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À mes parents,
Fatime et Ahmed

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LISTE DES ABRÉVIATIONS, DES SIGLES ET DES ACRONYMES

24-HOC	24-Hydroxycholesterol
AA	Acide arachidonique
ABC	*ATP binding cassette*
ADN	Acide désoxyribonucléique
ADNc	Acide désoxyribonucléique complémentaire
ApoD	Apolipoprotéine D
ApoE	Apolipoprotéine E
APRE	*Acute phase responsive element*
ARNm	Acide ribonucléique messager
BBP	*Bilin Binding Protein*
BDNF	*Brain-Derived Neurotrophic Factor*
BHE	Barrière hémato-encéphalique
Biot-apoD	*Biotinylated apoD*
BSA	*Bovine serum albumin*
BSG	Basigine
CETP	*Cholesteryl ester transfer protein*
CNS	*Central nervous system*
Cox-2	Cyclooxygenase-2
CypA	Cyclophiline A
DMEM	*Dulbecco's modified Eagle's medium*
EDTA	Acide éthylène-diamine tétraacétique
ELISA	*Enzyme-linked immunosorbent assay*
EMMPRIN	*Extracellular Matrix Metalloproteinase Inducer*

ERE	*Estrogen response element*
FGF-2	*Fibroblast Growth Factor-2*
FITC	*Fluorescein isothiocyanate*
GAP-43	*Growth associated protein 43*
GAPDH	*Glyceraldehyde 3-phosphate dehydrogenase*
GCDFP-24	*Gross cystic disease protein-24 kDa*
GFAP	*Glial fibrillary acidic protein*
GFP	*Green fluorescent protein*
Glaz	*Glial Lazarillo*
GRE	*Glucocorticoid response element*
H-apoD	*Human apoD*
H-apoD Tg	Souris transgénique exprimant H-apoD
HEK293T	*Human embryonic kidney 293T*
HG-BSG	*High glycoform-Basigin*
HRP	*Horseradish peroxidase*
IL	Interleukine
Ka	Constante d'association
KA	Acide kaïnique
Kd	Constante de dissociation
KO	*Knock-out*
LCAT	Lécithine cholestérol acyltransférase
LCR	Liquide céphalorachidien
LDL	*Low density lipoprotein*
LG-BSG	*Low glycoform-Basigin*
LPS	Lipopolysaccharide
M-apoD	*Mouse apoD*, apoD murine
MCT	*monocarboxylate transporters*
MMP	Matrix metalloproteinase

NF-kB	*Nuclear factor kappa B*
NFT	*Neurofibrillary tangle*
NPC	Niemann-Pick de type C
PARP-1	*Poly(ADP-ribose) polymerase-1*
PBS	*Phosphate buffered saline*
PFA	Paraformaldéhyde
PKC	Protein kinase C
PLA2	Phospholipase A2
PMCA2	*Plasma membrane calcium ATPases 2*
PPAR	*Peroxisome proliferator-activated receptor*
PPIA	*Peptidylprolyl isomerase A*
PRE	*Progesterone response element*
PVDF	*Polyvinylidene difluoride*
RAR	*Retinoic acid receptor*
RBP	*Retinol-Binding Protein*
ROS	*Reactive oxygen species*
SDS-PAGE	*Sodium dodecylsulfate-polyacrylamide gel electrophoresis*
SNC	Système nerveux central
SNP	Système nerveux périphérique
SR-BI	*Scavenger receptor class BI*
Tf	*Transferrin*
Tg	Transgénique
TGF-β	*Transforming growth factor beta*
TMA	Traumatisme médullaire aigu
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	*Tumor necrosis factor alpha*
UV	Ultra-violet
VLDL	*Very low density lipoprotein*

VSMC	*Vascular smooth muscle cells*
WT	*Wild-type*
ZSV	Zone sous-ventriculaire

RÉSUMÉ

L'apolipoprotéine D (apoD), largement synthétisée dans le système nerveux central (SNC), est une lipocaline dont le rôle principal est le transport de petites molécules hydrophobes. Son expression est augmentée suite à des lésions et des dégénérescences, telles que les maladies d'Alzheimer, de Parkinson et de Niemann-Pick. Cette importante modulation de l'expression de l'apoD s'établit spécifiquement au niveau des sites de lésions dans le SNC, suggérant ainsi un possible rôle de l'apoD dans la protection des neurones en situation de stress. Plusieurs études démontrent d'ailleurs que la surexpression de l'apoD protège la souris contre des dégénérescences induites par un stress inflammatoire et oxydatif. Il est donc important de déterminer la relation fonctionnelle qui existe entre l'expression de l'apoD dans le SNC et le processus de neuroprotection dans un contexte neuropathologique et lésionnel impliquant de nombreux mécanismes d'aggravation de l'état neurodégénératif (par exemple le phénomène d'excitoxicité). C'est pourquoi nous nous sommes intéressés, dans cette thèse, à mieux comprendre les mécanismes impliqués dans l'effet neuroprotecteur de l'apoD face à une lésion neurodégénérative.

Ces travaux ont tout d'abord permis de mettre en évidence, à l'aide de souris transgéniques surexprimant l'apoD humaine dans leur SNC, de nouveaux mécanismes de neuroprotection suite à l'excitotoxicité induite par injection de kaïnate. Ainsi, nous avons pu établir que la surexpression de l'apoD dans le SNC induit une résistance accrue aux convulsions et atténue de manière significative la réponse inflammatoire, conférant ainsi une protection contre l'apoptose induite par le kainate. De plus, nos résultats suggèrent que cette protection de l'apoD pourrait être attribuée à sa capacité i) de moduler l'expression de protéines clés dans le mécanisme d'excitotoxicité (PMCA2 et NR23B) dans l'hippocampe et ii) de réguler la distribution du cholestérol dans les neurones, surtout que l'internalisation de l'apoD est accentuée dans les neurones matures et en condition de stress. Par la suite, nos travaux ont permis de démontrer que l'internalisation de l'apoD nécessite la présence d'un récepteur. En effet, nos résultats ont montré que la modulation de la basigine affecte le processus d'internalisation de l'apoD, suggérant que la basigine serait potentiellement le récepteur de l'apoD. Par ailleurs, certaines mutations localisées dans la région de liaison de l'apoD ont affecté son processus d'internalisation.

L'ensemble des travaux présentés dans cette thèse ont donc permis de confirmer l'effet neuroprotecteur de l'apoD face à la neurodégénérescence en élucidant en partie les mécanismes impliqués dans cette neuroprotection.

Mots clés : apolipoprotéine D, excitotoxicité, acide kaïnique, cholestérol, neurone, basigine

INTRODUCTION

Les maladies neurodégénératives constituent un groupe de maladies dégénératives induisant un dysfonctionnement du système nerveux de manière progressive. Ce processus délétère aboutit généralement à une altération du fonctionnement des cellules nerveuses, menant ainsi à leur mort. De par leur diversité, ces neuropathologies constituent un redoutable fléau pouvant affecter autant le cerveau que la moelle épinière, autant les fonctions motrices que supérieures, autant l'enfant et le jeune adulte que de faire partie des maladies liées au vieillissement. Ces maladies peuvent affecter l'ensemble des constituants du système nerveux (les neurones comme la myéline) mais peuvent également se limiter à des régions très précises. Malgré la grande variété de leurs symptômes, toutes ces maladies neurodégénératives ont un point commun qui fait finalement leur unité : toutes sont caractérisées par une dégénérescence progressive et une absence de substances thérapeutiques, ce qui en fait un fléau redoutable. A un moment de sa vie, un Canadien sur trois (soit environ 10 millions de Canadiens) sera touché par un trouble neurologique ou psychiatrique. L'augmentation de l'espérance de vie et le vieillissement de la population ne feront, malheureusement, qu'aggraver ce bilan.

L'apolipoprotéine D est une lipocaline largement exprimée dans le système nerveux. Son expression se trouve être augmentée dans de nombreuses maladies neurodégénératives telles que les maladies d'Alzheimer, de Parkinson, de Niemman Pick de type C et les scléroses multiples. De nombreuses études suggèrent que l'apoD permettrait de promouvoir la réparation du tissu nerveux.

Ainsi, l'utilisation de l'apoD, comme un outil thérapeutique potentiel pour traiter les

maladies neurodégénératives, pourrait être envisagée. Pour cela, il reste encore à définir, d'une part, si cet effet neuroprotecteur de la surexpression de l'apoD est également observé au niveau d'autres mécanismes bien établis dans un grand nombre de maladies neurodégénératives et d'autre part, les mécanismes moléculaires responsables de cet effet bénéfique de l'apoD.

CHAPITRE I

ETAT DES CONNAISSANCES

L'apolipoprotéine D (apoD) est une glycoprotéine appartenant à la famille des lipocalines, dont le rôle principal est le transport de petites molécules hydrophobes. Plusieurs études ont permis de mettre en évidence la forte expression de cette protéine dans de nombreux organes autres que le foie et les intestins qui sont habituellement les organes producteurs des apolipoprotéines (Provost et al., 1991b; Seguin et al., 1995; Smith et al., 1990). De par sa large distribution tissulaire et son association avec une grande variété de ligands (tels que plusieurs stéroïdes, le cholestérol, la bilirubine et l'acide arachidonique), l'apoD semble donc être une protéine multifonctionnelle dont le rôle pourrait varier d'un organe à un autre (Rassart et al., 2000).

1.1 Caractéristiques de l'apolipoprotéine D

L'apoD a été initialement découverte par McConathy et Alaupovic en 1973 (McConathy and Alaupovic, 1973). Il s'agit d'une glycoprotéine, qui a principalement été associée aux lipoprotéines de haute densité (HDL). D'autre part, selon les caractéristiques de sa structure primaire, l'apoD est une apolipoprotéine atypique et appartient en fait à la famille des lipocalines.

1.1.1 Structure de l'apoD

1.1.1.1 Structure du gène

Le gène de l'apoD humaine est localisé au niveau du chromosome 3 (Drayna et al., 1987) tandis que, chez la souris, il se retrouve sur le chromosome 6 (Warden et al., 1992). Le promoteur de l'apoD humaine présente plusieurs éléments régulateurs. Parmi ceux-ci, figurent les éléments de réponse aux hormones stéroïdes, telles que l'œstrogène, la progestérone et les glucocorticoïdes (respectivement connus comme ERE, PRE et GRE), mais également les éléments de réponse aux acides gras, au sérum, à la phase aigüe ainsi qu'à des facteurs de transcription impliqués dans la réponse inflammatoire, tels que NF-kB (Nuclear factor kappa B) (Do Carmo et al., 2002; Do Carmo et al., 2007; Lambert et al., 1993). La présence de ces nombreux éléments régulateurs sur le promoteur de l'apoD humaine reflète la complexité de la régulation de l'expression de cette protéine.

1.1.1.2 Structure de la protéine

La séquence en acides aminés de l'apoD ne montre pas de similarité avec d'autres apolipoprotéines, mais présente plutôt une grande homologie avec les membres de la famille des lipocalines. En effet, parmi ces lipocalines, on note 25% d'homologie entre l'apoD et la Retinol-Binding Protein (RBP) ainsi que 30 à 40% d'homologie avec la Bilin Binding Protein (BBP) qui est une protéine d'insecte (Drayna et al., 1987; Weech et al., 1991). La séquence déduite à partir de l'ADN complémentaire (ADNc) de l'apoD révèle la présence d'un peptide signal de 20 acides aminés, suivi d'une protéine mature de 169 acides aminés (Drayna et al., 1987). Le poids moléculaire de l'apoD calculé à partir de son ADNc est de 18 kDa. Cependant, à cause de la présence de deux sites de glycosylation sur les résidus d'asparagine en position 45 et 78, le poids moléculaire apparent de l'apoD varie entre 20 et 32 kDa

(Rassart et al., 2000; Schindler et al., 1995; Weech et al., 1991; Yang et al., 1994). Cette glycosylation constitue environ 18% de la masse de la protéine (Drayna et al., 1987). La structure tertiaire de l'apoD révèle la présence de huit brins de feuillets β antiparallèles formant une cavité hydrophobe ouverte au sommet et dont la base fermée est de forme conique (Figure 1.1) (Eichinger et al., 2007; Peitsch and Boguski, 1990). Cette structure en forme d'entonnoir permet le transport ou la liaison des différents ligands de l'apoD qui viennent se loger dans sa cavité hydrophobe.

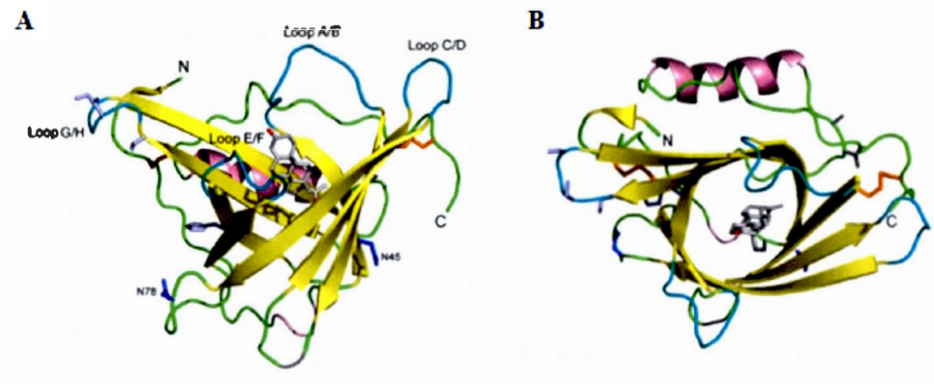

Figure 1.1 Représentation de la structure tertiaire de l'apoD formant un complexe avec la progestérone.
(**A**). Représentation transversale. L'analyse cristallographique de l'apoD montre (vue transversale A) qu'elle est constituée de 8 brins de feuillets β antiparallèles (en jaune) formant une cavité hydrophobique. Ces brins sont reliés entre eux par quatre boucles à l'entrée de cette cavité (en bleu clair) connues sous le nom de 'loop' A/B, 'Loop' C/D, 'Loop' E/F et 'Loop' G/H. D'autres boucles (en vert) sont également présentes du côté opposé de l'entrée de la cavité. On retrouve aussi deux hélices alpha (en mauve), dont une située sur le côté de la cavité et l'autre à l'extrémité opposée de l'entrée de la cavité, permettant la fermeture de cette cavité. Les sites de glycosylation (indiqués en bleu) sont localisés sur les résidus asparagine à la position 45 (N45) et 78 (N78).(**B**). Représentation axiale. Cela illustre l'intérieur de la cavité dans laquelle se logent les ligands de l'apoD tels que la progestérone (en gris).
Figure adaptée de (Eichinger et al., 2007).

Les feuillets sont reliés entre eux, à l'entrée de cette cavité, par quatre boucles connues sous le nom de 'loop' A/B, C/D, E/F et GH (Figure 1). Trois de ces quatre boucles, c'est-à-dire les boucles 'loop' A/B, 'loop' E/F et 'loop' G/H, présentent des chaînes hydrophobes. Il a été suggéré que ces boucles hydrophobes exposées pourraient permettre à l'apoD de s'insérer dans la phase lipidique des HDL ou au niveau de la membrane des cellules (Eichinger et al., 2007) grâce à la présence de résidus de cystéine. L'apoD humaine contient 5 résidus cystéine dont 4 forment des ponts disulfures intramoléculaires. Le cinquième résidu cystéine reste libre pour former des ponts disulfures avec d'autres protéines associées aux HDL dont l'apoA-I, l'apoA-II et la lécithine cholestérol acyl-transferase (LCAT) (Blanco-Vaca et al., 1992; Fielding and Fielding, 1980; Weech et al., 1991; Yang et al., 1994). L'extrémité opposée de l'ouverture de la cavité de l'apoD, qui est de forme conique, pourrait quant à elle constituer le site d'interaction de l'apoD avec son éventuel récepteur (Eichinger et al., 2007).

1.1.2 Ligands de l'ApoD

1.1.2.1 Les hormones stéroïdiennes

L'apoD, constituant la protéine majeure du fluide kystique mammaire humain (initialement appelée GCDFP-24), a été tout d'abord caractérisée comme ayant des propriétés de liaison spécifique avec diverses classes de stéroïdes. Cette protéine se lie notamment avec une forte affinité à la progestérone et la prégnénolone, mais également avec une faible affinité à l'œstrogène (Balbin et al., 1990; Lea, 1988; Pearlman et al., 1973; Vogt and Skerra, 2001). Toutefois, une étude récente (Ruiz et al., 2013) a noté une absence d'interaction entre l'apoD et l'œstradiolβ, suggérant que l'oestrogène ne constitue pas un ligand fonctionnel de l'apoD. De plus, la liaison de la prégnénolone n'est plus possible avec une apoD recombinante (produite chez la bactérie) non glycosylée (Vogt and Skerra, 2001), suggérant que le niveau de

glycosylation de l'apoD est important dans son interaction avec certains de ses nombreux ligands.

1.1.2.2 L'acide arachidonique

Le ligand physiologique avec lequel l'apoD a la plus forte affinité est l'acide arachidonique (Morais Cabral et al., 1995). En effet, il a été montré que l'apoD se lie avec l'acide arachidonique (dont la constante d'association Ka est de l'ordre de 10^8 M^{-1}) avec une affinité beaucoup plus importante que celle de la progestérone (Ka de 10^6 M^{-1}) (Morais Cabral et al., 1995). Toutefois, cette différence d'affinité de liaison entre l'acide arachidonique et la progestérone pour l'apoD semble disparaître en absence de glycosylation de l'apoD (Vogt and Skerra, 2001). En effet, l'acide arachidonique et la progestérone se lient à l'apoD recombinante produite chez la bactérie avec une affinité relativement similaire (Vogt and Skerra, 2001). Un fait intéressant est que l'acide arachidonique est le précurseur de la synthèse des eicosanoïdes (prostaglandines et leucotriènes) (Kuehl and Egan, 1980). Produits localement dans l'organisme en réponse à différents stimuli, ces agents sont impliqués dans de nombreux phénomènes biologiques tels que l'inflammation, l'agrégation des plaquettes et la régulation cellulaire (Kuehl and Egan, 1980). En fait, sous l'action de la phospholipase A2 (PLA2), l'acide arachidonique, présent dans les membranes, est libéré et devient ainsi le substrat d'enzymes telles que la lipoxygénase et la cyclo-oxygénase (notamment Cox-2) conduisant à la formation des prostaglandines et des leucotriènes en situation inflammatoire (Smith et al., 2000). Ceci permet de penser que l'apoD pourrait agir comme transporteur de l'acide arachidonique qui serait alors mobilisé à des fins de régulation et de protection cellulaire.

1.1.2.3 Le cholestérol

Le cholestérol est un constituant majeur des membranes cellulaires et est impliqué dans la régulation de nombreuses fonctions cellulaires notamment des fonctions neuronales (Simons and Toomre, 2000). L'apoD est généralement identifiée comme un composant des HDL. Ainsi en association avec LCAT, apoA-I ou CETP (Cholesteryl Ester Transfer Protein), cette protéine pourrait être impliquée dans le transport du cholestérol des tissus périphériques vers le foie pour qu'il y soit métabolisé (Blanco-Vaca et al., 1992; Spreyer et al., 1990; Weech et al., 1991; Yang et al., 1994). Une augmentation de l'activité d'estérification de la LCAT a effectivement été observée en présence de l'apoD (Fielding and Fielding, 1980; Steyrer and Kostner, 1988). Cela suggère que l'apoD formerait un complexe avec la LCAT et pourrait alors jouer un rôle de stabilisateur pour cette enzyme. Cette association avec la LCAT a amené à considérer le cholestérol et ses esters comme étant les principaux ligands de l'apoD (Drayna et al., 1986). Une liaison de l'apoD avec le cholestérol a bien été mise en évidence mais cette liaison a été démontrée comme étant de faible affinité (Patel et al., 1997). Cette faible affinité de l'apoD pour le cholestérol pourrait expliquer la raison pour laquelle l'interaction directe entre l'apoD et le cholestérol est controversée. En effet, d'autres études n'ont pas pu détecter cette liaison entre l'apoD et le cholestérol (Morais Cabral et al., 1995; Ruiz et al., 2013). Cette observation d'absence d'interaction directe pourrait s'expliquer par la sensibilité des techniques utilisées pour mesurer la liaison entre l'apoD et le cholestérol. Cela pourrait également être dû au fait que l'apoD pourrait interagir avec le cholestérol de manière indirecte, via la sphingomyéline qui a été identifiée comme étant un ligand de l'apoD (Ruiz et al., 2013).

1.1.2.4 Autres ligands

Des études de modélisation moléculaire révèlent que des composants dérivés de l'hème, tels que la bilirubine, constitueraient des ligands plus probables de l'apoD que le cholestérol et ses esters (Peitsch and Boguski, 1990). Ces données ont été confirmées par une étude mettant en évidence une liaison entre l'apoD et la bilirubine (Ka de 3.10^7 M^{-1}). Cette liaison favoriserait ainsi l'interaction de la bilirubine avec les HDL (Goessling and Zucker, 2000). Comme pour la prégnénolone, l'affinité de l'apoD pour la bilirubine semble être également dépendante du niveau de glycosylation de l'apoD (Vogt and Skerra, 2001). D'autres ligands pour l'apoD ont également été proposés, tels que l'acide E-3 méthyl-2-héxénoïque (E-3M2H) qui est une composante odorante des sécrétions axillaires (Zeng et al., 1996), l'acide rétinoïque impliqué dans la différenciation cellulaire, la sphingomyéline et les sphingolipides qui sont des constituants importants des particules des HDL et de la membranes plasmique (Breustedt et al., 2006; Rhinn and Dolle, 2012; Ruiz et al., 2013; Vance, 2012). Toutefois, comme pour le cholestérol, la liaison de l'apoD avec certains de ces ligands tels que la bilirubine et le E-3M2H reste controversée (Ruiz et al., 2013). De plus, l'association de l'apoD à son ligand peut varier dépendamment des conditions et du site d'expression, lui permettant ainsi d'exercer différentes fonctions.

1.1.3 Expression de l'ApoD

1.1.3.1 Expression tissulaire de l'ApoD

La large distribution tissulaire de l'expression du gène de l'apoD semble refléter son importance et son rôle comme protéine multifonctionnelle (Tableau 1.1). Depuis sa découverte, l'apoD a été caractérisée chez cinq espèces de mammifères (rat, souris,

cochon d'inde, macaque et homme) et a également été détectée chez le poulet (Ganfornina et al., 2005). De proches homologues de l'apoD ont également été retrouvés chez la bactérie *Escherichia coli* (Bishop et al., 1995), chez la drosophile (Sanchez et al., 2006) ainsi que chez les plantes (Frenette Charron et al., 2002).

Préférentiellement associée avec les lipoprotéines plasmatiques, l'apoD aviaire est présente dans l'ovocyte en croissance rapide. Cela suggère qu'elle y joue un rôle dans le transport et/ou la mobilisation des lipides et des molécules régulatrices durant l'embryogenèse chez les espèces ovipares (Vieira et al., 1995; Yao and Vieira, 2002). Chez l'homme, l'apoD est faiblement exprimée dans le foie et les intestins, deux organes correspondant à des sites majeurs de synthèse d'autres apolipoprotéines. Une importante expression de l'apoD a aussi été notée dans le pancréas, le placenta, le rein, les testicules, les ovaires, le placenta et le système nerveux (dont le cerveau, le fluide cérébrospinal et les nerfs périphériques) (Drayna et al., 1986). Chez le singe (Smith et al., 1990), le lapin (Provost et al., 1990; Provost et al., 1991b), et le cochon d'Inde (Provost et al., 1995), la distribution de l'apoD est similaire à celle retrouvée chez l'humain (Drayna et al., 1986). Néanmoins, chez la souris et le rat, l'analyse par buvardage de type northern montre que la distribution tissulaire de l'apoD diffère de celle de l'homme, du singe et du lapin. En effet, chez ces rongeurs, la distribution de l'apoD est majoritairement restreinte au système nerveux. On la retrouve notamment dans la moelle épinière, le cervelet et le cerveau (Boyles et al., 1990b; Seguin et al., 1995).

Tableau 1.1 Distribution tissulaire de l'ARNm et de la protéine de l'apoD dans différentes espèces (modifié de (Rassart et al., 2000)).

	Moelle épinière	Cervelet	Cerveau	Ovaires	Surrénales	Testicules	Muscles	Thymus	Poumon	Foie	Reins	Cœur	Intestin	Rate	Pancréas	Placenta	Sérum
Humain (ARNm)																	√
Rhésus (protéine)																	√
Lapin (ARNm)																	√
Souris (ARNm)																	x
Rat (ARNm)																	
Rat (protéine)																	√
Cochon d'inde (ARNm)																	

√, détectée

x, non détectée

nt, non testée

1.1.3.2 Distribution cellulaire de l'apoD

L'ARNm de l'apoD est essentiellement exprimé par les fibroblastes et, plus spécifiquement, par les fibroblastes à proximité des vaisseaux sanguins (Provost et al., 1991a; Smith et al., 1990). Dans le système nerveux central (SNC), l'expression de l'apoD est principalement observée dans les cellules gliales de la matière blanche, plus particulièrement dans les astrocytes et les oligodendrocytes. Par contre, dans le système nerveux périphérique (SNP), l'apoD est synthétisée par les fibroblastes endoneuraux (Boyles et al., 1989; Boyles et al., 1990b; Provost et al., 1991a). De plus, en conditions normales de culture i*n vitro*, l'apoD a été démontrée comme étant sécrétée, puis internalisée pour se retrouver au niveau de la région périnucléaire dans les fibroblastes de souris NIH/3T3. Les mécanismes impliqués dans l'internalisation de l'apoD restent cependant encore incertains. Il est intéressant de noter qu'en situation de stress, tels que l'arrêt de croissance ou le stress inflammatoire, l'apoD se trouve à être localisée essentiellement au niveau nucléaire (Do Carmo et al., 2007). De plus, il a été noté dans cette étude que l'apoD nucléaire était dérivée de la protéine sécrétée. De par sa large distribution tissulaire et de son association avec une grande variété de ligands, l'apoD semble donc être une protéine multifonctionnelle dont le rôle varie d'un organe à l'autre et qui est impliquée dans de nombreuses pathologies (Rassart et al., 2000).

1.1.4 Modulation de l'apoD en conditions physiologiques

Chez des individus ne présentant aucun signe pathologique, la concentration estimée d'apoD dans le plasma varie entre 5 à 23 mg/100mL (Camato et al., 1989). Mais divers facteurs biologiques, mis en évidence par de nombreuses études, sont impliqués dans la modulation de cette protéine. Ces études démontrent la complexité

de la régulation de l'expression de l'apoD, mais également son implication dans de nombreux processus physiologiques et pathologiques.

1.1.4.1 Implication de l'apoD dans la croissance et la différenciation cellulaire

Différentes études mettent en évidence l'existence d'une corrélation inverse entre l'expression de l'apoD et la prolifération cellulaire. En effet, l'induction de l'expression est observée sur des cultures de cellules immortalisées ou primaires ayant subi un arrêt de croissance via la déprivation de sérum, la sénescence ou la confluence (Do Carmo et al., 2002; Provost et al., 1991a). Parallèlement, des études réalisées sur des lignées cellulaires du cancer du sein et de la prostate indiquent que l'expression de l'apoD est inhibée par les œstrogènes alors qu'elle est stimulée par les androgènes (Simard et al., 1990; Simard et al., 1991). Cette expression de l'apoD est inversement reliée à la prolifération cellulaire (Simard et al., 1990; Simard et al., 1991). De plus, certaines lignées cellulaire de cancers mammaires voient leur prolifération inhibée en présence d'interleukine-1α (IL-1α) et d'acide rétinoïque alors que leur sécrétion de l'apoD est stimulée (Blais et al., 1994; Lopez-Boado et al., 1994). Finalement, une étude de Sarjeant et de ses collaborateurs révèle que l'apoD peut bloquer de manière sélective la prolifération (induite par des facteurs de croissance) des cellules de muscles lisses vasculaires (Sarjeant et al., 2003). Cette suppression se fait via un mécanisme empêchant la translocation nucléaire de la forme active de la protéine ERK1/2 (extracellular signal-regulated kinase 1/2). Toutefois, cette relation entre l'expression de l'apoD et la prolifération cellulaire n'est pas présente dans certaines situations telles qu'en cas de traitement avec l'interleukine 6 (IL-6). En effet, malgré son effet inhibiteur sur la prolifération, l'IL-6 diminue l'expression de l'apoD (Blais et al., 1995).

Outre la prolifération cellulaire, l'expression de l'apoD peut également être liée à la différenciation cellulaire. En effet, deux médiateurs de la différenciation, l'acide

rétinoïque et la 1,25-dihydroxyvitamin D3, induisent l'expression de l'apoD dans les cellules de cancers mammaires (Lopez-Boado et al., 1994; Lopez-Boado et al., 1997). Cette induction de l'apoD est médiée par les récepteurs nucléaires à l'acide rétinoïque (RAR) qui induisent une inhibition significative de la prolifération cellulaire. Ceci suggère que l'apoD pourrait être un marqueur biochimique de la différenciation et de l'arrêt de prolifération médiés par les RAR dans les cellules de cancers mammaires (Lopez-Boado et al., 1996). Cette relation entre l'induction de l'apoD, la différenciation et l'arrêt de croissance a également été suggérée dans des tissus humains de prostate (Aspinall et al., 1995). En effet, cette étude montre que l'apoD est principalement localisée dans les cellules de l'épithélium glandulaire prostatique ce qui suggère qu'elle pourrait être associée à l'état non-prolifératif ou différencié de ces cellules.

Certaines conditions de stress, tels que les stress oxydatif et inflammatoire ainsi que les UV, peuvent également activer l'expression de l'apoD (Do Carmo et al., 2007). Cette induction de l'expression de l'apoD est observée spécifiquement à des concentrations et des doses de peroxyde (inducteur de stress oxydatif) et d'UV entraînant l'arrêt de croissance. Cette induction de l'apoD peut être activée par des facteurs nucléaires impliqués dans ces stress. En effet, plusieurs facteurs nucléaires, tels que PARP-1 (Poly(ADP-ribose) polymerase-1) et APEX (Apurinic/Apyrimidinic Endonuclease-I) (facteurs qui sont régulés à la hausse dans les cellules en arrêt de croissance) peuvent se lier aux promoteurs de l'apoD et induire son activation dans les cellules NIH/3T3 en situation d'arrêt de croissance (Levros et al., 2010). APEX et PARP-1 sont connus pour être induits dans plusieurs situations pathologiques incluant le stress oxydatif et les conditions neurodégénératives (Duan et al., 2007; Fritz et al., 2003).

1.1.4.2 Contrôle de l'homéostasie énergétique par l'apoD

Il existe une corrélation positive entre la masse adipeuse corporelle, les niveaux circulants de la leptine et l'expression de l'apoD hypothalamique (Liu et al., 2001). Toutefois, cette corrélation avec l'adiposité corporelle disparaît chez les souris obèses ob/ob (déficitaire en leptine) et db/db (présentant une mutation du récepteur de la leptine Ob/R). En effet, ces souris voient leur niveau d'apoD hypothalamique réduit par rapport aux souris de type sauvage. De plus, cette même étude met en évidence la présence d'une interaction directe et spécifique de l'apoD et du récepteur Ob/R, au niveau de la portion cytoplasmique de la forme longue de ce récepteur (OB/Rb) (Liu et al., 2001). Ceci suggère que l'apoD hypothalamique est impliquée dans les voies de signalisation du récepteur Ob-Rb de la leptine qui contrôle l'accumulation de gras lors d'une diète riche en gras.

L'apoD se trouve également modulée dans certaines pathologies associées à un défaut métabolique, telles que le déficit familial en HDL ou la maladie de Tangier (Alaupovic et al., 1981), le déficit familial en LCAT (Albers et al., 1985) et le diabète de type 2 (Baker et al., 1994; Hansen et al., 2004). Le lien entre l'apoD et la majorité de ces maladies pourrait être relié à un défaut du métabolisme lipidique. De plus, la relation de l'apoD avec le diabète de type 2, l'obésité et l'hyperinsulinémie pourrait dépendre des récepteurs hépatiques de type X ou de l'activation des voies inflammatoires et pro-angiogéniques qui modifient le métabolisme de l'acide arachidonique (Hummasti et al., 2004).

La maladie de Tangier est due à une mutation du gène de transporteur ATP-binding cassette A1 (ABCA1), qui est impliqué dans l'efflux du cholestérol (Bodzioch et al., 1999). La perte de fonction d'ABCA1 entraine une accumulation de cholestérol dans les tissus périphériques et une augmentation de risque de développement de

l'athérosclérose (Bodzioch et al., 1999). De plus, l'analyse du profil protéique des particules de HDL isolées de patients atteints d'athérosclérose (dont le taux est faible) révèle un enrichissement de l'apoD dans les HDL, ce qui pourrait faire partie d'un mécanisme de compensation face à la dérégulation de l'homéostasie du cholestérol observée chez ces patients (Vaisar et al., 2007). Outre l'accumulation de l'apoD dans les particules de HDL, la présence de l'apoD dans les athéromes, qui sont des plaques essentiellement composées de lipides dans les artères, est associée avec une accumulation excessive du cholestérol dans ces plaques observées chez les patients atteints d'athérosclérose et dans des modèles murins d'athérosclérose (Perdomo and Henry Dong, 2009; Sarjeant et al., 2003). Ceci peut être corrélé avec le fait que l'apoD, qui a la capacité de se lier au cholestérol, facilite l'élimination de l'excès de cholestérol des cellules. En effet, l'excès de cholestérol, dont le transport est facilité par les HDL, peut être éliminé dans le foie (Pfrieger, 2003), qui est considéré comme le mécanisme à travers lequel les HDL protègent l'organisme contre le développement de l'athérosclérose (Tall, 2008).

L'implication de l'apoD dans le métabolisme des lipides a été confirmée dans des modèles de souris transgéniques surexprimant l'apoD humaine dans le système nerveux central (Do Carmo et al., 2009b). L'expression de l'apoD humaine dans ces souris a en effet été également détectée dans les tissus périphériques tels que le foie. Il a été rapporté dans cette étude que les souris transgéniques âgées développent une stéatose hépatique et une résistance à l'insuline. De plus, ces souris ne sont ni obèses ni diabétiques. Toutefois, on ignore si l'accumulation de lipides induite par l'apoD dans le foie pourrait être due à la lipogenèse *de novo* ou à des lipides provenant des tissus périphériques provenant des tissus périphériques.

1.1.4.3 Rôle de l'apoD dans le développement

Une modulation de l'expression de l'apoD est également notée durant les périodes de gestation et de développement embryonnaire. En effet, des niveaux faibles de transcrits de l'apoD ont été observés chez le cochon d'inde au stade fœtal (Provost et al., 1991a). Chez la souris, l'analyse du patron d'expression de l'apoD (par hybridation *in situ*) au cours de l'embryogenèse indique que cette expression, amorcée entre le 8ème et le 9ème jour embryonnaire, se trouve être sélectivement modulée tout au long du développement embryonnaire, et ce jusqu'à la naissance (Sanchez et al., 2002). L'induction de l'apoD, durant le développement et durant la période néonatale précoce chez le rat, est associée à la maturation et coïncide avec la période de myélinisation active et avec la synaptogénèse (Ong et al., 1999). L'apoD est également présente dans le vitellus de l'ovocyte en croissance rapide. Elle y est associée avec le transport et la mobilisation des lipides durant l'embryogenèse chez les espèces ovipares (Vieira et al., 1995).

Chez l'humain, l'apoD est fortement exprimée dans l'endomètre pendant la fenêtre d'implantation de l'embryon et dans le placenta (Drayna et al., 1986; Kao et al., 2002) Au cours de la grossesse, les niveaux d'apoD plasmatique diminuent, phénomène qui se trouve être accentué chez les femmes avec un gain de poids excessif (Do Carmo et al., 2009a).

1.1.4.4 Implication de l'apoD dans le cancer

Une modulation de l'expression de l'apoD est présente dans plusieurs cancers. Celle-ci se trouve être surexprimée dans de nombreux types de carcinomes, tels que les carcinomes de sein, des ovaires, de la prostate, de la peau et du système nerveux central. Cette augmentation du niveau d'expression de l'apoD dans les cancers du sein et du système nerveux central est essentiellement liée au caractère hautement différencié, non-invasif et non-métastatique de ces carcinomes (Hunter et al., 2002;

Porter et al., 2003; Serra Diaz et al., 1999). Cette surexpression de l'apoD est également associée à une diminution de possibilité de rechute, et donc à des meilleures chances de survie, pour les patients atteints par ces types de cancers (Diez-Itza et al., 1994). Certaines études mettent de l'avant l'hypothèse que l'arrêt de croissance dû à la différenciation pourrait expliquer la présence de l'apoD dans ces cancers (Diez-Itza et al., 1994; Porter et al., 2003; Serra Diaz et al., 1999). Toutefois, dans les cancers de la prostate, du pancréas et de la peau, le niveau élevé d'expression de l'apoD est associé à la nature invasive de ces cancers (Ashida et al., 2004; Aspinall et al., 1995; Hall et al., 2004; Zhang et al., 1998). L'augmentation de l'expression de cette protéine pourrait alors être un facteur défavorable de la progression de la maladie (Iacobuzio-Donahue et al., 2002; Miranda et al., 2003; Ryu et al., 2001; West et al., 2004). La transformation maligne dans ces types de cancers favoriserait donc l'augmentation simultanée de l'apoD et de la croissance tumorale, abolissant ainsi la corrélation inverse entre le niveau élevé d'expression de l'apoD et la prolifération cellulaire (Hall et al., 2004). Il reste cependant à déterminer si l'expression de l'apoD est une cause ou une conséquence de ces transformations cellulaires.

1.1.5 Rôle de l'apoD dans le système nerveux

L'apoD est exprimée par les cellules de Schwann dans le système nerveux périphérique (Boyles et al., 1990b; Patel et al., 1995; Provost et al., 1991a). Chez le rat, les fibres nerveuses périphériques présentent une faible quantité d'ARNm d'apoD (Boyles et al., 1990b). Cependant, dans un contexte pathologique, cette expression subit d'importantes variations. Le tissu nerveux périphérique est capable de synthétiser localement certaines apolipoprotéines telles que l'apoD et l'apolipoprotéine E (apoE). Il est donc probable que le système nerveux périphérique soit doté d'un système de lipoprotéines qui lui soit propre. Cela permettrait donc un transport lipidique entre les cellules et un maintien de l'homéostasie du cholestérol (Boyles et al., 1989). En effet, lors d'une lésion des nerfs périphériques, la

dégradation de la myéline libère une grande quantité de lipides (notamment le cholestérol), qui sont ensuite stockés pour être réutilisés durant la régénération. De ce fait, le transport lipidique devrait être plus actif lors d'une lésion de nerfs périphériques (Rawlins et al., 1970). Une étude a mis en évidence que le degré de variation du niveau du transcrit de cette protéine est en fonction de la nature des lésions nerveuses périphériques (Spreyer et al., 1990). En effet, le taux de transcrits augmente de manière modérée dans les nerfs sectionnés de rat dont la régénération a été volontairement interrompue par un processus de ligature des extrémités axonales; tandis que lorsqu'il s'agit de fibres nerveuses comprimées en régénération, le niveau d'ARNm et de la protéine de l'apoD augmente considérablement au moment de la croissance axonale (Boyles et al., 1990; Spreyer et al., 1990). Une nette augmentation de l'apoD a également été observée chez d'autres espèces, telles que le lapin et le singe marmouset, en situation de compression de nerfs périphériques (Boyles et al., 1990). L'apoD pourrait donc être impliquée dans ce processus de régénération via le transport des lipides (notamment du cholestérol), permettant ainsi le maintien de l'homéostasie et le recyclage des lipides lors de la repousse axonale dans le système nerveux périphérique. Cette hypothèse a été confirmée par une étude réalisée dans un modèle de lésions de nerfs périphériques chez des souris déficientes en apoD et des souris transgéniques surexprimant l'apoD humaine (Ganfornina et al., 2010). En effet, ces études ont montré que la régénération et la remyélinisation axonales sont retardées en absence de l'apoD et, à l'inverse, la présence de l'apoD stimule ces processus de régénération axonale et facilite la récupération de la fonction locomotrice suite à la lésion des nerfs.

Dans le système nerveux central, l'expression de l'apoD est observée dans les astrocytes, les oligodendrocytes et quelques neurones (Boyles et al., 1990b; Patel et al., 1995; Provost et al., 1991b). Chez l'homme, l'apoD est essentiellement présente dans le cytosol des cellules gliales dans la matière blanche (Hu et al., 2001; Navarro

et al., 1998). Dans la substance grise, l'apoD est localisée au niveau des astrocytes protoplasmiques et dans certains neurones (Navarro et al., 1998; Navarro et al., 2004). L'apoD a également été détectée dans le cytosol des cellules péri-vasculaires et dans les lysosomes des péricytes au niveau des parois des vaisseaux sanguins du néocortex (Hu et al., 2001). Ceci suggère un rôle potentiel de l'apoD dans le transport des stérols et des petites particules hydrophobes dans les cellules gliales et (ou vers) les vaisseaux sanguins dans le système nerveux central. Fait intéressant, une augmentation de l'expression de l'apoD a été observée dans le cortex cérébral de patients âgés. Cette augmentation est probablement due à une hausse du nombre d'astrocytes réactifs (del Valle et al., 2003; Kalman et al., 2000). L'induction de l'apoD a également été rapportée dans de nombreuses lésions et maladies neurodégénératives telles que la maladie d'Alzheimer.

1.2 Implication de l'ApoD dans les maladies neurodégénératives

1.2.1 Maladie d'Alzheimer

Bien qu'une hausse du niveau de l'apoD constitue un phénomène physiologique dans le cerveau vieillissant, une augmentation encore plus importante de l'expression a été constatée chez des souris PDAPP âgées qui constituent un modèle de la maladie d'Alzheimer. Ceci peut représenter une réponse compensatoire des cellules gliales face à l'accumulation de la β-amyloïde (Thomas et al., 2001c). En effet, les souris PDAPP sont des souris transgéniques exprimant une forme mutée de la protéine précurseur de l'amyloïde humain APP associée à la forme familiale de la maladie d'Alzheimer (Games et al., 1995). Ces souris présentent de nombreux dépôts β-amyloïdes, une perte synaptique, une astrogliose et une microgliose (Chen et al., 1998; Masliah et al., 1996). En effet, dans le cerveau de patients atteints de la maladie d'Alzheimer, il existe deux types de lésions, qui sont bien caractérisées (Terry et al., 1994) :

- Les dégénérescences neurofibrillaires (NFT) qui sont des lésions intraneuronales correspondant à des enchevêtrements formés de filaments en double hélice. Elles sont principalement constituées de la protéine Tau sous forme hyperphosphorylée.
- Les plaques séniles correspondant à des lésions situées dans l'espace entre les cellules. Le cœur des plaques est constitué de dépôts anormaux de la protéine β-amyloïde.

Ces deux types de lésions rendent compte de la mort cellulaire et de l'apparition de la démence dans la maladie d'Alzheimer.

Chez des patients atteints de la maladie d'Alzheimer, une importante augmentation de la protéine apoD a été observée dans le liquide céphalorachidien, l'hippocampe et le cortex temporal (Desai et al., 2005; Kalman et al., 2000; Terrisse et al., 1998). De plus, il a été noté que ce taux d'apoD est corrélé avec le nombre de dégénérescences neurofibrillaires mais pas avec celui des plaques séniles (Belloir et al., 2001; Glockner and Ohm, 2003). Néanmoins, une co-localisation de l'apoD et ces dégénérescences neurofibrillaires (NFT) est rarement observée dans un même neurone, suggérant ainsi que l'expression de l'apoD est augmentée dans les neurones stressés avant même qu'ils accumulent ces lésions intraneuronales. Mais une fois les NFT formées, la transcription de l'apoD est déjà altérée (Belloir et al., 2001). Une étude a montré que l'apoD est présente dans les plaques séniles mais qu'elle est localisée préférentiellement autour ou près des dépôts amyloïdes, alors que l'apolipoprotéine E (apoE) est toujours localisée au cœur des plaques amyloïdes (Navarro et al., 2003).

L'augmentation de l'apoD dans la maladie d'Alzheimer est corrélée avec le stade Braak de dégénération (Glockner and Ohm, 2003), mais est indépendante des concentrations protéiques de l'apoE (Terrisse et al., 1998). De plus, cette induction de

l'apoD est corrélée avec le génotype d'apoE dans l'hippocampe et le liquide céphalorachidien (Glockner and Ohm, 2003). Une étroite relation entre la maladie d'Alzheimer et le génotye de l'apoE a été clairement établie : l'isoforme E4 constitue un risque de facteur majeur de développement de la maladie (Henderson and Finch, 1989). Chez les souris déficientes en apoE, le niveau de cholestérol dans le cerveau n'est pas altéré, une conséquence possible de l'importante augmentation d'expression de l'apoD et de ABCA1 comme processus compensatoire permettant de maintenir l'homéostasie du cholestérol dans le cerveau (Jansen et al., 2009). À l'inverse, l'expression de l'apoD est faiblement augmentée dans le cerveau de souris 'knock in' exprimant les isoformes E3 et E4, et qui affichent une accumulation significative du cholestérol et de ses métabolites (Jansen et al., 2009). Toutefois, il a été montré que, contrairement à l'isoforme E2, les isoformes E3 et E4 de l'apoE, qui peuvent se lier sur le promoteur de l'apoD, répriment l'activation de celui-ci dans des cellules de gliobastome humain U87 (Levros et al., 2013). Cette faible augmentation de l'apoD chez les modèles de souris exprimant l'apoE3 et l'apoE4 pourrait être la cause de l'addition de l'effet répressif direct de l'apoE3 et de l'apoE4 et de l'effet activateur des conséquences de l'accumulation du cholestérol.

Ainsi, au début de la maladie, l'augmentation de l'apoD en présence d'un allèle E4 du gène de l'apoE peut être interprétée comme un mécanisme compensatoire et pourrait indiquer une ré-innervation en cours plutôt que des dommages ou de la mort cellulaire. Cependant, cette corrélation disparaît lors de la progression de la maladie révélant que l'expression de l'apoD serait sous le contrôle de mécanismes différents (Belloir et al., 2001; Kalman et al., 2000; Thomas et al., 2003c).

