very low, indicating that hApoD was rapidly cleared from the circulation. Electrophoresis of urine and brain protein samples in denaturing conditions confirmed that the radiation detected corresponded to native hApoD molecules migrating at 30 kDa (Figure 1B).

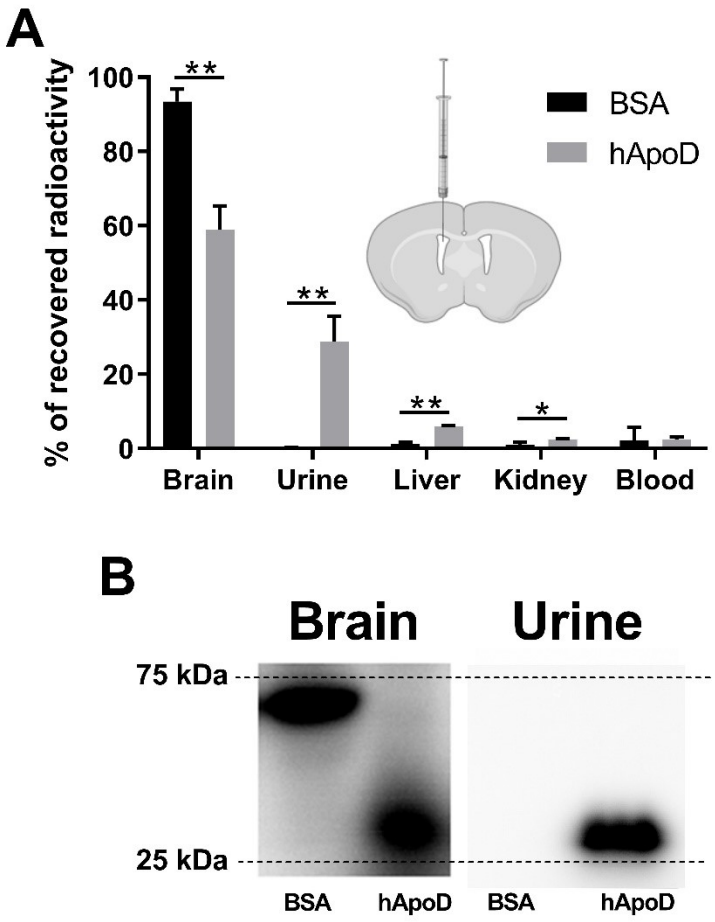


Figure 1: Human Apolipoprotein D (hApoD) injected in the brain accumulates in urine and liver. Radiolabeled proteins (Bovine Serum Albumin (BSA) and hApoD) were injected in mouse cerebral ventricles. Blood, urine, and—after perfusion—organs were collected 3 h post injection. (A) Radioactivity recovered in fluids and tissue homogenates (%) compared to the total radioactivity recovered in the whole body. Statistical significance was evaluated via a Student t-test: * $p < 0.05$, ** $p < 0.01$ ($n = 3$ animals). (B) Radiolabeled proteins extracted from brain and urine and visualized by radiography. A representative electrophoresis gel is shown.

2.3.2 hApoD Accumulates in Specific Tissues

ICV injections were limited to a total volume of 10 µL (5 µL per ventricle), adding up to approximately 2.5 µg of radiolabeled proteins. Under these experimental restrictions, hApoD could only be shown to accumulate in the kidneys and liver (Figure 1A). The radiation found in other organs (muscles, pancreas, spleen, intestines, and others) was too close to background radiation levels to confidently affirm that hApoD had accumulated in these organs. To determine if hApoD can target other tissues, we injected a greater amount of radiolabeled protein (approximately 10 µg) directly in the bloodstream. Note that this higher dose of hApoD was still

within physiological parameters for circulating ApoD (Rassart et al. 2000). Albumin was again chosen as a control because of its propensity to remain in the circulation (Andersen et al. 2014). Again, the blood and its radioactive content were eliminated by perfusion with a saline solution before tissue samples were taken. The amount of recovered radiolabeled hApoD was evaluated 3 and 6 h post injection (Figure 2A) and as a function of the albumin control (Figure 2B). Accumulation of hApoD occurred at a higher rate than albumin in the kidneys (5.6-fold), liver (30.0-fold), pancreas (3.1-fold), caecum (2.8-fold), spleen (3.3-fold), mesenteric adipose tissue (MAT; 4.2-fold), and muscles (2.4-fold) at least at either 3 or 6 h. The kidneys emerged as the tissue that most readily accumulated hApoD with 14% of recovered radioactivity per gram of tissue. This accumulation was probably due to hApoD within the nephrons since a major part of the radioactivity was found in urine (Figure 3A). The biggest difference between albumin and hApoD accumulation; however, was found in the liver (Figure 2B), indicating a very strong specificity of hApoD for this tissue. Interestingly, the accumulation of hApoD in muscles was stronger than albumin 3 h after injection. However, after 6 h, hApoD levels diminished (from 1.6 to 1.0% per gram of tissue), while albumin levels increased (from 0.7 to 1.4% per gram of tissue). Conversely, hApoD accumulation in the MAT appeared to be more persistent than albumin. MAT radiation from hApoD only diminished by 0.10% per gram of tissue in 3 h. In comparison, radiation from albumin was reduced by 0.96% per gram of tissue in that same interval. Very interestingly, hApoD within the bloodstream did not appear to enter the brain, suggesting that hApoD can only cross the BBB in one direction.

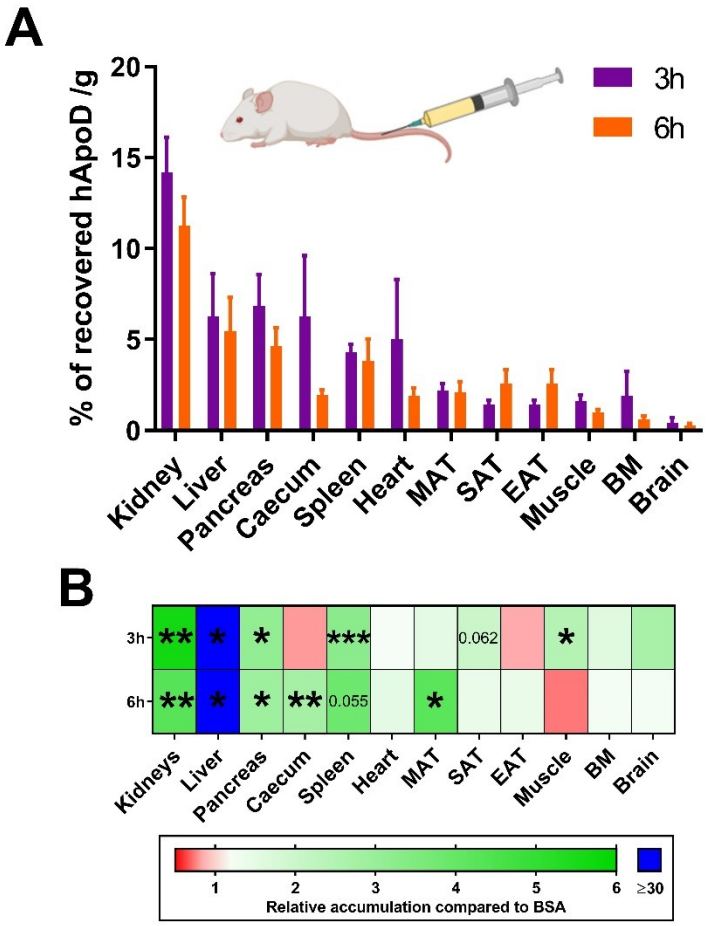


Figure 2: Circulating hApoD accumulates in specific peripheral organs. Radiolabeled proteins (Bovine Serum Albumin (BSA) and hApoD) were injected intravenously in mice. Blood, urine, and—after perfusion—organs were collected 3 and 6 h post-injection. (A) Results are presented as the percentage of radioactivity recovered per gram of tissues. EAT: epididymal adipose tissue, SAT: subcutaneous adipose tissue, MAT: mesenteric adipose tissue, and BM: bone marrow. (B) The heat map shows the accumulation of hApoD relative to albumin. Statistical significance was evaluated via a Student t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n = 4 animals).

To establish a general portrait of hApoD biodistribution when it exits the bloodstream, we extrapolated the total radioactivity found in each organ (or fluid) according to their total weight (or volume). Since we did not measure the total weight of adipose tissues nor of bone marrow, these tissues were excluded from this analysis. Albumin was mainly found in circulation at both 3 (91%) and 6 h (83%) after injection (Figure 3A). As expected from our previous results, hApoD exited the bloodstream at a higher rate than albumin. After 6 h, half of the injected hApoD had left the circulation and a significant fraction was again found in urine (33%; Figure 3A). Globally, hApoD accumulation level in tissues was higher at 3 h (25.2%) compared to 6 h (18.1%), suggesting a progressive shift of hApoD from blood to tissues and finally to urine. The principal target of hApoD

at 3 h (Figure 3B) was the muscles (10.67%) followed by the liver (8.93%), kidneys (3.09%), pancreas (1.24%), and spleen (0.41%). Because of their mass, muscles accounted for a large proportion of hApoD accumulation despite their poor apparent affinity for hApoD (Figure 2). For instance, while the liver accumulated hApoD at a much higher rate (6.0% of recovered radioactivity per gram of tissue), it was still outclassed in total hApoD accumulation by the muscles despite their low accumulation rate (1.6% of recovered radioactivity per gram of tissue; see Figure 2A).

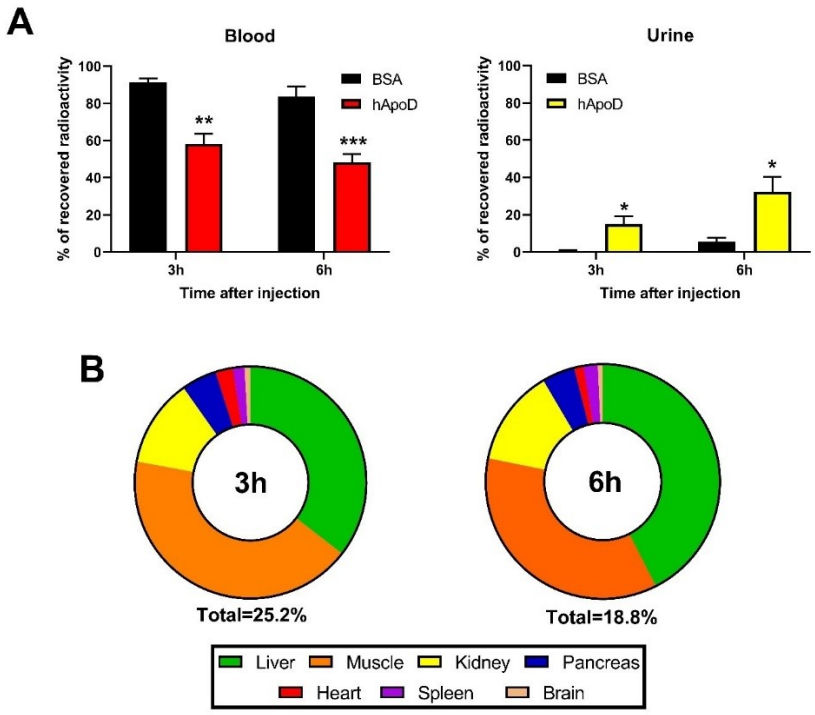


Figure 3: Relative accumulation of hApoD in fluids and tissues. Radiolabeled proteins (Bovine Serum Albumin (BSA) and hApoD) were injected intravenously in mice. Blood, urine, and—after perfusion—organs were collected 3 and 6 h post-injection. Results are presented for each (A) fluid and (B) organs as their average respective percentage of the total radioactivity recovered in the animals. Statistical significance was evaluated via a Student t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n = 4 animals).

2.3.3 hApoD Transcytoses through bEnd.3 Brain Endothelial Barrier Cells

Since hApoD can exit the brain compartment, we explored the possibility that hApoD might be able to pass through a BBB model consisting of a monolayer of the endothelial bEnd.3 cells. The impermeability of this monolayer model to passive diffusion was validated by electrical resistance measurement (≥35 Ohms/cm2) and Dextran permeability assay (Figure S2). Expression of Claudin-5 between adjacent cells was also validated, confirming the formation of tight junctions

(Figure 4A). Within 24 h, exogenous hApoD applied to the top compartment of the monolayer was internalized (Figure 4A) and was found in the bottom medium (Figure 4B), indicating that hApoD is subject to transcytosis through bEnd.3 cells in a dose-dependent manner. However, bEnd.3 cells are not polarized [41,42]. Therefore, our results may have underestimated the amount of hApoD that actually underwent transcytosis since part of the internalized protein could be released back to the top compartment where it was first internalized and not contribute to the buildup of ApoD concentration in the bottom compartment.

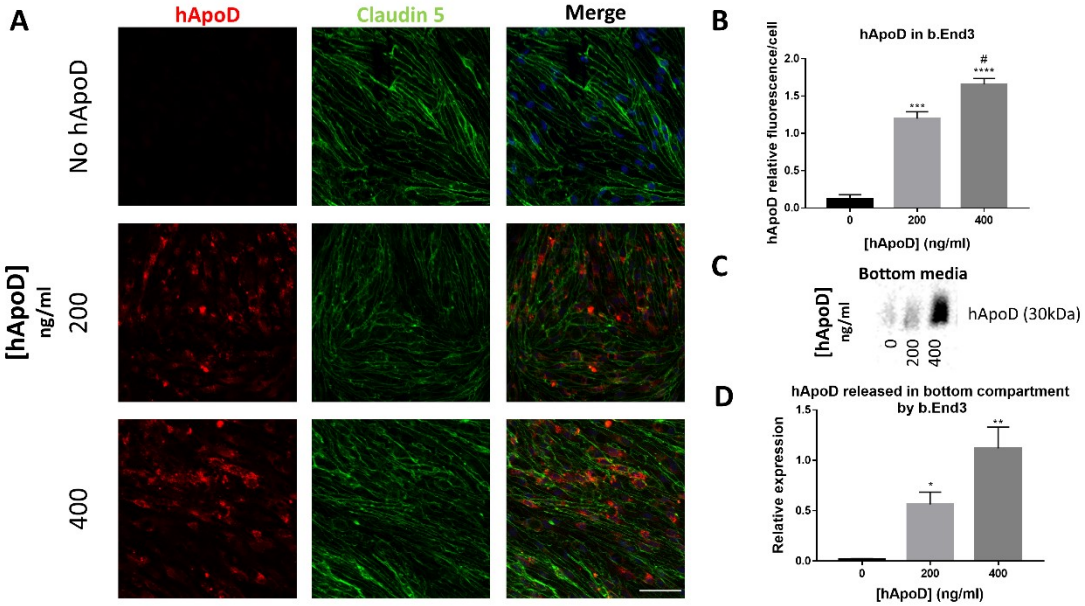


Figure 4: hApoD is subject to endocytosis and transcytosis through bEnd.3 cells. The top side of bEnd.3 cell monolayers was exposed to different concentrations of hApoD for 24 h. (A) Cells were immunostained for hApoD (red) and a tight junction marker (Claudin-5, green). Nuclei were stained with Hoechst (blue). Scale bar: 50 µm. (B) Average hApoD signal per cell (5 images taken for each monolayer). Fluorescence was normalized by the number of cell nuclei (C) Bottom media proteins immunoblotted for hApoD. (D) Quantification by immunoblot of hApoD released in bottom compartment by bEnd.3. Representative images are shown (n = 3 independent experiments). Statistical significance for panels B and D was evaluated via a Student t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ against the 0 ng/mL (*) and 200 ng/mL (#) conditions.

2.3.4 Cyclophilin A Competition Does Not Reduce hApoD Internalization in Brain Endothelial Barrier Cells

Having confirmed that hApoD can cross through bEnd.3 brain endothelial cells, we next endeavored to determine if the BSG receptor was implicated in this process. We used cyclophylin A, a BSG ligand known to compete with ApoD for BSG-dependent internalization [14]. We first confirmed that cyclophilin A was non-toxic at the concentrations used (Figure 5A). Surprisingly,

internalization and transcytosis of hApoD by bEnd.3 monolayers were unchanged despite the presence of various molar ratios of competing cyclophilin A (up to 10-fold excess relative to hApoD; Figure 5B–D). We then determined if BSG was expressed in bEnd.3 cells in comparison to control tissues (brain and liver). Our analysis showed that BSG was expressed in bEnd.3 cells at a comparable level to the liver, the tissue with the highest specific affinity for hApoD. Interestingly, BSG expression was much stronger in bEnd.3 than in whole brain lysate. Additionally, the level of BSG glycosylation varied greatly between samples (Figure 5E). BSG glycosylation can influence its protein–protein interactions [31]. Interestingly, the ratio of lowly-glycosylated BSG (LG-BSG) relative to total BSG expression appeared to align with our observed cellular affinity for hApoD internalization in vivo (Figures 2A and 5F). The liver, one of the tissues that most readily accumulates hApoD, had a high LG-BSG /total ratio, while the bEnd.3 cells had a lower ratio and needed 24 h to internalize and excrete hApoD to the bottom media. This suggested a greater role of BSG in peripheral tissues than in endothelial cells for hApoD internalization.

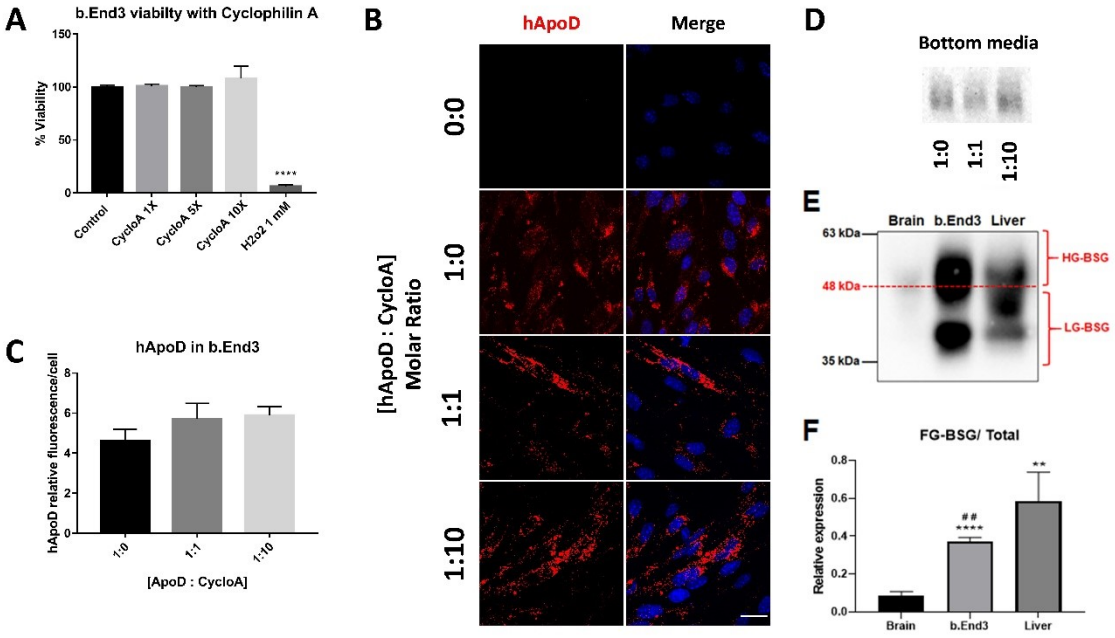


Figure 5: Cyclophilin A competition does not reduce hApoD internalization in bEnd.3 cells. (A) Viability of bEnd.3 cells in the presence of cyclophilin A, at 1, 5, and 10 times the molar concentration of hApoD (13.33 nM) used in the experiment. H2O2 was used as a control for the loss of cell viability. (B) The top side of bEnd.3 cell monolayers were exposed to hApoD (13.33 nM) for 24 h. Cyclophilin A was included from the beginning of the experiment at a molar equivalent (1:1) or in molar excess (1:10) relative to hApoD. Cells were immunostained for hApoD (red). Nuclei were stained with Hoechst (blue). Scale bar: 20 µm. Representative

confocal sections are presented. (C) Average hApoD signal per cell (5 images taken for each monolayer). Fluorescence was normalized by the number of cell nuclei. (D) Bottom media proteins immunoblotted for hApoD. (E) Immunoblot of BSG in bEnd.3 and control tissues (HG-BSG, Highly glycosylated BSG; LG, lowly glycosylated BSG). Panels D and E are representative results. (F) Quantification of LG-BSG expression relative to total BSG expression as compared to the brain (*) and liver (#). Statistical significance for panels A, C, and F was evaluated via a Student t-test: ** and ## $p < 0.01$, **** $p < 0.0001$ (n = 3 independent experiments).

2.3.5 Relationship between BSG Glycosylation and hApoD Accumulation

To further explore if BSG glycosylation was linked to the degree of tissue hApoD internalization, we studied the expression of the multiple glycosylated forms of BSG in mice tissues. BSG expression, per mg of tissue, was heterogeneous between tissues, with the liver and heart presenting an especially high overall BSG expression and adipose tissues having a low expression (Figure 6A). BSG bands were observed at multiple size and in varying patterns according to the tissue type. The SAT, muscles, and the brain only expressed the highly-glycosylated form of BSG (HG-BSG, >48 kDa). The liver and pancreas, however, expressed multiple forms of BSG (Figure 6A). We next performed correlations between BSG expression by glycosylation levels and the specific accumulation of hApoD in each tissue. As expected, the total expression of BSG in tissues was positively correlated with hApoD accumulation. However, the correlation was only statistically significant for the 3-h time point (Figure 6B). Interestingly, expression of the HG-BSG form was not found to be associated with hApoD-specific accumulation. In opposition, LG-BSG expression was correlated with hApoD specific accumulation at both time points, but especially 6 h after injection ($p = 0.0047$ **). Kidneys were outliers relative to all other tissues investigated and were therefore excluded from these correlation analyses (presented as separate data set) (Figure 6B). It is very likely that hApoD does not require to be internalized in kidney cells during urine excretion and it is probably passively filtered through the glomerular pores (discussed later).

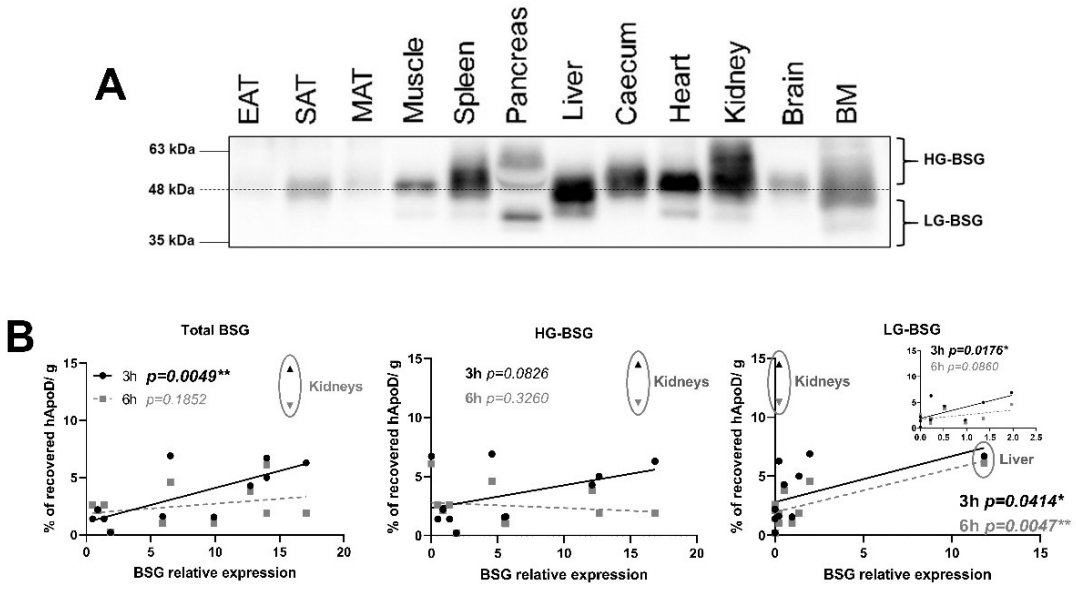


Figure 6: hApoD tends to accumulate in tissues expressing underglycosylated Basigin. (A) Immunoblot of Basigin (BSG) in its multiple glycosylated forms (HG, highly glycosylated; LG, lowly glycosylated) in various tissues, including epididymal adipose tissue (EAT), subcutaneous adipose tissue (SAT), mesenteric adipose tissue (MAT), and bone marrow (BM). Each well contained whole protein extracts from 20 µg of tissue. A representative immunoblot is shown (n = 4 animals). (B) Correlations between average hApoD accumulation (% of recovered radioactivity per gram of tissue) and average expression level of different classes of glycosylated Basigin in various tissues, 3 (black) and 6 h (grey) after IV injection. Inset: liver data eliminated from the correlation. Pearson's product moment correlations are presented: * p < 0.05, ** p < 0.01 (n = 4 animals).

2.4 Discussion

Our results demonstrated that injected hApoD can exit the brain and reach peripheric tissues. These observations were made by comparing the amount of hApoD that escaped the CNS to an albumin control. The rate at which hApoD leaves the brain was observed to be higher than that of albumin. Albumin is a natural component of the cerebro-spinal fluid (CSF). Its concentration in the CSF is lower than 1/125th of the one found in circulation (CSF/serum ratio < 8 × 10−3) [43] and the CNS barriers greatly limit albumin's ability to transfer bilaterally between the brain and the circulation. Similarly to other components of the CSF, albumin is constantly eliminated from the brain by the brain's waste clearance system [43,44]. The elimination of albumin from the CNS is a passive process that does not require an active transport across CNS barrier. In contrast, the hApoD's higher exit rate strongly suggested that it might be actively transported.

The two major circulating hApoD recipient organs are the kidneys/urine and the liver, the first being implicated in lipid metabolism and recycling and the latter being mostly implicated in waste removal. This corroborated our previous hypothesis that cerebral hApoD reaches the liver [8]. We previously showed that hApoD overexpression in the brain results in the development of an hepatic and muscular steatosis in mice [19]. The presence of hApoD as well as an increase in ARA proportion in the liver suggested that hApoD derived from the brain was responsible for the added efflux of ARA into the liver [8,19,22].

Surprisingly, a large part of the hApoD recovered outside the CNS was found in the urine. It was previously shown that ApoD can pass through the kidneys and accumulate in urine in monomeric and dimeric form [45]. Multiple factors including protein size and charge determine which molecules can pass through the kidneys by glomerular filtration. It is generally believed that proteins and polymers in the range of 30–50 kDa can pass through the glomerular pores. However, the diameter and shape of the molecules are also important as elongated molecules such as 350–500 kDa nanotubes with 1 nm diameter have been shown to be efficiently cleared by glomerular filtration [46]. Glomerular pores have been described as cuboids with an average dimension of 4 (40 Å) by 14 nm (140 Å) in a cross section, and 7 nm (70 Å) in length [47]. Albumin is a 66–69 kDa flexible, ellipsoid-shaped protein with a 3.8 nm diameter and a length of 15 nm. Despite its size, approximately 3.3 g of albumin is filtered daily in the human kidney [48]. Therefore, it was not surprising to find albumin in urine. ApoD is a 29–32 kDa, 4.5 nm (45 Å) by 4.0 nm (45 Å) protein [49]. Considering its size, it is likely that hApoD is also able to directly pass through the glomerular pores and end up in urine without the assistance of active transport. This idea was supported by the fact that kidneys were outliers in our correlation between BSG expression and hApoD accumulation in tissues (Figure 6B). This indicated that BSG was likely not implicated in this process or that another mechanism was far more important for hApoD accumulation in kidney\urine. However, the physiological relevance ApoD’s capacity to be filtered by the kidneys remain elusive.

One possible interpretation is that ApoD could also participate in the elimination of harmful waste products (oxidized lipids) and pro-inflammatory molecules (ARA) from the CNS or other targeted tissues. ApoD is overexpressed in periods of neurodegenerative stress [15,50–55]. ApoD is known to facilitate myelin clearance, extracellular matrix remodeling and axon regeneration after nerve injury [17]. A large quantity of hydrophobic and pro-inflammatory molecules is released from damaged neurons and myelin sheets after nerve injury. ARA and lysophospholipids (LPC) are two of the major products resulting from PLA2-mediated myelin cleavage and are both ApoD

ligands. In fact, ApoD-null mice have higher basal levels of free ARA and LPC in intact nerves as well as a lower free LPC level than wildtype mice in injured nerves [17]. The lack of ApoD during nerve injury appears to cause a heightened inflammatory response, delayed inflammation resorption, and slower healing process [17]. It is believed that this occurs, at least in part, because of a lack of proper management and clearance of free ARA and LPC. Previous studies on ApoD's role in managing these substances only looked at the possibility that these harmful compounds were sequestered by ApoD and reabsorbed locally by cells in the CNS. Considering our present results, however, it is possible that ApoD might redirect part of the released lipids away from the neurons and into the kidneys and liver during these episodes. This may prevent further damage caused by peroxidized lipids or pro-inflammatory ARA-derived eicosanoids in the brain [17,56].

Our results also showed that hApoD is rapidly depleted from the circulation in favor of tissular accumulation or excretion via the kidneys/urine. Interestingly, while ApoD is known to be increased in the CSF of patients with Alzheimer's disease [51], its plasmatic levels do not appear to be affected [57]. This could be due to the quick pace at which hApoD is depleted from plasma. Unfortunately, no data exist on ApoD concentration in the urine and peripheral tissues from Alzheimer and other neurodegenerative diseases patients.

In addition to tissues with lipid recycling and excretory functions, a number of other tissues were targeted by circulating ApoD. The skeletal muscles were also an important reservoir of hApoD accumulation, despite their poor specific affinity for hApoD (Figure 3A) and low expression of the highly glycosylated BSG (Figure 6A). They only remained an important hApoD reservoir because of their large mass. Additionally, accumulation of hApoD over albumin was only significant 3 h post-injection and tended to be lower than albumin at 6 h post-injection. It is worth mentioning that, while albumin largely stays in circulation, it can also accumulate in muscles [58] and intestinal cells [59]. This could explain why hApoD accumulation was stronger than albumin in these tissues at only one time point. Another evidence that ApoD targets the skeletal muscles is that transgenic mice overexpressing hApoD in their CNS develop muscle steatosis [19]. The function of ApoD in the skeletal muscles is likely unrelated to ApoD's hypothetical function of removing harmful molecules from the CNS. Other authors described ApoD as a lipid transporter with a very large diversity of ligands. In fact, ApoD can bind cholesterol and pregnenolone, bringing these essentials lipids in various organs where they can be used. Previous studies also mentioned that increased expression of ApoD in the CNS during brain injury can increase the availabilities of essential lipids for the synthesis of new cellular membranes (Reviewed in Rassart et al., 2020 [1]).

As of now, the literature provides little insight into ApoD's potential role in skeletal muscles. One study reported that ApoD, followed by the leptin receptor (LEPR), are the two most upregulated transcripts in muscle disuse atrophy. Meanwhile, genes responsible for energy metabolism, mitochondrial function, cell cycle regulation, stress response, sarcomere structure, cell growth/death, and protein turnover were downregulated [60]. ApoD is also upregulated in age-related skeletal muscle cells senescence, where it is expected to play anti-oxidative and anti-inflammatory roles [1,9,61]. Our results indicate that circulating ApoD could also be implicated in these mechanisms.

The spleen also appears to be targeted by circulating hApoD. The spleen is the major site of heme recycling from senescent red blood cells. There, heme is converted to bilirubin and then transported to the liver to be secreted into bile in the intestines [62]. Interestingly, ApoD can bind to bilirubin [63] and has long been suspected to participate in heme recycling [5].

hApoD appeared to also have affinity for the MAT. We previously showed that ApoD protein levels in adipose tissue are linked to improved metabolic parameters in obese women. Specifically, protein levels of ApoD in the human MAT were associated with reduced circulating TNF-α and improved insulin sensitivity (QUICKY index). These correlations did not exist with ApoD mRNA expression [64] suggesting that circulating ApoD, after adipose tissue internalization, was specifically responsible for this association. This hypothesis is reinforced by our present results, which indicate that circulating hApoD can accumulate in the MAT. However, the role of exogenous ApoD in adipose tissues remains unclear. In this tissue, ApoD may exert anti-inflammatory effects through its ability to modulate ARA metabolism [7,8,22], to reduce oxidative stress [9,10,65–67], and to disrupt osteopontin function, a protein implicated in macrophage recruitment [1,68].

We used an in vitro model of the endothelial part of the BBB, bEnd.3 cell monolayers, to confirm that hApoD can be subject to internalization and transcytosis. Beside the BBB, there are also other barriers between the periphery and the CNS through which ApoD could cross. The blood-CSF barrier (BCSFB), for instance, separates the blood from the CSF. It is formed by epithelial cells and the tight junctions of the choroid plexus. ApoD could potentially cross this barrier, enter the bloodstream, and then be distributed to peripheral organs. However, the BBB area in the human brain is 10 times greater than the BCSFB area [69]. Furthermore, since ApoD is mainly secreted by glial cells [1], in proximity to the BBB, its preferred exit route is likely to be the BBB. This motivated the use of the bEnd.3 cell line as our in vitro model.

Considering that ApoD internalization is a BSG-dependent, cyclophilin A-sensitive mechanism in neurons and HEK 293T [14], we expected BSG to be implicated in hApoD internalization by

bEnd.3 cells. Though BSG is expressed by bEnd.3 cells, cyclophilin A did not limit hApoD internalization/transcytosis in our hands. Interestingly, the BSG glycosylation pattern in bEnd.3 cells is different from the one reported in 293T cells. The 293T cells appear to possess a greater amount of LG-BSG (especially 30–35 kDa). Furthermore, hApoD internalization by 293T cells was reported as soon as 4 h after administration [14], while that process necessitated 24 h to be detectable in our bEnd.3 cell assays (data not shown). Our in vivo data showed that HG-BSG expression is not correlated to hApoD internalization in tissues, contrary to the LG-glycosylated forms. Higher levels of glycosylation present on a protein can hinder its protein–protein interactions [70]. In agreement with this observation, deglycosylation of BSG was reported to increase its interaction with Caveolin-1 [31]. It is likely that a similar phenomenon occurs between BSG and ApoD. This was supported by the fact that underglycosylated BSG expression is strongly associated with hApoD specific accumulation in peripheral tissues (Figure 6B). Taken together, these results suggested that, while BSG may be an important factor in peripheral hApoD cellular internalization, it may not be implicated in hApoD's capacity to exit the brain.

These results may also imply that BSG is not the exclusive receptor for ApoD. Inhibiting LDLR has previously been shown to reverse the synaptogenic effects of ApoD in dorsal root ganglion cell cultures [71]. LDLR is present at the BBB as well as in bEnd.3 cells [35] and is a major ApoE receptor in the brain [72,73]. LDLR could therefore also participate in the passage of the ApoD through the BBB. The lack of competition by cyclophilin A could be due to an LDLR-mediated internalization of hApoD instead of a BSG-mediated one in bEnd.3 endothelial cells.

While we showed the passage of hApoD through endothelial cell monolayers, our model lacked the full complexity of the BBB. Though endothelial cells are the main components of the BBB, the mechanisms regulating the crossing of proteins through the BBB are more complex and involve other cell types. The BBB is also composed of pericytes and astrocytes in an architecture that is difficult to achieve in vitro. In fact, addition of glial cells to bEnd.3 cells in a co-culture model enhances the barrier function of the endothelial monolayer [38]. Of note, the presence of hApoD in the plasma was rapidly detectable in vivo (3 h), a kinetic that was not replicable in our in vitro model. It appears highly probable that other cell types are implicated in hApoD exit from the brain.

In our experiments, we used free soluble hApoD monomer purified from cystic fluid. However, in physiological conditions, it is not excluded that ApoD may be able to pass through the BBB via extracellular vesicles (EVs). Indeed, ApoD is found in EVs [24–26], including EVs from serum and CSF [24]. Recently, EVs secreted from astrocytes and containing ApoD were shown to be internalized in neurons and to contribute to the survival of neurons under oxidative stress [26].

The passage of EVs from the periphery to the CNS has also been shown, notably in a drug delivery context where EVs were shown to cross the BBB in both directions [74]. Therefore, the high rate at which hApoD was able to exit the brain in vivo may be due to an inclusion on ApoD into EVs by glial cells prior to its passage through the BBB. This could also be a factor explaining the slow rate of internalization observed in vitro in endothelial bEnd.3 cells, where glial cells were absent.

