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Endothélium, inflammation et cognition : focus sur le BDNF

Martin Pedard

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Université de Bourgogne

UFR Sciences de Santé

Ecole doctorale « Environnement-Santé »

Laboratoire INSERM U1093 – Cognition, Action et Plasticité Sensorimotrice

Endothélium, inflammation et cognition : focus sur le BDNF

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« Seul l'exercice soutient l'esprit et maintient la vigueur de l'esprit. »

Cicéron (-65 avant JC)

A mon père.

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Abstract

BDNF (brain-derived neurotrophic factor) has been discovered in the brain and is widely implicated in neuroplasticity, memory and cognition through the activation of neuronal TrkB (tropomyosin receptor kinase B) receptors. We have recently shown that the cardiovascular system contained as much BDNF as the brain and that exogenous BDNF was able to induce endothelium-dependent vascular relaxation. Other studies have suggested that activation of endothelial TrkB receptors by BDNF is involved in atherosclerotic processes. Our laboratory suspects a close interaction between endothelial NO and BDNF and has even considered the possibility of involvement of BDNF secreted by cerebral microvessel endothelium in cognition. Patients suffering from rheumatoid arthritis (RA), an inflammatory disease of autoimmune origin, are at risk of cardiovascular disease and have an impairment of cognition, including a higher risk of depression. Surprisingly, the effect of RA on BDNF is poorly documented. The only available studies report an increase of BDNF in the blood and synovial fluid in RA. Our hypothesis is that a reduction in endothelial expression of BDNF may contribute to the cardiovascular risk and cognitive deficit associated with RA. Thus, in our work, we studied vascular and cerebral BDNF and its TrkB receptor on the rat model of adjuvant-induced arthritis.

Our main findings show that arthritis leads to 1) a decrease in BDNF levels in aortas independently of the severity of inflammatory symptoms but dependent on endothelial function, 2) a decrease in brain BDNF levels independent of the severity of inflammatory symptoms, but link with endothelial function, 3) a decreased expression of BDNF and its activated TrkB receptor at both neuronal and endothelial levels in the brain regions involved in cognition, 4) a positive correlation between endothelial expression of BDNF and neuronal expression of activated TrkB, 5) a lack of correlation between serum BDNF levels and its cerebral or vascular levels, but on the other hand the existence of a positive correlation between serum BDNF levels and inflammation, whether clinical or biological.

All of these data support the hypothesis that endothelial BDNF may be involved in atherosclerotic risk and cognitive impairment associated with arthritis. Inflammation should be considered as a confounding factor when circulating levels of BDNF are used as a reflection of levels present in the brain.

Key words: BDNF, Endothelial function, NO, Cognition, TrkB, Inflammation

Résumé

Le BDNF (brain-derived neurotrophic factor) a été découvert dans le cerveau et est largement impliqué dans la neuroplasticité, la mémoire et la cognition via l'activation des récepteurs TrkB (tropomyosin receptor kinase B) neuronaux. Nous avons récemment montré que le système cardiovasculaire contenait autant de BDNF que le cerveau et que le BDNF exogène était capable d'induire une relaxation vasculaire dépendante de l'endothélium. D'autres études ont suggéré que l'activation des récepteurs TrkB endothéliaux par le BDNF était impliquée dans les processus athérosclérotiques. Notre laboratoire soupçonne une interaction étroite entre NO et BDNF endothélial et a même envisagé la possibilité d'une implication du BDNF sécrété par l'endothélium des microvaisseaux cérébraux dans la cognition. Les patients souffrant de polyarthrite rhumatoïde (PR), une maladie inflammatoire d'origine auto-immune, sont à risque cardiovasculaire et présentent une altération de la cognition avec notamment un risque plus élevé de dépression. Étonnamment, l'effet de la PR sur le BDNF est peu documenté. Les seules études disponibles rapportent une élévation du BDNF dans le sang et le liquide synovial en cas de PR. Notre hypothèse est qu'une réduction de l'expression endothéliale du BDNF pourrait contribuer au risque cardiovasculaire et au déficit cognitif associés à la PR. Ainsi, dans notre travail, nous avons étudié le BDNF vasculaire et cérébral et son récepteur TrkB sur le modèle rat d'arthrite induite à l'adjuvant.

Nos principaux résultats montrent que l'arthrite conduit à 1) une réduction des taux aortiques de BDNF indépendamment de la sévérité des symptômes inflammatoires mais dépendante de la fonction endothéliale, 2) une diminution des taux cérébraux en BDNF indépendamment de la sévérité des symptômes inflammatoires mais en lien avec la fonction endothéliale, 3) une diminution de l'expression du BDNF et de son récepteur TrkB activé au niveau tant neuronal qu'endothélial dans les régions cérébrales impliquées dans la cognition, 4) une corrélation positive entre l'expression du BDNF par l'endothélium vasculaire et l'expression neuronale des TrkB activés, 5) une absence de corrélation entre les taux sériques de BDNF et ses taux cérébraux ou vasculaires mais en revanche l'existence d'une corrélation positive entre les taux sériques de BDNF et l'inflammation qu'elle soit clinique ou biologique.

L'ensemble de ces données est en faveur de l'hypothèse selon laquelle le BDNF endothélial pourrait être impliqué dans le risque athérosclérotique et le déficit cognitif associé à l'arthrite. L'inflammation doit être considérée comme un facteur confondant lorsque les taux circulants de BDNF sont utilisés comme un reflet des taux présents dans le cerveau.