1.2.2 Schizophrénie

La schizophrénie est un trouble psychotique sévère et chronique qui peut être dû à un déséquilibre de l'activité des neurotransmetteurs affectant les systèmes

sérotoninergiques, glutaminergiques et dopaminergiques (Jones and Pilowsky, 2002; Konradi and Heckers, 2003). Cette pathologie neuropsychiatrique est caractérisée par une altération du métabolisme des phospholipides (avec un défaut d'incorporation d'acide arachidonique dans les plaquettes et les érythrocytes) qui pourrait contribuer à l'altération de l'activité des neurotransmetteurs (Horrobin, 1998; Laugharne et al., 1996; Yao et al., 1996).

Le plasma et le cerveau post-mortem de patients atteints de la schizophrénie présentent une altération de l'expression de l'apoD (Mahadik et al., 2002; Thomas et al., 2001b; Thomas et al., 2003a). Plusieurs études ont mis en évidence une élévation du taux de l'apoD dans le cerveau post-mortem de patients chroniques et de rongeurs traités à la clozapine, qui est un médicament antipsychotique atypique (Mahadik et al., 2002; Thomas et al., 2001a). Ceci suggère donc que l'apoD a un rôle dans l'action pharmacologique de la clozapine. Ce médicament antipsychotique est efficace dans le traitement des patients atteints de schizophrénie (Ciapparelli et al., 2000). Chez ces patients schizophrènes traités, la clozapine augmente le taux d'acide arachidonique dans la membrane érythrocytaire (Vaddadi, 1992; Walker et al., 1999). Or, l'acide arachidonique a été identifié comme le ligand ayant la plus forte affinité pour l'apoD (Morais Cabral et al., 1995). Une étude a montré que l'apoD stabilise les niveaux d'acide arachidonique dans les membranes de cellules embryonnaires de rein humain 293T (HEK 293T) (Thomas et al., 2003b). En d'autres termes, une augmentation accrue de l'apoD, induite par les antipsychotiques atypiques, pourrait faciliter l'incorporation de l'acide arachidonique, permettant ainsi de stabiliser le taux de phospholipides dans les membranes des cellules. Des déficiences en acide arachidonique ont en effet été constatées chez des patients atteints de schizophrénie (Thomas et al., 2003b; Yao et al., 2005). L'augmentation d'apoD induite par les médicaments neuroleptiques serait donc bénéfique à ces patients. Cette étroite

relation entre apoD/acide arachidonique et la fonction de la clozapine a été confirmée chez des souris déficientes en apoD (Thomas and Yao, 2007).

1.2.3 Maladie de Niemann-Pick de type C

La maladie de Niemann-Pick de type C (NPC), caractérisée par un déficit de l'homéostasie lipidique, présente également une importante modulation de l'apoD. Il s'agit d'une maladie génétique qui implique des anomalies de transport intracellulaire du cholestérol, aboutissant à une accumulation de cholestérol non estérifié dans les endosomes tardifs et les lysosomes (Kolodny, 2000). Une importante augmentation d'apoD a été observée dans le plasma et le cerveau dans un modèle murin de la maladie de Niemann-Pick de type C (souris NPC). Des niveaux élevés d'apoD sont également présents dans les tissus périphériques tels que le cœur, le tissu adipeux et le thymus (Suresh et al., 1998; Yoshida et al., 1996). Bien que cette altération de transport du cholestérol soit observée à la fois dans les cellules périphériques et nerveuses, les principales caractéristiques de la maladie de NPC sont associées à une neurodégénérescence progressive, suggérant une plus grande vulnérabilité des cellules nerveuses face à la perturbation du trafic intracellulaire du cholestérol (Sevin et al., 2007; Suresh et al., 1998). En comparaison avec les tissus périphériques, le cerveau est fortement enrichi en cholestérol qui se trouve dans la gaine de myéline entourant les axones et facilitant la transmission nerveuse. De plus, le taux de cholestérol dans la membrane peut également influencer l'activité nerveuse (Dietschy and Turley, 2004). Ainsi, l'expression de l'apoD (dont un des ligands est le cholestérol) pourrait être augmentée afin de pallier à la dérégulation du cholestérol intracellulaire chez les souris NPC.

1.2.4 Autres neuropathologies et lésions neurodégénératives

Une modulation de l'expression de l'apoD a été observée dans d'autres situations neuropathologiques et neurodégénératives. Chez des patients atteints de la maladie de Parkinson, on note une augmentation évidente de l'expression de l'apoD par les cellules gliales dans la substance noire (Ordonez et al., 2006). Une accumulation de l'apoD a également été retrouvée dans le liquide céphalorachidien de patients suite à un accident vasculaire cérébral. Le taux d'apoD est également élevé dans le fluide céphalorachidien de patients atteints de la sclérose en plaques (Reindl et al., 2001). Suite à une atteinte cérébrale aiguë chez les rongeurs, l'expression de l'apoD est augmentée dans les astrocytes, mais est également retrouvée dans les neurones. C'est le cas, par exemple, dans l'hippocampe de rat suite à l'injection d'acide kaïnique (Montpied et al., 1999; Ong et al., 1997) ou suite à la lésion du cortex entorhinal (Terrisse et al., 1999). Dans un modèle d'ischémique cérébrale chez le rat, l'apoD est fortement induite dès les premières heures et continue d'augmenter jusqu'à 48h (Rickhag et al., 2006). Cette induction de l'apoD est détectée dans les neurones pyramidaux en dégénérescence dans la région lésée, autour de cette région mais aussi dans les oligodendrocytes et cette induction persiste au-delà d'une semaine (Rickhag et al., 2008).

Cette surexpression de l'apoD dans le cerveau vieillissant et dans ces conditions neuropathologiques soulève la possibilité que l'apoD joue un rôle important dans le maintien et dans la protection des neurones face à des lésions neurodégénératives.

1.2.5 Fonctions neuroprotectrices de l'apoD

Plusieurs études corroborent l'hypothèse d'un rôle neuroprotecteur de l'apoD face à une neurodégérescence induite par différentes neuropathologies, incluant le stress oxydatif, le stress inflammatoire et l'excitotoxicité (Do Carmo et al., 2008;

Ganfornina et al., 2008). L'excitotoxicité désigne la mort des neurones survenant à cause d'un excès d'activation entraînée par le glutamate ou d'autres agonistes excitateurs, tels que l'acide kaïnique (ou kaïnate KA); un puissant excitateur neuronaux (Olney et al., 1971; Watkins and Evans, 1981). L'excitotoxicité s'est révélé être un processus pathologique majeur impliqué dans de nombreuses affections neurologiques dégénératives du système nerveux central, aussi bien aiguës (comme les accidents vasculaires cérébraux) que chroniques (dont la sclérose latérale amyotrophique, la maladie de Parkinson, la maladie d'Alzheimer et certaines formes d'épilepsies) (Albin and Greenamyre, 1992; Lipton and Rosenberg, 1994).

Des souris transgéniques, surexprimant l'apoD dans le cerveau, présentent un meilleur taux de survie et moins de sensibilité face à un stress oxydatif induit dans le cerveau par injection d'un générateur de radicaux oxygénés libres (ROS), le paraquat, en exhibant une augmentation atténuée du taux de peroxydation lipidique. A l'inverse, chez les souris déficientes en apoD (apoD-/-), une élévation de la sensibilité face au stress oxydatif ainsi qu'une déficience de certaines taches cognitives ont été notées après injection du paraquat (Ganfornina et al., 2008). Parallèlement, la perte de l'homologue de l'apoD connu sous le nom de glial lazarillo (Glaz) chez la drosophile augmente la peroxydation lipidique et la sensibilité au stress oxydatif (Sanchez et al., 2000). Ces drosophiles mutantes ont une longévité plus réduite (Sanchez et al., 2006). À l'inverse, la surexpression de cette protéine induit une importante résistance contre le stress oxydatif et une augmentation de 29% de la longévité chez la drosophile (Hull-Thompson et al., 2009; Ruiz et al., 2012; Walker et al., 2006). Ces mêmes effets sont également observés avec la surexpression de l'apoD humaine chez les drosophiles, indiquant que le rôle de l'apoD est conservé durant l'évolution (Muffat et al., 2008). De plus, il a été démontré que cet effet anti-oxydant de l'apoD est médié par le résidu Met^{93} qui prévient la peroxydation lipidique (Bhatia et al., 2012; Oakley et al., 2012). Cet effet neuroprotecteur de l'apoD, observé à la suite d'un stress

oxydatif, a également été obtenu après induction d'un stress inflammatoire (Do Carmo et al., 2008). En effet, l'injection intracérébrale du virus OC43, un coronavirus humain capable de causer une inflammation démyélinisante du SNC rappelant la sclérose en plaques (Jacomy and Talbot, 2003), entraîne une activation accrue des processus inflammatoires (qui peuvent être délétères) et de la glie mais surtout un important taux de mortalité chez des souris de type sauvage. Toutefois, la surexpression d'apoD chez les souris transgéniques résulte en une augmentation significative du taux de survie de ces souris ainsi qu'en une atténuation de certains processus inflammatoires (tels que l'infiltration des cellules immunitaires) face à l'infection induite par OC43. De plus, cette protection semble être corrélée avec une restriction de l'activité de la PLA2. Cela suggère que l'apoD protège de l'encéphalopathie induite par OC43, via la voie de signalisation des PLA2. Cet effet protecteur de l'apoD pourrait résulter de la capacité de l'apoD à stabiliser l'acide arachidonique dans la membrane cellulaire ou à le séquestrer empêchant ainsi sa conversion en molécules pro-inflammatoires (Thomas et al., 2003a; Thomas et al., 2003b). Un effet neuroprotecteur de l'apoD face à l'excitotoxicité induite par l'acide kaïnique a également été mis en évidence, *in vitro*, sur un modèle organotypique d'hippocampe de souris. En effet, un pré-traitement avec l'apoD purifiée prévient la mortalité neuronale et la peroxydation de l'acide arachidonique et du cholestérol dans les cultures d'hippocampes de type organotypique exposées au kaïnate (He et al., 2009).

L'ensemble de ces données suggère donc un possible rôle de l'apoD dans la réponse cellulaire face à la progression de ces maladies, en agissant comme un facteur protecteur des neurones en situation de stress.

PROBLÉMATIQUE

De par sa large distribution tissulaire et son association avec une grande variété de ligands, l'apoD semble être une protéine multifonctionnelle dont le rôle varie d'un organe à un autre. L'apoD est largement synthétisée dans le SNC et son expression s'y trouve augmentée dans certaines neuropathologies telles que les maladies d'Alzheimer, de Parkinson et de Niemann-Pick de type C. Cette importante modulation d'expression s'établit spécifiquement au niveau des sites de lésions dans le SNC, suggérant ainsi un possible rôle de l'apoD dans la protection des neurones en situation de stress. Il a été préalablement démontré que la surexpression de l'apoD, chez les souris, protège celle-ci de dégénérescences induites par des stress inflammatoire et oxydatif. Ainsi, l'utilisation de l'apoD pourrait être envisagée comme un outil thérapeutique potentiel pour traiter les maladies neurodégénératives. Toutefois, selon le type de neuropathologies, de nombreux mécanismes, dont ceux induits par les stress oxydatif et inflammatoire, sont mis en jeu. Parmi ces mécanismes, certains induisent une aggravation de l'état neurodégénératif : c'est le cas du phénomène d'excitotoxicité. Afin de mieux comprendre le rôle de l'apoD dans les neuropathologies, il reste encore à définir, d'une part, si cet effet neuroprotecteur de la surexpression de l'apoD est également observé au niveau d'un mécanisme bien établi, tel que l'excitotoxicité, et d'autre part, les mécanismes moléculaires responsables de cet effet bénéfique de l'apoD.

Notre premier objectif était donc de déterminer si la surexpression de l'apoD dans le SNC protège de la neurodégénérescence induite par excitotoxicité. Pour cela, nous avons utilisé des souris transgéniques surexprimant l'apoD humaine dans les neurones et nous les avons soumises à l'excitotoxicité par injection avec l'acide

kaïnique, un analogue du glutamate. Il s'agissait donc, tout d'abord, de déterminer si la surexpression de l'apoD atténue la dégénérescence induite par le kaïnate par une étude comportementale (convulsions) et une analyse biochimique et cellulaire (apoptose et inflammation). Nous avons également vérifié si la surexpression de l'apoD favorisait un environnement non permissif au développement de l'excitotoxicité en mesurant les paramètres clés impliqués dans l'induction de l'excitotoxicité.

Outre l'induction de son expression suite à un stress, l'effet neuroprotecteur de l'apoD pourrait impliquer qu'elle soit internalisée dans les cellules. Notre deuxième objectif consistait donc de vérifier, tout d'abord, si l'internalisation de l'apoD nécessitait la présence d'un récepteur spécifique par une étude de liaison à des cellules en culturec . Une fois la présence du récepteur déterminée, il s'agit par la suite de purifier le complexe apoD/récepteur et d'identifier ce récepteur par spectrométrie de masse. Une fois identifié, l'implication de cette protéine sur l'internalisation de l'apoD sera vérifiée.

Considérée comme une protéine multi-ligand et multi-fonctionnelle, la fonction de l'apoD pourrait donc dépendre du ligand qu'elle transporte. Notre dernier objectif consiste à déterminer si l'effet neuroprotecteur de l'apoD dépend de sa capacité à se lier avec certains ligands impliqués dans les processus neurodégénératifs tels que l'excitotoxicité. Pour cela, nous avons généré plusieurs mutants de l'apoD portant des mutations sur les acides aminés situés dans la région de liaison avec son ligand. Nous avons, par la suite, vérifié leur capacité à être internalisé dans les cellules.

CHAPITRE II

OVEREXPRESSION OF APOLIPOPROTEIN D PROTECTS AGAINST KAINATE-INDUCED NEUROTOXICITY IN MICE

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Avant propos

Des travaux antérieurs ont démontré un effet neuroprotecteur de l'apoD face un stress oxydatif et inflammatoire induit dans le SNC. Cet article a donc pour objectif de vérifier l'effet neuroprotecteur de l'apoD dans un autre phénomène impliqué dans les maladies neurodégénératives, l'excitotoxicité. Pour ce faire, l'excitotoxicité a été induite par injection intrapéritonéale de l'acide kaïnique (un analogue du glutamate) chez les souris surexprimant l'apoD humaine dans les neurones sous le contrôle du promoteur du gène Thy-1 (H-apoD Tg). L'effet de la surexpression de l'apoD sur le phénomène d'excitotoxicité a ensuite été vérifié en mesurant plusieurs paramètres impliqués dans cette dégénérescence (incluant les convulsions, l'inflammation).

Dans le phénomène d'excitotoxicité, l'induction de la mort neuronale est la conséquence d'une cascade de processus dépendant de l'influx de sodium, de l'influx de calcium et de l'exocytose de glutamate. L'importante stimulation des récepteurs AMPA (α-amino-3-hydroxy5-methyl-4-isoxazolepropionic acid)/kainate (récepteurs glutaminergiques ionotropiques) par l'acide kaïnique (un puissant agoniste excitateur) induit une suractivation des récepteurs NMDA (N-methyl-D-aspartate), qui constituent les principaux acteurs du processus neurotoxique; ceci entraînant une entrée massive de calcium dans les neurones. Cette augmentation de calcium intracellulaire est également accentuée par l'inversion de la pompe Na+/Ca2+ et la libération de calcium stocké dans le réticulum endoplasmique (Mody and MacDonald, 1995). Cette accumulation excessive de calcium intracellulaire induit plusieurs mécanismes toxiques tels que l'activation de PLA2, la production de radicaux libres et l'induction de l'apoptose (Mody and MacDonald, 1995; Patel et al., 1996).

L'excès de calcium intracellulaire a également pour conséquence la libération dans le milieu extracellulaire des constituants des vésicules synaptiques contenant

essentiellement du glutamate, contribuant ainsi à une augmentation des concentrations du glutamate dans le milieu extracellulaire (Benveniste et al., 1984). Ces fortes concentrations extracellulaires de glutamate présentes au niveau du site principal (lésion primaire) peuvent alors se propager vers les neurones avoisinants ayant pour conséquence de fragiliser des neurones qui n'avaient pas été affectés par la lésion primaire.

Ce chapitre, présenté sous forme d'article scientifique, a été soumis pour publication dans le journal 'Experimental Neurology'. Les références de cet article se trouvent à la fin de la thèse. J'ai été impliquée dans toutes les facettes expérimentales des travaux présentés dans cet article, dans le montage des figures et dans la rédaction de l'article à l'exception de l'expérience liée à l'évaluation de l'effet de la surexpression de l'apoD sur l'apoptose induite par le kaïnate. Elle a été réalisée par Azadeh Alikashani. Sonia Do Carmo a été impliquée dans la correction de cet article. Le Dr Eric Rassart a supervisé le projet et corrigé l'article.

2.1 Abstract

Excitotoxicity due to the excessive activation of glutamatergic receptors leads to neuronal dysfunction and death. Excitotoxicity has been implicated in the pathogenesis of a myriad of neurodegenerative diseases with distinct etiologies such as Alzheimer's and Parkinson's. Numerous studies link apoD, a secreted glycoprotein highly expressed in CNS, to maintenance and protection of neurons in various mouse models of acute stress and neurodegeneration. Here, we used a mouse model overexpressing human apoD in neurons (H-apoD Tg) and tested the neuroprotective effects of apoD in the kainic acid (KA)-lesioned hippocampus. Our results show that apoD overexpression in H-apoD Tg mice induces an increased resistance to KA-induced seizures, significantly attenuates inflammatory responses and confers protection against KA-induced cell apoptosis in the hippocampus. The apoD-mediated protection against KA-induced toxicity is imputable in part to increased plasma membrane Ca2+ ATPase type 2 expression (1.7 fold), decreased NMDA receptor subunit NR2B levels (30%) and modified lipid metabolism. Indeed, we demonstrate that ApoD can attenuate intracellular cholesterol content in primary hippocampal neurons and in brain of H-apoD Tg mice. In addition, apoD can be internalized by neurons and this internalization is accentuated in aging and injury conditions. In summary, our results provide additional clues on the mechanisms involved in apoD-mediated neuroprotection in neurodegenerative conditions.

Keywords: apolipoprotein D (apoD), kainic acid, seizure, apoptosis, excitotoxicity, cholesterol metabolism, inflammation, calcium.

2.2 Introduction

Apolipoprotein D (apoD) is a secreted glycoprotein and a member of the lipocalin family of proteins, which bind and transport small lipophilic molecules (Rassart et al., 2000). In human, this atypical apolipoprotein is highly expressed in several tissues including the central nervous system (CNS), unlike other apolipoproteins, which are essentially produced in liver and intestine (Drayna et al., 1986; Rassart et al., 2000; Weech et al., 1991). In rodents, apoD expression is found primarily in the CNS. In the brain, high apoD expression is observed in the hippocampus, the prefrontal cortex and the substantia nigra (Elliott et al., 2010), mainly in glial cells (astrocytes and oligodendrocytes) but also in a few neurons (Hu et al., 2001; Provost et al., 1991b; Rickhag et al., 2008; Smith et al., 1990).

The upregulation of apoD in the aging brain (Kalman et al., 2000) and in multiple neurological conditions, including Alzheimer's (Terrisse et al., 1998), Parkinson's (Ordonez et al., 2006), Niemann-Pick's type C (NPC) (Yoshida et al., 1996) diseases, multiple sclerosis (Reindl et al., 2001), nervous system injuries including stroke (Boyles et al., 1990; Franz et al., 1999; Terrisse et al., 1999). This raises the possibility that apoD plays a prominent role in neuronal maintenance and protection against injury.

Supporting this hypothesis, studies revealed that overexpression of apoD (or its orthologs) in transgenic mice, Drosophila and Arabidopsis led to increased resistance to oxidative stress (Charron et al., 2008; Ganfornina et al., 2008; Muffat et al., 2008) and inflammation (Do Carmo et al., 2008). Conversely, apoD inactivation in mice, plants and flies resulted in decreased resistance and ultimately reduced survival in response to oxidative stress (Charron et al., 2008; Ganfornina et al., 2008; Sanchez et al., 2006). It was further established that apoD specifically prevents lipid peroxidation but not protein carbonylation in response to oxidative insults (Ganfornina et al., 2008).

This antioxidant effect of apoD on lipids is mediated through a highly conserved methionine residue (Met_{93}), converting reactive to non-reactive lipid hydroxides (Bhatia et al., 2012; Oakley et al., 2012). ApoD may also influence inflammatory pathways via the regulation of arachidonic acid (AA) signalling and metabolism (Do Carmo et al., 2008). Indeed, it was suggested that apoD could stabilize AA into the cell membrane or sequester AA, reducing the availability of free AA and preventing its conversion into pro-inflammatory molecules (Thomas et al., 2003a; Thomas et al., 2003b). Another way for apoD to prevent neurotoxicity lies in its capacity to bind several ligands involved in neurological injuries. ApoD can bind AA, progesterone and sphingomyelin, with high affinity, as well as cholesterol, bilirubin and estradiol, with lower affinity or through indirect interactions.

Despite increasing evidence indicating that apoD accumulation in neuropathological conditions is potentially protective, little is known on the response of apoD to excitotoxic insults and the pathways involved. Earlier work demonstrated increased apoD levels in the hippocampus of kainic acid-treated rats (Montpied et al., 1999; Ong et al., 1997). Kainic acid (KA), an analog of glutamate, can induce the over-activation of glutamate receptors and is widely used as a model to explore excitotoxic processes in neurodegenerative injury (Zheng et al., 2011). Because of its high density of kainate receptors, the hippocampus is more sensitive to KA-induced neurotoxicity (Darstein et al., 2003). Also, glutamatergic pathways seem to be particularly affected in apoD knockout mice with a 20% decrease in the density of kainate receptors in the CA 2-3 subfields of the hippocampus, a global decrease in AMPA receptors and a global increase in muscarinic M2/M4 receptors (Boer et al., 2010). These changes may contribute to impairments in learning memory, motor tasks and orientation-based tasks observed in these animals (Ganfornina et al., 2008), all of which involve glutamatergic neurotransmission.

Therefore, using transgenic mice overexpressing human apoD in their neurons, we sought to better understand the role of apoD in excitotoxic injury induced by KA treatment. Here we demonstrate that overexpression of apoD in neurons protects mice against KA-induced seizures and cellular apoptosis. We also found that apoD protection confers not only through its anti-inflammatory properties but also through the regulation of cholesterol distribution in neurons, and by affecting the levels of proteins known for their involvement in limiting excitotoxic effects.

2.3 Material and Methods

2.3.1 Mice

Human apoD transgenic (H-apoD Tg) mice express the human apoD (H-apoD) cDNA under the control of the Thy-1.2 promoter, as previously described (Do Carmo et al., 2008; Ganfornina et al., 2008). In H-apoD Tg mice, H-apoD is expressed in neuronal cells in all regions of the nervous system (Do Carmo et al., 2008). Because the FVB/N mouse strain is more vulnerable to KA lesions (McLin and Steward, 2006), all mice were backcrossed into the FVB/N background (Charles River, Canada) for at least 10 generations. Genotyping was performed by PCR on tail biopsies as described previously (Ganfornina et al., 2008). In all experiments, 12-16 weeks old males WT and H-apoD Tg littermates were used. The animals were housed under standard conditions at constant temperature (20°C to 22°C) and humidity (50% to 60%), under a 12h light/dark cycle and had free availability to water and food. All mice were euthanasied by CO2 asphyxiation after being anesthetised with isoflurane (PPC, Richmond Hill, ON, Canada). The experimental procedures were approved by the Animal Care and Use Committee of Université du Québec à Montréal.

2.3.2 Kainate administration and assessment of behavioural seizures

Kainic acid (KA) (Sigma-Aldrich, St Louis, MO) (15 mg/kg, prepared in phosphate-buffered saline, PBS) or vehicle (PBS) were injected intraperitoneally (i.p.) in WT and H-apoD Tg mice. Results of preliminary dose-response experiments revealed consistent seizures with a mortality of less than 10 % in animals injected with a KA dose of 15 mg/kg. This dose was used in all following experiments. For behavioural assessment, animals (17 mice for each group) were monitored every 5 min for behavioural seizures during 2h and the seizure intensity were blind-scored following a modified Racine's classification (Racine, 1972): 'stage 0', no seizure and normal

behaviour; 'stage 1', immobilisation; 'stage 2', myoclonic jerk and head nodding; 'stage 3', bilateral forelimb clonus and rearing; 'stage 4', continuous rearing and falling; 'stage 5', loss of posture; 'stage 6', running and jumping seizure. For each mouse, the sum of all seizure intensity was also calculated by adding the seizure scores observed in all 5 min intervals during 2h following KA injection. The latency to 'stage 2', 'stage 3' and 'stage 4' seizure was also monitored. Animals that did not present these 'stage' seizures were given a value of 120 min.

2.3.3 Protein extraction and Western blot analysis

Non-injected (4-5 WT and 4 H-apoD Tg mice) and 3 or 7 days post-injected (4-5 WT and H-apoD Tg mice) animals were euthanized and hippocampi were collected, frozen in dry ice, and conserved at -80°C until protein extraction. Hippocampi were then homogenized in cold lysis buffer (50 mM Tris-HCL, pH 7.3, 150 mM NaCl, 5 mM EDTA, 0.2% (v/v) Triton X-100 complemented with complete protease inhibitor (Roche Molecular Diagnostics, Mannheim, Germany). After 30 min incubation at 4°C, homogenates were sonicated and cleared by centrifugation. Protein concentration was assessed by the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Canada). Proteins (30 μg per sample) were separated on 12% (w/v) SDS-polyacrylamide gels and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Ontario, Canada). To analyse NMDAR receptor subunits and PMCA (Plasma membrane calcium ATPases) expression, proteins were separated on 8% (w/v) SDS-polyacrylamide gels. Membranes were incubated with primary antibodies: human apoD mouse monoclonal antibody (2B9), 1:10000 (Terrisse et al., 1998), produced as described in (Weech et al., 1986); mouse apoD goat polyclonal antibody (Santa Cruz biotechnology, Dallas, USA), 1:1000; GFAP rabbit polyclonal antibody (Cell signalling, Whitby, Canada), 1:10000; Mac-2 rat monoclonal antibody (ATCC, Cedarlane, Burlington, Canada), 1:1000; COX-2 rabbit polyclonal antibody (Cell Signaling), 1:1000; synaptophysin rabbit polyclonal antibody (Abcam, Toronto,

Canada), 1:10000, NMDAR1 rabbit monoclonal antibody (Cell Signaling), 1:1000; NMDAR2A rabbit polyclonal antibody (Cell Signaling), 1:1000; NMDAR2B rabbit monoclonal antibody (Cell Signaling), 1:1000; PMCA2 rabbit monoclonal antibody (Sigma-Aldrich), 1:1000; β-Actin mouse monoclonal antibody (Sigma-Aldrich), 1:10000. Then, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and visualized by chemiluminescence (ECL, GE Healthcare, Quebec, Canada) using a Fusion FX7 system (Vilber Lourmat, France).

2.3.4 TUNEL assay

To detect apoptosis-induced nuclear DNA fragmentation, we used the ApoAlert DNA Fragmentation Assay kit (Clontech Laboratories, Montain View, CA), a fluorescence terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) based assay. Briefly, 3 days post-injection, brains of WT (n=4) and H-apoD Tg mice (n=4) were dissected and placed in 4% paraformaldehyde (PFA) at 4°C for 24h. The fixed brains were then embedded in OCT freezing medium (Fisher Scientific, Ottawa, Canada). Eight μm sagittal sections were cut using a cryostat (Leica, Ontario, Canada), and mounted onto silane-coated slides. The fixed sections were then permeabilized with proteinase K at room temperature for 10 min before being incubated with equilibrium buffer (200mM potassium cacodylate pH 6.6, 25 mM Tris-HCL, 0.2 mM DTT, 0.25 mg/mL BSA, 2.5 Mm cobalt chloride) for 10 min at room temperature. The sections were then incubated with equilibrium buffer containing nucleotide mix and TdT terminal transferase at 37°C for 60 min. The reaction was stopped with 2X SSC and the sections were washed with PBS. The sections were stained with propidium iodide (PI) (2.5 μg/mL, Sigma-Aldrich) for nuclei labeling. TUNEL-positive nuclei were visualized by fluorescence microscopy.

Mean pixel TUNEL and PI fluorescence intensity were measured in hippocampus using Image J software.

2.3.5 PMCA2 Immunohistochemistry

Fixed brains of 4 non-injected WT and H-apoD Tg mice embedded in OCT freezing medium (Fisher Scientific) were cut in 8 μm sagittal sections, permeabilized and blocked for 1h in 0,1% triton X-100 (w/v), 10% goat serum (v/v) and 10% bovine serum albumin (BSA) (w/v). After blocking, sections were incubated overnight at 4°C in humid atmosphere with a primary antibody against PMCA2 (rabbit monoclonal antibody, 1:250, Sigma-Aldrich). After washing in PBS (3X,10 min), the sections were then incubated with goat anti rabbit IgG-Alexa 488 (1:1000; Life Technologies, Burlington, Canada) at room temperature for 1h. Sections were rinsed again with PBS and for nuclei staining, the last wash contained 100 ng/mL 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). PMCA2 fluorescence was visualized by confocal microscopy.

2.3.6 Subcellular Fractionation of brain tissue

Whole brains of 4 non-injected WT and H-apoD Tg mice were homogenized in 10 volumes of cold homogenization buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, 2 mM EDTA and 10% (w/v) complete protease inhibitor (Roche)) using a motorised glass-teflon homogenizer. The homogenates (H) were centrifuged at 1000 g for 15 min. The nuclear pellets (P1) were removed and the S1 supernatants were centrifuged at 10000 g for 30 min. Supernatants (S2, cytosol fraction) were separated from pellets (P2). To remove any cytosol contaminations, the pellets P2 were resuspended in homogenization buffer and centrifuged again at 100000 g for 30 min. The supernatants (S'2) were removed and the pellets (P'2, membrane fractions) were resuspended in homogenization buffer.

2.3.7 Cholesterol quantification

Cholesterol levels were assessed using the Amplex Red cholesterol assay (Life Technologies) in whole brain homogenates (H), membrane (P'2) and cytosol (S2) fractions of mouse brain. Samples, diluted in reaction buffer, were mixed with an equivalent volume of Amplex Red working solution containing 300 μM Amplex Red reagent, 2 U/mL cholesterol oxidase, 0.2 U/mL cholesterol esterase and 2 U/mL horseradish peroxidase (HRP). After incubation at 37°C for 30 min, fluorescence was measured at 568 nm using fluorescence spectroscopy (Tecan Infinite M1000, Tecan US, NC, USA). Cholesterol levels were normalized by protein concentration.

2.3.8 24(S)-Hydroxycholesterol quantification

Brain and plasma 24(S)-hydroxycholesterol (24-HOC) levels were assessed using a 24(S)-Hydroxycholesterol ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA). Brains (100 mg) of 4 non-injected WT and H-apoD Tg mice were homogenized in ethanol 95% (1 mL). After centrifugation, the pellets were resuspended in 1 mL of Ethanol:dichloromethane (1:1 v/v) and then re-centrifuged. The supernatants (obtained after the 2 centrifugations) were combined and evaporated to dryness with a rotary evaporator. The samples were then rehydrated at room temperature with ethanol 95% (16 μL) and assay buffer (484 μL). After diluting samples in assay buffer, 24-HOC quantification in plasma (1:1000) and brain homogenates (1:100) was performed according to manufacturer's instructions.

2.3.9 Hippocampal culture preparation

WT (FVB/N strain) mouse embryos, at 18 days of gestation (from 4 WT females), were used to prepare primary hippocampal neuronal cultures as previously described (Brewer et al., 1993). Briefly, hippocampi were dissected in HBSS (without Ca^{2+} and

Mg^{2+}) supplemented with 1 mM sodium pyruvate and 10 mM HEPES, and dissociated by trituration with a fire-polished Pasteur pipette. Supernatants were then centrifuged for 1 min at 1000 g. The pellets were resuspended in HBSS. The cell suspensions were plated at a density of 5 x $10^5/cm^2$ in neurobasal medium (Invitrogen) supplemented with 0.5 mM L-glutamine and B27 supplement (Invitrogen) and grown on glass coverlips pretreated with poly-D-lysine (Sigma-Aldrich)

2.3.10 Filipin staining

Hippocampal primary neurons (10 days) were incubated for 24h with H-apoD (250 ng/mL) purified from breast cyst fluid as described in (Norfeldt et al., 1981). Staining of intracellular cholesterol was achieved using filipin, a fluorescent polyene antibiotic with high affinity for cholesterol (Coxey et al., 1993). Hippocampal neurons were fixed in 4% paraformaldehyde (PFA) in PBS containing 2% sucrose for 15 min, washed with PBS and permeabilized with 0,1% Triton X-100. The cells were incubated with filipin (125 µg/mL; Sigma-Aldrich) in PBS for 2h at room temperature. Filipin-cholesterol complexes were visualized by confocal microscopy. For GAP-43 (neuronal marker) immunohistochemistry, neurons were blocked in PBS with 10% goat serum, 10% bovin serum albumin (BSA), and 0,1% triton X-100 for 1h and incubated overnight at 4°C in humid atmosphere with a primary antibody against GAP-43 (rabbit polyclonal antibody, 1:250, Abcam). After washing in PBS (3X for 10 min), the cells were exposed to a goat anti rabbit IgG-Alexa 568 (1:1000; Life Technologies) at room temperature for 1h, washed, and mounted on slide with Prolong Gold antifade (Life Technologies). Gap-43 immunoreactivity was visualized by confocal microscopy.

2.3.11 Cholesterol treatment

Hippocampal primary neurons (10 days) were pre-incubated for 3h with water-soluble cholesterol (10 ug/mL; Sigma). The cells were then exposed or not to purified H-apoD (250 ng/mL) for 24h. Cholesterol uptake was visualized by filipin staining using confocal microscopy. Mean pixel filipin fluorescence intensity was measured in the soma of cells using image J software.

2.3.12 ApoD internalization

Purified H-apoD uptake by hippocampal neurons was assessed in aging and KA-induced excitotoxic conditions. For KA toxicity, hippocampal neurons (at 10 days) were pre-incubated with or without purified H-apoD (250 ng/mL) for 24h. Then, cells were exposed to KA (100 μM) for 24h. For aging conditions, hippocampal neurons were cultured for 28 days and then incubated with purified H-apoD (250 ng/mL) for 24h.

KA-treated and mature neurons were fixed in 4% PFA and processed for immunohistochemistry using an antibody against H-apoD (1:100; mouse monoclonal antibody, 2B9) and a goat anti-mouse IgG-Alexa 488 (1:1000; Life Technologies) as described here above (in section 2.3.10). H-apoD and DAPI fluorescent labelings were visualized by confocal microscopy.

2.3.13 Statistical analyses

Statistical differences were determined using One-way ANOVAs, Two-way ANOVAs followed by Bonferroni post hoc tests and unpaired t test, as indicated in the legend. Statistical analyses were performed with GraphPadPrism Software for statistical differences. $p<0.05$ was defined as the significance threshold. All data are presented as mean ± SEM.

2.4 Results

2.4.1 Endogenous M-apoD induction in the KA-lesioned mouse hippocampus coincides with astrocytic activation

Kainate (KA)-induced apoD expression has previously been reported in the rat hippocampus (Montpied et al., 1999; Ong et al., 1997). To investigate the time course of endogenous apoD expression in the mouse hippocampus following KA treatment, WT mice were injected with KA (15 mg/kg; i.p.) and the hippocampus was processed for the detection of apoD protein at days 1, 3 and 7 post-injection. In parallel, the expression of the classic reactive astrocyte marker GFAP, known as a neurotoxicity marker after brain injury (Eng and Ghirnikar, 1994), was also assessed (Chen et al., 2002). Endogenous apoD protein started to accumulate 1 day following KA injection and peaked at day 3 (2-fold induction) compared to non-injected mice (NI) (Fig. 2.1A). Hippocampal apoD expression returned to baseline levels 7 days post-injection. In a similar manner, the relative hippocampal expression of GFAP protein (Fig. 2.1B) began to increase on day 1 post-injection, with its highest expression at day 3 (3-fold induction), corresponding to the neurotoxicity peak. As for apoD, GFAP levels started to decrease at day 7, although they remained significantly higher than in non-injected animals. Overall, these data show that KA treatment induces apoD accumulation in the hippocampus of mice, a phenomenon which is concomitant with astrocyte activation.

Since endogenous M-apoD appears upregulated after KA insult and as H-apoD overexpression in neurons has demonstrated protective effects against oxidative stress (Bajo-Graneras et al., 2011; Ganfornina et al., 2008), encephalitis (Do Carmo et al., 2008) and nerve damage (Ganfornina et al., 2010), we next aimed at evaluating a potential protective role for apoD against KA-induced excitotoxicity.

2.4.2 H-apoD Tg mice are more resistant to KA-induced seizures

KA affects limbic structure and induces epileptic discharges (Ben-Ari et al., 1981). To investigate the effect of H-apoD overexpression on the generation of KA-induced seizures, WT and H-apoD Tg mice were injected with KA and their behavior was recorded for 2h post-injection. Seizure behaviors were scored according to a modified Racine's rating scale (Racine, 1972). KA injection induced significantly lower behavioural seizures scores in H-apoD Tg mice compared to WT animals (Fig. 2.2A). In addition, the sum of all seizure scores (cumulative score) that mice experienced over that time was decreased by 2-fold in H-apoD Tg mice (26.3 ± 3.06) compared to WT animals (50.12 ± 4.12; Fig. 2.2B). We also assessed the latency to reach 'stage 2' (myoclonic jerks of the head), 'stage 3' (bilateral forelimb clonus and rearing) and 'stage 4' (continuous rearing and falling) seizures. If the animal did not present these 'stage' seizures, the latency was considered as 120 min. Compared to WT mice, the latency to seizure onset was significantly higher in H-apoD Tg mice (Fig. 2.2C). This onset was delayed by 34±14.5 min for 'stage 4'. In 'stage 3' occurrence, this difference was further emphasized. Indeed, the onset of 'stage 3' seizures was observed 57± 13 min later in H-apoD Tg mice than in WT animals. No significant difference was observed for the latency to seizure 'stage 2' between Wt and H-apoD Tg mice. These results suggest that H-apoD overexpression in transgenic mice induces an increased resistance to KA-induced seizures. We thus explored the mechanisms involved in such protection.

2.4.3 Transgenic mice expressing H-apoD are more resistant to KA-induced apoptosis

Because H-apoD Tg mice were more resistant to KA-induced seizures, we investigated whether apoD overexpression attenuated the pathological consequences of KA-excitotoxicity at the cellular level. KA-induced cell apoptosis, a consequence

of seizures, was assessed in sagittal sections from WT and H-apoD Tg mice using TUNEL labeling, at day 3 post-injection (corresponding to the astrogliosis and apoD peaks) (Fig. 2.3). In WT mice, TUNEL labeling was present in different hippocampal subfields (CA1, CA3, hilus of the dentate gyrus) (Fig. 2.3C). In contrast, in the hippocampus of H-apoD Tg mice, TUNEL staining was decreased in the different subfields (Fig. 2.3D). Moreover, at day 3 post-KA injection, the overall level of cell damage, quantified as total TUNEL fluorescence in the H-apoD Tg hippocampus was significantly reduced compared to WT mice (Fig. 2.3E). These results indicate that H-apoD expression confers protection against KA-induced cell apoptosis.

2.4.4 H-apoD reduces the activation of inflammatory processes in the hippocampus

KA-induced neurodegeneration is associated with glial activation (microglia and astrocytes) and inflammatory mediators production (Chen et al., 2005). Since that apoD production is accompanied by GFAP activation following KA challenge (Fig. 2.1B) and based on the significant resistance of H-apoD Tg mice against seizures and hippocampal KA-induced apoptosis, we examined the presence of inflammatory processes in the hippocampus of WT and H-apoD Tg mice at day 3 post-injection. In the hippocampus of WT mice, KA injection resulted in significant astrocytic (GFAP; 3-fold) (Fig. 2.1B, 2.4A,B) and microglial activation (Mac-2; 3-fold) (Fig. 2.4A,C). It also induced expression of COX-2, an inflammatory mediator which promotes the recurrence of seizures and neuronal loss (Kim et al., 2001) (Fig. 2.4A,D). Interestingly, in response to KA treatment, H-apoD Tg mice display lower levels of Mac-2 and COX-2 than their WT counterparts. However, H-apoD Tg mice also display robust GFAP activation in their hippocampus, comparable to levels detected in WT animals. Together, these data demonstrate that the expression of H-apoD significantly attenuates inflammatory responses consequent to KA-induced excitotoxic neurodegeneration, except for the activation of astrocytes.

2.4.5 Effect of H-apoD on PMCA2 expression in hippocampus.

We then hypothesized that the neuronal expression of H-apoD induces favourable conditions that may attenuate KA-induced lesions. Plasma membrane calcium ATPases (PMCA), major calcium pumps, play an important role in regulating Ca^{2+} intracellular levels. KA reduces the levels of PMCA2, a major isoform present in the brain, leading to Ca2+ dyshomeostasis (Kurnellas et al., 2010). We thus analyzed the expression of PMCA2 in the hippocampus of WT and H-apoD Tg mice. PMCA2 expression in baseline, non KA-induced conditions was 1.7-fold higher in H-apoD Tg mice compared to WT animals (Fig. 2.5A,B). Immunohistological analyses confirmed that hippocampal PMCA2 was increased in H-apoD Tg mice (Fig. 2.5D) compared to WT animals (Fig. 2.5C). These data reveal that neuronal H-apoD overexpression leads to increased PMCA2 expression.

2.4.6 Effect of H-apoD on NMDA receptors in hippocampus.

Since the excitotoxicity process is induced by over-activation of glutamate receptors, we assessed the protein level of NMDA receptor subunits (NR1, NR2A and NR2B) in WT and H-apoD Tg mice (Fig. 2.6). WT and H-apoD Tg animals presented similar hippocampal protein levels of NR1 and NR2A (Fig. 2.6A, 2.6B, C), although a trend for NR2A downregulation in H-apoD Tg mice was observed (Fig. 2.6A, C). In contrast, NR2B levels were significantly reduced in H-apoD Tg mice (Fig. 2.6A,D) (30% decrease). Because NMDA receptors are essential mediators of brain plasticity, we also examined the effect of neuronal H-apoD over-expression on synaptophysin, a widely used marker of synaptic plasticity (Reddy et al., 2005). Interestingly, synaptophysin was not altered in the hippocampus of H-apoD Tg compared to WT animals (Fig. 2.6A, 2.6E). This data indicates that the protection against KA-induced toxicity observed H-apoD Tg mice might be imputable in part to decreased NR2B levels in these mice.

2.4.7 H-apoD is involved in cholesterol metabolism.

A role for apoD in lipid metabolism has been suggested by several studies (Do Carmo et al., 2009b; Perdomo and Henry Dong, 2009; Perdomo et al., 2010). Defects in cholesterol metabolism are associated with various neurological injuries, such as kainate-induced excitotoxicity (He et al., 2006; Ong et al., 2003).

We first evaluated whether neuronal expression of human apoD modulates cholesterol levels in the mouse brain. These were measured in membrane, cytosol and whole brain fractions of WT and H-apoD Tg mice, using the Amplex Red Cholesterol assay. Interestingly, the cytosolic brain fraction of H-apoD Tg mice had significantly less cholesterol (40% decrease) compared to WT mice (Fig. 2.7B). However, in the whole homogenate (Fig. 2.7A) and membrane fractions (Fig. 2.7C), the cholesterol levels remained unchanged.

Because neurons can convert excess cholesterol to 24(S)-hydroxycholesterol (24-HOC) which crosses the blood brain barrier to be eliminated by the liver via the plasma (Bjorkhem and Meaney, 2004), we examined if the decreased cholesterol levels in the cytosolic brain fraction were associated with increased 24-HOC levels in the brain and plasma of H-apoD Tg mice. Despite a trend towards an increase, 24-HOC levels in the plasma (Fig. 2.7E) and brain (Fig. 2.7D) were not significantly different between WT and H-apoD Tg mice. Thus, 24-HOC pathway is not involved in the decreased cholesterol levels of the cytosolic brain fraction (Fig. 2.7B) in Tg-apoD mice.

Beside 24-HOC production, neurons can release their cholesterol by efflux pathways (Chen et al., 2013). To investigate the effect of apoD on intracellular cholesterol content, hippocampal neurons grown for 10 days were treated with purified H-apoD for 24h. The intracellular cholesterol was then stained with filipin, which can bind

cholesterol. Consistent with our observations in the brain of H-apoD Tg mice (Fig. 2.7), H-apoD-treated hippocampal neurons (Fig. 2.8D, 2.8F) showed less cholesterol/filipin staining than untreated neurons (Fig. 2.8A, 2.8C). Interestingly, the cholesterol/filipin content of neurites remained unchanged in H-apoD treated neurons (Fig. 2.8C', 2.8F'). To improve further our understanding of the processes involved in apoD-mediated reduction of cholesterol content, we then assessed the effect of purified H-apoD on the neuronal uptake of exogenous cholesterol. Hippocampal neuronal cultures were preincubated for 3h in the presence of cholesterol and further exposed or not to purified human apoD for 24h. Cholesterol uptake was stained by filipin. Filiping staining showed cholesterol accumulation in hippocampal neurons preincubated with exogenous cholesterol (Fig. 2.8G). Interestingly, this accumulation was decreased by 50% following purified H-apoD incubation (Fig. 2.8H, I).

Overall, these results show that apoD is involved in the regulation of intraneuronal cholesterol content.