In our study, circulating hApoD did not appear to enter the brain from the circulation. This could indicate that hApoD transport through the CNS barriers is unidirectional or simply follows its concentration gradient (from brain to blood) under normal conditions. It is also possible that the quick pace at which hApoD is internalized by other tissues depletes the circulating stocks of hApoD in a manner that prevents its return to the brain. It is worth mentioning that our data do not definitely prove that ApoD is incapable of entering the brain from the circulation. Our study was aimed at characterizing the biodistribution of hApoD upon release from the brain. Injected radiolabeled proteins that do enter the brain often do so at a low percentage (e.g., approximately 1% of the total injected amount for transferrin) [75]. Therefore, studies centered on the brain often employ methodologies different to that used here. These include the use of a positive control and the injection of proteins into the left jugular vein instead of the tail vein [76], with a possible recirculation of radiolabeled proteins [77]. Further steps will be required to formally ascertain if ApoD can enter the brain from the circulation. ApoD exist in different forms. Much like BSG, ApoD also possess multiple glycosylation levels, which are likely to influence its protein–protein interactions [2]. Glycosylation plays an important role in the thermostability, folding, as well as the overall charge of a protein and can influence its protein–protein interactions [70]. The injection of different forms of ApoD, including monomers, dimers, tetramers, and EV containing ApoD—as well as differently glycosylated ApoD—could also give different results.

In conclusion, our study shows for the first time that hApoD can efficiently exit the brain, most likely by active passage through the BBB and reach peripheral tissues with functions related to lipid and glucose homeostasis (liver, muscles, pancreas, adipose tissues, and intestines, together known as metabolic organs) as well as excretion (Kidneys/urine). The characterization of hApoD's distribution pattern represents an important advance in the understanding of ApoD function and correlates well with its known functions. The high amount of hApoD found in the liver and urine in our experiments suggests that ApoD may have a role in the excretion and recycling of brain lipids. Our results also suggest that ApoD accumulation in peripheral tissues is likely dependent on BSG and influenced by its glycosylation levels. ApoD's capacity to leave the brain is likely more

complex and might not depend on BSG. However, it remains unclear what receptor other than BSG participates in this process. Furthermore, ApoD's own glycosylation and incorporation into EVs is also likely to influence ApoD cell internalization and tissue distribution.

2.5 Materials and Methods

2.5.1 Animals

Experiments were carried out on C57BL/6 male mice of 3 to 4 months of age. Animals were housed under standard conditions at constant temperature (20–22 °C) and humidity (50–60%), under a 12 h light/dark cycle with free access to water and food (standard rodent chow; Charles River #5075). Experimental procedures were approved by the Animal Care and Use Committee of Université du Québec à Montréal (protocol #962, reference number 0319-962-0320, 24 April 2019).

2.5.2 Protein Radiolabelling

Human ApoD (hApoD), purified from breast cyst fluid [4] and bovine serum albumin (BSA) were both radiolabeled with iodine-125 in the form of iodine monochloride following the method described by A.S. McFarlane [78]. Unbound Iodine-125 was removed by exclusion chromatography using Bio-Spin 6 gel columns (BioRad #732-6002, Mississauga, Ontario, Canada). The eluted protein concentration was assessed by Bradford assay [79]. Specific radioactivity ranged from 0.11 to 0.16 µCi/µg of protein. Radiolabeled proteins were visualized after denaturing gel electrophoresis followed by revelation using a Molecular Dynamics Storage Phosphor Screen (Kodak Storage Phosphor Screen SO230, Rochester, New-York, USA) and Typhoon FLA9500 (Figure S1).

2.5.3 Intracerebroventricular and Intravascular Injections

For intracerebroventricular (ICV) injection, ketamine/xylazine anesthetized animals were placed on a stereotaxic table (Stoelting #51600). Approximately 2.5 µg of radiolabeled hApoD or BSA (5 µL volume; 7 × 105 CPM) was bilaterally injected into the lateral ventricles at a rate of 0.2 µL/min (Hamilton syringe 1701 N) as previously described [80,81]. The bregma coordinates used for injection were: 1.0 mm lateral, −0.3 mm posterior and −2.5 mm below, as previously described [82]. The needle was gently removed 5 min after the end of each injection. For intravascular injections, 100 µL PBS (pH 7.4) containing 3 × 106 CPM (1.35 µCi) of either radiolabeled hApoD or BSA was administered via the tail vein (26G syringe).

2.5.4 **Tissue and Fluid Sample Preparation**

To collect urine, the bladder was emptied with a 26G needle and syringe. Blood was collected by cardiac exsanguination from the left ventricle with a 22G needle and syringe without damaging the inferior vena cava. Immediately after, the blood was flushed from the circulation by performing a whole-body perfusion. Briefly, a catheter was inserted into the inferior vena cava from the right auricle of the heart and maintained in place with chirurgical sutures. The lower part of the right ventricle was perforated to allow the blood to escape. A buffer solution (128 mM NaCl, 4.0 mM KCl, 0.62 mM H2PO4, 1.1 mM NaH2PO4, 11.1 mM Dextrose, 10.1 mM HEPES, 1.1 mM MgCl2, 0.42 mM MgSO4, 1.5 mM CaCl2, pH 7.4, 37 °C) was then circulated with a peristaltic pump at 2 mL/min for at least 5 min, until the buffer-diluted blood coming out was clear. Organs and tissues were then collected: brain, liver, adductor and medial hamstring muscles, kidneys, heart, spleen, pancreas, caecum, bone marrow, as well as omental, mesenteric, and subcutaneous adipose tissues.

Plasma was prepared by centrifugation (15 min; 2000× g) of 500 µL blood mixed with 50 µL EDTA 10%. Whole organ weight was determined whenever possible (for brain, liver, kidneys, heart, spleen, pancreas, and caecum). Because only samples of adipose tissues and bone marrow were obtained, their whole organ weight was not determined. Tissue samples were homogenized in a lysis buffer (2 µL/mg of tissue) suitable for tissues with high a lipid content (50 mM Tris-HCl pH 7.4, sucrose 250 mM, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM EDTA, 1 mM DTT, 1 mM sodium vanadate, 1 mM PMSF) using a Dounce tissue grinder (Wheaton, 357421). Tissue homogenate and fluid (plasma and urine) radioactivity was measured by scintillation counting. Individual organ radioactivity was extrapolated by multiplying the specific radioactivity (CPM/g of tissue sample) by the weight of the organ. To extrapolate blood radioactivity, plasma was considered to account for 58% of the blood volume (Feher, 2012) and the total blood volume was considered to be 2 mL for a 25 g mouse [83]. Pelleted blood cells had radioactive levels approximately half of that of the plasma itself according to Geiger counter readings. Therefore, blood cell specific radioactivity (BCSR) was estimated from the plasma specific radioactivity according to the following equation:

$$BCSR = \frac{Plasma\ specific\ radioactivity}{2}$$

Total blood radioactivity (TBR) was calculated as follows:

$$BR = \big((Plasma\ specific\ radioactivity \times 0.58) + (BCSR \times 0.42)\big) \times \frac{2\ mL}{25\ g} \times Body\ weight$$

Similarly, whole muscle radioactivity was extrapolated from the combined adductor and medial hamstring muscles specific radioactivity and the animal body weight. Total wet skeletal muscle mass was considered to be 6.9 g for a 25 g mice, consisting of 1.8 g of dry weight [84] and 5.1 g of water (water composition of 75%) [85]. Total muscle radioactivity was calculated as follows:

$$Total\ muscle\ radioactivity = Muscle\ radioactivity\ \times \frac{6.9\ g}{25\ g} \times Body\ weight$$

In addition to measuring radioactivity from the urine contained in the bladder, the bottom of each cage was swabbed with a humid absorbing paper and this radioactivity (measured by scintillation counting) was added to the urine fraction. The total recovered radioactivity was determined by adding all tissues and fluids together.

2.5.5 **Cell Culture**

The bEnd.3 brain endothelial cell line was obtained from the American Type Culture Collection (ATCC, CRL-2299, Manassas, Virginia, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS), 1% sodium pyruvate (1 mM), and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) from Wisent Bioproducts, in a humidified incubator at 37 °C with 5% CO2.

To generate monolayers, 30,000 bEnd.3 cells were seeded on the upper surface of Falcon cell culture inserts (Corning, #08770, Corning, New-York, USA) placed within the wells of a 24-well plate (Corning, #09761146). The culture medium (top and bottom compartments) was replaced every two days. After 7–10 days, monolayer imperviousness was systematically validated via transendothelial electrical resistance (TEER) (Figure S2) measurements using a voltage and resistance meter (EVOM2, World Precision Instruments Inc., Sarasota, Florida, USA) equipped with a cell culture cup chamber electrode (Endohm-6, World Precision Instruments Inc. Sarasota, Florida, USA). The background value measured on a cell-free insert (insert resistance) was subtracted from each raw TEER measurement. Resistivity (Ohms·cm2) was calculated as follows:

$$TEER = \left(Resistance\ (\Omega) - Insert\ Resistance\ (\Omega)\right) \times Surface\ area\ (cm^2)$$

Monolayers with TEER values of at least 35 Ohms/cm2 (Figure S2) were retained for transcytosis experiments (Wuest, Wing, and Lee 2013; Clark and Davis, 2015).

The monolayers were also systematically validated by assessing permeability to Dextran-FITC (10 kD, Sigma-Aldrich, St-Louis, Missouri, USA). Briefly, 10 µL of Dextran-FITC (1 mg/mL; in serum-free, phenol red-free culture media) were added to the media on the top of the insert. After

a 1 h incubation, 150 µL of culture medium was sampled from the bottom compartment and analyzed on a 96-well plate reader (Tecan, Switzerland). Apparent permeability index (Papp) was calculated from the following equation:

$$P_{app} = \frac{V_R \Delta C_R}{\Delta t \, S_{ins} C_D}$$

With Papp apparent permeability (cm·s−1); VR, volume of receiving compartment (cm3), ΔCR, change in concentration in the receiving compartment (µM); Δt, time in seconds (s); Sins: surface of the insert (cm2) and CD: concentration in the donor compartment (µM) [38]. Monolayers with Dextran-FITC apparent permeability values (Papp) below 10–6 cm·s−1 were used for transcytosis experiments [86]. Monolayers were further validated following transcytosis experiments by verifying the presence of Claudin 5-positive tight junctions between cells [38].

For our transwell assays, monolayer cells were treated 24 h on the top side with hApoD (purified from human cystic fluid) at 200 or 400 ng/mL with or without recombinant human cyclophilin A (R&D systems, 3589-CAB). Cell viability (Figure S2) was confirmed using a resazurin-based kit (Sigma-Aldrich, TOX8, St-Louis, Missouri, USA).

2.5.6 **Immunofluorescence**

Monolayer cells were fixed with 4% paraformaldehyde 15 min at room temperature, permeabilized with 0.2% Triton X-100 in PBS and blocked with 5% no-fat dry milk in PBS. Primary antibodies rabbit anti-Claudin 5 (Thermofisher #34-1600, Waltham, Massachusetts, USA) and mouse anti-ApoD (2b9 1:100) [87] were used with the dilution (1:100). Secondary antibodies rabbit Alexa Fluor 488 (Invitrogen #A11008, Carlsbad, California, USA) and mouse Alexa Fluor 594 (Invitrogen #A11005) were used with the dilution 1:500. The nuclei were stained with Hoechst 33258 (10 µg/mL, Sigma-Aldrich #861405) 15 min at room temperature. Then, inserts were placed on a slide with mounting medium (Prolong™ Gold Antifade Mountant). Mounted inserts were examined on a Zeiss LSM780 system confocal microscope equipped with a 30-mW, 405-nm diode laser, a 25-mW 458/488/514 argon multiline laser, a 20-mW DPSS 561-nm laser, and a 5-mW HeNe 633-nm laser mounted on Zeiss Axio Observer Z1, as well as a Plan-Apochromat 63× oil DIC 1.4NA objective. Images (1.6× zoom scans) were acquired with Zen 2011 software (Zeiss, Germany). Images were analyzed with ImageJ software (version 1.52p, National Institutes of Health, Bethesda, Maryland, USA).

2.5.7 **Immunoblotting**

For tissue sample analysis, lysates were incubated 30 min at 4 °C, cleared by centrifugation (10,000× g, 15 min) and the lipid layer was discarded. For bEnd.3 transwell media analysis, media proteins from top and bottom compartment were concentrated with Amicon Ultra-0.5 10 kDa centrifugal filters (Millipore Sigma #UFC501024, Burlington, Massachusetts, USA).

For both sample types, protein concentration was assessed by Bradford assay [79]. Proteins (20 µg, unless otherwise indicated) were separated on 10% SDS-PAGE gels and transferred on PVDF membranes. Blocking was performed using 5% milk, 1 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4 °C. Antibodies used were against Basigin (Abcam, ab188190 1:5000), ApoD (MyBioSource #2003092 1:1000), and goat anti-rabbit HRP conjugated IgG (Sigma-Aldrich #A6154 1:10,000 or Abcam ab6721 1:4000). Bands were visualized using chemiluminescent HRP substrate (Millipore, WBKLS0500, Burlington, Massachusetts, USA) in a FUSION FX7 Imaging system and analyzed with the gel analyzer function of Image J software (National Institutes of Health, Bethesda, Maryland, USA).

2.5.8 **Statistics**

Our histogram results are presented as mean ± standard error of the mean. The statistical analysis were performed with the GraphPad 7 software. For histograms, statistically significant differences from control values were determined by using a one-tailed Student's t-test and a Welch's correction was applied when variances between groups were unequal. Variance equivalence between groups was determined by Fisher's f-test. Associations between variables of interest were quantified by linear regression using Pearson's product moment correlation coefficients analysis. Statistical significance was considered reached if the p-value was <0.05.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1.

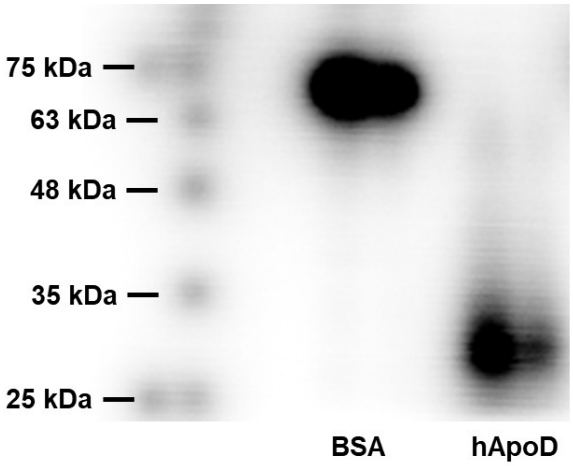
Author Contributions: F.D., V.H., G.R., M.P., and G.F.-D. performed the experiments; F.D., V.H., and K.-F.B. wrote the manuscript; C.M. C.R., K.-F.B., and E.R. edited the manuscript and supervised the study; F.D. and V.H. are co-first authors; C.R. and C.M. are co-last authors. All authors have read and agreed to the published version of the manuscript.

Funding: FD was supported by a Fonds de recherche du Québec-Nature et Technologie (FRQNT) fellowship (dossier number 269948)

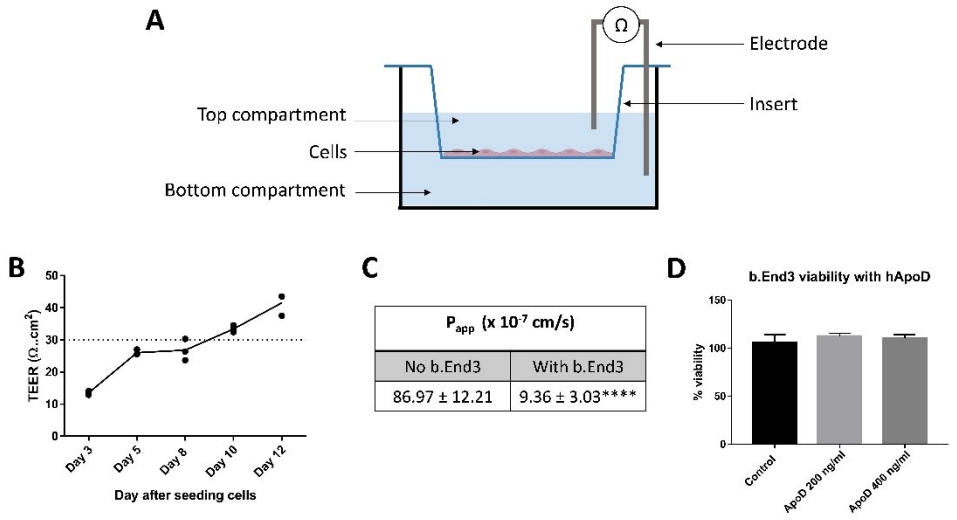
Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Animal Care and Use Committee of Université du Québec à Montréal (protocol #962).

Acknowledgments: We are grateful for the financial support provided by the NSERC, the Louise & André Charron Research Chair on Alzheimer disease and the Quebec Network on Aging-RQRV (CR). We are also grateful for the work accomplished by our interns: Doriane Gisquet, Annabelle Boudy, and Audrey Girardeau.

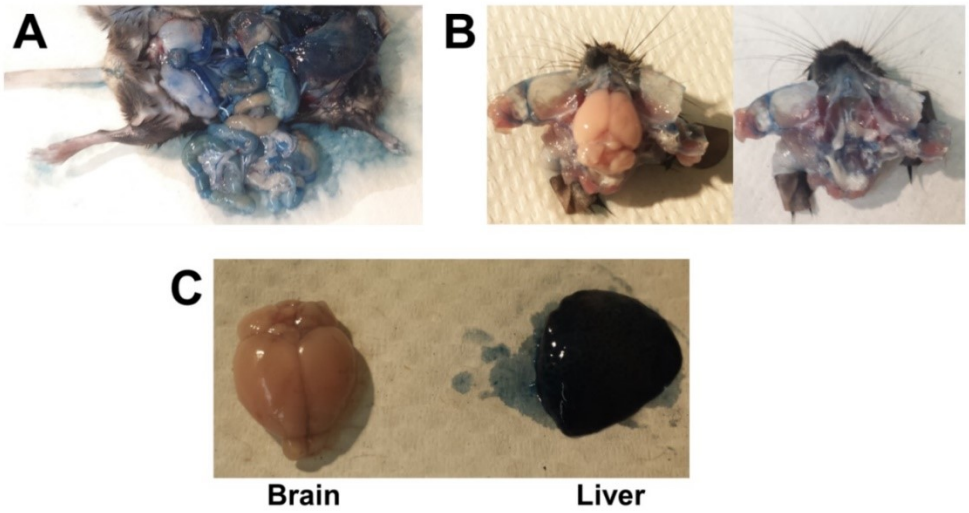
Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.



Supplementary Figure 1. Protein integrity after radiolabeling



Supplementary Figure 2. b.End.3 model validation. A) Representative schematic of TEER measurement in a bEnd.3 endothelial monolayer model disposed on insert. B) Transen-dothelial electrical resistance (TEER) of bEnd.3 cells after seeding. TEER value for bEnd.3 cells above 30 Ω.cm2 means that the monolayer is established. C) Permeability coefficient (Papp) values of Dextran 10kDa measured in bEnd.3 culture or without cells. Values are taken 12 days after seeding. D) Viability of bEnd.3 in presence of ApoD at 200 ng/ml and 400 ng/ml for 24h. Statistical significance for panels C and D was evaluated via a Student t-test: ****p<0.0001. n=3 independent experiments for all panels.



Supplementary Figure 3. HEPES circulation protocol validation. HEPES was circulated with a peristaltic pump at 2 mL/min for at least 5 minutes, until the buffer-diluted blood coming out was clear. An Evans Blue buffer was then circulated for 5 minutes, showing a proper coloring of tissues and circulatory system A-C) with exception to the brain B) andC) which remained clear.

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3 INTRACEREBRAL INJECTION OF STREPTOZOTOCIN IN APOLIPOPROTEIN D-DEFICIENT MICE: EFFECTS ON THE WORKING AND SPATIAL MEMORY, APOLIPOPROTEINS E, J, SYNAPTOPHYSIN LEVELS AND ANTIOXIDANT CAPACITY

Injection intracérébrale de streptozotocine chez des souris déficientes en Apolipoprotéine D : Effets sur la mémoire de travail et spatial, les niveaux d'Apolipoprotéines E, J, de synaptophysine et la capacité antioxydante.

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Article à soumettre dans le journal *Life Science*

Résumé en français

L'Apolipoprotéine D (ApoD) pourrait jouer des rôles clés dans le maintien et la protection des neurones des conditions de stress ou de neurodégénération. L'injection intracérébroventriculaire de streptozotocine (icv-STZ) chez les souris est connue pour induire plusieurs aspects de la maladie d'Alzheimer (MA).

But : Evaluer les effets d'une déficience d'ApoD sur le comportement cognitif, l'Apolipoprotéine E (ApoE) et J (ApoJ), une protéine synaptique et des marqueurs de stress oxydatif suivant une icv-STZ.

Méthode principale : Les souris sauvages, ApoD$^{+/-}$ et ApoD$^{-/-}$ âgées de 8 mois ayant reçues des icv-STZ (3mg/kg). L'entraînement et l'apprentissage de la mémoire spatiale ont été évaluées par les tests comportementaux : le piège en Y et la piscine de Morris. L'ApoE, l'ApoJ et la synaptophysine ont été analysées dans l'hippocampe et le striatum par Western-Blot et les marqueurs de stress oxydatifs par carbonylation des protéines et essais ABTS.

Découvertes clés : Le test de la piscine de Morris suggère des déficiences de mémoire spatiale dépendante de l'hippocampe chez les souris ApoD$^{+/-}$ et ApoD$^{-/-}$ ayant reçu de la STZ. Dans le striatum, le niveau d'ApoJ était plus haut chez les souris ApoD$^{-/-}$ et sauvages avec icv-STZ. Dans l'hippocampe, les niveaux d'ApoE sont plus bas chez les souris ApoD$^{+/-}$ et ApoD$^{-/-}$ que chez les sauvages et ont été diminué avec l'icv-STZ. Le niveau de synaptophysine était plus haut dans le striatum chez les souris ApoD$^{-/-}$ sans aucun effet de l'icv-STZ. Les souris ApoD$^{+/-}$ et ApoD$^{-/-}$ ont une réduction de leur capacité antixoydante dans l'hippocampe, le striatum et le cortex frontal sans effet de l'icv-STZ.

Conclusion : Nous fournissions l'évidence que l'ApoD participe à la mémoire de travail et spatial, la régulation de l'ApoE et l'ApoJ, de la synaptophysine et sur les capacités antioxydantes dans le cerveau.

Contribution des auteurs :

Dans cet article, j'ai participé à chaque étape du projet une fois que les souris sont arrivées dans l'animalerie, cela a été organisé par Morgane Perrotte. Les souris nous ont été données par le Pr. Eric Rassart de l'UQAM. Avec Morgane Perrotte, nous avons réalisées ensemble les injections de streptozotocine, les tests comportementaux, ainsi que du génotypage. Les injections intracérébrales et les tests comportementaux nous ont été enseignés par le Pr. Jonathan Brouillette et Chloé Provost. La suite des dissections et expériences a exclusivement été réalisé par moi-même. J'ai effectué les tests statistiques et écrit cet article que Charles Ramassamy a corrigé.

Lien entre l'article ou les articles précédents et le suivant :

Nous avons vu dans l'article précédent que l'apoD retrouvée dans le cerveau avait principalement une origine cérébrale. Ainsi lors de dommages cérébraux, c'est l'apoD synthétisée dans le cerveau qui va agir dans le mécanisme de neuroprotection. L'apoD montre des effets neuroprotecteurs, cependant son rôle dans la mémoire spatiale n'a pas encore été déterminé. Il serait intéressant d'observer si le manque d'apoD pourrait agir comme un facteur de risque de

l'apparition de troubles de la mémoire spatiale. C'est pour répondre à ces questions que nous avons injecté de la STZ en icv chez des souris apoD$^{-/-}$. La dose choisie de STZ est connue pour ne pas causer de troubles de la mémoire spatiale chez les souris mais des bouleversements biochimiques (modification de la potentialisation à long-terme). Cette étude va permettre, en lien avec la précédente de montrer l'importance que possède l'apoD dans le cerveau au niveau de la préservation de la mémoire spatiale mais aussi dans le stress oxydatif et l'influence qu'elle possède en son absence avec les deux autres apoliprotéines bien connues du cerveau : l'apoE et l'apoJ.

3.1 Abstract

Apolipoprotein D (ApoD) is suspected to play key roles in the maintenance and protection of neurons under stress or neurodegeneration conditions. The intracerebroventricular injection of streptozotocin (icv-STZ) in mice is known to induce many aspects of sporadic Alzheimer's disease (AD).

Aims: To evaluate the effects of the ApoD deficiency on cognitive behavior, apolipoproteins E (ApoE) and J (ApoJ), synaptic protein, and oxidative stress makers following an icv-STZ.

Main methods: Wild type (WT), $ApoD^{+/-}$ and $ApoD^{-/-}$ mice at 8-month-old received an icv-STZ (3mg/Kg). Training and spatial learning memory were evaluated by Y-Maze and Morris Water Maze (MWM) behavioral tests. ApoE, ApoJ, and synaptophysin were analyzed in hippocampus and striatum by Western Blot and oxidative markers by protein carbonylation and ABTS assays.

Key findings: The MWM test suggest an impairment in hippocampal-dependent memory in $ApoD^{+/-}$ and $ApoD^{-/-}$ mice receiving STZ. In striatum, the level of ApoJ was higher in $ApoD^{-/-}$ and in WT mice with icv-STZ. In hippocampus, the levels of ApoE were lower in $ApoD^{+/-}$ and $ApoD^{-/-}$ than in WT mice and were significantly decreased with an icv-STZ. The level of synaptophysin was higher in striatum from $ApoD^{-/-}$ mice without any effect of the icv-STZ. $ApoD^{+/-}$ and $ApoD^{-/-}$ mice have a reduction in the antioxidant capacity in hippocampus, striatum and frontal cortex without any effect of an icv-STZ.

Significance: We provide the evidence that ApoD participates in the working and spatial memory, on the regulation of ApoE, ApoJ, synaptophysin and on the antioxidant capacity in the brain.

Highlights:

- Lack of ApoD in mice induce a spatial memory deficit following and icv-STZ
- ApoJ increased in striatum and ApoE decreased in hippocampus in $ApoD^{-/-}$ mice
- Antioxidant capacities were reduced in the $ApoD^{-/-}$ and in controls mice with an icv-STZ.
- Synaptophysin was decreased in $ApoD^{-/-}$.

3.2 Introduction

Apolipoprotein D (ApoD) is an atypical apolipoprotein, mainly present in high-density lipoprotein (HDL) (1). ApoD is a transporter of lipids, arachidonic acid, cholesterol and triglycerides, several small hydrophobic molecules in various tissues (2, 3). Its functions are mostly associated to lipid metabolism and trafficking, regeneration, inflammation, antioxidative and neuroprotection. In rodent and humans, ApoD expression is highest in the brain (4, 5) (see review by Rassart et al.,(3)) where its level increased during aging (6) and in some neurodegenerative disorders (7-12). In Alzheimer's disease (AD), ApoD is highly expressed in the brain, the cerebrospinal fluid (CSF), hippocampus and enthorhinal cortex (11, 13, 14). ApoD level is also increased in several animal models of brain injury indicating the important role of ApoD in neuroprotection (15-21). ApoD deficient mice- have reduced locomotors, exploratory activities compared to WT mice (19, 22). These effects could be due to the induction of some genes involved in synaptic function and myelin homeostasis by ApoD (23, 24). However, the role of ApoD in spatial memory has not yet been established.

The presence of ε4 ApoE isoform is one of the main risk factors for AD (25). Apolipoproteins E, J and D are closely regulated (26, 27). Although ApoD is mainly located in glial cells and ApoJ expression preferentially occurs in neurons (28), their immunostainings show a positive correlation with the progression of AD suggesting a possible complementarity role between both proteins (28, 29).

In addition to be a lipid transporter, ApoD has antioxidant and neuroprotective functions (19, 30-32). The role of ApoD in the resistance to oxidative stress was confirmed in other species such as Drosophila and Arabidopsis (33-35).

The intracerebroventricular (icv) injection of streptozotocin (N-(Methylnitrosocarbamoyl)-α-D-glucosamine; STZ) is a well-known model to involve neuroinflammation, oxidative stress, and can mimic some pathological aspects of the sporadic form of AD (36, 37). In this study, we have chosen a dose of STZ that causes biochemical alterations while preserving the alterations of the spatial memory in WT mice (38). We thus evaluated the effect of the ApoD deficiency on learning, spatial memory and oxidative stress in WT, ApoD+/- and ApoD-/-mice in the presence of an icv injection of STZ (icv-STZ). Our results indicate that ApoD contributes in the preservation of the spatial memory, modifies the expression ApoE and J in hippocampus and striatum and provide a protective redox status.

3.3 Experimental procedures

3.3.1 Animals Ethics statement

The experimental procedures were approved by the Institutional Animal Care and Use Ethics Committee (IACUC) of the Institut National de la Recherche Scientifique (INRS) and are in accordance with the Canadian Council on Animal Care (CCAC) guidelines (Protocol number #1412-01). The ApoD-/- mice and ApoD+/- mice were kindly provided by Pr. E Rassart (Université du Québec à Montréal). (19). The ApoD gene was silenced using a targeting vector with neomycin phosphotransferase (Neo) gene interrupting the exon 6 of the ApoD gene. The background of mice was C57BL6/J.

Animals were housed at 24 ± 1°C in a 12h light / 12h dark cycle with free access to water and feed with “Teklad global 18% protein rodent diet” (cat. # 2018) from Envigo, Montréal.

3.3.2 Surgical Procedures

Briefly, 8-month-old mice were anesthetized with an intraperitoneal injection of ketamin/xylazin mixture (100mg/kg and 10mg/kg, respectively) and placed into a stereotaxic table. Each mouse received a single icv injection of 3.0mg/kg STZ in 3.0 µl of 0.9% saline into the left ventricle of the brain (icv-STZ) while control mouse received an icv injection of 3.0 µl of 0.9% saline (icv-saline). The injection was performed with the Hamilton syringe (Model 1701 N) with the following stereotaxic coordinates: −1.0 mm lateral from midline, −0.3 mm posterior to the bregma and −2.5 mm below the skull surface (39) at a rate of 0.2 µl/min and the needle was slowly removed 5 min after the end of the injection. The experimental design of the study was summarized in Fig 1.

3.3.3 Forced Alternation Test (Y-Maze)

A three-arm Y-maze test was performed 3 weeks after the surgery. Three arms are equally distributed at 120° angle. Each arm had an internal dimension 405mm (length) x 150 mm (width) and 300 mm (height). Visual cues are placed at the top of the end of each arm. For the first trial, mice were placed at the end of one arm of the maze (start arm) and allowed to explore freely two arms while the third is closed during the training test (3 min) (Fig 2A). The blocked arm was alternated between each mouse. Mice were returned to their home cage for 5 min and then reintroduced into the same start arm to explore all three accessible arms for 3 min (test trial). Time spent in the new arm and the average time spent in the other arms were recorded by a camera placed above the maze. The videos were analyzed with the Smart software (Panlab, Harvard

Apparratus). The duration of exploration of the new arm was calculated, as well as the percentage of mice in each group who have chosen the new arm.

3.3.4 **Morris Water Maze (MWM)**

The MWM task was conducted 7 days after the Y-maze test. The MWM test was performed in a 1.4 m diameter pool. The platform (14 cm in diameter) was submerged 1 cm below the surface of water maintained at 24 ± 1 °C and has been made opaque by the addition of non-toxic white painting. The learning trials were conducted over 4 training days. During these days, mice learned the location of the submerged platform using visuo-spatial cues installed around the internal side of the pool walls. The submerged platform remained in the centre of the same quadrant throughout all training days. Mice were given three trials of 60s per day with 30 min inter-trial interval during training days. If the platform was not found within 60 s, mice were guided to it and remained there for 10 s before removal. During the learning phase, the time and the distance to reach the platform were extracted by the Smart software (Harvard Apparatus). Mice start at different location for each trial on the same day. During the probe trial on the fifth day, the platform was removed, and mice were allowed to explore the pool during 45 s. The mean latency to reach the platform area, the number of entries and the traveled distance in the former platform area were calculated with Smart software (Panlab, Harvard Apparratus).

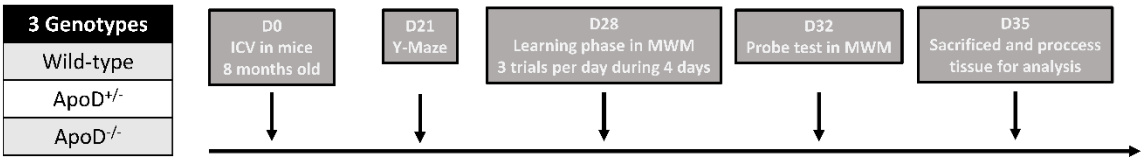

Figure 1: Experimental design. At 8-month-old, mice received an icv injection of saline or streptozotocin (STZ). Three genotypes were tested: wild-type, ApoD+/-, ApoD-/-. Y-Maze assay was performed on the 21th day (D21) after the icv injection, the Morris Water Maze on the 28th (D28), and the probe test on 32th (D32). Mice were sacrificed at 35th day and brains were collected.

3.3.5 **Brain protein extraction**

35 days after the injection, mice were anesthetized with a ketamine/xylazine cocktail, and perfused intracardially with 20 mL of a 0.9% saline solution. Brains were collected and immersed in cold isopentane for 1 to 2 minutes and transferred to -80°C before use. Hippocampus, striatum, frontal cortex and temporal cortex were dissected on ice. Samples were crushed with a potter in RIPA buffer (5 µl per mg) with protease inhibitor cocktail (1:100, Sigma-Aldrich, #P8340) and

phosphatase inhibitor (1:50, Sigma-Aldrich, #P0044). The homogenate was centrifuged for 10 minutes at 3000 rpm at 4°C. Protein samples were collected and measured with a kit (Pierce™ BCA Protein Assay Kit #23225, ThermoFisher).

3.3.6 **Cell Culture**

The Neuro2a, neuron cell line was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Flask T25 Cell+ (Sarstedt, Germany) in Eagle's minimal essential medium (EMEM), with 10 % fetal bovine serum (FBS), 1% sodium pyruvate (1 mM) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) from Wisent Bioproducts, in a humidified incubator at 37°C with 5% CO2 until 80% confluence. Cells were treated 24h with hApoD (purified from human cystic fluid) at 200 ng/ml during 24h. Cells were detached and centrifuged for 6 minutes at 2 100 rpm. The pellet was incubated 45min at 4°C with 125µl of RIPA buffer and protease inhibitor. Protein concentration was measured as previously.

3.3.7 **Western blot**

20µg of proteins from striatum or hippocampus homogenates were separated on a 10% SDS-polyacrylamide gel by electrophoresis then transferred onto a PVDF membrane using Trans-Blot Turbo System (Bio-Rad, Hercules, CA, USA). The membranes were then blocked during 1h at room temperature with TBS (Tris-buffered saline) containing 5% Bovine Serum Albumin (BSA), followed by an overnight incubation at 4°C with either primary antibodies: Synaptophysin (1:2000, abcam #32127) or ApoJ (1:2000, Mybiosource #768013), ApoE (1:200, Santa Cruz Biotechnology sc-393774) in TBS 5% BSA. The membranes were then washed three times with TBS-Tween 0.1% (TBS-T) during 5 minutes, incubated 1h at room temperature with a secondary antibody HRP-conjugated (1:10 000, Sigma Aldrich, #A6154).

For the protein carbonylation detection, after the transfer to the PVDF membrane, the carbonyl groups were derivatized with a solution of 1mM di-nitrophenol hydrazine (DNPH) in 2M hydrochloric acid solution for 1h then blocked at 4°C overnight with TBS-T. The membrane was then incubated with an anti-dinitrophenol hydrazine (DNP) antibody (D9356 sigma, 1:2000 in TBS with 5% BSA) which recognizes the previously derivatized carbonyl group followed by an incubation with the secondary antibody coupled with HRP. Between each step, membranes were washed 5 times for 5 minutes with TBS-T. The membrane was revealed with ECL substrate (Bio-Rad laborarories, Inc., Hercules, CA, USA) and the luminescence was analyzed by the FluorChem system. The bands were analyzed with the Image J software (40).