Mots clés : BDNF, Fonction endothéliale, NO, Cognition, TrkB, Inflammation

Table des matières

<i>Remerciements</i>	3
<i>Abstract</i>	4
<i>Résumé</i>	5
<i>Table des matières</i>	6
<i>Liste des tableaux</i>	10
<i>Liste des figures</i>	10
<i>Abréviations</i>	11
INTRODUCTION GENERALE	12
ETAT DE L'ART	15
Chapitre 1 Fonction & dysfonction endothéliales	16
I) Introduction	17
II) Rôle physiologique de l'endothélium	18
1) Endothélium et perméabilité capillaire	18
2) Endothélium et débit sanguin régional	18
a) Vasodilatation dépendante du flux	18
b) Vasodilatation rétrograde propagée.....	19
3) Endothélium et contrôle de la fluidité du sang	20
4) Endothélium et contrôle de l'angiogénèse	20
5) Endothélium et contrôle de la prolifération des cellules musculaires lisses	21
a) Remodelage vasculaire et pression pariétale	21
b) Remodelage vasculaire et processus artériosclérotique.....	21
6) Endothélium et contrôle de l'extravasation des globules blancs	22
a) Extravasation des globules blancs en cas de lésion d'un tissu	22
b) Extravasation des globules blancs lors du processus athérosclérotique	22
III) Dysfonction endothéliale	23
1) Définition	23
2) Synthèse endothéliale du NO	23
a) Concentration cytosolique en Ca ²⁺	24
b) Phosphorylation de l'enzyme	24
c) Dimérisation de l'enzyme	24

d)	S-nitrosylation de l'enzyme.....	25
3)	Mécanisme d'action du NO d'origine endothéliale.....	25
4)	Mécanismes de la dysfonction endothéliale	25
a)	Diminution de la production de NO	25
b)	Inactivation du NO	26
5)	Origines de la dysfonction endothéliale.....	27
6)	La dysfonction endothéliale, une maladie systémique	28
7)	Exploration de la fonction endothéliale.....	29
a)	Chez l'Homme	29
b)	Chez l'animal	30
	Chapitre 2 Risque cardiovasculaire & cognition	32
I)	Le constat.....	33
1)	Chez l'Homme	33
2)	Chez l'animal.....	33
II)	Liens mécanistiques entre risque CV et cognition.....	34
1)	Altération du DSC.....	34
2)	Altération de la neuroplasticité/neurogénèse.....	35
a)	Altération de la neuroplasticité.....	35
b)	Altération de la neurogénèse	35
3)	Altération de la perméabilité de la BHE	36
	Chapitre 3 Le BDNF	37
I)	Introduction.....	38
II)	BDNF cérébral.....	38
	Article 1	39
III)	Le BDNF circulant.....	55
1)	Origine du BDNF circulant	55
2)	Variabilité inter-individuelle des taux circulants de BDNF	56
3)	Intérêt de la mesure du BDNF circulant.....	57
a)	BDNF circulant, un marqueur de la santé cérébrale ?.....	57
b)	BDNF circulant, un marqueur de la santé cardiovasculaire ?.....	58

Chapitre 4 La polyarthrite rhumatoïde	60
I) Généralités	61
II) Les modèles animaux d'arthrite	63
1) Choix du modèle AIA	63
2) Immunisation du rat AIA	64
3) Evolution des signes cliniques chez le rat AIA	64
a) Inflammation chez le rat AIA.....	64
b) Douleur chez le rat AIA	66
III) PR & risque cardiovasculaire	66
1) Le constat	66
2) PR & dysfonction endothéliale	67
a) Mise en évidence chez l'Homme	67
b) Etudes chez le rat AIA	67
c) Mécanismes impliqués dans la dysfonction endothéliale	68
IV) PR & déficit cognitif	69
1) Le constat	69
a) Chez l'Homme	69
b) Chez l'animal	69
2) Mécanismes	70
a) Inflammation systémique	70
b) Dysfonction endothéliale.....	70
c) Origine iatrogène.....	71
V) PR & BDNF	71
1) PR & BDNF dans les articulations	71
2) PR & BDNF circulant	71
TRAVAUX PERSONNELS	73
Article 2	74
Résumé des travaux	75
a) Contexte & hypothèse	75
b) Protocole expérimental.....	75
c) Principaux résultats	75
Article 3	85
Résumé des travaux	86

a) Contexte & hypothèse	86
b) Protocole expérimental.....	86
c) Principaux résultats	87
Article 4	98
Résumé des travaux.....	99
a) Contexte & hypothèse	99
b) Protocole expérimental.....	99
c) Principaux résultats	99
Article 5	107
Résumé des travaux.....	108
a) Contexte & hypothèse	108
b) Protocole expérimental.....	108
c) Principaux résultats	108
<i>DISCUSSION GENERALE & PERSPECTIVES</i>	<i>121</i>
1) Dysfonction endothéliale et BDNF endothélial.....	122
2) Dysfonction endothéliale cérébrale et PR	123
3) Dysfonction endothéliale cérébrale et déficit cognitif en cas de PR.....	124
4) BDNF circulant et PR	125
5) Retombées cliniques de nos études	127
<i>Liste des productions scientifiques liées au projet de thèse</i>	<i>128</i>
<i>Liste des productions scientifiques non liées au projet de thèse</i>	<i>130</i>
<i>REFERENCES BIBLIOGRAPHIQUES.....</i>	<i>131</i>

Liste des tableaux

Tableau 1: Avantages et inconvénients des techniques les plus couramment utilisées pour évaluer la fonction endothéliale.....	30
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Liste des figures