2.4.8 H-apoD internalization by neurons is increased following KA treatment.

ApoD is a secreted protein which can be internalized by various cell lines (Do Carmo et al., 2007; Leung et al., 2004; Sarjeant et al., 2003; Thomas et al., 2003b) and by primary mouse astrocytes (Bajo-Graneras et al., 2011). It was also proposed that neurons, which express very little or no apoD, can import it from surrounding glial cells (Ordonez et al., 2006). However, apoD internalization by neurons in normal and stress conditions was never demonstrated. To address this question, hippocampal neuronal cultures (at 10 days) were preincubated for 24h with purified H-apoD and further exposed to KA (100 μM) or control conditions for 24h. The exogenous apoD internalized in neurons was detected by immunocytochemistry, using specific antibody against human apoD. Under physiological conditions, purified human apoD was detected mostly in the perinuclear area into vesicular compartments in soma and

neurites (Fig. 2.9B). Kainate treatment provoked an important intracellular and nuclear accumulation of H-apoD (Fig. 2.9C). As some studies reported an accumulation of apoD in the aging brain (Kalman et al., 2000; Kim et al., 2009; Loerch et al., 2008), we then assessed the internalization of exogenous apoD in mature neurons (at 28 days). Compared to young neurons (Fig. 2.9B), H-apoD internalization in mature neurons was increased and distributed in all cells including along the neurites (Fig. 2.9D). However, apoD internalization in mature neurons is less dramatic compared to kainate treated neurons (Fig. 2.9C). Overall this data suggests that apoD can be internalized in neurons and this internalization is accentuated in aging and injury conditions.

2.5 Discussion

The excessive activation of glutamatergic receptors can cause excitotoxicity due to increased Ca^{2+} influx into cells. This leads to a number of deleterious consequences and ultimately to neuronal dysfunction and death. Excitotoxicity was reported in various neurodegenerative conditions with distinct etiologies such as Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis (Dong et al., 2009; Van Den Bosch et al., 2006). This suggests that excitotoxicity may be a common pathway underlying neurological pathogenesis. Understanding the mechanisms associated with excitotoxicity regulation should provide potential targets for neuroprotective strategies.

In this study, we provide additional clues on the mechanisms involved in apoD-mediated neuroprotection. We demonstrate that overexpression of human apoD in neurons protects against KA-induced lesions *in vivo*, decreasing the severity of seizures, cellular apoptosis and reactive gliosis in KA-treated animals. This neuroprotection appears to be linked to the control of calcium levels, glutamate receptor subunits and cholesterol metabolism.

In accordance with previous studies (Montpied et al., 1999; Ong et al., 1997), acute KA treatment induced a transient apoD upregulation in the mouse hippocampus that peaked at day 3 post-injection and was resolved after 7 days. Such peak of apoD production corresponds to that of astrocyte activation and proliferation, as documented by increased GFAP levels. The concordance in time of apoD and GFAP levels is in line with the fact that astrocytes are one of major sites of apoD production in the CNS (Rassart et al., 2000). In addition, increased synthesis of apoD in astrocytosis has been observed following enthorinal cortex lesion (Terrisse et al., 1999), coronavirus OC43-induced encephalitis (Do Carmo et al., 2008), experimental stroke (Ruscher et al., 2010) and aging processes (del Valle et al., 2003). However, it

remains to be determined if apoD production is a cause or consequence of astrocytosis. Nevertheless, astrocytes may not be the sole source of apoD production as after KA injury, apoD was also detected in pyramidal neurons of the hippocampus (Ong et al 1997). The peak of apoD production also correlated with increased apoptosis, as assessed by TUNEL labeling. Increased apoD expression has been reported in pathologic conditions with increased apoptosis and a potential role of apoD on the death-survival balance was suggested (Bajo-Graneras et al., 2013).

Epileptiform seizures can be induced by the systemic or intracerebral injection of KA in rodents (Ben-Ari, 1985). Seizures are generated in the CA3 region of the hippocampus, where pyramidal cells are more sensitive to KA treatment (Ben-Ari and Cossart, 2000), then propagate to other limbic structures (Ben-Ari and Cossart, 2000; Miles and Wong, 1983). These recurrent seizures are associated with excitotoxic cell death cascades (Zheng et al., 2011). However, the molecular mechanisms involved in seizure-induced cell death are not clearly understood. However, a tight relationship between COX-2 and seizures severity and induced cell death was reported in both *in vivo* and *in vitro* models of excitotoxicity (Kim et al., 2001; Kunz and Oliw, 2001; Sayyah et al., 2003; Takemiya et al., 2003).

Induction of pro-inflammatory processes after kainate treatment, including upregulation of cyclooxygenase enzymes, prostaglandins, cytokine production and microglia activation (Jankowsky and Patterson, 2001; Vezzani and Granata, 2005) contributes to cell damage. KA treatment was shown to immediately induce COX-2 expression thus facilitating the recurrence of hippocampal seizures (Kim et al., 2001; Takemiya et al., 2007). Interestingly, the COX-2 induction was reduced in KA-injected H-apoD Tg mice. The effect of apoD on COX-2 down-regulation might be mediated through the control of arachidonic acid (AA) levels. First, apoD could influence the availability of free AA, its preferential ligand, in the cell by

binding/chelation, limiting its use in enzymatic pathways such as COX-2 (He et al., 2009; Thomas et al., 2003a). Moreover, apoD could attenuate PLA2 activity triggered by excitotoxic conditions, again reducing free AA levels (Do Carmo et al., 2008). Our results further demonstrate that the anti-inflammatory effects of apoD in KA-response are mediated by limiting microglial but not astrocytic activation. It has already been established that inhibition of microglial activation exerts neuroprotective actions against KA-induced injury (Byun et al., 2010; Penkowa et al., 2005). The effects of apoD on microglia activation might be COX-2-dependent considering that COX-2 contributes to microglial activation (Palumbo and Bosetti, 2013; Vijitruth et al., 2006). However, as COX-2 regulates both microglia and astrocytic activation, the fact that H-apoD Tg mice display, at day 3 after KA treatment, similar GFAP but decreased Mac-2 levels compared to WT mice suggests that the response to COX-2 reduction due to apoD overexpression is faster in microglia than in astrocytes.

Another pathway by which apoD could be protective is by increasing the Ca^{2+} buffering capacity of neurons. Excessive influx of calcium into neurons, as generated by the over-activation of glutamate receptors by KA plays a critical role in seizure genesis (McNamara, 1992; Meyer, 1989) and triggers diverse cellular death mechanisms (Pellegrini-Giampietro et al., 1997; Zipfel et al., 1999). Intracellular calcium homeostasis requires several mechanisms, regulation of Ca^{2+} efflux, which can be carried out by plasma membrane Ca^{2+} ATPases (PMCA). The expression of the most abundant isoform in the CNS, PMCA-2, is decreased after KA exposure (Kurnellas et al., 2010; Tempel and Shilling, 2007). It is likely that, by stimulating the production of PMCA-2, the presence of H-apoD in neurons contributes to lower intracellular calcium levels and consequently the detrimental effects of excitotoxicity in H-apoD Tg mice. However, the mechanism by which apoD influences PMCA-2 levels remains to be determined. As increases of intracellular Ca^{2+} content by excitotoxicity stimulate the generation of reactive oxygen species (Rothman and

Olney, 1986) and lipid peroxidation, which in turn downregulate rapidly PMCA levels (Chiarello et al., 2014), it is possible that apoD affects PMCA-2 levels through its anti-oxidant properties. Indeed, apoD and its orthologs have been shown to protect from pro-oxidative insults in several organisms (Charron et al., 2008; Ganfornina et al., 2008; Muffat et al., 2008; Sanchez et al., 2006).

Our results also support the hypothesis that apoD limits excitotoxicity by controlling NMDA receptors (NMDAR) subunit composition. It is well established that the activation of NMDAR-mediated neuronal death or survival pathways depends on its subunit composition and subcellular localisation (Hardingham et al., 2002; Liu et al., 2007; Szydlowska and Tymianski, 2010; Zhou and Baudry, 2006). Indeed, activation of NR2B containing NMDARs increases neuronal apoptosis and in contrast, the action of NR2A-containing NMDARs promotes neuronal survival pathways in rat models (*in vitro* and *in vivo*) of ischemic stroke (Liu et al., 2007; Taghibiglou et al., 2009). The down-regulation of NR2B levels in H-apoD Tg mice hippocampus, with unchanged levels of NR1 and NR2A subunits, might contribute to limit seizure severity and detrimental effects such as apoptosis in response to KA challenge. However, NR2B downregulation in surface membrane at synaptic sites of rat striatal neurons has been reported to produce synaptic plasticity alterations (Mao et al., 2009). Of relevance, this NR2B down-regulation does not affect the expression of the presynaptic vesicle protein synaptophysin (Liu et al., 2013; Martinez et al., 1997; Rosenbrock et al., 2005), suggesting normal synaptic plasticity mechanisms. However, how apoD controls NR2B levels is yet unknown and needs further attention.

Finally, apoD-mediated neuroprotection after KA challenge can also be related to its role in lipid metabolism. A direct association between dysregulation of cholesterol homeostasis and neurodegeneration has been clearly demonstrated in several

neurodegenerative diseases, such as Alzheimer's, Huntington's and Niemann-Pick type C disease (Vance, 2012), diseases which also show an upregulation of apoD. Cholesterol, an integral component of neural membranes, is crucial for the neuronal functions such as axon guidance and synaptic transmission (Dietschy and Turley, 2001). Cholesterol is mostly synthesized in astrocytes and transported to neurons by apolipoprotein E-containing lipoproteins. However, neurons can also produce cholesterol in their cell bodies (Vance, 2012).

Our results show that H-apoD Tg mice harbor decreased levels of cytosolic cholesterol but similar total cholesterol levels in their brain compared to WT mice. This suggests that apoD favours cholesterol redistribution, to membranes or for elimination. Although we could not detect significant differences in the levels of membrane-bound cholesterol or in brain and plasmatic 24-OH cholesterol levels, a cholesterol derivative that can cross the blood brain barrier for elimination (Bjorkhem et al., 1997; Lund et al., 2003). The involvement of apoD in lipid metabolism is clearly established (Do Carmo et al., 2009b; Perdomo et al., 2010). Mice lacking apoD display an increase of specific fatty acids in their brain with a decreased ratio of cholesterol:total phospholipids in the membrane compartment (Thomas and Yao, 2007).

In accordance with cholesterol dysmetabolism in neurodegenerative diseases, it was reported that intracerebroventricular injections of KA in rats resulted in an increase in immunoreactivity to cholesterol in the affected CA fields of the hippocampus (Ong et al., 2003). Interestingly, our results show an important increase in the internalization of H-apoD in KA treated primary mouse neurons. In addition, the addition of exogenous H-apoD to healthy primary neurons reduced their cholesterol content. Altogether, our study suggests an important role for apoD in regulating cholesterol metabolism in the brain upon injury.

In sum, this study provides additional clues on the emerging mechanisms underlying the neuroprotective role of apoD upregulation in CNS injury. As a result of its lipid-binding properties, apoD might reverse the excitotoxicity-induced accumulation of cholesterol and potentially other lipids in neurons and most importantly their presence as free, cytosolic species, limiting their availability for deleterious reactions. In the same way, apoD limits pro-inflammatory reactions such as microglial activation and this might be related to its capacity of binding/chelating free lipids. Apart from its functions in lipid metabolism, apoD could limit the effects of acute excitotoxicity by controlling NMDAR subunit composition and intracellular Ca^{2+} levels.

2.6 Acknowledgements

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2.7 Figure legends

Figure 2.1 Correlation between M-apoD protein expression and astrogliosis after KA treatment. Endogenous apoD (M-apoD) (A) and astrocyte activation GFAP (B) protein expression were analysed by western blot of hippocampal homogenates from non-injected (NI) and WT mice injected with kainic acid (15 mg/kg) 1, 3 and 7 days post-injection. GFAP is used as a marker of astrocyte activation. Values were normalized by β-Actin protein expression: the ratio in non-injected mice was given an arbitrary value of 1. Normalized values are presented as mean ± SEM (n=3 to 5 animals for each group). One-way Anova followed by Bonferroni post-test: *$p<0.05$, ***$p<0.001$ compared to non-injected mice; ###: $p<0.001$ compared to 1 day post-injected mice; δ: $p<0.05$, δδ: $p<0.01$ compared to 3 days post-injected mice.

Figure 2.2 H-apoD Tg mice are more resistant to KA-induced seizures. (A) Seizure intensity was evaluated for 2h, according to a modified Racine's scale, in WT and H-apoD Tg mice injected with kainic acid (15 mg/kg). Unpaired t test: ***$p=0.0001$ compared to WT. (B) Cumulative seizure score was assessed as the sum of the scores recorded each 5 min over a 2h period after KA injection in WT and H-apoD Tg mice. Unpaired t test: ***$p<0.0001$ compared to WT. (C) The latency to 'stage 2', 'stage 3' and 'stage 4' seizures was measured in WT and H-apoD Tg mice. Animals that did not present these 'stage' seizures were given a value of 120 min. Two-way Anova following by Bonferroni post-test: *$p<0.05$, ***$p<0.001$ compared to WT. Values are means ± SEM of 17 animals per group.

Figure 2.3 H-apoD Tg mice are more resistant to KA-induced apoptosis. Representative images of nuclear (PI, red) (A, B) and TUNEL staining (green) (C, D) of sagittal sections (dorsal hippocampus is shown) from WT (A, C) and H-apoD Tg (B, D) mice, 3 days post-KA-injection. E, Quantification of apoptotic cells in the hippocampus of KA-treated mice. The number of TUNEL-positive cells was divided by the number of total cells (PI-positive nuclei) and normalized by the WT, which was given an arbitrary value of 100%. Normalized values are presented as mean (4 animals per group) ± SEM. Unpaired t test: **p=0.0063 compared to WT.

Figure 2.4 KA-induced inflammation is reduced in H-apoD Tg mice. (A) Western blot analysis of H-apoD, GFAP (astrocyte marker), Mac-2 (microglia marker) and COX-2 in hippocampal homogenates of vehicle (c) and KA-injected WT and H-apoD Tg mice, 3 days post-injection. β-Actin is used as loading control. Quantification of GFAP (B), Mac-2 (C) and COX-2 (D) protein expression by densitometry. Values were normalized by β-Actin protein expression and by vehicle-injected WT mice values, which were given an arbitrary value of 1. Normalized values are presented as mean ± SEM (n=3 to 5 animals for each group). Two-way Anova following by Bonferroni post-test: **p<0.01, ***p <0.001 compared to vehicle-injected WT; #p<0.05 compared to KA-injected WT mice. (C) mice injected with PBS (vehicle). KA: mice injected with kainate (15 mg/kg).

Figure 2.5 PMCA2 levels are up-regulated in H-apoD Tg mice. (A) Western blot analysis of PMCA2 in hippocampal homogenates of WT and H-apoD Tg mice. β-Actin is used as loading control. (B) Quantification of PMCA2 protein expression. Values were normalized by β-Actin protein expression and by WT values, which were given an arbitrary value of 1. Normalized values are presented as mean ± SEM (n=3 to 4 animals for each group). Unpaired t test: ***p=0.0006 compared to WT mice. (C-D) PMCA2 immunostaining (green) in hippocampus sections of WT (C) and H-apoD Tg (D) mice. Nuclei were stained with DAPI (blue).

Figure 2.6 NMDA receptor subunit expression in H-apoD Tg mice. (A) Western blot analysis of NMDAR subunits (NR1, NR2A, NR2B) and synaptophysin in hippocampal homogenates of WT and H-apoD Tg mice. β-Actin is used as loading control. Quantification of NR1 (B), NR2A (C), NR2B (D) and synaptophysin (E) protein expression. Values were normalized by β-Actin protein expression and by WT values (with an arbitrary value of 1). Note that only NR2B expression in H-apoD Tg was statistically different from WT. Normalized values are presented as mean ± SEM (n=3 to 4 animals for each group). Unpaired t test: *p=0.0181 compared to WT mice.

Figure 2.7 Cholesterol metabolism in H-apoD Tg mice. Cholesterol levels were assessed in whole (A), cytosolic (B) and membrane (C) fractions of brain homogenates in WT and H-apoD Tg mice using the Amplex red assay. Values were normalized by the total protein content of each fraction. Each graph represents the mean values ± SEM (n=3 to 6 animals for each group), normalized by WT mice, which was given an arbitrary value of 100%. Note that only the cytosolic fraction of H-apoD Tg brain was statistically different from WT. Unpaired t test: *p=0.0283 compared to WT mice. Quantification of 24-hydroxycholesterol (24-OHC) in brain homogenates (D) and plasma (E) of WT and H-apoD Tg mice. Each graph represents the mean values ± SEM (n=3 to 4 animals for each group), normalized by WT mice, which was given an arbitrary value of 100%.

Figure 2.8 H-apoD affects cholesterol distribution in neurons. Hippocampal neurons (10 days), treated (D, E, F) or not (A, B, C) with purified human ApoD for 24h, were stained with filipin (A, D) (gray) and anti-GAP43 antibodies (red) (B, E) and analysed by confocal microscopy. Enlarged view of boxed segment shows decreased filipin labeling intensity in the soma of neurons treated with purified H-apoD (F) compared to non-treated neurons (C). Note that the filipin staining extended to the neurites and that H-apoD-treated (F') and non-treated neurons (C') show similar filipin staining. Scale bar = 10 μm. G-I, Filipin staining was assessed in hippocampal neuronal (10 days) preincubated for 3h in the presence of cholesterol and further exposed (H) or not to purified H-apoD (G) for 24h. Quantification of filipin staining (I). Values were normalized by filipin-staining of neurons non-treated with H-apoD (arbitrary value of 100%). Normalized values are presented as mean ± SEM (n=3). Unpaired t test: **p=0.0011 compared to neurons non-treated with H-apoD.

Figure 2.9 H-apoD internalization is increased in KA-treated neurons. Hippocampal neurons (10 days) preincubated for 24h with purified H-apoD and further exposed to control conditions (B) or KA (100 μM) (C) for 24h were immunostained with anti-H-apoD (green) and anti-GAP43 antibodies (red) and analysed by confocal microscopy. As a control, staining was also assessed in cells without H-apoD pre-incubation, before KA treatment (A). Nuclei were labeled with DAPI (blue). (D) H-apoD internalization in mature hippocampal neurons (28 days). H-apoD (green) and GAP43 (red) immunoreactivity was assessed in mature hippocampal neurons incubated with purified H-apoD for 24h. Note that important H-apoD internalization was observed in KA-treated neurons (B) and mature neurons (C) compared to neurons non-treated with KA (A). Scale bar = 10 μm.

2.8 Figures

Figure 2.1

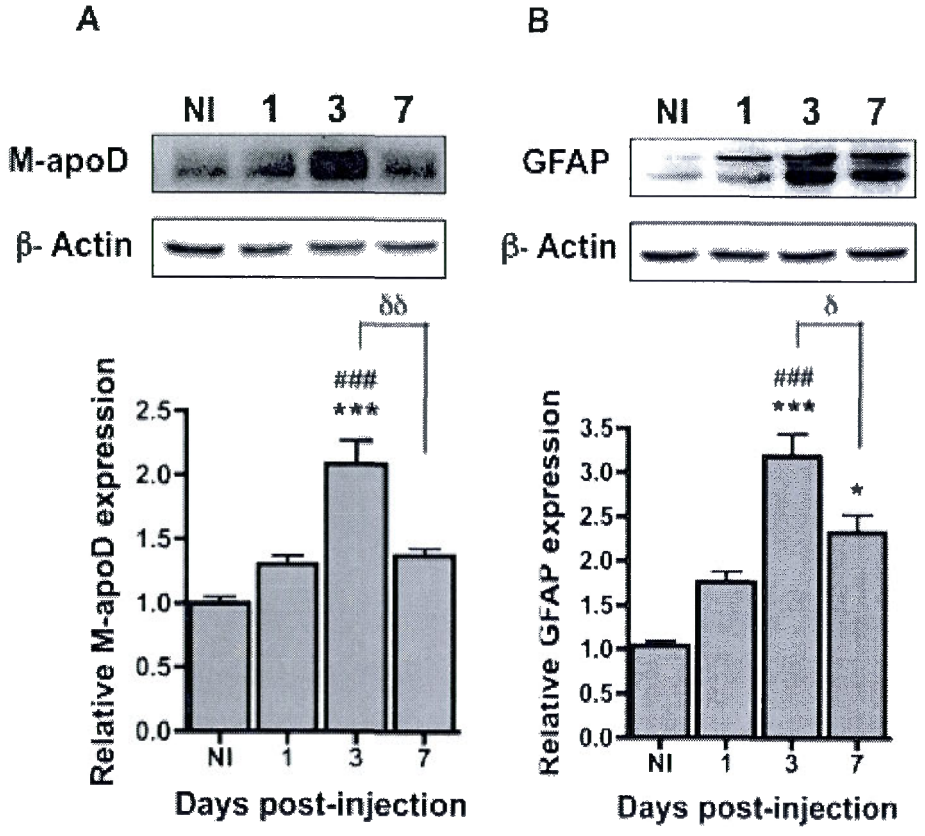


Figure 2.2

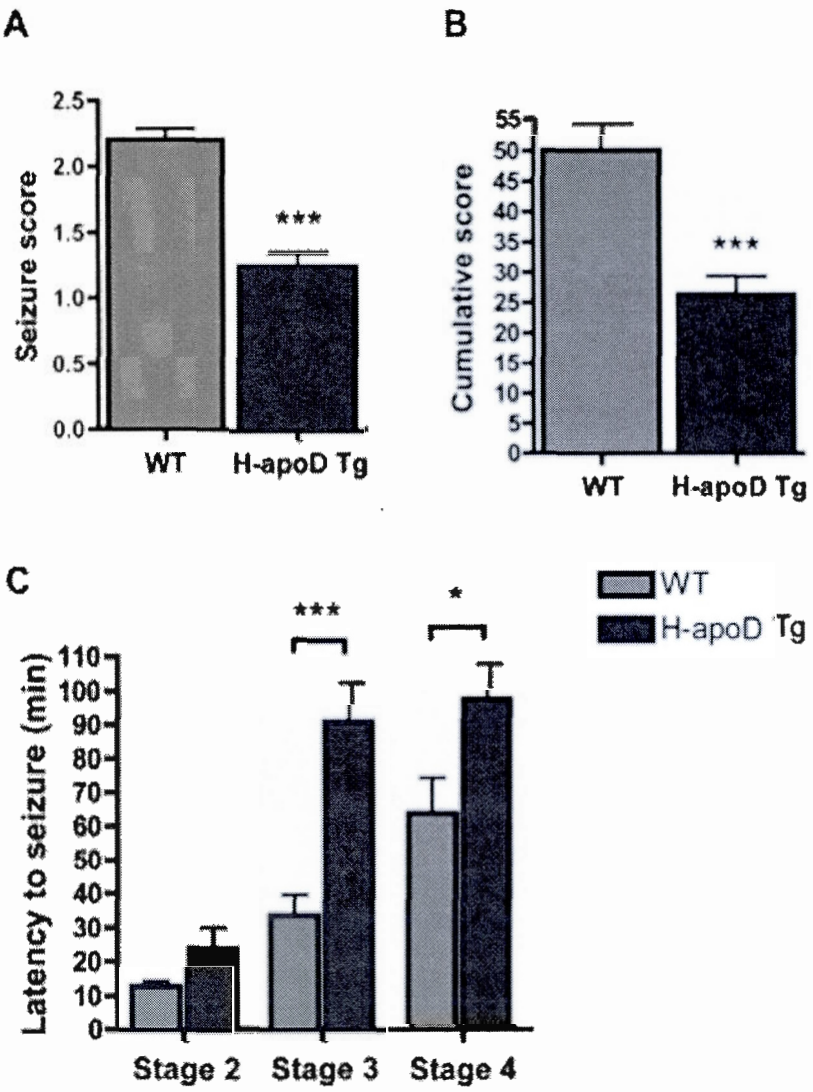

A
Seizure score
2.5
2.0
1.5
1.0
0.5
0.0
***
WT
H-apoD Tg
B
Cumulative score
55
50
45
40
35
30
25
20
15
10
5
0
***
WT
H-apoD Tg
C
Latency to seizure (min)
110
100
90
80
70
60
50
40
30
20
10
0
***
*
WT
H-apoD Tg
Stage 2
Stage 3
Stage 4

Figure 2.3

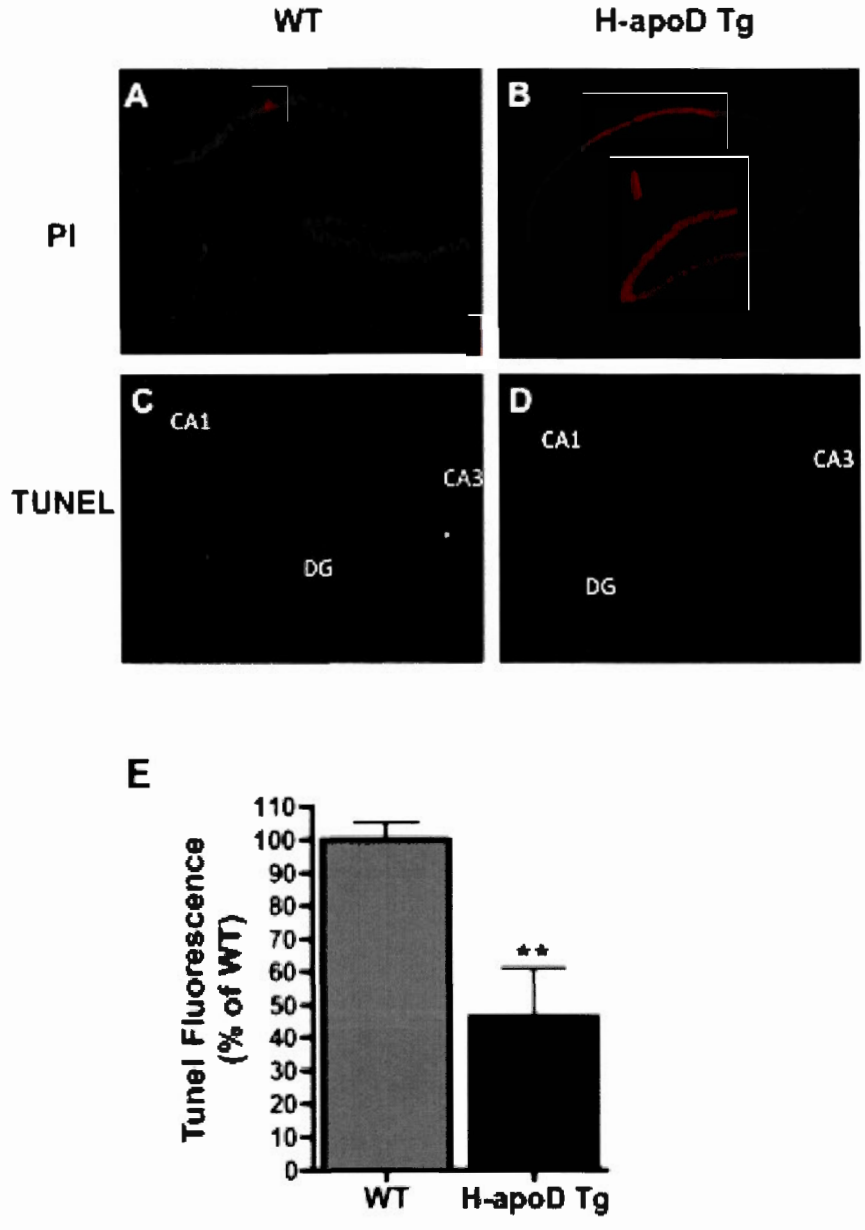

WT
H-apoD Tg
A
B
PI
C
CA1
CA3
DG
TUNEL
D
CA1
CA3
DG
E
Tunel Fluorescence (% of WT)
110
100
90
80
70
60
50
40
30
20
10
0
**
WT
H-apoD Tg

Figure 2.4

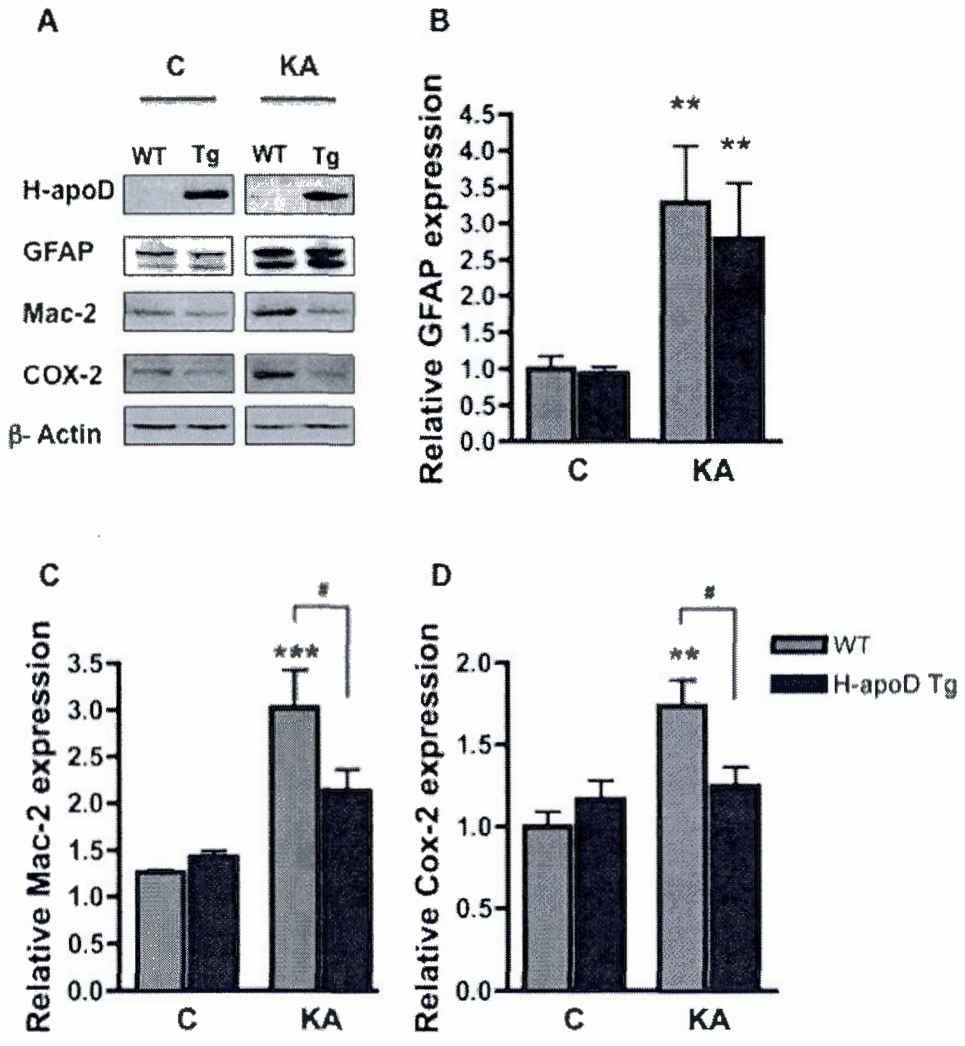

A
C
KA
WT
Tg
WT
Tg
H-apoD
GFAP
Mac-2
COX-2
β- Actin
B
Relative GFAP expression
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
C
KA
C
Relative Mac-2 expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
#
***
C
KA
D
Relative Cox-2 expression
2.0
1.5
1.0
0.5
0.0
#
**
C
KA
WT
H-apoD Tg

Figure 2.5

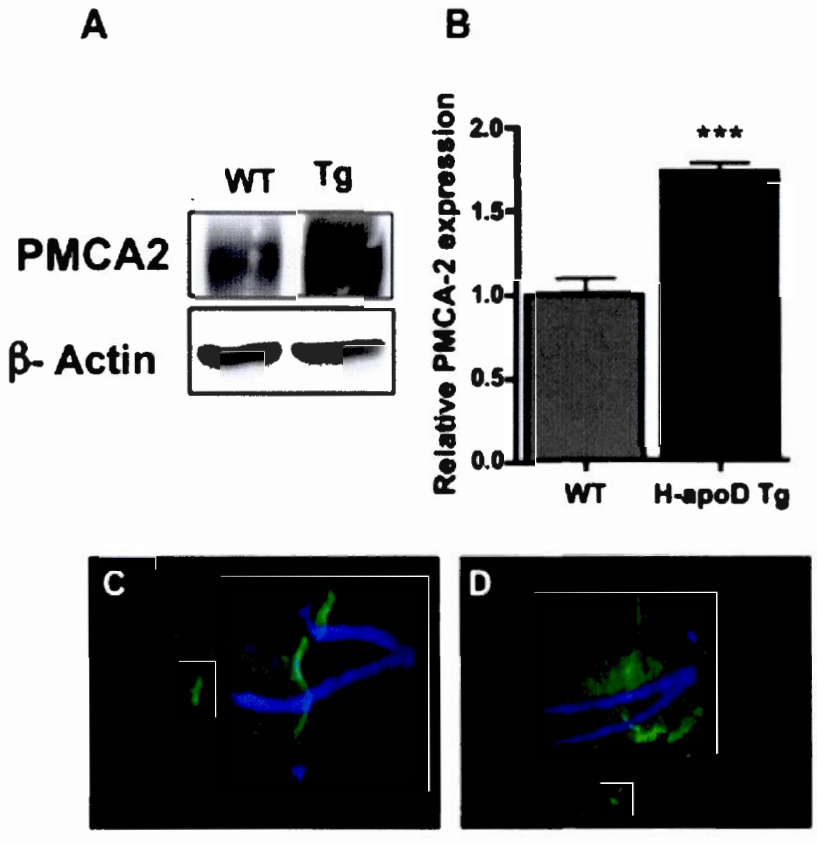

A
B
WT
Tg
PMCA2
β- Actin
Relative PMCA-2 expression
2.0
1.5
1.0
0.5
0.0
***
WT
H-apoD Tg
C
D

Figure 2.6

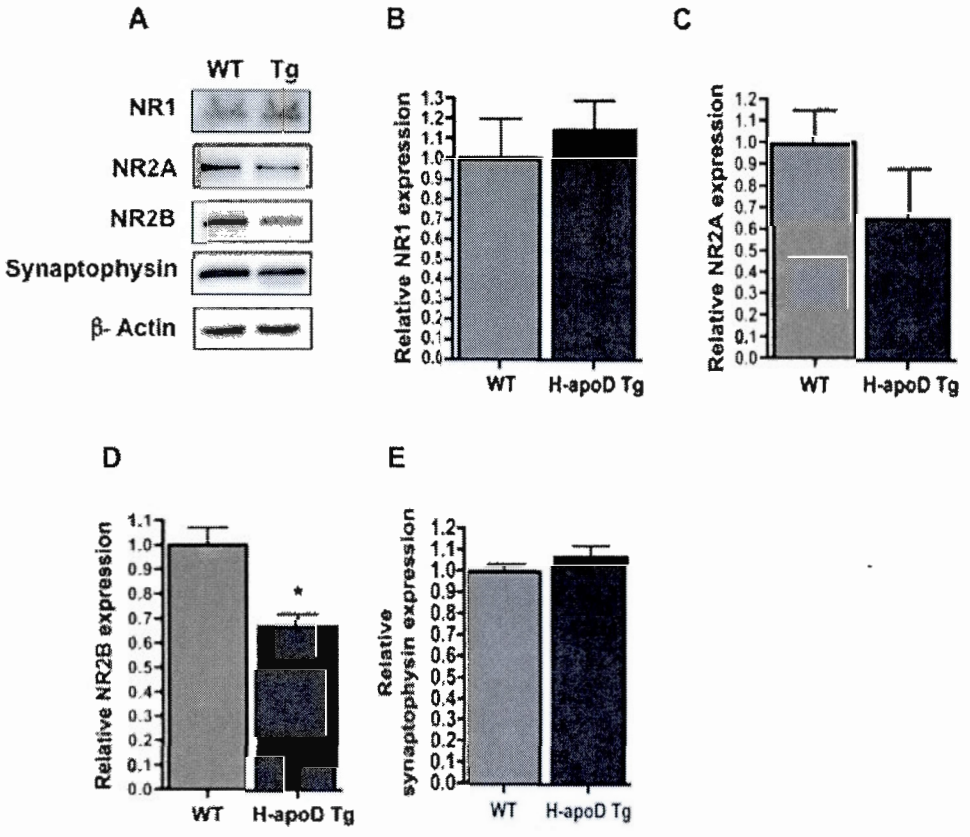

A
WT
Tg
NR1
NR2A
NR2B
Synaptophysin
β- Actin
B
Relative NR1 expression
WT
H-apoD Tg
C
Relative NR2A expression
WT
H-apoD Tg
D
Relative NR2B expression
*
WT
H-apoD Tg
E
Relative synaptophysin expression
WT
H-apoD Tg

Figure 2.7

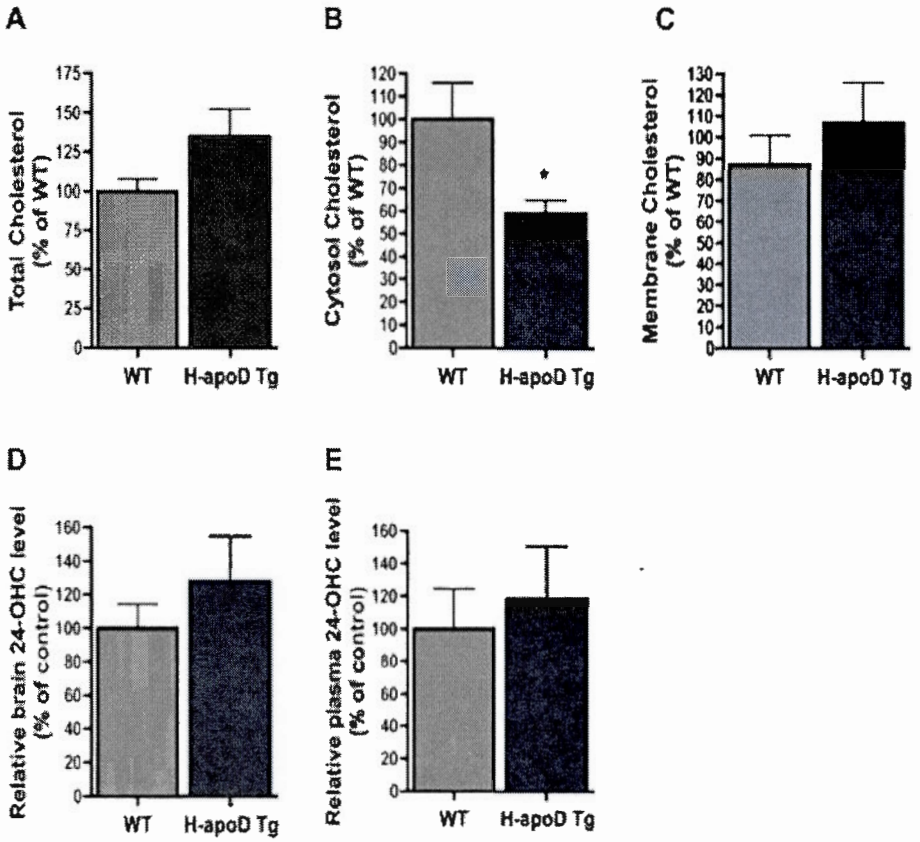

A
Total Cholesterol (% of WT)
175
150
125
100
75
50
25
0
WT
H-apoD Tg
B
Cytosol Cholesterol (% of WT)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
WT
H-apoD Tg
C
Membrane Cholesterol (% of WT)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
WT
H-apoD Tg
D
Relative brain 24-OHC level (% of control)
160
140
120
100
80
60
40
20
0
WT
H-apoD Tg
E
Relative plasma 24-OHC level (% of control)
160
140
120
100
80
60
40
20
0
WT
H-apoD Tg

Figure 2.8

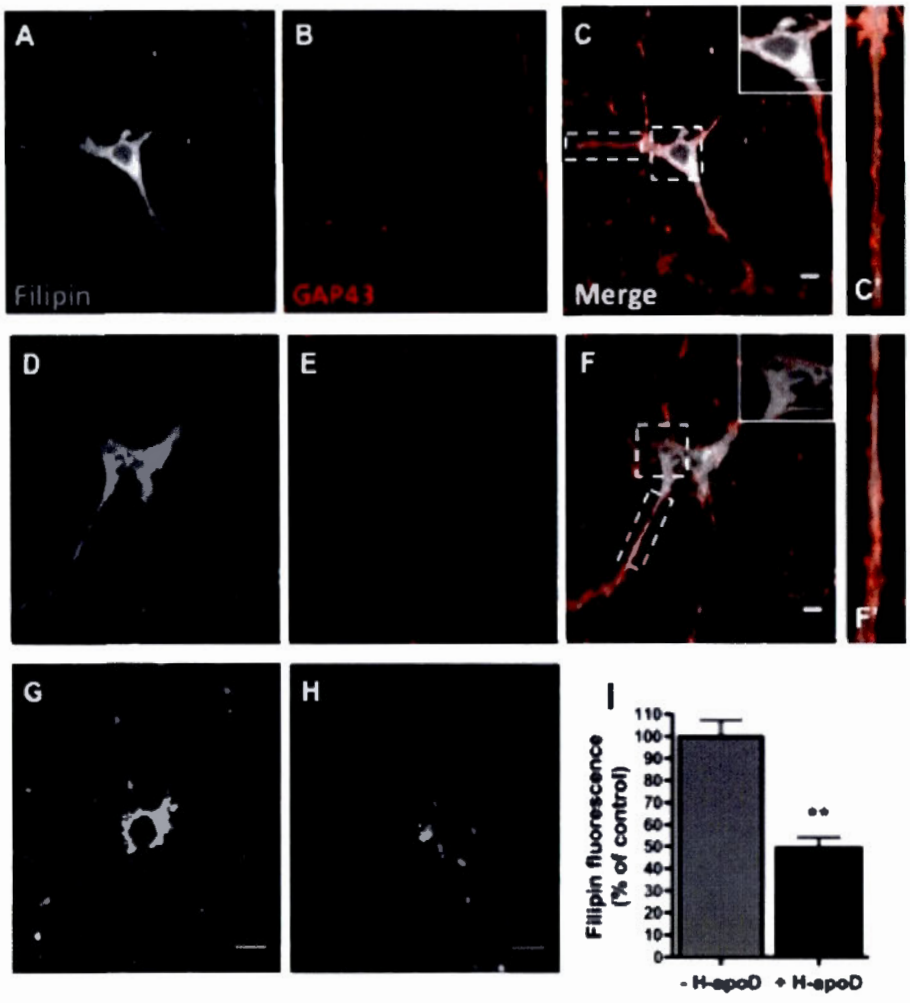
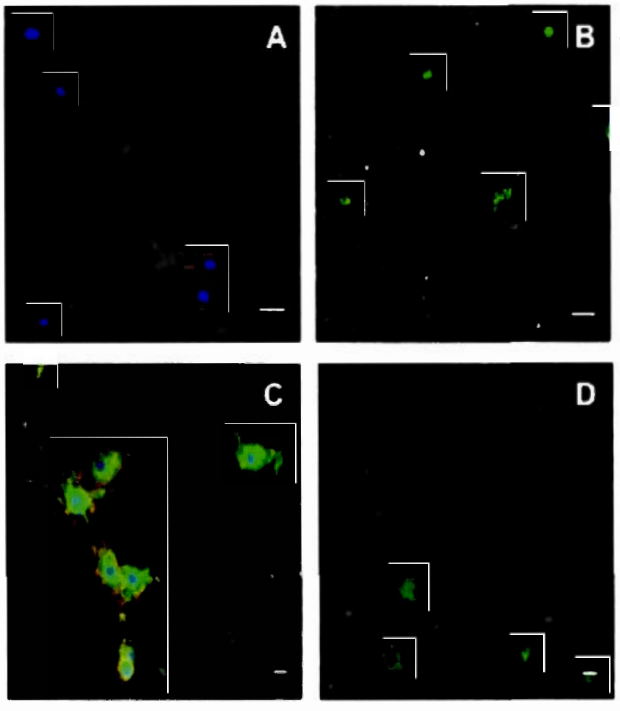

A
B
C
C'
Filipin
GAP43
Merge
D
E
F
F'
G
H
I
Filipin fluorescence (% of control)
110
100
90
80
70
60
50
40
30
20
10
0
**
- H-apoD
+ H-apoD

Figure 2.9



CHAPITRE III

APOLIPOPROTEIN D INTERNALIZATION IS A BASIGIN DEPENDANT MECHANISM

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Avant propos

L'étude présentée en chapitre II nous a permis d'analyser l'effet neuroprotecteur de l'apoD dans un modèle de neurodégénérescence induite par excitotoxicité. Cette étude a également mis en évidence la capacité d'internalisation de l'apoD dans les cellules neuronales et l'accentuation de cette capacité en situation de stress. L'objectif de ce chapitre a donc été de déterminer le mode d'internalisation de l'apoD dans les cellules, en utilisant comme modèle des cellules HEK 293T, une lignée cellulaire de l'épithélium rénal embryonnaire humain.

Ce chapitre est présenté sous forme d'article scientifique qui est publié dans le journal 'The Journal of Biological Chemistry'. Les références de cet article se trouvent à la fin de la thèse. J'ai été impliquée dans toutes les facettes expérimentales des travaux présentés dans cet article ainsi que dans le montage des figures et la rédaction de l'article. La première partie de ce projet (concernant la vérification de l'existence d'un récepteur dans l'internalisation de l'apoD) a été conçue et élaborée avec la collaboration du Dr Louise Brissette qui a également corrigé l'article. La supervision du projet et la correction de l'article ont été réalisés par le Dr Eric Rassart.

3.1 Abstract

Apolipoprotein D (apoD), a member of the lipocalin family, is a 29-kDa secreted glycoprotein which binds and transports small lipophilic molecules. Expressed in several tissues, apoD is upregulated under different stress stimuli and in many pathologies. Numerous studies revealed that overexpression of apoD led to neuroprotection in various mouse models of acute stress and neurodegeneration. This multifunctional protein is internalized in several cells types but the specific internalization mechanism remains unknown. In this study, we demonstrate that the internalization of apoD involves a specific cell surface receptor in 293T cells identified as the transmembrane glycoprotein basigin (BSG; CD147), more particularly its low glycosylated form. Our results show that internalized apoD colocalizes with BSG into vesicular compartments. Downregulation of BSG disrupted the internalization of apoD in cells. In contrast, overexpression of basigin in SH-5YSY cells which poorly express BSG restored the uptake of apoD. Cyclophilin A, a known ligand of BSG, reduced competitively apoD internalization confirming that BSG is a key player in the apoD internalization process. In summary, our results demonstrate that basigin is very likely the apoD receptor and provide additional clues on the mechanisms involved in apoD-mediated functions, including neuroprotection.