3.3.8 Radical Scavenging ability

The radical scavenging ability of protein samples (from hippocampus, striatum, frontal cortex, temporal cortex) was evaluated with a colorimetric assay based on the inhibition of the formation of 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS.+), a stable free radical, from the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), as described by (41) with some modifications. In this assay, the conversion of ABTS (7mM) into the radical ABTS•+ by 2.5mM potassium persulfate was monitored at 734 nm for 10-min after the addition of 10 µl of protein samples to 90 µl of the ABTS solution, The extent of decolorization as a percentage inhibition of the ABTS•+ radical cation formation was determined as a function of concentration and time and calculated relative to the reactivity of the Trolox standard curve (0–100 µM) obtained under the same conditions. The ABTS radical scavenging activity was expressed as µM Trolox equivalent/µg of protein was calculated with Trolox standard curve.

3.3.9 Statistical analysis

Results were presented as mean ± standard error mean. Statistical analysis was performed with GraphPad 7 software. Statistically significant differences (p-value: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$) were determined by two-ANOVA followed by Fisher's LSD test. The Chi-squared test was used to calculate statistical differences for the Fig 2C. The number of animals used for each experiment is indicated in the legends of each figure.

3.4 Results

3.4.1 Effect of icv-STZ on the hippocampal-dependent learning and memory in ApoD +/- and ApoD -/- mice

Three weeks after the ICV-STZ injection, behavioral tests were carried out to investigate the working and spatial memory in WT, ApoD+/-, and ApoD-/- mice. In YM test, WT-icv-saline mice spent more time exploring the new arm (the previously blocked arm) as compared to the STZ injected-mice ($p \leq 0.01$), indicating an induction by STZ of the deficit in working memory (Fig. 2B). The Y-maze test was equivalent between ApoD+/- or ApoD-/- mice, treated or not with STZ. These four groups indeed spent the same time than the WT-icv saline exploring the new arm which suggest a preservation of the working memory independently of the presence of ApoD and STZ injection (Fig. 2B). At least 50% of mice choose the new arm as the first choice. These results

illustrate that the deficiency in ApoD did not affect the working memory which was also not affected by STZ treatment (Fig. 2C).

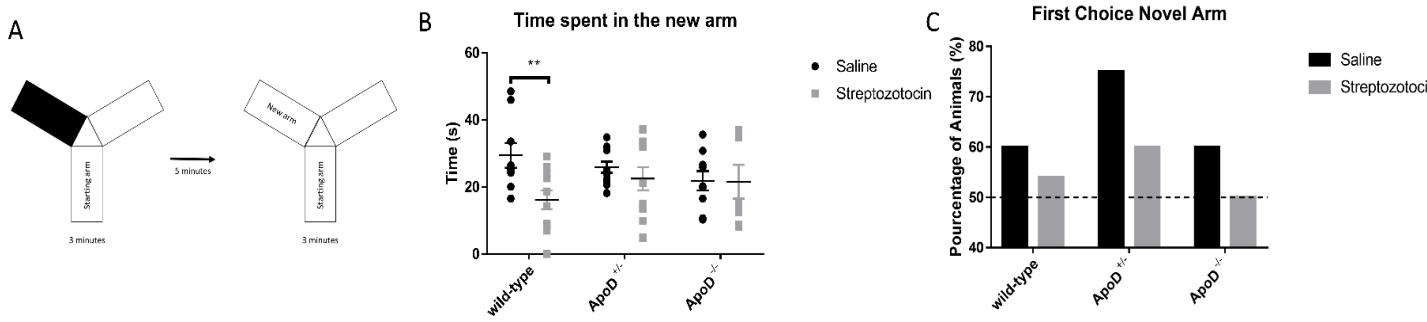

Figure 2: Forced Alternation (Y-Maze). The Y-Maze test was performed 3 weeks after the icv. Mice were first placed in a Y-Maze where one of the three arms is blocked for 3 minutes. After 5 minutes out of the maze, mice were place again at the same starting point, with all arms free. A- Experimental design of Y-Maze procedure. B –Time spent in the new arm in the first minute of the test. C – The percentage of animals selecting the novel arm as the first-choice mice for each group, quantitative values are mean + S.E.M and data groups were compared with two-way ANOVA followed by Fisher's LSD test with N=8. ** p<0.01.

Four weeks after the saline injection, spatial memory was further tested in the MWM. During the learning phase, the latency to find the platform progressively decreased over training days to reach 20s to 30s in WT and in ApoD+/- and ApoD-/- groups (Fig. 3). In icv-STZ mice, learning was not different for WT, ApoD+/- and ApoD-/- groups as the time to reach the platform between the first and last trial did not decrease significantly (Fig. 3).

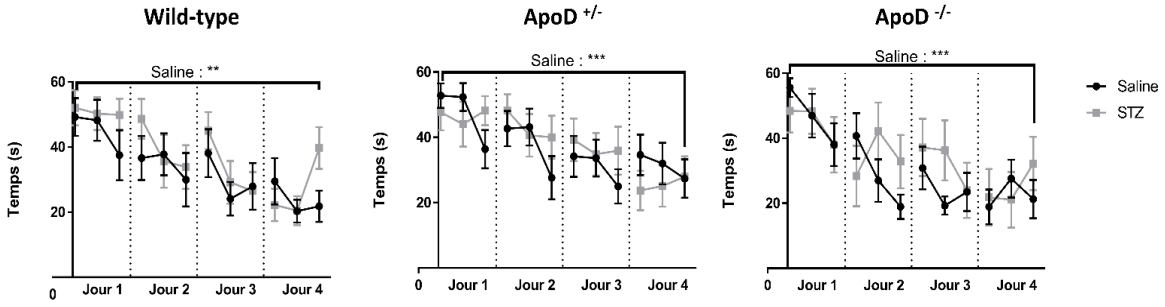

Figure 3: During the first 4 days, mice learn the location of a hidden platform, at the rate of 3 one-minute trials per day. Mice start in different places in each test andt evaluates the learning capacity. Mouse learning stage over 4 days in the Morris Water Maze (3 trials per day), to learn with the help of clues in the room, the location of the platform, in randomized the starting point to each mice. n>8 mice for each group, values are mean + S.E.M and data groups were compared with two-way ANOVA followed by Fisher's LSD test. ** p<0.01, *** p<0,001.

On the fifth day, the platform was removed. The number of times and the time mice spent in the quadrant where the platform was located (platform quadrant) are indicative of the spatial memory. The time latency of the first entrance in the platform area was not different between icv-saline and icv-STZ mice except for the ApoD-/- group (25.9s ±6.8 versus 7.7s ± 2.7) (Fig. 4A). Moreover, the number of entries in the platform quadrant was significantly decreased in ApoD+/-- and ApoD-/- with icv-STZ (Fig. 4B). Besides, WT with icv-STZ mice enter significantly more often in the platform quadrant than ApoD-/- with icv-STZ mice. The percentage of distance swum in the platform area by ApoD-/- - with icv-saline was almost two-fold higher than ApoD-/--with icv-STZ mice (Fig. 4C) while it was similar for the WT and the ApoD+/- groups. When examining the mouse swimming trajectories, WT mice showed preference around the platform quadrant while the ApoD-/- with icv-STZ mice goes around the platform quadrant without searching it (Fig. 4D). In the probe test, we verified that mice swam the same distance on average for each assay, to ensure equivalent locomotion across genotypes and treatments (Supp Fig.1). Altogether, the MWM test suggest an impairment in hippocampal-dependent memory in ApoD+/- and ApoD -/- mice receiving STZ.

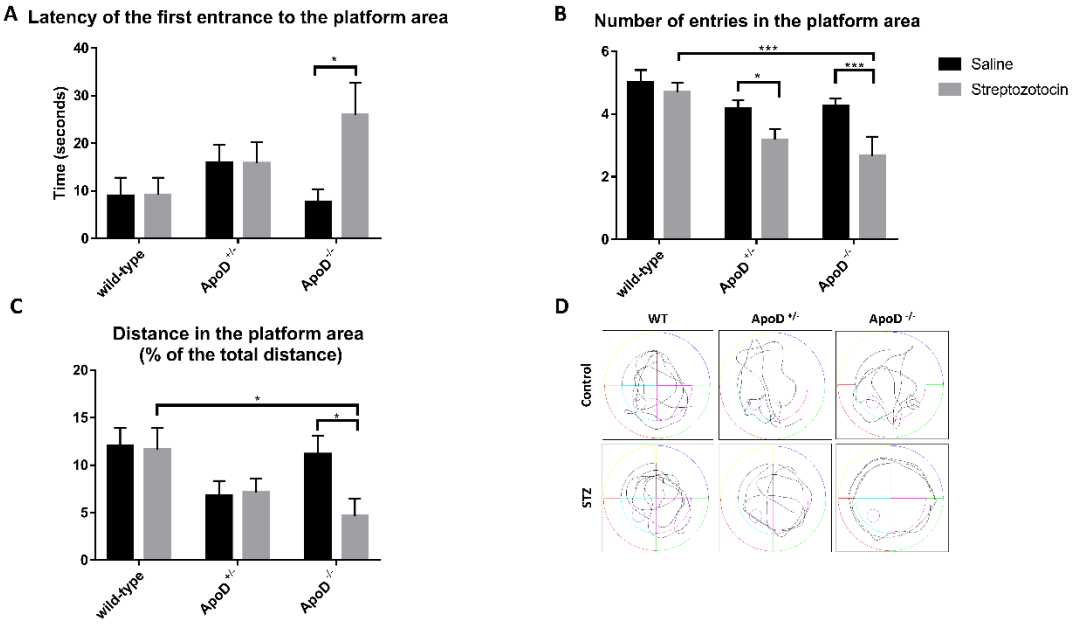

Figure 4: Spatial memory test of Morris Water Maze. The platform was removed, and mice have 45 seconds in the maze. Trajectories and times in each part of the pool are analyzed with a tracking software (Smart – Harvard Apparatus) A – Time to reach the platform area for the first time (time in seconds); B – Percentage of the distance swum in the platform in relation to the total distance travelled. C – The number of entries in the platform zone. area more often than ApoD-/- mice injected with STZ. D – Representative examples of trajectories of 6 mice (1 for each group) during the probe test. n>8 mice to each group, values are mean + S.E.M and data groups were compared with two-way ANOVA followed by Fisher's LSD test. * p<0.05, *** p<0,001.

3.4.2 **The role of ApoD on the expression of ApoE and ApoJ**

We have then analyzed the role of ApoD on the levels of ApoE and ApoJ. Interestingly, in hippocampus, we found that ApoE levels were lower in ApoD+/- and in ApoD-/- than in WT with with icv-saline (Fig. 5A). Moreover, STZ treatment induced a huge decrease of the expression of ApoE in WT and in ApoD+/- but not in ApoD-/- likely because it is already very low with icv-saline (Fig. 5A). To note, the level of ApoE is similar in all icv-STZ mice. However, the expression of ApoE in striatum and of ApoJ in hippocampus are independent of the genotypes and treatment (Fig. 5B, C). Interestingly, in striatum, the level of ApoJ was significantly upregulated (about 4 times) in WT with icv-STZ compared to WT with icv-saline while in ApoD+/- and ApoD-/-, the ApoJ protein was not induced by STZ treatment (Fig. 5D). Besides, in ApoD-/- - mice with icv-saline mice, the level of ApoJ is 3 times higher than WT with icv-saline ($p<0.05$).

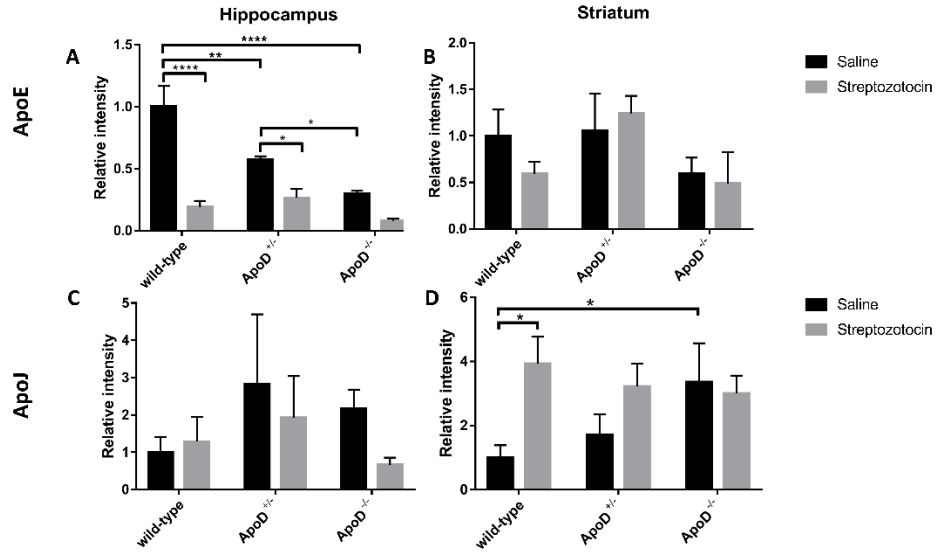

Figure 5: Apolipoprotein E and Apolipoprotein J levels in hippocampus and striatum analyzed by western-blot. The level of protein was normalized using total protein. n=5 mice to each group, values are mean + S.E.M and data groups were compared with two-way ANOVA followed by Fisher's LSD test. * $p<0.05$, ** $p<0.01$, **** $p<0,0001$.

3.4.3 **The role of apoD on the expression of the synaptic protein synpatophysin**

Five weeks after the icv injection, proteins were extracted from hippocampus and striatum. In hippocampus, the presynaptic protein synaptophysin is not different between the genotypes and treatments (Fig. 6A). In the striatum from WT, the synaptophysin level in icv-saline was two-fold higher than in icv-STZ mice (Fig. 6B). Interestingly, its levels were lower in the striatum from ApoD+/- and ApoD-/- as compared to WT mice with icv-saline ($p<0.05$) and was not modified following STZ treatment.

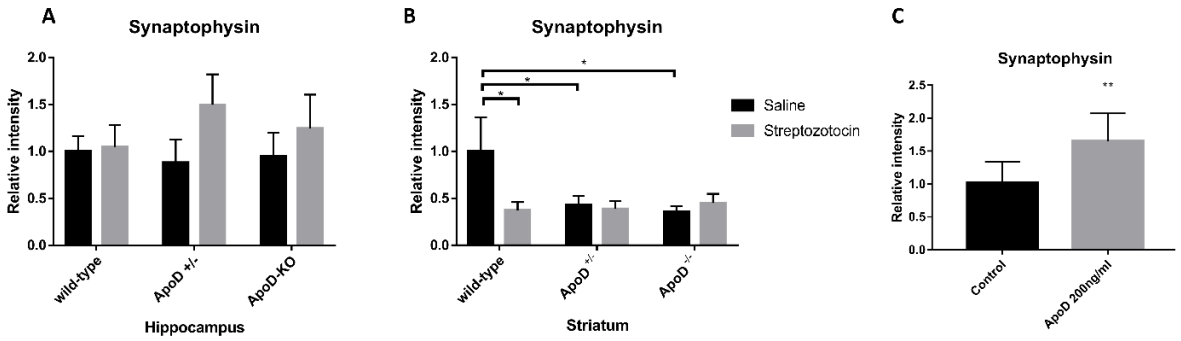

Figure 6: A-B; Synaptophysin levels in hippocampus, striatum and in Neuro2a cells analyzed by Western-blot. The level of each marker was normalized using total protein. n=5 mice to each group, values are mean + S.E.M and data groups were compared with two-way ANOVA followed by Fisher's LSD test. * $p<0.05$. C-Synaptophysin expression in Neuro2A after hApoD treatment, 200 ng/ml during 24 hours, n=3. ** $p<0.01$ compared to control condition.

In order to study the role of ApoD on the expression of the synpatic protein, we have treated the neuroblastoma cells Neuro2a cells with 200ng/ml of hApoD for 24 hours. We found a 50% increase of synaptophysin level in Neuro2a treated with hApoD compared to untreated (Fig. 6C).

3.4.4 Influence of ApoD deficiency and STZ injection on oxidative stress markers

The antioxidant capacity of different brain structures was determined by the ABTS assay. It is lower in hippocampus, striatum and frontal cortex from ApoD+/- and ApoD-/- as compared to WT mice with icv-saline. The STZ treatment induced a reduction of the antioxidant capacity in WT mice but not in ApoD+/- and ApoD-/- except in the temporal cortex (Fig.7A-D).

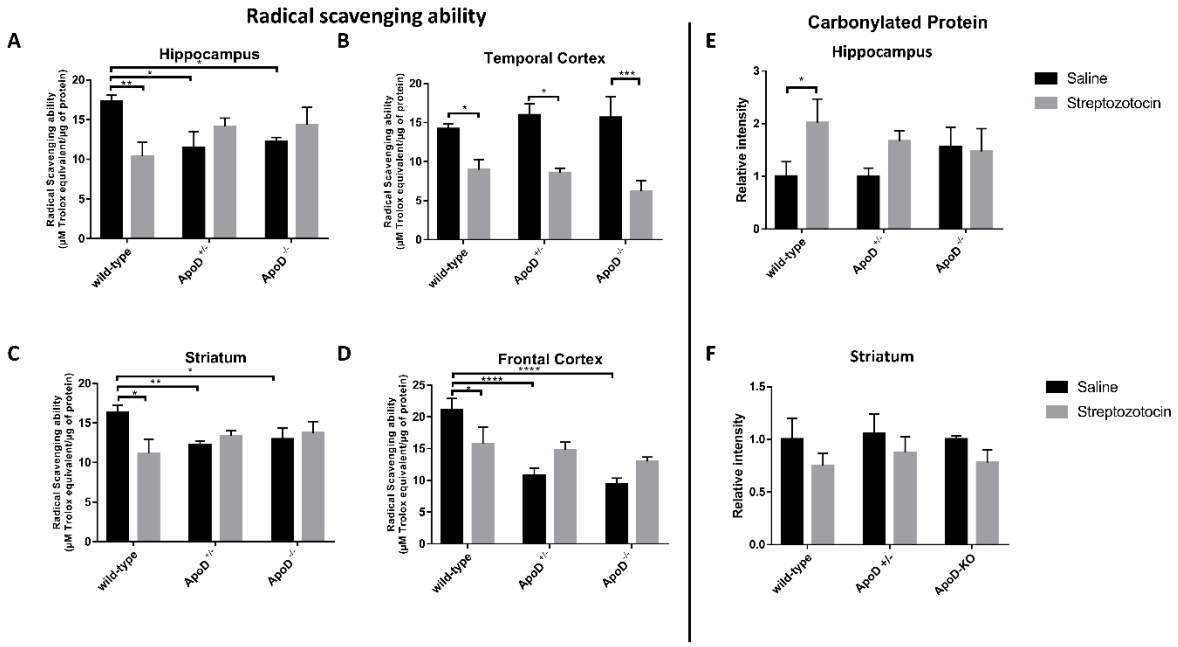

Figure 7: Oxidative stress activity. A-B: Protein carbonylation levels in hippocampus and striatum revealed by western-blot and normalized by total protein. C-F: Radical scavenging activity of protein sample from hippocampus, frontal cortex, striatum, temporal cortex. Radical scavenging activity was determined by ABTS assay. The activity is exprimed in µM Trolox equivalent/µg of protein. n=5 mice to each group, values are mean + S.E.M and data groups were compared with two-way ANOVA followed by Fisher's LSD test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0,0001.

We have then analyzed the levels of protein carbonylation, which is an index oxidative marker, in hippocampus and striatum. In both structures, the levels of protein carbonylation were not different between groups of mice with icv-saline. The STZ treatment induced a twofold increase in hippocampus from WT mice but not in ApoD+/- and ApoD-/- and no effect of STZ was observed in striatum (Fig.7 E, F).

3.5 Discussion

In this study, we have investigated the role of ApoD, with or without icv-STZ, on the working and spatial memory, on the levels of ApoE and ApoJ, both apolipoproteins involved in synaptic remodeling, on the synaptic protein synaptophysin, and on the antioxidant capacities of different structures of the brain. In WT mice, we found that icv-STZ mice spent less time in the new arm of the Y-Maze assay. In ApoD+/- and in ApoD-/- mice, the icv-STZ injection induced a spatial memory impairment but did not have any effect on the learning period assessed by the MWM and on the Y-maze tests. Time to find the platform decreased between the first and the last try for icv-saline mice in the MWM assay, with no effect of the genotype. Our results also demonstrated that the expressions of ApoE and ApoJ in different regions of the brain are dependent on the presence

of ApoD, with a reduction of the levels of ApoE in hippocampus and an induction of ApoJ in striatum in ApoD-/- mice. Also, the icv-STZ injection induced a huge decrease of ApoE levels in hippocampus independently of the presence of ApoD while no effect was observed in striatum. These results demonstrated that ApoE and ApoJ are differently regulated by ApoD and may vary with the structure of the brain. Interestingly, in hippocampus the expression of synaptophysin is not dependent on the presence of ApoD and was not altered following an icv-STZ. In striatum, the level of synaptophysin was reduced in WT mice with an icv-STZ indicating that STZ induced a decrease of synaptic terminals in this structure. The role of ApoD on synaptophysin was confirmed on the neuronal cell culture model as previously described in dorsal root ganglia cells (24). We also showed that the icv-STZ injection induced a decrease of the ability of different structures from WT mice to scavenge free radicals with an increase of the levels of protein carbonyls. Our results also showed that the free radical scavenging property is decreased in the absence of ApoD, except in the temporal cortex, which is in line with the property of ApoD to maintain the redox status in the brain (42, 43).

Icv-STZ is one of the well-known experimental models to mimic sporadic AD (44) and a single icv-STZ (3 mg/kg) injection was found to be the most appropriate for the induction of long term cognitive impairment, induces phosphorylation of tau protein (44), alterations of glial and neuronal cells in different regions of the hippocampus especially in the CA3 area (44, 45). Moreover, the single dose was chosen in this study because the mortality was found to be increased by 25% when STZ was injected twice (3 mg/kg) (46). We have studied the effect of an icv-STZ on the hippocampal-dependent learning and memory in both the MWM and Y-maze tests with one arm closed during training. The Y-maze was used to assess short term memory and a mouse that shows no preference for any of the arms during the testing session is an indication of an impaired spatial memory and functioning of the hippocampus (47). As expected, WT mice entered more frequently into and spent more time in the novel, previously unvisited arm of the Y-maze than the icv-STZ mice. Surprisingly, icv-STZ did not have any effect on ApoD+/- or ApoD-/- mice probably due to the preservation of the levels of ApoJ in hippocampus which could boost memory and reduce brain inflammation (48).

MWM test represents one of the most widely used test of spatial learning for rodents across repeated trials and reference memory determined by preference for the platform area when the platform is absent (49, 50). The MWM has proven to be a robust and reliable test that is strongly correlated with hippocampal synaptic plasticity (49, 51). and is independent of locomotor effects. As previously described, the absence of ApoD influence the learning abilities in aged mice (22

months-old) (22) but icv-STZ accelerates the onset of spatial memory impairments in ApoD-/-, and to a lesser degree in ApoD+/- mice. The spatial memory deficit was assessed with the probe test (5th day) which is known to be dependent on the hippocampus (52, 53). The dose injected in our study (3mg/kg) does not induce spatial memory deficits in WT mice in the MWM assay but we cannot exclude biochemical changes in some structures of the brain (38).

The complex hippocampal-striatum is very important for the spatial navigation in behavioural tests (54). The striatum plays a vital role in learning because it receives input from the cortex and thalamus and sends outputs that ultimately relay information back to the cortex via the thalamus (see review by (55)). Moreover, the striatum is a site where glutamatergic input from many brain regions converges with dense innervation from midbrain dopamine (DA) neurons (55, 56). In striatum, we observed a reduction synaptophysin level in ApoD+/-- and in ApoD-/- with or without icv-STZ probably because dopaminergic neurons of the nigrostriatal circuit are especially vulnerable to axonal degeneration due to their arborized, long, thin and poorly myelinated axons (57). The reduction of the synapses density following icv-STZ was previously observed (38). The absence of ApoD could decrease the transport of cholesterol between glial and neuronal cells, and thus a decrease in synaptic plasticity(58). We also confirm this effect of ApoD on synaptophysin levels on the neuronal cell line following hApoD treatment. These results and others on peripheral neurons (24) confirm that ApoD is important for the maintenance of synaptic terminals. Accordingly, axonal regeneration and remyelination were found to be delayed in ApoD-null mice, whereas it was stimulated in mice overexpressing ApoD (59).

For the first time, we demonstrated the regulation between ApoE, ApoJ and ApoD, three keys apolipoproteins involved in synaptic remodeling (12). We have previously demonstrated that ApoE binds to the ApoD promoter and repress the ApoD activity (26). Accordingly, in ApoE deficient mice, the expression of ApoD was found to be strongly upregulated (27). Here, we demonstrated that in hippocampus, ApoE levels are dependent on the presence of ApoD. Interestingly, in hippocampus, the absence of ApoD and the reduction of ApoE are compensated by the maintenance of the levels of ApoJ. In striatum, the ApoE levels are independent of the presence of ApoD while there is a compensatory elevation of ApoJ to counterbalance the ApoD deficiency. Both apolipoproteins are suspected to play complementary role in the presence of neurodegeneration as observed during the progression of AD and Braak's stages (28).

Dopaminergic neurons in the striatum are especially susceptible to oxidative stress, due to the numerous oxidants produced during enzymatic and non-enzymatic reactions that dopamine undergoes when released from synaptic vesicles into the synaptic cleft or the cytosol, which

include H2O2•(60). Thus, the induction of ApoJ in the striatum may represent a protective mechanism against oxidative stress because they are many sites of response to oxidative stress on the promoter of ApoJ gene (61, 62). For instance, NF-□B, a transcription factor which play a key role in oxidative stress (63), is known to regulate the expression of ApoJ (64). It was found that NF-□B is activated in rat brain after STZ injection (65), and in organ treated with STZ (66). Also, in astrocytes and in human fibroblasts, ApoJ expression is strongly induced after oxidative injury (67, 68). Thus, in this study the induction of ApoJ may be a protective mechanism against oxidative stress. Interestingly, the human ApoD have also NF-□B binding site and stress response elements (APRE) on his promoter region (69).

STZ is known to induce mitochondrial alterations and then causes oxidative stress (70). The icv-STZ is a model of metabolic disorder simulating certain characteristics found in AD such as insulin resistance and oxidative stress (71-73). We found that in hippocampus from WT mice, the induction of oxidative stress, analyzed by protein carbonylation, evolves inversely with the antioxidant capacity while in ApoD-/- and ApoD+/- mice, the antioxidant capacity is decreased but remain stable with icv--STZ. The decrease of scavenging ability in these two structures can participate to the decline of memory and biochemical deregulations. The absence of ApoD does not seem to influence the antioxidant capacities in temporal cortex, in contrast to other brain structures studied. These results confirm that both ApoD+/- and ApoD-/- mice are more vulnerable to oxidative stress because they have reduced antioxidant capacities and particularly in regions of the brain essentially involved in learning and memory.

3.6 Conclusion

ApoD is one of the few genes with a consistent and evolutionarily conserved up-regulation in the aged brain and in some neurodegenerative disorders. However, the effects of ApoD expression in animal model which mimic the sporadic form of AD remain to be studied. As previously reported, icv-STZ did not induce spatial memory deficits in WT mice, but biochemical differences were observed (38). In this study, the efficiency of STZ injection was validated by the biochemical modifications observed in different structures of the brain. These results lead to the hypothesis that this dose of STZ dose induce alterations equivalent to those observed at the pre-symptomatic stage of AD. This study demonstrates the complementarity of these apolipoproteins in the brain and confirms that the sensitivity to oxidative stress is dependent on the presence of ApoD.

Author contributions

V.H., M.P. and C.R. designed the project; V.H, M.P. performed experiments; V.H. analyzed the data; V.H. and C.R. wrote the paper; J.B and C.P. assisted in the implementation of behavioural tests; and E.R. gave the genetic mice model and give advise on the biology of ApoD.

Funding

This work was supported by the Chaire Louise & André Charron on Alzheimer's disease, Fondation Armand Frappier and the Quebec Network for Research on Aging (CR).

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4 DISCUSSION GÉNÉRALE ET CONCLUSION

4.1 Discussion générale

Les travaux réalisés dans le cadre de cette thèse ont permis de mettre en évidence le passage de l'apoD à travers la BHE, du cerveau vers le sang, pour atteindre les tissus métaboliques. Dans un second temps, nous avons mis en évidence l'importance de l'apoD dans la protection de la mémoire spatiale et plus particulièrement son importance vis-à-vis des agressions oxydatives. Nos résultats ouvrent la voie pour de nouvelles perspectives (fondamentale, clinique et curative) d'expériences détaillées plus tard dans la discussion.

4.1.1 L'apoD cérébral traverse la BHE et s'accumule dans les tissus périphériques

4.1.1.1 L'apoD cérébrale est retrouvée dans les tissus périphériques

Physiologiquement, chez l'humain, l'apoD est retrouvée dans plusieurs tissus périphériques de l'organisme, principalement dans les reins et le foie (Rassart *et al.*, 2000; Rassart *et al.*, 2020). Dans le SNC, elle est exprimée par les astrocytes et est aussi retrouvée dans les fluides tels que le plasma et le LCS. À ce jour, aucune étude n'a permis de démontrer un passage entre le sang et le cerveau de notre protéine d'intérêt. Pour l'étudier, nous avons injecté dans un premier temps chez des souris sauvages, de l'apoD marquée à l'iode 125, dans les ventricules cérébraux, dans le but d'observer si celle-ci pouvait se retrouver en périphérie. À la suite de l'injection, l'apoD radioactivement marquée a été principalement retrouvée dans les reins et le foie ainsi que dans les fluides (urine et sang). L'apoD cérébrale peut donc se retrouver en périphérie confirmant l'hypothèse qu'elle peut influencer l'apparition de stéatoses hépatiques dans les tissus périphériques comme cela a été vu chez les souris surexprimant de l'apoDh dans les neurones (Desmarais *et al.*, 2019; Labrie *et al.*, 2015).

4.1.1.2 Implication d'un transport actif pour le passage à travers la BHE

L'apoDh se retrouve de manière importante en périphérie après son injection dans les ventricules cérébraux. Nous émettons l'hypothèse que ce transport est actif, étant donné que l'apoD est retrouvée à une quantité bien plus importante à celle de l'albumine qui est reconnue comme capable de traverser la BHE de manière passive (Jessen *et al.*, 2015; Tumani *et al.*,

2017). Ce transport actif pourrait être dépendant de la BSG, un récepteur identifié récemment qui participe à l'internalisation de l'apoD (Najyb *et al.*, 2015). La BSG étant présente à la surface de la BHE, celle-ci pourrait être impliquée dans son passage du cerveau vers la périphérie (Zuchero *et al.*, 2016). Cependant, dans notre étude, chez une lignée de cellules endothéliales de souris bEnd.3, reconnue pour mimer les caractéristiques des cellules endothéliales de la BHE murine (Rahman *et al.*, 2016; Watanabe *et al.*, 2013; Yang *et al.*, 2017), l'utilisation de la cyclophylline A, un ligand de la BSG, ne diminue pas l'internalisation de l'apoDh dans ces cellules. Cela suggère que chez les bEnd.3, la BSG n'intervient pas exclusivement dans le processus d'internalisation de l'apoD, et que d'autres récepteurs pourraient être impliqués. L'état de glycosylation de la BSG influence l'affinité avec certains de ces ligands et plus la BSG est glycosylée, moins son ligand est capable de se lier. En effet, la déglycosylation de la caveoline-1 entraîne une augmentation de son interaction avec la BSG (Tang *et al.*, 2004). *In vivo*, après l'injection de l'apoDh dans la veine caudale, nous observons une corrélation entre le degré de glycosylation de la BSG et la radio-iodination liée à l'apoDh dans chacun des organes. Plus la BSG est glycosylée, moins l'internalisation est efficace. Cette corrélation inverse confirme, l'hypothèse que l'apoD pourrait moins efficacement se lier à la BSG hautement glycosylée. Cette observation a déjà été faite avec la calvéoline qui possède une efficacité d'interaction diminuée quand la BSG est pleinement glycosylée (Tang *et al.*, 2004). Lors du traitement d'apoDh sur les cellules endothéliales bEnd.3, l'apoDh ne se lie pas efficacement à la BSG, celle-ci étant hautement glycosylée (comparable aux niveaux retrouvés dans le cerveau) et donc ayant une affinité amoindrie avec l'apoDh. L'utilisation de la cyclophylline A, ne montre donc aucune différence, l'internalisation de l'apoD par le mécanisme BSG-dépendant n'étant que peu ou pas utilisée. Cela suggère, que chez la lignée de cellules bEnd.3, d'autres récepteurs pourraient être impliqués. Les récepteurs aux apolipoprotéines comme LRP1 et LRP2 pourraient aussi être associés au transport de l'apoD, ces récepteurs sont présents au niveau de la BHE, et sont déjà impliqués dans le transport d'autres apolipoprotéines comme l'apoE et l'apoJ (Deane *et al.*, 2004; Shibata *et al.*, 2000). Il a déjà été rapporté qu'un inhibiteur des apolipoprotéines annulait l'effet de l'apoD *in vitro* dans une culture de neurones du ganglion de la racine dorsale provenant de rats (Kosacka *et al.,* 2009). Ceci mettrait en évidence une interaction entre ces récepteurs et l'apoD, cette interaction pouvant induire une signalisation cellulaire, ou l'internalisation de l'apoD. Dans la MA, l'expression de LRP est réduite (Deane *et al.*, 2008). Si l'interaction entre l'apoD et cette protéine est confirmée, alors la diminution de LRP dans la MA pourrait induire un passage à travers la BHE moins important et une séquestration de l'apoD dans le cerveau. Ceci pourrait expliquer l'augmentation des

niveaux d'apoD vu uniquement dans le cerveau et non dans le plasma (Perrotte *et al.*, 2019; Terrisse *et al.*, 1998).

4.1.1.3 **La glycosylation de la BSG**

Bien dans le chapitre 2, nous mettons en évidence une corrélation entre l'internalisation de l'apoD et la présence de BSG faiblement glycosylée, nous ne le montrons pas directement. Nous avons remarqué que l'apoD était internalisée dans les organes exprimant la BSG faiblement glycosylée. Pour confirmer que le passage de l'apoD dans les organes périphériques se fait par le biais de la BSG, nous pourrions réaliser une injection d'apoD radio-iodiné dans le sang, chez des souris déficientes en BSG. Cela permettrait d'observer si la distribution dans les organes est différente particulièrement pour le foie et les reins. Dans ces cas-là, l'apoD s'internaliserait dans le foie de manière moins importante, et sa distribution pourrait être rééquilibrée dans les autres organes comme le cerveau.

Nous avons émis l'hypothèse que la BSG hautement glycosylée induisait une interaction plus faible avec l'apoD, se traduisant par une internalisation moins efficace. Pour confirmer cette hypothèse, l'utilisation d'une enzyme de déglycosylation à la surface de la lignée cellulaire bEnd.3 pourrait être utilisée. Une comparaison des niveaux d'internalisation de l'apoD avec les cellules traitées ou non traitées permettra de distinguer si la déglycosylation de la BSG induit une augmentation de l'internalisation de l'apoD. Une autre manière de démontrer ceci, serait de réaliser des mutagenèses dirigées de la BSG au niveau de ses sites de glycosylations. Il a déjà été montré que la mutation de trois sites de *N*-glycosylation (N44Q, N152Q et N186Q) diminuait grandement la glycosylation des formes hautement et faiblement glycosylées (Tang *et al.*, 2004). Des études d'interactions entre l'apoD et la BSG mutée et non mutée pourraient être faites par résonance plasmodique de surface (ou SPR) permettant de définir des coefficients d'affinité entre l'apoD et tous les mutants de la BSG. De plus, ces constructions génétiques pourraient être intégrées par recombinaison homologue à la place du gène de la BSG dans les cellules bEnd.3. Cela pourrait aussi se faire pas le système Crispr-cas9. Des études d'internalisation permettraient d'observer l'importance de ces sites de glycosylations dans l'affinité avec l'apoD dans les cellules.

Ces études nécessaires permettront de mettre en lien la glycosylation de la BSG et l'internalisation de l'apoD et permettraient d'expliquer pourquoi celle-ci ne se fait pas dépendamment de la BSG dans le cerveau et la BHE.