Figure 1: Valeurs des contraintes de cisaillement exercées par le sang sur l'endothélium vasculaire.....	19
Figure 2: Récapitulatif des mécanismes conduisant à une diminution de la biodisponibilité en NO secondaire à une exposition des cellules endothéliales aux facteurs de risque cardiovasculaire ou à l'inflammation systémique.....	28
Figure 3: Origine du BDNF plasmatique.....	55
Figure 4: Facteurs influençant les taux circulants de BDNF	57
Figure 5: Recommandations de la HAS sur la démarche diagnostique et de prise en charge initiale de la PR...	61
Figure 6: Recommandations de la HAS concernant le bilan et la prise en charge de la PR.	62
Figure 7: Photographies de pattes postérieures de rats non malades (A) et AIA (B) et de pattes antérieures de rats non malades (C) et AIA (D) prises au jour 27 post-immunisation.....	64
Figure 8: Changements radiographiques de la patte arrière observés chez un rat non malade (a) et AIA (b et c) au jour 195 post-immunisation. A noter l'ostéof ormation dans le tarse et le calcaneum (b) et dans l'extrémité inférieure du tibia (c).....	65
Figure 9: Evolution du score arthritique chez le rat AIA.	65
Figure 10: Effets de la diminution de la production endothéliale de BDNF sur le processus athéromateux et la cognition en cas d'arthrite et boucle reliant production endothéliale de NO et de BDNF en cas d'arthrite.....	126

Abréviations

ACh : Acétylcholine	NGF : Nerve growth factor
ADMA : Asymmetric dimethylarginine	NO : Nitric oxide
AIA : Adjuvant-induced arthritis	nor-NOHA : N ω -hydroxy-nor-Arginine.
AINS : Anti inflammatoire non stéroïdien	p75 ^{NTR} : Pan 75 neurotrophin receptor
Akt : Protéine kinase B	PA : Pression artérielle
ANG-II : Angiotensine II	PAI-1 : Plasminogen activator inhibitor-1
ApoE : Apolipoprotéine E	PDGF- β : Platelet derived growth factor- β
ARNm : Acide ribonucléique messenger	PGI-2 : Prostacycline
BACE-1 : Beta-secretase 1	P-gp : Glycoprotéine P
BDNF : Brain-derived neurotrophic factor	PI3K : Phosphoinositide 3-kinase
BH2 : Dihydrobioptérine	PKA : Protéine kinase dépendante de l'AMPc
BH4 : Tetrahydrobioptérine	PR : Polyarthrite rhumatoïde
BHE : Barrière hémato-encéphalique	PV : Pression veineuse
CAMKII : Protéine kinase Ca ²⁺ /calmoduline-dépendante	RA : Résistance artériolaire
CCP : Cyclic citrullinated peptide	RCP : Résumé des caractéristiques du produit
CIA : Collagen-induced arthritis	SC : Sous-cutanée
COX : Cyclooxygénase	SCORE : Systematic coronary risk estimation
CO ₂ : Dioxyde de carbone	SNC : Système nerveux central
CRP : C reactive protein	SOD : Superoxyde dismutase
DAS 28 : Disease activity score 28	TGF β : Tumor growth factor β
DSC : Débit sanguin cérébral	TNF α : Tumor necrosis factor α
EDHF : Endothelium-derived hyperpolarizing factor	TNFR : TNF α receptor
EDRF : Endothelium-derived relaxing factor	t-PA : Tissue plasminogen activator
ELISA : Enzyme-linked immunosorbent assay	TrkB : Tropomyosin-related kinase B
eNOS : Endothelial nitric oxide synthase	TXA2 : Thromboxane A2
ET-1 : Endothéline-1	u-PA : Urokinase
EVA : Echelle visuelle analogique	VCAM-1 : Vascular cell adhesion molecule-1
FMD : Flow-mediated dilatation	VEGF : Vascular endothelial growth factor
GMPc : Guanosine monophosphate cyclique	vs : Vitesse de sédimentation
GTP : Guanosine triphosphate	vWF : von Willebrand Factor
HAS : Health assessment questionnaire	WB : Western blot
HAS : Haute autorité de santé	
HSP90 : Heat shock protein 90	
ICAM-1 : Intercellular adhesion molecule-1	
IFN- α : Interféron α	
IFN- γ : Interféron γ	
IL-1 β : Interleukine-1 β	
IM : Intramusculaire	
KO : Knock-out	
LDL : Low-density lipoprotein	
L-NAME : N(ω)-nitro-L-arginine methyl ester	
LPS : Lipopolysaccharide S	
LTP : Long-term potentiation	
MTX : Méthotrexate	
NADPH : Nicotinamide adénine dinucléotide phosphate	
NF- κ B : Nuclear factor-kappa B	

INTRODUCTION GENERALE

Depuis sa découverte dans le cerveau fin des années 1980, la neurotrophine BDNF (brain-derived neurotrophic factor) a fait l'objet d'une multitude de travaux centrés sur le neurone. A ce jour, le BDNF est considéré comme un médiateur indispensable de la neurogénèse et la synaptogenèse et son implication dans l'apprentissage, la mémoire et la cognition est unanimement reconnue. En fait, l'activation des récepteurs TrkB neuronaux par le BDNF synthétisé et sécrété par le neurone explique le couplage existant entre activité neuronale et efficacité synaptique. Récemment, notre laboratoire a montré que le système cardiovasculaire adulte contenait autant de BDNF que l'hippocampe, la région du cerveau la plus riche en BDNF, et que l'expression vasculaire du BDNF était confinée aux cellules endothéliales. Cependant, le rôle et le métabolisme du BDNF d'origine endothéliale ont été peu étudiés et pratiquement tout reste à découvrir sur ce nouveau facteur dérivé de l'endothélium.