Keywords: apolipoprotein D (apoD), basigin, internalization, cyclophilin A, human neuroblastoma line cell.

3.2 Introduction

Apolipoprotein D (apoD) was first described in 1973 as a protein associated with lipoproteins in human plasma, more specifically with high density lipoproteins (HDL) (McConathy and Alaupovic, 1973) Since its discovery, apoD has been characterized as a 29-kDa secreted glycoprotein comprising eight-stranded antiparallel β-barrels that form an hydrophobic conically shaped cavity that is referred to as the pocket of apoD binding to its ligands (Eichinger et al., 2007). It is a member of the family of lipocalins responsible for the binding and transport of small lipophilic molecules (Drayna et al., 1986; Flower, 1996; Rassart et al., 2000). ApoD is known to bind more specifically arachidonic acid (AA), progesterone and sphingomyelin with high affinity, but also to interact directly or indirectly with cholesterol, bilirubin and estradiol (Morais Cabral et al., 1995; Rassart et al., 2000; Ruiz et al., 2013; Vogt and Skerra, 2001). In many species, apoD is widely expressed during the embryonic, postnatal and adult life. In human, it is mainly expressed in the testes, brain, placenta, kidneys, spleen, lungs, ovaries and pancreas (Drayna et al., 1986; Ganfornina et al., 2005; Provost et al., 1991a; Sanchez et al., 2002; Seguin et al., 1995; Smith et al., 1990; Vieira et al., 1995). However, unlike most apolipoproteins, apoD is poorly expressed in the human liver and intestine. In addition, it is found primarily in the CNS in rodents, suggesting an important role for this protein in this tissue (Drayna et al., 1986).

At the cellular level, apoD is internalized in different cell types, including NIH/3T3 cells (Do Carmo et al., 2007), vascular smooth muscle cells (VSMC) (Leung et al., 2004; Sarjeant et al., 2003) and murine astrocytes (Bajo-Graneras et al., 2011) by an unknown mechanism and is translocated to the nucleus in response to stress such as serum deprivation, oxidative molecules and pro-inflammatory factors (Blais et al., 1994; Do Carmo et al., 2002; Do Carmo et al., 2007; Provost et al., 1991a). In

addition, its expression is upregulated during aging or in several pathological conditions such as atherosclerosis, different types of cancer and neurological diseases, including Alzheimer's disease and multiple sclerosis (Perdomo and Henry Dong, 2009; Reindl et al., 2001; Terrisse et al., 1998). Thus, apoD may play a protective role in response to stressful stimuli. This hypothesis was confirmed by several *in vivo* studies. Indeed, neuronal overexpression of apoD in transgenic mice led to an increased resistance to oxidative stress (Ganfornina et al., 2008) and inflammation (Do Carmo et al., 2008). In contrast, apoD deletion in mice resulted in a decreased resistance and survival in response to oxidative stress in the brain (Ganfornina et al., 2008). Moreover, it has been reported that apoD could specifically prevent lipid peroxidation through a highly conserved methionine residue (Met_{93}), converting reactive to non-reactive lipid hydroxides (Bhatia et al., 2012; Oakley et al., 2012). Studies have also suggested that apoD could influence inflammatory pathways or prevent toxicity by interacting with its multiple ligands. For example, apoD binds AA and regulates its signalling and metabolism (Do Carmo et al., 2008; Thomas et al., 2003a; Thomas et al., 2003b). Therefore, given its multiple partners and expression patterns, apoD has been proposed as a multiligand and multifunctional protein.

Even if several studies have highlighted the potential protective role of apoD in neurological diseases, the exact molecular mechanisms involved in this process are still unclear. However, the potential protective role of apoD involves its uptake into the cells (Bajo-Graneras et al., 2011; Do Carmo et al., 2007), possibly through a receptor dependent mechanis. Therefore, we sought to determine how apoD was internalized into the cells to better understand the function of apoD in physiological and pathological conditions. We identified basigin as a cell surface receptor important for apoD internalization in 293T cells. Additionally, we demonstrated that its downregulation impairs exogenous apoD internalization. Moreover, cyclophilin A, a

natural ligand of basigin, blocked the apoD uptake. Thus, our findings clearly demonstrated that basigin can be proposed as the apoD receptor.

3.3 Material and Methods

3.3.1 Cell culture

Human Embryonic Kidney cells (HEK293T) and human neuroblastoma cells (SH-5YSH) were obtained from ATCC (American Type Cell Culture, Manassas, VA). HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Wisent, St-Bruno, QC, Canada) and SH-SY5Y cells in RPMI (Wisent) supplemented with 10% inactivated fetal bovine serum, glutamine (2nM), penicillin G (100 units/mL) and streptomycin (100 µg/mL). The cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

3.3.2 Radiolabeling of H-apoD

Human apoD (H-apoD), purified from breast cyst fluid (Norfeldt et al., 1981), was iodinated according to the iodine monochloride method, as described by Brodeur et *al.* (Brodeur et al., 2008). Briefly, sodium 125 iodide (400 µCi) were incubated with H-apoD (400 µg) in 0.5 M glycine pH10. Free iodine was removed using gel filtration on Sephadex G-25, followed by dialysis in phosphate buffered saline (PBS). ^{125}I-apoD concentration was assessed by the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Canada). The specific activity ranged from 300,000 to 350,000 cpm/µg protein.

3.3.3 H-apoD binding assay

HEK293T cells were seeded at 2 x 10^5 cells per well onto 24-well plates (Sarstedt, Montreal, Canada). After 24h, the cells were washed twice with 1 mL of PBS and incubated for 2h at 4°C with a range of concentrations of ^{125}I-apoD (1-20 µg/mL) in a total volume of 250 µL buffer (pH 7.4) containing 4% bovine serum albumin (BSA),

25mM HEPES, and 125 µL of Dulbecco's modified Eagle's medium (2X), for total binding. Nonspecific binding was measured by addition of 20-fold excess of unlabeled H-apoD. The cells were washed once with PBS followed by two washes with PBS containing 0.2% BSA. Cells were then solubilized in 750 µL of NaOH (0.1N) and counted with a Cobra II counter (Canberra-Packard, Ramsey, MN, USA). Protein concentration was assessed by the Bio-Rad protein assay (Bio-Rad Laboratories). Specific binding, defined as the difference between total binding and nonspecific binding, was obtained with GraphPad Prism 4 software. Nonlinear saturation binding data were transformed into linear data (ratio of cell bound to free ^{125}I-apoD versus cell-bound ^{125}I-apoD plots), according to the Scatchard method (Scatchard, 1949). The equilibrium dissociation constant (K_d) and maximum binding capacities (B_{max}) were calculated using GraphPad Prism software.

3.3.4 H-apoD biotinylation

H-apoD was biotinylated with N-hydroxysuccinidobiotin (NHS-D-Biotin; Sigma-Aldrich, St Louis, MO). H-apoD (10 mg/mL in 0.1 M sodium carbonate buffer, pH 9.5) was incubated overnight at 4°C with 10 % NHS-D-Biotin (11 mg/mL). The reaction solution was then dialyzed with PBS. Biotinylated-H-apoD concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories). The H-apoD biotinylation was confirmed by Western Blot using horseradish peroxidase (HRP)-conjugated streptavidin (GE Healthcare, Quebec, Canada).

3.3.5 293T cell membranes preparation

293T cell membranes were prepared according to the modified technique of Kawaguchi et *al.* (Kawaguchi et al., 2007). Briefly, 293T cells were lysed in PBS containing 8.6% sucrose and 10% (w/v) of complete protease inhibitors (Roche Molecular Diagnostics, Mannheim, Germany) with a Polytron homogenizer (Fisher

Scientific, Ottawa, Canada) for 30s on ice. The homogenate was layered onto 40% sucrose (diluted in PBS) and centrifuged at 25,000g for 30 min at 4°C. The membrane fraction, collected at the interphase, was diluted in PBS and then centrifuged at 25,000g for 30 min at 4°C. The pellet (membrane fraction) was resuspended in PBS and briefly sonicated. The membrane proteins concentration was determined by the Bio-Rad protein assay.

3.3.6 Biotinylated H-apoD binding to 293T membrane proteins

Biotinylated H-apoD was used to pull down the protein complex composed of apoD and its putative receptor. 293T membranes (250 μg protein) were incubated at room temperature for 30 min with 20 μg biotinylated H-apoD (biot-H-apoD) in a final reaction volume of 700 μL. The negative control (non-specific binding) was performed in the presence of 10-fold excess of unlabeled H-apoD. Because it was important to maintain the stability of this complex along the pull down assay, we used the glutaraldehyde crosslinker which offers the possibility to cross-link ε-amino groups within a distance of 8 Å (Peters and Richards, 1977). Thus, to crosslink apoD to its receptor, the reaction mixture diluted in 20mM HEPES buffer (pH 7.5) was treated with 0.11% glutaraldehyde for 5 min at 37°C. The reaction was stopped by the addition of 0.1M Tris-HCl, pH 8.0. The mixture was then centrifuged twice at 25000g for 30 min at 4°C and the membrane pellet resuspended in PBS.

3.3.7 Purification of biotinylated-HapoD/receptor complex

The apoD/membrane protein complex was purified using streptavidin magnetic beads (New England Biolabs, Toronto, Canada). The membrane pellet was solubilized in 2% (v/v) Triton X-100 in PBS for 30 min at 4°C followed by the removal of the detergent by gel filtration through a sephadex G-50 column (New England Biolabs).

Streptavidin beads (400 µg) were washed in PBS and incubated overnight at 4°C with the solubilized membrane proteins, containing the covalent apoD-receptor complex. The complex was further washed five times in PBS and eluted from the streptavidin beads with 40 µl of 0.1% (w/v) SDS in PBS. The eluate was mixed with SDS-loading buffer, migrated on SDS-PAGE (12%) and visualized by silver staining. Finally, the protein bands present in the positive sample and absent in the negative control were defined as bands of interest that could contain the apoD/membrane protein complex. These were excised and analyzed by mass spectrometry (LC-MS/MS) at the Institute for Research in Immunology and Cancer (IRIC, Montreal, Canada) for protein identification. To eliminate identification of false positives, mass spectrometry analysis was also done on corresponding pieces of gel excised from the negative control lane.

3.3.8 Cell transfections and apoD internalization

293T cells were plated at 5 x 10^4 cells in 24-well plates (Sarstedt). After 24h, the cells were transfected with pre-designed siRNA (20nM) for human basigin (siBSG) (Ambion, Life Technologies, Burlington, Canada) and negative control (siCtrl) (GFP-22 siRNA; Qiagen, Toronto, Canada). Transfection with siRNA was carried out using Lipofectamine RNAiMAX transfection Reagent (Life Technologies) according to the manufacturer's instructions. To overexpress basigin, SH-5YSH cells (8 x 10^4) were plated in 24-well plates (Sarstedt). After 24h, the cells were transfected with pCMV6 plasmid containing the human basigin coding region (BSG (NM_198589) Human cDNA ORF Clone, OriGene, Rockville, MD, USA) or pCMV6 empty plasmid (for negative control) using Lipofectamine 2000™ reagent (Life Technologies), according to the manufacturer' instructions. After 48h, the cells were exposed to biotinylated H-apoD (biot-H-apoD) (250 ng/mL) for 24h. The BSG protein downregulation/overexpression and biot-HapoD internalization were evaluated by

western blot analysis and immunocytochemistry as described in the following sections. To confirm the specificity of BSG knock down effect on apoD internalization, 293T cells were transfected with siBSG for 48h (as described above), and incubated with tetramethylrhodamine-conjugated human transferrin (Tf) (Life Technologies) for 24h and analyzed by confocal microscopy.

3.3.9 Protein extraction and Western blot analysis

Basigin protein expression and apoD internalization levels were evaluated by Western blot analysis. 293T cells (transfected by siBSG or siCtrl) exposed to biot-HapoD for 24h were washed twice with ice-cold PBS and homogenized in cold lysis buffer (50 mM Tris-HCL, pH 7.3, 150 mM NaCl, 5 mM EDTA, 0.2% (v/v) Triton X-100 complemented with complete protease inhibitors (Roche Molecular Diagnostics, Mannheim, Germany). Homogenates were incubated for 30 min at 4°C, sonicated and protein concentrations were assessed by the Bio-Rad protein assay. Proteins (30 µg per sample) were separated on 12% (w/v) SDS-polyacrylamide gels and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Ontario, Canada). Membranes were further incubated with horseradish peroxidase (HRP)-conjugated streptavidin (1:10,000; GE Healthcare), anti-human basigin mouse monoclonal antibody (IgG) (1:10,000; Ancell, Minnesota, USA) and anti-β-Actin mouse monoclonal antibody (IgG) (1:10,000; Sigma-Aldrich). The membranes were, thereafter, incubated with HRP-conjugated secondary antibody (1:10,000; GE Healthcare, Quebec, Canada) (for basigin and β-actin detection) and visualized by chemiluminescence (ECL, GE Healthcare) using a Fusion FX7 system (Vilber Lourmat, France).

3.3.10 Immunocytochemistry

After incubation with biot-H-apoD for 24h, 293T and SH-SY5Y cells (transfected and non-transfected) were then washed twice with ice-cold PBS and fixed for 15 min in 4% paraformaldehyde in PBS containing 2% sucrose, washed with PBS and processed for immunocytochemistry. The cells were permeabilized and blocked for 1h at room temperature in PBS containing 0.1% (w/v) Triton X-100 and 10% (w/v) BSA. After blocking, the cells were incubated overnight at 4°C in a humid atmosphere with FITC-conjugated anti-basigin (1:1000; Ancell) and Alexa Fluor 568 streptavidin conjugate (1:1000; Life Technologies). Thereafter, the cells were washed twice for 10 min with PBS and once with PBS containing 100 ng/mL of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for nuclei staining. The cells were mounted on slides with Prolong Gold antifade (Life Technologies).

3.3.11 Cyclophilin A/PPIA competition assay

We used cyclophilin A, a natural ligand of BSG, as a competitor to confirm the specific role of BSG in apoD internalization. 293T cells were incubated with H-apoD (250 ng/mL) in the presence or not of 1-fold or 20-fold excess of recombinant human cyclophilin A (peptidyl-prolyl isomerase, PPIA; Cederlane, Ontario, Canada) for 4h. The cells were then washed twice with PBS, fixed in 4% PFA and processed for immunocytochemistry as described previously. The apoD internalization was detected using a specific antibody against H-apoD (1:100; 2B9 mouse monoclonal antibody (Terrisse et al., 1998)) and a goat anti-mouse IgG-Alexa 488 (1:1000; Life Technologies). H-apoD and DAPI labellings were visualized by confocal microscopy. 293T cells were also incubated for 4h with tetramethylrhodamine-conjugated human transferrin (250 ng/mL) in presence of 20-fold excess of PPIA or not. The transferrin internalization was analyzed by confocal microscopy.

3.3.12 Statistical analyses

Statistical differences were determined using two-way ANOVAs followed by Bonferroni post hoc tests. Statistical analyses were performed with Prism Software (GraphPad 4). Results were considered significant when $P < 0.05$. The P-value was calculated with reference to the control data. All experiments were performed in triplicate.

3.4 Results

3.4.1 Receptor-mediated binding and uptake of human apolipoprotein D (H-apoD)

ApoD is a secreted protein, which can be internalized by various cell types (Bajo-Graneras et al., 2011; Do Carmo et al., 2007; Leung et al., 2004; Sarjeant et al., 2003). It has been reported that exogenous apoD can prevent uptake of arachidonic acid (AA) by 293T cells (Thomas et al., 2003b), but there is no information on the mechanisms of internalization. In this study, we demonstrated that, under normal conditions, endogenous expression of apoD was not showed by immunocytochemistry in 293T cells (Fig. 3.1A). However, when 293T cells were incubated for 24h with H-apoD, we observed apoD internalization, possibly through a receptor-dependent mechanism (Bajzer et al., 1989), into vesicular compartments in the perinuclear area (Fig. 3.1B). 293T cells were incubated for 2h with increasing concentrations of ^{125}I-labeled purified H-apoD (^{125}I-apoD) (total ^{125}I-apoD binding) in the presence or not of excess of unlabeled apoD in excess (non-specific ^{125}I-apoD binding) to assess specific binding of apoD. This binding assay (Fig. 3.1C) revealed that ^{125}I-apoD binding to 293T cells was concentration-dependent and saturable. Moreover, Scatchard analyses of the specific binding (Fig. 3.1D) showed a single class of binding sites on the cell surface, with a K_D at 9.38 ± 2.45 μg/mL and a B_{max} of 0.25 ±0.02 ng/μg cell proteins. Overall, these data indicates that the binding and internalization of apoD in 293T cells is a receptor dependent mechanism.

3.4.2 Identification of the apoD receptor

In order to identify the apoD receptor, we designed a strategy to stabilize the interaction between the apoD protein and its receptor in order to purify the complex

with magnetic beads. Once purified, the apoD/receptor complex was visualized by SDS-PAGE and silver staining (Fig. 3.2A). The specific bands 1, 2, 3, 4 and 5 (Fig. 3.2A), that were only present in the extract purified from cells incubated with biot-apoD compared to the negative control (in presence of 10-fold excess of unlabelled apoD), were then analyzed by mass spectrometry. ApoD was detected in bands 1 and 2 only (Fig. 3.2B), suggesting that these bands, observed at approximately 60 and 120 kDa, correspond to the covalent protein complex comprising apoD and its receptor. Several potential candidates that could act as the apoD receptor were identified in bands 1 and 2 (Fig. 3.2B). Ionic pumps very likely do not mediate the apoD endocytosis. This is the case for the sodium/potassium-transporting ATPase (identified in bands 1 and 2, Fig. 3.2B), an integral membrane protein that hydrolyses ATP and transports sodium and potassium across the cell plasma membrane to maintain ionic gradients (Blais et al., 1994). Latrophilin-2 (190 kDa) (Silva and Ushkaryov, 2010) and leucine-rich repeat neuronal protein 4 (79 kDa) (Fukamachi et al., 2002) cannot constitute the covalent apoD complex observed at about 60 kDa in band 1 (Fig. 3.2A). The last candidate, known as basigin (BSG), is a cell surface transmembrane glycoprotein which plays important role in several cellular activities including growth, differentiation, survival and adhesion (Muramatsu and Miyauchi, 2003). BSG is widely expressed in many cell lines, including 293T cells and in multiple glycosylated forms, high (HG-BSG, at about 45-65 kDa) and low (LG-BSG, detected at about 32kDa) glycoforms (Tang et al., 2004). Therefore, the protein linked to apoD in the 60 kDa complex (band 1, Fig. 3.2A) is very likely the low glycosylated form of BSG.

3.4.3 Impact of basigin concentrations on apoD internalization in 293T and SH-SY5Y cells

To investigate if BSG is involved in the apoD internalization process, we assessed the impact of BSG downregulation (using specific siRNA) on apoD internalization. The

BSG protein is mainly localized on the plasma membrane of 293T cells (Fig. 3.3Aa). However, in 293T cells treated with biot-apoD, BSG staining was detected on the plasma membrane as well but also appeared in vesicular compartments (Fig. 3.3Ad, 3.3Af) along with apoD being internalized (Fig. 3.3Ae, 3.3Af). These data suggest that exposure of 293T cells with exogenous apoD involves the redistribution of BSG. Interestingly, apoD internalization was strongly reduced in siBSG-treated cells (Fig. 3.3Be, 3.3Bf) compared to cells transfected with siCtrl (Fig. 3.3Bb, 3.3Bc). Such apoD internalization was diminished by 60% following a significant 40% decrease of the expression of all glycosylated forms of BSG (HG-BSG and LG-BSG) (Fig. 3.3C, 3.3D). Furthermore, BSG downregulation had no effect on transferrin internalization (Fig. 3.S1A). This process is known to be independent of BSG (Hopkins and Trowbridge, 1983), confirming that the decreased internalization of apoD is a direct consequence of the downregulated expression of BSG.

To confirm its role, the effect of BSG on apoD internalization was also evaluated in SH-SY5Y human neuroblastoma cells. These cells display low BSG expression levels compared to HeLa (Nahalkova et al., 2010) and 293T cells (Fig 3.4A). In addition, it has been reported that apoD can promote cell survival and differentiation in SH-SY5Y cells, but only under the presence of neuronal differentiation factors (Ruiz et al., 2013; Sasaki et al., 2009). Indeed, we detected low apoD internalization levels in SH-SY5Y cells (Fig. 3.4Ba-c). Interestingly, the overexpression of BSG in SH-SY5Y cells induced an important increase of intracellular exogenous apoD levels (Fig. 3.4Bd-f) compared to their counterparts cells transfected with the empty vector (Fig. 3.4Bb-Bc). Overall these results demonstrate that BSG is a key player in the apoD internalization process.

3.4.4 H-apoD internalization is blocked by cyclophilin A

It was previously reported that BSG binds cyclophilin A (also known as peptidyl-prolyl isomerase, PPIA), a protein secreted in response to inflammatory stress that triggers signalling and chemotactic activities (Pushkarsky et al., 2001; Yurchenko et al., 2002). To further verify the specificity of BSG effect on apoD internalization, we performed a competition assay between apoD and cyclophilin A. 293T cells were incubated for 4h with purified human apoD in the presence or not of cyclophilin A (Fig. 3.5). As previously described, exogenous apoD is internalized into vesicular compartments of 293T cells (Fig. 3.5A-C). Treatment with cyclophilin A at the same concentration as that of exogenous apoD had no effect on apoD internalization (Fig. 3.5D-F). However, the addition of cyclophilin A in excess (20X) had no effect on the endogenous expression of apoD (Fig. 3.5J-L), but completely blocked the exogenous apoD internalization (Fig. 3.5G-I). Moreover, as a control, transferrin internalization, which is internalized through another receptor, was not affected by the presence of cyclophilin A in excess confirming that internalization process was still perfectly functional in cyclophilin treated cells (Fig. 3.S1B). Overall, these results demonstrate that apoD internalization is blocked by cyclophilin A.

3.5 Discussion

ApoD is a secreted glycoprotein which can be internalized by various cells types (Bajo-Graneras et al., 2011; Do Carmo et al., 2007; Leung et al., 2004; Sarjeant et al., 2003). However, the quantity of apoD internalized depends on the cell type and the extent of stress conditions (Bajo-Graneras et al., 2011; Do Carmo et al., 2007), suggesting the involvement of a specific mechanism responsible for this internalization. In this study, we clearly demonstrated that the internalization of apoD is not a passive diffusion through the plasma membrane but rather a specific cell surface receptor-mediated uptake by BSG.

Also named CD147 and EMMPRIN (extracellular matrix metalloproteinase inducer), BSG is a multifunctional cell surface transmembrane glycoprotein which has two extracellular Ig domains (Biswas et al., 1995; Toole, 2003). It has been reported that BSG is widely expressed in many tissues including the brain (Igakura et al., 1998; Kosugi et al., 2014; Muramatsu and Miyauchi, 2003; Yuasa et al., 2001). In the brain, BSG is strongly expressed in the limbic system (including hippocampus, olfactory system, amygdala and enthorinal cortex), in the cortex and in the cerebellum (Fan et al., 1998). A similar tissue distribution has been observed for apoD (Provost et al., 1991b) in concordance with the interactions observed in this study between apoD and BSG. Interestingly, BSG-deficient mice were shown to suffer from many disorders related to female fertility, spermatogenesis, retinal development and, more importantly, nervous system function (Hori et al., 2000; Igakura et al., 1998; Kuno et al., 1998; Naruhashi et al., 1997). Indeed, these mice have learning and memory task deficits (Naruhashi et al., 1997). Most interestingly, apoD knockout mice also have impaired learning, memory, orientation-based and motor tasks (Ganfornina et al., 2008). Thus BSG and apoD could have similar or related functions. Indeed, the expressions of both BSG and apoD are increased in several inflammatory diseases including atherosclerosis (Choi et al., 2002; Siwik et al., 2008; Yoon et al., 2005),

ischemia (Boulos et al., 2007; Waldow et al., 2009), multiple sclerosis and experimental autoimmune encephalomyelitis (Agrawal et al., 2011; Perdomo and Henry Dong, 2009; Reindl et al., 2001; Rickhag et al., 2008; Tsukamoto et al., 2013), suggesting that apoD and BSG could very well be involved in common pathways in these pathologies.

ApoD is associated with reduced invasive and proliferative activity of several cancer cell types (Campisi, 2005; Provost et al., 1991a; Soiland et al., 2007). The role of BSG in cancer progression has also been well demonstrated. BSG is known to interact with several tumor and inflammation-induced molecules including monocarboxylate transporters (MCT), cyclophilins, integrins and caveolin-1 (Chen et al., 2010; Dai et al., 2009; Tang and Hemler, 2004; Yurchenko et al., 2002). In addition, in tumor cells, elevated BSG expression enhances tumor invasion by stimulating the secretion of multiple matrix metalloproteinases (MMP), this function being dependent upon the glycosylation levels (Beesley et al., 2008; Guo et al., 1997; Sun and Hemler, 2001). Indeed, the tumorigenic properties of BSG is mainly due to the high glycosylated form of BSG, HG-BSG (Beesley et al., 2008; Sun and Hemler, 2001; Tang et al., 2004; Tang and Hemler, 2004). However, the low glycosylated form of BSG, LG-BSG, is presumed to be a precursor for HG-BSG formation in the endoplasmic reticulum (Huang et al., 2013; Tang et al., 2004). Both HG-BSG and LG-BSG are expressed on the plasma membrane of many cell types and the high HG/LG ratio is highly correlated with lymphatic metastasis abilities of hepatocarcinoma cell lines (Jia et al., 2006). Moreover, the over-glycosylation of BSG is associated with multidrug resistance in human leukemia (Beesley et al., 2008). In addition, BSG promotes MMP-2 and MMP-3 production in breast cancer cells, which display increased tumoral invasion and metastasis capacities (Caudroy et al., 2002; Zucker et al., 2001). This can however be abolished by the upregulation of caveolin-1 (Hino et al., 2003; Tang and Hemler, 2004), which can bind to LG-BSG

and block its conversion to HG-BSG, resulting in an increase of LG-BSG on the cell surface and a decrease of the HG/LG ratio (Tang et al., 2004). Interestingly, our results suggest that apoD needs to form a complex with LG-BSG to be internalized. Therefore the increase of LG-BSG on the cell surface, promoted by caveolin-1, could induce apoD internalization, thereby reducing the risk of tumorigenesis. Although a parallel induction of apoD and caveolin-1 by oxidative stress has been demonstrated in NIH/3T3 cells (Do Carmo et al., 2007), the relation between apoD and caveolin-1 has not yet been established. However, the impairment of spatial memory tasks (Gioiosa et al., 2008; Niesman et al., 2014) and the inflammation induced by traumatic brain injury (Niesman et al., 2014) in caveolin-1 deficient mice suggest a tight relationship between apoD and caveolin-1.

Overexpression of human apoD in transgenic mice neurons reduces as well T cells infiltration and production of pro-inflammatory cytokines following coronavirus-induced neurodegeneration (Do Carmo et al., 2008). In this study, we demonstrated that an excess of cyclophilin A decreases apoD uptake into the cells. Interestingly, BSG has been identified as an essential signalling receptor for cyclophilins, which are secreted in response to inflammatory stress (Pushkarsky et al., 2001; Yurchenko et al., 2002; Yurchenko et al., 2006). Cyclophilin-mediated signalling results to chemotaxis of neutrophils, eosinophils and T cells (Allain et al., 2002; Sherry et al., 1992; Xu et al., 1992; Yurchenko et al., 2002). Moreover, it has been shown that cyclophilin A, secreted by prion-infected mice brains, induces the release of cytokines by microglia and astrocytes *in vitro* (Tribouillard-Tanvier et al., 2012). These studies suggest that both BSG and apoD might influence inflammatory pathways, possibly through the regulation of AA signalling and metabolism (Do Carmo et al., 2008). Indeed, apoD could stabilize or sequester its preferential ligand AA into the cell membrane, reducing the availability of free AA and preventing its conversion into pro-inflammatory molecules (Thomas et al., 2003a; Thomas et al., 2003b). Therefore the

binding of apoD to BSG could reduce its interaction with cyclophilin A and, as a result, attenuate the inflammatory process. In addition, our results show that apoD internalization is followed by the endocytosis of BSG, leading to the redistribution of BSG. The apoD endocytotic transport pathway remains unknown. Nevertheless, it has been already reported that BSG is internalized by a clathrin-independent endocytosis and recycling pathway (Eyster et al., 2009; Maldonado-Baez et al., 2013). The co-localization of exogenous apoD internalized and basigin suggests that apoD can follow the same endocytosis pathway as basigin for its internalization process.

In summary, the current study is the first to identify BSG, more particularly LG-BSG, as receptor essential for the apoD internalization. Binding of apoD to LG-BSG probably involves a specific region of apoD referred to as the spike forming in the back of ligand binding pocket (Eichinger et al., 2007; Flower et al., 2000). However, the exact mechanisms of interaction between apoD and BSG remain to be determined. Nonetheless the strong relationship between BSG and apoD highlighted in this study provides additional clues on the mechanisms involved in the apoD functions, including its neuroprotective effect.

3.6 Acknowledgements

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3.7 Figure legends

Figure 3.1 Receptor-mediated binding and internalization of H-apoD by 293T cells. (A, B) H-apoD internalization. 293T cells were untreated (A) or presence of purified human H-apoD for 24h (B) and stained with anti-H-apoD 2B9 antibody (green). Nuclei were labeled with DAPI (blue). Note that apoD staining is not detected in 293T cells without exogenous apoD incubation. Scale bar = 10 μm. (C, D) Saturation curves of binding of ^{125}I labeled H-ApoD (^{125}I-apoD) to 293T cells. Binding was conducted at 4°C for 2 h. Affinity binding of ^{125}I-apoD to 293T cells (C) was measured as follows: 293T cells were incubated with increasing concentrations of ^{125}I-apoD in the absence (total binding □) and presence (non-specific binding Δ) of excess unlabelled H-apoD. The difference between total binding and non-specific binding corresponded to specific binding (•). Scatchard analysis of equilibrium binding (D) revealed a single population of binding sites, with the calculated equilibrium dissociation constant (Kd) and maximum number of binding sites (Bmax) being 9.38±2.45 μg/mL and 0.25± 0.02 ng/μg cell protein respectively. Data points represent the mean ± SEM of 3 experiments performed each in duplicate.

Figure 3.2 Purification and identification of membrane protein binding to apoD. (A) Membrane proteins from 293T cells were incubated with biot-apoD and, as negative control, in the presence of 10-fold excess of unlabelled H-apoD. The purification of crosslinked apoD/membrane protein complexes was performed with streptavidin magnetic beads and visualized by SDS-PAGE and silver staining. Note that the band, observed at about 28 kDa, corresponds to the free biot-apoD. Bands 1, 2, 3, 4 and 5 in the apoD/membrane protein complex (lane 1), which disappear in the negative control (lane 2), were analyzed by mass spectrometry (LC-MS/MS). (B) Mass spectrometry analysis has identified several putative candidates as apoD receptors. Proteins from bands 3, 4 and 5 which did not contain apoD were not analyzed.

Figure 3.3 Basigin downregulation affects apoD internalization. (A) Confocal analysis of 293T cells incubated with (Ad-f) or without biot-apoD (Aa-c). The BSG protein endogenous and internalized exogenous apoD were stained with anti-BSG antibodies (green) (Aa, Ad) and Alexa Fluor 568 streptavidin (red) (Ab, Ae), respectively. Nuclei were labeled with DAPI (blue). The doted boxes represent enlarged view showing the co-localization (yellow) of BSG and internalized apoD in vesicular compartments of biot-apoD-treated cells (Af) compared to non-treated cells (Ac). Note that the exposure to apoD induces the redistribution of basigin localization (Ad) compared to non-treated 293T cells (Aa). (B) Knock down of BSG expression using specific siRNA (siBSG). 293T cells were transfected with siBSG (Bd-Bf) or siCtrl (negative control) (Ba-Bc) for 48h followed by incubation with biot-apoD for 24h and analyzed by confocal microscopy. Decreased BSG expression (Bd) was confirmed by staining with anti-BSG antibody (green) compared to siCtrl-transfected cells (Ba). Biot-apoD internalization was assessed using Alexa Fluor 568 streptavidin (red) in siBSG-transfected (Be) compared to siCtrl-transfected (Bb) cells. Note that biot-apoD internalization is decreased in siBSG-transfected cells (Be) compared to siCtrl-transfected (Bb) and non-transfected 293T cells (Ae). Scale bar = 10 μm. (C, D) Western blot analysis (C) and quantification (D) of BSG expression and biot-apoD internalization in extracts of siBSG and siCtrl–transfected cells, treated with biot-apoD for 24h. β-Actin was used as a loading control. Values were normalized on β-Actin protein expression and on siCtrl values, which were given an arbitrary value of 100. Normalized values are presented as mean ± SEM (each experiment was performed in triplicate). Two-way Anova following by Bonferroni post-test: ***$p<0.001$ compared to siCtrl-transfected cells.

Figure 3.4 ApoD internalization in SH-SY5Y cells. (A) BSG expression (green) in human neuroblastoma SH-SY5Y cells and 293T cells were assessed by confocal microscopy. Note that SH-SY5Y cells display low BSG protein expression compared

to 293T cells. (B) Effect of biot-apoD internalization on SH-SY5Y cells, with or without overexpression of BSG. SH-SY5Y cells, transfected with plasmid expressing human basigin (pBSG) (Bd-Bf) or with the empty plasmid (vehicle) (Ba-Bc) for 48h, were exposed to biot-apoD for 24h. The BSG protein expression and biot-apoD internalization were assessed by confocal microscopy with anti-BSG antibody (green) (Ba, Bd) and Alexa Fluor 568 streptavidin (red) (Bb, Be), respectively. Nuclei were labeled with DAPI (blue). Note that biot-apoD internalization (Ab) was decreased in low BSG expressing SH-SY5H cells (Aa) but improved by overexpression of BSG protein (Bd). Scale bar = 10 μm.

Figure 3.5 ApoD internalization is inhibited by cyclophilin A. 293T cells were incubated for 4h with H-apoD (250 ng/mL) followed by the addition of 250 ng/mL of cyclophilin (PPIA) in equimolar amounts (H-apoD:PPIA (1:1)) (D, E) or 20-fold excess of PPIA, (H-apoD:PPIA (1:20)) (G, H). As controls, the cells were incubated with H-apoD only (H-apoD:PPIA (1:0)) (B, C) or PPIA only in 20-fold excess (H-apoD:PPIA (0:20)) (J, K). Internalized H-apoD was revealed by immunostaining (green) and analyzed by confocal microscopy. Nuclei were labeled with DAPI (blue). Scale bar = 10 μm.

Figure 3.S1 Transferrin internalization is not altered by BSG inhibition. (A) Downregulation of BSG has no effect on transferrin (Tf) uptake in 293T cells. 293T cells were transfected with siBSG for 48h and incubated with tetramethylrhodamine-conjugated human transferrin (Tf) for 24h. The Tf staining (red) (Ab, Ac) was analysed by confocal microscopy. BSG expression was immunostained with anti-BSG (green). (B) Cyclophilin A does not compete with transferrin. Confocal analysis

of Tf internalization (red staining) in 293T cells which were incubated for 4h with tetramethylrhodamine-conjugated human Tf (250 ng/mL) followed by the addition of equimolar amounts or 20-fold excess of PPIA, (Tf:PPIA (1:1) (A,C) or (1:20), respectively (D, F)). Nuclei were labeled with DAPI (blue). Scale bar = 10 μm.

3.8 Figures

Figure 3.1

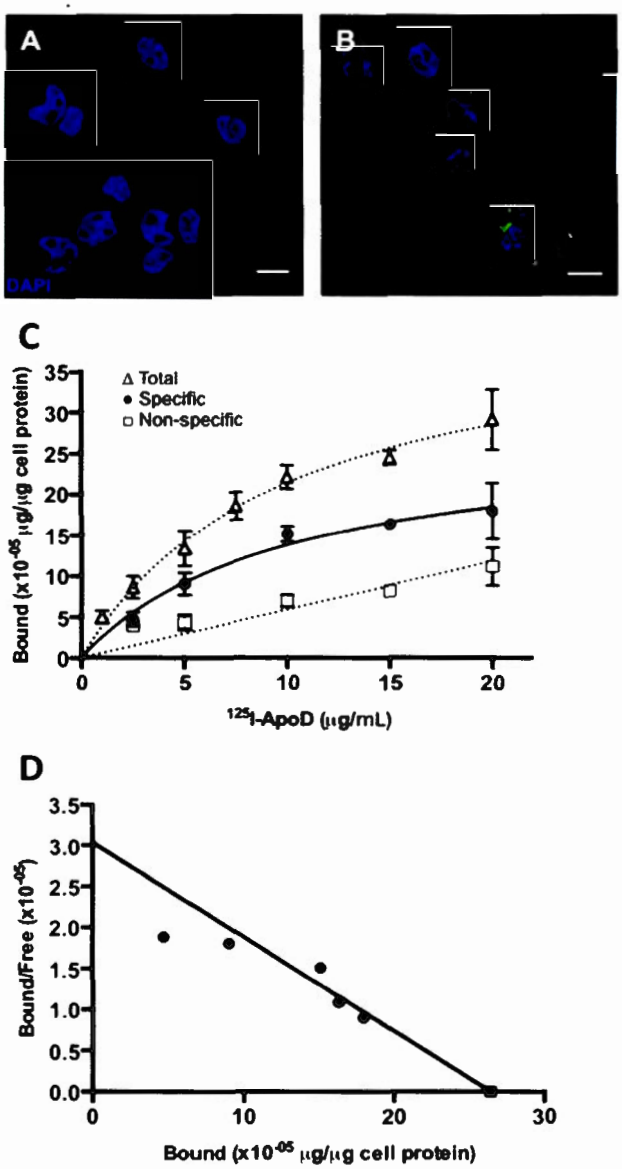
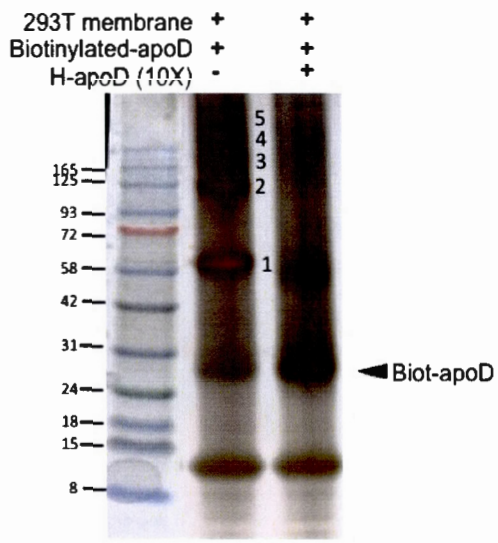


Figure 3.2

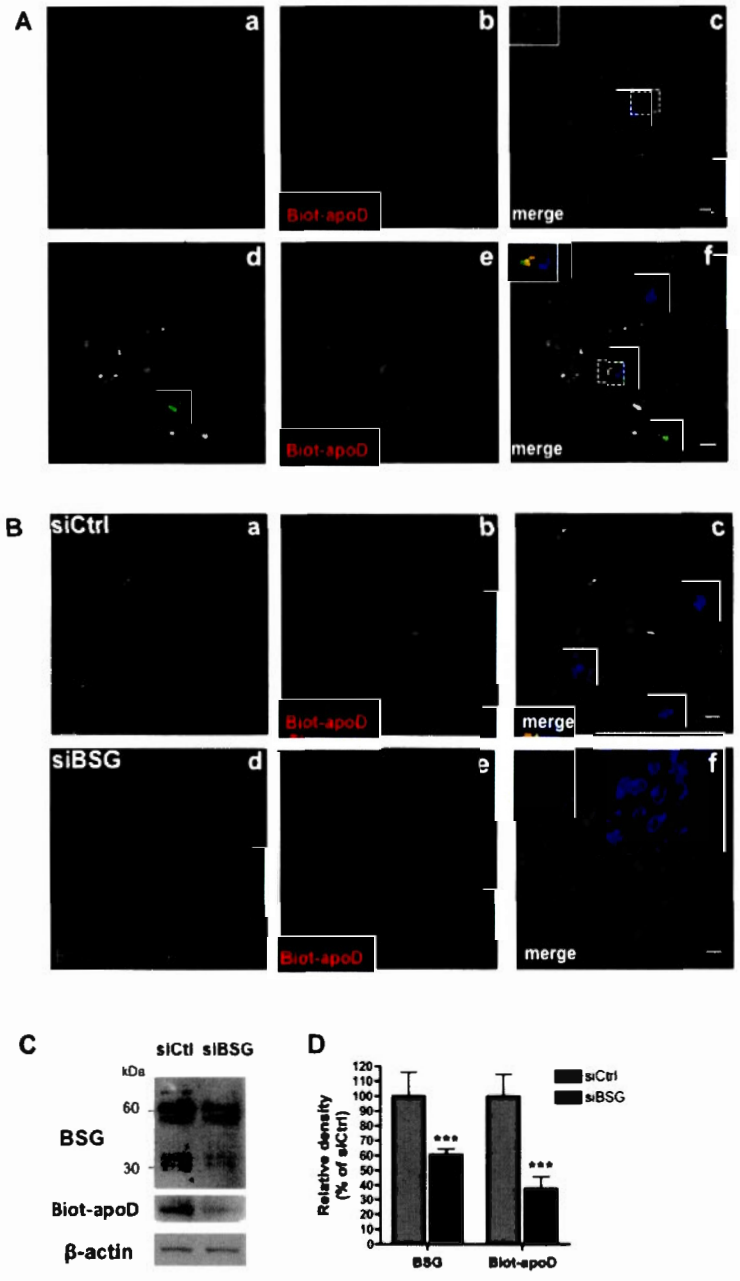
A



B

Band No (on the gel)	Protein Identification	Accession No (Uniprot)	Score	Peptide no
#1	Apolipoprotein D	C9JF17	163	3
	Latrophilin-2	H0Y3V3	31	1
	cDNA FLJ60077; higly similar to Sodium/ potassium-transporting ATPase alpha-1 chain	B7Z3V1	262	2
	Basigin	I3L4S8	46	1
	Leucine-rich repeat neuronal protein 4	Q8WUT4	39	1
#2	Apolipoprotein D	C9JF17	39	1
	cDNA FLJ59543; higly similar to Sodium/ potassium-transporting ATPase alpha-3 chain	*B7Z358*	56	1

Figure 3.3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b
c
Biot-apoD
merge
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e
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merge
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f
Biot-apoD
merge
C
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kDa
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30
BSG
Biot-apoD
β-actin
D
siCtrl
siBSG
Relative density (% of siCtrl)
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60
50
40
30
20
10
0
***
***
BSG
Biot-apoD

Figure 3.4

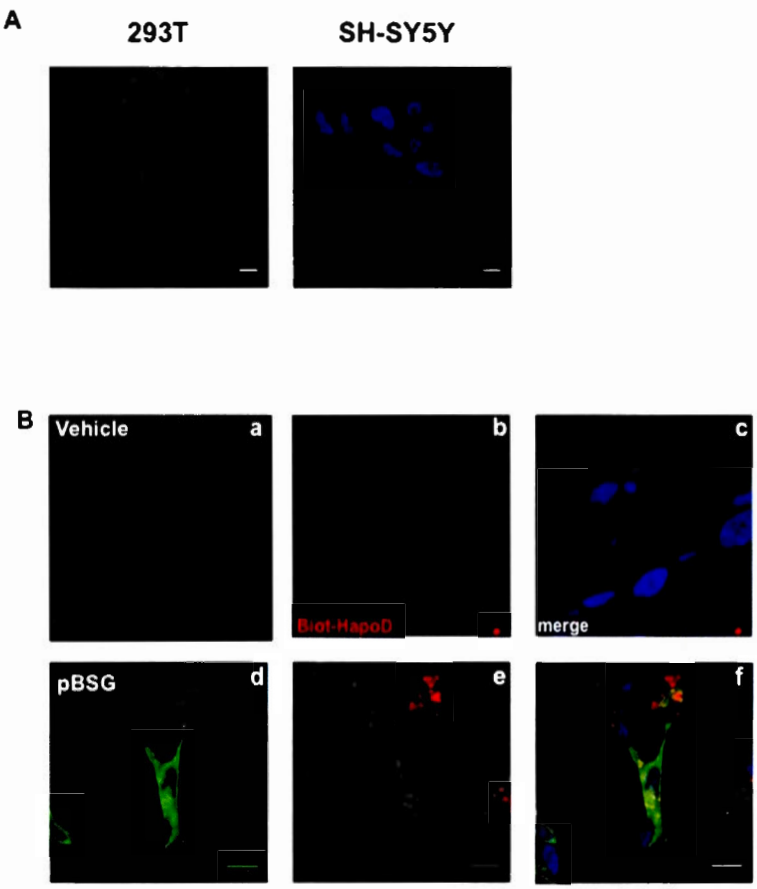
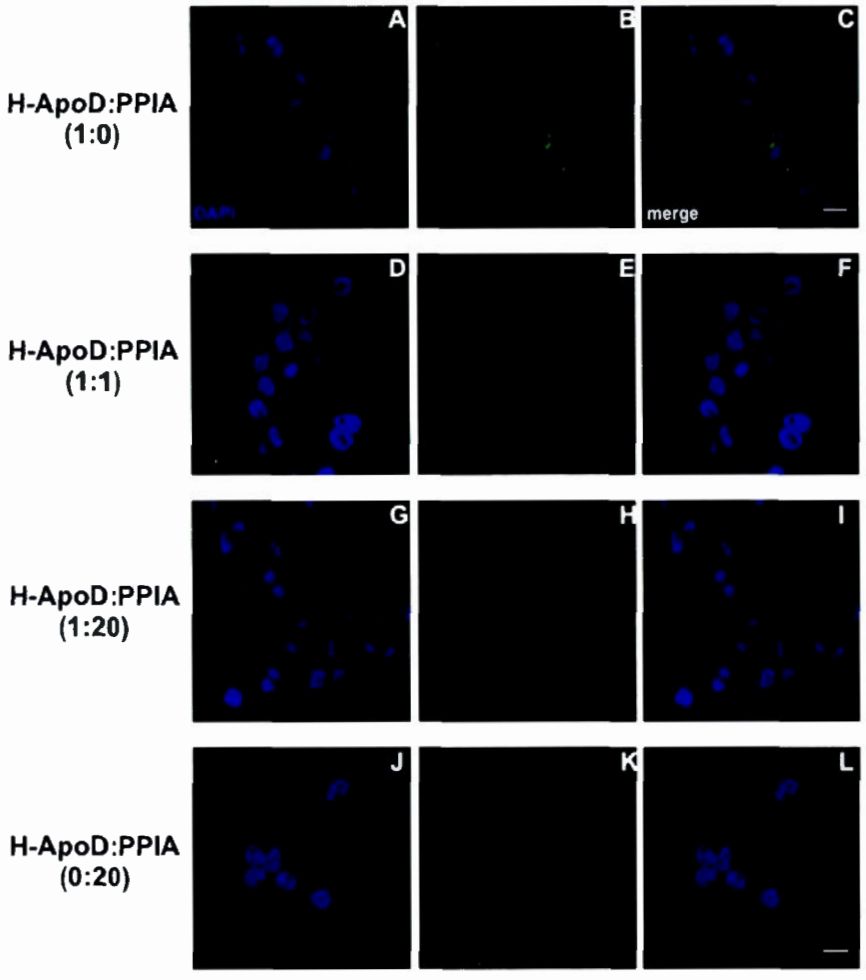


Figure 3.5


H-ApoD:PPIA
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B
C
DAPI
merge
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D
E
F
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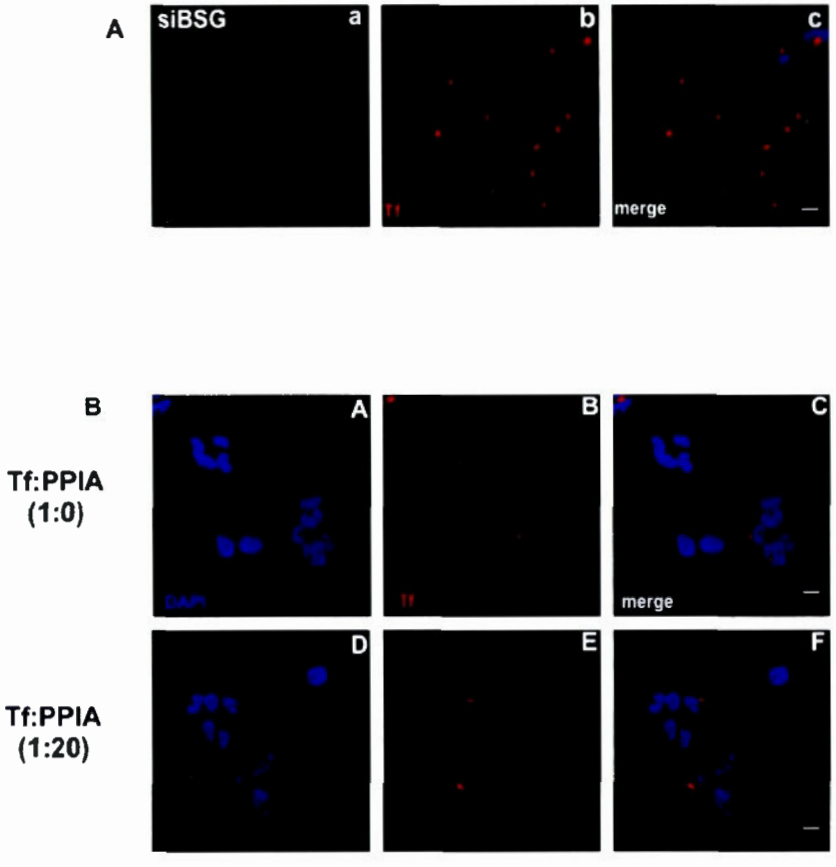
(1:20)
G
H
I
H-ApoD:PPIA
(0:20)
J
K
L

Figure 3.S1



CHAPITRE IV

APOLIPOPROTEIN D PREVENTS INTRANEURONAL CHOLESTEROL ACCUMULATION IN KAINATE-TREATED NEURONS

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Avant propos

Comme nous avons vu dans le chapitre II, l'un des mécanismes pour expliquer l'effet neuroprotecteur de l'apoD pourrait être la participation de l'apoD dans le processus d'efflux du cholestérol dans les neurones. Il a également été proposé dans d'autres études que la protection induite par l'apoD face à l'inflammation pourrait résulter de sa capacité à stabiliser l'acide arachidonique dans la membrane cellulaire ou de le séquestrer, empêchant ainsi sa conversion en molécules pro-inflammatoires. Notre dernier objectif consistait donc à vérifier si cet effet neuroprotecteur de l'apoD dépend de sa capacité à se lier avec certains ligands impliqués dans les processus neurodégénératifs tels que l'excitotoxicité. Pour cela, nous avons d'abord vérifié que l'apoD régule les niveaux de cholestérol intracellulaire dans les neurones en situation de stress. Par la suite, pour vérifier l'implication de la liaison de l'apoD avec son ligand dans le mécanisme de neuroprotection, plusieurs mutants de l'apoD portant des mutations sur les acides aminés situés dans la région de liaison avec son ligand ont été produits. Puisque l'absence de glycosylation peut affecter l'affinité de liaison de l'apoD avec son ligand, nous avons donc choisi de produire ces mutants dans les cellules eucaryotes 293T afin de les purifier. Une fois produites, nous avons vérifié si les mutations effectuées dans la région de liaison du ligand de l'apoD pouvaient affecter l'internalisation de l'apoD dans les cellules.