4.1.1.4 Etude des récepteurs impliqués dans l'internalisation de l'apoD

Nous avons vu l'internalisation de l'apoDh dans plusieurs organes périphériques, principalement les reins et le foie lors d'une injection périphérique et cérébrale. Nous avons lié leur internalisation à une plus faible glycosylation de la BSG dans ces organes. De plus, nous avons montré que la quantité d'apoD internalisée dans les cellules endothéliales bEnd.3 n'était pas différente lorsque ces cellules étaient préalablement traitées avec de la cyclophylline A (compétiteur du site de liaison de l'apoD sur la BSG) ou non. Cela suppose que la BSG, n'est pas le récepteur exclusif de l'apoD permettant son internalisation.

Il serait intéressant d'approfondir cette hypothèse en étudiant de potentiels récepteurs qui pourraient être impliqués dans cette internalisation. Par exemple, LRP-1 et LRP-2 sont déjà impliqués dans l'internalisation de l'apoE et l'apoJ et présents en grande quantité dans le cerveau. Il a déjà été vu que l'effet de l'apoD était annihilé lorsqu'un inhibiteur aux récepteurs aux apolipoprotéines était utilisé dans une lignée de cellules nerveuses de la moelle épinière (Kosacka *et al.*, 2009). Pour étudier ces récepteurs, la diminution de leur expression *via* l'utilisation de siRNA dirigés contre les gènes de LRP1 et LRP2, permettrait d'observer si l'inhibition transitoire de l'expression de LRP1 et LRP2 induit une diminution de l'internalisation de l'apoD.

De plus l'apoD peut aussi se retrouver sous sa forme non libre, liée aux HDL. Ces derniers de petite taille sont capables de traverser la BHE (Feingold & Grunfeld, 2000; Koch *et al.*, 2001). L'apoD pour entrer dans le cerveau pourrait emprunter cette voie d'internalisation. L'apoA-I, capable de se lier aux HDL emprunte ce chemin et a été retrouvée dans le LCS. Il a été démontré que son origine provenait des HDL sanguins, celle-ci n'étant pas synthétisée dans le SNC (Demeester *et al.*, 2000; Koch *et al.*, 2001). Une purification de HDL plasmatiques d'origine humaine pourrait être entreprise suivie d'une immunoprécipitation dirigée contre l'apoD pour s'assurer de récupérer seulement les HDL contenant de l'apoD. Ces HDL seraient ensuite déposés sur les cellules bEnd.3, ainsi nous mettrions en évidence la capacité de l'apoD à être internalisée par des cellules endothéliales de la BHE *in vitro* via les HDL. Il faudrait ensuite confirmer cette hypothèse *in vivo*. Pour ce faire, une injection périphérique des HDL précédemment purifiés pourra se faire chez la souris, ensuite le cerveau sera récupéré et un co-immunomarquage dirigé contre l'apoDh et un marqueur des HDL. Si une co-localisation est retrouvée, cela pourra montrer que l'apoDh à travers les HDL peut être internalisée dans le cerveau depuis le sang.

Il a aussi été montré précédemment que l'apoD pouvait être incorporée dans les VEs, notamment dans son transport entre les astrocytes et les neurones (Pascua-Maestro *et al.*, 2018). Elle est présente en grande quantité dans ces vésicules (Cheow *et al.*, 2016; Jiang *et al.*, 2019; Pascua-Maestro *et al.*, 2018). De façon similaire aux expériences suggérées pour les HDL, une isolation des VEs plasmatiques pourrait être réalisée. Après vérification de la présence de l'apoD dans ces VEs, elles seraient déposées sur des cellules endothéliales mimant les caractéristiques de la BHE comme la lignée cellulaire bEnd.3. L'avantage de cette lignée cellulaire est qu'elle est d'origine murine, donc l'apoD d'origine humaine pourra être distinguée aisément de l'apoD endogène par immunodétection. Cette expérience réalisée dans un système d'insert pourrait permettre aussi d'observer sous quelle forme l'apoDh est ensuite sécrétée (libre ou liées aux VEs).

Une approche plus générale pourrait aussi permettre d'identifier des récepteurs ayant une affinité pour l'apoD. Pour se faire, à partir du lysat de protéines cellulaires totales, une co-immunoprécipitation dirigée contre l'apoD pourrait être effectuée. Les protéines récupérées seraient ensuite analysées par spectrométrie de masse pour les identifier et obtenir une liste de protéines candidates capables de se lier à l'apoD. Cette expérience a déjà été effectuée dans l'étude montrant le mécanisme d'internalisation de l'apoD dépendant de la BSG mais effectuée sur des cellules HEK 293T qui est une lignée humaine de rein donc non cérébrale (Najyb *et al.*, 2015). Faire cette même expérience dans une lignée de cellules neuronales pourrait ajouter des informations sur les récepteurs de l'apoD dans le SNC. Par exemple dans cet article cité précédemment (Najyb *et al.*, 2015), parmi les protéines retrouvées en immunoprécipitation, il y a la *leucine-rich repeat neuronal protein 4* qui est en partie exprimée dans cerveau (Uhlen *et al.*, 2015). Cette protéine appartient à la famille des protéines LRR (*leucine rich repeat*) qui fonctionnent comme les récepteurs aux tyrosines kinases et dont la liaison avec l'apoD pourrait induire une cascade de signalisation, comme l'apoE peut agir en fonction de ces allèles (Huang *et al.*, 2017).

4.1.1.5 L'apoD périphérique ne semble pas traverser la BHE pour se retrouver dans le cerveau

Dans le chapitre 2, nous avons étudié le passage de l'apoDh du système périphérique au cerveau. Nos expériences n'ont pas mis en évidence la présence d'apoDh radio-iodiné dans le cerveau des souris. Ceci nous permet de conclure que l'apoDh ne traverse pas efficacement la

BHE dans le sens du sang vers le cerveau. Cependant notre expérience possède quelques limitations.

Tout d'abord, nous injectons l'apoDh dans la veine caudale de la souris. L'apoD se retrouve donc diluée très rapidement dans la circulation sanguine et pourrait être internalisée dans le foie rapidement. Ainsi son passage à travers la BHE serait minime ou inexistant et non détectable dans l'appareil à scintillation. De plus, pour confirmer que l'apoD ne traverse pas la BHE du sang vers le cerveau, un contrôle positif serait nécessaire pour valider notre expérience. Les apolipoprotéines E et J seraient des candidates idéales, cependant, elles n'ont pas montré de résultats concluants pour démontrer qu'elles traversaient la BHE (de Retana *et al.*, 2019; Liu *et al.*, 2012).

Ensuite, dans le cadre de notre expérience, l'apoD injectée est sous forme libre. Dans la littérature, l'apoD a été décrite pour se retrouver sous différentes formes : libre, dimérisée, liée aux HDL, dans les VEs (Blanco-Vaca *et al.*, 1992; Kielkopf *et al.*, 2018; McConathy & Alaupovic, 1973; Pascua-Maestro *et al.*, 2018). Elle peut être à l'intérieur des VEs qui sont capables de traverser la BHE (Alvarez-Erviti *et al.*, 2011). L'apoD dans les VEs a été retrouvée dans le cerveau, notamment dans son transport entre les astrocytes et les neurones (Pascua-Maestro *et al.*, 2018). Il serait possible que les VEs plasmatiques contenant de l'apoD puissent traverser la BHE pour rentrer dans le cerveau. Dans ce cas, le mécanisme d'internalisation n'impliquerait pas la BSG, mais celui des VEs.

En revanche nos études *in vitro*, réalisées avec les cellules endothéliales immortalisées de souris (bEnd.3), ont montré que l'apoDh pouvait être internalisée. De plus, dans un système d'inserts, permettant la création de deux compartiments séparés par une monocouche de cellules endothéliales bEnd.3, l'apoD peut être relarguée dans le compartiment opposé à celui traité initialement. Cependant, cette expérience, est réalisée *in vitro*, dans un modèle simplifié de la BHE, ce modèle ne contient pas les péricytes et les astrocytes, permettant le maintien structural de la BHE (Yang *et al.*, 2017). De plus, ce modèle *in vitro* de BHE n'est pas un modèle polarisé. Ainsi il n'est pas possible de véritablement distinguer dans ce modèle le compartiment sang du compartiment cerveau (Brown *et al.*, 2007; Helms *et al.*, 2016). L'utilisation de péricytes et astrocytes, en plus de notre lignée cellulaire endothéliale dans une chambre micro-fluidique permettrait de distinguer ces deux compartiments dans une organisation 3D de la BHE *in vitro* (Wang *et al.*, 2016). Cette organisation pourrait confirmer nos résultats *in vivo* montrant principalement un passage du cerveau vers le sang. Une alternative à l'utilisation de cellules bEnd.3 serait l'utilisation de la lignée cellulaire hCMEC/D3 qui est une lignée humaine (Weksler

et al., 2013) et qui montre une polarisation de quelques récepteurs comme ceux des glycoprotéines (Tai *et al.*, 2009).

Enfin, dans le cadre de notre étude, nous avons étudié le passage de l'apoD à travers la BHE dans un modèle où la barrière n'est pas altérée. Dans plusieurs maladies, comme la MA, la BHE se retrouve altérée, et sa perméabilité est augmentée permettant le passage d'un plus grand nombre de protéines (Zlokovic, 2008). Dans un modèle de barrière détériorée, il serait intéressant d'observer si le passage de l'apoD est modifié. Plusieurs modèles de souris possédant une BHE altérée existent, notamment les souris 5xFAD qui possèdent 5 mutations sur l'APP (*Amyloid Precursor Protein*) et la PPS1. Il s'agit d'un modèle génétique de la MA, mais dont la BHE à partir de l'âge de 4 mois devient défaillante (Liu *et al.*, 2020). La récupération du cerveau après perfusion périphérique permettrait de déterminer si l'apoDh, lorsque la BHE est détériorée, peut se retrouver dans le cerveau, et donc la traverser. Si la radioactivité n'est pas observée dans le cerveau dans le contexte d'une barrière détériorée, alors l'augmentation d'apoD observée dans ces cas pathologiques, serait expliquée, par la simple induction d'expression d'apoD par les astrocytes dans le cerveau, destinée aux neurones, ceci étant un mécanisme de protection (Pascua-Maestro *et al.*, 2018).

4.1.2 L'absence d'apoD augmente la sensibilité dans un modèle mimant des caractéristiques de la Maladie d'Alzheimer

Pour la première fois nous avons montré que l'apoD d'origine cérébrale représentait la grande majorité d'apoD dans le cerveau, nous nous sommes concentrés sur son rôle dans celui-ci. Plusieurs études ont mis en évidence le rôle de l'apoD dans un mécanisme de protection neuronale (Ganfornina *et al.*, 2008; Najyb *et al.*, 2016). Cependant, ces études n'ont pas étudié l'impact de l'absence d'apoD sur la mémoire spatiale dans des conditions neuropathologiques. Ainsi, nous avons utilisé des souris déficientes en apoD ($apoD^{-/-}$) auxquelles nous avons injecté de la streptozotocine (STZ). Cette toxine est bien connue pour induire une neuroinflammation, du stress oxydatif et des altérations biochimiques (Zhang *et al.*, 2020). Il s'agit d'un modèle non génétique et utilisé dans des phases précliniques de tests de thérapies pharmacologiques pour la MA (Grieb, 2016). Bien que le mécanisme d'action directe de l'icv-STZ n'est pas encore bien établi, ces effets ont déjà été documentés. Notamment, dépendamment de la dose, l'icv-STZ peut induire des troubles de la mémoire spatiale chez les rongeurs, mais aussi un bouleversement de la potentialisation à long-terme (Li *et al.*, 2016a). Chez les souris icv-STZ, une neuroinflammation est retrouvée (Chen *et al.*, 2013). De plus, cette injection peut induire une accumulation de peptide Aβ (Knezovic *et al.*, 2015), une augmentation de la phosphorylation de Tau (Chen *et al.*, 2013),

ainsi que du stress oxydatif dans le cerveau des animaux causé par une augmentation des niveaux de malondialdéhyde et une diminution des niveaux de glutathion (Grunblatt *et al.*, 2004; Tota *et al.*, 2010). La STZ induit également des changements sur le métabolisme du glucose (Deng *et al.*, 2009; Grunblatt *et al.*, 2004). La dose injectée induit des altérations synaptiques mais pas de déficits de la mémoire spatiale chez des souris sauvages (Li *et al.*, 2016a).

L'icv-STZ induisant une neuroinflammation, il aurait été aussi pertinent de l'évaluer chez les souris déficientes en apoD et de les comparer aux souris sauvages. Pour ce faire, l'activation des microglies et les astrocytes auraient pu être étudiés en mesurant respectivement Iba1 et GFAP par microscopie à fluorescence. Ceci pourrait se faire particulièrement dans l'hippocampe où une concentration plus importante de ces types cellulaires impliqués dans l'inflammation pourrait être vue. Cette neuroinflammation pourrait être aussi mise en évidence en mesurant plusieurs cytokines pro-inflammatoires comme IL-1β, IL-6, ou TNF-α. Elle est d'autant plus importante à étudier qu'il s'agit d'un aspect important de la MA qui évolue entre les différents stades de la maladie (Fan *et al.*, 2015; Femminella *et al.*, 2016).

4.1.2.1 L'apoD préserve la mémoire spatiale dans le cadre d'une induction de troubles de mémoire spatiale

Le rôle protecteur de l'apoD dans un contexte de neurodégénération a déjà été montré auparavant chez la souris (Do Carmo *et al.*, 2008; Ganfornina *et al.*, 2008; Najyb *et al.*, 2016). Cependant, son implication dans la mémoire spatiale n'a pas encore été mise en évidence. L'objectif de cette étude était d'observer si l'absence d'apoD intensifie les bouleversements biochimiques et si des déficits de mémoire spatiale sont retrouvés.

Dans notre étude, nous avons testé deux types de mémoire. Lors du test du Y-Maze, c'est la mémoire à court terme qui est analysée, le temps de repos entre les deux essais étant de 5 minutes (Cowan, 2008). L'absence d'apoD n'a pas permis de mettre en évidence des différences dans cette catégorie de mémoire.

En revanche, lors du test de la piscine de Morris, c'est la mémoire à moyen terme qui a été testée, le temps entre le dernier essai d'apprentissage et la phase de test étant de 24 h. De plus, lors de ce test, c'est plus particulièrement la mémoire spatiale qui est visée. En effet, c'est à l'aide d'indices disposés autour de la piscine que la souris va tenter de retrouver la zone où se trouvait la plateforme. Nous avons montré que l'absence d'apoD lors d'icv-STZ, induisait des troubles de la mémoire spatiale, non observés chez des souris sauvages. Par contre, chez les

souris apoD $^{-/-}$ injectées avec la solution contrôle, aucune différence de capacité de la mémoire spatiale n'a été observée. Ainsi l'absence d'apoD chez des souris âgées de 9 mois se fait sentir lors de l'icv-STZ affectant seulement la mémoire à long terme.

4.1.2.2 L'absence d'apoD induit des modifications d'expression des apoE et apoJ

Pour mieux comprendre ces différences au niveau de la mémoire spatiale, nous avons analysé principalement deux structures : l'hippocampe et le striatum. L'hippocampe est la structure cérébrale principale impliquée dans la mémoire. Il permet l'encodage des informations puis les envois dans une autre structure, pour stocker ces informations. Le complexe hippocampe-striatum est très important dans la navigation spatiale, ce que nous avons étudié avec la piscine de Morris (Goodroe *et al.*, 2018). Pour rappel, dans le cerveau, l'apoD est principalement synthétisée par les astrocytes, et son expression est augmentée dans un contexte de stress cellulaire (Provost *et al.*, 1991b; Rassart *et al.*, 2000; Rassart *et al.*, 2020). Les apolipoprotéines J et E sont déjà bien documentées et sont retrouvées augmentées dans le cerveau de patients atteints de la MA (Foster *et al.*, 2019; Kim *et al.*, 2009a). Elles participent entre autres à l'élimination du peptide Aβ, un des deux marqueurs histopathologiques de la MA (DeMattos *et al.*, 2004; Holtzman, 2001; Oda *et al.*, 1995). L'apoE et l'apoJ sont aussi connues pour avoir un rôle dans la neuroprotection, ainsi que dans la plasticité synaptique (Foster *et al.*, 2019; Kim *et al.*, 2014). Dans le cadre de notre étude, nous avons vu que les souris déficientes en apoD avaient des niveaux diminués d'apoE dans l'hippocampe et des niveaux d'apoJ augmentés dans le striatum. Nous avons aussi vu ces observations chez les souris sauvages injectées avec de la STZ. La diminution des niveaux d'apoE pourrait avoir pour conséquence un bouleversement dans l'hippocampe et notamment un défaut dans la synaptogénèse et la plasticité synaptique. D'ailleurs, nous avons observé une diminution des niveaux de synaptophysine dans le striatum en absence d'apoD, ainsi que dans les icv-STZ chez les souris sauvages. De plus, *in vitro*, le traitement d'apoDh sur une lignée cellulaire immortalisée de neurones murins, les N2a, induit une augmentation d'expression de synaptophysine, confirmant des travaux réalisés sur une lignée de neurones de la moelle épinière (Kosacka *et al.*, 2009). Ces déficits induiraient des problèmes de mémoire spatiale tel qu'observés lors de l'injection de STZ chez les souris apoD $^{-/-}$. De manière intéressante, chez des souris apoE $^{-/-}$, une augmentation de l'apoD avait déjà été observée au niveau du cerveau (Jansen *et al.*, 2009; Terrisse *et al.*, 1999). Ceci suggère un mécanisme de compensation quand l'apoE est absente. Dans le cadre de notre

expérience, nous observons une diminution d'apoE chez les souris apoD$^{-/-}$. Des liens d'expression entre l'apoE et l'apoD ont déjà été montrés, en effet l'apoE peut se lier sur la région promotrice de l'apoD, avec plus ou moins d'efficacité en fonction de l'allèle (Levros *et al.*, 2013).

L'expression d'apoJ est augmentée en cas de stress oxydatif (Charnay *et al.*, 2008; Frippiat *et al.*, 2001), son promoteur possédant plusieurs sites de réponses au stress oxydatif (Jin & Howe, 1997; Wong *et al.*, 1993). Ainsi, nos observations pourraient être expliquées par un stress oxydatif plus important chez les souris icv-STZ et déficientes en apoD.

4.1.2.3 **L'absence d'apoD entraîne une augmentation de marqueurs oxydatifs**

Nous avons observé une augmentation de la carbonylation des protéines dans l'hippocampe mais aussi une diminution du piégeage des radicaux libres dans le cortex frontal, l'hippocampe et le striatum. Cette diminution du piégeage des espèces réactives oxygénées traduit une diminution de l'activité antioxydante. On peut souligner que l'absence d'apoD chez la souris influence plusieurs structures cérébrales. Le striatum est responsable en grande partie des activités de locomotion comme en témoignent les maladies de Parkinson et Huntington, où cette structure est particulièrement touchée (Pisa *et al.*, 1980). Les différences vues dans le striatum peuvent expliquer les observations montrant que les souris apoD$^{-/-}$ étaient moins actives dans le test de l'*open-field*, qui est un test qui permet de mesurer l'anxiété, les niveaux d'activité de locomotion générale ainsi que la volonté d'explorer (Ganfornina *et al.*, 2008). Ces activités diminuées chez les apoD$^{-/-}$, pourraient s'expliquer par une accumulation du stress oxydant. Dans notre étude, nous montrons une augmentation de marqueurs du stress oxydant (protéines carbonylées et diminution de la capacité du piégeage des radicaux libres) dans plusieurs structures cérébrales. Notamment cette augmentation est retrouvée dans l'hippocampe, qui est le siège de l'encodage de la mémoire et donc pourrait expliquer les défauts de mémoire spatiale observées dans la piscine de Morris pour les souris apoD$^{-/-}$ injectées avec de la STZ. Dans l'hippocampe, l'augmentation des protéines carbonylées peut aussi être expliquée par la diminution des capacités antioxydantes. D'autres paramètres comme la diminution d'apoE peuvent aussi expliquer ces observations, cette dernière, agit dans de nombreux mécanismes contre le stress oxydant mais aussi de neuroprotection et synaptogénèse contribuant indirectement à lutter contre le stress oxydatif (Kim *et al.*, 2014).

Cette augmentation du stress oxydatif pourrait être la cause du déclin cognitif observé chez les souris apoD$^{-/-}$ injectées avec de la STZ. En effet, il a été vu dans plusieurs modèles de

souris utilisés pour étudier la MA que l'apparition de stress oxydatif était plus précoce que l'apparition de mémoire spatiale (Belkacemi & Ramassamy, 2012). Bien qu'aucune corrélation directe n'ait été montrée, une étude plus profonde permettrait de valider cette hypothèse. Un autre aspect qui pourrait expliquer ces déficits est le manque d'apoD et donc de transporteur de cholestérol permettant la remyélination après une dégénération. Comme mentionné en introduction, il a déjà été montré que chez les souris apoD$^{-/-}$ il y avait un retard de remyélination et régénération axonale après une lésion du nerf sciatique (Ganfornina *et al.*, 2010). On pourrait penser ainsi que ce même phénomène est observé dans le cerveau de ces souris et qu'à la suite de l'icv-STZ, la régénération axonale est moins efficace chez les souris apoD-/- conduisant à une mémoire spatiale en déclin comparé aux souris sauvages.

4.1.2.4 L'absence d'apoD influe sur la sensibilité vis-à-vis de dommages oxydatifs cérébraux

En conclusion de cet article, nous montrons que l'absence d'apoD induit des déficits de mémoire spatiale lors de l'icv-STZ. Dans un cas physiologique, les souris déficientes en apoD ne montrent pas de trouble de mémoire spatiale à 9 mois d'âge. En revanche des différences d'expressions protéiques sont retrouvées notamment pour les apoJ et apoE ainsi que la synaptophysine. Il faut souligner aussi que les souris sans apoD possèdent des capacités antioxydantes moins performantes que les souris sauvages.

Tout ceci tend à montrer que les souris apoD déficientes montrent une sensibilité accrue à une induction de stress oxydatif. Cette étude montre l'importance de l'apoD dans la protection antioxydante et celle de la mémoire spatiale dans des conditions neuropathologiques.

Il aurait été intéressant d'essayer de contrer cette attaque en injectant de l'apoD exogène dans le cerveau quotidiennement et observer si les effets de déficits de mémoire spatiale apparaissent tout de même. Cela permettrait de savoir si le manque d'apoD durant la vie de l'animal conditionne cette sensibilité plus importante à des déficits de mémoire spatiale ou si un apport supplémentaire contrerait ces attaques.

4.1.3 L'apoD et différence dans les sexes

Dans nos études *in vivo,* nous avons utilisé des souris mâles, dans le but d'approfondir les connaissances sur l'apoD. Cependant, il serait intéressant d'observer des différences d'expression d'apoD dans les deux sexes ou de différencier les effets d'une déficience d'apoD chez les souris mâles et femelles. L'apoD possède sur son promoteur plusieurs éléments de

réponses, dont ceux des progestérones et œstrogènes, deux hormones sexuelles. De plus, c'est aussi une protéine de liaison de la progestérone (Pearlman *et al.*, 1973).

Cet état de la littérature suggère que l'apoD pourrait jouer un rôle dans le métabolisme chez les femmes. Ceci pourrait être confirmé chez des souris apoD$^{-/-}$, en analysant les niveaux de progestérones et œstrogènes au cours de la vie de l'animal. De plus, chez des souris sauvages où ces hormones seraient données dans l'alimentation, des taux d'apoD sanguins pourraient être mesurés pour ainsi observer un effet sur sa concentration. Si l'apoD agit différemment entre les sexes alors des études plus approfondies sur l'apoD et la mémoire spatiale pourraient être entreprises pour y voir une éventuelle différence entre les sexes. De plus le passage à travers la BHE et son accumulation dans les différents organes pourraient aussi être étudiés. La distribution pourrait être retrouvée différente.

4.2 Perspectives

4.2.1 Acide Arachidonique et ApoD

L'acide arachidonique est le ligand à ce jour avec la meilleure affinité pour l'apoD. Il s'agit d'un des lipides retrouvés en grande quantité dans le cerveau. Le groupe IVA (GIVA) de phospholipase A2 (PLA2) possède une très forte spécificité de substrat pour l'acide arachidonique (Kudo & Murakami, 2002), et est exprimé chez les neurones (Kishimoto *et al.*, 1999; Sanchez-Mejia *et al.*, 2008; Sandhya *et al.*, 1998) et peut être induit dans les cellules gliales (Clemens *et al.*, 1996). L'activité de PLA2 est particulièrement élevée dans l'hippocampe et coïncide avec les hauts niveaux de GIVA (Sanchez-Mejia *et al.*, 2008). Plusieurs études suggèrent que PLA2 et l'acide arachidonique jouent des rôles importants dans la signalisation synaptique et la potentialisation à long terme, ainsi que dans l'apprentissage et la mémoire. L'inhibition de PLA2 chez des rats, induits des déficits de mémoire spatiale vus dans l'essai *probe* (similaire à celui effectué dans le chapitre 3) de la piscine de Morris (Holscher *et al.*, 1995). Bien qu'il ait déjà été montré que les niveaux d'acide arachidonique étaient similaires chez des souris sauvages et déficientes en apoD (Thomas & Yao, 2007). Un déficit de l'apoD, induirait un transport d'acide arachidonique moins important, et pourrait contribuer à des défauts de mémoire. L'acide arachidonique favorise la potentialisation à long terme dans des neurones hippocampiques de rats (Williams *et al.*, 1989). D'autre part, chez des souris transgéniques exprimant l'APP humaine (APPh), une augmentation de l'acide arachidonique est observée. Les auteurs suggèrent que cette augmentation est due au niveau d'Aβ (Sanchez-Mejia *et al.*, 2008). L'acide arachidonique pourrait être impliqué dans le processus de neuroinflammation dans la

maladie d'Alzheimer (Glass *et al.*, 2010; Wyss-Coray, 2006; Wyss-Coray & Mucke, 2002). Plus particulièrement les sous-produits de l'acide arachidonique (Balboa & Balsinde, 2006). Comme soulevé précédemment, le stress oxydatif augmente dans le cerveau de patients atteints de la maladie d'Alzheimer (Mattsson *et al.*, 2009; Pratico *et al.*, 1998; Querfurth & LaFerla, 2010). L'isoprostane est un de ces produits d'oxydation de l'acide arachidonique qui est relâché par PLA2. De plus les isoprostanes sont retrouvés augmentés dans la maladie d'Alzheimer ainsi que dans les souris APPh (Mattsson *et al.*, 2009; Pratico *et al.*, 2001; Pratico *et al.*, 1998; Querfurth & LaFerla, 2010). Un autre sous-produit bien décrit dans la littérature est le 4-HNE, qui est lui aussi un produit de l'oxydation de l'acide arachidonique, et est retrouvé en quantité élevée dans la maladie d'Alzheimer (Mark *et al.*, 1997). Cette augmentation est due à une plus grande activité de PLA2, qui va libérer l'acide arachidonique des phospholipides. De plus, une diminution des phospholipides est retrouvée aussi chez les patients atteints de la maladie d'Alzheimer, comparé aux patients contrôles (Prasad *et al.*, 1998). L'importance de l'acide arachidonique a ainsi déjà été bien démontrée, cependant l'importance du duo apoD et acide arachidonique n'est pas encore bien étudiée. Une absence d'apoD chez les souris APPh pourrait conduire à une limitation du transport de l'acide arachidonique. De plus en l'absence d'apoD, celle-ci ne permettrait pas de limiter la peroxydation lipidique, tout particulièrement pour l'acide arachidonique et contribuerait à une augmentation du stress oxydatif.

4.2.2 **Etude du rôle de l'apoD dans la neuroprotection**

4.2.2.1 **Rôle de l'apoD dans la neurogenèse**

Pour combattre les maladies neurodégénératives, un des objectifs est de lutter contre la mort neuronale. Pour ce faire, activer la neurogenèse est une des pistes d'étude. Il s'agit d'un processus permettant la formation de neurones fonctionnels à partir de cellules souches neurales. Bien que ce processus soit principalement actif chez l'embryon et l'enfant, il existe aussi une neurogenèse adulte. Il serait intéressant de déterminer si l'apoD pourrait être impliqué dans ce processus de neurogenèse adulte.

Il a été montré que la clozapine, un médicament antipsychotique augmentait la neurogenèse dans l'hippocampe de rat adulte (Halim *et al.*, 2004). De plus, elle induit l'expression d'apoD chez les patients atteints de schizophrénie, mais aussi chez les rongeurs. (Mahadik *et al.*, 2002; Thomas *et al.*, 2001a). Comme dans le chapitre 3, nous pourrions injecter la streptozotocine dans les ventricules latéraux, mais à une dose plus élevée connue pour induire

des troubles de la mémoire spatiale (Li *et al.*, 2016a). Un groupe de souris pourrait recevoir de la clozapine de manière journalière pour observer si son administration permet de lutter contre l'apparition des troubles de la mémoire. De plus, une observation de la neurogenèse associée à l'expression de l'apoD pourrait être observée en histologie. Nous pourrions dénombrer les neurones dans la région de l'hippocampe, en observant NeuN et analyser une co-localisation avec l'apoD (une protéine spécifique des neurones). D'autre part, pour dénombrer les neurones néo-formés, nous pourrions injecter le BrdU (5-bromo-2'-deoxyuridine) en intrapéritonéal. Il s'agit d'un marqueur de la synthèse d'ADN couramment utilisé pour étudier la neurogenèse (Cameron & McKay, 2001). Des coupes d'immunohistochimie permettront ensuite d'analyser ainsi que dénombrer les cellules néoformées à l'aide de marqueurs gliaux et neuronaux (GFAP et NeuN).

Nous pourrons ensuite comparer les souris ayant reçu le traitement de clozapine et celle ne l'ayant pas reçu. Au point de vue biochimique, nous pourrions analyser la capacité antioxydante chez ces souris, mais aussi les effets directs du stress oxydatif, comme la carbonylation des protéines et la peroxydation lipidique. Si ce traitement montre des effets positifs sur la mémoire et les altérations biochimiques, il pourrait être transposé à l'humain. Il a l'avantage d'être déjà commercialisé pour les patients schizophrènes.

4.2.2.2 **Dans un contexte de neurodégénération**

Dans le cas de notre étude (Chapitre 3), nous avons utilisé un modèle de souris déficiente en apoD. Nous aurions pu aussi utiliser un modèle de surexpression d'apoDh dans les neurones : Thy-1-apoDh. Dans ce cas-là, la concentration de STZ injectée aurait été plus importante pour permettre l'apparition de déficits de mémoire spatiale chez les souris sauvages. L'apoD étant principalement exprimée par les astrocytes, puis sécrétée pour être internalisée par les neurones, le modèle de souris Thy-1-apoDh permet d'observer directement le rôle de l'apoD dans les neurones. Les souris Thy1-apoDh ont été précédemment étudiées. Après des injections de paraquat et d'acide kaïnique, ces souris ont montré une protection au stress oxydatif plus importante que les souris sauvages (Ganfornina *et al.*, 2008; Najyb *et al.*, 2016). Des études comportementales comme effectuées dans le chapitre 3 pourraient être entreprises afin d'observer si la surexpression d'apoDh contribue à protéger les capacités de mémoires spatiales des souris. Ensuite des études de synaptogénèse, mais aussi sur les niveaux des apoJ et apoE pourront être réalisées pour comparer les résultats avec notre étude dans le cas d'une déficience d'apoD.

4.2.2.3 **Neuroprotection dans le cas d'une infection virale**

A plus large échelle que les maladies neurodégénératives, l'apoD pourrait jouer aussi un rôle dans d'autres maladies développant des symptômes liés au SNC. Par exemple, l'infection chez l'humain du SARS-CoV-2 induit des pertes d'odorat et de goût causé par des dysfonctions cérébrales chez certains patients (Carignan *et al.*, 2020). Ce virus est responsable de la pandémie mondiale qui a débuté à la fin de l'année 2019. Il est intéressant de noter que l'apoD a montré un effet bénéfique contre un précédent coronavirus, OC-43 (Do Carmo *et al.*, 2008), il s'agit d'un des 7 coronavirus qui infecte les humains selon l'Organisation Mondiale de la Santé (Killerby *et al.*, 2018). Il a été vu précédemment que lors d'une infection avec OC-43 que les animaux surexprimant l'apoDh au sein des neurones résistaient mieux à l'infection. Pour le SARS-CoV-2, l'induction d'apoD pourrait permettre de limiter ces dysfonctions. Pour vérifier cela, comme pour l'expérience avec OC-43 (Do Carmo *et al.*, 2008), une injection cérébrale du SARS-CoV-2 pourrait être réalisée chez des souris déficientes en apoD, mais aussi chez les souris Thy-1-apoDh exclusivement dans les neurones. L'observation d'une résistance neuronale chez les souris Thy-1 confirmerait cette hypothèse. Chez des souris sauvages après une injection du SARS-Cov-2, une administration de clozapine qui induit une augmentation de l'apoD dans le cerveau (Thomas *et al.*, 2001a), pourrait être effectuée en vue d'un possible traitement contre l'anosmie et d'agueusie dans le cas d'une infection du coronavirus chez l'humain.

4.2.2.4 **Lutte contre le stress oxydatif**

Les études chez les souris surexprimant l'apoDh dans les neurones ont montré une protection accrue vis-à-vis de toxines comme le paraquat et l'acide kaïnique (Ganfornina *et al.*, 2008; Najyb *et al.*, 2016). Ceci a été confirmé par notre étude décrite dans le chapitre 3, montrant une sensibilité plus importante à la STZ, un inducteur de stress oxydatif entrainant des dommages de la mémoire spatiale ainsi qu'une augmentation d'un déséquilibre entre les capacités pro-oxydantes et antioxydantes chez les souris apoD$^{-/-}$. Cette capacité pourrait être due à la présence de la Met93 qui contribue à limiter la formation des sous-produits de la peroxydation lipidique tels que le 4-HNE ou l'acroléine. Plus précisément, l'apoD réduit les lipides hyperoxydés grâce à sa Met93, cette dernière se retrouve modifiée en méthionine sulfoxyde (MetSO) (Bhatia *et al.*, 2012), ainsi les lipides sous cette forme ne sont pas susceptibles de former des sous-produits toxiques. Pour observer le rôle de cette Met93 *in vivo,* une modification de la Met93 par un autre acide aminé pourrait être effectuée chez des souris, afin d'observer si chez des souris âgées une différence d'accumulation de marqueurs oxydatifs est vue.

4.2.2.5 **Inaction de l'apoD dans le contexte de la MA**

Nous avons vu dans le chapitre 3 que la déficience d'apoD amènerait à une fragilité plus importante des capacités cognitives. Dans la MA, l'apoD est retrouvée augmentée dans le cerveau de patients, cette augmentation ne contribue pas à protéger de manière efficace ces patients contre des troubles de la mémoire, premiers symptômes apparaissant dans la MA. Cette augmentation ne serait pas suffisante, l'une des hypothèses nous amène à admettre que l'apoD est inactive. Comme vu précédemment l'apoD participe à diminuer la peroxydation lipidique *via* sa Met93. Les enzymes MetSO réductases réduisent les méthionines et leur permettent de revenir à leur état non oxydé et donc à nouveau de limiter la peroxydation lipidique. Cependant, l'activité de ces enzymes est réduite dans la MA (Gabbita *et al.*, 1999), ainsi la réduction de la MetSO en position 93 de l'apoD est moins efficace dû à l'activité diminuée. En parallèle, chez les patients atteints de la MA, une augmentation des dimères de l'apoD est observée dans l'hippocampe (Bhatia *et al.*, 2013). Celle-ci est retrouvée associée avec les niveaux des lipides conjugués (Bhatia *et al.*, 2013). Dans la MA, l'apoD serait devenue inactive dû à son activité de lutte contre la peroxydation lipidique, favorisant l'apparition de dimère d'apoD, et participant à l'accélération de la progression de la maladie (Oakley *et al.*, 2012). Ainsi l'augmentation de l'apoD ne serait pas efficace, car celle-ci pourrait être sous une forme inactive pour lutter contre la peroxydation lipidique.