Nous et d'autres avons montré que le BDNF exogène était capable d'induire une relaxation vasculaire qui nécessitait la présence de l'endothélium d'où la possibilité d'un contrôle autocrine du tonus vasculaire par le BDNF endothélial. D'autres études, bien que peu nombreuses, ont suggéré que l'activation des TrkB endothéliaux était impliquée dans les processus athérosclérotiques. Notre laboratoire a même envisagé la possibilité d'une implication du BDNF sécrété par l'endothélium des microvaisseaux cérébraux dans la cognition. L'ensemble de ces données identifie le BDNF endothélial comme un marqueur potentiel et jusque-là insoupçonné non seulement du risque cardiovasculaire mais aussi du risque cognitif. Or, le nombre de travaux montrant que les patients à risque cardiovasculaire sont également à risque cognitif est croissant. Aussi, notre idée est que le BDNF endothélial pourrait être le lien entre maladie cardiovasculaire et déficit cognitif. Plus précisément, la dysfonction endothéliale s'accompagnerait d'une moindre production de BDNF par l'endothélium vasculaire d'où l'augmentation du risque athéromateux et de troubles cognitifs. En faveur de cette idée, nous avons récemment montré au laboratoire 1) que l'hypertension artérielle réduisait et l'exercice aérobie augmentait les expressions endothéliale (aussi bien périphérique que centrale) et neuronale (cérébrale) du BDNF, alors que la fonction endothéliale est altérée par l'hypertension et améliorée par l'exercice, 2) que l'exposition de vaisseaux isolés à un donneur de NO augmentait leur production de BDNF.

La polyarthrite rhumatoïde (PR), une maladie inflammatoire d'origine auto-immune, est comme l'hypertension, le diabète et la sédentarité associée à une augmentation non seulement du risque cardiovasculaire mais aussi du risque de déficit cognitif. Ainsi, la mortalité d'origine cardiovasculaire est plus élevée et les capacités cognitives sont moindres chez le patient arthritique en comparaison aux individus non malades du même âge. Cependant, les mécanismes en cause restent spéculatifs même si l'inflammation et la dysfonction endothéliale sont largement incriminées. Notre hypothèse est qu'une anomalie de l'expression endothéliale du BDNF pourrait contribuer aux risques cardiovasculaire et cognitif associés à la PR. Aussi, dans notre travail, **nous avons étudié l'effet de l'arthrite sur les taux vasculaires, cérébraux et circulants de BDNF et le lien existant entre ces paramètres et l'inflammation/la dysfonction endothéliale.** Les études ont été conduites chez le rat sur le modèle d'arthrite induite à l'adjuvant (AIA).

Le manuscrit comprend :

- Un état de l'art articulé autour de 4 axes :
 - La fonction/dysfonction endothéliale
 - Le lien entre risques cardiovasculaire et cognitif
 - Le BDNF
 - La polyarthrite rhumatoïde
- Les résultats expérimentaux présentés dans nos publications
- Une discussion générale

ETAT DE L'ART

Chapitre 1

Fonction & dysfonction endothéliales

I) Introduction

L'endothélium vasculaire est constitué d'une monocouche de cellules épithéliales en contact avec le sang. Son épaisseur ne dépasse pas 1 μm et sa surface est voisine de 4000 m^2 chez l'Homme (6 terrains de football). Il repose sur une lame basale, portion acellulaire du tissu conjonctif sous-jacent. L'endothélium, qu'il appartienne à la circulation périphérique ou cérébrale, à la macro ou microcirculation, régule une multitude de fonctions via la synthèse et la sécrétion de multiples facteurs notamment :

- Des facteurs vasorelaxants dont le monoxyde d'azote (NO), la prostacycline (PGI-2) et les facteurs hyperpolarisants dérivés de l'endothélium (EDHF) et des facteurs vasoconstricteurs dont l'endothéline-1 (ET-1), le thromboxane A₂ (TXA₂) et l'angiotensine II (ANG-II)
- Des facteurs capables d'induire l'agrégation des plaquettes comme le facteur von Willebrand (vWF) ou à l'inverse exerçant un effet antiagrégant plaquettaire comme le NO et la PGI-2
- Des facteurs qui contribuent à la lyse du caillot de fibrine comme le NO, l'activateur tissulaire du plasminogène (t-PA) ou qui s'opposent à la fibrinolyse comme l'inhibiteur de l'activateur du plasminogène (PAI-1)
- Des facteurs proangiogéniques dont le NO, des métalloprotéases, le facteur de croissance transformant (TGF- β) et le facteur de croissance endothélial vasculaire (VEGF) ou antiangiogéniques dont la thrombospondine-1, la canstatine, l'arrestine et la tumstatine
- Des facteurs capables de contrôler positivement la prolifération cellulaire dont l'ET-1 et l'ANG-II ou de s'y opposer dont le facteur de croissance dérivé des plaquettes β (PDGF- β) et le NO
- Des facteurs qui contrôlent l'adhésion des globules blancs circulants à la surface luminale des cellules endothéliales comme le NO

Parmi ces facteurs, le NO est le mieux caractérisé tant sur le plan de sa synthèse que de ses effets. On note que le NO est impliqué dans de nombreuses fonctions de l'endothélium. Aussi, l'incapacité de l'endothélium à produire suffisamment de NO est l'indice le plus largement utilisé pour définir la dysfonction endothéliale, anomalie à l'initiation du processus athérosclérotique et de plus en plus incriminée dans l'altération de la cognition.

II) Rôle physiologique de l'endothélium

1) Endothélium et perméabilité capillaire

Les capillaires représentent le site exclusif des échanges entre le plasma et le liquide interstitiel qui entoure les cellules des tissus vascularisés. La perméabilité des capillaires aux substances nutritives, aux déchets métaboliques de faible poids moléculaire et aux solutés hydrosolubles de haut poids moléculaire dépend de la nature de l'endothélium dont on distingue 4 types : continu, fenestré, discontinu et de type barrière hématoencéphalique (BHE). Pour les 2 premiers types, les cellules endothéliales sont reliées entre elles par des jonctions serrées de type *fascia occludens* et le passage des substances se fait en vrac par voie transcellulaire (transcytose et pores ioniques), la vitesse d'échange étant plus rapide pour l'endothélium fenestré (glandes, rein et intestin) que continu (peau et muscles). Pour l'endothélium de type discontinu (foie et moelle osseuse), la lame basale et les jonctions intercellulaires sont incomplètes, expliquant la grande perméabilité de ce type d'endothélium capillaire aux protéines (foie) et aux cellules (moelle osseuse). La BHE est quant à elle une barrière à perméabilité sélective qui correspond sur le plan anatomique à la paroi des capillaires sanguins du système nerveux central (SNC). Son histologie est détaillée dans la revue page 42.