Ce chapitre consiste en une étude préliminaire présentée sous forme d'article. Les références de cet article se trouvent en fin de thèse. J'ai été impliquée dans toutes les facettes expérimentales des travaux présentés dans cet article, dans le montage des figures et la rédaction de l'article. La construction des clones qui ont permis de produire les différents mutants a été réalisée par Christian Trahan. La supervision du projet et la correction de l'article ont été réalisées par le Dr Eric Rassart.

4.1 Abstract

Cholesterol is a major component of the plasma membrane of most eukaryotic cells and plays a key role in neuronal functions. Dysregulation of cholesterol homeostasis has been observed in several neurodegenerative diseases, such as Alzheimer's and Niemann-Pick's type C diseases. These diseases also present an upregulation of apolipoprotein D (apoD), a secreted glycoprotein highly expressed in CNS. Numerous studies link apoD to maintenance and protection of neurons in various mouse models of acute stress and neurodegeneration. We have recently demonstrated that apoD favours cholesterol redistribution in physiological conditions by modulating its transport to the membrane or its efflux. Here, we show that apoD binds cholesterol with low affinity. Moreover, it decreases the intracellular cholesterol accumulation induced by kainate treatment in primary hippocampal neurons, suggesting that the mechanism underlying the neuroprotective effect of apoD involves the regulation of intraneuronal cholesterol levels. Also, mutations in the ligand-binding pocket of apoD reveal that binding to its ligand cholesterol in 293T cells can be essential for its internalization and, thus, for its role in neuroprotection. Overall, we demonstrate the existence of another mechanism for the regulation of intraneuronal cholesterol homeostasis, by which apoD protects neurons against injury.

Keywords: apolipoprotein D (apoD), cholesterol, kainic acid, internalization, apoD mutants, Niemann-Pick type C, Alzheimer.

4.2 Introduction

Apolipoprotein D (apoD) is a 29kDa secreted lipocalin that binds and transports small lipophilic molecules. It is highly expressed in several human tissues, including the central nervous system (CNS) (Drayna et al., 1986; Rassart et al., 2000; Weech et al., 1991). In rodents, apoD expression is found primarily in the CNS (Seguin et al., 1995). In the CNS of mammals, this atypical apolipoprotein is mainly expressed in glial cells (astrocytes and oligodendrocytes), but also in a few neurons (Hu et al., 2001; Provost et al., 1991b; Rickhag et al., 2008; Smith et al., 1990). In the aging brain and in several neurological diseases, apoD is upregulated (Franz et al., 1999; Kalman et al., 2000; Ordonez et al., 2006; Reindl et al., 2001; Terrisse et al., 1998; Terrisse et al., 1999; Yasojima et al., 1999), suggesting that apoD is involved in neuronal maintenance and protection against injury as confirmed by *in vivo* studies. Indeed, neuronal overexpression of apoD in transgenic mice leads to an increased resistance to oxidative stress (Ganfornina et al., 2008) and inflammation (Do Carmo et al., 2008) whereas apoD deletion in mice reduces the resistance and survival in response to oxidative stress in the brain (Ganfornina et al., 2008). Moreover, a recent study in our laboratory (Najyb et al, submitted; Chapitre II) revealed that apoD overexpression in neurons of transgenic mice induces an increased resistance to kainate-induced seizures and cell apoptosis. These effects could be mediated by a significant attenuation of inflammatory responses in the hippocampus.

ApoD is comprised of eight-stranded antiparallel β-barrels structure enclosing a hydrophobic conically shaped cavity that is referred to as the apoD binding pocket (Eichinger et al., 2007). Through this cavity, apoD can bind with high affinity multiple ligands such as arachidonic acid (AA), progesterone and sphingomyelin, but can also interact with cholesterol, bilirubin and estradiol with low affinity or through indirect interactions (Morais Cabral et al., 1995; Patel et al., 1997; Rassart et al., 2000;

Ruiz et al., 2012). Thus, apoD appears to be a multiple functional protein that could influence inflammatory pathways and prevent neurotoxicity through different mechanisms such as the regulation of AA signalling and metabolism (Do Carmo et al., 2008). It was notably suggested that apoD could stabilize AA into the cell membrane or sequester it, reducing the availability of free AA and preventing its conversion into pro-inflammatory molecules (Thomas et al., 2003a; Thomas et al., 2003b). Moreover, apoD has been shown to specifically prevent lipid peroxidation in the mouse brain (Ganfornina et al., 2008), by converting reactive to non-reactive lipid hydroxides in response to oxidative stress through a highly conserved methionine residue (Met_{93}) (Bhatia et al., 2012; Oakley et al., 2012).

In our laboratory, we recently demonstrated that apoD favours cholesterol redistribution in physiological conditions by modulating its transport to the membrane or its efflux (Najyb et al, submitted; Chapitre II). Therefore, the neuroprotective effect of apoD could be mediated by the regulation of another of its ligand, cholesterol. In this study, we clearly demonstrate that apoD decreases the intracellular cholesterol accumulation induced by kainate toxicity in primary hippocampal neurons, suggesting that apoD plays a neuroprotective effect. The involvement of cholesterol, as an apoD ligand, in neuroprotection was assessed by creating apoD mutants in the ligand-binding pocket and further measure their ability to bind ligands such as AA or cholesterol. Preliminary results indicate that these mutations impaired the internalization of apoD in 293T cells, suggesting that the binding of apoD to its ligands is required for its internalization.

4.3 Materiel and Methods

4.3.1 Hippocampal culture preparation

WTFVB/N mouse (Charles River, Canada) embryos from 3 females (at 18 days of gestation) were used to prepare primary hippocampal neuronal cultures as previously described (Brewer et al., 1993). Briefly, hippocampi were dissected in HBSS (without Ca^{2+} and Mg^{2+}) supplemented with 1 mM sodium pyruvate and 10 mM HEPES and dissociated by trituration with a fire-polished Pasteur pipette. Tissue homogenates were then centrifuged for 1 min at 1000 g at room temperature. The cell pellets were recovered and resuspended in HBSS. Primary neurons were plated at a density of 5 x 10^5 cells/cm^2 on glass coverlips pretreated with poly-D-lysine (Sigma-Aldrich) in Neurobasal medium (Invitrogen) supplemented with 0.5 mM L-glutamine and B27 supplement (Invitrogen).

4.3.2 Filipin staining

After 10 days of culture, hippocampal primary neurons were pre-incubated with purified H-apoD (250 ng/mL; purified from breast cyst fluid) for 24h. Then, neurons were incubated in medium with or without Kainic Acid (KA) (100 μM) for 3h. Staining of intracellular cholesterol was achieved using filipin, a fluorescent polyene antibiotic with high affinity for cholesterol (Coxey et al., 1993). Hippocampal neurons were fixed for 15 min in PBS containing 4% paraformaldehyde (PFA) and 2% sucrose, washed with phosphate buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS. The cells were then incubated with filipin (125 μg/mL; Sigma-Aldrich) for 2h at room temperature. Filipin-cholesterol complexes were visualized by confocal microscopy (Nikon, Mississauga, Canada).

To achieve exogenous human apoD staining, neurons were treated for 1h with PBS containing 10% goat serum, 10% bovin serum albumin (BSA) and 0.1% Triton and incubated overnight at 4°C in a humid atmosphere with a primary antibody against H-apoD (Terrisse et al., 1998) (1:100; 2B9 mouse monoclonal antibody, produced as described in (Weech et al., 1986)). After three washes of 10 min in PBS, the cells were exposed to a goat anti-mouse IgG-Alexa 488 (1:1000; Life Technologies) at room temperature for 1h, washed three times with PBS and mounted on slides with Prolong Gold antifade (Life Technologies). Finally, filipin and H-apoD staining were visualized by confocal microscopy (Nikon, Mississauga, Canada).

4.3.3 Dot Blot analysis

The binding of H-apoD to cholesterol and 24-hydroxycholesterol (24-HOC) was analyzed by dot blot as previously described (Lin et al., 2013). Briefly, different amounts of cholesterol (Sigma-Aldrich, St Louis, MO) and 24-HOC (Enzo) (0 - 20 nmol) were dotted onto a PVDF (polyvinylidene difluoride) membrane (Millipore, Ontario, Canada) placed in a Bio-Dot SF apparatus (Bio-Rad Laboratories, Mississauga, Canada). After a blocking step with 3% BSA for 1h, the membrane was incubated with purified H-apoD (5 µg/mL) for 1h at room temperature. The membrane was then washed three times with PBS and stained with a mouse monoclonal antibody (2B9) against human apoD (1:10000). Thereafter, the membrane was incubated with a horseradish peroxidase (HRP) conjugated goat anti-mouse (1:10000; GE Healthcare, Quebec, Canada) visualized by chemiluminescence (ECL, GE Healthcare, Quebec, Canada) using a Fusion FX7 system (Vilber Lourmat, France).

4.3.4 Generation of apoD mutants

Several mutations in the ligand-binding pocket were realized using PCR mediated site specific mutagenesis (Sarkar and Sommer, 1990). The amino acids to be mutated were chosen because (i) they were conserved among five species (human, rabbit, guinea pig, rat, mouse), (ii) they were likely to be involved in the ligand binding function and (iii) they were unlikely to induce drastic changes in the overall apoD structure. The amino acids residues, which were substituted, were Phe^{36} by Tyr (F36Y), Val^{56} by Ala (V56A), Gln^{69} by Asn (Q69N), Leu^{61} by Ile (L61I), Glu^{73} by Asp (E73D), Ala^{83} by Val (A83V) and Ile^{117} by Leu (I117L).

4.3.5 Cell culture and transfections

HEK293T cells (Human Embryonic Kidney cells), obtained from ATCC (American Type Cell Culture, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (Wisent, St-Bruno, QC, Canada) supplemented with 10% inactivated fetal bovine serum (FBS), glutamine (2nM), penicillin G (100 units/mL) and streptomycin (100 μg/mL). The cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. 293T cells were transfected for 48h with pcDNA3.1-His Tag (Life Technologies) containing native human apoD or mutants using Polyfect transfection reagent (Qiagen) according to the manufacturer's instructions. Expression of ApoD-His and mutants was assessed by western blot as described in the following section.

4.3.6 Western blot analysis

ApoD-His and mutants transfected-293T cells were lysed in a lysis buffer (50 mM Tris-HCL, pH 7.3, 150 mM NaCl, 5 mM EDTA, 0.2% (v/v) Triton X-100 complemented with complete protease inhibitors (Roche Molecular Diagnostics, Manheim, Germany)). Homogenates were incubated for 30 min at 4°C and

centrifuged at 20,000g for 10 min at 4°C. The protein concentrations were assessed in the supernatants by the Bio-Rad protein assay (Biorad). The conditioned media (20 µL) and cell lysates (30 µg per sample) were separated on 12% (w/v) SDS-polyacrylamide gels and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Ontario, Canada). Membranes were further incubated with primary antibodies against His-tag mouse monoclonal antibody (1:1,000, Qiagen) and β-Actin mouse monoclonal antibody (1:10,000; Sigma-Aldrich). The membranes were, thereafter, incubated with a HRP-conjugates antibody (1:10,000; GE Healthcare, Quebec, Canada) and visualized by chemiluminescence (ECL, GE Healthcare) using a Fusion FX7 system (Vilber Lourmat, France).

4.3.7 Purification/concentration of recombinant proteins

ApoD-His and mutants, secreted from transfected-293T cells, were concentrated by Ni-NTA purification system. Conditioned media from apoD-His and mutant-transfected 293T cells were diluted in phosphate buffer containing 300 mM NaCl, 30 mM of imidazole, 0.4% (v/v) NP40 complemented with complete protease inhibitors (Roche Molecular Diagnostics, Mannheim, Germany) and incubated overnight at 4°C with Ni-NTA His-Bind resin (Novagen, EMD Millipore, Ontario, Canada). After three washes, the bound proteins were eluted with the Elution buffer (300 mM imidazole, 300mM NaCl and complete protease inhibitors). At each step, aliquots of fractions were assessed by western blot for the presence of apoD-His or mutants, using antibody against His Tag. The protein-containing fractions were pooled and dialyzed against PBS. The concentrations of apoD-His and mutants were assessed by indirect ELISA method as described below.

4.3.8 Indirect ELISA

96-well ELISA plates (Thermo Scientific, Waltham, USA) were coated overnight at 4°C with purified human apoD (H-apoD) standards (within range concentration of 0 - 100 ng/µL) and samples (apoD-His and mutants) diluted in 0.1 M sodium carbonate buffer pH 9. The coated wells were blocked with 5% BSA diluted in PBS and were incubated for 1h at room temperature with an antibody against human apoD (1:1000, 2B9). After three washes in PBS, the wells were treated with HRP-conjugated goat anti-mouse antibody (1:1,0000; GE Healthcare) for 1h at room temperature. After three washes with PBS, peroxidase substrate TMB (3,3',5,5'-Tetramethylbenzidine) solution (100 µL, Fitzgerald, MA, USA) was applied to each well for 30 min at room temperature. Then, the reaction was stopped by adding 50 µL of 1 M phosphoric acid. Absorbance values (at 450 nm) were obtained with an Elisa Plate Reader (Tecan Infinite M1000, Tecan US, NC, USA).

4.3.9 Immunocytochemistry

Native and mutant apoD internalization was assessed by immunocytochemistry. 293T cells were incubated with 200 ng/mL of apoD-His and mutants for 24h. After two washes with ice-cold PBS, the cells were fixed for 15 min in 4% paraformaldehyde (PFA) in PBS containing 2% sucrose. The cells were permeabilized and blocked for 1h at room temperature in PBS containing 0.1% (w/v) triton X-100 and 10% (w/v) BSA. Then, the cells were incubated overnight at 4°C in a humid atmosphere with an anti-His-Tag mouse monoclonal antibody (1:100, Qiagen). After three washes with PBS, the cells were exposed to a goat anti mouse IgG-Alexa 488 (1:1000; Life Technologies) at room temperature for 1h, washed, and mounted on slides with Prolong Gold. His staining was visualized by fluorescence microscopy (Nikon).

4.4 Results

4.4.1 H-apoD attenuates cholesterol accumulation following KA treatment

Defects in cholesterol metabolism are associated with various neurological injuries, such as kainate-induced excitotoxicity (He et al., 2006; Ong et al., 2003; Sodero et al., 2012). We evaluated the effect of exogenous apoD on the intracellular levels of cholesterol induced by kainate (KA)-induced toxicity in primary neurons (Fig. 4.1A). In accordance with previous studies (He et al., 2006), we observed that KA-treated neurons displayed an important accumulation of cholesterol (Fig. 4.1Ab) compared to non-treated neurons (Fig. 4.1Aa). Interestingly, H-apoD-pretreated hippocampal neurons (Fig. 4.1Ac) showed less cholesterol/filipin staining than nonpre-treated neurons (Fig. 4.1Ab) in response to KA toxicity. As previously demonstrated, KA treatment induced an important uptake of exogenous apoD, which was co-localized with cholesterol (Fig. 4.1Ac) (Najyb et al., submitted; Chapitre II). Overall, our results suggest that internalized apoD attenuates the increased intraneuronal cholesterol content induced by KA treatment.

4.4.2 Direct interaction between H-apoD and cholesterol

The co-localization between apoD and intracellular cholesterol content suggests a putative interaction. To address this question, the binding of apoD to cholesterol and its oxysterol, 24-OHC, was assessed by dot blot (Fig. 4.1 B, C). As shown in Fig. 4.1B, purified H-apoD binds to cholesterol in a dose-dependent manner, but with low affinity since apoD staining was not detected with less than 2.5 nmol of cholesterol. In contrast, direct binding with 24-OHC was not detected for apoD (Fig. 4.1C). Overall, these data demonstrate that apoD binds cholesterol.

4.4.3 Effect of mutations on apoD internalization

It has been suggested that the neuroprotective role of apoD is linked to its capacity to bind a number of ligands involved in neurological injuries, including AA (Thomas et al., 2003a; Thomas et al., 2003b) and cholesterol as demonstrated in the previous section. Mutations of residues present in the apoD ligand-binding pocket were performed to investigate whether the apoD neuroprotective effect is mediated by its capacity to bind a specific ligand. Western blot analyses revealed that apoD-His and mutants were expressed by transfected 293T cells (Fig. 4.1Ba) and were secreted into the extracellular medium (Fig. 4.1Bb). Interestingly, the secreted apoD-His and the mutant protein (Fig. 4.1Ba) differed from their intracellular form (Fig. 4.1Bb) which could be due to glycosylation. However, the mutant Y108F displayed a lower expression compared to apoD-His and others mutants. Moreover, the level of mutant secreted proteins F36Y (3), I117L (4) and A83V (8) seems to be higher than that of apoD-His (Fig. 4.2Ba).

To investigate if these mutations affect the uptake of apoD, we assessed the internalization of these mutants apoD by immunocytochemistry. To address this question, 293T cells were incubated with purified apoD-His and mutants for 24h. The native and mutants apoD internalized in cells were detected by immunocytochemistry, using specific antibody against His-Tag. The mutants E73D (Fig. 4.3B) and Q69N (Fig. 4.3F) display internalization levels similar to apoD-His (Fig. 4.3A) while the internalization levels of mutants L61I (Fig. 4.3E), V56A (Fig. 4.3G) and A83V (Fig. 4.3H) were decreased compared to apoD His (Fig. 4.3A). Interestingly, the uptake process seemed to fail for the mutants F36Y (Fig. 4.3C) and I117L (Fig. 4.3D). In these mutants, mutated residues were respectively localized in loops A/B and G/H (Fig. 4.2A). Overall, these preliminary data suggest that mutations in the binding pocket at position 56, 61 and 83, more precisely in loops A/B and G/H, decrease the

internalization of the protein. On the other hand, mutations in the β-barrel at position 67-76 do not impact the apoD internalization process.

4.5 Discussion

Previous studies revealed that overexpression of apoD lead to neuroprotection in oxidative and inflammation stress mediated neurodegeneration (Do Carmo et al., 2008; Ganfornina et al., 2008; He et al., 2009). We have previously demonstrated that apoD overexpression in neurons also protects mice against KA-induced seizures and cellular apoptosis, which could be associated with the regulation of cholesterol distribution (Najyb et al, submitted; Chapitre II). A direct association between dysregulation of cholesterol homeostasis and neurodegeneration has been observed in several neurodegenerative diseases, such as Alzheimer's and Niemann-Pick's type C diseases (Vance, 2012). Most interestingly, these diseases also present an upregulation of apoD (Ordonez et al., 2006; Terrisse et al., 1998; Yoshida et al., 1996). Regarding the interaction between apoD and cholesterol, several studies have reported discrepancies which could be explained by the low affinity of the ligands examined or by technical issues (Morais Cabral et al., 1995; Patel et al., 1997; Rassart et al., 2000; Ruiz et al., 2012). An indirect interaction between apoD and cholesterol has been suggested since the later was found to be co-transported with sphingomyelin (Ruiz et al., 2012). In this study, we clearly demonstrate that apoD binds cholesterol (although with low affinity) and prevents its accumulation into neurons in response to the excitotoxicity injury induced by KA treatments (He et al., 2006).

Cholesterol, a major component of the plasma membrane of most eukaryotic cells, is involved in the regulation of several cellular functions, especially neuronal functions (Simons and Toomre, 2000). Indeed, cholesterol, an important lipid constituent of the myelin sheath and the lipid rafts in neurons and astrocytes, plays a key role in brain development and neuronal functions, such as axon guidance and synaptic

transmission (Pfrieger, 2003). In the brain, cholesterol synthesis and metabolism requires tight interactions between astrocytes and neurons (Pfrieger, 2003). In adulthood, astrocytes are the main source of cholesterol for neurons although neurons can also produce cholesterol in their cell bodies (Dietschy and Turley, 2004; Pfrieger, 2003; Vance, 2012). Synthesized in astrocytes, cholesterol is then transported by apolipoprotein E-containing lipoproteins to neurons (Vance, 2012) where it is internalized and can be redistributed to the plasma membrane and organelles (Dietschy and Turley, 2004; Pfrieger, 2003). Excessive intraneuronal cholesterol is eliminated by its conversion to 24-hydroxycholesterol (24-OHC), which can cross the blood brain barrier and reach the liver for elimination (Bjorkhem and Meaney, 2004). This capacity to convert cholesterol to 24-HOC is specific to neurons (Bjorkhem and Meaney, 2004). However, in this study, we demonstrated that there is no direct interaction between 24-HOC and apoD. Moreover, previous results from our laboratory (Najyb et al, submitted; Chapitre II) indicate that 24-HOC pathway is not involved in the decreased cholesterol levels of the cytosolic brain fraction in transgenic mice that overexpress neuronal apoD. Thus, although the 24-HOC pathway is usually the major pathway for cholesterol elimination, apoD seems to utilize another pathway to regulate intraneuronal cholesterol levels.

Other cholesterol transporters, including the ATP binding cassette (ABC) transporters and high density lipoproteins (HDL), are involved in the regulation of cholesterol efflux (Chen et al., 2013). HDL increases cholesterol efflux in cortical neuronal cultures (Chen et al., 2013). This process can be mediated by the ABC transporter ABCG4 which is highly expressed in neurons. Indeed, the downregulation of ABCG4 leads to the decrease of cholesterol efflux (Chen et al., 2013). Although the relation between apoD and ATP binding cassette (ABC) transporters has not yet been defined, it was reported that apolipoprotein E deficient mice display an increased expression of both apoD and ABCA1. Indeed, the later is known to be involved in cholesterol

efflux process in astrocytes (Chen et al., 2013) as a compensatory mechanism to maintain the cholesterol homeostasis in the brain (Jansen et al., 2009). Thus, apoD associated with HDL-like particles found in the cerebrospinal fluid (CSF) (Koch et al., 2001) could be involved in the cholesterol efflux mediated by the ABCG4 pathway.

The neuroprotective effect of apoD could also be mediated by its interaction with other ligands (Morais Cabral et al., 1995; Rassart et al., 2000; Ruiz et al., 2013; Vogt and Skerra, 2001). Surprisingly, no information exists on whether the binding of apoD to its ligands is required for its internalization and, thus, its function. Therefore, our study is the first to show that mutations in the ligand-binding pocket of apoD, more particularly at positions 36 and 117, reduce the apoD internalization process in primary neuronal cells. Interestingly, the apoD uptake is completely blocked by these mutations in the 'loop' region suggesting that it is more important for apoD internalization than the β-barrels. These extended loops are localized at the entrance of the ligand-binding pocket (Eichinger et al., 2007). Thus, the effect of these mutations on apoD internalization could either interfere with the entrance of the ligand in the pocket or with the binding of apoD with its receptor at the plasma membrane.

In summary, our results suggest that the neuroprotective effect of apoD in KA-mediated toxicity could be mediated by the regulation of intraneuronal levels of cholesterol in addition to AA. Moreover, we showed that the binding capacity of apoD to its ligand influences its function and that the loop region is critical for this step. Therefore, this study provides additional clues on the exact protective mechanisms mediated by apoD through the regulation of cholesterol homeostasis in neurons.

4.6 Acknowledgements

We thank Denis Flipo for his help with the confocal microscopy analysis. We also thank Dr Évemie Dubé for helpful discussion. This work was supported by the Canadian Institutes of Health Research grant MOP-15677.

4.7 Figure legends

Figure 4.1 H-apoD affects intracellular cholesterol in neurons. (A) Hippocampal neurons (10 days) were pre-incubated with (+ H-apoD) or without (- H-apoD) purified human apoD for 24h and further treated with kainic acid (100 μM) for 3h (+KA) or not. Cells were subsequently stained with filipin (gray) and anti-H-apoD antibodies (green) and analyzed by confocal microscopy. Note that the filipin staining decreases in the soma of neurons treated with purified H-apoD (as indicated by arrow in Ac) compared to non-treated neurons following kainate treatment. Scale bar = 10 μm. B and C. Dot blot analysis of direct binding of H-apoD to various amounts of cholesterol (B) and 24-hydroxycholesterol (C). All experiments were performed in triplicate.

Figure 4.2 Expression of recombinant apoD and mutants. (A) Schematic 3D structure representation of human apoD (gray) complexed with progesterone (blue)(Eichinger et al., 2007). The positioning of the different mutated residues (Y108F, E73D, F36Y, I117L, L61I, Q69N, V56A, A83V) is shown in red (in the left panel). The sequence of human apoD protein (P05090) with the mutated residues is shown in red and depicted as red box (in the right panel). The green boxes represent the β-barrels and yellow boxes correspond to α-helix Modeling structure of apoD (protein data bank code: 2HZQ) was performed using Chimeria software. (B) Western blot analysis of apoD-His and mutants expresion in conditioned media and intracellular level of transfected 293T cells. As a negative control, His staining was also assessed in cells transfected with empty vector (Ctrl -). Cellular extract from apoD-His transfected cells (Ctrl +) served as positive control. β-Actin was used as a loading control for cellular extracts. All experiments were performed at least in triplicate.

Figure 4.3 Analysis of apoD-His and mutants internalization. 293T cells were incubated for 24h with 200 ng/mL of apoD-His or its mutants purified from conditioned media of apoD-his and mutants transfected cells. ApoD-His and the mutants internalization was revealed by using anti-his antibody (green). All experiments were performed in duplicate.

4.8 Figures

Figure 4.1

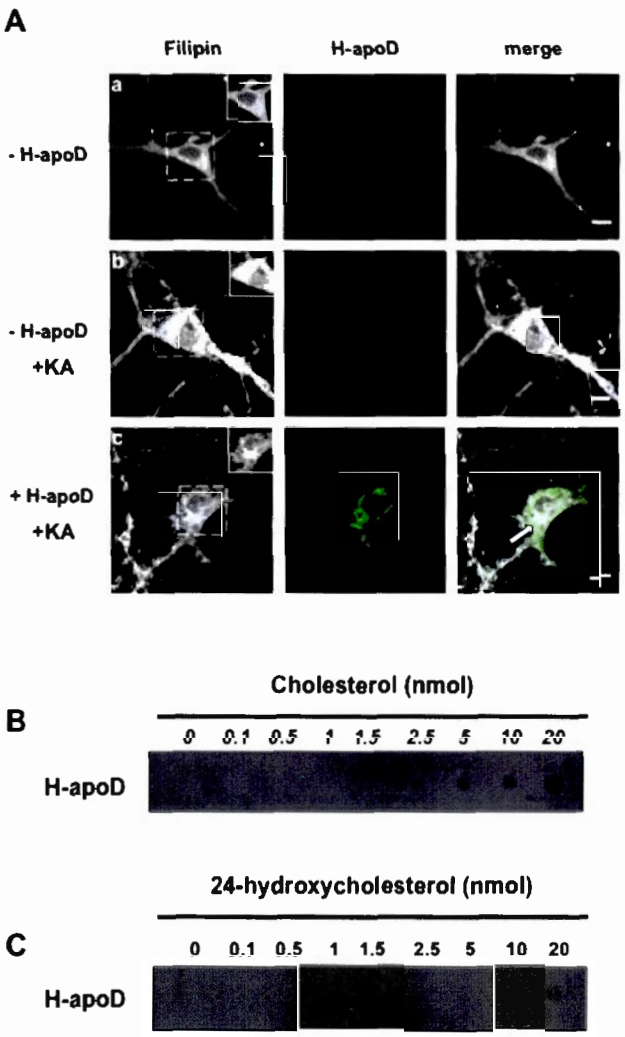
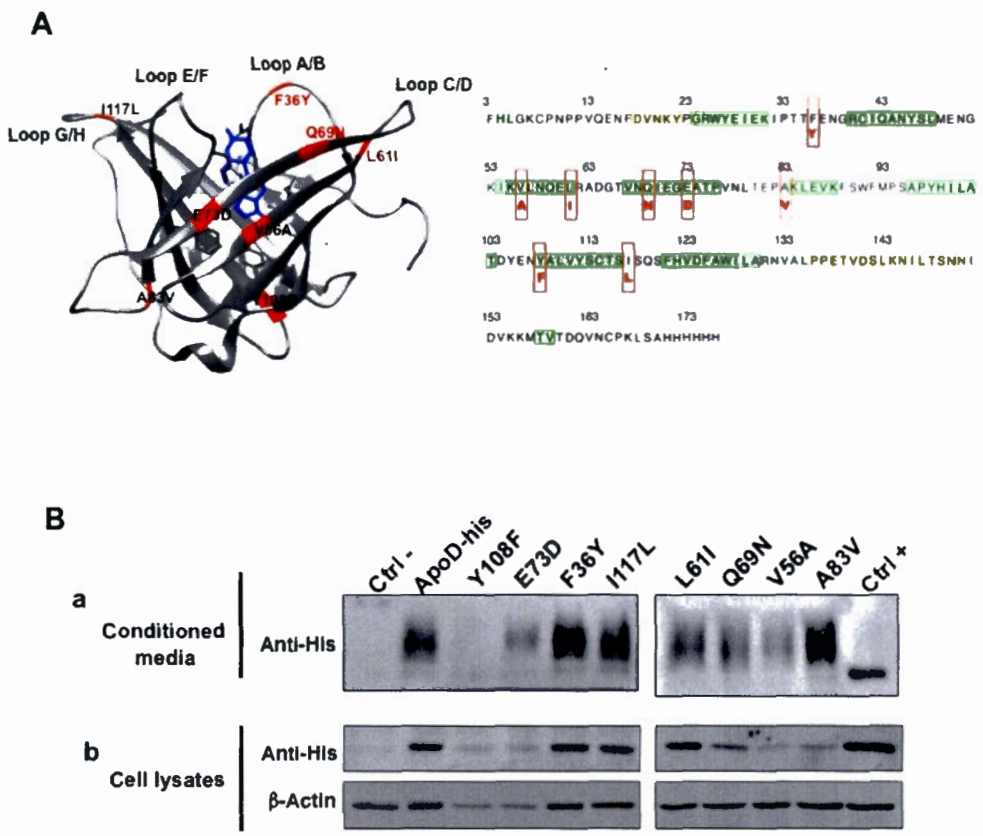


Figure 4.2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L61I
V56A
A83V
B
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ApoD-his
Y108F
E73D
F36Y
I117L
L61I
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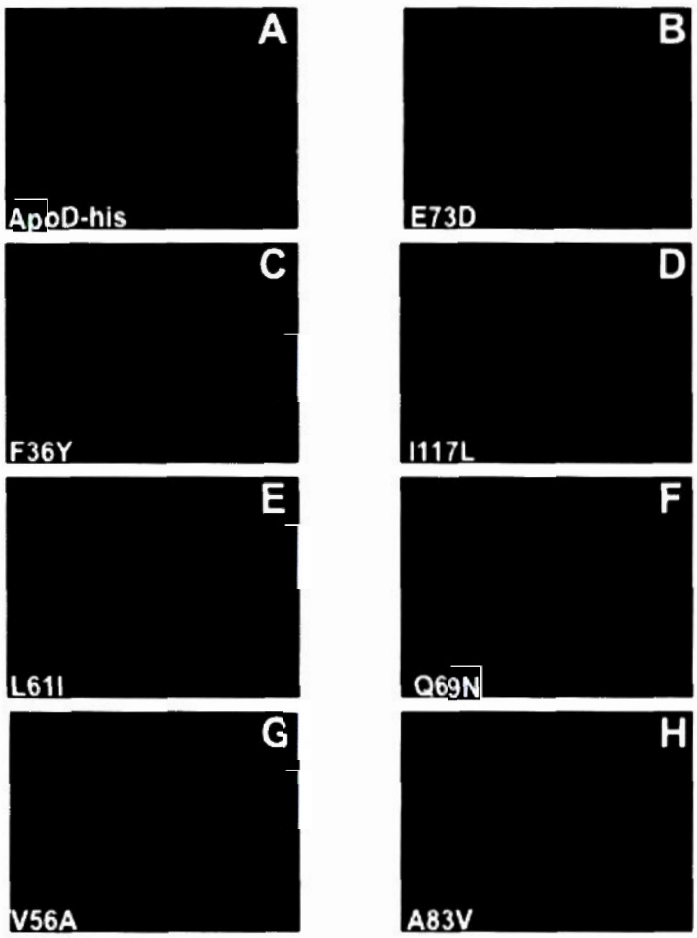
A83V
Ctrl +
a
Conditioned media
Anti-His
b
Cell lysates
Anti-His
β-Actin

Figure 4.3



CHAPITRE V

DISCUSSION

5.1 Conclusions générales

Les travaux réalisés dans le cadre de cette étude ont permis, dans un premier temps, de confirmer l'effet neuroprotecteur de l'apoD dans l'excitotoxicité en mettant en évidence de nouveaux mécanismes qui pourraient être impliqués dans ce processus. Dans un deuxième temps, ces travaux ont permis de démontrer pour la première fois l'existence d'un récepteur impliqué dans le processus d'internalisation de l'apoD.

5.1.1 Effet neuroprotecteur de l'apoD face à l'excitotoxicité

Malgré l'accumulation de preuves mettant en évidence l'effet neuroprotecteur de l'apoD dans des conditions neuropathologiques, on en sait encore peu sur l'effet de la surexpression de l'apoD et les mécanismes sous-jacents, notamment sur le mécanisme d'excitotoxicité. Nous avons donc utilisé un modèle de souris surexprimant l'apoD humaine dans le SNC (H-apoD Tg) pour tester les effets neuroprotecteurs de l'apoD face à l'excitotoxicité induite par injection de l'acide kaïnique (chapitre II). Cette étude nous a permis de démontrer que la surexpression de l'apoD entraîne une plus grande résistance face aux convulsions induites par le kaïnate, une baisse des réponses inflammatoires et confère une protection contre l'apoptose des cellules dans l'hippocampe, suggérant que la surexpression de l'apoD favorisait la présence d'un environnement non permissif à la propagation de

l'excitotoxicité. Il est connu que la mort neuronale induite par excitotoxicité est la conséquence d'une série d'événements qui se produisent en cascade, dont un des éléments-clés est l'accumulation intracellulaire de calcium dans les neurones (Mody and MacDonald, 1995). Il est donc possible que l'apoD crée un environnement neuroprotecteur en modulant les niveaux intracellulaires de calcium.

5.1.1.1 Éventuelle implication de l'apoD dans la régulation du calcium intracellulaire

Une concentration excessive de calcium intracellulaire peut avoir des conséquences néfastes sur la cellule neuronale (Patel et al., 1996). Il n'est donc pas étonnant que les résultats obtenus en chapitre II tendent à montrer que l'apoD module l'expression de deux protéines, la pompe PMCA2 et la sous-unité NR2B du récepteur NMDA, impliquées dans l'homéostasie du calcium intracellulaire.

Tout d'abord, tel que démontré dans le chapitre II, la surexpression de l'apoD induit une augmentation de l'expression de PMCA2, qui est une pompe participant à l'efflux du calcium en excès. Il a été démontré que la peroxidation lipidique affecte l'expression de PMCA2 (Chiarello et al., 2014). Ainsi, l'apoD pourrait moduler le niveau intracellulaire de calcium en augmentant l'expression de PMCA2 via la peroxidation afin de protéger les neurones contre l'excitotoxicité. Nous avons aussi observé une diminution d'expression de la sous-unité NR2B, mais pas des sous unités NR1 et NR2A, dans l'hippocampe de souris H-apoD Tg. Un récepteur NMDA fonctionnel est constitué obligatoirement de 2 sous-unités NR1 (qui lient la glycine, un co-agoniste du glutamate) et 2 sous-unités NR2 (qui lient le glutamate) (Kendrick et al., 1996). Il existe 4 types de sous-unités NR2 (A, B, C et D), NR2A et NR2B étant exprimés dans plusieurs régions du cerveau notamment au niveau de l'hippocampe, contrairement aux autres sous unités (Wenzel et al., 1997). Nos résultats suggèrent donc que l'apoD favorise la présence des récepteurs NMDA contenant peu ou pas de sous-unité NR2B. Il a été montré justement que, dans un

modèle animal d'ischémie cérébrale et sur des cultures neuronales de cortex *in vitro*, l'activation des récepteurs NMDA contenant NR2B augmente la mort neuronale induite par excitotoxicité. À l'inverse, l'activation des récepteurs NMDA contenant NR2A exerce une action neuroprotectrice en favorisant la survie neuronale (Liu et al., 2007). Ceci pourrait donc expliquer la diminution de l'apoptose cellulaire observée dans l'hippocampe de souris H-apoD Tg après injection au kaïnate. NR2B est également impliqué dans le processus de la plasticité synaptique (Mao et al., 2009). Nous n'avons cependant pas observé, dans l'hippocampe de souris WT et H-apoD Tg, des niveaux d'expression différents de la synaptophysine, un marqueur communément utilisé pour mesurer la plasticité synaptique (Martinez et al., 1997). Cela suggère donc que seule l'absence de l'apoD, et non pas sa surexpression, affecterait la plasticité synaptique et les capacités locomotrices et d'apprentissage tel qu'observé chez les souris apoD -/- (Ganfornina et al., 2008). Pour le vérifier, il serait intéressant de réaliser une étude comportementale basée sur la mémoire, qui est un processus dépendant de la plasticité synaptique, mesurant, par exemple, la mémoire spatiale des souris H-apoD Tg lors du test de la piscine de Morris (Liu et al., 2007).

La modulation de l'expression de ces 2 facteurs, NR2B et PMCA2, suggère donc que l'apoD pourrait exercer son rôle protecteur en régulant le niveau de calcium intracellulaire. Il serait intéressant de vérifier l'effet de l'apoD sur l'accumulation de calcium intracellulaire induite par le kaïnate à partir de cultures primaires neuronales ou de coupes organotypiques d'hippocampes issus de souris H-apoD Tg ou apoD (-/-) en utilisant la technique d'imagerie calcique (Barreto-Chang and Dolmetsch, 2009).

5.1.1.2 Régulation du cholestérol intracellulaire

Il est connu que le cholestérol est très impliqué dans la régulation de plusieurs fonctions neuronales dont la croissance axonale et la transmission synaptique (Dietschy and Turley, 2001). Une dérégulation de l'homéostasie du cholestérol et une modulation de l'expression de l'apoD ont d'ailleurs été observées dans plusieurs maladies neurodégénératives (Vance, 2012). L'excitotoxicité induit également une accumulation de cholestérol, à la fois *in vivo* et *in vitro*, dans les neurones de l'hippocampe de rat suite à un traitement avec le kaïnate (Ong et al., 2003). Nous avons remarqué (dans le chapitre IV) que cette accumulation du cholestérol induite, dans les cultures neuronales, par un traitement au kaïnate était cependant atténuée en présence d'apoD exogène. L'effet neuroprotecteur de l'apoD résiderait donc dans ses caractéristiques anti-inflammatoires, anti-oxydantes mais aussi dans le contrôle de l'homéostasie du cholestérol. Nous avons en effet observé que la surexpression de l'apoD (en chapitre II) diminue le niveau de cholestérol cytosolique sans affecter le niveau total et le niveau membranaire de cholestérol dans le cerveau de souris H-apoD Tg. Ceci suggère que l'effet neuroprotecteur de l'apoD observé (en chapitre II) chez les souris H-apoD Tg face à l'excitotoxicité pourrait impliquer des mécanismes de régulation du cholestérol intracellulaire. De plus, l'ajout de cholestérol exogène dans les cultures neuronales de l'hippocampe entraîne une importante accumulation de cholestérol dans la cellule, mais, après traitement avec de l'apoD exogène, cette accumulation est fortement diminuée, suggérant que l'apoD pourrait être impliquée dans la régulation de la distribution du cholestérol intracellulaire (Chapitre II).