Pour étudier un lien entre la MA et cette modification de la Met93, une analyse de spectrométrie de masse ciblée sur l'apoD pourrait être effectuée dans des échantillons humains, de plasma ainsi que du LCS enrichies en apoD par immunoprécipitation. Une étude comparative entre les différents groupes (sains, et les différents stades de la MA) serait réalisée. La spectrométrie de masse permettrait d'identifier de manière semi-quantitative les échantillons ayant une plus grande proportion d'apoD avec la Met93 oxydée. Ceci permettrait de confirmer que dans la MA cet acide aminé est impliqué dans la lutte contre la peroxydation lipidique et que son activité peut être inhibée par la présence de cette oxydation.

4.2.2.6 **Etude de l'activité synaptique et de l'apoD**

Dans le chapitre 3 nous avons montré que le traitement d'apoDh induisait *in vitro* une augmentation des niveaux de synaptophysine. Chez les souris apoD$^{-/-}$, nous avons vu une diminution de la synaptophysine, cependant, nous n'avons pas montré une activité de synaptogénèse plus grande due à l'apoD. De plus, il a déjà été montré que chez des souris apoD^{-}

$^{-/-}$, le recyclage de la myéline était limité, et ne permettait pas sa régénération, inhibant une bonne direction axonale et limitant la synaptogénèse. La myéline est la couche isolante autour des axones permettant l'accélération de la vitesse de conduction du potentiel d'action. Un défaut de celle-ci pourrait induirait un potentiel d'action fragile. Il pourrait être intéressant d'observer ce phénomène chez les souris apoD$^{-/-}$ mais également chez les souris Thy1-apoDh. Pour cela, un dénombrement des synapses sera effectué, en observant les co-localisations de protéines pré et post-synaptiques comme respectivement la synaptophysine et la PSD-95. De plus pour mesurer l'activité synaptique des expériences de potentialisation à long terme pourraient être effectuées. Cette expérience permettrait d'observer la réponse des synapses à un stimulus, mais aussi sa capacité à retrouver un niveau basal. Une observation de différence dans ces cas pourrait permettre d'étudier une différence de la plasticité synaptique chez ces souris, et d'associer l'apoD à ce phénomène.

4.2.3 **ApoD et maladies neurodégénératives**

4.2.3.1 **Comprendre l'impact des variants de l'apoD sur les maladies neurodégénératives**

L'apoDh possède plusieurs polymorphismes retrouvés dans plusieurs groupes ethniques espacés géographiquement. Certains ont été retrouvés pour être des facteurs de risques de la MA, notamment en Finlande, en Amérique du Nord et en Chine (Chen *et al.*, 2008; Desai *et al.*, 2003; Helisalmi *et al.*, 2004). Cependant, aucune étude n'est pas allée plus loin pour comprendre pourquoi ces polymorphismes induisaient un facteur de risque augmenté. Tout comme des études précédentes sur les allèles de l'apoE, il serait intéressant de remplacer chez la souris le gène de l'apoD endogène par ces différents variants. Ainsi une comparaison pourra être réalisée au niveau de la synaptogénèse. Les souris possédant des variants de l'apoD pourraient aussi être croisées avec des modèles génétiques de souris bien décrits de la MA. Une étude comme celle-ci pourrait permettre de mettre en avant l'importance de l'apoD, et d'observer si certaines mutations du gène induisent une sensibilité plus importante aux agressions biochimiques causant des déficits cérébraux. Elle validerait aussi les observations réalisées sur différents groupes ethniques mettant en évidence que la présence de certains variants de l'apoD comme facteur de risque. Nous avons montré dans notre étude que son absence induisait une sensibilité accrue aux dommages, des mutations pourraient induire ce même type d'effet.

4.2.3.2 **ApoD et Parkinson**

Contrairement à la MA, l'apoD est retrouvée augmentée dans le sang de patients atteints de la maladie de Parkinson (Waldner *et al.*, 2018). Il s'agit d'une maladie chronique neurologique qui est liée à une diminution de la production de dopamine dans le *substantia nigra.* Elle est caractérisée par des tremblements des muscles au repos, une rigidité, une lenteur des mouvements ainsi qu'une altération de l'équilibre. Une étude réalisée chez des patients sains et atteints de la maladie de Parkinson montre que les neurones dopaminergiques, responsables de la production de dopamine, ne contiennent pas d'apoD (Ordonez *et al.*, 2006). Cependant, chez les cellules gliales autour de ces neurones, l'apoD est bien présente, son signal augmente dans les cas de Parkinson (Ordonez *et al.*, 2006).

Bien que l'apoD soit produite par les cellules gliales, celle-ci ne pourrait pas être internalisée dans les neurones. Il serait intéressant de comprendre en quoi il y a un dysfonctionnement d'internalisation dans les neurones de cette pathologie. Pour cela, une étude pour permettre de récupérer des cellules fibroblastiques de patients atteints de la maladie de Parkinson, mais aussi de schizophrénie et de la MA pourrait être mise en place. Ces fibroblastes seraient dédifférenciés pour être redifférenciés en neurones avec la technologie des cellules souches pluripotentes induites. Ces neurones nouvellement formés pourraient être traités avec de l'apoD, son internalisation pourrait être différente dans chacun des neurones reliés à une maladie neurodégénérative. L'internalisation de l'apoD serait étudiée par immunofluorescence dirigée contre l'apoD. D'autre part ; et dans une approche plus globale, il serait intéressant de récupérer les protéines cellulaires totales suite au traitement de l'apoD dans chacune de nos conditions. Ces protéines totales pourraient ensuite être analysées par spectrométrie de masse, pour une analyse protéomique. L'objectif de cette expérience serait d'observer différents mécanismes d'activation ou d'inhibition que l'apoD pourrait effectuer dans chacune des pathologies ainsi que dans les cellules issues d'un individu sain. Une liste de protéines candidates serait obtenue et permettrait après analyse bioinformatique d'observer des effets de l'apoD dans ces pathologies. De plus, cette étude permettrait d'observer l'expression de la BSG et des autres récepteurs susceptibles d'intervenir dans l'internalisation de l'apoD. D'autre part, cette étude permettrait de savoir si l'apoD possède un effet commun dans ces 3 maladies neurodégénératives ou si elle agit différemment.

4.2.3.3 **ApoD et dépistage clinique plasmatique dans l'Alzheimer**

L'apoD montre une augmentation dans le sang de patients atteints dans la maladie de Parkinson, et pourrait être un candidat intéressant dans le diagnostic de cette maladie (Waldner *et al.*, 2018). Dans la MA, sa présence augmente au niveau cérébral, mais dans le plasma l'apoD libre, bien que retrouvée en grande quantité, ne montre pas d'évolution entre les stades de la maladie ainsi qu'en comparant avec des patients sains (Perrotte *et al.*, 2019; Terrisse *et al.*, 1998). Cependant récemment au laboratoire, l'étude des VEs périphériques provenant de patients atteints de la MA a montré une augmentation de l'apoD dans le contexte pathologique (Ben Khedher *et al.*, 2021). Ceci montre que dépendant de l'état de l'apoD (libre, liée aux HDLs ou dans les VEs), sa concentration semble différente. L'approfondissement de ces données permettrait ainsi d'établir des tests cliniques non invasifs simples pour permettre d'établir un diagnostic rapide et efficace de la MA. D'autre part, comme nous l'avons vu dans le chapitre 2, l'apoD cérébrale est capable d'être internalisée dans les reins, pour se retrouver intacte dans les urines. Ainsi dans des cas pathologiques comme dans la MA, l'augmentation de l'apoD pourrait aussi être observé dans les urines, pour ainsi être éliminer comme dans le cas du chapitre 2 où l'apoD a été injecté pour être retrouvée en excès. D'ailleurs, des études sur l'urine de patients atteints de la MA, en ciblant des sous-produits métaboliques ont montré des résultats encourageants montrant que l'étude de l'urine de patients peut être prometteuse (Kurbatova *et al.*, 2020). Ce mécanisme d'élimination par les urines pourrait s'effectuer aussi pour l'apoD dans la MA, bien que l'augmentation d'apoD ne soit pas vue dans le plasma sous sa forme libre, ce constat pourrait être différent dans les urines. L'apoD dans le sang demeurerait de manière très transitoire dans sa forme libre et serait distribuée très rapidement dans les organes. Une récente étude a montré que l'apoD dans une cohorte de patients atteints de la MA, augmentait dans leurs urines (Watanabe *et al.*, 2020). Bien que dans une seconde cohorte les auteurs n'ont pu confirmer cette augmentation, il serait intéressant d'approfondir cette méthode de dépistage clinique. De plus la forme dimérisée a déjà été retrouvée dans les urines, cette forme qui est retrouvée augmentée chez les patients atteints de la MA. (Bhatia *et al.*, 2013; Blanco-Vaca & Pownall, 1993).

4.3 Conclusion générale

Dans cette thèse nous avons montré pour la première fois que l'apoD retrouvée dans le cerveau avait principalement comme origine celui-ci. De plus nous avons montré que l'apoD cérébrale était capable de traverser la BHE pour se retrouver dans les tissus périphériques ainsi

que les fluides. Ensuite, nous avons par le biais de nos résultats émis l'hypothèse de l'existence d'autres récepteurs pour l'apoD, notamment dans le cerveau et la BHE. Notre second volet d'étude a montré la sensibilité importante due au manque d'apoD chez la souris. Cette sensibilité s'est traduite par une apparition de déficits de mémoire spatiale chez des souris apoD$^{-/-}$ après l'icv-STZ. Enfin, elle a montré une diminution des capacités anti-oxydantes et une augmentation du stress oxydatif. C'est la première fois qu'on peut considérer que le manque d'apoD peut être un facteur de risque de l'apparition de déficits de mémoire spatiale. Notre travail original a permis d'élargir et confirmer les connaissances sur cette protéine d'intérêt.

L'expression de l'apoD est modifiée dans plusieurs neuropathologies (Alzheimer, Parkinson, Schizophrénie, etc.). L'augmentation de son expression serait un mécanisme de réaction à l'augmentation de stress oxydatif, mais aussi aux dommages neuronaux. Une meilleure compréhension de cette augmentation semble essentielle pour comprendre la fonction de celle-ci. De plus, il faudrait approfondir les raisons pour lesquelles l'augmentation de l'apoD dans ces maladies ne contribuent pas efficacement à protéger les patients. Ceci permettra de mieux identifier le rôle de cette protéine, mais dans à une plus grande échelle permettre d'approfondir les connaissances sur ces maladies. Comme approfondi en perspectives, l'apoD est une protéine d'intérêt pour l'étude de ces maladies neurodégénératives. Elle peut être étudiée sur différents volets : fondamental, clinique et curatif.

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6 ANNEXE 1 : GLUTATHIONE : AN OLD AND SMALL MOLECULE WITH GREAT FUNCTIONS AND NEW APPLICATIONS IN THE BRAIN AND IN ALZHEIMER'S DISEASE

ANTIOXIDANTS & REDOX SIGNALING
Volume 35, Number 4, 2021
© Mary Ann Liebert, Inc.
DOI: 10.1089/ars.2020.8129

FORUM REVIEW ARTICLE

Glutathione: An Old and Small Molecule with Great Functions and New Applications in the Brain and in Alzheimer's Disease

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Abstract

Significance: Glutathione (GSH) represents the most abundant and the main antioxidant in the body with important functions in the brain related to Alzheimer's disease (AD).
Recent Advances: Oxidative stress is one of the central mechanisms in AD. We and others have demonstrated the alteration of GSH levels in the AD brain, its important role in the detoxification of advanced glycation end-products and of acrolein, a by-product of lipid peroxidation. Recent *in vivo* studies found a decrease of GSH in several areas of the brain from control, mild cognitive impairment, and AD subjects, which are correlated with cognitive decline.
Critical Issues: Several strategies were developed to restore its intracellular level with the L-cysteine prodrugs or the oral administration of γ-glutamylcysteine to prevent alterations observed in AD. To date, no benefit on GSH level or on oxidative biomarkers has been reported in clinical trials. Thus, it remains uncertain if GSH could be considered a potential preventive or therapeutic approach or a biomarker for AD.
Future Directions: We address how GSH-coupled nanocarriers represent a promising approach for the functionalization of nanocarriers to overcome the blood/brain barrier (BBB) for the brain delivery of GSH while avoiding cellular toxicity. It is also important to address the presence of GSH in exosomes for its potential intercellular transfer or its shuttle across the BBB under certain conditions. *Antioxid. Redox Signal.* 35, 270–292.

Keywords: advanced glycation end-products, ferroptosis, glutaredoxin, thioredoxin, nanoparticles

Introduction

CURRENTLY, 50 MILLION people suffer from dementia worldwide, and about two-thirds of them are affected by Alzheimer's disease (AD) (11). AD is clinically characterized by cognitive impairment and memory loss disrupting daily life. Generally, these clinical signs appear a few decades after the initiation of cellular damages in the brain. The sporadic form of AD accounts for over 95% of cases, while the familial form represents only 5%. Both forms share the same neuropathological hallmarks with the presence of the amyloid-beta (Aβ) plaques and the neurofibrillary tangles (NFTs), which spread through the brain as the disease progresses. Synaptic and neuronal loss, astrogliosis, neuroinflammation, and oxidative stress are also observed in several areas of the brain (208).

Multiple lines of evidence suggest that the Aβ cascade, with the overproduction and structural modifications of the Aβ peptide to form harmful oligomers, is central in AD. Indeed, the presence of metal ions (iron, zinc, and copper) and reactive oxygen species (ROS) can accelerate the oligomerization and aggregation of the Aβ peptide (35), which causes the formation of oxidative markers with deleterious effects in the vicinity of the Aβ plaques (139). The mechanisms underlying the decrease in Aβ clearance remain to be clarified, but could involve the failure of microglial cells and

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macrophages to adequately degrade the extracellular Aβ deposits and the apolipoprotein E (ApoE). The APOE4 isoform does not alter Aβ synthesis, but can dramatically increase Aβ deposition in AD animal models (87). Unlike Aβ, there is little overlap between ApoE-immunoreactive neurons and NFT-positive neurons (81). Collectively, ApoE4 is considered a major causative or contributing factor for AD by acting as a chaperone for Aβ, which affects the clearance and deposition of Aβ, ultimately contributing to Aβ plaque formation, promoting tau-induced neurodegeneration and brain atrophy (186, 187).

NFTs are constituted by highly phosphorylated tau proteins that are present in axons and the best-established functions of tau are to stabilize microtubules. In AD, no tau mutations have been identified but its hyperphosphorylation negatively regulates its binding to microtubules leading to paired helical filament (PHF-tau) formation and the disruption of the axonal transport. Interestingly, NFT density, but not Aβ plaques, is correlated with cognitive decline in AD (76). Tau hyperphosphorylation requires the activation of different kinases mainly glycogen synthase kinase-3β (GSK-3β), which was found to be activated by oxidative stress [see recent review by Rana and Singh (172)].

Oxidative stress is considered the main contributor to aging and represents one of the major risk factors for AD (29). It shares common mechanisms between the divergent pathogenic pathways of AD and its progression. The implication of oxidative stress in the AD physiopathology emerged from abundant cellular and animal model data and strengthened by abundant studies in patients (23, 163, 170, 171, 190). Cells regulate oxidative stress by the action of housekeeping nonenzymatic and enzymatic antioxidants. Among them, glutathione (GSH) represents the most abundant and the main antioxidant with multiple functions. In this review, we summarize how GSH homeostasis is tightly linked to other redox mechanisms, its important roles in the brain, its multiple functions notably in the detoxification of proteins and lipid peroxidation, and how it can be involved in the pathophysiology and therapeutics of AD in innovative tools as a ligand of nanocarriers.

GSH Synthesis and Homeostasis

GSH is a small tripeptide consisting of the amino acids L-glutamate (Glu), cysteine (Cys), and glycine (Gly). The GSH system comprises NADPH, GSH reductase (GRX), and GSH (Fig. 1). GSH is synthesized by the sequential actions of the glutamate-cysteine ligase (GCL) (also known as γ-glutamylcysteine [γ-GC] synthetase) and γ-l-glutamyl-l-cysteine: glycine ligase (also known as GSH synthetase), both cytosolic enzymes (74) (Fig. 1). GSH is mainly synthesized in the cytoplasm where the bulk of the GSH is retained but its presence is also required in different organelles such as in the nucleus, mitochondria, endoplasmic reticulum (ER), vacuoles, and the extracellular medium [see review by Lushchak (124)]. Its nuclear accumulation plays crucial roles in the cell cycle, in proliferation, in oxidative signaling, in the control of the redox state of critical protein sulfhydryls, which are necessary for DNA repair and expression (14), on the activity of redox transcription factors, or on the expression of some genes (67).

As its cytosolic pool, the mitochondrial matrix and intermembrane space (IMS) pools of GSH are mostly in a reduced state. The IMS pool of GSH, which is $\sim$10–14 mM, is in equilibrium with the cytosolic GSH due to the presence of porins that allow free passage of reduced GSH (GSH) and oxidized GSH (GSSG) across the outer mitochondrial membrane (31). In contrast to the mitochondrial, cytosolic, and nucleus, the ER GSH/GSSG ratio pool is lower than in the cytoplasm and ranges from 3:1 to 1:1 (181). This is quite low compared with the cell overall ratio, which is greater than 30:1 and usually 100:1. Extracellular GSH/GSSG concentrations, ranging from 0.14 to 2.80 μM, are also usually 100 to 1000 times less than the intracellular ones being between 1 and 11 mM (181, 194), while the extracellular concentration of its cysteine precursor is much higher, with values ranging from 40 to 50 μM (140).

Cellular GSH homeostasis is regulated by its biosynthesis, oxidation, reduction, protein glutathionylation, adduction with toxic compounds, and export from the cells. The GSH biosynthetic capacity of various cells and tissues throughout

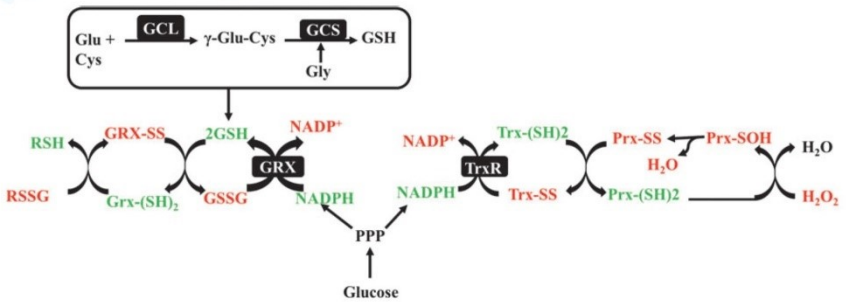

FIG. 1. GSH synthesis and its interactions with different redox systems. GSH is synthesized from Glu, Cys, and Gly by the sequential actions of the GCL (also known as γ-glutamylcysteine synthetase) and γ-l-glutamyl-l-cysteine:glycine ligase (also known as GSH synthetase), both cytosolic enzymes. The GSH system comprises the couple GSH/GSSG, NADPH/NADP$^+$, and GRX. GSH homeostasis is dependent on the availability of NADPH derived from the PPP or its reduction by GRX or by the TrxR/Prx couples. γGluCys, γ-glutamylcysteine; Cys, cysteine; GCL, glutamate-cysteine ligase; Glu, glutamate; Gly, glycine; GRX, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; PPP, pentose phosphate pathway; Prx, peroxiredoxin; PrxSOH, peroxiredoxin sulfenate; RSSG, glutathionylated protein; TrxR, Trx reductase. Color images are available online.

the body is under the control of multiple factors, including the intracellular substrate availability (L-cysteine), the activity of GCL, the rate-limiting enzyme in GSH synthesis, and the feedback inhibition of GSH on γ-glutathione synthetase (γ-GCS). GCL is composed of a catalytic (GCLC) and a modifier (GCLM) subunit, which are regulated at multiple levels. The posttranslational modifications of the GCLC subunit by phosphorylation or by the lipid peroxidation product 4-hydroxynonenal can also affect the activity of the enzyme (15). In addition, the γ-GCS activity can also be modulated by phosphorylation and nitrosation (74). Once synthesized, GSH can undergo transport across biological membranes, notably across the plasma membrane for intercellular distribution. Some studies have suggested the presence of GSH transporters (62) and the first one identified was the multidrug resistance-associated proteins, a subclass of the ATP-binding cassette transporter superfamily. However, these efflux pumps have a broad substrate specificity with a low affinity for GSH than for GSH conjugates (17).

At basal levels of oxidative and nitrosative stress, GRX activity and NADPH availability are sufficient to maintain the ratio [GSH]/[GSSG] > 100 (6, 157). However, under stress conditions or in the presence of other factors, which limit the GRX reaction (*e.g.*, glucose-6-phosphate dehydrogenase deficiency may limit NADPH synthesis), GSSG may accumulate, leading to the cellular shift of the thiol redox status that activates oxidant response transcriptional elements (185). Also, GSSG may be preferentially secreted from the cells (5) and degraded extracellularly because GSSG is not taken up by cells.

The impairment of the ratio GSH/GSSG and consequently of the cell redox balance may affect the activity of some redox-sensitive transcription factors such as the nuclear factor erythroid 2-related factor (Nrf2), the major regulator of the antioxidant response element (ARE) pathway, defined as the Nrf2-ARE system (125) (Fig. 2). Genes coding for several key enzymes linked to GSH metabolism, including the catalytic and regulatory subunits of GCL, the GSH synthase, the X_c^- system, GRX, and glutathione peroxidase (GPX) familiesm are transcriptionally regulated by Nrf2 (104, 203) (Figs. 2 and 3). Hence, Nrf2 represents a key transcriptional regulator of GSH metabolism and homeostasis.

The nuclear factor-κB (NF-κB) is another redox-sensitive transcription factor involved in GSH synthesis (141). Compared with Nrf2, NF-κB is a rapid response factor. The 5′-flanking region of the human γ-GCSH gene contains putative NF-κB, activator protein-1 (AP-1), AP-2, antioxidant, electrophile response elements (ARE/EpRE), and Sp-1 binding sites within 1500 bp of the transcription start site (149) (Fig. 3).

The activating transcription factor 4 (ATF4) also plays a role in GSH metabolism (82). ATF4 is a member of the ATF/cAMP response element-binding protein (CREB) group of the bZIP transcription factor family. Its binds to a variety of

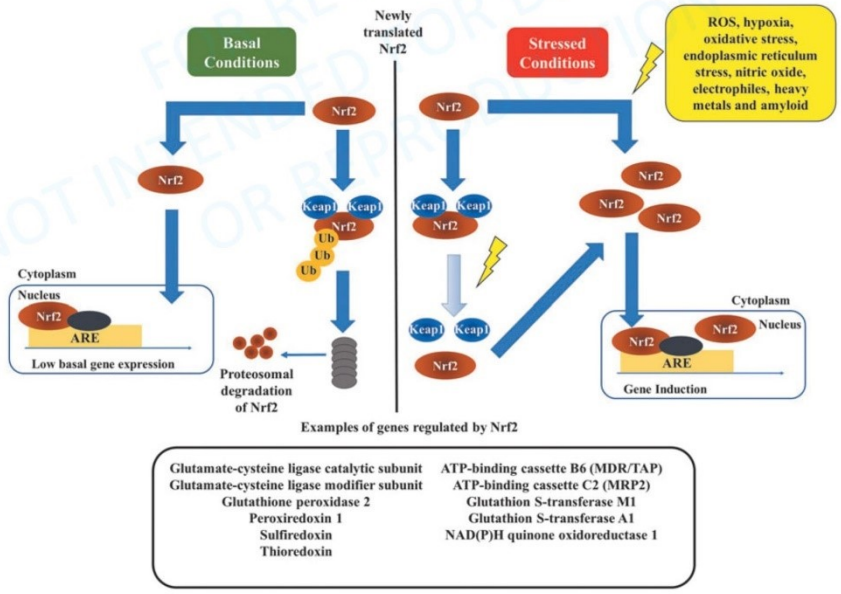

FIG. 2. Regulation of Nrf2 by Keap1 under basal and stressed conditions. Under basal condition, the Nrf2 protein undergoes a rapid ubiquitylation and degradation in the cytoplasm with a minor fraction of Nrf2 translocated to the nucleus to maintain a low basal level of ARE-driven gene expression. Under stressed condition, Keap1 is unable to bind Nrf2, which translocated into the nucleus and bound to the AREs to activate target genes. ARE, antioxidant response element; Nrf2, nuclear factor erythroid 2-related factor. Color images are available online.

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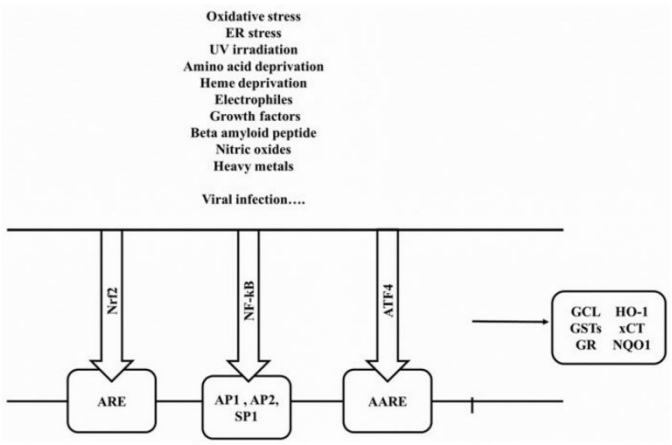

FIG. 3. Nrf2, NF-κB, and ATF4 regulate genes involved in GSH synthesis. Various stimuli induce the transciptional activation of Nrf2, NF-κB, and ATF4. Nrf2 binds to AREs, NF-κB to AP1, AP2, and SP1, and ATF4 to the AARE. The activation of these transcription factors leads to the transcription of diverse genes involved in GSH synthesis and homeostasis. AARE, amino acid-response element; ATF4, activating transcription factor 4; GRX, glutathione reductase; GSTs, glutathione-S-transferases; HO-1, heme oxygenase-1; NF-κB, nuclear factor-κB; NQO1, NAD(P)H: quinone acceptor oxidoreductases; xCT, cystine/glutamate antiporter (SLC7A11).

genes involved in amino acid import, GSH biosynthesis, and resistance to oxidative stress (82). ATF4 also plays a key role in regulating the basal GSH levels in neuronal and non-neuronal cells, induces the expression of the specific subunit xCT of the glutamate/cystine antiporter system X_c^- in response to cystine starvation (179). Basal xCT expression is determined by ATF4, whereas Nrf2 represents an inducer of xCT (178, 188) (Fig. 3). Thus, the intracellular GSH/GSSG concentrations are much higher than the extracellular ones being mainly under the reduced GSH form, and the cellular GSH synthesis and homeostasis depend on multiple factors and the stress conditions.

Cellular Functions of GSH

As one of the most important small-molecule antioxidants, GSH plays a central role in maintaining cellular redox homeostasis (181). Indeed, GSH and GSH-associated metabolism represent the major line of defense for the protection of cells from oxidative and other forms of toxic stress. GSH can scavenge free radicals, reduce peroxides, form adduct with electrophilic compounds (for instance, acrolein), thereby eliminating both ROS and their toxic by-products.

Many cellular redox couples work together to maintain the redox environment. However, the intracellular GSSG/2GSH couple is the most abundant and represents an important indicator of the redox environment, while the extracellular redox state is mainly maintained by cysteine/cystine (Cys/CySS) (97). Changes in the redox potential of various thiols, disulfides, GSSG/2GSH could be considered a series of nanoswitches, a cellular switchboard correlated with the biological status of the cells. Thus, by changing the reduction potential of redox couples, a series of nanoswitches are activated that could move the cell from proliferation to various stages of differentiation into apoptosis or necrosis. Necrosis represents the complete loss of the ability to activate and/or respond to changes in these nanoswitches.

GSSG/2GSG together with NAD^+/NADH and $NADP^+$/NADPH (219) play a key role in the regulation of the intracellular redox environment with a more reducing environment associated with cell proliferation, and a more oxidizing environment associated with differentiation. GSH plays also a role in signal transduction, in apoptosis, and in the expression of some genes such as those encoding for heat shock proteins, two components of AP-1 (Fos and FosB), positive regulators of NF-κB (TRAF6, FADD, EDG2, and tumor necrosis factor-a [TNF-α]), and of death receptor pathways (14, 42, 67, 96, 213). Thus, as the most abundant redox buffer in cells, GSH plays an important role in controlling the oxidoreductive nanoswitches, the status of the protein thiol/disulfide equilibrium, and thereby drives the cell biological status.

Glutathion, Thioredoxin, and Glutaredoxin Pathway Cross Talk

The three redox systems $NADP^+$/NADPH, GSSG/2GSH, and TrxSS/Trx(SH)2, the major cellular thiol-dependent antioxidant, are not functionally isolated systems (Fig. 1). The Trx and GSH systems with NADPH are thermodynamically connected although the Trx levels are 100- to 1000-fold less than GSH (181). The thioredoxin (Trx) pathway composed of Trx, Trx reductase (TrxR), and the GSH/GRX system is two cellular disulfide reductase systems dependent on the NADPH/$NADP^+$ ratio (Fig. 1). Trx is a ubiquitous small (12 kDa) selenoenzyme with a -CGPC- motif within its active site, which usually develops intramolecular disulfides in contrast to GSH that forms intermolecular disulfides. There are three forms of mammalian TrxRs: cytosolic TrxR1, mitochondrial TrxR2, and testis-specific TrxR3. The reduction of the disulfide back to the dithiol form is catalyzed by TrxR and by NADPH (86). In addition to the GSH/GSSG and Trx systems, mammal cells also express the Trx glutathione reductase, also known as TXNRD3/TrxR3 a fusion protein of TrxR and Grx domains, able to reduce Trx, GSSG, and GSH-mixed disulfide (121). Trx and GSH systems have some overlapping functions allowing maintenance of redox regulation, but in many cases, cross talk occurs between both systems where components of one system serve as a backup for the other (59). For instance, in stressed neurons, GSH can

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serve as a backup molecule for TrxR to keep Trx in a reduced state (28). In contrast, Trx2 has also been demonstrated to reduce GSSG, which further stresses the importance of cross talk between both systems (148).

The GSH-glutaredoxin (Glrx) system is another disulfide reduction system in cells. Glrxs, a part of the Trx protein family, can catalyze the reduction of substrates via a monothiol mechanism. In mammalian cells, there are two major forms of Glrx: Glrx1, present in the cytosol, and Glrx2, located in the mitochondria and nuclei (123). Glrx1 accepts electrons from GSH, while Glrx2 can obtain electrons from both GSH and TrxR2 (95).

Although peroxiredoxins, GSH-PX, and catalase are more efficient to degrade H_2 O_2 than GSH (122), their activities and concentrations in the brain are lower than GSH (43, 50, 197). In brain cells under oxidative conditions, GSH, Trx, and Glrxs are important redox regulatory pathways and the dysregulation of their crossroad could be important events in AD pathogenesis (8, 13). Thus, these disulfide reductase systems represent critical antioxidant systems actively involved in the maintenance of the redox balance, an activity essential for the critical biological events in various areas of the brain.

Regulation and Functions of GSH in the Brain

Important roles of the antiporter glutamate/cysteine X_c^- on the GSH synthesis and homeostasis

The brain, one of the most metabolically active organs, is highly vulnerable to oxidative stress (37). Thus, the optimal redox homeostasis is crucial to limit oxidative-induced damages leading to some neurodegenerative disorders. In the brain, GSH is the most abundant thiol-containing molecule and one of the most important antioxidants with concentrations being around 2–3 m*M* (200). However, its distribution was found to be variable with the highest level in the cortex, followed by the cerebellum, hippocampus, and striatum, and is lowest in the substantia nigra (98). Its low levels in the susbtantia nigra dopaminergic neurons probably contribute to the oxidation of dopamine and early nigral degeneration in Parkinson's disease (56).

In the brain, GSH is mainly synthesized locally because, due to its rapid metabolization in the blood (109), its plasma levels are much lower (2–20 μM) than those in the brain. Also, due to its hydrophilic property, its permeability through the blood/brain barrier (BBB) by passive diffusion is very limited (41) and it is as yet unclear whether a direct GSH transport system exists at the BBB.

Astrocytes, as secretory cells of the brain (212), have a high capacity of production, storage, and release of GSH into the extracellular medium in response to oxidative stress, via the multidrug resistance transporter protein (57, 85). In the extracellular space, GSH could be degraded by the membrane-associated enzyme γ-glutamyl transpeptidase into γ-glutamyl moiety and the dipeptide cysteine glycine (CysGly) could be hydrolyzed to glycine and cysteine. This latter is oxidized to form cystine, its dimeric form. CysGly and cystine are, respectively, taken up by the peptide transporter PepT2 and the X_c^- system in astrocytes for the GSH resynthesis (57, 58, 188, 210) (Fig. 4). For this, cystine is reduced to cysteine by GSH or TRX-reductase 1 (TRX-R1) (130). The X_c^- system is an amino acid antiporter that mediates the import of L-cystine and the export of L-glutamate at a ratio of 1:1 across the cellular plasma membrane (Fig. 4). Although the X_c^-; system can also transport both amino acids in both directions (11), since the intracellular pool of cystine is negligibly small because it is rapidly reduced and the intracellular glutamate concentration is generally higher than the extracellular space, the X_c^- system generally imports cystine while exporting glutamate.

Thus, L-glutamat can affect L-cystine uptake and its availability for GSH synthesis (Fig. 4). The X_c^- expression is observed in specific regions of the adult brain, notably in subpopulations of astrocytes, although with different intensities (180), and is highly involved in the cysteine maintenance and thus on the intracellular GSH synthesis (18, 19, 156).

Neurons do not express cystine transport and depend on cysteine transport for the synthesis and maintenance of the intracellular GSH levels. For these cells, cysteine is taken up by the excitatory amino acid transporter 3 (EAAT3/EAAC1) (Fig. 4) and their survival, activities, and the maintenance of their intracellular GSH levels are greatly dependent on this transporter. Thus, neuronal cells in culture can only survive when they are cocultured with the X_c^- system expressing cells to provide cysteine to neuronal recipient cells (84, 92). However, many cell types, particularly neurons, express xCT, the light chain subunit of the X_c^- system, encoded by the *SLC7A11* gene under the regulation of the redox-sensitive transcription factor Nrf2 (178). Nrf2 and the ATF4 are activated under amino acid depletion, ER stress, or oxidative stress and induce cystine transport (178, 217). The X_c^- system was found to be implicated in memory and behavior. For example, mice deficient for the xCT subunit have lower extracellular hippocampal glutamate, impaired spatial memory, which was more pronounced in younger mice, and susceptibility to limbic seizures (49). Recently, X_c^- was shown to contribute to the neuroprotective and the improvement of the cognitive impairments of the N-methyl-D-aspartate (NMDA) receptor antagonist memantine in AD (155).

It is interesting to note that the Nrf2-target genes in neurons are quite different from those in astrocytes. For instance, in neuronal cells, the Nrf2 activator tert-butylhydroquinone (tBHQ) induced the upregulation of genes related to mitochondrial activation, calcium binding and transport, cell adhesion, integrin signaling synaptic activity (106), the catalytic and regulatory subunits of GCL, Srxn1 (sulfiredoxin), and xCT, which are dynamically regulated by the synaptic activity (159). In astrocytes, most of Nrf2-dependent upregulated genes are detoxification enzymes, including glutathione-S-transferases (GSTs), NAD(P)H:quinone acceptor oxidoreductases (NQO1), ferritin, GSH synthetase, and those involved in glucose metabolism-related genes. In addition, neuronal Nrf2 is less responsive to tBHQ than astrocytes probably because neuronal cells express 30- to 1000-fold less Nrf2 protein (24, 93, 188) and contain greater Cul3-dependent Nrf2 degradation capacity than astrocytes (4, 93). Thus, the Nrf2 induction is considered the major mechanism by which astrocytic cells protect nearby neuronal cells.

Thus, to survive, neurons receive strong antioxidant support from surrounding glial cells, particularly astrocytes (24, 53, 57). Although the GSH synthesis in neuronal cells is highly dependent on the GSH precursors provided by astrocytes, neuronal cells are also able to control GSH homeostasis by reducing GSH through different pathways such as GRX, Trx, and peroxiredoxins (Figs. 1 and 4).