2) Endothélium et débit sanguin régional

L'apport sanguin aux organes, c'est-à-dire l'apport en substances nutritives au niveau de leurs réseaux capillaires dépend de la pression de perfusion de l'organe. Cette pression de perfusion est égale à la différence entre la pression artérielle (Pa) et la pression veineuse (Pv) rapportée aux résistances artériolaires de l'organe (RA) selon la formule :

$$\text{Débit sanguin} = (\text{Pa} - \text{Pv}) / \text{RA}$$

Les RA diminuent proportionnellement à l'activité métabolique de l'organe qui conduit à la production de métabolites capables de relaxer le muscle lisse artériolaire indépendamment de l'endothélium. L'augmentation de débit qui en résulte déclenche 2 processus, quant à eux dépendants de l'endothélium : la vasodilatation dépendante du flux et la vasodilatation rétrograde propagée.

a) Vasodilatation dépendante du flux

La vasodilatation dépendante du flux (flow mediated dilatation en anglais ou FMD) est la relaxation quasi-immédiate (Lehoux et al., 2006) du muscle lisse vasculaire induite par l'augmentation des contraintes de cisaillement (forces de friction) exercées sur l'endothélium par le déplacement du

sang. Ces contraintes de cisaillement (τ , Dyn / cm²) sont dépendantes du débit (Q, mL/s), de la viscosité sanguine (η , 0,009 Poise) et du rayon intérieur du vaisseau (r, cm) selon la formule :

$$\text{Contraintes de cisaillement } \tau = 4\eta Q / \Pi r^3$$

Aussi, les contraintes de cisaillements sont plus élevées au niveau des vaisseaux de petit que de gros calibre (Figure 1).

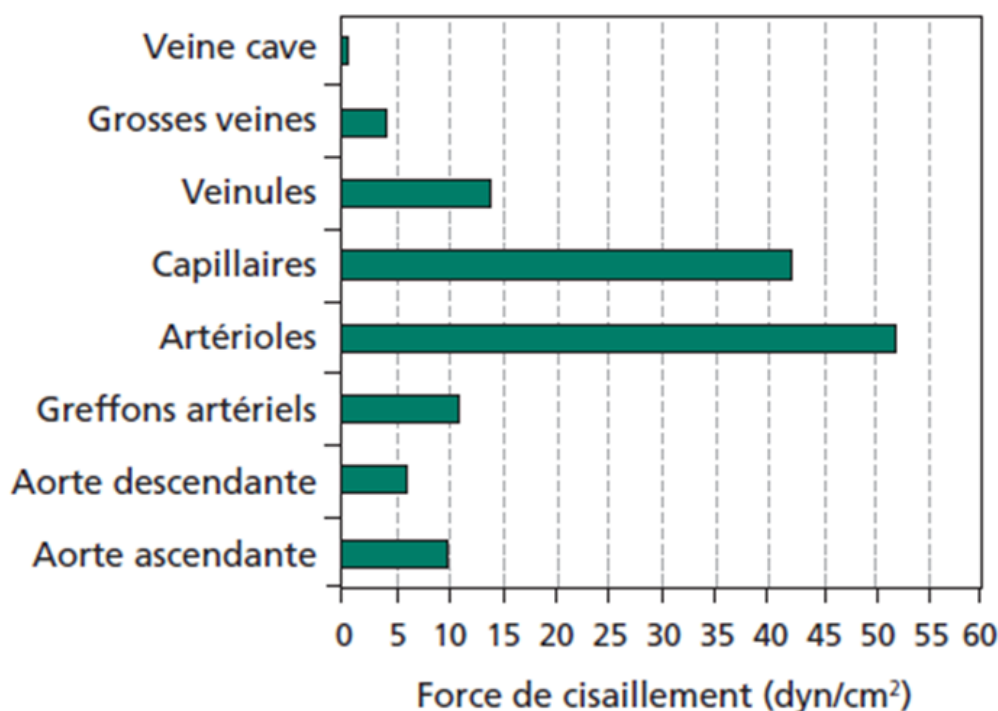


Figure 1: Valeurs des contraintes de cisaillement exercées par le sang sur l'endothélium vasculaire d'après Chatzizisis et al. (2007).

De façon importante, un vaisseau désendothélialisé (Thorin-Trescases and Bevan, 1998, Ward et al., 2000, Drouin and Thorin, 2009) ou dont l'endothélium est incapable de produire du NO (Golding et al., 2002, You et al., 2005, Drouin and Thorin, 2009) ne se relâche plus en réponse à une augmentation du flux. Ces données donnent la preuve que la vasodilatation dépendante du flux est un processus dépendant du NO d'origine endothéliale. Les mécanismes impliqués seront présentés page 25.

b) Vasodilatation rétrograde propagée

La vasodilatation rétrograde propagée est une vasodilatation qui se propage rapidement dans le sens opposé au flux sanguin de l'artériole aux vaisseaux situés en amont. Ce phénomène a d'abord été mis en évidence par Hilton qui a montré que la contraction du muscle soléaire s'accompagnait d'une augmentation de calibre de l'artère fémorale (Hilton, 1959) et explique l'augmentation du flux qui est

observée dans des territoires ne présentant pas d'hyperactivité métabolique. Par exemple, chez le rat, la stimulation des vibrisses induit une vasodilatation des artérioles piales situées bien en amont des artérioles présentes dans les aires corticales activées (Cox et al., 1993, Erinjeri and Woolsey, 2002).