La voie majeure d'élimination du cholestérol dans le SNC est sa conversion en 24-hydroxycholestérol (24-HOC) qui peut traverser la barrière hémato-encéphalique (BHE) pour être éliminer dans le foie (Vance, 2012). Puisque la surexpression de l'apoD n'affecte pas les niveaux de 24-HOC dans le cerveau et dans le plasma de souris H-apoD Tg, il est donc probable que le cholestérol, un des ligands de l'apoD,

soit transporté par celle-ci vers la membrane ou vers les lipoprotéines telles que les HDL en vue de son efflux. Le cerveau des souris apoD -/- montre d'ailleurs une diminution du ratio cholestérol : phospholipide dans les extraits membranaires (Thomas and Yao, 2007). Toutefois, l'interaction directe entre l'apoD et le cholestérol est controversée. Nous avons donc vérifié cette interaction en utilisant une méthode différente de celle utilisée auparavant. Nos résultats (en chapitre IV) indiquent que l'apoD peut se lier au cholestérol, mais avec une faible affinité. Mais, étant donné que le cholestérol est présent en grande quantité dans l'organisme notamment dans le système nerveux, il serait possible qu'il soit un ligand important malgré sa faible affinité. Il a d'ailleurs été proposé que cette faible affinité de l'apoD pour le cholestérol faciliterait l'incorporation du cholestérol dans les membranes ou dans les HDLs (Patel et al., 1997). Pour vérifier si cet efflux du cholestérol se fait par l'intermédiaire de l'apoD, les mutations décrites dans le chapitre IV, qui possiblement interfèrent avec la liaison de l'apoD au cholestérol, pourraient être utilisées avec des cultures primaires neuronales traitées ou non avec le kainate. De plus, une étude a montré que ABCG4 (un transporteur à cassette de liaison de l'ATP très fortement exprimé dans les neurones) et les HDLs augmentent l'efflux du cholestérol dans les cultures corticales de neurones (Chen et al., 2013). Il serait donc intéressant de vérifier les niveaux de lipoprotéines ainsi que l'expression de certains transporteurs de type ABC dont ABCG4 et ABCA1 (impliqués dans l'efflux du cholestérol dans les astrocytes) dans les cerveaux des souris Tg-apoD et apoD -/-. De la même façon, l'efflux du cholestérol dans les astrocytes est médié par l'apoE et ABCA1 (Chen et al., 2013).Il serait donc intéressant de vérifier l'effet de l'apoD sur l'efflux du cholestérol dans les astrocytes. Il a déjà été démontré *in vitro* que l'apoD exogène pouvait être internalisée dans les astrocytes, plus particulièrement dans les astrocytes issus de souris apoD -/- où le taux d'apoD internalisée est plus élevé (Bajo-Graneras et al., 2011). Cette augmentation de l'entrée de l'apoD pourrait donc permettre de pallier à la dérégulation du niveau de certains lipides (incluant le cholestérol et l'acide

arachidonique) dans les extraits membranaires issus de cerveaux de souris apoD -/- (Thomas and Yao, 2007).

5.1.2 Internalisation de l'apoD médiée par la basigine

Le rôle de l'apoD dans notre modèle murin suggérait que cette protéine pouvait être internalisée dans les neurones. Nous avons donc confirmé (en chapitre II) que l'apoD est capable d'entrer dans les neurones et que cette internalisation s'intensifie en réponse à un stress induit par le kaïnate ou dans des neurones matures. Ainsi, l'augmentation de l'apoD dans les neurones en situation neuropathologique ou dans les cerveaux de patients âgés pourrait être non seulement due à l'induction de l'expression de l'apoD, mais également à l'augmentation de l'internalisation de l'apoD produite essentiellement par les cellules gliales. Il est cependant essentiel de mieux comprendre les mécanismes d'internalisation de l'apoD afin de mieux comprendre l'impact de la surexpression de l'apoD.

Nos résultats (Chapitre III) prouvent que l'internalisation de l'apoD nécessite la présence d'un récepteur. Ce récepteur pourrait très probablement être la basigine, connue également sous le nom de CD147 ou EMMPRIN. La basigine est une glycoprotéine membranaire de la famille des immunoglobulines impliquée dans de nombreuses pathologies, tel que le cancer ou la maladie d'Alzheimer (Iacono et al., 2007). Nous avons démontré (en chapitre III) que la cyclophiline A, un ligand naturel de la basigine, pouvait bloquer l'internalisation de l'apoD. Ceci suggère que le site de liaison de la cyclophiline A sur la basigine pourrait être identique ou très proche de celui de l'apoD. Il a été reporté que le site de liaison de la cyclophiline A serait localisé au niveau de l'ectodomaine de la basigine (Song et al., 2011), suggérant donc que le site de liaison de l'apoD serait également localisé au niveau de l'ectodomaine de la basigine (Figure 5.1).

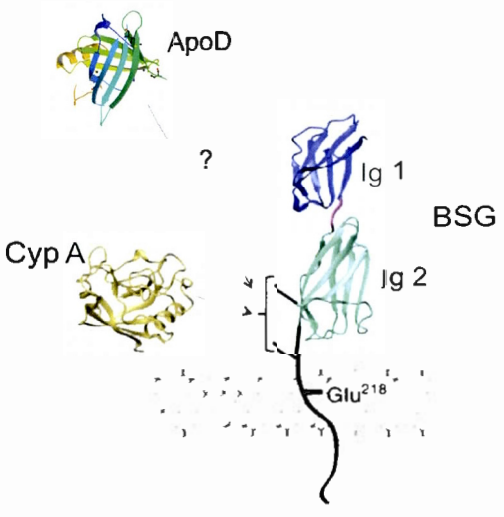


Figure 5.1 Représentation schématique de site de liaison hypothétique de l'apoD avec la basigine. CypA, cyclophiline A ; BSG, basigune ; ApoD, apolipoprotéine D (figure adaptée de (Yurchenko et al., 2010).

Nos résultats révèlent clairement que la basigine est fortement impliquée dans le processus d'internalisation de l'apoD, suggérant une étroite relation entre la basigine et l'apoD. En réponse à un stress ou dans certaines pathologies, la cyclophiline A est sécrétée et interagit avec la basigine pour entrainer une activation des médiateurs pro-inflammatoires (incluant les cytokines et les métalloprotéinases) et une infiltration des leucocytes pro-inflammatoires vers les sites de lésions (Arora et al., 2005; Damsker et al., 2009; Song et al., 2011). De plus, il a été montré que l'inhibition de la basigine dans un modèle murin de sclérose en plaques diminue l'infiltration des leucocytes dans la moëlle épinière, permettant ainsi de diminuer la sévérité des signes cliniques (Agrawal et al., 2011). Il est donc intéressant de noter que la surexpression de l'apoD chez les souris H-apoD Tg diminue l'infiltration des lymphocytes T induite par injection intracérébrale du virus OC43 (Do Carmo et al., 2008). Ceci suggère donc que l'interaction de l'apoD avec la basigine pourrait constituer un mécanisme

additionnel qui expliquerait la diminution d'infiltration des leucocytes et de la production de cytokines chez les souris H-apoD Tg injectées avec le virus OC43.

De plus, l'apoD, sécrétée dans le milieu extracellulaire, peut entrer dans la cellule à partir du milieu extracellulaire (Do Carmo et al., 2007). Cette apoD sécrétée présente cependant un niveau de glycosylation très important. Mais il apparait que le niveau de glycosylation n'affecte pas le processus d'internalisation de l'apoD (Chapitre IV). Ceci suggère donc que l'interaction de l'apoD avec son récepteur ne dépend pas de son niveau de glycosylation contrairement à sa capacité à se lier avec son ligand (Chapitre IV). En effet, le site de liaison de l'apoD avec son ligand est au sein d'une cavité hydrophobe (Eichinger et al., 2007; Peitsch and Boguski, 1990). Des mutations réalisées sur les acides aminés formant cette cavité hydrophobe nous a permis de diminuer la capacité d'internalisation de l'apoD dans la cellule (Chapitre IV), suggérant que la liaison de l'apoD avec son ligand favoriserait son interaction avec son récepteur et donc son internalisation. Il serait donc intéressant de vérifier l'effet de ces mutations sur la liaison de l'apoD avec ses ligands (tels que l'acide arachidonique, la progestérone et le cholestérol) pour confirmer si l'apoD doit nécessairement se lier avec son ligand pour être internalisée. De la même façon, des mutations réalisées sur les boucles A/B et G/H, localisées à l'entrée de la cavité hydrophobe, bloquent l'internalisation de l'apoD (Chapitre IV), suggérant que ces régions pourraient être impliquées dans l'interaction de l'apoD avec son récepteur.

Il est indéniable que l'apoD joue un rôle neuroprotecteur suite à un stress induit dans le SNC. En effet, l'ensemble de nos travaux met en évidence l'implication de l'apoD dans le processus de neuroprotection face à différents mécanismes participant à la neurodégénérescence. Ces travaux mettent également en avant qu'en plus de protéger contre le stress oxidatif et le stress inflammatoire, la surexpression de l'apoD dans notre modèle de souris transgéniques (H-apoD Tg) protège également contre

l'excitotoxicité qui accompagne de nombreuses neuropathologies comme la maladie d'Alzheimer, celle de Parkinson ou la sclérose en plaque. Ceci suggère que l'effet neuroprotecteur de l'apoD n'est pas spécifique à un stress en particulier, mais protège plutôt la cellule contre une grande majorité de mécanismes neurodégénératifs impliqués dans les maladies neurodégénératives, d'où son induction dans de nombreuses neuropathologies. L'apoD constitue donc une cible thérapeutique potentielle dans toutes ces neuropathologies.

5.2 Perspectives

Les travaux présentés dans cette thèse ont permis de mieux comprendre le rôle neuroprotecteur de l'apoD. Toutefois, les résultats présentés dans cette thèse soulèvent plusieurs questions, notamment sur le mode d'action de l'apoD mais également sur son implication dans d'autres processus tel que la neurogenèse ou la régénérescence axonale. Les expériences proposées dans cette partie permettraient d'approfondir encore plus nos connaissances sur les fonctions de l'apoD.

5.2.1 ApoD et la barrière hémato-encéphalique

De par sa large distribution tissulaire, l'apoD est trouvée à la fois dans le sang, dans le cerveau et dans le liquide céphalo-rachidien (LCR). Suite à une lésion neurodégénérative, l'apoD se trouve être augmentée dans le SNC. Il est vrai que cette augmentation est due à l'induction de son expression ; mais peut-on trouver de l'apoD produite en périphérie dans le cerveau ou le LCR et inversement ? Ceci implique que l'apoD soit dotée de la capacité à traverser la barrière hémato-encéphalique (BHE). L'apoD est detectée dans les cellules périvasculaires et les péricytes au niveau du cortex (Hu et al., 2001; Navarro et al., 2004).

La barrière hémato-encéphalique (BHE), est une barrière qui sépare le SNC du sang, le protégeant ainsi du reste de l'organisme. Régulant tous les échanges entre le sang et le cerveau, elle est constituée de cellules endothéliales, de péricytes et de pieds astrocytaires (Ballabh et al., 2004). Dû à la présence de jonctions serrées entre les cellules endothéliales, le passage de molécules reste très limité. Les échanges entre le sang et le SNC se font donc par l'intermédiaire de différents mécanismes de transport au niveau des cellules endothéliales (Moody, 2006).

Le passage de molécules à travers la BHE peut donc être médié par un transport actif (via des transporteurs de type ABC) ou par transcytose (via un récepteur spécifique) (Kusuhara and Sugiyama, 2005). La transcytose, impliquée dans le transport de molécules de plus haut poids moléculaire, permet à une molécule d'être internalisée dans la cellule endothéliale par l'intermédiaire de son récepteur puis de sortir au niveau du pôle opposé par exocytose (de Lange, 2012). Les HDL peuvent traverser la BHE par transcytose médiée par SR-BI (Scavenger receptor class BI) localisé à la surface des cellules endothéliales (Balazs et al., 2004). L'apoD pourrait traverser la BHE grâce à sa capacité de s'associer aux HDL. De plus, nous avons vu, en chapitre III, que le basigine semble être le récepteur de l'apoD. La basigine a été détectée dans les cellules endothéliales de la BHE (Sameshima et al., 2000). Ceci suggère donc la présence d'un transport de l'apoD à travers la BHE médiée par la basigine.

La capacité de l'apoD de traverser la BHE pourra donc se vérifier par injection systémique d'apoD humaine purifiée chez la souris. L'utilisation de l'apoD humaine permet de la différencier de l'apoD endogène chez la souris. Le cerveau et le LCR seront ensuite prélevés pour analyser la présence de l'apoD humaine. Pour vérifier le transport de l'apoD du cerveau vers le sang, une injection stéréotaxique de l'apoD humaine au niveau des ventricules cérébraux sera réalisée. La présence de l'apoD exogène sera ainsi évaluée dans le plasma.

5.2.2 Implication de l'apoD dans la neurogenèse

Un autre mécanisme envisagé pour pallier à la perte neuronale qui s'opère au cours des maladies neurodégénératives est l'utilisation de cellules souches neurales. La régénération par remplacement de neurones à partir de progéniteurs endogènes, ou communément appelée neurogenèse, permettrait de remédier à différents problèmes cliniques, notamment les rejets de greffes. Ces neurones néo-formés sont produits par des cellules souches neurales résidant dans des régions restreintes du SNC : le gyrus denté de l'hippocampe et la zone sous-ventriculaire (ZSV) qui borde les ventricules cérébraux (Kaplan and Hinds, 1977; Lois and Alvarez-Buylla, 1994). Contrairement aux neurones néo-formés dans le gyrus denté, les neurones nouvellement formés dans la ZSV doivent migrer sur de longues distances vers le bulbe olfactif où ils se différencient en neurones bulbaires (Taupin and Gage, 2002). Ceci soulève l'espoir de les exploiter pour réparer le tissu nerveux endommagé ou perdu au cours d'une maladie neurologique ou d'un traumatisme. Il serait donc intéressant de déterminer si l'apoD est impliquée dans le processus de neurogenèse.

Des traitements avec Ro25-6981, un antagoniste de la sous-unité NR2B du récepteur NMDA, induit une augmentation de la neurogenèse et de la survie des neurones néo-formés dans le gyrus denté de souris, entrainant une amélioration des performances dans les tâches cognitives dépendantes de la mémoire (Hu et al., 2008). Il est démontré en chapitre II que la surexpression de l'apoD dans le SNC des souris transgéniques diminue l'expression de la sous-unité NR2B, suggérant ainsi que l'apoD pourrait être impliquée dans l'activation de la neurogenèse. De plus, il a été montré que la clozapine, un médicament antipsychotique atypique qui induit l'expression de l'apoD (Mahadik et al., 2002; Thomas et al., 2001a), augmente la neurogenèse dans l'hippocampe de rat adulte (Halim et al., 2004). L'effet de l'apoD sur le processus de neurogenèse, *in vivo*, pourrait être ainsi déterminé en utilisant des souris déficientes en apoD (apoD -/-) et surexprimant l'apoD humaine dans les

neurones (H-apoD Tg). Le principe consisterait donc à faire une étude comparative chez ces souris de la prolifération des cellules et de la survie des neurones nouvellement formés. Pour vérifier la prolifération cellulaire, les souris WT, H-apoD Tg et apoD -/- seraient injectées, en intrapéritonéal, avec du BrdU (5-bromo-2'-déoxyuridine) pour 24h pour analyser la prolifération cellulaire et 4 semaines (avec des injections quotidiennes de BrdU) pour vérifier la survie des cellules nouvellement formées. Le BrdU est un marqueur de la synthèse de l'ADN communément utilisé pour l'analyse de le neurogenèse (Cameron and McKay, 2001). Le nombre de cellules marquées et leur phénotype serait analysé par immunohistochimie à partir de coupes de cerveaux des souris injectées grâce à l'utilisation de marqueurs neuronal et glial.

5.2.2 Régénération axonale dans le SNC

L'apoD est fortement induite lors de la croissance axonale dans les fibres nerveuses périphériques comprimées (Boyles et al., 1990; Spreyer et al., 1990). Il a été rapporté que l'apoD stimule le processus de régénération et de remyélinisation axonale suite à une lésion du nerf sciatique, en facilitant l'élimination de la myéline dégradée (Ganfornina et al., 2010), suggérant un rôle bénéfique de l'apoD dans la régénération et dans la croissance axonale dans le système nerveux périphérique (SNP). Cet effet bénéfique de l'apoD pourrait également être dû à la capacité de l'apoD de transporter les lipides tels que le cholestérol. Toutefois, les axones dans le SNP se régénèrent plus rapidement tandis que dans le système nerveux central (SNC), ce processus de régénération est aboli. Une des raisons expliquant cette différence d'habilité de régénération entre le SNP et le SNC est l'environnement dans lequel se déroule cette lésion. Le point majeur qui différencie la régénération axonale du SNP de celle du SNC est l'efficacité et la vitesse d'élimination des débris de myéline, engendrés lors de lésion axonale (Bignami and Ralston, 1969; George and Griffin, 1994). A la fois dans le SNC et le SNP, les débris de myéline contiennent plusieurs molécules

inhibitrices de la régénération axonale (He and Koprivica, 2004). Toutefois, dans le SNP, l'environnement extracellulaire dans lequel se déroule la dégénérescence de l'axone favorise cette régénération axonale. A l'inverse, dans le SNC, la présence prolongée de débris de myéline, mal éliminés, contribuent à l'inhibition de cette régénération. Autrement dit, le SNC crée un environnement non-permissif à la régénération axonale et une élimination plus rapide et efficace de ces débris de myéline (identique à celle qui se produit dans le SNP) permettrait de promouvoir cette croissance axonale. Bien que la régénération axonale périphérique soit favorisée par la présence de l'apoD, l'effet de l'apoD sur la régénération axonale dans le SNC reste encore inconnu. Il s'agirait donc de déterminer si l'apoD promeut la réparation et la croissance axonales dans le SNC, en utilisant comme modèle expérimental de dégénérescence axonale dans le SNC le modèle de traumatisme médullaire aigu (TMA) chez les souris apoD -/- et H-apoD Tg.

Le traumatisme médullaire correspond à une lésion de la moelle épinière, entraînant, dépendamment de la localisation de la lésion, une paralysie de la partie du corps située au-dessous de cette lésion (Tator, 1995). Lors d'un TMA, 2 évènements chronologiques apparaissent :

(1) la lésion primaire (interruption physique de l'axone par section) : elle se caractérise par une dégradation de l'axone et de la gaine de myéline en aval de la section et l'inhibition de la repousse axonale, à cause des débris de la myéline, entrainent la mort du neurone ;

(2) la lésion secondaire (conséquence de la lésion primaire) : elle correspond à l'induction de plusieurs mécanismes neurodégénératifs incluant l'excitotoxicité, l'apoptose et le stress inflammatoire suite à la mort neuronale induite par la lésion primaire (Schwab et al., 2006; Tator and Fehlings, 1991; Tator, 1995).

L'excitotoxicité apparait très rapidement après la lésion primaire induite par un TMA et contribue à la propagation de la dégénérescence dans la moelle épinière (Hulsebosch, 2002; Privat, 2005; Schwab et al., 2006). La réaction inflammatoire, quant à elle, est déclenchée plus tard mais s'intensifie au bout de quelques heures et dure plusieurs jours: la microglie est donc activée; les monocytes et lymphocytes sanguins franchissent la barrière hémato-encéphalique (BHE) lésée (Schwab et al., 2006) . Nous avons observé (en chapitre II) que la surexpression de l'apoD procure un environnement qui ne favorise pas la propagation de l'excitotoxicité induite par le kaïnate. De plus, l'injection intracérébrale du coronavirus HCoV-OC43 résulte en une diminution de l'infiltration des lymphocytes T CD4 dans la moëlle épinière de souris H-apoD Tg (Do Carmo et al., 2008). Chez les souris transgéniques surexprimant l'apoD humaine dans le SNC (H-apoD Tg), l'apoD humaine a bien été détectée dans la moelle épinière (Do Carmo et al., 2008). Ceci suggère que la surexpression de l'apoD dans la moëlle épinière des souris H-apoD Tg pourrait atténuer et retarder les effets de la lésion secondaire et favoriserait la régénération axonale dans la moëlle épinière suite au TMA. À l'inverse, l'absence de l'apoD chez les souris apoD -/- accélèrerait l'activation des mécanismes impliqués dans la lésion secondaire. Pour vérifier cette hypothèse, le traumatisme médullaire serait induit par contusion au niveau de vertèbres thoraciques chez des souris WT, apoD (-/-) et H-apoD Tg, comme décrit précédemment (Kuhn and Wrathall, 1998). La performance locomotrice des souris serait mesurée à l'aide d'une échelle d'évaluation.

Parallèlement à l'étude comportementale, plusieurs mécanismes déclenchés lors de la lésion secondaire du TMA seront analysés:

- l'infiltration des neutrophiles, des macrophages et des lymphocytes,
- la production de cytokines pro- et anti-inflammatoires,
- l'activation de la microglie et des astrocytes,
- l'expression des facteurs neurotrophiques (tels que BDNF, FGF-2),
- l'apoptose,
- le profil lipidique.

APPENDICE A

AUTRE CONTRIBUTION – COLLABORATION AVEC Dr. CATHERINE MOUNIER (UQAM)

TRANSGENIC MICE OVEREXPRESSING APOLIPOPROTEIN D DEVELOP HEPATIC STEATOSIS THROUGH ACTIVATION OF PPARγ AND FATTY ACID UPTAKE

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Avant propos

Cette étude fait suite à une étude antérieure montrant que notre modèle de souris transgéniques (H-apoD Tg) présente des troubles métaboliques notamment une résistance à l'insuline et une stéatose hépatique. Le but de cet article était donc de déterminer les mécanismes impliqués dans cette stéatose hépatique.

La rédaction et le montage des figures ont été effectués par Maryline Labrie. J'ai participé aux expériences de ce projet et à la correction de l'article. Ces travaux ont été supervisés par les Dr Catherine Mounier et Eric Rassart qui ont également corrigé le manuscrit.

A.1 Abstract

Transgenic mice (Tg) overexpressing human apolipoprotein D (apoD) in the brain are resistant to neurodegenerescence, but slowly develop hepatic steatosis. We showed that PPARγ expression in the liver of the Tg mice is increased by 2-fold compared to wild type (WT) mice. Consequently, PPARγ target genes Plin2 and Cide A/C are overexpressed, leading to increased lipid droplets formation. Expression of the fatty acid transporter CD36, another PPARγ target, is also increased in Tg mice associated with elevated fatty acid uptake as measured in primary hepatocytes. Activation of AMPK in the liver of Tg leads to phosphorylation of acetyl CoA carboxylase while fatty acid synthase expression is induced. However, hepatic lipogenesis measured *in vivo* is not significantly different between WT and Tg mice. In addition, expression of carnitine palmitoyl transferase 1, the rate-limiting enzyme of β-oxydation, is slightly upregulated. Finally, we showed that overexpressing apoD in HepG2 cells in presence of arachidonic acid (AA), the main ligand of apoD, increases the transcriptional activity of PPARγ. Taken together, our results demonstrate that the hepatic steatosis observed in apoD Tg mice is a consequence of increased PPARγ transcriptional activity by AA leading to increased fatty acid uptake by the liver.

Key words: Apolipoprotein; Liver; Nuclear receptors/PPAR; Arachidonic acid; Lipid droplets, Apolipoprotein D, fatty acid metabolism, PPARγ, lipogenesis, hepatic steatosis

A.2 Introduction

Apolipoprotein D (apoD), a 29 kDa glycoprotein, is a member of the lipocalin super family (Drayna et al., 1987). It transports several small hydrophobic compounds such as arachidonic acid (AA), progesterone, pregnenolone, bilirubin, cholesterol and E-3-methyl-2-hexenoic acid (Dilley et al., 1990; Morais Cabral et al., 1995; Patel et al., 1997; Peitsch and Boguski, 1990; Terrisse et al., 2001; Zeng et al., 1996). In human, apoD is found in the plasma fraction, associated with high-density lipoprotein (HDL). It is highly expressed in the brain, adrenal glands, kidneys, pancreas and placenta but poorly expressed in intestine and liver (Drayna et al., 1986; Drayna et al., 1987; McConathy and Alaupovic, 1973; McConathy and Alaupovic, 1976). In contrast, the murine expression of the apoD gene is almost exclusively expressed in the central nervous system (CNS) (Cofer and Ross, 1996; Seguin et al., 1995).

We have previously shown that transgenic mice (Tg) overexpressing human apoD (H-apoD) in the brain are protected against neurodegeneration and injuries (Do Carmo et al., 2008; Ganfornina et al., 2008) suggesting that apoD could be a good therapeutic target for neurodegenerative diseases. Unfortunately, these mice develop, with age, insulin resistance, glucose intolerance as well as hepatic and muscular steatosis (Do Carmo et al., 2009b).

Our previous observations showed that the peroxisome proliferator-activated gamma (PPARγ) mRNA expression is increased in the liver of H-apoD Tg mice (Do Carmo et al., 2009b). PPARγ is a nuclear receptor implicated in adipocyte differentiation. Two isoforms exist: PPARγ1 is ubiquitously expressed while PPARγ2 is almost exclusive to adipose tissue (Tontonoz et al., 1994a; Tontonoz et al., 1994b). When activated by one of its ligands, PPARγ heterodimerizes with retinoid X receptor α (RXRα) and binds to the peroxisome proliferator response elements (PPRE) on the promoter of target genes (Tontonoz and Spiegelman, 2008; Zoete et al., 2007).

PPARγ regulates positively its own transcription and induces transcription of the CCAAT/enhancer-binding protein α (C/EBPα), which also activates PPARγ gene expression (Darlington et al., 1998; Li et al., 2010). Many natural PPARγ ligands have been discovered including AA, prostaglandins, oxidized fatty acid (FA) and some polyunsaturated fatty acid (PUFA) (Forman et al., 1995; Kliewer et al., 1995; Kliewer et al., 1997; Krey et al., 1997; Nagy et al., 1998).

Activation of hepatic PPARγ leads to an upregulation of free FA (FFA) uptake by increasing the expression of fatty acid transporter CD36 (Feng et al., 2000). PPARγ is also involved in lipid droplets (LD) formation through increased expression of LD associated proteins such as perilipin 2 (Plin2) and cell death-inducing DFFA-like effectors (Cide) A and C (Chawla et al., 2001; Rogue et al., 2010; Rogue et al., 2011). These LD-associated proteins down-regulate LD lipolysis by reducing association of lipases with the surface of LD (Jinno et al., 2010; Keller et al., 2008; Listenberger et al., 2007). On the other end, hepatic PPARα regulates energy combustion (Pyper et al., 2010) by activating the mitochondrial and the peroxisomal β-oxidation pathways as well as the microsomal ω-oxidation pathway (Reddy and Hashimoto, 2001). Paradoxically, PPARα also activates lipogenesis by regulating the sterol regulatory element binding protein-1 (SREBP-1c) and liver X receptor α expression (LXRα) (Hebbachi et al., 2008).

Many studies have demonstrated a link between elevated PPARγ expression and hepactic steatosis. Adenoviral over-expression of PPARγ1 in PPARα knockout (KO) mice that display reduced fatty acid oxidation in liver induces ectopic fat accumulation and lipogenesis leading to hepatic steatosis (Gavrilova et al., 2003). In *Ob/Ob* and lipoatrophic mice, elevated expression of PPARγ2 is associated with non-alcoholic fatty liver disease (NAFLD) while inhibition of PPARγ expression reduces

hepatic steatosis through downregulation of lipogenesis and inhibition of LD formation (Matsusue et al., 2003; Rahimian et al., 2001; Zhang et al., 2006).

Lipogenesis is regulated at various levels. SREBP-1c and LXRα are the main transcription factors responsible for the induction of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) expression, the two rate-limiting enzymes of lipogenesis. These enzymes produce non-esterified FA (NEFA) that are subsequently desaturated by the stearoyl-CoA desaturase (SCD). These NEFA are further esterified to form the triglycerides (TG) implicating enzyme such as the diglyceride acyltransferase (DGAT) (Ameer et al., 2014). Lipogenesis can be inhibited by AMP-activated protein kinase (AMPK) through phosphorylation and inhibition of both ACC and SREBP-1c (Viollet et al., 2006).

In the present study, we demonstrate that H-apoD Tg mice develop hepatic steatosis through over-expression and activation of PPARγ1. Consequently, the expressions of Plin2, Cide A and C are increased leading to stabilization of LD. In addition, we observed an increased CD36 expression associated with elevation of fatty acid uptake. In these conditions, lipogenesis remains unaffected despite an increased expression of FAS and a phosphorylation of ACC as a result of increased AMPK activity. Overexpressing ApoD in HepG2 cells in presence of AA suggested that the apparition of hepatic steatosis in Tg mice is the result of PPARγ activation by AA, one of the main ligand of apoD. Our work reveals a novel mechanism of apoD action in lipid metabolism.

A.3 Material and Methods

A.3.1 Materials

Cell culture medium was purchased from Wisent (Wisent, St-Bruno, Qc, Canada). Bodipy 493/503, Prolong Gold antifade reagent, Galacto-light™ beta-galactosidase reporter gene assay system, Trizol Reagent and mouse anti-myc monoclonal antibody were purchased from Invitrogen (Invitrogen, Burlington, ON, Canada). AA, anti-mouse horseradish peroxidase-conjugated secondary antibody, luciferin and propidium iodure were obtained from Sigma (Sigma-Aldrich, Oakville, ON, Canada). Anti-PPARγ (C26H12), anti-AMPKα, anti-phospho-AMPKα (Thr172) (40H9), HPRT and β-actin antibodies were from Cell signaling (cell signaling technology, Danvers, MA, USA). Anti-ACC and anti-phospho-ACC (Ser79) antibodies were from Millipore (Millipore, Billerica, MA, USA). Anti-Plin2 antibody was from Novus Biological (Novus Biologicals, Littleton, CO, USA) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and Bradford reagent were from Bio-rad (Life Science Bio-rad, Mississauga, Ontario, Canada). H-ApoD monoclonal and polyclonal antibodies have already been described (Terrisse et al., 1998; Terrisse et al., 2001). Complete Protease Inhibitor Cocktail Tablets were purchased from Roche (Laval, PQ, CAN). Collagenase Type I was from Worthington (Lakewood, NJ). Gal4-PPARγ and UAS-Luciferase plasmids were generously provided by Dr. Maurizio Crestani (University of Milano, Italia).

A.3.2 Animals

All the experimental procedures were approved by the Animal Care and Use Committee of Université du Québec à Montréal. Animals were housed at 24 ± 1°C in a 12h light dark cycle and fed a standard rodent chow *ad libitum* with free access to water. The H-apoD Tg mice in a C57BL/6 background overexpress the H-ApoD gene

under the control of the neuron-specific Thy-1 promoter (Do Carmo et al., 2008; Do Carmo et al., 2009b; Ganfornina et al., 2008). All experiments were carried out on 12 months old male.

A.3.3 Preparation of primary hepatocytes

Primary hepatocytes were isolated by *in situ* liver perfusion and collagenase digestion as previously described (Truong et al., 2000).Briefly, mice were anaesthetized by intraperitoneal injectionof pentobarbital and the portal vein was cannulated. The liver was then perfused with perfusion buffer (10 mM HEPES, 142mM NaCl, 6,7mM KCl; pH 7,85) containing 0,6mM EGTA and 1,5 U/mL heparin and subsequently digested with 30 000U collagenase type I (Worthington) dissolved in 150 mL of perfusion buffer containing 5 mM calcium. Hepatic cells were gently released from the Glisson capsule and incubated for 1h at room temperature with 5X Wash solution consisting of DMEM/F12 (Life technologies, Gibco) with 10% fetal bovine serum (Life technologies, Gibco), 500 U/mL penicillin, 500 µg/mL streptomycin and 1,25 µg/mL Fungizone (Life Technologies) on an orbital shaker. Approximately 1x106 millions of cells were seeded on collagen-pretreated plates (Corning Costar) in DMEM/F12 media containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. The next day, culture media was removed and renewed with serum-free DMEM/F12 containing the same antibiotics and the cells were starved for 48h prior to the experiments.

A.3.4 Transfection of HepG2 cells

The human hepatocarcinoma cells (HepG2) were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS. Cells were then transfected with Fugene HD with a UAS-Luciferase construct (a luciferase reporter plasmid containing five PPAR response elements) in combination with a human myc-

tag apoD cDNA construct (Terrisse et al., 1998) (or an empty vector) in the presence or not of Gal4-PPARγ (containing the PPARγ cDNA and the DNA binding domain of GAL4). 48h after transfection, cells were incubated with 7μM of AA bound to BSA for 4h. Cells were then harvested and cellular extracts were prepared for luciferase (de Wet et al., 1987) and β-galactosidase assays (Invitrogen).

A.3.5 RNA extraction and semi-quantitative RT-PCR

Tissues were collected, frozen in dry ice and kept at −80°C until further use. Total RNA was extracted with the TRIZOL reagent according to the manufacturer instructions. Total RNA was then reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit and amplified with a Taq DNA polymerase and specific primers. HPRT was used as control.

A.3.6 Immunoblotting

Tissues or cultured cells were homogenized in cold lysis buffer (50 mM Tris·HCl pH 7.3, 150 mM NaCl, 5 mM EDTA, 0.2% Triton X-100, 2 mM sodium orthovanadate and 10% Complete protease inhibitor). Lysates were then incubated 30 min at 4°C, cleared by centrifugation and stored at –80°C until further use. Based on Bradford assay (Bradford, 1976), 50 μg of protein of each sample were separated on SDS-PAGE and transferred to PVDF membranes. After blocking with 5 % milk 1h at room temperature, the membrane was incubated with the primary antibodies overnight at 4°C. Dilutions of the primary antibodies were: 1:1000 for PPARγ (C26H12), 1:1000 for total AMPKα antibody; 1:1000 for phospho-AMPKα (Thr172) (40H9) antibody; 1:1000 for ACC antibody; 1:300 for anti-phospho-ACC (Ser79) antibody; 1:5000 for Plin2 antibody, 1:10000 for apoD antibody (Terrisse et al., 1998), 1:10000 for HPRT antibody and 1:100000 for β-actin antibody. Primary antibodies were then detected with a goat anti-rabbit horseradish perioxidase-

conjugated secondary antibody (1:10000) and visualized by chemiluminescence. Amidoblack staining was used as loading control. Briefly, membranes were stained for 20 min in amidoblack solution (0,1 % Amidoblack, 40 % v/v methanol and 10 % v/v acetic acid) and rinsed 10 min twice in decoloration solution (40 % v/v methanol and 10 % v/v acetic acid). Western blot were quantified by densitometry using the image J software.

A.3.7 Lipid staining

Liver samples were incubated overnight at 4°C in 4% paraformaldehyde, frozen in dry ice and kept at −80°C until further use. Four micro thick longitudinal sections were cut with a cryostat and incubated 5 minutes in a solution of PBS containing 0.04 mg/ml propidium iodide and 0.1 µg/ml Bodipy493/503. After 3 washes of 5 min in PBS, coverslips were mounted onto slides using Prolong Gold antifade reagent and observed within 24h with a laser scanning confocal microscope (Nikon TE300) (original magnification X60).

A.3.8 In vivo lipogenesis

1-year-old mice were fasted for 24h and then injected intraperitoneally with 7 µCi of $^{3}H_2O$. One-hour later animals were killed and blood and liver were collected. To evaluate plasma water specific radioactivity 20 µl of plasma was dissolved in 4 ml of Scintillator (Ultima Gold, from Perkin Elmer) and counted with a scintillation counter (TRi Carb 2800TR). To evaluate the fatty acid specific radioactivity, 1g of liver was dissolved in 30% KOH at 70°C. Then 3 ml of ethanol 96% was added, heated at 70°C for 2h and acidified with 3 ml of sulfuric acid 9 M. Lipids were extracted 3 times with 10 ml of light petroleum, fractions were collected, washed 3 times with 10 ml of water, and dried at RT. Lipids were then dissolved in 15 ml of scintillator and counted as described for the plasma. Fatty acid specific radioactivity was expressed

as cpm/g of liver and counted. The rate of lipogenesis was calculated by dividing the fatty acid specific radioactivity by the plasma water specific activity.

A.3.9 ^{3}H-oleate fatty acid uptake

Primary hepatocytes of WT and Tg mice were serum starved for 48h in a serum free media. Fatty acid uptake using ^{3}H-oleate was performed as previously described (Stremmel and Berk, 1986) with some modifications. Briefly, cells were incubated in serum free DME/F12 media containing 50 µM oleate dissolved in BSA (fatty acid/BSA ratio: 2:1) and 0.68 µCi/mL of ^{3}H-oleate for 10 min at RT. The reaction was stopped by adding 200 µM of ice-cold phloretin solution for 2 min. Cells were then washed 3 times with PBS and lysed in 0,1 N NaOH for 30 min at RT. Lysates were counted in 10 mL Ultima-Gold solution (Tri-Carb 2800TR, Perkin Elmer) and protein quantified (Bradford Assay, BioRAD).

A.3.10 Statistical analysis.

Results are expressed as means ± SD. Statistical analysis was performed with GraphPad 5 software. The statistical significance from control values was determined by Student's t-test. Values were considered to be significant at $P < 0.05$.

A.4 Results

A.4.1 PPARγ and C/EBP expression in liver of H-apoD Tg mice

We previously demonstrated that PPARγ mRNA was increased in H-apoD Tg mice liver (Do Carmo et al., 2009b). In the present study, we showed that both PPARγ1 and γ2 are increased (1.37-fold and 1.16-fold Tg *vs* WT respectively) (Fig. A.1A). At the protein level, PPARγ1 was increased by 2.24-fold in Tg mice while PPARγ2 was poorly detectable (Fig. A.1B). The expression of C/EBPα mRNA, an early marker of adipogenic-like phenotype was also increased (Fig. A.1C) while C/EBPβ remained unchanged (Fig. A.1D).

A.4.2 Lipid droplets formation

We measured the expression level of key proteins known to be involved in LD formation. The expression of the PPARγ target gene Plin2 (Targett-Adams et al., 2005), a protein implicated in LD formation, was increased by 1.98-fold in Tg mice (Fig. A.2A). Similar observations were made regarding Cide A and Cide C (1.47 and 1.45-fold respectively), two targets of PPARγ that are implicated in LD fusion (Xu et al., 2012) (Fig. A.2B &D). A well-documented independent gene of PPARγ regulation, Cide B remained unchanged (Fig. A.2C). Conversely, the expression of genes coding for several lipases (ATGL, HSL and MGL) as well as the ATGL coactivator CGI-58 remained unchanged (*data not shown*). In agreement with an elevated expression of proteins involved in hepatic LD formation and fusion, we found that the size of the LD in H-apoD Tg mice was drastically increased (5.45-fold) compared to WT. However, we did not observe a significant modification in the number of LD between the mice (Fig. A.2E).

A.4.3 Hepatic FFA uptake

We previously demonstrated that circulating FFA, cholesterol and TG remained unchanged in H-apoD Tg mice compared to WT (Do Carmo et al., 2009b). Here, we showed that the mRNA expression of two enzymes implicated in lipoprotein metabolism, lipoprotein lipase (LPL) and hepatic lipase (HL) remained unaffected in Tg mice (*Data not shown*). In contrast, the expression of CD36, a target of PPARγ and the main transporter of hepatic FFA in cells, is significantly increased (1.2-fold) in liver of Tg mice (Fig. A.3A). To evaluate the impact of CD36 increased expression on FA uptake in the liver of Tg mice, we prepared primary hepatocytes from both WT and Tg animals. Incubation of cells with ^{3}H-oleate showed a 30% increase in oleate uptake in Tg mice (Fig. A.3B). This confirmed that the upregulation of CD36 provides a functional role in those mice.

A.4.4 Hepatic lipogenesis

We previously demonstrated that the mRNA levels of SREBP-1c and FAS were increased in H-apoD Tg mice liver compared to WT mice (Do Carmo et al., 2009b). Since elevated lipogenesis has also been associated with hepatic steatosis, we evaluated the expression levels and the activity of several key proteins involved in hepatic lipogenesis. We showed that the AMPK expression was increased by 1.82-fold in the liver of Tg mice. The AMPK activity is also increased as documented by an increased phosphorylation of the kinase on Thr172 residue (2.63 fold) (Fig. A.4A). Consequently, one of the target proteins, ACC displayed an increased phosphorylation on Ser79 (2.42 fold) suggesting a reduced activity of the first enzyme of lipogenesis (Fig. A.4B). On the other hand, a significant increase in FAS protein expression was observed (1.97-fold) corroborating our previous observation at the mRNA level (Do Carmo et al., 2009b) (Fig. A.4C). The mRNA expression of

ACC, SCD1, DGAT and LXRα mRNA remained unaffected in H-apoD Tg mice liver compared to WT mice (Fig. A.4D).

The observation of an increased phosphorylation of ACC and an augmented expression of FAS seemed contradictory. Hence, we measured *de novo* lipogenesis *in vivo* by $^{3}H2O$ injection in mice. As shown in Fig. A.4E, the level of *de novo* lipid synthesis in the liver is not significantly different between Tg and WT mice suggesting that hepatic steatosis cannot be attributed to a modification of *de novo* lipid synthesis.

A.4.5 Hepatic β-oxydation

We previously demonstrated that PPARα mRNA was increased in H-apoD Tg mice liver suggesting an increase in lipid β-oxydation in the liver (Do Carmo et al., 2009b). A similar increase was observed at the protein level (2.73 fold) (Fig. A.5A). PPARα is known to regulate expression of genes involved in the β-oxidation pathway, so we examined the expression of two key proteins involved in this process. The mRNA level of PGC-1α, a co-activator of PPARα remained unchanged (Fig. A.5B). However, the mRNA expression of CPT-1, the rate limiting enzyme of the mitochondrial lipid transfer where β-oxidation of lipids takes place, is increased by 1.26-fold in the liver of H-apoD Tg mice compared to WT (Fig. A.5C). This might be associated to an upregulation of lipid β-oxidation in the liver of H-apoD Tg mice.

A.4.6 Effect of ApoD overexpression on PPARγ activation by AA

To understand the link between apoD and PPARγ over-expression and activation, we evaluated the potential role of apoD as an AA transporter, one of the main ligand of PPARγ. We used the human hepatocarcinome HepG2 cell line as a well-characterized model for the study of hepatic lipid metabolism. Cells were transfected with a

construct containing the cDNA of human apoD. In absence of any transfection or by transfection with the empty vector, no apoD was detected in the HepG2 cells. Transfection with the H-apoD cDNA showed a strong expression of the protein (Fig. A.6A). We next co-transfected the H-apoD cDNA construct with a construct containing five peroxisomes proliferator-activator receptor elements (PPRE) linked to a luciferase reporter gene. Thereafter, cells were incubated with 7 μM of AA in presence or absence of apoD. At this concentration, AA does not induce any cell toxicity (Notarnicola et al., 2011) despite a slight decrease in apoD expression (Fig. A.6A). In these conditions, over-expression of apoD increases PPARγ transcriptional activity (3.7 fold, Fig. A.6B). Addition of AA alone also increased PPARγ transcriptional activity to a similar level (3.9-fold). Very interestingly, a combination of AA and apoD over-expression has a strong synergistic transactivation of almost 9-fold on PPARγ transcriptional activity (Fig. A.6B).

Taken together, our study show that overexpressing H-apoD in mice leads to increased PPARγ expression and subsequent activation of those proteins involved in LD formation. This is also associated with increased fatty acid uptake while lipogenesis remained unaffected. Besides, the mild metabolic syndrome showed by these mice is probably due to a slight increase in mitochondrial β-oxydation as a compensatory mechanism. Experiences performed in HepG2 cells suggest that our observations in mice are probably the result of increase transport of AA by apoD in the liver resulting in PPARγ transcriptional activation and downstream effects.

A.5 Discussion

The goal of this study was to characterize the molecular mechanisms leading to TG accumulation in the liver of adult H-apoD Tg mice (one year old). We used a model of Tg mice overexpressing human apoD essentially in the CNS originally generated to study the role of apoD in several neurodenerative disorders (Do Carmo et al., 2008; Ganfornina et al., 2008). However, we observed that these mice develop hepatic steatosis and insulin resistance at one year of age (Do Carmo et al., 2009b). In these mice, apoD is mainly expressed in the CNS while in human, it is expressed in several organs although at different levels (Drayna et al., 1987; Seguin et al., 1995). As expected, analysis of the expression pattern of human apoD mRNA in Tg compared to WT mice, clearly showed a higher in the CNS, however, an expression in the liver is also detected (Do Carmo et al., 2009b). This expression is mild and may explain at least in part the phenotype of Tg mice where clear hepatic steatosis and insulin-resistance is only observed after one year of age. In addition, apoD is a circulating protein that can be uptake by the liver. Therefore, increased plasmatic apoD may lead to increase expression in the liver (Do Carmo et al., 2009b). Taken together, even if these mice were not designed to study the effect of apoD in liver, they display a mild increase of hepatic H-apoD expression that explains the observed phenotype.

In the present study, we showed that increased expression of H-apoD in the liver activates the nuclear receptor PPARγ, leading to hepatic fat accumulation. This is the result of an increase in FA uptake rather than an upregulation of lipogenesis. Our *in vitro* studies performed on HepG2 cells also strongly suggested that apoD acts as an AA transporter leading to the activation of PPARγ. AA is the preferential ligand of apoD (Morais Cabral et al., 1995) and a precursor for prostaglandins, which are also natural PPARγ activators (Forman et al., 1995; Kliewer et al., 1995). We showed that activation of PPARγ by AA in HepG2 cells is significantly potentiated by the increased in apoD concentration in cells. In agreement with our study, Thomas *et al.*

(Thomas et al., 2003b) demonstrated in cultured embryonic kidney (HEK) 293T cells that apoD stabilizes AA at the plasma membrane and inhibits the release of AA in the extracellular media.

Challenging our observations, Perdomo *et al.(Perdomo et al., 2010)* showed that mice injected with an adenovirus expressing apoD, the activation of LPL leads to a decrease in circulating TG-rich lipoproteins. The authors did not observe any accumulation of ectopic fat in the liver. The difference between this study and our results could be attributed to the fact that they used younger mice (six weeks instead of one year old animals) and mostly because the half-life of the adenovirus in mice is certainly too short to allow appearance of steatosis. Our results also showed that variation of hepatic PPARγ expression is observed as early as 3 months in H-apoD Tg mice, a stage where hepatic steatosis is not yet developed (*C. Mounier, unpublished data*). The authors also used a different genetic background that may influence the results. Finally, the use of adenoviruses to overexpress apoD using may lead to a different level of apoD expression in the liver.

In the liver of H-apoD Tg mice, we observed a strong increase in PPARγ expression associated with an activation of its transcriptional activity. Interestingly, PPARγ and c/EBPα both induce each other's expression maintaining a positive feedback loop for development of an adipocyte like phenotype (Darlington et al., 1998; Li et al., 2010; Rosen et al., 2002). In agreement with these data, we observed an increase in C/EBPα expression while C/EBPβ remained unchanged. Another consequence of elevated PPARγ expression is the increase of CD36 expression. However, the LPL and HSL levels remained constant at least at the mRNA level. Previous studies demonstrated that activation of PPARγ in the liver increases expression of LPL and long chain fatty acid (LCFA) transporter CD36 (Rogue et al., 2010; Rogue et al., 2011). LPL hydrolyzes TG-rich circulating lipoproteins and CD36 transports LCFA inside the

hepatocytes (Goldberg, 1996; Hirano et al., 2003; Love-Gregory et al., 2008). However, in H-apoD Tg mice, circulating FFA, TG and cholesterol levels remain similar to those in control mice (Do Carmo et al., 2009b). This may suggest that only the FA uptake is affected without any increase in lipoproteins hydrolysis. Similar observations associating increased CD36 expression and FA uptake were made in cardiac cells (Menard et al., 2010).