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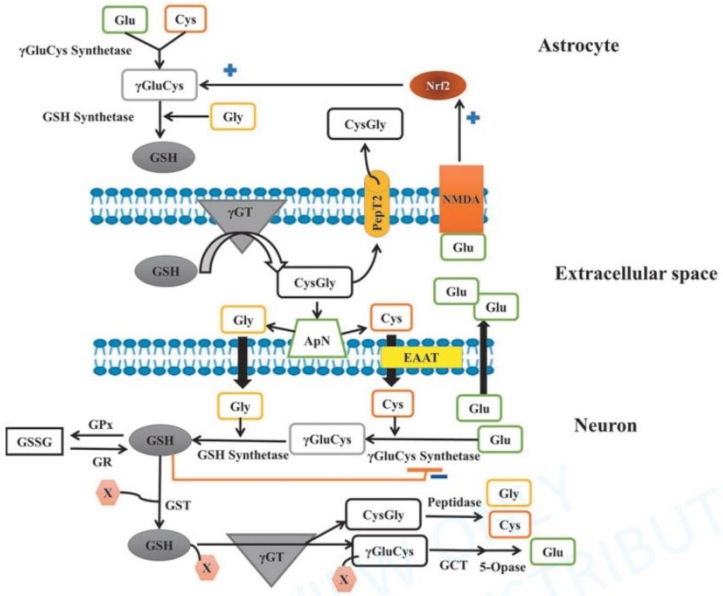

FIG. 4. Neuronal-astrocyte communication to maintain GSH homeostasis. In the extracellular compartment, GSH could be degraded by the membrane-associated enzymes γGT into γ-glutamyl moiety and CysGly. CysGly could be hydrolyzed to cysteine and glycine. Cysteine could be oxidized to form cystine, its dimeric form. CysGly is taken up by the peptide transporter PepT2 in astrocytes for the GSH resynthesis and cystine by the X_c^- system, which is then reduced to cysteine by GSH or TRX-R1 needed for GSH synthesis. In neurons, cysteine is taken up by the EAAT3/EAAC1 for the synthesis of GSH. It can form adducts with electrophiles (X). These conjugations are transformed by γGT, peptidase, γ-glutamylcylotransferase, and 5-opase to glutamate, cysteine, and glycine. ApN, aminopeptidase; CysGly, dipeptide cysteine glycine; EAAT, excitatory amino acid transporter; EAAT3/EAAC1, excitatory amino acid transporter 3; GCT, γ-glutamylcylotransferase; γGT, γ-glutamyl transpeptidase; TRX-R1, TRX-reductase 1. Color images are available online.

Cross talk between neurons and astrocytes contributes to maintain GSH homeostasis in the brain

The interaction between astrocytes and neurons is important for neuronal GSH homeostasis.

For instance, the chronic activation of astrocytic NMDA receptors, a Ca^{2+} subtype of ionotropic glutamate receptor, can activate Nrf2 (93). Indeed, the application of NMDA directly to astrocytes triggers Cdk5-mediated Nrf2 phosphorylation and induction of Nrf2-dependent gene expression with the capacity to confer neuroprotection on nearby neurons (93). In contrast, NMDA receptor blockade *in vivo* causes a reduction in brain GCL expression, activity, and thus GSH levels (21). These results strongly suggest that the neuronal glutamate release could control astrocytic Nrf2 (93). Accordingly, stimuli that promote the synaptic activity or neuronal depolarization can increase the astrocytic nuclear accumulation of Nrf2 (78). Increased synaptic activity causes an immediate elevation of GSH utilization (83) and the capacity of neurons to synthesize and recycle GSH (21). In contrast, the blockade of the neuronal activity is associated with neurodegeneration, which can be ameliorated by supplying the brain with a cell-permeable form of γ-GC, the product of GCL catalysis (21).

In microglial cells, GSH plays also an important role. In both human microglia and astrocytes, the depletion of GSH by the treatment with L-buthionine sulfoximide induced a dose-dependent release of proinflammatory cytokines such as interleukin-6 (IL-6) and TNF-α, as well as nitrite, a marker for iNOS induction (110). This loss of GSH correlated with the activation of the proinflammatory p38 MAP kinase, c-jun N-terminal kinase (JNK), and NF-κB pathways (90).

Neuronal and astrocytes have clearly distinct homeostatic mechanisms related to GSH. However, it is not clear to what extent neuronal activity influences the transcriptome of the surrounding glial cells. A better understanding of reciprocal interactions between neuronal/glial cells is required to gain a better picture of brain redox homeostasis. The cooperative nature of the interaction is depicted in Figure 4.

Role of GSH in the S-Glutathionylation of Proteins

Under oxidative or nitrosative stress conditions, GSH can protect cells against toxic proteins by forming a mixed disulfide between reactive thiols and GSH, a process called S-glutathionylation. Similar to S-nitrosylation, protein S-glutathionylation is a well-controlled reversible reaction

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that alters protein functions in physiological conditions in the brain. S-glutathionylated proteins could be deglutationylated by the Glrx enzymes. Although several proteins, including Trxs (73), protein disulfide isomerase (161), and sulfiredoxin (64), have deglutathionylase activity under different conditions, Glrxs are considered to be the major deglutathionylase enzymes due to their high affinity and selectivity for glutathionylated proteins (95).

Protein S-glutathionylation induced an important signal in neuronal cell death via the effects of abnormal protein polymerization and protein degradation. For example, actin was reported to be constitutively glutathionylated in the brain (196) and the Glrx1-catalyzed deglutathionylation of actin is crucial for its polymerization, a key event required to maintain the cellular dynamics, and to protect neurons from the accumulation of disarranged actin filaments (160).

In AD, there is a significant increase in the level of S-glutathionylated in the inferior parietal lobule tissues compared with the level observed in age-matched controls (153). For instance, S-glutathionylated tau protein rapidly undergoes polymerization to form filaments (54). Redox proteomics in the AD brain has identified specific S-glutathionylated proteins, including those involved in glucose and energy metabolism (153), deoxyhemoglobin, α-crystallin B, glyceraldehyde phosphate dehydrogenase, and α-enolase (45, 46). However, S-glutathionylated can also protect the integrity of the sulfhydryl groups of some proteins such as p53 from an irreversible modification that occurs in the progression of AD, leading to its permanent inactivation (52, 211). Selective glutathionylation of p53 in the AD brain could prevent the formation of aggregates involved in oxidative stress conditions and neurodegeneration (52). For instance, in the transgenic mice model of AD (APP/PS1), the S-glutathionyl levels in the blood and brain increased with aging compared with wild-type mice with proteins at 8.4–20.3 and 13.2–37.2 kDa being most susceptible to S-glutathionylation (222). Thus, the accumulation of abnormal protein S-glutathionylation could potentially be considered early biomarkers to predict AD.

The GSH Depletion Is a Key Player for Oxytosis and Ferroptosis

GSH depletion can induce different forms of cell death in the brain (Fig. 5). GSH depletion is a common feature of apoptotic cell death triggered by a wide variety of stimuli, including activation of death receptors or stress inducers (66), and excessive GSH depletion can switch apoptotic to necrotic cell death (206). The combination of the depletion of GSH or its inhibition, the accumulation of ROS production, lipid peroxidation, lipooxygenase activation, and calcium influx can induce neuronal cell death through the pathway named oxytosis (20). On the contrary, the accumulation of lipid peroxidation by-products triggered by the inhibition of GSH biosynthesis and the GSH-dependent antioxidant enzyme GSH peroxidase 4 (GPX4), the exacerbation of iron causes

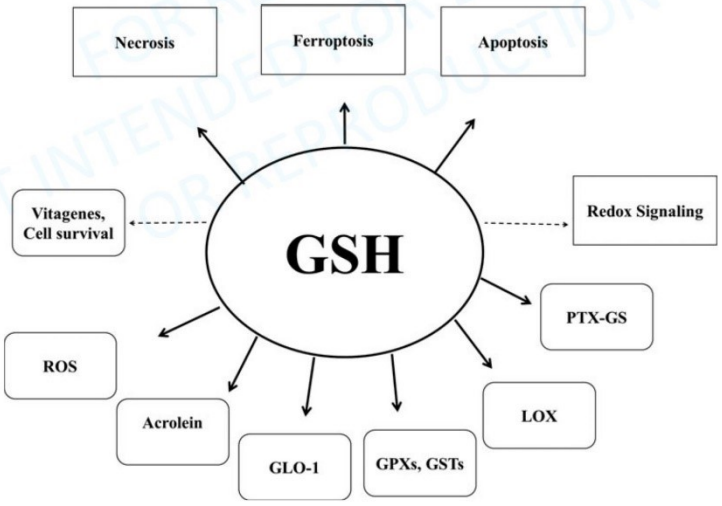

FIG. 5. Summary of different cellular functions of GSH. GSH is the most important ROS scavenger, and can form adduct with nucleophilic compounds (acrolein), a direct or indirect regulator of various enzymes such as GLO-1, GPXs, and GST. GSH reduces the intracellular level of lipid peroxidation (LOX) and protects against toxic PTX-GS. Depletion of GSH could induce apoptosis, necrosis, or ferroptosis in the presence of a decrease of the GPX4 activity. GSG together with NAD^+/NADH and $NADP^+$/NADPH regulates the intracellular redox environment, which controls the activity of redox-sensitive transcription factors or redox signaling pathways. GSH could promote adaptative changes and activate prosurvival pathways, which are under the control of protective genes called vitagenes. GLO-1, glyoxalase-1; GPXs, glutathione peroxidase; LOX, lipooxygenase; PTX-GS, S-glutathionylated proteins; ROS, reactive oxygen species.

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cell death by ferroptosis. Oxytosis and ferroptosis are distinct from apoptosis, classic necrosis, autophagy, and other forms of cell death. Both oxytosis and ferroptosis represent very similar (or even the same) forms of regulated cell death (112) characterized by ROS generation and GPX4 inactivation (162). Thus, the central endogenous suppressor of ferroptosis is the selenoenzyme GPX4, which requires GSH as substrate (1). Both oxytosis and ferroptosis are also prevented by radical scavengers such as ferrostatin-1, liproxstatin-1, and endogenous vitamin E (34, 39, 55, 91, 111, 126, 128, 198) (Fig. 6).

Another key initiating step for ferroptosis is the inhibition of the cellular uptake of cystine. As the X_c^- system is among the cystine transport systems into cells, its inhibition can induce cell death by ferroptosis (150). Thus, ferroptosis appears similar in several aspects to glutamate toxicity, a phenotype observed in certain neuronal cell lines treated with high concentrations of glutamate to inactivate the system X_c^- and deprive cells of cystine/cysteine.

The concentration of iron is another factor that control ferroptosis. In the brain, higher concentrations of iron are preferentially found in the nucleus accumbens, substantia nigra, deep cerebellar nuclei, and parts of the hippocampus (77, 192). Lipid peroxidation, a hallmark feature of ferroptosis, is considered an early event in the pathology of AD (173, 190). The conditional ablation of GPX4 in the forebrain (cerebral cortex and hippocampus) of mice (Gpx4BIKO) resulted in AD-like cognitive impairment (spatial learning and memory) accompanied by hippocampal neurodegeneration, elevated lipid peroxidation (enhanced 4-hydroxynonenal adducts observed in the cerebral cortex), and neuroinflammation (80). These phenotypes were further exacerbated in mice fed with a diet deficient in the lipid-soluble antioxidant tocopherol (80). These observations are in line with the depletion of GSH in the frontal cortex and hippocampus, which correlates with the decline in cognitive functions (129, 131). Taken together, these data indicate that ferroptosis is one of the consequences of the GSH decline in AD (80, 126, 129, 136, 198).

Roles of GSH in AD

As described above, brain GSH levels decrease with age and a loss of GSH can impact on the cognitive function. A decrease in GSH is also associated with microglial activation and endothelial dysfunction, both of which can contribute to the impairments of brain function. Here, we describe that several changes in brain function in AD are directly or indirectly linked to GSH metabolism.

There are very limited data available in the determination of GSH in *post mortem* tissues from the AD brain. Altogether, these studies indicate that GSH levels in frontal, occipital lobes, or hippocampus were broadly not altered in AD (16, 101, 164, 199). One of the limitations of these studies was the absence of the determination of the ApoE genotype because we and others have demonstrated that the levels of GSH and other oxidative markers were lower in the hippocampus only in $\varepsilon 4$ allele of ApoE carriers (145, 171). This reduction appears to be specific to the hippocampus because it is not reduced in the frontal cortex (170). Using *in vivo* proton resonance spectroscopy to measure GSH levels in the hippocampi and frontal cortices from control, mild cognitive impairment (MCI), and AD subjects, an AD-dependent decrease in GSH was found in both regions that correlated with the decline in cognitive functions (129, 131). Moreover, levels of hippocampal GSH could discriminate between healthy controls and MCI subjects, while cortical GSH levels could discriminate between MCI and AD patients. Very recently, the quantification of the *in vivo* brain GSH levels in the cingulate cortex (CC) showed a significant GSH depletion in AD and MCI control patients (189). Moreover, receiver operator characteristic analysis of GSH level in the CC differentiated between MCI and normal control groups with an accuracy of 82.8% and 73.5% between the AD and normal control groups (189). However, we cannot exclude the possibility that the decrease of GSH could promote adaptative changes of neurons to better tolerate subsequent stress to maintain cognitive health, as a "neurohormseis" concept (137, 176), because cellular stress response activates pro-survival pathways, which are under the control of protective genes called vitagenes (GSH, heat shock proteins, sirtuins, Trxs…) (32, 48),

In cells, tissues, and plasma, the reactivity of GSH leads to several forms such as glutathionylation, the formation of adducts with electrophilic compounds such as by-products of lipid peroxidation (*i.e.*, acrolein). Thus, the determination of its levels in tissues does not consider its different forms and redox states. Therefore, the analysis of the global thiol–disulfide redox status in tissues and cells is a challenging task because of the following: (i) thiols easily undergo auto-oxidation during the preanalytical phase of sample preparation; (ii) disulfides can be reduced back to thiols enzymatically during cell manipulation; and (iii) basal levels of disulfides (GSSG and oxidized peroxiredoxin [PSSG]) are very low compared with GSH. Also, a large part of ROS/reactive nitrogen species-mediated signal transduction relies on modifications of cysteine thiol (-SH) within proteins. Irreversible thiol modification (sulfonic acid, $-SO_3H$) has been considered a hallmark for various pathological conditions and usually leads to permanent functional loss and degradation of the protein. Reversible thiol modifications, such as S-nitrosylation (-SNO), S-sulfenylation (sulfenic acid, -SOH), S-glutathionylation (-SSG), disulfide formation (-S-S-), S-sulfhydration (-S-SH), and sulfinic acid ($-SO_2H$), are involved in redox signaling (Fig. 7).

GSH disulfide, formed upon oxidation, is referred to as "oxidized GSH" (GSSG), but further oxidation products are also formed from GSH, for example, sulfonates. Other forms of disulfide are of the mixed type, a major class of biologically interesting ones being GSH-cysteinyl disulfides on proteins. Thus, proteins can be glutathionylated or, as sometimes referred to, thiolated. These reactions demonstrate that P-SH contributes to the cellular antioxidant network, thereby influencing its redox environment.

Another challenge is the compartmentation of GSH and GSSG, which may be at a disequilibrium steady state to each other. Measurement of the total content of GSH and GSSG in cells would represent an overall redox of the cytosol, not the redox environment of the various compartments such as the ER, nucleus, or mitochondria. However, the mtGSH pool plays an important role in AD because its depletion specifically can account for the increased susceptibility to the Aβ peptide. First, mtGSH levels determine the sensitivity of brain mitochondria to Aβ-mediated oxidative stress and the

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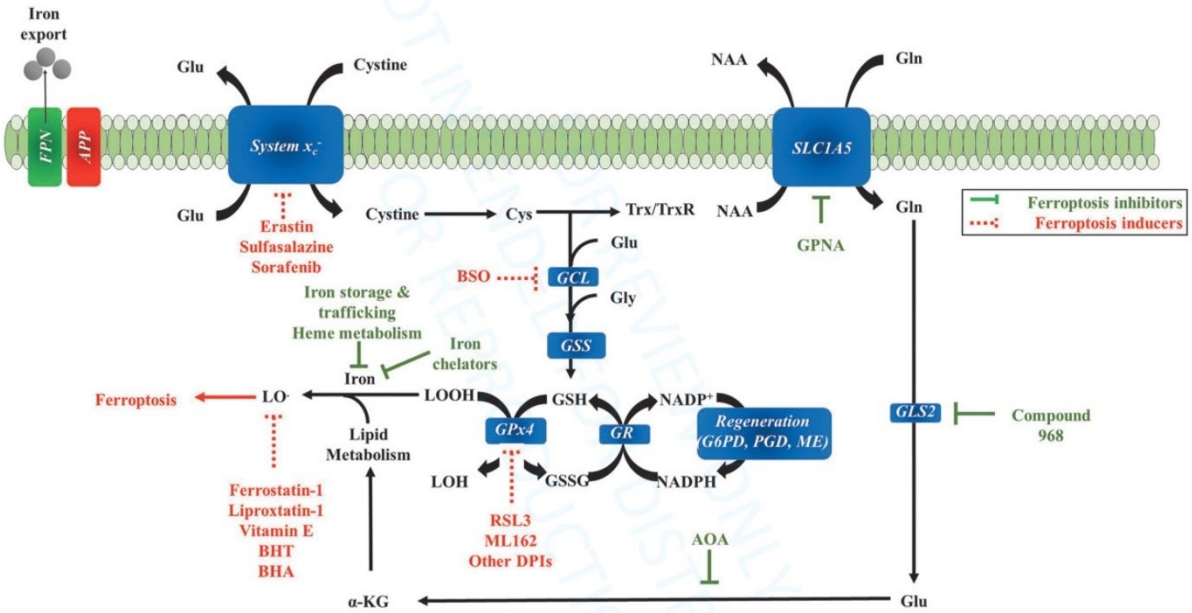

FIG. 6. Ferroptosis and its molecular regulation by the GSH system. The reduction of the activity of GPX4 by the depletion of GSH leads to iron-mediated generation and propagation of lipid peroxidation and culminates in ferroptosis. Inductors of ferroptosis (in *red*). Ferroptosis can be experimentally induced by inhibitors of Gpx4 such as RSL3, ML 162, or DPIs, by BSO-induced depletion of GSH, limiting cysteine availability by erastin, sulfasalazine, or sorafenib (inhibition of the antiporter X_c^-). Inhibitors of ferroptosis (in *green*): Ferroptosis can be experimentally inhibited at different steps by blocking the entry of Gln by GPNA (inhibitor of the glutamine transporter SLC1A5), by compound 968 (an inhibitor of GLS2), or by AOA to block the formation of α-KG. Ferroptosis can also be reduced in limiting iron availability (heme metabolism, iron chelators, or iron export). α-KG, alpha-ketoglutarate; AOA, oxyacetate; BSO, buthionine sulfoximine; FPN, ferroportin; G6PD, glycose-6-phosphate dehydrogenase; Gln, glutamine; GLS2, glutaminase 2; GPNA, L-g-glutamyl-p-nitroanilide; LOOH, lipid peroxidation; PGD, phosphogluconate dehydrogenase. Color images are available online.

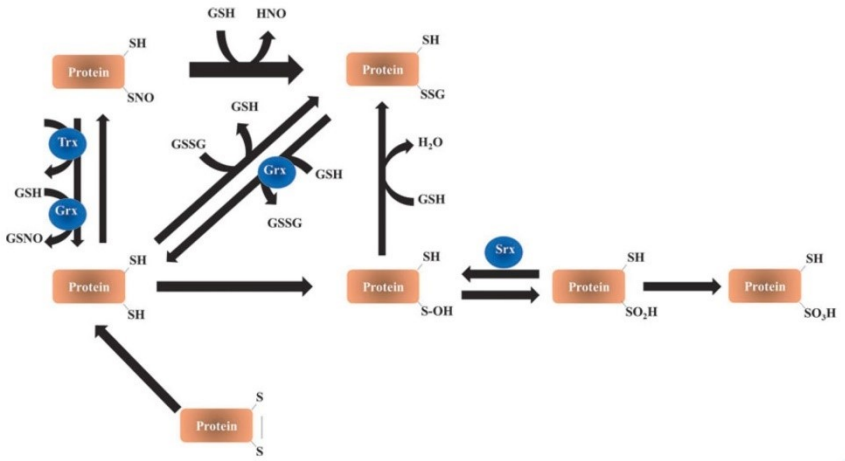

FIG. 7. Different thiol modifications regulated by Trx and GSH systems. Cysteine in proteins can be modified to lead to the formation of sulfenic acid (-SOH), sulfonic acid (-SO_3H), or disulfide (-S-S-). Nitric oxide can react with thiols to form S-nitrosylation (-SNO), and GSSG can form S-glutathionylation (-SSG) with reactive thiols. Trx, thioredoxin. Color images are available online.

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release of apoptotic proteins (63). Second, the depletion of the mtGSH pool induced neuroinflammation and neuronal damages (63). Collectively, these results suggest that a possible therapeutic approach to slow disease progression could be to replenish mtGSH (133).

The determination of oxidative stress markers and antioxidants in blood from subjects with AD or those with MCI highlights potential interactions between peripheral redox changes and the brain pathology. The extent of oxidation in peripheral blood is greater than the changes in the brain for most markers [see review by Schrag *et al.* (184)]. For instance, an increase of oxidative stress in serum, erythrocytes, and circulating lymphocytes with lower GSH/GSSG ratio in lymphocytes (7) and low-molecular-weight thiols was observed in MCI and AD [(25, 44) and review by Schrag *et al.* (184)]. A decrease in GSH/GSSG ratio may be due to the reduction of GSH synthesis, GSH depletion, or a lower regeneration of GSH from GSSG, or a combination of all. The challenges when dealing with fluids such as blood to assess the redox environment is relatively complex because the levels of GSH are different from one cell type to another. The decrease in GSH content in red blood cells from male AD patients was associated with reduced activities of GCL and GSH synthase with no change in the amount of oxidized GSH or the activity of GRX.

Oxidative markers are higher in blood than in the brain and raise the possibility that systemic derangements leading to a pro-oxidative environment may be upstream of brain injury in AD. The observation of increased risk of dementia in patients who have systemic proinflammatory/pro-oxidative diseases such as diabetes, obesity, and hypercholesterolemia supported this hypothesis.

In the following sections, we describe two potential mechanisms of action implicated in AD pathophysiology requiring GSH: one is related to inflammation and the formation of advanced glycation end-products (AGEs) and the second is related to the accumulation of acrolein, a by-product of lipid peroxidation, both can exacerbate each other.

Roles of GSH in the Detoxification of AGEs

AGEs arise from the nonenzymatic addition of sugars to proteins. Glycation can directly affect the protein structure, function, and aggregation. Although the enhanced formation of glycated proteins was initially associated with diabetes, it is now recognized that glycation of proteins is also increased in normal aging as well as in AD (30, 51, 214).

Glucose is often associated with AGE formation, but methylglyoxal (MG) or glyoxal (GO) could highly contribute to the generation of the intra- and extracellular AGEs because MG is more reactive than glucose (51). Thus, the most potent glycating agent and the major source of AGEs is the dicarbonyl MG, a by-product of glycolysis, glucose autooxidation, and lipid peroxidation. In mammalian cells, MG and GO are formed nonenzymatically from the triose phosphate glycolytic pathway intermediates, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (165).

The main pathway for the removal of MG is the glyoxalase system, which is composed of two enzymes, glyoxalase-1 (GLO-1) and glyoxalase-2 (GLO-2), and GSH (205) (Fig. 8). GLO-1 is the rate-limiting enzyme for the system and is dependent on GSH. GLO-1 and GLO-2 successively convert MG into S-D-lactoylGSH and D-lactate. Indeed, in cells, experimental depletion of GSH can induce an accumulation of MG-modified proteins (127) leading to the formation of various AGEs. Thus, GLO-1 activity is tightly dependent on cell and tissue GSH levels. GLO-1 activity is also regulated by the posttranslational modifications, including glutathionylation (27) of a critical cysteine residue caused by GSSG (27). This finding suggests that GLO-1 activity is also

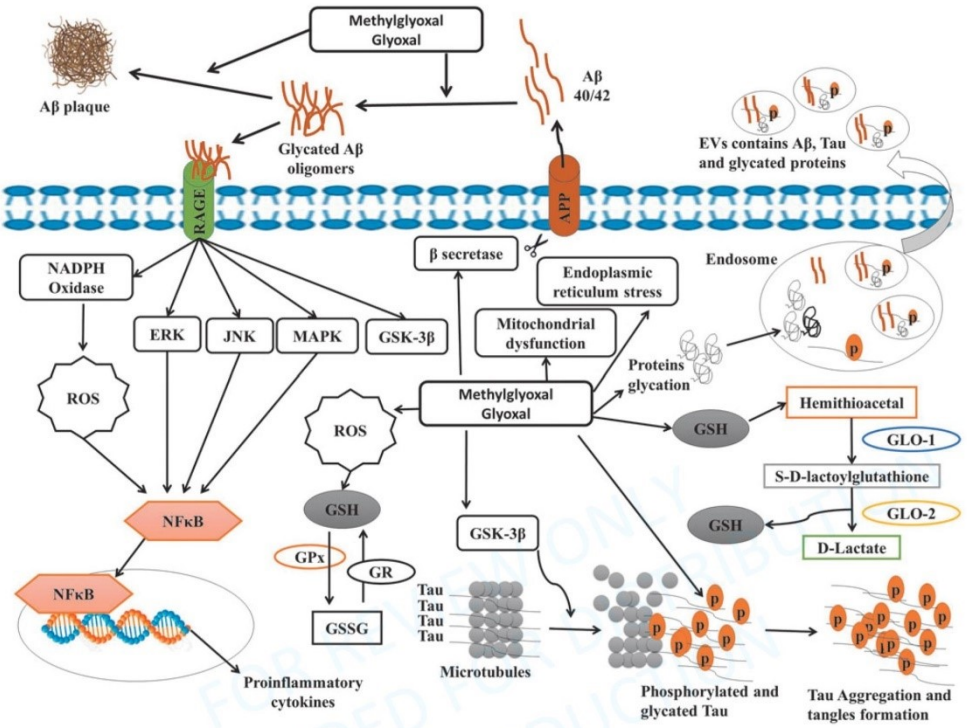

FIG. 8. Role of GSH in the restoration of cellular stress induced by AGEs and their precursors. MG or glyoxal contributes to the generation of most endogenous intra- and extracellular AGEs, which can glycate Aβ, accelerate Aβ oligomerization and Aβ plaque formation. AGEs can interact with RAGE, promote ROS production with the NADPH oxidase, and activate downstream mechanisms that further affect neuronal functions such as some signaling pathways (ERK, JNK, MAPK, GSK-3β) and NF-κB. AGEs can also glycate phosphorylated tau proteins and accelerate tau aggregation leading to neurofibrillary tangles. MG and glyoxal are degraded by the glyoxalase system, GLO-1 and GLO-2. GLO-1 is the rate-limiting enzyme for the system and is absolutely dependent on GSH. GLO-1 and GLO-2 convert MG into S-D-lactoylGSH first and then D-lactate. MG, glyoxal, and AGEs can induce mitochondria dysfunction and ER stress. Some AGEs or their precursors could be entrapped in EVs and released in extracellular space. AGEs, advanced glycation end-products; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; EVs, extracellular vesicles; GLO-2, glyoxalase-2; GSK-3, glycogen synthase kinase-3; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MG, methylglyoxal; RAGE, receptors for advanced end-products. Color images are available online.

dependent on the cellular redox state, the GSH/GSSG ratio. GLO-1 is differentially expressed in different cell types in the human brain (107) with a biphasic variation, an increase up to 55 years of age, and a progressive decrease thereafter (107). In SAMP8 mice, a model of accelerated aging, GLO-1 activity and the GSH/GSSG ratio in the brain decrease with age and lead to the accumulation of MG (168).

Interestingly, higher serum levels of MG-modified proteins (22) or pentosidine (216) were associated with a faster rate of cognitive decline in nondemented elderly subjects (>80 years). Recently, we found that the serum levels of a by-product of MG and GO, N-(1-carboxymethyl)-L-lysine (CML), were negatively correlated with the clinical cognitive scores, they were higher in the early stage of AD while the levels of pentosidine, another AGE, remained unchanged (79). We have also demonstrated that the levels of CML in the circulating extracellular vesicles (EVs) were lower in the moderate stage of AD (79). This accumulation of CML is probably due to the reduction of the glyoxalase system during the development of AD. This reduction was not attributable to low levels of the limiting enzyme GLO-1 because it is upregulated in the early and middle stages of AD (108) but probably caused by the decrease of its essential cofactor GSH in AD. Also, GSSG was found to be able to inactivate GLO-1 through covalent modification (27). Interestingly, in APP/PS1 mouse, the administration of ψ-GSH, a synthetic cofactor of glyoxalase, completely reversed the development of the spatial memory and long-term cognitive/cued-recall impairment, the Aβ deposition, and oxidative stress indicators (171). Therefore, the restoration of the glyoxalase system through GSH is a plausible mechanism for the attenuation of oxidative stress in AD.

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The binding of AGEs to their receptors for advanced end-products (RAGE) induced the activation of multiple downstream mechanisms, which further affect neuronal functions, such as lipid metabolism impairment, activation of protein kinase-C (PKC), oxidative stress, inflammation, and apoptosis through the activation of the transcription factor NF-κB (75), inhibition of AKT-mediated activation of GSK-3, Erk1/2, and p38 kinases, as well as the upregulation of RAGE (114) (Fig. 8). In the brain, RAGE activation has been shown to induce inflammation, ROS formation, and impaired microglia (105), astrocytes (26), cerebrovascular (113), and neuronal cell viability (218). Thus, the depletion of GSH through AGEs and their precursors MG or GO can also directly or indirectly increase inflammatory responses and reduce neuronal viability (65). Besides, the formation of AGEs due to the depletion of GSH could induce the glycation of phospho-tau and Aβ oligomers, which exacerbate the *in vitro* and *in vivo* phospho-tau and Aβ toxicity (60, 94, 115). Thus, the depletion of GSH can indirectly contribute to neurodegeneration through the glyoxalase pathway, which represents an interesting pharmacological target to prevent or attenuate the toxic effects induced by AGEs or AGE derivatives in AD.

Roles of GSH in the Detoxification of Acrolein and Implication in AD

Acrolein is an α, β-unsaturated aldehyde with a molecular weight of 56 Da (CH_2=CH-CHO) and two reactive and functional groups: the aldehyde group and the carbon/carbon double bond. It is the strongest electrophile among the unsaturated aldehydes and therefore displays strong reactivity with nucleophile compounds such as GSH (61). Indeed, acrolein can form adducts with amino acid residues such as lysine, histidine, and cysteine (61, 135). As cysteine is more nucleophilic than lysine and histidine, it is thus the immediate target for acrolein.

We have previously demonstrated that acrolein induces rapid depletion of the neuronal and glial levels of GSH, reaching 50% only after 30 minutes but rebounded to control levels after 24 h (47, 152, 191). These data demonstrate the important role of the intracellular thiols in the removal of toxic aldehyde products resulting from lipid peroxidation or polyamine metabolism.

The depletion of GSH induced by acrolein due to alkylation reactions could be responsible for the modification of the ratio GSH/GSSG and thus the cellular redox state toward a more oxidizing condition. The thiol-acrolein adducts are very stable with a dissociation constant being 10 to 10,000 times lower than those with other aldehydes (61, 70, 103). Thus, thiol-acrolein can accumulate and induce cell death (47, 152, 191). The reaction between acrolein and GSH occurs spontaneously at neutral pH (2), at the third carbon, generating a glutathionylpropionaldehyde (GS-propionaldehyde). This compound can in turn elicit superoxide anion or hydroxyl radical formation in the presence of xanthine oxidase and be responsible for the induction of lipid peroxidation (2). GS-propionaldehyde can also be metabolized by aldehyde dehydrogenase (142). Glutathiolation can facilitate the aldehyde reduction by the aldose reductase, an enzyme implicated in the cellular response against oxidative stress (169).

It is now well recognized that by-products of lipid peroxidation such as 4-hydroxynonenal, F4-isoprostane, and acrolein are involved in AD pathogenesis (36, 47, 88, 134, 143, 144, 146, 147, 151, 152, 170, 171, 182, 184, 190, 195). In MCI and AD brain, levels of acrolein were found to be significantly higher in vulnerable brain regions such as the hippocampus and amygdala (120, 215). In AD, acrolein is associated with proteins detected in NFTs and dystrophic neuritis surrounding senile plaques (33) and levels of acrolein-conjugated protein in plasma and CSF were significantly higher than those from control subjects (207). By reacting with GSH, acrolein alters the ratio GSH/GSSG supporting the formation of S-glutathionylation or S-glutathiolation. Accordingly, an increase in S-glutathionylation proteins was found in the inferior parietal lobule of the AD brain (153). Thus, in AD, the depletion of GSH synthesis could render cells particularly susceptible to acrolein toxicity (12, 118).

Role of EVs/Exosomes in the Secretion of GSH

Astrocytes are known to actively release divergent secretory organelles, including synaptic-like microvesicles and exosomes. Exosomes are released from astrocytes notably in response to oxidative and heat stress (201) and also in pathological conditions. Proteomic analysis revealed that exosomes released from different types of cultured cells contain various proteins. It is interesting to note that five target genes of the Nrf2-ARE are among the top 50 most commonly identified proteins in exosome preparation. Peripheral and platelet-derived EVs can, respectively, contain GSH synthase and GRX (102, 116, 166) but their content in neuronal or astroglial-derived EVs remains to be determined (Fig. 9). Also, the demonstration of the presence of GSH in exosomes will be interesting and could represent a new mechanism for the intercellular transfer of GSH.

GSH Pathways As a Therapeutic Approach

Given the implication of GSH in different pathways related to AD, several strategies to improve its plasma and brain bioavailability have been investigated to restore its intracellular level to prevent alterations observed in AD.