Là encore, l'altération de l'endothélium supprime le phénomène de vasodilatation rétrograde propagée (Horiuchi et al., 2002, Dietrich et al., 2009) même si les mécanismes impliqués ne sont pas bien connus. Un courant hyperpolarisant se propagerait le long de l'endothélium et conduirait par l'intermédiaire des jonctions myoendothéliales à une relaxation du muscle lisse sous-jacent (Horiuchi et al., 2002, Dietrich et al., 2009).

3) Endothélium et contrôle de la fluidité du sang

L'endothélium permet le maintien de la fluidité du sang non seulement en prévenant la formation du clou plaquettaire, première étape de l'hémostase, mais aussi en permettant la lyse du caillot sanguin une fois constitué. En effet, les cellules endothéliales empêchent l'adhérence, l'agrégation et l'activation des plaquettes (Michiels, 2003, Verhamme and Hoylaerts, 2006) via la sécrétion de NO et PGI-2. L'endothélium via la sécrétion de t-PA, une sérine protéase, permet également la fibrinolyse. En effet, cette enzyme transforme le plasminogène circulant en plasmine qui à son tour dégrade la fibrine du caillot sanguin en produits de dégradation de la fibrine solubles. Le t-PA est sécrété par les cellules endothéliales de manière constitutive ou en réponse à une élévation des contraintes de cisaillement ou à des agonistes tels que la thrombine ou la bradykinine. Une autre protéase d'origine endothéliale permet la formation de plasmine. Il s'agit de l'u-PA qui est synthétisé et sécrété par l'endothélium de la circulation rénale en réponse à des cytokines proinflammatoires ou encore à des facteurs de croissance (Pearson, 1999, Arnout et al., 2006, Brown, 2006).

4) Endothélium et contrôle de l'angiogénèse

L'angiogénèse adulte, qu'elle soit physiologique ou pathologique, se définit comme le bourgeonnement de microvaisseaux à partir de l'endothélium des vaisseaux préexistants. Il s'agit d'un processus complexe impliquant notamment la division et la migration des cellules endothéliales ainsi que la maturation des nouveaux microvaisseaux, reflet d'un équilibre étroit entre apoptose et survie des nouvelles cellules endothéliales (Watson et al., 2017).

La cellule endothéliale joue un rôle important dans la régulation de l'angiogénèse. En effet, la dégradation de la matrice extracellulaire, étape préliminaire à l'angiogénèse, est médiée et régulée par plusieurs familles de protéases sécrétées par l'endothélium. Sous l'action de ces protéases, de nombreux facteurs de croissance endothéliaux tels que le TGF- β et le VEGF ayant des propriétés angiogéniques sont libérés dans la matrice extracellulaire. Inversement, et dans l'esprit d'un

phénomène étroitement régulé, les inhibiteurs de l'angiogenèse tels que la thrombospondine-1, la canstatine, l'arrestine et la tumstatine sont également libérés par l'endothélium,

5) Endothélium et contrôle de la prolifération des cellules musculaires

lisses

En condition physiologique, les cellules musculaires lisses de la média ont un faible taux de renouvellement. Deux évènements majeurs initiés par la dysfonction endothéliale s'accompagnent de la prolifération des cellules musculaires lisses: l'hypertension artérielle et le processus artériosclérotique.

a) Remodelage vasculaire et pression pariétale

Il est bien documenté que l'hypertension artérielle et l'augmentation de la tension pariétale qui en résulte constitue un stimulus mécanique qui induit l'hyperplasie et l'hypertrophie des cellules musculaires lisses. Cette réponse des cellules endothéliales met en jeu entre autre un déficit en NO. Ainsi, les études sur co-culture de cellules endothéliales/cellules musculaires lisses (Peiro et al., 1995) ou sur vaisseau désendothélialisé (Lamb et al., 1984, Jacot and Wong, 2008) ont bien montré l'importance de l'endothélium dans le contrôle de la prolifération des cellules musculaires. En faveur de l'implication du NO, l'inhibition pharmacologique ou génétique de la NO synthase endothéliale (eNOS) prévient la prolifération des cellules musculaires lisses induite par les contraintes de cisaillement (Kang et al., 2010). On peut préciser ici que le remodelage d'une artère de conductance conduit à une perte d'élasticité (marquée pour l'aorte) et une augmentation de sa rigidité alors qu'au niveau d'une artère de résistance, le remodelage est associé à une réduction du diamètre de la lumière (donc une augmentation des résistances), à une raréfaction du lit artériolaire et une moindre capacité de l'endothélium à produire du NO d'où une potentialisation de la dysfonction endothéliale. La rigidité artérielle (ou plus exactement l'analyse de la vitesse de l'onde de pouls) ainsi que l'épaisseur de l'intima-media sont mesurées en clinique et considérées comme des prédicteurs fiables du risque cardiovasculaire (Anderson, 2006, Halcox et al., 2009).

b) Remodelage vasculaire et processus artériosclérotique

Le processus artériosclérotique, qui peut se définir par les changements liés au vieillissement qui se produisent dans les vaisseaux sanguins comme l'épaississement des parois, la perte d'élasticité du tissu conjonctif et le durcissement de la paroi vasculaire, s'accompagne de la migration des cellules musculaires lisses de la média vers l'intima, site de leur prolifération. Migration et multiplication nécessitent une dédifférenciation cellulaire. Les cellules musculaires lisses perdent leur propriété contractile au bénéfice d'une augmentation de leur capacité de synthèse (protéines de la matrice

extracellulaire). Ainsi, les cellules musculaires lisses contribuent non seulement à la formation des plaques mais aussi à la formation de leur chape fibreuse prévenant ainsi leur rupture et les évènements thrombotiques qui en résultent. Certaines cellules musculaires lisses acquièrent même le phénotype de macrophage et produisent des cytokines proinflammatoires qui pourraient être impliquées dans le maintien de l'activation endothéliale, se définissant par un état pro-inflammatoire et procoagulant des cellules endothéliales (Li et al., 2016b). Cependant, il a été récemment suggéré que c'est l'apoptose des cellules musculaires lisses qui déclencherait l'inflammation vasculaire (Bennett et al., 2016). Il est admis que le NO d'origine endothéliale exerce un effet antiprolifératif sur les cellules musculaires lisses. Ainsi, la formation de la néointima chez le lapin hypercholestérolémique est stimulée par l'inhibition de la production de NO (Naruse et al., 1994) et inhibée par le transfert *in vivo* du gène eNOS (Von Der Leyen et al., 1995, Channon et al., 1996, Channon et al., 1998).