Another mechanism by which PPARγ may be implicated in hepatic lipid accumulation could be by the induction of LD formation and maturation (Chawla et al., 2001; Rogue et al., 2010; Rogue et al., 2011). In the present study, we demonstrated that Plin2 protein expression was increased in H-apoD Tg mice. Listenburger *et al (Listenberger et al., 2007)* showed that Plin2 lowers the rate of TG turnover in LD by reducing the association of ATGL with LD and therefore the hydrolysis of TG (Bickel et al., 2009). Our analysis also revealed an increased expression of Cide A and C, two proteins implicated in the fusion of LD (Gong et al., 2009; Nishino et al., 2008; Puri et al., 2008; Yonezawa et al., 2011). The fusion of LD reduces lipolysis explaining the 5-fold increase in LDs' size in the liver of H-apoD Tg mice. The formation and the maturation of LD leading to TG accumulation is probably mediated by PPARγ since mRNA expression of Cide B, which is not a target gene of PPARγ, was unaltered in our study.

Several studies showed that activation of PPARγ induces lipogenesis (Matsusue et al., 2003; Rahimian et al., 2001; Zhang et al., 2006). Since we previously showed that SREBP-1c and FAS mRNA expressions were increased in H-apoD Tg mice liver (Do Carmo et al., 2009b), we measured the mRNA levels of key lipogenic enzymes including LXRα, a transcription factor that induces lipogenic gene transcription (Chu et al., 2006; Glass and Rosenfeld, 2000; Joseph et al., 2002; Liang et al., 2002; Peng et al., 2008). We did not observe any change in the mRNA levels of ACC, SCD1,

DGAT and LXRα. However, we confirmed our previous published data (Do Carmo et al., 2009b) showing that, in the liver of H-apoD Tg mice, the increase of FAS mRNA level is reflected by an increase in FAS protein. We also observed an elevation of AMPK expression and activity leading to increased phosphorylation and inhibition of ACC (Ha et al., 1996). Interestingly, Mao *et al (Mao et al., 2006)* showed that inhibition of ACC1 in mouse liver induces expression of FAS and finally reduced TG accumulation. These data could explain why in our conditions, we observed an increase in FAS expression. However, by directly measuring *de novo* lipogenesis *in vivo* in mice using 3H2O, we showed the over-expression of H-apoD has no significant effect on the lipogenic pathway in 1-year-old animals. A similar observation was made in 3-month-old mice (*data not shown*)

We observed that AMPK is overexpressed and activated in the liver of H-apoD Tg mice. Since AMPK is activated by LCFA, it is likely that its activation is due to the increased FFA uptake. Since glycolysis is the most important pathway producing acetyl-CoA, the down-regulation of glucose formation through gluconeogenesis might be a compensatory mechanism. In fact, AMPK inhibits the transcription of the rate-limiting gluconeogenic enzymes G6Pase and PEPCK (Foretz et al., 2005). In agreement with these observations, we showed that G6Pase and PEPCK mRNA expression levels are slightly but significantly decreased in the liver of H-apoD Tg mice (*data not shown*). Inhibition of gluconeogenesis might protect the Tg mice against hyperglycemia thus reducing hyperinsulinemia that often appears in association with hepatic steatosis (Yki-Jarvinen, 2010).

PPARα is also activated by LCFA (Desvergne and Wahli, 1999; Mandard et al., 2004). We previously demonstrated that hepatic PPARα mRNA is increased in H-apoD Tg mice liver (Do Carmo et al., 2009b). PPARα is a nuclear receptor that induces transcription of several genes implicated in the mitochondrial β-oxidation of

lipids (Mandard et al., 2004). Its elevated expression is associated with an increased expression of CPT1, the rate limiting-enzyme of the mitochondrial β-oxidation (Kerner and Hoppel, 2000). Since CPT-1 is normally inhibited by malonyl-CoA that is produced by ACC (McGarry and Brown, 1997), inhibition of ACC by AMPK in the liver of H-apoD Tg mice is associated with an increased expression of CPT-1 strongly suggesting an activated β-oxidation. However, this increased expression is mild and does not appear to be sufficient to reverse the progression of hepatic steatosis in the H-apoD Tg mice. In agreement with this, the steatosis only appears after one year of age.

In conclusion, our study describes for the first time a role for apoD in the regulation of PPARγ and downstream activation of metabolic pathways leading to hepatic steatosis. In Tg mice, elevated apoD expression will lead to stabilization of AA to the plasma membrane and subsequent activation and increased expression of the nuclear receptor PPARγ. As a result, PPARγ target genes such as CD36, Plin2, Cide A and Cide C become overexpressed increasing LCFA uptake by the hepatocytes and protecting LD against lipolysis by blocking access to lipases. As a result, lipids accumulate in H-apoD Tg mice liver leading to hepatic steatosis. By a compensatory mechanism, LCFA transported into the hepatocytes activate AMPK and PPARα increasing CPT1 expression which activates mitochondrial β-oxidation. Activated AMPK also reduces hyperglycemia by repressing of G6Pase and PEPCK gene expression. However, the activation of these compensatory pathways is insufficient to fully inhibit the accumulation of ectopic fat in the liver, but it certainly helps to reduce the progression of hepatic steatosis explaining why H-apoD Tg mice develop hepatic steatosis and insulin resistance only after one year of age. Overall our study highlights a new role for apoD as an AA transporter regulating lipid accumulation in the liver.

A.6 Acknowledgements

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A.7 Figure Legends

Figure A.1 PPARγ and C/EBP expression in the liver of H-apoD Tg mice. Semi-quantitative RT-PCR (A) and Western blot (B) analysis of PPARγ expression in liver and skeletal muscle of WT and H-apoD Tg mice. A- Graphs represent the mRNA expression level normalized by HPRT. A representative gel is presented above. B- The graph represents the level of PPARγ protein expression standardized by amidoblack staining. Muscle tissue was used for PPARγ1/PPARγ2 positive control. Semi-quantitative RT-PCR analysis of C/EBPα (C) and C/EBPβ (D) mRNA expression. The graphs represent the level of mRNA expressions normalized by HPRT. Values are expressed relatively to the WT mice and are the means ± SD of 4 mice per group. *P<0.05 and **P<0.01 vs WT mice.

Figure A.2 Lipid droplets formation in the liver of H-apoD Tg mice.
(A) Western blot analysis of Plin2 expression. The graph represents the level of Plin2 protein expression standardized by amidoblack staining. A representative gel is presented. Semi-quantitative RT-PCR analysis of Cide A (B), Cide B (C) and Cide C (D) mRNA expression. The graphs represent the level of mRNA expressions normalized by HPRT. Values are expressed relatively to the WT mice and are the means ± SD of 4 mice per group.(E) Confocal analysis of lipid droplets in liver tissues of WT and H-apoD Tg mice. Lipid droplets are stained with bodipy (in green) and nucleus with propidium iodide (in red). Graphs represent the quantification of 18 images. *P<0.05, **P<0.01, P<0.001 vs WT mice.

Figure A.3 FFA uptake in the liver of H-apoD Tg mice. (A) Semi-quantitative RT-PCR analysis of CD36 expression in liver tissue from WT and H-apoD Tg mice. Graph represents the mRNA expression levels normalized by HPRT. Representative gels are presented. Values are expressed relatively to the WT mice and are the means ± SD of 4 mice per group. (B) 3H-oleate uptake was evaluated in primary hepatocytes prepared from WT and Tg mice. Results are expressed as CPM of 3H per mg of hepatic protein and represent the mean of 3 independent experiments. **P<0.01 *vs* WT mice.

Figure A.4 Lipogenesis in the liver of H-apoD Tg mice. Western blot analysis of total and phospho-AMPKα (A), total and phospho-ACC (B) and FAS (C) protein expression in the liver of WT and H-apoD Tg mice. The graphs represent the levels of protein expressions standardized by amidoblack staining. Representative gels are presented. (D) Semi-quantitative RT-PCR analysis of ACC, SCD1, DGAT and LXRα mRNA expression. The graph represents the level of mRNA normalized by HPRT. Representative gels are presented. (E) *In vivo* lipogenesis measured in 1 year old mice. The values represent the amount of $^{3}H2O$ incorporated into triglycerides. Values are expressed relatively to the WT mice and are the means ± SD of 4 mice per group. *P<0.05, **P<0.01 vs WT mice.

Figure A.5 Analysis of genes involved in β-oxydation in the liver of H-apoD Tg mice. (A) Western blot analysis of PPARα protein expression. The graph represents the level of PPARα protein expression standardized by amidoblack staining. A representative gel is presented. Semiquantitative RT-PCR analysis of PGC-1α (B) and CPT1 (C) expression in liver of WT and H-apoD Tg mice. PGC1α and CPT1 gene expression was normalized by HPRT. For each graph, the H-apoD Tg values were normalized by the WT values and are the means ± SD of 4 mice per group. *P<0.05 and **P<0.01 *vs* WT mice.

Figure A.6 Measure of PPARγ transcriptional activity in presence of AA and/or apoD.

(A) HepG2 cells were either non transfected (NT) or transfected with a myc-Tag apoD-cDNA or empty vector (EV) construct and incubated with BSA or arachidonic acid (AA). The level of H-apoD expression was evaluated by Western blot using a specific H-apoD antibody. (B) HepG2 cells were transfected with UAS-Luc, GAL4-PPARγ, β-galactosidase and with either an empty vector or a myc-Tag apoD-cDNA construct. After transfection, cells were treated with 7μM AA for 4h. Luciferase activity represents data normalized by β-galactosidase activity. The data represent the mean ± SD (n=3). *P<0.05 and **P<0.01 *vs* the non-stimulated control without apoD. The gel presented below showed the expression of apoD in transfected cells using a myc antibody.

A.8 Figures

Figure A.1

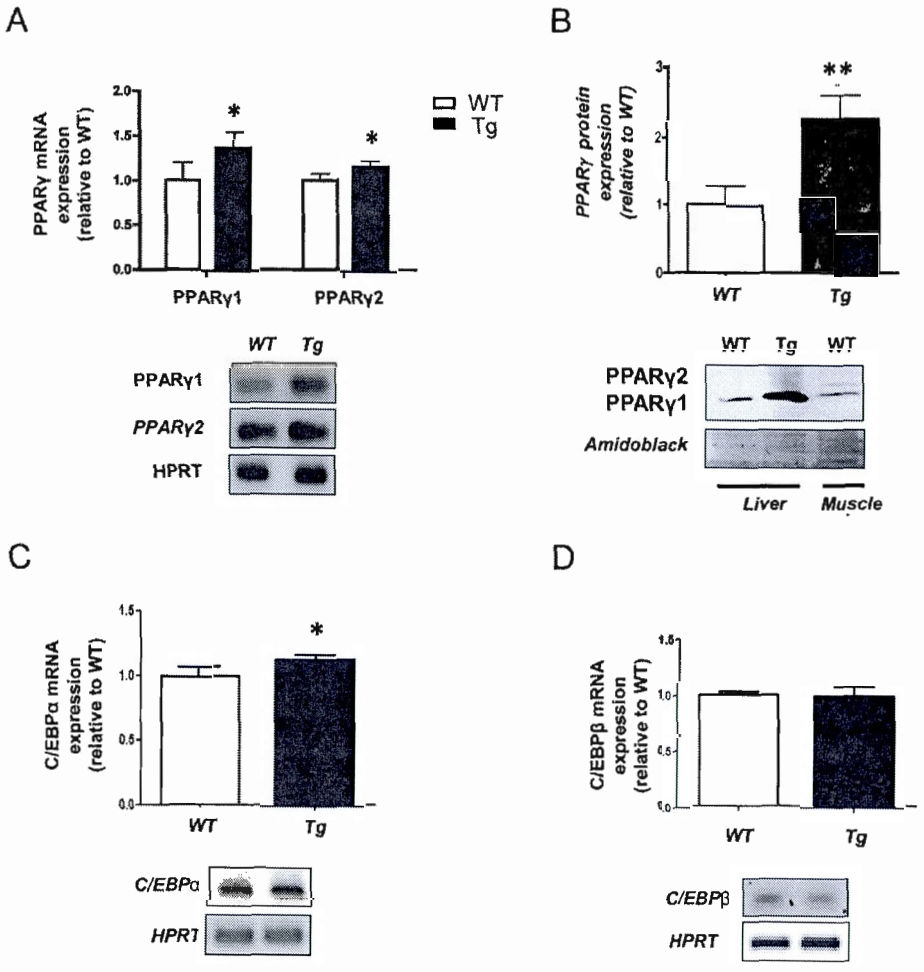


Figure A.2

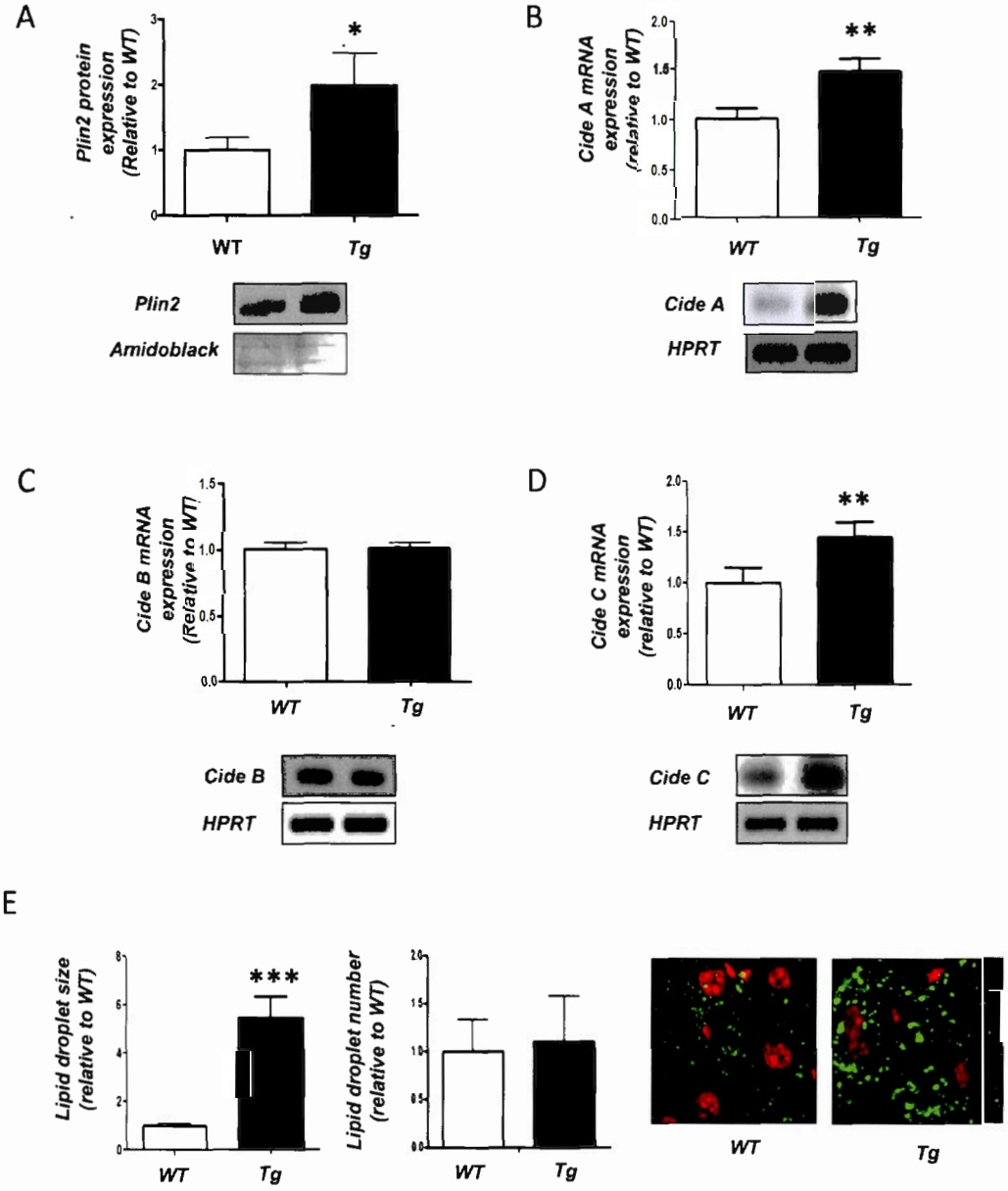

A
Plin2 protein expression (Relative to WT)
*
WT
Tg
Plin2
Amidoblack
B
Cide A mRNA expression (relative to WT)
**
WT
Tg
Cide A
HPRT
C
Cide B mRNA expression (Relative to WT)
WT
Tg
Cide B
HPRT
D
Cide C mRNA expression (relative to WT)
**
WT
Tg
Cide C
HPRT
E
Lipid droplet size (relative to WT)
***
WT
Tg
Lipid droplet number (relative to WT)
WT
Tg
WT
Tg

Figure A.3

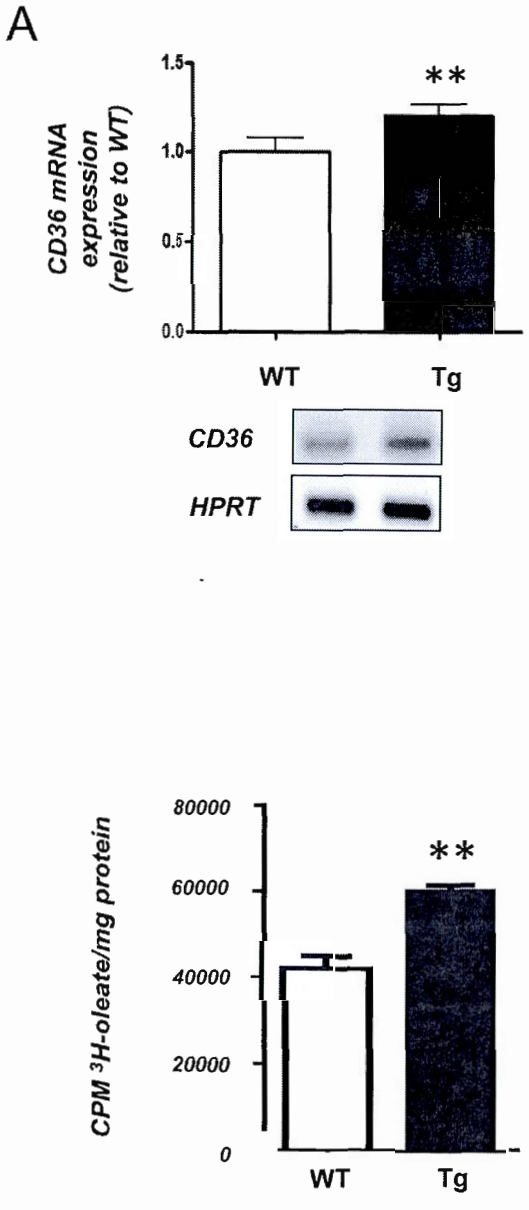
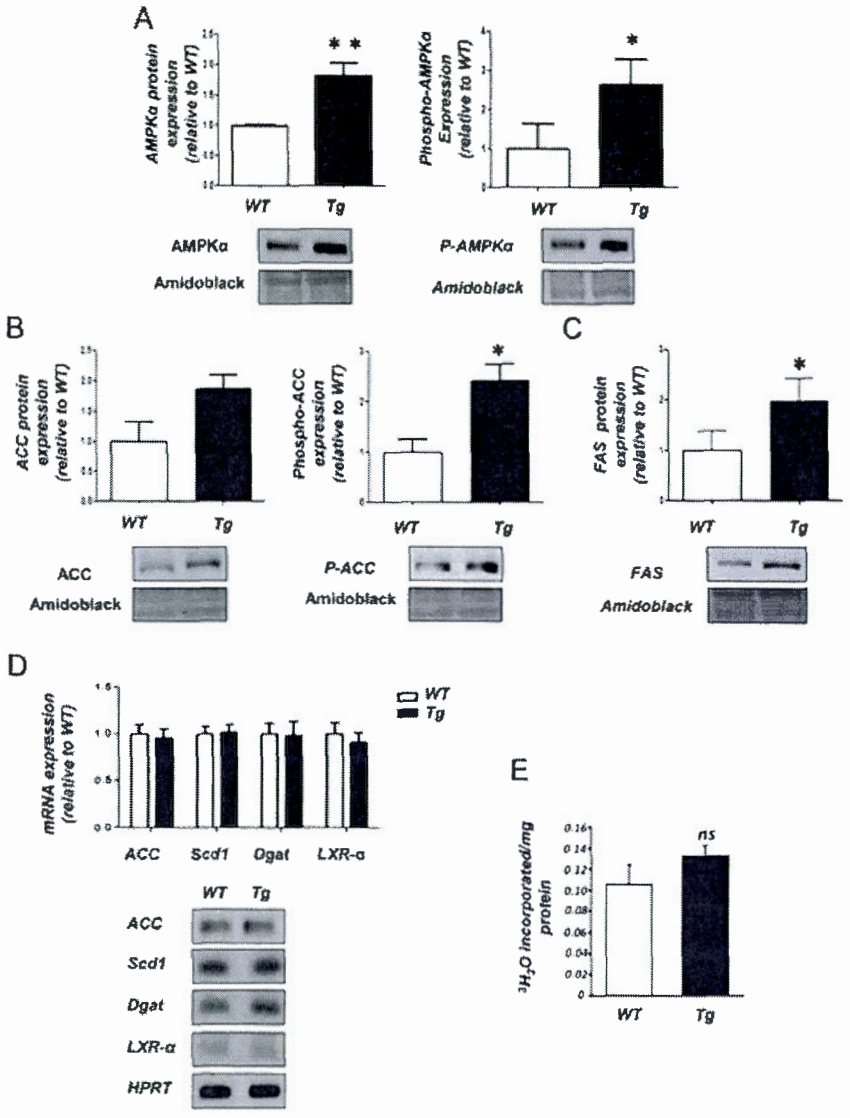

A
CD36 mRNA expression (relative to WT)
1.5
1.0
0.5
0.0
**
WT
Tg
CD36
HPRT
B
CPM 3H-oleate/mg protein
80000
60000
40000
20000
0
**
WT
Tg

Figure A.4


A
AMPKα protein expression (relative to WT)
Phospho-AMPKα Expression (relative to WT)
WT
Tg
AMPKα
Amidoblack
P-AMPKα
Amidoblack
B
ACC protein expression (relative to WT)
Phospho-ACC expression (relative to WT)
ACC
Amidoblack
P-ACC
Amidoblack
C
FAS protein expression (relative to WT)
FAS
Amidoblack
D
mRNA expression (relative to WT)
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Scd1
Dgat
LXR-α
HPRT
E
ns

Figure A.5

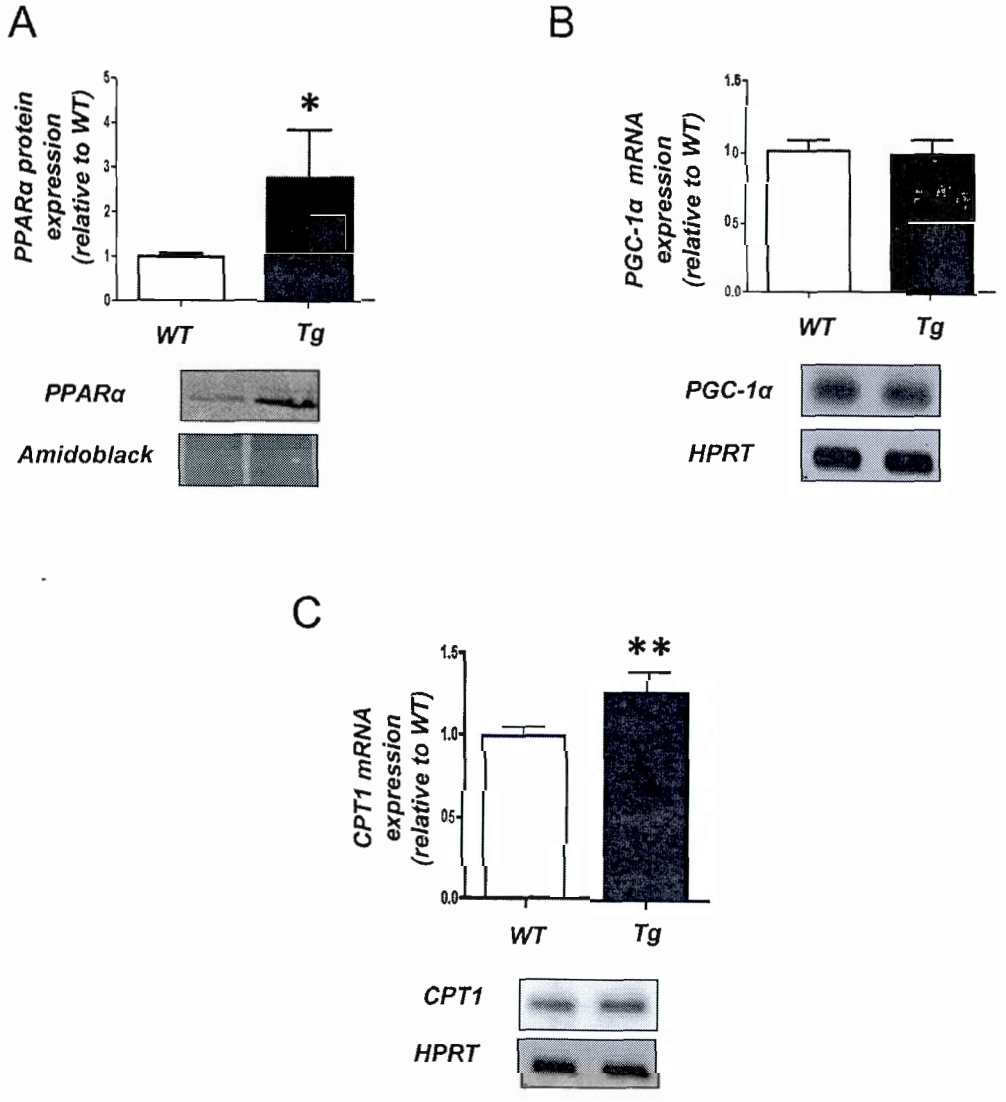
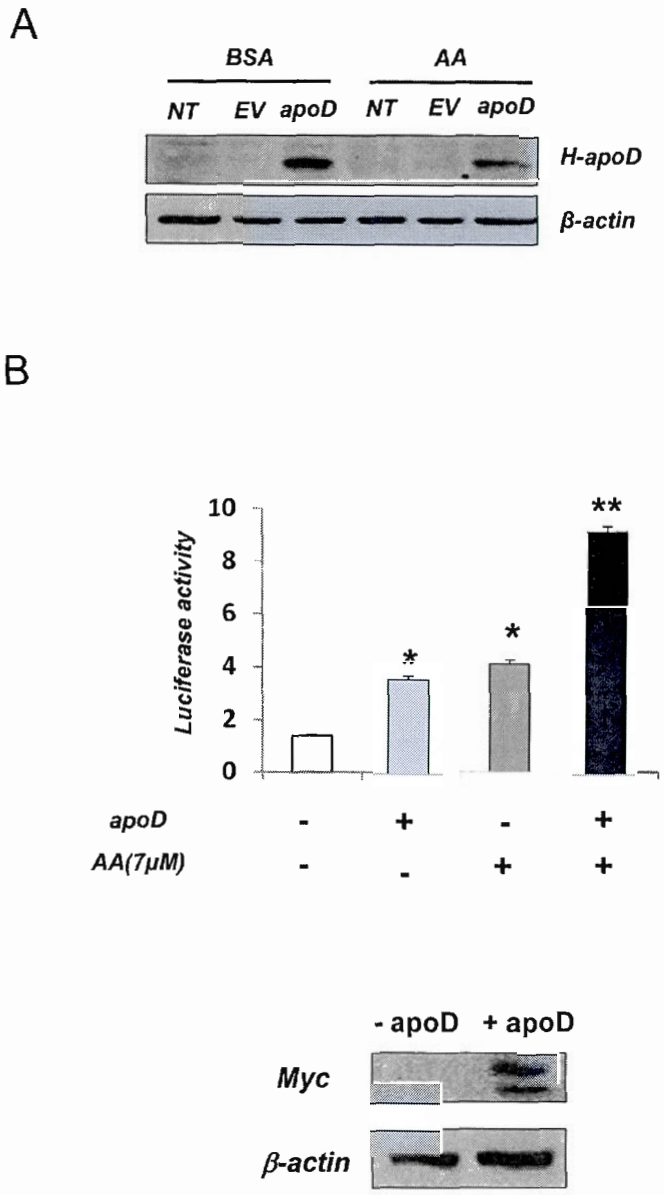

A
PPARα protein expression (relative to WT)
*
WT
Tg
PPARα
Amidoblack
B
PGC-1α mRNA expression (relative to WT)
WT
Tg
PGC-1α
HPRT
C
CPT1 mRNA expression (relative to WT)
**
WT
Tg
CPT1
HPRT

Figure A.6


A
BSA
AA
NT
EV
apoD
NT
EV
apoD
H-apoD
β-actin
B
Luciferase activity
10
8
6
4
2
0
*
*
**
apoD
-
+
-
+
AA(7μM)
-
-
+
+
- apoD
+ apoD
Myc
β-actin

APPENDICE B

APOLIPOPROTEIN D-NULL FEMALE MICE SHOW A LOW BONE MASS PHENOTYPE

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À soumettre

Abstract

Apolipoprotein D (ApoD) is a member of the lipocalin family which is known to transport small hydrophobic ligands. A major site of ApoD expression in mice is the central nervous system and there is accumulating evidence suggesting that ApoD plays a protective role in the nervous tissue. Recently, gene expression of *ApoD* was reported in bone-forming osteoblasts but its role in bone metabolism is yet to be documented. We investigated the regulation of *ApoD* gene expression in osteoblasts and documented the bone phenotype of *ApoD*-/- (null) mice. Gene expression of *ApoD* was evidenced in the murine osteoblast cell line MC3T3-E1 and its expression was enhanced under condition of low cell proliferation following serum deprivation and contact inhibition. Moreover, culture condition promoting osteogenic differentiation enhanced *ApoD* gene expression and associated with increased cellular and secreted levels of ApoD protein. Gene expression of *ApoD* was confirmed in primary cultures of bone marrow mesenchymal cells, with higher expression level in female mice over males. *ApoD*-null female mice showed a low bone mass phenotype which was accentuated with aging, resulting in 18 to 50% reduction of trabecular bone volume and 13 to 23% reduction of cortical bone volume between 3 to 9 months of age. In contrast, trabecular bone volume of *ApoD*-null male mice was not different compared to wild-type mice whereas cortical bone volume was reduced by an average of 24%. Low bone mass phenotype in *ApoD*-null female mice was associated with reduced trabecular thickness and number of trabeculae. Analysis of trabecular bone sections from *ApoD*-null female mice indicated lower osteoblast number whereas osteoclast number was not affected. Our results indicate that ApoD contribute to the regulation of bone metabolism in mice.

Key words: apolipoprotein D; osteoblast; low bone mass

RÉFÉRENCES

Agrawal, S.M., et al., 2011. EMMPRIN: a novel regulator of leukocyte transmigration into the CNS in multiple sclerosis and experimental autoimmune encephalomyelitis. J Neurosci. 31, 669-77.

Alaupovic, P., et al., 1981. Plasma apolipoprotein concentrations in familial apolipoprotein A-I and A-II deficiency (Tangier disease). Metabolism. 30, 805-9.

Albers, J.J., et al., 1985. Defective enzyme causes lecithin-cholesterol acyltransferase deficiency in a Japanese kindred. Biochim Biophys Acta. 835, 253-7.

Albin, R.L., Greenamyre, J.T., 1992. Alternative excitotoxic hypotheses. Neurology. 42, 733-8.

Allain, F., et al., 2002. Interaction with glycosaminoglycans is required for cyclophilin B to trigger integrin-mediated adhesion of peripheral blood T lymphocytes to extracellular matrix. Proc Natl Acad Sci U S A. 99, 2714-9.

Ameer, F., et al., 2014. De novo lipogenesis in health and disease. Metabolism.

Arora, K., et al., 2005. Extracellular cyclophilins contribute to the regulation of inflammatory responses. J Immunol. 175, 517-22.

Ashida, S., et al., 2004. Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs. Cancer Res. 64, 5963-72.

Aspinall, J.O., et al., 1995. Differential expression of apolipoprotein-D and prostate specific antigen in benign and malignant prostate tissues. J Urol. 154, 622-8.

Bajo-Graneras, R., et al., 2011. Apolipoprotein D mediates autocrine protection of astrocytes and controls their reactivity level, contributing to the functional maintenance of paraquat-challenged dopaminergic systems. Glia. 59, 1551-66.

Bajo-Graneras, R., et al., 2013. Expression and potential role of apolipoprotein D on the death-survival balance of human colorectal cancer cells under oxidative stress conditions. Int J Colorectal Dis. 28, 751-66.

Bajzer, Z., Myers, A.C., Vuk-Pavlovic, S., 1989. Binding, internalization, and intracellular processing of proteins interacting with recycling receptors. A kinetic analysis. J Biol Chem. 264, 13623-31.

Baker, W.A., et al., 1994. Apolipoprotein D gene polymorphism: a new genetic marker for type 2 diabetic subjects in Nauru and south India. Diabet Med. 11, 947-52.

Balazs, Z., et al., 2004. Uptake and transport of high-density lipoprotein (HDL) and HDL-associated alpha-tocopherol by an in vitro blood-brain barrier model. J Neurochem. 89, 939-50.

Balbin, M., et al., 1990. Apolipoprotein D is the major protein component in cyst fluid from women with human breast gross cystic disease. Biochem J. 271, 803-7.

Ballabh, P., Braun, A., Nedergaard, M., 2004. The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis. 16, 1-13.

Barreto-Chang, O.L., Dolmetsch, R.E., 2009. Calcium imaging of cortical neurons using Fura-2 AM. J Vis Exp.

Beesley, A.H., Weller, R.E., Kees, U.R., 2008. The role of BSG (CD147) in acute lymphoblastic leukaemia and relapse. Br J Haematol. 142, 1000-2.

Belloir, B., et al., 2001. Altered apolipoprotein D expression in the brain of patients with Alzheimer disease. J Neurosci Res. 64, 61-9.

Ben-Ari, Y., et al., 1981. Electrographic, clinical and pathological alterations following systemic administration of kainic acid, bicuculline or pentetrazole: metabolic mapping using the deoxyglucose method with special reference to the pathology of epilepsy. Neuroscience. 6, 1361-91.

Ben-Ari, Y., 1985. Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. Neuroscience. 14, 375-403.

Ben-Ari, Y., Cossart, R., 2000. Kainate, a double agent that generates seizures: two decades of progress. Trends Neurosci. 23, 580-7.

Bhatia, S., et al., 2012. Selective reduction of hydroperoxyeicosatetraenoic acids to their hydroxy derivatives by apolipoprotein D: implications for lipid antioxidant activity and Alzheimer's disease. Biochem J. 442, 713-21.

Bickel, P.E., Tansey, J.T., Welte, M.A., 2009. PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. Biochim Biophys Acta. 1791, 419-40.

Bignami, A., Ralston, H.J., 3rd, 1969. The cellular reaction to Wallerian degeneration in the central nervous system of the cat. Brain Res. 13, 444-61.

Bishop, R.E., et al., 1995. Stationary phase expression of a novel Escherichia coli outer membrane lipoprotein and its relationship with mammalian apolipoprotein D. Implications for the origin of lipocalins. J Biol Chem. 270, 23097-103.

Biswas, C., et al., 1995. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. Cancer Res. 55, 434-9.

Bjorkhem, I., et al., 1997. Importance of a novel oxidative mechanism for elimination of brain cholesterol. Turnover of cholesterol and 24(S)-hydroxycholesterol in rat brain as measured with 18O2 techniques in vivo and in vitro. J Biol Chem. 272, 30178-84.

Bjorkhem, I., Meaney, S., 2004. Brain cholesterol: long secret life behind a barrier. Arterioscler Thromb Vasc Biol. 24, 806-15.

Blais, Y., et al., 1994. Potent stimulatory effect of interleukin-1 alpha on apolipoprotein D and gross cystic disease fluid protein-15 expression in human breast-cancer cells. Int J Cancer. 59, 400-7.

Blais, Y., et al., 1995. Interleukin-6 inhibits the potent stimulatory action of androgens, glucocorticoids and interleukin-1 alpha on apolipoprotein D and GCDFP-15 expression in human breast cancer cells. Int J Cancer. 62, 732-7.

Blanco-Vaca, F., et al., 1992. Characterization of disulfide-linked heterodimers containing apolipoprotein D in human plasma lipoproteins. J Lipid Res. 33, 1785-96.

Bodzioch, M., et al., 1999. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat Genet. 22, 347-51.

Boer, S., et al., 2010. Decreased kainate receptors in the hippocampus of apolipoprotein D knockout mice. Prog Neuropsychopharmacol Biol Psychiatry. 34, 271-8.

Boulos, S., et al., 2007. Evidence that intracellular cyclophilin A and cyclophilin A/CD147 receptor-mediated ERK1/2 signalling can protect neurons against in vitro oxidative and ischemic injury. Neurobiol Dis. 25, 54-64.

Boyles, J.K., et al., 1989. A role for apolipoprotein E, apolipoprotein A-I, and low density lipoprotein receptors in cholesterol transport during regeneration and remyelination of the rat sciatic nerve. J Clin Invest. 83, 1015-31.

Boyles, J.K., Notterpek, L.M., Anderson, L.J., 1990. Accumulation of apolipoproteins in the regenerating and remyelinating mammalian peripheral nerve. Identification of apolipoprotein D, apolipoprotein A-IV, apolipoprotein E, and apolipoprotein A-I. J Biol Chem. 265, 17805-15.

Boyles, J.K., et al., 1990b. Identification, characterization, and tissue distribution of apolipoprotein D in the rat. J Lipid Res. 31, 2243-56.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72, 248-54.

Breustedt, D.A., Schonfeld, D.L., Skerra, A., 2006. Comparative ligand-binding analysis of ten human lipocalins. Biochim Biophys Acta. 1764, 161-73.

Brewer, G.J., et al., 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res. 35, 567-76.

Brodeur, M.R., et al., 2008. Scavenger receptor of class B expressed by osteoblastic cells are implicated in the uptake of cholesteryl ester and estradiol from LDL and HDL3. J Bone Miner Res. 23, 326-37.

Byun, J.S., et al., 2010. Neuroprotective effects of stanniocalcin 2 following kainic acid-induced hippocampal degeneration in ICR mice. Peptides. 31, 2094-9.

Camato, R., et al., 1989. Protein polymorphism of a human plasma apolipoprotein D antigenic epitope. J Lipid Res. 30, 865-75.

Cameron, H.A., McKay, R.D., 2001. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol. 435, 406-17.

Campisi, J., 2005. Suppressing cancer: the importance of being senescent. Science. 309, 886-7.

Caudroy, S., et al., 2002. EMMPRIN-mediated MMP regulation in tumor and endothelial cells. Clin Exp Metastasis. 19, 697-702.

Charron, J.B., et al., 2008. The plant Apolipoprotein D ortholog protects Arabidopsis against oxidative stress. BMC Plant Biol. 8, 86.

Chawla, A., et al., 2001. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol Cell. 7, 161-71.

Chen, H., et al., 2010. Co-expression of CD147/EMMPRIN with monocarboxylate transporters and multiple drug resistance proteins is associated with epithelial ovarian cancer progression. Clin Exp Metastasis. 27, 557-69.

Chen, J., et al., 2013. Cholesterol efflux is differentially regulated in neurons and astrocytes: implications for brain cholesterol homeostasis. Biochim Biophys Acta. 1831, 263-75.

Chen, K.S., et al., 1998. Neurodegenerative Alzheimer-like pathology in PDAPP 717V-->F transgenic mice. Prog Brain Res. 117, 327-34.

Chen, Z., et al., 2002. Excitotoxic neurodegeneration induced by intranasal administration of kainic acid in C57BL/6 mice. Brain Res. 931, 135-45.

Chen, Z., et al., 2005. Increased microglial activation and astrogliosis after intranasal administration of kainic acid in C57BL/6 mice. J Neurobiol. 62, 207-18.

Chiarello, D.I., et al., 2014. Effect of hypoxia on the calcium and magnesium content, lipid peroxidation level, and Ca(2)(+)-ATPase activity of syncytiotrophoblast plasma membranes from placental explants. Biomed Res Int. 2014, 597357.

Choi, E.Y., et al., 2002. Upregulation of extracellular matrix metalloproteinase inducer (EMMPRIN) and gelatinases in human atherosclerosis infected with Chlamydia pneumoniae: the potential role of Chlamydia pneumoniae infection in the progression of atherosclerosis. Exp Mol Med. 34, 391-400.

Chu, K., et al., 2006. Stearoyl-coenzyme A desaturase 1 deficiency protects against hypertriglyceridemia and increases plasma high-density lipoprotein cholesterol induced by liver X receptor activation. Mol Cell Biol. 26, 6786-98.

Ciapparelli, A., et al., 2000. Clozapine for treatment-refractory schizophrenia, schizoaffective disorder, and psychotic bipolar disorder: a 24-month naturalistic study. J Clin Psychiatry. 61, 329-34.

Cofer, S., Ross, S.R., 1996. The murine gene encoding apolipoprotein D exhibits a unique expression pattern as compared to other species. Gene. 171, 261-3.

Coxey, R.A., et al., 1993. Differential accumulation of cholesterol in Golgi compartments of normal and Niemann-Pick type C fibroblasts incubated with LDL: a cytochemical freeze-fracture study. J Lipid Res. 34, 1165-76.

Dai, J.Y., et al., 2009. The interaction of HAb18G/CD147 with integrin alpha6beta1 and its implications for the invasion potential of human hepatoma cells. BMC Cancer. 9, 337.

Damsker, J.M., et al., 2009. Targeting the chemotactic function of CD147 reduces collagen-induced arthritis. Immunology. 126, 55-62.

Darlington, G.J., Ross, S.E., MacDougald, O.A., 1998. The role of C/EBP genes in adipocyte differentiation. J Biol Chem. 273, 30057-60.

Darstein, M., et al., 2003. Distribution of kainate receptor subunits at hippocampal mossy fiber synapses. J Neurosci. 23, 8013-9.

de Lange, E.C., 2012. The physiological characteristics and transcytosis mechanisms of the blood-brain barrier (BBB). Curr Pharm Biotechnol. 13, 2319-27.

de Wet, J.R., et al., 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol Cell Biol. 7, 725-37.

del Valle, E., et al., 2003. Apolipoprotein D expression in human brain reactive astrocytes. J Histochem Cytochem. 51, 1285-90.

Desai, P.P., et al., 2005. Apolipoprotein D is a component of compact but not diffuse amyloid-beta plaques in Alzheimer's disease temporal cortex. Neurobiol Dis. 20, 574-82.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev. 20, 649-88.

Dietschy, J.M., Turley, S.D., 2001. Cholesterol metabolism in the brain. Curr Opin Lipidol. 12, 105-12.

Dietschy, J.M., Turley, S.D., 2004. Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. J Lipid Res. 45, 1375-97.

Diez-Itza, I., et al., 1994. Expression and prognostic significance of apolipoprotein D in breast cancer. Am J Pathol. 144, 310-20.

Dilley, W.G., et al., 1990. Immunologic and steroid binding properties of the GCDFP-24 protein isolated from human breast gross cystic disease fluid. Breast Cancer Res Treat. 16, 253-60.

Do Carmo, S., et al., 2002. Modulation of apolipoprotein D and apolipoprotein E mRNA expression by growth arrest and identification of key elements in the promoter. J Biol Chem. 277, 5514-23.

Do Carmo, S., Levros, L.C., Jr., Rassart, E., 2007. Modulation of apolipoprotein D expression and translocation under specific stress conditions. Biochim Biophys Acta. 1773, 954-69.

Do Carmo, S., et al., 2008. Neuroprotective effect of apolipoprotein D against human coronavirus OC43-induced encephalitis in mice. J Neurosci. 28, 10330-8.

Do Carmo, S., et al., 2009a. Modulation of Apolipoprotein D levels in human pregnancy and association with gestational weight gain. Reprod Biol Endocrinol. 7, 92.

Do Carmo, S., et al., 2009b. Human apolipoprotein D overexpression in transgenic mice induces insulin resistance and alters lipid metabolism. Am J Physiol Endocrinol Metab. 296, E802-11.

Dong, X.X., Wang, Y., Qin, Z.H., 2009. Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. Acta Pharmacol Sin. 30, 379-87.

Drayna, D., et al., 1986. Cloning and expression of human apolipoprotein D cDNA. J Biol Chem. 261, 16535-9.

Drayna, D.T., et al., 1987. Human apolipoprotein D gene: gene sequence, chromosome localization, and homology to the alpha 2u-globulin superfamily. DNA. 6, 199-204.

Duan, Y., Gross, R.A., Sheu, S.S., 2007. Ca2+-dependent generation of mitochondrial reactive oxygen species serves as a signal for poly(ADP-ribose) polymerase-1 activation during glutamate excitotoxicity. J Physiol. 585, 741-58.

Eichinger, A., et al., 2007. Structural insight into the dual ligand specificity and mode of high density lipoprotein association of apolipoprotein D. J Biol Chem. 282, 31068-75.

Elliott, D.A., Weickert, C.S., Garner, B., 2010. Apolipoproteins in the brain: implications for neurological and psychiatric disorders. Clin Lipidol. 51, 555-573.

Eng, L.F., Ghirnikar, R.S., 1994. GFAP and astrogliosis. Brain Pathol. 4, 229-37.

Eyster, C.A., et al., 2009. Discovery of new cargo proteins that enter cells through clathrin-independent endocytosis. Traffic. 10, 590-9.

Fan, Q.W., et al., 1998. Expression of basigin, a member of the immunoglobulin superfamily, in the mouse central nervous system. Neurosci Res. 30, 53-63.

Feng, J., et al., 2000. Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-gamma. J Lipid Res. 41, 688-96.