GSH synthesis is often limited by L-cysteine availability. Cysteine can be obtained by the breakdown of GSH or from proteins by autophagy, synthesized endogenously by cystathionine γ-lyase, or from the diet. This lead to the investigation of the relationship between diet and GSH tissue levels (202), to the development of the pharmacological L-cysteine prodrugs (167), to the delivery of the GSH prodrug derivatives with more favorable physicochemical properties, the study of L-cyst(e)ine transport (204), and the oral administration of γ-GC (220). This latter approach leads to the FDA-approved cysteine precursor N-acetyl-cysteine. However, clinical trials did not show significant restoration of the GSH stocks (38, 183). On the contrary, a sublingual administration form of GSH had demonstrated some benefits over oral supplementation as it bypasses the gastrointestinal tract and the first-pass metabolism (183). The therapeutic potential of exogenous γ-GCS was also developed to raise GSH levels by circumventing the age-related dysregulation of the rate-limiting step of GSH synthesis. The oral administration of γ-GC is effectively absorbed via the intestinal lumen, similar to that observed for GSH (89), and should therefore become

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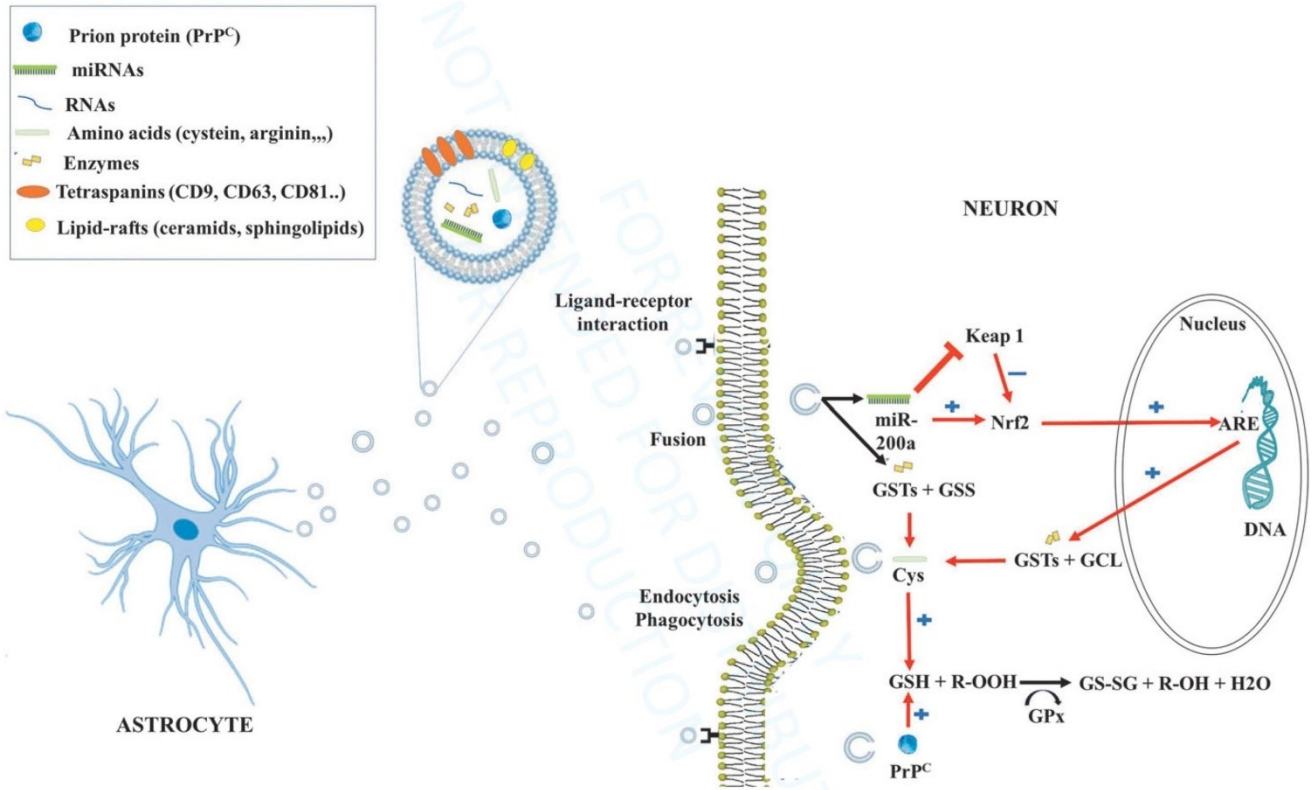

FIG. 9. Molecular components of astrocyte-derived-EVs as potential mediators of neuronal glutathione metabolism. Astroglial-derived EVs or exosomes may contain several functional miRNAs such as the mir-200a, which promote Nrf2-mediated expression of antioxidant enzymes in neurons by inhibiting Keap1. Therefore, inactivation of Keap1, *inter alia* by mir-200a, results in Nrf2 nuclear translocation, binding to ARE, and activation of cytoprotective gene transcription. EVs may contain numerous amino acids such as cysteine as well as antioxidative stress enzymes/proteins such as glutathione synthetase and the prion protein (PrP^C) (Exocarta, 2019). PrP^C reportedly protects neuronal cells from oxidative stress damage. Previous studies have shown increased production of GSH (by 78%) and the activities of GRX (64%) and SOD (21%) in the presence of PrP^C suggesting that PrP^C regulates the activities of specific detoxifying enzymes. Color images are available online.

systemically available, taken up by cells to increase the GSH synthesis but the its bioavailability remains to be determined. Recently, supplementation with γ-GC for 3 months can reduce brain oxidative stress, neuroinflammation, and maintain the antioxidant status in an AD mouse model (119). Moreover, the administration of γ-GC was sufficient to protect against Aβ burden and prevent deficits in spatial memory (119). Thus, the administration of γ-GC may have the potential as an adjunct for treating diseases associated with chronic GSH depletion.

It was found that γ-GC treatment increased the activity of selected matrix metalloproteinases (MMP2, MMP9) and MMP-9 has been shown to degrade Aβ deposits, which are dependent on GSH status (10). In opposite, GSSG can inhibit the insulin-degrading enzyme, the enzyme involved in the degradation of Aβ, by reacting with one or more of its cysteines (40).

Considering the crucial roles of GSH in various biological activities, a new pharmacological approach to raise or to maintain its levels should be encouraged. To date, there is no clinical trial with GSH restoration associated with cognitive improvement. Therefore, it is necessary to study the effect of GSH restoration in MCI and AD patients to evaluate its efficacy as a preventive or therapeutic potential.

Nanotechnology at the Rescue of GSH Deficit in Brain Cells and GSH-Pegylated Liposomes/ Nanoparticles As Innovative Tools to Enhance Brain Delivery of Active Compounds

Several studies suggest that the restoration of the intracellular levels of GSH may have a disease-modifying effect (167). Thus, oral supplementation has been proposed to alleviate these deficits. However, oral delivery of GSH is a pharmacological challenge because GSH is a charged peptidic hydrophilic molecule with a low cell membrane permeability and low cellular barrier translocation. Moreover, GSH is very sensitive to degradation, with a high enzymatic degradation rate in the gastrointestinal tract, decreasing strongly its oral bioavailability. However, the daily consumption of GSH supplements was effective at increasing body compartment stores of GSH (174), but no benefit of oral uptake on GSH stock level or oxidation biomarkers has been reported in clinical trials (9). The design of the new delivery systems appears as a more promising avenue to overcome these challenges. Encapsulation in nanosized particles has been proposed to improve GSH bioavailability through protection from redox reaction or microbiome and gastrointestinal tract epithelial cell enzymatic degradation. Numerous oral liposomal forms of GSH have been commercialized with positive effects on the intracellular GSH stock and neuroprotection *in vitro* (221), GSH plasma level, and immune function *in vivo* (193). Other types of GSH-loaded nanoparticles (NPs) have been proposed for the oral route but none of them reached the clinical stage and few had presented the pharmacokinetic results in animal models (71).

One of the most important limitations for drug delivery to the brain is the presence of the BBB. Nanotechnology-based GSH has also been proposed to overcome the BBB to enhance the brain delivery of GSH based on nanocarriers. These carrier systems include, for example, liposomes and polymeric NPs that can potentially protect drugs from peripheral degradation and facilitate their transport into the brain. For this, poly(ethylene glycol) (PEG) is used as a surface modifier to reduce the toxicity, increase stability, and prolong circulation time. Coating nanocarriers or liposomal surface with ligands is also used to achieve active drug transport into the brain by targeting specific receptors located on the BBB. Different studies have also used GSH for stabilizing NPs (132).

The addition of GSH to a potential substrate for a transporter expressed at the BBB (99, 100), at the surface of liposomes/nanocarriers, can potentially enhance their brain delivery. For instance, PEGylated liposomes containing GSH (GSH-PEG) were found to enhance the brain delivery and the effects of doxorubicin and methylprednisolone in animal models with brain tumors and multiple sclerosis, respectively (69, 209). The pharmacokinetics of drugs changed greatly by encapsulation in GSH-PEG liposomes with more than 40-fold prolongation of the half-life, from several minutes to almost 7 h. Thus, GSH-PEG liposomes offer a promising system for enhancing and prolonging the delivery of drugs to the brain (117). Besides, the GSH coating does not interfere with the drug release (72).

Several teams reported enhanced delivery of nanocarriers' drug cargo into the brain via interaction between GSH-decorated drug-loaded NPs and vascular endothelial cells. GSH PEGylated liposome ("G-technology"®) enhanced the penetration of a fluorescent reporter and model drug into rat brain over untargeted liposome (138, 175). Similarly, the delivery of single-domain antibody fragments directed to Aβ was found to increase in mice when associated with GSH-modified liposome (177).

We have efficiently synthesized poly-lactic-co-glycolic acid 50:50 NPs (PLGA-NPs) coated with PEG and GSH (GSH-NPs) loaded with curcumin (GSH- NPs-Cur), using thiol-maleimide click reaction. For this, the GSH moiety was installed on the backbone of PEG-PLGA using the free thiol end group of GSH. We found that GSH functionalization did not affect the drug loading efficiency, the size, the polydispersity index, the zeta potential, the release profile, and the stability of the formulation. GSH-conjugation increases the neuronal uptake of these NPs. We have recently demonstrated that the presence of reduced GSH on the surface of the formulations exhibits a better neuroprotective property against acrolein. The neuronal internalization of GSH- NPs-Cur was higher than with free curcumin. We found that GSH-conjugation modifies the route of internalization enabling them to escape the uptake through macropinocytosis toward clathrin/caveolae-mediated endocytosis and therefore avoiding the lysosomal degradation (158). The GSH-coupled nanocarriers thus represent a promising approach for the functionalization of nanocarriers to efficiently cross the BBB for the delivery of drugs to the brain while avoiding cellular toxicity. Thus, the choice of ligand for ligand-modified NPs could be one of the potential strategies when developing a novel formulation against brain disorders such as AD.

Conclusions

Aging is the primary risk factor for AD. With aging, considerable oxidative stress contributes to the progression of neurodegeneration. GSH is the major antioxidant in the body with a particularly high concentration in the brain.

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Several studies have demonstrated that the levels of GSH are reduced with aging and in AD. In addition to its antioxidant effect, GSH is involved in various important pathways involved in detoxification and cell survival. The regulation of its homeostasis is challenging due to the complexity of the regulation of different enzymes involved.

Preclinical work supports a potential therapeutic role for γ-GC in the treatment of diseases associated with chronic oxidative stress and the cysteine precursor derivatives are FDA approved.

In the brain, astrocytes express complex exocytotic machinery that is associated with several types of secretory vesicles involved in the secretion of a wide variety of proteins, lipids, nucleic acid, neurotransmitters, and neurotransmitter precursors. To date, little research effort has been focused on exploring the role of exosomes on the transfer of GSH or its precursors to raise and replenish intracellular GSH and particularly to neuronal cells. More research is required to better understand the role of exosomes on the protection against oxidative stress and potentially their role in neuroprotection including the transfer of GSH. Nanotechnology appears as a promising tool to increase the GSH pool in brain cells; however, several challenges are yet to be overcome. GSH has also the potential as a targeting ligand for nanocarrier translocation across the BBB to treat various brain dysfunctions (3, 68, 154).

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Authors' Contributions

Conceptualization, writing, editing, and validation: C.R. Writing—original draft: M.H. (50%), V.H. (20%), M.R.B.K. (15%), and J.-M.R. (15%).

Author Disclosure Statement

No competing financial interests exist.

Funding Information

The authors gratefully acknowledge the financial support from the Research Chair Louise & André Charron on Alzheimer's disease (C.R.), the Foundation Armand-Frappier (C.R., V.H., M.R.B.K.), Fonds de Recherche du Québec–Nature et Technologies (FRQNT) (C.R.), and support from NSERC (C.R., J.-M.R.).

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Date of first submission to ARS Central, June 19, 2020; date of final revised submission, February 12, 2021; date of acceptance, February 15, 2021.

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Abbreviations Used

α-KG = alpha-ketoglutarate
γ-GC = γ-glutamylcysteine
γGluCys = γ-glutamylcysteine
γGT = γ-glutamyl transpeptidase
AD = Alzheimer's disease
AGEs = advanced glycation end-products
AOA = oxyacetate
ApN = aminopeptidase
AP-1 = activator protein-1
ApoE = apolipoprotein E
ARE = antioxidant response element
ARE/EpRE = antioxidant and electrophile response elements
ATF4 = activating transcription factor 4
Aβ = amyloid-beta
BBB = blood/brain barrier
BSO = buthionine sulfoximine
CC = cingulate cortex
CML = N-(1-carboxymethyl)-L-lysine
CREB = cAMP response element-binding protein
Cys = cysteine
CysGly = dipeptide cysteine glycine
EAAT = excitatory amino acid transporter
EAAT3/EAAC1 = excitatory amino acid transporter 3
ER = endoplasmic reticulum
ERK = extracellular-signal-regulated kinase
EVs = extracellular vesicles
FPN = ferroportin
G6PD = glycose-6-phosphate dehydrogenase
GCL = glutamate-cysteine ligase
GCS = glutathione synthetase
GCT = γ-glutamylcylotransferase
GLO-1 = glyoxalase-1
GLO-2 = glyoxalase-2
Glrxs = glutaredoxins
GLS2 = glutaminase 2
Glu = glutamate
Gly = glycine
GO = glyoxal
GPNA = L-g-glutamyl-p-nitroanilide
GPX = glutathione peroxidase
GPX4 = glutathion peroxidase 4
GRX = glutathione reductase
GSH = glutathione
GSK-3 = glycogen synthase kinase-3
GS-propionaldehyde = glutathionylpropionaldehyde
GSSG = oxidized GSH
GST = glutathione-S-transferase
HO-1 = heme oxygenase-1
IL-6 = interleukin-6
IMS = intermembrane space
JNK = c-jun N-terminal kinase
LOOH = lipid peroxidation
LOX = lipooxygenase
MAPK = mitogen-activated protein kinase
MCI = mild cognitive impairment
MG = methylglyoxal
NFTs = neurofibrillary tangles
NF-κB = nuclear factor-κB
NMDA = N-methyl-D-aspartate
NPs = nanoparticles
NQO1 = NAD(P)H:quinone acceptor oxidoreductases
Nrf2 = nuclear factor erythroid 2-related factor
PEG = poly(ethylene glycol)
PepT2 = peptide transporter
PGD = phosphogluconate dehydrogenase
PKC = protein kinase-C
PLGA-NPs = poly-lactic-co-glycolic acid 50:50 NPs
PPP = pentose phosphate pathway
Prx = peroxiredoxin
PrxSOH = peroxiredoxin sulfenate
PSSG = oxidized peroxiredoxin
RAGE = receptor of glycation end-products
ROS = reactive oxygen species
RSSG = glutathionylated protein
tBHQ = tert-butylhydroquinone
Srxn1 = sulfiredoxin
TNF-α = tumor necrosis factor-a
Trx = thioredoxin
TrxR = thioredoxin reductase
TRX-R1 = TRX-reductase 1

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7 ANNEXE 2: POLYPHENOL-PEPTIDE INTERACTIONS IN MITIGATION OF ALZHEIMER'S DISEASE: ROLE OF BIOSURFACE-INDUCED AGGREGATION

Journal of Alzheimer's Disease 81 (2021) 33–55
DOI 10.3233/JAD-201549
IOS Press

Review

Polyphenol-Peptide Interactions in Mitigation of Alzheimer's Disease: Role of Biosurface-Induced Aggregation

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Accepted 19 February 2021
Pre-press 15 March 2021

Abstract. Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder, responsible for nearly two-thirds of all dementia cases. In this review, we report the potential AD treatment strategies focusing on natural polyphenol molecules (green chemistry) and more specifically on the inhibition of polyphenol-induced amyloid aggregation/disaggregation pathways: in bulk and on biosurfaces. We discuss how these pathways can potentially alter the structure at the early stages of AD, hence delaying the aggregation of amyloid-β (Aβ) and tau. We also discuss multidisciplinary approaches, combining experimental and modelling methods, that can better characterize the biochemical and biophysical interactions between proteins and phenolic ligands. In addition to the surface-induced aggregation, which can occur on surfaces where protein can interact with other proteins and polyphenols, we suggest a new concept referred as "confinement stability". Here, on the contrary, the adsorption of Aβ and tau on biosurfaces other than Aβ- and tau-fibrils, e.g., red blood cells, can lead to confinement stability that minimizes the aggregation of Aβ and tau. Overall, these mechanisms may participate directly or indirectly in mitigating neurodegenerative diseases, by preventing protein self-association, slowing down the aggregation processes, and delaying the progression of AD.

Keywords: Alzheimer's disease, amyloid, blood cells, computer simulation, polyphenols, tau

INTRODUCTION

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder, responsible for nearly two-thirds of all dementia cases [1, 2] including Lewy body dementia, vascular dementia, and frontotemporal dementia [3, 4]. The prevalence of AD is about 5–10% above the age of 60 years but increases up to 40–50% above the age 85 years [5]. With the aging of the population [6], dementia affects nearly 50 million people worldwide, and is predicted to increase to 152 million by 2050 [7]. The estimated annual healthcare cost is US $1 trillion, which could double by 2030 [7].

The multifactorial disorders of AD, considering genetic and non-genetic components, are clinically

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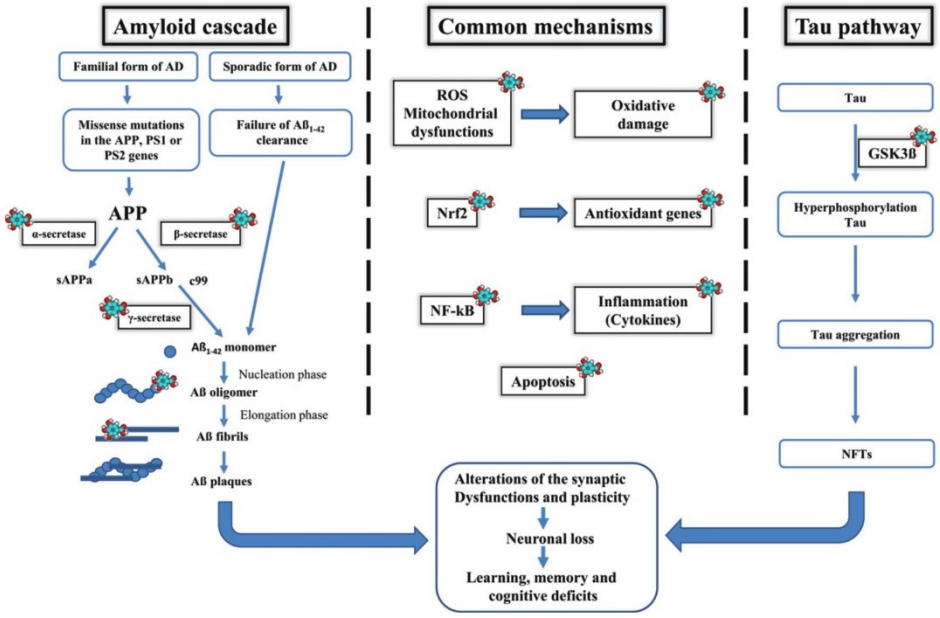


Fig. 1. Multiple targets of polyphenols in the integrative amyloid-cascade and tau pathway. Both Aβ and tau pathology are independent and dependent pathways leading to AD. In familial form of AD, different mutations on *APP*, *PS1*, and *PS2* genes lead $A\beta_{1-42}$ overproduction while in sporadic form of AD, failure of the $A\beta_{1-42}$ clearance under physiological conditions induced its gradual rising. The net result is to enhance the production of the putatively neurotoxic $A\beta_{1-42}$ monomer at the expense of the putatively neuroprotective $A\beta_{40}$. $A\beta_{1-42}$ accumulation into soluble oligomers induce oxidative damage, inhibit the activity of Nrf2 and thus the antioxidant genes, activate NF-κB and the production of cytokines and finally cause apoptosis. $A\beta_{1-42}$, ROS, and oxidative damage can activate glycogen synthase kinase 3β (GSK3β) which phosphorylates tau protein. Both the formation of NFTs due to the hyperphosphorylation of tau and the amyloid cascade lead to synaptic dysfunction, neuronal loss, and finally to learning and cognitive impairment. ( ) Represent different pathways targeted by polyphenolic compounds.

characterized by memory dysfunction, loss of lexical access, spatial and temporal disorientation, and impairment of judgement. Although the molecular mechanisms of AD have not been fully elucidated yet, compelling evidence indicates that abnormal proteins accumulation in the brain, such as the intracellular aggregation of the tau [8–10] and extracellular deposition of amyloid-β (Aβ) [11, 12] leads to neuronal loss [13]. However, the heterogeneous nature of neurodegenerative disorders increases the challenges to understand the underlying mechanisms from the initial phases to the progression of AD. Over the years, several pathways have been studied including the Aβ cascade and deposition, abnormal tau aggregation, oxidative stress, mitochondrial dysfunctions, lysosomal alterations, neuroinflammation, and metabolic disorders where all of these converge to neurodegeneration (Fig. 1).

The amyloid-β pathway

Since 1991, the amyloid-cascade hypothesis has provided the main framework to understand the pathogenesis of AD [14, 15]. The basis for this hypothesis was the discovery of autosomal dominant mutations in three genes—*APP*, *PSEN1*, and *PSEN2* (the latter two encoding presenilin 1 and 2, respectively)—that induce pathogenic Aβ aggregation into neuritic plaques [14, 15]. The amyloid-β protein precursor (AβPP), a type I transmembrane protein, contains a large extracellular domain [16]. In familial AD with APP mutations, the amyloid cascade leads to early onset of cognitive deficits and dementia, likely through complex age-dependent cellular and molecular changes, including the spreading and deposition of neurofibrillary tangles (NFTs). Although mutations in the above three genes do not occur in sporadic AD, similar neuropathological

changes in Aβ and tau were observed in both familial and sporadic AD [17–19].

Over the years, the amyloid-cascade hypothesis involved into an integrative model that provides a general framing for other disease mechanisms, e.g., immunoreactivity, microgliosis, mitochondrial dysfunction, oxidative stress, and dysregulation of protein homeostasis [20, 21].

The amyloid-β and tau integrative pathways

It is now thought that Aβ and tau pathologies can follow both independent and dependent pathways leading to AD. Aβ preferentially accumulates in brain regions with high metabolic demand (such as association cortices) and spreads from neocortex to allocortex to brainstem, eventually reaching the cerebellum [22–24]. Tau pathology, by contrast, first becomes evident in the (trans)entorhinal cortex from which it spreads to limbic areas, and from there to the neocortex [25–28]. The finding that Aβ and tau pathologies initially start in different brain regions, referred to as the 'spatial paradox', argues against the idea that tau pathology is driven by amyloid pathology occurring in the same local brain area. Aβ and tau pathologies also follow distinct temporal sequences because Aβ in the neocortex is already present 10–20 years before the emergence of clinical AD symptoms and the rate of Aβ accumulation attenuates during the clinical stage of AD [29]. In addition, the extent and locations of Aβ deposition are only modestly correlated with the brain areas affected by neurodegeneration [30, 31]. Both spatially and temporally, tau pathology correlates much more strongly with neurodegeneration and cognitive impairment than Aβ pathology. Increased tau PET signal is associated with worse cognitive performance in both cognitively normal individuals and patients with clinical AD [26, 27, 32, 33]. The spread of tau pathology was associated with a specific gene-expression profile of 'axon-related' genes, whereas the spread of Aβ was linked to a different profile of 'dendrite-related' genes. A third subset of 'lipid metabolism-related' genes was linked to increased spread of both Aβ and tau pathology [34].

Learning, memory, and cognitive deficits characterize AD patients, whereas memory deficits are a hallmark of amnestic mild cognitive impairment. These altered functions largely originate from synaptic dysfunction involving altered synaptic proteomes [35, 36] with the particular contribution of $A\beta_{42}$ oligomers [35, 37]. These oligomers cause oxidative damage to synaptic membranes [38], suggesting the relation between oligomer-induced oxidative damage and synaptic dysfunction.

Role of the amyloid-β oligomers

Many oligomeric Aβ species have been described [39]. Large oligomers of $A\beta_{42}$ are relatively less toxic whereas small $A\beta_{42}$ oligomers (e.g., dimers or trimers that easily enter lipid bilayers) appear highly toxic to synapses [40]. Thus, in the absence of amyloid plaques, soluble Aβ oligomers from AD brains have been showed to impair hippocampal synaptic plasticity, decrease synapses, induce tau hyperphosphorylation and neuritic dystrophy, activate microglial inflammation, and impair memory in normal adult rodents [40]. Together, the soluble fraction of high molecular weight oligomeric Aβs are far less bioactive than the smaller oligomers in AD brain. The composition of Aβ plaques is both fibrillar and soluble high molecular weight oligomeric Aβs. Therefore, it is important to target diffusible Aβ species that are highly bioactive in AD brain. Thus, the neutralization of the toxicity of oligomeric Aβ species was suggested as a chronic therapy for AD [41, 42].

Amyloid-β-targeted therapies

To date, there is no approved drugs that can either revert or cure the AD. The amyloid cascade hypothesis has also guided most drug discovery efforts in both familial and sporadic AD, where the objective was the removal of various forms of cerebral Aβ. Unfortunately, if Aβ-targeted therapies tested in phase III clinical trials (bapineuzumab, gantenerumab, solanezumab, crenezumab, lanabecestat, atabecestat, verubecestat, and elenbecestat) [43] can effectively reduce Aβ load in AD brains, they were unsuccessful in slowing cognitive decline either with mild cognitive impairment or AD patients. In addition, two phase III clinical trials of Aβ-targeted therapy, aducanumab, were halted in March 2019 [44]. Interestingly, in one of the two trials, cognitive decline was attenuated in patients receiving high dose aducanumab, [44]. However, the AD drug candidate aducanumab took a beating from FDA advisors (Science, Nov. 6, 2020) and the FDA's decision is expected by March 2021 [45].

The lack of beneficial effects on cognitive outcome from these trials could be due to different reasons such as the timing of the interventions, as the studies

involved patients in clinically advanced stages of AD, and insufficient dosing or the wrong Aβ species being targeted [46]. Alternatively, the failure of these trials could indicate that removing Aβ from the brain is not sufficient to halt cognitive decline suggesting that tau pathology, tau-mediated neurodegeneration, and other mechanisms in AD are driven partially by Aβ pathways. Most of the unsuccessful therapeutic approaches for AD had focused on Aβ or tau pathways. Interestingly, Hammond et al. [47] suggested that AD treatments may also need to be disease stage-oriented with Aβ and tau as targets in early AD and glucose metabolism as a target in later AD. Recently, the EU-US Clinical Trials on Alzheimer's Disease (CTAD) Task Force reported that effective treatments should include new biomarkers intervening in early stages of AD, and of combination therapies [44]. This led to the diversification of the development of the drug portfolio.

Protection against amyloid-β by polyphenols

In this regard, several polyphenols have been investigated to promote neuroprotection by targeting Aβ oligomers and attenuated Aβ-induced-reactive oxygen species production, restore the Aβ-induced reduction of antioxidants activities or mitochondrial dysfunctions (Fig. 1) [48–51]. Numerous flavonoids can interact and destabilize Aβ peptide structures, function of ligand:Aβ molar ratio [52]. Most polyphenols that are Aβ-aggregation inhibitors share a catechol moiety, also related to anti-oxidative stress, such as: (+)-taxifolin, myricetin, quercetin, (+)-catechin, epigallocatechin gallate (EGCG), anthocyanins [53], OH-tyrosol, tyrosol [54–57], and rosmarinic acid or grape-derived polyphenols [58–61]. As a result, these compounds can efficiently destabilize the β-sheet structures of Aβ and prevent the elongation of Aβ oligomers.

Interestingly, some antioxidant polyphenols, such as resveratrol or quercetin-3-O-glucoside [62], are able to enter the brain to a limited extent and have shown promise for AD treatment [63]. Another mechanism by which polyphenols could exert protective properties is by generation of a hormetic response to their use [64–66], i.e., they generate a mild oxidative stress that the body tries to mitigate by upregulating protective genes. This often leads to increases in the levels of antioxidants such as glutathione and HO-1, mediated by activation of the transcription nuclear factor erythroid 2-related factor 2 (Nrf2) [66, 67] (Fig. 1).

Among the 560 anthocyanins (ACNs) identified in nature, the common anthocyanidin aglycones, e.g., pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, can form covalent conjugates with sugars and organic acids to generate a plethora of ACNs. Some ACNs have the ability to cross the blood-brain barrier and reach the brain [68, 69], particularly the hippocampus [70–73]. The type and concentration of ACNs differ widely among different fruits and vegetables, ranging from 1.4 mg/g to 800 mg/g of dry weight [74]. Youdim et al. [75] reported the citrus flavonoids, hesperetin, naringenin, and their relevant *in vivo* metabolites, as well as the dietary ACNs and *in vivo* forms, cyanidin-3-rutinoside and pelargonidin-3-glucoside, are taken up by two brain endothelial cell lines from mouse (b.END5) and rat (RBE4).

In this review, we first discussed the biochemical aspects of Aβ and tau which are relevant to β-amyloidopathy and tauopathy. We then reported the potential of AD treatment strategies focusing on natural polyphenol molecules (green chemistry) and specifically on the inhibition of polyphenol-induced amyloid and/or tau aggregation or disaggregation pathways. We discussed how polyphenols can potentially alter the structure and/or delaying Aβ and tau self-association at the very early stage of AD, hence, potentially playing a key role in the progression of neurodegenerative disorders. In addition, we suggest a new concept, referred as "confinement stability", where the adsorption of Aβ and tau on biosurfaces other than Aβ- and tau-fibrils, e.g., membranes, vessels, red blood cells (RBCs) etc., can either lead to confinement stability or to surface-induced aggregation, depending on the affinity of Aβ and tau to these surfaces.

BIOCHEMICAL ASPECTS OF $A\beta_{40}$ AND $A\beta_{42}$

The molecular weights of $A\beta_{40}$ and $A\beta_{42}$ monomers are 4.33 and 4.51 kDa, respectively. $A\beta_{42}$ with two additional hydrophobic residues (Ile41 and Ala42) at the C-terminus shows a greater propensity to induce fibrils formation than $A\beta_{40}$ [76]. The predicted solubility is higher for $A\beta_{40}$ than $A\beta_{42}$ being 0.4 μM and 0.04 μM, respectively [77], consistent with $A\beta_{42}$ experimental solubility of 0.04 μM [78]. Both $A\beta_{40}$ and $A\beta_{42}$ monomers have a hydrodynamic radius (Rh) of 0.9 ± 0.1 nm [79], i.e., below the colloidal domain (~5 nm to 5 μm) [80]. The total

mass of Aβ is estimated to 6.5 mg in cortical grey matter of AD brain compared to 1.7 mg in control brains [81]. For example, an Aβ rate of mass accumulation of 30 ng/h is enough to place a person on the trajectory to accumulate 5 mg of Aβ in the brain over a 20-year time frame [81]. The *in vivo* fractional production and clearance rates of Aβ in the human CNS was reported to be 7.6% per hour and 8.3% per hour, respectively [82]. Importantly, Aβ fibrils from human brains are right-hand twisted, quite different from *in vitro* fibrils [83]; this emphasizes the preferred use of human AD brains in future investigations. Additional biochemical aspects of Aβs can be found in [84].

BIOCHEMICAL ASPECTS OF TAU

In the perspective of understanding tau aggregation mechanisms, the following describe some biochemical and biophysical properties of tau protein implicated in AD: structures, domains, phosphorylated and binding residues, solubility, ionic charge, prone to or suppression of aggregation, etc.

The accumulation of misfolded and aggregated forms of tau protein in the brain is a neuropathological hallmark of tauopathies observed in neurodegenerative diseases (NDs), including AD and Pick's disease [85–87]. The microtubule-associated protein tau (MAPT), identified in mid-1970s [88, 89], encodes the protein tau [90]. Tau is notably characterized by the presence of three or four (according to the isoforms) imperfect repetitions of a motif of about 30 residues, known as the microtubule-binding repeats (MTBRs), and where the N-terminal to the MTBR is a proline-rich region (PRR) [91]. Moreover, tau can be characterized into four sections: N-terminal projection, proline-rich domain, microtubule-binding domain (MBD), and a C-terminal [92]. Tau full length monomer (hTau40wt(441)) has a molecular weight of 45.8 kDa, a radius of gyration (Rg) of 6.5 ± 0.3 nm and a hydrodynamic radius (Rh) of 5.3 nm [93], within the colloidal domain.

Tau is a natively unfolded protein largely found in axons, where it serves to stabilize microtubules that have a diameter of ~25 nm [94], and shows little tendency for aggregation [95]. Phosphorylation triggers tau-tau self-assembly [96]. Tau pseudophosphorylation on some sites found preceding residue 208 mainly suppresses tau aggregation whereas tau pseudophosphorylation at sites in the C-terminal region preferentially promotes tau self-association, particularly S422 [97, 98]. Tau phosphorylation by GSK3β induced tau aggregation [99, 100]. Two hexapeptides, ^{275}VQJINK^{280} and ^{306}VQJVYK^{311}, are effective in generating β-sheet structures while processing tau aggregation [101, 102].

Abnormal folding of the MAPT results into paired helical filaments (PHFs) and NFTs [103]. The cores of PHFs and straight filament are composed of eight β-sheets (β1-8) that run along the length of the protofilament, adopting a C-shaped architecture [104]. Hydrophobic clustering, aliphatic stacking (V339, L344, V350, I354), and aromatic stacking (F346) stabilize the interior of β-helix [104]. The existence of *in vitro* twisted ribbon-like assemblies of tau fibrils was observed, showing corrugations with periodicities of 17.4 ± 2.7 nm ($n = 16$) in fibrils of human tau40 [105].

In human AD cortex, soluble Aβ dimers induced tau hyperphosphorylation and neuritic degeneration [106]. Therefore, Aβ is upstream of tau in AD pathogenesis and triggers the conversion of tau from a normal to a toxic state, but there is also evidence that toxic tau enhances Aβ toxicity via a feedback loop.

The above biochemical and biophysical properties of Aβ and tau nucleation and growth provide key fundamental molecular insights which are the basis for effective mechanisms in delaying neurodegenerative bioprocesses.

GENERAL STRUCTURE OF POLYPHENOLS

Among 8,000 known polyphenolic compounds, more than 5,000 flavonoids are widely distributed in plants [107–109], e.g., tannins, in particular proanthocyanidins with more than 1000 derivatives identified to date. Tannins can be classified into two groups: hydrolysable tannins and condensed tannins [110, 111]. The condensed tannins, also referred as proanthocyanidins, are the most abundant. Hydrolysable gallotannins contain gallic acid (GA) substituents esterified with a polyol residue (mainly D-glucose). The biosynthetic pathway, starting from D-glucose and after the galloylation reaction, yields di-, tri-, tetra, penta-, hexa-, hepta-, and octagalloylglucoses. Gallotannin with 10 (up to 12) units of GA esterified to a single glucose moiety are having many phenolic OHs, e.g., tannic acid (TA) with 25 phenolic OHs. The majority of polyphenols have more than two aromatic rings, essential for π-π stacking with aromatic amino acid residues of Aβ and at least three phenolic hydroxyl groups that can form hydrogen bonds with hydrophilic residues of Aβ [112].

MECHANISMS AND DELAYING AGGREGATION OF Aβ AND TAU

There are various mechanisms for delaying Aβ and tau aggregation in the context of AD. Natural polyphenols are known to strongly associate with these proteins, thus have the potential to prevent protein self-association and the formation of toxic oligomers, fibrils, and plaques. Different aggregation mechanisms occur in human brain, such as: salt-induced aggregation, bulk aggregation, and surface-induced aggregation (either on Aβ and tau fibrils from secondary nucleation, or on biosurfaces other than Aβ- and tau-fibrils). Interestingly, polyphenols are stable in high conductivity environments, such as physiological conditions.

Nucleated polymerization processes are involved in many growth phenomena in nature [113]. For example, the biology of human brain involves molecular and macromolecular growth bioprocesses. This includes different Aβ and tau structures, conformations and shapes, such as aggregate, cluster, dimer, fibril, fiber, monomer, neurofilament light, NFTs, oligomers, PHF, plaque, protofibril, and straight filament. Some of the above are structures within the colloidal domain (~5 nm to 5 μm) [80]. Smaller molecules with less than ten or fifteen amino acids (aas) are below this range, but protein oligomers of $A\beta_{40/42}$ and larger macromolecules such as tau protein with more than about 100 aas (e.g., tau441), are likely within this range. Nevertheless, the interactions in both ranges are diffusion controlled (perikinetic).

Aggregate denotes dimers, trimers, and higher order assemblies. The term oligomer often refers to aggregates of 2–20mers [114]. Amyloid fibrils are linear aggregates with a repetitive cross-beta structure. Primary nuclei can form during the lag phase from monomers in bulk solution. Then, the proliferation of new aggregates takes place on fibrils catalytic surfaces, referred as secondary nucleation [114, 115]. The lag phase can be minimized by addition of pre-formed nuclei or seeds [116]. Interestingly, a significant delay on the onset of $A\beta_{40}$ fibrils formation was reported at a low apolipoprotein E3 (apoE3) concentration (40 nM), equivalent to an apoE3:Aβ molar ratio of 1:1000 [117].

Salt-induced aggregation and critical association concentration

The formation of Aβ fibrils and other polypeptide aggregates strongly depends on the physiological and chemical environment, e.g., the type and salt concentration [118]. Jain et al. [119] reported the impact of NaCl on the kinetics of Aβ fibril formation and β-rich oligomer formation, where the aggregation rate significantly increased up to ~100 mM NaCl and reaches a plateau at about ~200 mM NaCl. Interestingly, the sodium concentration in human cerebrospinal fluid (CSF) and in serum was reported to be 145.3 to 147.7 mM [120]. Moreover, the aggregation kinetics and thermodynamics measurements yield the following order of chloride cations for $A\beta_{40}$ peptide aggregation: $Mg^{2+} > Li^+ > Na^+ > K^+$, while sodium anions are ranked as: $SO_2^- > I^- > Cl^- > NO^- \approx ClO^-$ [118]. These series are known as lyotropic series and are predicted from an extension of classical colloidal coagulation theory, by including the effects of electroviscous drag [121, 122].