6) Endothélium et contrôle de l'extravasation des globules blancs

Pour quitter le compartiment vasculaire, les globules blancs doivent d'abord adhérer à l'endothélium avant de le traverser que ce soit par voie paracellulaire ou, moins fréquemment, par voie transcellulaire. L'adhésion des globules blancs à l'endothélium n'est possible que si les cellules endothéliales expriment au sein de leur membrane luminale des molécules d'adhérence que sont les E- et P-sélectines, la molécule d'adhérence intercellulaire-1 (ICAM-1) et la molécule d'adhérence cellulaire vasculaire-1 (VCAM-1) (Imhof and Aurrand-Lions, 2004, Min et al., 2005, Leon and Ardavin, 2008). L'extravasation des globules blancs s'observe en cas de lésion d'un tissu et du processus inflammatoire qui en découle ou lors du processus athérosclérotique, qui se caractérise par le dépôt d'une plaque essentiellement composée de lipides (athérome) sur la paroi des artères.

a) Extravasation des globules blancs en cas de lésion d'un tissu

Lors de la lésion d'un tissu, les cellules en souffrance secrètent des cytokines proinflammatoires telles que le TNF α ou l'IL-1 β qui induisent l'expression de molécules d'adhérence par les cellules endothéliales au niveau des veinules postcapillaires. On parle alors d'activation endothéliale. L'endothélium va alors pouvoir fixer les globules blancs circulants, première étape de la diapédèse. Après infiltration du tissu lésé par les globules blancs, ceux-ci via la sécrétion de cytokines proinflammatoires vont renforcer l'activation endothéliale et entretenir la diapédèse.

b) Extravasation des globules blancs lors du processus athérosclérotique

Lors du processus athérosclérotique, c'est l'accumulation passive de lipoprotéines de basse densité (low density lipoprotein, LDL) dans l'intima des artères, suivie de leur oxydation qui est à l'origine de l'activation endothéliale. Il en résulte une entrée des monocytes circulants dans la paroi vasculaire et

leur transformation en macrophages. Ceux-ci phagocytent les LDL oxydées (macrophages spumeux) et secrètent des cytokines qui à leur tour induisent une activation endothéliale. Les neutrophiles et les mastocytes vont également infiltrer le tissu sous-endothélial. Les premiers contiennent de grandes quantités de myéloperoxydases, de NADPH oxydase et de lipoxigénases qui vont contribuer au stress oxydatif et à l'oxydation des LDL. Les seconds, quant à eux, favorisent l'athérosclérose en libérant le contenu de leurs granules riches en protéases et cytokines proinflammatoires (Sun et al., 2007).

III) Dysfonction endothéliale

1) Définition

La dysfonction endothéliale se définit comme un déséquilibre entre la production des facteurs anti- et pro-coagulants, inducteurs et inhibiteurs de la croissance cellulaire, anti- et pro-inflammatoires ainsi qu'entre la production des facteurs endothéliaux vasoconstricteurs et vasodilatateurs. Cependant, en pratique, la dysfonction endothéliale est le plus souvent définie par une incapacité de l'endothélium à produire suffisamment de NO.

2) Synthèse endothéliale du NO

La découverte du NO d'origine endothéliale s'est faite en 2 étapes. La première a été la démonstration en 1980 par Furchgott et Zawadzki (1980) que l'acétylcholine exerçait un effet vasodilatateur via la sécrétion par l'endothélium d'un facteur qu'ils nomment alors facteur relaxant dérivé de l'endothélium (EDRF). La seconde étape est la démonstration par Palmer (1987) et Ignarro (1989) que cet EDRF était le NO, un espèce radicalaire gazeuse et liposoluble.

Le NO est synthétisé et secrété en continu par l'endothélium à partir de la NO synthase endothéliale, eNOS (ou NOS III), une enzyme qui dégrade la L-arginine en NO et L-citrulline en présence de son cofacteur, la tetrahydrobioptérine (BH4). L'enzyme est localisée sous forme de monomères et dimères dans les cavéoles (radeaux lipidiques enrichis en glycosphingolipides et cholestérol situé à proximité de la membrane plasmique). Seule la forme dimérisée est capable de produire du NO (Andrew and Mayer, 1999). La synthèse endothéliale de NO dépend non seulement de la quantité d'enzymes présente mais aussi de son activité. Un des principaux facteurs physiologiques qui contrôle l'expression de l'enzyme est le flux sanguin. En effet, l'élévation des contraintes de cisaillement via un mécanisme dépendant du Ca^{2+} modulé par la voie PI3K/Akt (Malek et al., 1999) induit l'expression du gène eNOS (Ranjan et al., 1995, Uematsu et al., 1995, Nadaud et al., 1996). L'activité de l'enzyme est quant à elle influencée par de multiples facteurs que nous allons décrire car leur connaissance est un prérequis à la compréhension des mécanismes qui conduisent à la dysfonction

endothéliale. Il s'agit de la concentration cytosolique en calcium et de la phosphorylation, dimérisation et S-nitrosylation de l'enzyme.