Fielding, P.E., Fielding, C.J., 1980. A cholesteryl ester transfer complex in human plasma. Proc Natl Acad Sci U S A. 77, 3327-30.

Flower, D.R., 1996. The lipocalin protein family: structure and function. Biochem J. 318 (Pt 1), 1-14.

Flower, D.R., North, A.C., Sansom, C.E., 2000. The lipocalin protein family: structural and sequence overview. Biochim Biophys Acta. 1482, 9-24.

Foretz, M., et al., 2005. Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. Diabetes. 54, 1331-9.

Forman, B.M., et al., 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell. 83, 803-12.

Franz, G., et al., 1999. Increased expression of apolipoprotein D following experimental traumatic brain injury. J Neurochem. 73, 1615-25.

Frenette Charron, J.B., et al., 2002. Molecular and structural analyses of a novel temperature stress-induced lipocalin from wheat and Arabidopsis. FEBS Lett. 517, 129-32.

Fritz, G., et al., 2003. APE/Ref-1 and the mammalian response to genotoxic stress. Toxicology. 193, 67-78.

Fukamachi, K., et al., 2002. Neuronal leucine-rich repeat protein-3 amplifies MAPK activation by epidermal growth factor through a carboxyl-terminal region containing endocytosis motifs. J Biol Chem. 277, 43549-52.

Games, D., et al., 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F beta-amyloid precursor protein. Nature. 373, 523-7.

Ganfornina, M.D., et al., 2005. Molecular characterization and developmental expression pattern of the chicken apolipoprotein D gene: implications for the evolution of vertebrate lipocalins. Dev Dyn. 232, 191-9.

Ganfornina, M.D., et al., 2008. Apolipoprotein D is involved in the mechanisms regulating protection from oxidative stress. Aging Cell. 7, 506-15.

Ganfornina, M.D., et al., 2010. ApoD, a glia-derived apolipoprotein, is required for peripheral nerve functional integrity and a timely response to injury. Glia. 58, 1320-34.

Gavrilova, O., et al., 2003. Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. J Biol Chem. 278, 34268-76.

George, R., Griffin, J.W., 1994. Delayed macrophage responses and myelin clearance during Wallerian degeneration in the central nervous system: the dorsal radiculotomy model. Exp Neurol. 129, 225-36.

Gioiosa, L., et al., 2008. Altered emotionality, spatial memory and cholinergic function in caveolin-1 knock-out mice. Behav Brain Res. 188, 255-62.

Glass, C.K., Rosenfeld, M.G., 2000. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. 14, 121-41.

Glockner, F., Ohm, T.G., 2003. Hippocampal apolipoprotein D level depends on Braak stage and APOE genotype. Neuroscience. 122, 103-10.

Goessling, W., Zucker, S.D., 2000. Role of apolipoprotein D in the transport of bilirubin in plasma. Am J Physiol Gastrointest Liver Physiol. 279, G356-65.

Goldberg, I.J., 1996. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. J Lipid Res. 37, 693-707.

Gong, J., Sun, Z., Li, P., 2009. CIDE proteins and metabolic disorders. Curr Opin Lipidol. 20, 121-6.

Guo, H., et al., 1997. Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. J Biol Chem. 272, 24-7.

Ha, J., et al., 1996. Cloning of human acetyl-CoA carboxylase-beta and its unique features. Proc Natl Acad Sci U S A. 93, 11466-70.

Halim, N.D., et al., 2004. Effects of chronic haloperidol and clozapine treatment on neurogenesis in the adult rat hippocampus. Neuropsychopharmacology. 29, 1063-9.

Hall, R.E., et al., 2004. Apolipoprotein-D: a novel cellular marker for HGPIN and prostate cancer. Prostate. 58, 103-8.

Hansen, L., et al., 2004. Expression profiling of insulin action in human myotubes: induction of inflammatory and pro-angiogenic pathways in relationship with glycogen synthesis and type 2 diabetes. Biochem Biophys Res Commun. 323, 685-95.

Hardingham, G.E., Fukunaga, Y., Bading, H., 2002. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci. 5, 405-14.

He, X., et al., 2006. Lovastatin modulates increased cholesterol and oxysterol levels and has a neuroprotective effect on rat hippocampal neurons after kainate injury. J Neuropathol Exp Neurol. 65, 652-63.

He, X., et al., 2009. Apolipoprotein D modulates F2-isoprostane and 7-ketocholesterol formation and has a neuroprotective effect on organotypic hippocampal cultures after kainate-induced excitotoxic injury. Neurosci Lett. 455, 183-6.

He, Z., Koprivica, V., 2004. The Nogo signaling pathway for regeneration block. Annu Rev Neurosci. 27, 341-68.

Hebbachi, A.M., et al., 2008. Peroxisome proliferator-activated receptor alpha deficiency abolishes the response of lipogenic gene expression to re-feeding: restoration of the normal response by activation of liver X receptor alpha. J Biol Chem. 283, 4866-76.

Henderson, V.W., Finch, C.E., 1989. The neurobiology of Alzheimer's disease. J Neurosurg. 70, 335-53.

Hino, M., et al., 2003. Caveolin-1 as tumor suppressor gene in breast cancer. Surg Today. 33, 486-90.

Hirano, K., et al., 2003. Pathophysiology of human genetic CD36 deficiency. Trends Cardiovasc Med. 13, 136-41.

Hopkins, C.R., Trowbridge, I.S., 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. J Cell Biol. 97, 508-21.

Hori, K., et al., 2000. Retinal dysfunction in basigin deficiency. Invest Ophthalmol Vis Sci. 41, 3128-33.

Horrobin, D.F., 1998. The membrane phospholipid hypothesis as a biochemical basis for the neurodevelopmental concept of schizophrenia. Schizophr Res. 30, 193-208.

Hu, C.Y., et al., 2001. Immunocytochemical localization of apolipoprotein D in oligodendrocyte precursor-like cells, perivascular cells, and pericytes in the human cerebral cortex. J Neurocytol. 30, 209-18.

Hu, M., et al., 2008. Negative regulation of neurogenesis and spatial memory by NR2B-containing NMDA receptors. J Neurochem. 106, 1900-13.

Huang, W., et al., 2013. Modulation of CD147-induced matrix metalloproteinase activity: role of CD147 N-glycosylation. Biochem J. 449, 437-48.

Hull-Thompson, J., et al., 2009. Control of metabolic homeostasis by stress signaling is mediated by the lipocalin NLaz. PLoS Genet. 5, e1000460.

Hulsebosch, C.E., 2002. Recent advances in pathophysiology and treatment of spinal cord injury. Adv Physiol Educ. 26, 238-55.

Hummasti, S., et al., 2004. Liver X receptors are regulators of adipocyte gene expression but not differentiation: identification of apoD as a direct target. J Lipid Res. 45, 616-25.

Hunter, S., et al., 2002. Differential expression between pilocytic and anaplastic astrocytomas: identification of apolipoprotein D as a marker for low-grade, non-infiltrating primary CNS neoplasms. J Neuropathol Exp Neurol. 61, 275-81.

Iacobuzio-Donahue, C.A., et al., 2002. Exploring the host desmoplastic response to pancreatic carcinoma: gene expression of stromal and neoplastic cells at the site of primary invasion. Am J Pathol. 160, 91-9.

Iacono, K.T., et al., 2007. CD147 immunoglobulin superfamily receptor function and role in pathology. Exp Mol Pathol. 83, 283-95.

Igakura, T., et al., 1998. A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. Dev Biol. 194, 152-65.

Jacomy, H., Talbot, P.J., 2003. Vacuolating encephalitis in mice infected by human coronavirus OC43. Virology. 315, 20-33.

Jankowsky, J.L., Patterson, P.H., 2001. The role of cytokines and growth factors in seizures and their sequelae. Prog Neurobiol. 63, 125-49.

Jansen, P.J., et al., 2009. Absence of ApoE upregulates murine brain ApoD and ABCA1 levels, but does not affect brain sterol levels, while human ApoE3 and human ApoE4 upregulate brain cholesterol precursor levels. J Alzheimers Dis. 18, 319-29.

Jia, L., et al., 2006. Deglycosylation of CD147 down-regulates Matrix Metalloproteinase-11 expression and the adhesive capability of murine hepatocarcinoma cell HcaF in vitro. IUBMB Life. 58, 209-16.

Jinno, Y., et al., 2010. Cide-a and Cide-c are induced in the progression of hepatic steatosis and inhibited by eicosapentaenoic acid. Prostaglandins Leukot Essent Fatty Acids. 83, 75-81.

Jones, H.M., Pilowsky, L.S., 2002. Dopamine and antipsychotic drug action revisited. Br J Psychiatry. 181, 271-5.

Joseph, S.B., et al., 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. J Biol Chem. 277, 11019-25.

Kalman, J., et al., 2000. Apolipoprotein D in the aging brain and in Alzheimer's dementia. Neurol Res. 22, 330-6.

Kao, L.C., et al., 2002. Global gene profiling in human endometrium during the window of implantation. Endocrinology. 143, 2119-38.

Kaplan, M.S., Hinds, J.W., 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 197, 1092-4.

Kawaguchi, R., et al., 2007. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science. 315, 820-5.

Keller, P., et al., 2008. Fat-specific protein 27 regulates storage of triacylglycerol. J Biol Chem. 283, 14355-65.

Kendrick, S.J., Lynch, D.R., Pritchett, D.B., 1996. Characterization of glutamate binding sites in receptors assembled from transfected NMDA receptor subunits. J Neurochem. 67, 608-16.

Kerner, J., Hoppel, C., 2000. Fatty acid import into mitochondria. Biochim Biophys Acta. 1486, 1-17.

Kim, E.J., et al., 2001. Differential roles of cyclooxygenase isoforms after kainic acid-induced prostaglandin E(2) production and neurodegeneration in cortical and hippocampal cell cultures. Brain Res. 908, 1-9.

Kim, W.S., et al., 2009. Apolipoprotein-D expression is increased during development and maturation of the human prefrontal cortex. J Neurochem. 109, 1053-66.

Kliewer, S.A., et al., 1995. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell. 83, 813-9.

Kliewer, S.A., et al., 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A. 94, 4318-23.

Koch, S., et al., 2001. Characterization of four lipoprotein classes in human cerebrospinal fluid. J Lipid Res. 42, 1143-51.

Kolodny, E.H., 2000. Niemann-Pick disease. Curr Opin Hematol. 7, 48-52.

Konradi, C., Heckers, S., 2003. Molecular aspects of glutamate dysregulation: implications for schizophrenia and its treatment. Pharmacol Ther. 97, 153-79.

Kosugi, T., et al., 2014. CD147 (EMMPRIN/Basigin) in kidney diseases: from an inflammation and immune system viewpoint. Nephrol Dial Transplant.

Krey, G., et al., 1997. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol. 11, 779-91.

Kuehl, F.A., Jr., Egan, R.W., 1980. Prostaglandins, arachidonic acid, and inflammation. Science. 210, 978-84.

Kuhn, P.L., Wrathall, J.R., 1998. A mouse model of graded contusive spinal cord injury. J Neurotrauma. 15, 125-40.

Kuno, N., et al., 1998. Female sterility in mice lacking the basigin gene, which encodes a transmembrane glycoprotein belonging to the immunoglobulin superfamily. FEBS Lett. 425, 191-4.

Kunz, T., Oliw, E.H., 2001. The selective cyclooxygenase-2 inhibitor rofecoxib reduces kainate-induced cell death in the rat hippocampus. Eur J Neurosci. 13, 569-75.

Kurnellas, M.P., et al., 2010. Reduced expression of plasma membrane calcium ATPase 2 and collapsin response mediator protein 1 promotes death of spinal cord neurons. Cell Death Differ. 17, 1501-10.

Kusuhara, H., Sugiyama, Y., 2005. Active efflux across the blood-brain barrier: role of the solute carrier family. NeuroRx. 2, 73-85.

Lambert, J., et al., 1993. Structure of the human apolipoprotein D gene promoter region. Biochim Biophys Acta. 1172, 190-2.

Laugharne, J.D., Mellor, J.E., Peet, M., 1996. Fatty acids and schizophrenia. Lipids. 31 Suppl, S163-5.

Lea, O.A., 1988. Binding properties of progesterone-binding Cyst protein, PBCP. Steroids. 52, 337-8.

Leung, W.C., et al., 2004. Apolipoprotein D and platelet-derived growth factor-BB synergism mediates vascular smooth muscle cell migration. Circ Res. 95, 179-86.

Levros, L.C., Jr., et al., 2010. Characterization of nuclear factors modulating the apolipoprotein D promoter during growth arrest: implication of PARP-1, APEX-1 and ERK1/2 catalytic activities. Biochim Biophys Acta. 1803, 1062-71.

Levros, L.C., Jr., et al., 2013. Binding and repressive activities of apolipoprotein E3 and E4 isoforms on the human ApoD promoter. Mol Neurobiol. 48, 669-80.

Li, H.X., et al., 2010. Review: Epigenetic regulation of adipocyte differentiation and adipogenesis. J Zhejiang Univ Sci B. 11, 784-91.

Liang, G., et al., 2002. Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. J Biol Chem. 277, 9520-8.

Lin, C.J., et al., 2013. Inhibition of Helicobacter pylori CagA-Induced Pathogenesis by Methylantcinate B from Antrodia camphorata. Evid Based Complement Alternat Med. 2013, 682418.

Lipton, S.A., Rosenberg, P.A., 1994. Excitatory amino acids as a final common pathway for neurologic disorders. N Engl J Med. 330, 613-22.

Listenberger, L.L., et al., 2007. Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. J Lipid Res. 48, 2751-61.

Liu, Y., et al., 2007. NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J Neurosci. 27, 2846-57.

Liu, Y., et al., 2013. Downregulation of caveolin-1 contributes to the synaptic plasticity deficit in the hippocampus of aged rats. Neural Regen Res. 8, 2725-33.

Liu, Z., Chang, G.Q., Leibowitz, S.F., 2001. Apolipoprotein D interacts with the long-form leptin receptor: a hypothalamic function in the control of energy homeostasis. FASEB J. 15, 1329-31.

Loerch, P.M., et al., 2008. Evolution of the aging brain transcriptome and synaptic regulation. PLoS One. 3, e3329.

Lois, C., Alvarez-Buylla, A., 1994. Long-distance neuronal migration in the adult mammalian brain. Science. 264, 1145-8.

Lopez-Boado, Y.S., Tolivia, J., Lopez-Otin, C., 1994. Apolipoprotein D gene induction by retinoic acid is concomitant with growth arrest and cell differentiation in human breast cancer cells. J Biol Chem. 269, 26871-8.

Lopez-Boado, Y.S., et al., 1996. Retinoic acid-induced expression of apolipoprotein D and concomitant growth arrest in human breast cancer cells are mediated through a retinoic acid receptor RARalpha-dependent signaling pathway. J Biol Chem. 271, 32105-11.

Lopez-Boado, Y.S., et al., 1997. Growth inhibition of human breast cancer cells by 1,25-dihydroxyvitamin D3 is accompanied by induction of apolipoprotein D expression. Cancer Res. 57, 4091-7.

Love-Gregory, L., et al., 2008. Variants in the CD36 gene associate with the metabolic syndrome and high-density lipoprotein cholesterol. Hum Mol Genet. 17, 1695-704.

Lund, E.G., et al., 2003. Knockout of the cholesterol 24-hydroxylase gene in mice reveals a brain-specific mechanism of cholesterol turnover. J Biol Chem. 278, 22980-8.

Mahadik, S.P., et al., 2002. Elevated plasma level of apolipoprotein D in schizophrenia and its treatment and outcome. Schizophr Res. 58, 55-62.

Maldonado-Baez, L., et al., 2013. Microtubule-dependent endosomal sorting of clathrin-independent cargo by Hook1. J Cell Biol. 201, 233-47.

Mandard, S., Muller, M., Kersten, S., 2004. Peroxisome proliferator-activated receptor alpha target genes. Cell Mol Life Sci. 61, 393-416.

Mao, J., et al., 2006. Liver-specific deletion of acetyl-CoA carboxylase 1 reduces hepatic triglyceride accumulation without affecting glucose homeostasis. Proc Natl Acad Sci U S A. 103, 8552-7.

Mao, L.M., et al., 2009. Stability of surface NMDA receptors controls synaptic and behavioral adaptations to amphetamine. Nat Neurosci. 12, 602-10.

Martinez, G., et al., 1997. MAP2, synaptophysin immunostaining in rat brain and behavioral modifications after cerebral postischemic reperfusion. Dev Neurosci. 19, 457-64.

Masliah, E., et al., 1996. Comparison of neurodegenerative pathology in transgenic mice overexpressing V717F beta-amyloid precursor protein and Alzheimer's disease. J Neurosci. 16, 5795-811.

Matsusue, K., et al., 2003. Liver-specific disruption of PPARgamma in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. J Clin Invest. 111, 737-47.

McConathy, W.J., Alaupovic, P., 1973. Isolation and partial characterization of apolipoprotein D: a new protein moiety of the human plasma lipoprotein system. FEBS Lett. 37, 178-82.

McConathy, W.J., Alaupovic, P., 1976. Studies on the isolation and partial characterization of apolipoprotein D and lipoprotein D of human plasma. Biochemistry. 15, 515-20.

McGarry, J.D., Brown, N.F., 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem. 244, 1-14.

McLin, J.P., Steward, O., 2006. Comparison of seizure phenotype and neurodegeneration induced by systemic kainic acid in inbred, outbred, and hybrid mouse strains. Eur J Neurosci. 24, 2191-202.

McNamara, J.O., 1992. The neurobiological basis of epilepsy. Trends Neurosci. 15, 357-9.

Menard, S.L., et al., 2010. Abnormal in vivo myocardial energy substrate uptake in diet-induced type 2 diabetic cardiomyopathy in rats. Am J Physiol Endocrinol Metab. 298, E1049-57.

Meyer, F.B., 1989. Calcium, neuronal hyperexcitability and ischemic injury. Brain Res Brain Res Rev. 14, 227-43.

Miles, R., Wong, R.K., 1983. Single neurones can initiate synchronized population discharge in the hippocampus. Nature. 306, 371-3.

Miranda, E., et al., 2003. Apolipoprotein D expression in cutaneous malignant melanoma. J Surg Oncol. 83, 99-105.

Mody, I., MacDonald, J.F., 1995. NMDA receptor-dependent excitotoxicity: the role of intracellular Ca2+ release. Trends Pharmacol Sci. 16, 356-9.

Montpied, P., et al., 1999. Hippocampal alterations of apolipoprotein E and D mRNA levels in vivo and in vitro following kainate excitotoxicity. Epilepsy Res. 35, 135-46.

Moody, D.M., 2006. The blood-brain barrier and blood-cerebral spinal fluid barrier. Semin Cardiothorac Vasc Anesth. 10, 128-31.

Morais Cabral, J.H., et al., 1995. Arachidonic acid binds to apolipoprotein D: implications for the protein's function. FEBS Lett. 366, 53-6.

Muffat, J., Walker, D.W., Benzer, S., 2008. Human ApoD, an apolipoprotein up-regulated in neurodegenerative diseases, extends lifespan and increases stress resistance in Drosophila. Proc Natl Acad Sci U S A. 105, 7088-93.

Muramatsu, T., Miyauchi, T., 2003. Basigin (CD147): a multifunctional transmembrane protein involved in reproduction, neural function, inflammation and tumor invasion. Histol Histopathol. 18, 981-7.

Nagy, L., et al., 1998. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell. 93, 229-40.

Nahalkova, J., et al., 2010. CD147, a gamma-secretase associated protein is upregulated in Alzheimer's disease brain and its cellular trafficking is affected by presenilin-2. Neurochem Int. 56, 67-76.

Naruhashi, K., et al., 1997. Abnormalities of sensory and memory functions in mice lacking Bsg gene. Biochem Biophys Res Commun. 236, 733-7.

Navarro, A., et al., 1998. Pattern of apolipoprotein D immunoreactivity in human brain. Neurosci Lett. 254, 17-20.

Navarro, A., et al., 2003. Immunohistochemical study of distribution of apolipoproteins E and D in human cerebral beta amyloid deposits. Exp Neurol. 184, 697-704.

Navarro, A., Del Valle, E., Tolivia, J., 2004. Differential expression of apolipoprotein d in human astroglial and oligodendroglial cells. J Histochem Cytochem. 52, 1031-6.

Niesman, I.R., et al., 2014. Traumatic brain injury enhances neuroinflammation and lesion volume in caveolin deficient mice. J Neuroinflammation. 11, 39.

Nishino, N., et al., 2008. FSP27 contributes to efficient energy storage in murine white adipocytes by promoting the formation of unilocular lipid droplets. J Clin Invest. 118, 2808-21.

Norfeldt, P.I., et al., 1981. Isolation and partial characterization of the lipoprotein families A and A-I from high-density lipoproteins of human serum. Eur J Biochem. 118, 1-8.

Notarnicola, M., et al., 2011. Polyunsaturated fatty acids reduce fatty acid synthase and hydroxy-methyl-glutaryl CoA-reductase gene expression and promote apoptosis in HepG2 cell line. Lipids Health Dis. 10, 10.

Oakley, A.J., et al., 2012. Molecular dynamics analysis of apolipoprotein-D-lipid hydroperoxide interactions: mechanism for selective oxidation of Met-93. PLoS One. 7, e34057.

Olney, J.W., Adamo, N.J., Ratner, A., 1971. Monosodium glutamate effects. Science. 172, 294.

Ong, W.Y., et al., 1997. Differential expression of apolipoprotein D and apolipoprotein E in the kainic acid-lesioned rat hippocampus. Neuroscience. 79, 359-67.

Ong, W.Y., et al., 1999. Apolipoprotein D gene expression in the rat brain and light and electron microscopic immunocytochemistry of apolipoprotein D

expression in the cerebellum of neonatal, immature and adult rats. Neuroscience. 90, 913-22.

Ong, W.Y., et al., 2003. Increase in cholesterol and cholesterol oxidation products, and role of cholesterol oxidation products in kainate-induced neuronal injury. Brain Pathol. 13, 250-62.

Ordonez, C., et al., 2006. Apolipoprotein D expression in substantia nigra of Parkinson disease. Histol Histopathol. 21, 361-6.

Palumbo, S., Bosetti, F., 2013. Alterations of brain eicosanoid synthetic pathway in multiple sclerosis and in animal models of demyelination: role of cyclooxygenase-2. Prostaglandins Leukot Essent Fatty Acids. 89, 273-8.

Patel, M., et al., 1996. Requirement for superoxide in excitotoxic cell death. Neuron. 16, 345-55.

Patel, R.C., et al., 1997. Probing the structure of the ligand binding cavity of lipocalins by fluorescence spectroscopy. Protein Eng. 10, 621-5.

Patel, S.C., et al., 1995. Astrocytes synthesize and secrete the lipophilic ligand carrier apolipoprotein D. Neuroreport. 6, 653-7.

Pearlman, W.H., Gueriguian, J.L., Sawyer, M.E., 1973. A specific progesterone-binding component of human breast cyst fluid. J Biol Chem. 248, 5736-41.

Peitsch, M.C., Boguski, M.S., 1990. Is apolipoprotein D a mammalian bilin-binding protein? New Biol. 2, 197-206.

Pellegrini-Giampietro, D.E., et al., 1997. The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. Trends Neurosci. 20, 464-70.

Peng, D., et al., 2008. Antiatherosclerotic effects of a novel synthetic tissue-selective steroidal liver X receptor agonist in low-density lipoprotein receptor-deficient mice. J Pharmacol Exp Ther. 327, 332-42.

Penkowa, M., et al., 2005. Metallothionein reduces central nervous system inflammation, neurodegeneration, and cell death following kainic acid-induced epileptic seizures. J Neurosci Res. 79, 522-34.

Perdomo, G., Henry Dong, H., 2009. Apolipoprotein D in lipid metabolism and its functional implication in atherosclerosis and aging. Aging (Albany NY). 1, 17-27.

Perdomo, G., et al., 2010. A role of apolipoprotein D in triglyceride metabolism. J Lipid Res. 51, 1298-311.

Peters, K., Richards, F.M., 1977. Chemical cross-linking: reagents and problems in studies of membrane structure. Annu Rev Biochem. 46, 523-51.

Pfrieger, F.W., 2003. Cholesterol homeostasis and function in neurons of the central nervous system. Cell Mol Life Sci. 60, 1158-71.

Porter, D., et al., 2003. Molecular markers in ductal carcinoma in situ of the breast. Mol Cancer Res. 1, 362-75.

Privat, A., 2005. [Pathophysiology and treatment of spinal cord injury]. Bull Acad Natl Med. 189, 1109-17; discussion 1117-8.

Provost, P.R., et al., 1990. Molecular characterization and differential mRNA tissue distribution of rabbit apolipoprotein D. J Lipid Res. 31, 2057-65.

Provost, P.R., et al., 1991a. Apolipoprotein D transcription occurs specifically in nonproliferating quiescent and senescent fibroblast cultures. FEBS Lett. 290, 139-41.

Provost, P.R., et al., 1991b. Localization of the major sites of rabbit apolipoprotein D gene transcription by in situ hybridization. J Lipid Res. 32, 1959-70.

Provost, P.R., et al., 1991a. Apolipoprotein D transcription occurs specifically in nonproliferating quiescent and senescent fibroblast cultures. FEBS Lett. 290, 139-41.

Provost, P.R., et al., 1991b. Localization of the major sites of rabbit apolipoprotein D gene transcription by in situ hybridization. J Lipid Res. 32, 1959-70.

Provost, P.R., et al., 1995. Guinea pig apolipoprotein D RNA diversity, and developmental and gestational modulation of mRNA levels. Mol Cell Endocrinol. 109, 225-36.

Puri, V., et al., 2008. Cidea is associated with lipid droplets and insulin sensitivity in humans. Proc Natl Acad Sci U S A. 105, 7833-8.

Pushkarsky, T., et al., 2001. CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A. Proc Natl Acad Sci U S A. 98, 6360-5.

Pyper, S.R., et al., 2010. PPARalpha: energy combustion, hypolipidemia, inflammation and cancer. Nucl Recept Signal. 8, e002.

Racine, R.J., 1972. Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol. 32, 281-94.

Rahimian, R., et al., 2001. Hepatic over-expression of peroxisome proliferator activated receptor gamma2 in the ob/ob mouse model of non-insulin dependent diabetes mellitus. Mol Cell Biochem. 224, 29-37.

Rassart, E., et al., 2000. Apolipoprotein D. Biochim Biophys Acta. 1482, 185-98.

Rawlins, F.A., et al., 1970. Reutilization of cholesterol-1,2-H3 in the regeneration of peripheral nerve. An autoradiographic study. Lab Invest. 22, 237-40.

Reddy, J.K., Hashimoto, T., 2001. Peroxisomal beta-oxidation and peroxisome proliferator-activated receptor alpha: an adaptive metabolic system. Annu Rev Nutr. 21, 193-230.

Reddy, P.H., et al., 2005. Differential loss of synaptic proteins in Alzheimer's disease: implications for synaptic dysfunction. J Alzheimers Dis. 7, 103-17; discussion 173-80.

Reindl, M., et al., 2001. Increased intrathecal production of apolipoprotein D in multiple sclerosis. J Neuroimmunol. 119, 327-32.

Rhinn, M., Dolle, P., 2012. Retinoic acid signalling during development. Development. 139, 843-58.

Rickhag, M., et al., 2006. Comprehensive regional and temporal gene expression profiling of the rat brain during the first 24 h after experimental stroke

identifies dynamic ischemia-induced gene expression patterns, and reveals a biphasic activation of genes in surviving tissue. J Neurochem. 96, 14-29.

Rickhag, M., et al., 2008. Apolipoprotein D is elevated in oligodendrocytes in the peri-infarct region after experimental stroke: influence of enriched environment. J Cereb Blood Flow Metab. 28, 551-62.

Rogue, A., et al., 2010. Gene Expression Changes Induced by PPAR Gamma Agonists in Animal and Human Liver. PPAR Res. 2010, 325183.

Rogue, A., et al., 2011. Comparative Gene Expression Profiles Induced by PPARgamma and PPARalpha/gamma Agonists in Human Hepatocytes. PLoS One. 6, e18816.

Rosen, E.D., et al., 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev. 16, 22-6.

Rosenbrock, H., et al., 2005. Effect of chronic intermittent restraint stress on hippocampal expression of marker proteins for synaptic plasticity and progenitor cell proliferation in rats. Brain Res. 1040, 55-63.

Rothman, S.M., Olney, J.W., 1986. Glutamate and the pathophysiology of hypoxic--ischemic brain damage. Ann Neurol. 19, 105-11.

Ruiz, M., et al., 2012. Grasshopper Lazarillo, a GPI-anchored Lipocalin, increases Drosophila longevity and stress resistance, and functionally replaces its secreted homolog NLaz. Insect Biochem Mol Biol. 42, 776-89.

Ruiz, M., et al., 2013. Lipid-binding properties of human ApoD and Lazarillo-related lipocalins: functional implications for cell differentiation. FEBS J. 280, 3928-43.

Ruscher, K., et al., 2010. Effects of chronic Clozapine administration on apolipoprotein D levels and on functional recovery following experimental stroke. Brain Res. 1321, 152-63.

Ryu, B., et al., 2001. Invasion-specific genes in malignancy: serial analysis of gene expression comparisons of primary and passaged cancers. Cancer Res. 61, 1833-8.

Sameshima, T., et al., 2000. Expression of emmprin (CD147), a cell surface inducer of matrix metalloproteinases, in normal human brain and gliomas. Int J Cancer. 88, 21-7.

Sanchez, D., et al., 2000. Characterization of two novel lipocalins expressed in the Drosophila embryonic nervous system. Int J Dev Biol. 44, 349-59.

Sanchez, D., Ganfornina, M.D., Martinez, S., 2002. Expression pattern of the lipocalin apolipoprotein D during mouse embryogenesis. Mech Dev. 110, 225-9.

Sanchez, D., et al., 2006. Loss of glial lazarillo, a homolog of apolipoprotein D, reduces lifespan and stress resistance in Drosophila. Curr Biol. 16, 680-6.

Sarjeant, J.M., et al., 2003. Apolipoprotein D inhibits platelet-derived growth factor-BB-induced vascular smooth muscle cell proliferated by preventing

translocation of phosphorylated extracellular signal regulated kinase 1/2 to the nucleus. Arterioscler Thromb Vasc Biol. 23, 2172-7.

Sarkar, G., Sommer, S.S., 1990. The "megaprimer" method of site-directed mutagenesis. Biotechniques. 8, 404-7.

Sasaki, Y., et al., 2009. p53 family members regulate the expression of the apolipoprotein D gene. J Biol Chem. 284, 872-83.

Sayyah, M., Javad-Pour, M., Ghazi-Khansari, M., 2003. The bacterial endotoxin lipopolysaccharide enhances seizure susceptibility in mice: involvement of proinflammatory factors: nitric oxide and prostaglandins. Neuroscience. 122, 1073-80.

Scatchard, G., 1949. The Attractions of Proteins for Small Molecules and Ions. Annals of the New York Academy of Sciences. 51, 660-672.

Schindler, P.A., et al., 1995. Site-specific detection and structural characterization of the glycosylation of human plasma proteins lecithin:cholesterol acyltransferase and apolipoprotein D using HPLC/electrospray mass spectrometry and sequential glycosidase digestion. Protein Sci. 4, 791-803.

Schwab, J.M., et al., 2006. Experimental strategies to promote spinal cord regeneration--an integrative perspective. Prog Neurobiol. 78, 91-116.

Seguin, D., Desforges, M., Rassart, E., 1995. Molecular characterization and differential mRNA tissue distribution of mouse apolipoprotein D. Brain Res Mol Brain Res. 30, 242-50.

Serra Diaz, C., et al., 1999. Expression and clinical significance of apolipoprotein D in male breast cancer and gynaecomastia. Br J Surg. 86, 1190-7.

Sevin, M., et al., 2007. The adult form of Niemann-Pick disease type C. Brain. 130, 120-33.

Sherry, B., et al., 1992. Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages. Proc Natl Acad Sci U S A. 89, 3511-5.

Silva, J.P., Ushkaryov, Y.A., 2010. The latrophilins, "split-personality" receptors. Adv Exp Med Biol. 706, 59-75.

Simard, J., et al., 1990. Regulation of progesterone-binding breast cyst protein GCDFP-24 secretion by estrogens and androgens in human breast cancer cells: a new marker of steroid action in breast cancer. Endocrinology. 126, 3223-31.

Simard, J., et al., 1991. Stimulation of apolipoprotein D secretion by steroids coincides with inhibition of cell proliferation in human LNCaP prostate cancer cells. Cancer Res. 51, 4336-41.

Simons, K., Toomre, D., 2000. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol. 1, 31-9.

Siwik, D.A., et al., 2008. EMMPRIN mediates beta-adrenergic receptor-stimulated matrix metalloproteinase activity in cardiac myocytes. J Mol Cell Cardiol. 44, 210-7.

Smith, K.M., Lawn, R.M., Wilcox, J.N., 1990. Cellular localization of apolipoprotein D and lecithin:cholesterol acyltransferase mRNA in rhesus monkey tissues by in situ hybridization. J Lipid Res. 31, 995-1004.

Smith, W.L., DeWitt, D.L., Garavito, R.M., 2000. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem. 69, 145-82.

Sodero, A.O., et al., 2012. Cholesterol loss during glutamate-mediated excitotoxicity. EMBO J. 31, 1764-73.

Soiland, H., et al., 2007. Emerging concepts of apolipoprotein D with possible implications for breast cancer. Cell Oncol. 29, 195-209.

Song, F., et al., 2011. Cyclophilin A (CyPA) induces chemotaxis independent of its peptidylprolyl cis-trans isomerase activity: direct binding between CyPA and the ectodomain of CD147. J Biol Chem. 286, 8197-203.

Spreyer, P., et al., 1990. Regeneration-associated high level expression of apolipoprotein D mRNA in endoneurial fibroblasts of peripheral nerve. EMBO J. 9, 2479-84.

Steyrer, E., Kostner, G.M., 1988. Activation of lecithin-cholesterol acyltransferase by apolipoprotein D: comparison of proteoliposomes containing apolipoprotein D, A-I or C-I. Biochim Biophys Acta. 958, 484-91.

Stremmel, W., Berk, P.D., 1986. Hepatocellular influx of [14C]oleate reflects membrane transport rather than intracellular metabolism or binding. Proc Natl Acad Sci U S A. 83, 3086-90.

Sun, J., Hemler, M.E., 2001. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. Cancer Res. 61, 2276-81.

Suresh, S., et al., 1998. Cellular cholesterol storage in the Niemann-Pick disease type C mouse is associated with increased expression and defective processing of apolipoprotein D. J Neurochem. 70, 242-51.

Szydlowska, K., Tymianski, M., 2010. Calcium, ischemia and excitotoxicity. Cell Calcium. 47, 122-9.

Taghibiglou, C., et al., 2009. Role of NMDA receptor-dependent activation of SREBP1 in excitotoxic and ischemic neuronal injuries. Nat Med. 15, 1399-406.

Takemiya, T., et al., 2003. Inducible brain COX-2 facilitates the recurrence of hippocampal seizures in mouse rapid kindling. Prostaglandins Other Lipid Mediat. 71, 205-16.

Takemiya, T., Matsumura, K., Yamagata, K., 2007. Roles of prostaglandin synthesis in excitotoxic brain diseases. Neurochem Int. 51, 112-20.

Tall, A.R., 2008. Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. J Intern Med. 263, 256-73.

Tang, W., Chang, S.B., Hemler, M.E., 2004. Links between CD147 function, glycosylation, and caveolin-1. Mol Biol Cell. 15, 4043-50.

Tang, W., Hemler, M.E., 2004. Caveolin-1 regulates matrix metalloproteinases-1 induction and CD147/EMMPRIN cell surface clustering. J Biol Chem. 279, 11112-8.

Targett-Adams, P., et al., 2005. A PPAR response element regulates transcription of the gene for human adipose differentiation-related protein. Biochim Biophys Acta. 1728, 95-104.

Tator, C.H., Fehlings, M.G., 1991. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg. 75, 15-26.

Tator, C.H., 1995. Update on the pathophysiology and pathology of acute spinal cord injury. Brain Pathol. 5, 407-13.

Taupin, P., Gage, F.H., 2002. Adult neurogenesis and neural stem cells of the central nervous system in mammals. J Neurosci Res. 69, 745-9.

Tempel, B.L., Shilling, D.J., 2007. The plasma membrane calcium ATPase and disease. Subcell Biochem. 45, 365-83.

Terrisse, L., et al., 1998. Increased levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients. J Neurochem. 71, 1643-50.

Terrisse, L., et al., 1999. Modulation of apolipoprotein D and apolipoprotein E expression in rat hippocampus after entorhinal cortex lesion. Brain Res Mol Brain Res. 70, 26-35.

Terrisse, L., et al., 2001. Structure-function relationships of human apolipoprotein D an immunochemical analysis. Life Sci. 70, 629-38.

Thomas, E.A., et al., 2001a. Clozapine increases apolipoprotein D expression in rodent brain: towards a mechanism for neuroleptic pharmacotherapy. J Neurochem. 76, 789-96.

Thomas, E.A., et al., 2001b. Increased CNS levels of apolipoprotein D in schizophrenic and bipolar subjects: implications for the pathophysiology of psychiatric disorders. Proc Natl Acad Sci U S A. 98, 4066-71.

Thomas, E.A., et al., 2001c. Apolipoprotein D mRNA expression is elevated in PDAPP transgenic mice. J Neurochem. 79, 1059-64.

Thomas, E.A., Copolov, D.L., Sutcliffe, J.G., 2003a. From pharmacotherapy to pathophysiology: emerging mechanisms of apolipoprotein D in psychiatric disorders. Curr Mol Med. 3, 408-18.

Thomas, E.A., George, R.C., Sutcliffe, J.G., 2003b. Apolipoprotein D modulates arachidonic acid signaling in cultured cells: implications for psychiatric disorders. Prostaglandins Leukot Essent Fatty Acids. 69, 421-7.

Thomas, E.A., et al., 2003a. Antipsychotic drug treatment alters expression of mRNAs encoding lipid metabolism-related proteins. Mol Psychiatry. 8, 983-93, 950.

Thomas, E.A., et al., 2003c. Apolipoprotein D levels are elevated in prefrontal cortex of subjects with Alzheimer's disease: no relation to apolipoprotein E expression or genotype. Biol Psychiatry. 54, 136-41.

Thomas, E.A., Yao, J.K., 2007. Clozapine specifically alters the arachidonic acid pathway in mice lacking apolipoprotein D. Schizophr Res. 89, 147-53.

Tontonoz, P., et al., 1994a. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 8, 1224-34.

Tontonoz, P., Hu, E., Spiegelman, B.M., 1994b. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell. 79, 1147-56.

Tontonoz, P., Spiegelman, B.M., 2008. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem. 77, 289-312.

Toole, B.P., 2003. Emmprin (CD147), a cell surface regulator of matrix metalloproteinase production and function. Curr Top Dev Biol. 54, 371-89.

Tribouillard-Tanvier, D., et al., 2012. Role of cyclophilin A from brains of prion-infected mice in stimulation of cytokine release by microglia and astroglia in vitro. J Biol Chem. 287, 4628-39.

Truong, T.Q., et al., 2000. Analysis of low-density lipoprotein catabolism by primary cultures of hepatic cells from normal and low-density lipoprotein receptor knockout mice. Biochim Biophys Acta. 1484, 307-15.

Tsukamoto, K., et al., 2013. Identification of apolipoprotein D as a cardioprotective gene using a mouse model of lethal atherosclerotic coronary artery disease. Proc Natl Acad Sci U S A. 110, 17023-8.

Vaddadi, K.S., 1992. Use of gamma-linolenic acid in the treatment of schizophrenia and tardive dyskinesia. Prostaglandins Leukot Essent Fatty Acids. 46, 67-70.

Vaisar, T., et al., 2007. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. J Clin Invest. 117, 746-56.

Van Den Bosch, L., et al., 2006. The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. Biochim Biophys Acta. 1762, 1068-82.

Vance, J.E., 2012. Dysregulation of cholesterol balance in the brain: contribution to neurodegenerative diseases. Dis Model Mech. 5, 746-55.

Vezzani, A., Granata, T., 2005. Brain inflammation in epilepsy: experimental and clinical evidence. Epilepsia. 46, 1724-43.

Vieira, A.V., et al., 1995. Identification of a circulatory and oocytic avian apolipoprotein D. Mol Reprod Dev. 42, 443-6.

Vijitruth, R., et al., 2006. Cyclooxygenase-2 mediates microglial activation and secondary dopaminergic cell death in the mouse MPTP model of Parkinson's disease. J Neuroinflammation. 3, 6.

Viollet, B., et al., 2006. Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. J Physiol. 574, 41-53.

Vogt, M., Skerra, A., 2001. Bacterially produced apolipoprotein D binds progesterone and arachidonic acid, but not bilirubin or E-3M2H. J Mol Recognit. 14, 79-86.

Waldow, T., et al., 2009. Prevention of ischemia/reperfusion-induced accumulation of matrix metalloproteinases in rat lung by preconditioning with nitric oxide. J Surg Res. 152, 198-208.

Walker, D.W., et al., 2006. Overexpression of a Drosophila homolog of apolipoprotein D leads to increased stress resistance and extended lifespan. Curr Biol. 16, 674-9.

Walker, N.P., Fox, H.C., Whalley, L.J., 1999. Lipids and schizophrenia. Br J Psychiatry. 174, 101-4.

Warden, C.H., et al., 1992. Localization of the gene for apolipoprotein D on mouse chromosome 16. Genomics. 12, 851-2.

Watkins, J.C., Evans, R.H., 1981. Excitatory amino acid transmitters. Annu Rev Pharmacol Toxicol. 21, 165-204.

Weech, P.K., et al., 1986. Apolipoprotein D and cross-reacting human plasma apolipoproteins identified using monoclonal antibodies. J Biol Chem. 261, 7941-51.

Weech, P.K., et al., 1991. Apolipoprotein D--an atypical apolipoprotein. Prog Lipid Res. 30, 259-66.

Wenzel, A., et al., 1997. NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. J Neurochem. 68, 469-78.

West, R.B., et al., 2004. Apo D in soft tissue tumors: a novel marker for dermatofibrosarcoma protuberans. Am J Surg Pathol. 28, 1063-9.

Xu, L., Zhou, L., Li, P., 2012. CIDE proteins and lipid metabolism. Arterioscler Thromb Vasc Biol. 32, 1094-8.

Xu, Q., et al., 1992. Leukocyte chemotactic activity of cyclophilin. J Biol Chem. 267, 11968-71.

Yang, C.Y., et al., 1994. Structure of human apolipoprotein D: locations of the intermolecular and intramolecular disulfide links. Biochemistry. 33, 12451-5.

Yao, J.K., van Kammen, D.P., Gurklis, J.A., 1996. Abnormal incorporation of arachidonic acid into platelets of drug-free patients with schizophrenia. Psychiatry Res. 60, 11-21.

Yao, J.K., et al., 2005. Association of plasma apolipoproteins D with RBC membrane arachidonic acid levels in schizophrenia. Schizophr Res. 72, 259-66.

Yao, Y., Vieira, A., 2002. Comparative 17beta-estradiol response and lipoprotein interactions of an avian apolipoprotein. Gen Comp Endocrinol. 127, 89-93.

Yasojima, K., et al., 1999. Distribution of cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins in human brain and peripheral organs. Brain Res. 830, 226-36.

Yki-Jarvinen, H., 2010. Liver fat in the pathogenesis of insulin resistance and type 2 diabetes. Dig Dis. 28, 203-9.

Yonezawa, T., et al., 2011. Which CIDE are you on? Apoptosis and energy metabolism. Mol Biosyst. 7, 91-100.

Yoon, Y.W., et al., 2005. Upstream regulation of matrix metalloproteinase by EMMPRIN; extracellular matrix metalloproteinase inducer in advanced atherosclerotic plaque. Atherosclerosis. 180, 37-44.

Yoshida, K., et al., 1996. Molecular cloning of the mouse apolipoprotein D gene and its upregulated expression in Niemann-Pick disease type C mouse model. DNA Cell Biol. 15, 873-82.

Yuasa, J., et al., 2001. Specific localization of the basigin protein in human testes from normal adults, normal juveniles, and patients with azoospermia. Andrologia. 33, 293-9.

Yurchenko, V., et al., 2002. Active site residues of cyclophilin A are crucial for its signaling activity via CD147. J Biol Chem. 277, 22959-65.

Yurchenko, V., Constant, S., Bukrinsky, M., 2006. Dealing with the family: CD147 interactions with cyclophilins. Immunology. 117, 301-9.

Yurchenko, V., et al., 2010. Cyclophilin-CD147 interactions: a new target for anti-inflammatory therapeutics. Clin Exp Immunol. 160, 305-17.

Zeng, C., et al., 1996. A human axillary odorant is carried by apolipoprotein D. Proc Natl Acad Sci U S A. 93, 6626-30.

Zhang, S.X., et al., 1998. Immunolocalization of apolipoprotein D, androgen receptor and prostate specific antigen in early stage prostate cancers. J Urol. 159, 548-54.

Zhang, Y.L., et al., 2006. Aberrant hepatic expression of PPARgamma2 stimulates hepatic lipogenesis in a mouse model of obesity, insulin resistance, dyslipidemia, and hepatic steatosis. J Biol Chem. 281, 37603-15.

Zheng, X.Y., et al., 2011. Kainic acid-induced neurodegenerative model: potentials and limitations. J Biomed Biotechnol. 2011, 457079.

Zhou, M., Baudry, M., 2006. Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. J Neurosci. 26, 2956-63.

Zipfel, G.J., Lee, J.M., Choi, D.W., 1999. Reducing calcium overload in the ischemic brain. N Engl J Med. 341, 1543-4.

Zoete, V., Grosdidier, A., Michielin, O., 2007. Peroxisome proliferator-activated receptor structures: ligand specificity, molecular switch and interactions with regulators. Biochim Biophys Acta. 1771, 915-25.

Zucker, S., et al., 2001. Tumorigenic potential of extracellular matrix metalloproteinase inducer. Am J Pathol. 158, 1921-8.