The critical association concentration of Aβ, or $(CAC)_{A\beta}$, represents the minimum concentration of Aβ leading to self-association, which depends among other parameters on the salt concentrations. For example, the experimental $(CAC)_{A\beta40}$ was reported to be: ~0.2 μM [123], ~0.7–1 μM [124], 0.88 ± 0.07 μM [125], and Hellstrand et al. [126] showed that spontaneous aggregation only occurs when $A\beta_{42}$ concentration is ~0.2 μM, all in the presence of salt. Novo et al. [127] reported that the *in vitro* $A\beta_{42}$ critical aggregation concentration, under physiological conditions, is about 0.091 ± 0.014 μM. However, since the experimental solubility of $A\beta_{42}$ is 0.04 μM [78], $(CAC)_{A\beta42}$ in the absence of salt should be less than 0.04 μM.

Bulk-induced nucleation and growth

This type of mechanism suggests that Aβ and tau association and aggregation occur in bulk solution, e.g., physiological fluid such as CSF and blood environment. In the case of $A\beta_{42}$ aggregation, the key amyloid formation steps have been reported [128, 129] as: 1) "the primary nucleation of new aggregates from monomers in solution" [130–132], 2) "the addition of monomers to fibrils ends resulting in their elongation" [76, 133, 134], and 3) "the secondary nucleation of monomers on the fibrils surface" [128, 135, 136]. All the above three microscopic processes occur during all three macroscopic phases (lag, growth and plateau), albeit at different rates, as governed by the rate constants, and concentrations of reacting species at each point on time [114, 137, 138].

In bulk solution, the secondary nucleation process shows a much lower energy barrier than primary

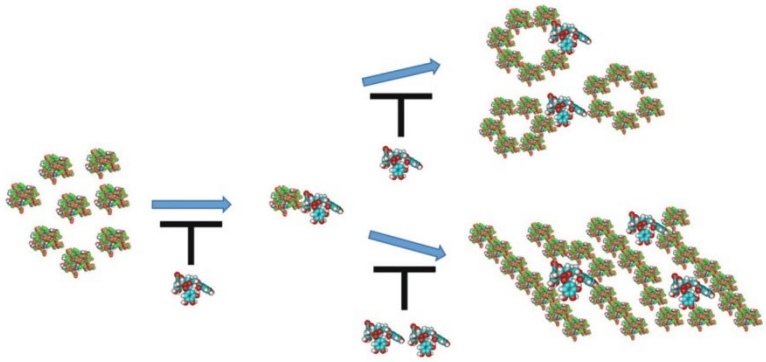


Fig. 2. Hypothesis of phenolic-induced altering and/or delaying amyloid-β (Aβ) self-association and the growth of oligomers/fibrils in bulk solution: (left) monomers of Aβ; (middle) Aβ/phenolic ligand complex; (right-top) Aβ-oligomers and complexed with phenolic ligands; and (right-bottom) Aβ-fibrils and complexed with phenolic ligands. The Aβ protein is green/red colored, and the phenolic ligand is blue/red colored. The black T-shape symbol refers to inhibition/delaying the growth of oligomers/fibrils. This cartoon is not to scale, i.e., the phenolic ligand is much smaller than Aβ-protein.

nucleation (~10 times) [139], where the surfaces of existing amyloid fibrils is catalyzing the formation of new pre-fibrillar aggregates from the soluble peptides [139, 140]. Moreover, Cohen et al. [129] reported the free-energy landscape for secondary nucleation.

Strikingly, even though the oligomers are the key source of fibrils, less than 10% of $A\beta_{42}$ oligomers successfully converted into fibrillar species, whereas the remaining 90% of the oligomers dissociated back to the monomeric form [141]. Michaels et al. [141] suggested that oligomer dissociation is 'spontaneous', whereas oligomer upconversion involves additional interactions with monomers, and may occur in bulk solution in contact with the fibril surface [142]. As for $A\beta_{42}$ and $A\beta_{40}$, the vast majority of oligomers do not form fibrils, but rather dissociates back to monomers. This type of mechanism has been ascribed to a non-classical nucleation process for $A\beta_{42}$ amyloid fibrils [141], but alternatively can be readily described by a dynamic equilibrium between oligomer formation and break-up. Aggregates break up with a certain inherent rate constant and are formed by collisions, which involve interactions between particles or molecules in solution. These processes are pH dependent, as are other aggregation processes of proteins in general.

Frankel et al. [143] investigated the mechanisms underlying $A\beta_{42}$ aggregation (0.8–10 μM) in human CSF through the kinetic experiments, though in healthy humans $A\beta_{42}$ concentration in CSF is around 250 pM. They also found that the aggregation process involves the same microscopic steps in CSF as in pure buffer, but the secondary nucleation rate constant is decreased [143].

Natural polyphenolic molecules can alter and/or delay Aβ self-association and the growth of oligomers/fibrils in bulk solution (Fig. 2).

It is noteworthy that using AFM and mica sheets functionalized with 1-(3-aminopropyl) silatrane (APS) showed that surface-induced aggregation occurs at a concentration at which no aggregation in solution is observed [144], e.g., likely below the critical association concentration of Aβ. The experiments were performed with full-size Aβ protein ($A\beta_{42}$, 0.1 μM), a decapeptide $A\beta_{14-23}$ (0.1 μM) and α-synuclein (0.01 μM); all three systems suggest a significant preference of the on-surface aggregation pathway compared to the aggregation in the bulk solution [144].

Surface-induced aggregation on biosurfaces other than Aβ- and tau-fibrils

So far, we have reported Aβ elongation/aggregation on fibril surfaces which is consistent with the related AD literature. However, other possible mechanisms must be highlighted since many more biosurfaces in human brain are available for Aβ/tau adsorption and aggregation, thus probably relevant to neurodegenerative diseases, such as AD.

In other words, aggregation can also occur on biosurfaces other than Aβ- and tau-fibrils, e.g., membranes, vesicles, endosomes, exosomes, micelles, erythrocytes/RBCs, platelets, albumin, and

blood vessels. The total length and surface area of human brain capillaries are ~600 km and ~20 m^2, respectively [145, 146]. For example, Aβ can be cleared via perivascular drainage pathways or deposited as neuritic plaques in the brain parenchyma or as cerebral amyloid angiopathy (CAA) along vessel walls [147]. When Aβ deposition occurs in brain capillaries (CAA type I), it tends to be widespread in the neocortex and hippocampus [148, 149], and associated with severe AD pathology [149, 150].

The human tau (hTau40) is also highly surface active and preferentially interacts with negatively charged membranes [151, 152]. Georgieva et al. [153] reported that lipid membranes efficiently facilitate *in vitro* tau aggregation. For their part, Yu et al. [154] reported that human islet amyloid polypeptide (hIAPP) aggregation was strongly enhanced by negatively charged membranes.

In addition, Aβ peptides interact with plasma proteins and RBC surface [155–158]. In the human body, 84% of the blood cells are RBCs and about 50% of the volume of blood (hematocrit) consists of RBCs [159], having an overall negative charge [160]. In human blood, circulating blood cells are exposed to nanomolar levels of soluble $A\beta_{40/42}$ [161]. Interestingly, Aβ deposits in the extracellular space of the brain and on the walls of cerebral blood vessels, mainly capillaries. A dynamic equilibrium between brain Aβ and plasma Aβ has been reported by DeMattos et al. [162]. Lan et al. [157] showed that 98% of AD peripheral RBCs were amyloid binding-positive. Kiko et al. [163] provided evidence that $A\beta_{40/42}$ were detected in RBCs. Moreover, $A\beta_{42}$ interacted with RBCs more avidly than $A\beta_{40}$ [164]; *in vitro* and *in vivo* experiments suggested that Aβ induces oxidative damage to RBCs [156, 164]. Morphological changes induced in RBCs, triggered by Aβ binding, was also observed [157, 165]. Remarkably, Aβ and tau as well as alpha-syn/Aβ and alpha-syn/tau heterocomplexes were also observed in RBCs [166, 167].

Interestingly, Koren et al. [168] postulated that circulating erythrocytes and likely also other blood cells might be coated by polyphenols from nutrients. The binding of polyphenols [168] and hydrolysable tannins [169] with RBC surface membrane have also been observed. RBCs and lipoproteins in blood showed to be reservoirs and transporters of polyphenols [170]. Harbi et al. [170] also determined the concentration of polyphenols associated with RBCs (intracellular+surface-bound); e.g., EGCG binds to RBC surface (33%) and intracellular (43%).

Moreover, Aβ peptides interact with platelet surfaces in a highly specific manner [171], with platelets being 4.9% of the number of cells in human body [159], and the main source of Aβ peptides in blood (~90%) [172, 173]. Wolozin et al. [171] also found that low levels of soluble Aβ (0.1–1 nM) augment adenosine-diphosphate(ADP)-dependent platelet aggregation. However, the ingestion of the polyphenol quercetin-4′-O-β-D-glucoside inhibited platelet aggregation in humans [174]. Nevertheless, the impact of polyphenols on aggregation might differ whether platelets are activated or not [175].

Biere et al. [176] found that the large majority of Aβ (~89%) is bound to albumin and specific lipoproteins in human plasma. Albumin is also an Aβ carrier [177]. Interestingly, Yeggoni et al. [178] showed that the natural polyphenol corilagin binds to human serum albumin and found the experimental binding constant of the complex to be $4.2 \pm 0.02 \times 10^5$/mol with a free energy of –7.6 kcal/mol, also supported by their computational MD results.

Real-time precise determination of the growth rates of protein aggregates on surfaces can be measured using a quartz crystal oscillator (surface) [179]. Although the quartz crystal microbalance with dissipation (QCMD) method [180] proved to be reliable to study Aβ aggregation [181, 182], there are very few publications on the interactions between polyphenols and Aβ on surfaces. For example, Wang et al. [183] showed a reduction of ~65% of the growth rates of Aβ monomer on curcumin-induced aggregates. By using QCMD combined with liquid AFM, Yu et al. [154] observations pointed toward a surface-involved pathway of protein adsorption and 2D amyloid aggregation. Surface plasmon resonance also showed to be an effective method to study the kinetic of $A\beta_{42}$ aggregation on carboxylated dextran-modified surfaces [184].

Surface-induced aggregation

Considering that Aβ [144] and tau [151–153] proteins are highly surface active and preferentially interact with negatively charged membranes, this can trigger adsorption on membranes followed by surface-induced aggregation where protein can interact with other proteins and polyphenols. Importantly, human physiological Aβ [143, 185, 186] and tau [187] concentrations are in the low nanomolar range. In 2017, Barnejee et al. [144] reported that on-surface *in vitro* aggregation of Aβ and α-synuclein occurs at a concentration at which no aggregation in bulk

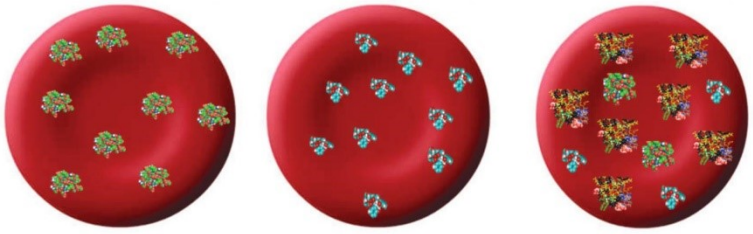


Fig. 3. A schematic of confinement stability: Adsorption of Aβ proteins (left), phenolic ligands (center) and Aβ/phenolic ligand complexes (right) on human RBC biosurface (red), results in stability by confinement, i.e., these proteins and ligands are unable to aggregate due to temporary or permanent confinement. This cartoon is not to scale, i.e., Aβ, ligand and complexes are much smaller than indicated (RBC diameter ~7.5–8.7 μm [268]).

solution is observed. Interestingly, in 2005 we proposed that surface-induced aggregation/clustering occurs, causing an induction period [188, 189].

Consequently, a strategy where surface-induced aggregation occurring below the critical aggregation concentration of Aβ and tau, treated at the earliest stage, might result in minimizing protein self-association, slowing down the aggregation processes and delaying the progression of AD.

Proposed concept: confinement stability

The adsorption of Aβ and tau on biosurfaces other than Aβ- and tau-fibrils, e.g., membranes, vessels, albumin, RBCs, etc., can lead to confinement stabilization, a new concept recently proposed [190]. For example, Fig. 3 shows that Aβ and/or tau proteins (left) and phenolic ligands (center) are confined and stabilized on RBC biosurfaces (red), which then prevents protein self-association and slows down the aggregation process, hence delaying the progression of AD. Moreover, polyphenolic ligands can associate with Aβ and/or tau proteins, forming complexes (right), and thus prevent or inhibit self-association and aggregation of these proteins.

The adsorption and confinement of soluble Aβ and tau on biosurfaces potentially influences a number of phenomena that might occur, such as structural changes, reconformation, increased local protein concentration, decreased protein entropy, alteration of physiological functions, and formation of toxic Aβ-oligomers and/or protofibrils, tau NFTs, etc. Moreover, these confined and stabilized proteins are unable to diffuse and collide with other proteins and/or cells in bulk solutions. Confinement also occurs in hydrogels and tissues where it modifies the folding and aggregation of proteins [191–193].

In AD, the impact of small molecules (proteins, polyphenols) and nanoparticles that are stabilized by RBCs/platelets/vessel walls/etc., through confinement stability, is still to be enlightened. Although RBC perturbation by Aβ protein has been reported [156], our hypothesis is that the overall impact of polyphenols will be beneficial since they will bind to Aβ and/or tau proteins, e.g., resulting in less oxidative damage.

We expect that our proposed concept of "confinement stability" highlights new and additional mechanisms implicating Aβ and tau pathways in the physiopathology of NDs, such as AD. Consequently, research is necessary not only to elucidate direct but also indirect pathways involving surface-induced processes occurring on biosurfaces other than Aβ- and tau-fibrils.

THERAPEUTIC STRATEGIES FOR DECREASING Aβ AND TAU PRODUCTION AND AGGREGATION

The complexity of Aβ/tau production and aggregation pathways, the role of the monomer/oligomers or aggregated forms of Aβ/tau, and the unknowns regarding the progression of AD highlight the challenges for the discovery of effective treatments to delay or prevent this disease. For the amyloidopathy, some strategies targeting the amyloidogenic pathways (e.g., β-secretase (BACE) inhibitor [194–197], the non-amyloidogenic pathways (e.g., α-secretase activator), as well as the γ-secretase inhibitors and modulators (e.g., [198]) were developed with limited success. For the tauopathy, some of the most promising therapies have been discussed by Simic et al. [199]: minimize tau phosphorylation, proteolysis, aggregation, clearance of intra- and extra-cellular

tau, and microtubules stabilization. Nevertheless, the need for fundamental understanding of Aβ and tau kinetics as well as aggregation inhibitors is imperative.

One strategy to mitigate AD may be the development of neuroprotective agents to prevent protein self-association, reduce Aβ and tau aggregation and/or induce the formation of non-toxic Aβ oligomers/tau NFTs. For example, polyphenolic ligand might prevent or delay Aβ and/or tau self-association at the earliest stage, e.g., below the critical association concentration of Aβ and tau proteins.

Table 1 shows molecular and pharmacological properties of some natural polyphenols and one commercial synthetic drug for comparison, e.g., donepezil (Aricept), an acetylcholinesterase inhibitor [200–203]. As expected, polyphenols are far less toxic than donepezil. For example, corilagin (β-1-O-Galloyl–3,6-(R)-hexahydroxydiphenoyl-D-Glucose) [204, 205] has an LD50 between 3500–5000 mg/kg b.wt. [206], suggesting a very low toxicity even at high dosages [206, 207] (Table 1). Moreover, corilagin, a hydrolysable tannin, shows numerous pharmacological properties [208–213]. Corilagin (more rigid) is a close analogue of 1, 3, 6-tri-O-galloy-β-D-glucose (TGG) (more flexible), with similar molecular properties (Table 1) [84, 204]. This type of molecules also has remarkable properties for stabilizing and/or destabilizing colloidal systems [188, 189]. For example, we proposed a surface-induced clustering mechanism where corilagin/poly(ethylene oxide) clusters slowly built on microcellulose surfaces (corresponding to the induction period) [189]. Very little or no research has been performed on the inhibiting effects of corilagin and TGG on the kinetics of Aβ and tau aggregation/disaggregation bioprocesses. Nonetheless, their complementary structures (rigid versus flexible) might be relevant in the context of AD [84]. For comparison, 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose (PGG) alone inhibited: i) $A\beta_{40}/A\beta_{42}$ fibril formation; ii) Aβ aggregation at low concentrations (IC50 = 3 μM); and iii) neurotoxic Aβ oligomer formation [214]. Interestingly, both TGG and PGG molecules share similar properties, e.g., hydrophobicity and flexibility [84, 204]. Consequently, both natural polyphenols corilagin and TGG have these physicochemical attributes, thus potential candidates for AD treatment.

In addition, the effective concentrations (EC50) of TA on the formation, extension, and destabilization of preformed Aβ fibrils ($fA\beta_{40}$ and $fA\beta_{42}$), are in the order of less than 0.1 μM [215], and the *in vitro* IC50 are 0.012 and 0.022 μM for $A\beta_{40}$ and $A\beta_{42}$, respectively [216]. TA also inhibited the *in vitro* aggregation of tau peptide R3, with an IC50 of 3.5 μM [217]; however, the inhibitor GA was less effective with an IC50 of 92 μM [217]. Moreover, TA shows low toxicity, e.g., LD50 of 2260 mg/kg (oral rat) (Table 1).

EGCG, with an LD50 of 2170 mg/kg (mice), remodels large oligomers/fibrils into less toxic off-pathway assemblies [218]. Among fifteen secondary metabolites from plants, EGCG, myricetin, silibinin, and luteolin lowered the Aβ aggregation below 40% [219]. Moreover, EGCG inhibited the *in vitro* tau aggregation [220], in addition both EGCG and curcumin facilitated clearance of hyperphosphorylated tau [218].

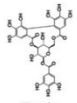
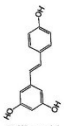
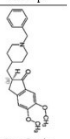
There is a wide variety of mechanisms by which polyphenols show neuroprotective effects [221]. For instance, earlier studies have reported IC50 values of myricetin against of $A\beta_{40}$ and $A\beta_{42}$ aggregation within 0.2 to 0.9 μM [222, 223]. Moreover, fisetin (3, 3′,4′,7-tetrahydroxyflavone) inhibited $A\beta_{42}$ aggregation [224]. Their results suggest that the 3′,4′-dihydroxyl group, but not the 3- or 7-hydroxyl group, is critical for the inhibitory effect on the formation of $A\beta_{42}$ fibrils [224]. The polyphenol isomers vescalagin and castalagin protect SH-SY5Y neuroblastoma cells by reducing the toxicity of $A\beta_{42}$ oligomers [225]. Vescalagin totally inhibited aggregation at an $A\beta_{42}$:polyphenol ratio of 1 : 1. Both vescalagin and castalagin decreased the amount of parallel β-sheets, and induced rearrangement of peptides into helix, anti-parallel β-sheets and other secondary structures [225]. Morin attenuates tau hyperphosphorylation by inhibiting GSK3β, implicated in AD pathogenesis, and showed the strongest inhibition in the GSK3β activity assay [226].

Potential strategies for effective anti-dementia drugs do not only focus on the inhibition of oligomer/fibril formation, but also the destabilization/disaggregation of pre-aggregated Aβ- and/or tau-fibrils, or a combination of them. Unsurprisingly, numerous polyphenols have this ability as evidenced in the following section.

DESTABILIZATION AND DISAGGREGATION OF Aβ AND TAU

Fibrils destabilization serves the dual purpose of deformed fibrils becoming non-neurotoxic themselves and further inhibiting the formation of

Table 1
Molecular and pharmacological properties of some natural polyphenols related to Aβ and tau aggregation/disaggregation and comparison with donepezil

Parameters	Corilagin	TGG	Tannic acid (TA)	EGCG	Gallic acid (GA)	Curcumin (Keto)	Resveratrol (RES)	Donepezil; Aricept
Molecular structure								
Type of molecule	Tannin	Tannin	Tannin	Flavonoid	Phenolic acid	Curcuminoid	Stilbenoid	Synthetic
Molecular formula	$C_{27}H_{22}O_{18}$	$C_{27}H_{24}O_{18}$	$C_{76}H_{52}O_{46}$	$C_{22}H_{18}O_{11}$	$C_7H_6O_5$	$C_{21}H_{20}O_6$	$C_{14}H_{12}O_3$	$C_{24}H_{29}NO_3$
Molar mass (g/mol)	634	636	1701	458	170	368	228	379
Inhibition of Aβ/Tau aggregation	Yes[a]/Yes	Yes[a]/Yes	Yes/Yes	Yes/Yes	Yes/?	Yes/Yes	Yes/Yes	Yes/Yes
Disaggregation of Aβ-/Tau-fibrils	NA/NA	NA/NA	Yes/?	Yes/?	Yes/?	Yes/?	Yes/Yes	?/?
Enzyme inhibition or enhancement	BACE1[b] (IC50 = 34 μM); PEP[c] (IC50 = 0.236 μM)	PEP[c] (IC50 = 0.157μM)	BACE	Enhances α-Secretase[d]	?	BACE	Promotes non-amyloidogenic pathways[e]	AChE, BACE1
Hydrolysable	Yes	Yes	Yes	Yes	?	Yes	Yes	No
Phenolic rings (#)	Yes (3)	Yes (3)	Yes (10)	Yes (3)	Yes (1)	Yes (2)	Yes (2)	No (0)
Phenolic-OH (#)	9	9	25	8	3	2	3	0
Hydrophobicity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Solubility in water (g/L)	5.0 in PBS @pH 7.2	1.6 @pH 5–7	Very high	~5	11.9 @20°C	0.4 @pH 7.3	~0.03	0.00293 @25°C
Biotransformation: metabolites/derivatives	EA, GA, M3[f]	GA	GA, PY	GA, EGC, EGC-M5	4-OMGA	DHC, THC	Trans-RES-3-O-glucuronide[g]	?
Bioavailability (Oral)	?	NA	NA	Low (~0.1–0.26%)	High	Low	Poor (< 1%)	Very high
Toxicity LD_{50} (mg/kg per b.weight)	3500–5000 (mice)	NA	2260 (Oral rat)	2170 (mice)	5000 (Oral rat)	> 2000 (mice)	7060 (rat)	32.6 (Oral rat)

Corilagin, β-1-O-Galloyl-3,6-(R)-hexahydroxydiphenoyl-D-Glucose; TGG, 1,3,6-Tri-O-Galloyl-β-D-Glucose; EGCG, epigallocatechin-3-gallate; [a]Binding with Aβ based on theoretical calculations (MD and HREX); MD, molecular dynamics; HREX, Hamiltonian Replica Exchange; [b][269]; [c]PEP, Propyl endopeptidase enzyme [270]; [d][218]; [e]Decreases the level of Aβ by inducing non-amyloidogenic cleavage of AβPP [271, 272]; [f]Corilagin hydrolyzed metabolites (EA, GA, and M3 (C20H18O14) [273, 274]); [g]Primary metabolite in human liver (Trans-RES-3-O-glucuronide) [274, 275]; NA, not available; EA, ellagic acid; EGC, epigallo-catechin; PY, pyrogallol; EGC-M5, 5-(3′, 4′, 5′-trihydroxyphenyl)-γ-valerolactone and 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone (human major urinary metabolites of tea polyphenols); PBS, phosphate-buffered saline; DHC, dehydrocurcumin; THC, tetrahydrocurcumin; 4-OMGA, 4-O-methylgallic acid.

higher-order aggregates [227, 228]. Freyssin et al. [229] reported the significant role of polyphenols on aggregation and disaggregation of amyloid peptides, tau, and α-synuclein, in line with dispersive properties of polyphenols such as tannins [230].

Bieschke et al. [231] showed EGCG to inhibit $A\beta_{42}$ fibrillogenesis, but also the ability to convert large, mature Aβ fibrils into smaller amorphous protein aggregates. Immuno-infrared sensor data are consistent with the degradation of Aβ fibrils induced by EGCG [232]. Gallic acid was shown to inhibit amyloid fibril formation (molar ratio Aβ:GA of 1:2) and to disaggregate preformed fibrils [233, 234]. Adding GA to the aggregated $A\beta_{42}$ fibrils for 2 h clearly reduced the $A\beta_{42}$ fibril particle size from predominantly 100 nm fibrils to ~60 nm [234]. Fujiwara et al. [214] showed, *in vitro* and *in vivo*, that PGG disaggregated preformed Aβ fibrils. After incubation of 25 μM of fresh $fA\beta_{40}$ and $fA\beta_{42}$ with 50 μM TA, Ono et al. [215] showed that TA destabilized preformed Aβ fibrils. Liu et al. [233] proposed that the gallate group in GA (and related compounds) is the structural motif that prevents fibril formation.

Curcumin showed *in vivo* [235], and *in vitro* [227], the ability to inhibit Aβ aggregation and to disaggregate preformed Aβ fibrils. The activity on insulin-degrading enzyme toward $A\beta_{42}$ in the presence of resveratrol results in a substantial increase in $A\beta_{42}$ fragmentation compared to the control [236]. Sun et al. [237] demonstrated that tau alone gave long fibrils, while 50 μM resveratrol induced the formation of short tau fibrils, and 200 μM resveratrol led to smaller aggregates. Vion et al. [238] showed that both resveratrol and trans ε-viniferin, at 1 μM, induced disaggregation of pre-aggregated $A\beta_{42}$ peptide. Caillaud et al. [239] also reported that trans ε-viniferin reduces the size and density of amyloid deposits and decreases reactivity of astrocytes and microglia.

Khan et al. [240] provided a synopsis on the relationship between quercetin and cognitive performance in AD. Quercetin and rutin inhibit the formation of Aβ fibrils and disaggregated Aβ-fibrils [195]. Moreover, quercetin displays fibril destabilizing effects on preformed fibrillar Aβ, reversing Aβ-induced neurotoxicity [241]. Dihydroquercetin (Taxifolin), also disassemble Aβ *in vitro*, reduced levels of Aβ oligomers *in vivo*, and restored decreased cerebral blood flow as well as cerebrovascular reactivity in Tg-SwDI mice [61].

Dihydromyricetin or anthocyanins/anthocyans also reduces fibrils formation and disaggregates preformed fibrils [242–244]. Interestingly, only amorphous aggregates were formed when the molar ratio of $A\beta_{40}$ to dihydromyricetin was 1:3 [243].

Although AD pathways and the related polyphenols are constantly investigated, the Gordian knot has not been untied yet. To solve this problem, we still need additional scientific knowledge and tools. For example, many questions related to NDs cannot be handled experimentally and could benefit by using advanced computational molecular methods.

COMPUTER SIMULATION OF PHENOLIC LIGANDS WITH AβAND TAU

After decades of experimental research where major advancements have been achieved, we still need to identify the real cause of AD. Consequently, computational methods, often named '*in silico*' approaches, can accelerate the development, where scientists can generate experimental data while molecular theoretical calculations are processing. However, even after almost 20 years of amyloid aggregation MD simulations, there are still limitations, more specifically: force field, protein concentration, and simulation length challenges [245].

Molecular modeling (MM) encompasses all theoretical and computational methods used to model or mimic molecular behavior. MM development began in the early 1960s, although the underlying math originated much earlier. The common feature of all MM methods is the atomistic-level insights it provides. Numerous methods exist: e.g., molecular mechanics [246], semi-empirical [247, 248], density functional theory [249], molecular dynamics (MD) [250], ab-initio [251], as well as sampling methods such as replica exchange molecular dynamics (REMD) [252–254], and molecular docking [255–257]. The selection of methods hinges on whether a quick answer from classical methods (e.g., molecular mechanics) is desired, as opposed to highly accurate results from quantum mechanics-based calculations (e.g., ab-initio molecular orbital calculations) but time-consuming.

The therapeutic area where computational methods impact most is in the small-molecule drug discovery area, e.g., polyphenols. Virtually all small molecule drugs work by binding to proteins, enzymes, receptors, and ion channels, and sometimes DNA or RNA. The binding of small molecule, or ligand, to the targeted protein induces a biological response. Small molecule drugs are popular, because they are easy to produce, distribute and administer,

and easily chemically modified to fine tune the effects of the drug. Modelling methods can guide chemists to synthesize molecules with improved binding to the protein, its activity. By improving the binding of the target molecule with the desired protein target and reducing binding with undesirable related protein targets, ligands can be made more selective, an attribute which reduces many side effects. Generally, pharmacokinetic properties can be predicted with an in-silico method, allowing researchers to avoid wasting resources on compounds that will either be too toxic, or have the wrong biological transport properties for a successful drug. In silico methods are a way of reducing the chemical search space, by helping to design experiments, and glean as much information as possible, from existing experimental data.

Given the experimental difficulties in terms of predictability from mouse to human *in vitro/in vivo* clinical experiments, computer modelling (in silico) has emerged as a reliable tool to elucidate brain chemistry. Due to their importance and size, both highly intrinsic disordered $A\beta_{40}$ and $A\beta_{42}$ proteins have been extensively studied. For example, using a coarse-grained force field coupled to Hamiltonian-temperature replica exchange MD simulations, the equilibrium structures of $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{40}$ (D23N) monomers [258], and dimers [259], were determined. They observed striking morphological differences [258], and they also showed that $A\beta_{42}$ dimer has a higher propensity than $A\beta_{40}$ dimer to form β-strands at the central hydrophobic core (residues 17–21) and C-terminal (residues 30–42), i.e., critical segments for Aβ oligomerization. Chiricotto et al. [260] applied the multi-scale Lattice Boltzmann Molecular Dynamics method (LBMD) to study the initial phases of the hydrophobic central core of amyloid peptide ($A\beta_{16–22}$; KLVFFAE) aggregation.

Using MD simulations, Zhao et al. [261] studied the early adsorption and conformational change of Aβ oligomers from dimer to hexamer on three different self-assembled monolayers (SAMs) terminated with CH3, OH, and COOH groups. Combining with experimental results, all SAM model surfaces exhibited a seeding effect for Aβ polymerization [261].

Results from the virtual oligomerization inhibition were in excellent agreement with the experimental results, of the performance of six known Aβ aggregation inhibitors: brazilin, curcumin, EGCG, ELND005, resveratrol, and tacrine [262]. However, only EGCG is still active at phase III, while some of them were terminated due to the lack of efficacy. The EGCG ligand strongly interacted with most residues of $A\beta_{16–22}$, notably F19 and F20. Interestingly, with EGCG the oligomerization time was significantly delayed: e.g., i) control (7, 20, and 48 ns), and EGCG-$A\beta_{16–22}$ (16, 50, and 106 ns), for dimer, trimer, and tetramer, respectively [262]. Using a combination of *in vitro* experimental measurements and in silico methods, Acharya et al. [232] found that the most favorable GlideScore (–6.9; docking site #1) was obtained when EGCG binds inside the fibril, stabilized by π-stacking interactions with residue F19 [232]. Zhan et al. [263] showed both EGCG and EGC disruptive capacity on the newly cryo-EM resolved LS-shaped $A\beta_{42}$ protofibrils by breaking the hydrogen bond between H6 and E11 through $\pi-\pi$ interactions with residues H14/Y10 and hydrogen-bonding interactions with E11 [263].

The interactions between dihydromyricetin and $A\beta_{40}$ trimer were mainly nonpolar, and where MD simulation showed the key $A\beta_{40}$ interacting residues are V18, A21, and D23 [243]. Mechanistic insights from MD suggest that morin can penetrate into the $A\beta_{42}$ hydrophobic core to disrupt the Asp23-Lys28 salt bridge and interfere with backbone hydrogen bonding [264]. Also, Lemkul et al. [264] reported that morin inhibits the early stages of Aβ peptide aggregation. Gargari and Barzegar [265] showed that flavonoids (myricetin, morin) exert dual and more effective functions against monomeric aggregation-prone state (fibrillogenesis suppression) and remodel the Aβ aggregation pathway (fibril destabilization).

Cyanidin-3-O-glucoside (Cy-3-G) inhibits $A\beta_{40}$ fibrillogenesis, disrupts the β-sheet structure, disaggregates preformed fibrils, and reduces amyloid cytotoxicity [266], e.g., when the $A\beta_{40}$:Cy-3G ratio was 1:3, the inhibitory effect on $A\beta_{40}$ fibril formation was about 95%. Cy-3G mainly interacted with: N-terminal region, central hydrophobic cluster and β-sheet region II via hydrophobic and electrostatic interactions [266].

In vitro and in silico results from Guéroux et al. [267], showed the ability of some polyphenols from the procyanidin family, to specifically bind the proline-rich region of tau. Interestingly, the galloylated procyanidins ECG and EGCG exhibit a higher affinity with respect to the non-galloylated procyanidins [267]. Theoretical and experimental results indicated that tau interacts with TA by forming a hairpin structure, hence, a feature for inhibiting tau polymerization [217].

More than ever, computing capacity and MM methods are transforming our understanding of the

brain chemistry and more specifically the underlying mechanisms of amyloid peptides aggregation and disaggregation involved in NDs, such as AD.

CONCLUSIONS

Physicochemical interactions between protein and natural ligands play a major role in numerous bioprocesses. Those have to be addressed to better understand the mechanisms and responses of natural polyphenolic ligands/protein complexation. The Aβ peptide is an endogenous compound involved in several NDs, such as AD which nucleates decades before a conclusive diagnostic. The amyloid-cascade hypothesis has provided the main framework for understanding the AD pathogenesis, where the basis is the pathogenic Aβ peptides aggregation into neuritic plaques. Yet, this hypothesis must integrate the contributions of other proteins such as tau, which is involved in more than 20 neurodegenerative diseases, including AD. Consequently, it is critical to intervene at the earliest stage (e.g., nucleation, induction period) of the disease and to select the effective chemistry with the right mechanisms. This complex task not only needs highly sophisticated medical experimental knowledge, but also theoretical molecular methods which helps to explore the underlying mechanisms. To achieve this, a multidisciplinary approach, combining experimental and theoretical methods, can be more effective to characterize the biochemical and biophysical interactions between proteins and phenolic ligands.

Many pathways have been investigated to mitigate and delay amyloid- and tau-opathies, e.g., targeting amyloidogenic pathways (e.g., BACE inhibitor), and non-amyloidogenic pathways (e.g., α-secretase activator) as well as γ-secretase inhibitors and modulators, inhibit the nucleation process of formation of oligomers/protofibrils/fibrils, alter/reduce oligomer/NFTs cytotoxicity, disaggregate pre-aggregated fibrils, etc. Nonetheless, one critical pathway is to prevent self-association at the early stages of AD (induction period), and below the Aβ and tau critical association concentrations.

The pathways described in this review are mainly related to amyloid- and tau-based surfaces (e.g., protofibrils, fibrils, fibers, NFTs, etc.). However, our review suggests a sub-ensemble which includes surface-induced processes occurring on biosurfaces other than Aβ- and tau-fibrils, e.g., membranes, vesicles, blood vessels, RBCs, platelets, and likely relevant to NDs. For example, the adsorption of Aβ and tau on RBC biosurfaces, can either lead to confinement stabilization or to surface-induced aggregation, depending on the affinity of Aβ and tau, and Aβ/phenolic ligand complexes, to these surfaces. Bioprocesses occurring on surfaces other than Aβ- and tau-fibrils, can be analyzed under a new concept, referred as confinement stability (Fig. 2). For example, adsorption of Aβ/tau proteins, phenolic ligands and Aβ/phenolic ligand complexes on human RBC biosurface, results in stability by confinement, i.e., these proteins, ligands, and complexes are unable to aggregate due to temporary or permanent confinement. Overall, this sub-ensemble may also participate indirectly in mitigating neurodegenerative diseases, by preventing protein self-association, slowing down the aggregation process, and delaying the progression of AD.

In any case, it is imperative to develop strategic pathways that will work at the very early stages and below the CAC of Aβ and tau, i.e., even before they form dimers, trimers, and oligomers.

ACKNOWLEDGMENTS

The authors are grateful for the generous financial support by the R. Howard Foundation and La Fondation Famille Lemaire. Professors Mousseau's, Ramassamy's and van de Ven and Dr. Gaudreault's work is also supported, in part, by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Research Chair Louise & André Charron on Alzheimer's disease.

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/20-1549r1).

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