a) Concentration cytosolique en Ca^{2+}

Le taux de calcium intracellulaire est un déterminant essentiel de l'activité de la eNOS car la fonction catalytique maximale d'eNOS nécessite la liaison de la calmoduline à l'un des domaines de l'enzyme afin de faciliter le transfert d'électrons entre les domaines réductase et oxygénase de l'enzyme (Chen and Wu, 2000). En effet, la liaison de la calmoduline à eNOS lève l'interaction inhibitrice existant entre la cavéoline-1 et eNOS (Michel et al., 1997). Ainsi, l'activation des récepteurs muscariniques endothéliaux (récepteurs couplés à une protéine G de type q) stimule la production de NO via une augmentation de la concentration intracellulaire en calcium.

b) Phosphorylation de l'enzyme

La phosphorylation de la eNOS est l'un des processus qui permet de contrôler l'activité de l'enzyme. La phosphorylation des sérines 1177, 635 et 617 augmente l'activité de l'enzyme alors que la phosphorylation de la thréonine 495 ou de la sérine 116 la diminue (Venema et al., 1996). Le site de phosphorylation le plus étudié est la sérine 1177 (Chen et al., 2014). Corson et al. (1996) ont montré qu'eNOS phosphorylée sur la sérine 1177 produisait 10 fois plus de NO que l'enzyme non phosphorylée. Cette phosphorylation est induite par l'activation des récepteurs endothéliaux au VEGF (Fulton et al., 1999, Michell et al., 2002), à la bradykinine (Fleming et al., 2001) et surtout par une augmentation des contraintes de cisaillement (Corson et al., 1996, Dimmeler et al., 1999) via un mécanisme dépendant de la voie Akt. C'est la phosphorylation d'eNOS sur la sérine 1177 qui est responsable de la vasodilatation dépendante du flux (Hutcheson and Griffith, 1991, Koller and Kaley, 1991, Niebauer and Cooke, 1996, Dimmeler and Zeiher, 2003).

c) Dimérisation de l'enzyme

Lorsque la eNOS est sous forme de dimère, l'un des domaines réductases d'un des deux monomères donne un électron au domaine oxygénase de l'autre monomère permettant ainsi la catalyse de la L-arginine en L-citrulline et NO. Sous sa forme monomère, eNOS devient incapable de délivrer des électrons à l'autre monomère et les électrons sont transférés sur une autre position du même monomère d'eNOS. Cela entraîne alors l'interaction de l'enzyme avec l'oxygène et la formation d'anions superoxydes (Xu et al., 2007, Schulz et al., 2008). La dimérisation de la eNOS est favorisée par BH4 (Venema et al., 1997, Cai and Harrison, 2000, Antoniadis et al., 2006, Chen et al., 2010a) et par la L-arginine bien que le rôle de l'arginine soit controversé (Venema et al., 1997, Chen et al., 2010a). Enfin, la protéine de choc thermique HSP90 (Xu et al., 2007, Ramírez et al., 2008) permettrait

de maintenir eNOS dans l'état dimérisé alors que l'interaction de la eNOS avec la protéine cavéoline 1 prévient sa dimérisation (Komers et al., 2006).

d) S-nitrosylation de l'enzyme

La S-nitrosylation de l'enzyme eNOS réduit son activité (Erwin et al., 2005, Erwin et al., 2006). De manière intéressante, Ravi et al. (2004) ont montré que le NO était capable de diminuer l'activité de la eNOS via la S-nitrosylation de l'enzyme indiquant que le NO est capable de contrôler sa propre production.

3) Mécanisme d'action du NO d'origine endothéliale

La demi-vie du NO ne dépasse pas 2 secondes. Aussi, le NO ne peut exercer que des effets autocrines et paracrines. Les cellules situées à proximité des cellules endothéliales capables de répondre au NO sont les cellules endothéliales elles-mêmes, les cellules musculaires lisses sous-jacentes, les éléments figurés et les neurones en tout cas pour le NO produit par les capillaires sanguins cérébraux. Le NO agit via l'activation de la guanylate cyclase soluble, une enzyme qui transforme le GTP en GMPc, un second messager aux multiples effets : effet endothélioprotecteur de type antiapoptotique (Chung et al., 2001), effet vasodilatateur via une moindre phosphorylation des chaînes légères de la myosine (Horowitz et al., 1996), effet antiprolifératif via une activation de la protéine kinase A (PKA) (Cornwell et al., 1994, 1993), effet antiagrégant plaquettaire via une réduction des taux cytosoliques en Ca^{2+} (Rao et al., 1990, Moro et al., 1996), effet anti-adhérent des globules blancs via l'inhibition du facteur nucléaire- κB (NF- κB) (Chen et al., 1999). L'action sur les neurones du NO dérivé de l'endothélium cérébral sera développée dans le 2^{ème} chapitre de la thèse.

4) Mécanismes de la dysfonction endothéliale

Les paragraphes qui suivent vont exposer les mécanismes qui concourent à une production/sécrétion insuffisante de NO par l'endothélium. Ils comprennent les processus qui conduisent à une moindre production de NO (réduction de la synthèse/activité de eNOS) et ceux qui conduisent à une inactivation du NO.

a) Diminution de la production de NO

La production de NO par l'endothélium dépend évidemment de sa capacité à synthétiser la eNOS et de l'activité de l'enzyme. De façon intéressante, la synthèse de eNOS est inhibée par les LDL oxydés (Liao et al., 1995) indiquant que le processus athéromateux peut entretenir la dysfonction endothéliale. De plus, il n'est pas exclu que les cytokines proinflammatoires puissent inhiber la

synthèse de l'enzyme. En effet, l'exposition de cellules endothéliales au lipopolysaccharide S (LPS) diminue les taux de ARNm codant eNOS (Lu et al., 1996).

Augmentation de la concentration en cavéoline-1

Tous les mécanismes capables d'augmenter les concentrations de cavéoline-1, comme l'hypercholestérolémie ou la présence de LDL oxydées diminuent donc la production de NO (Feron et al., 1999).

Découplage de la eNOS

Le découplage de la eNOS qui est objectivée le plus souvent par une réduction du rapport eNOS sous forme de dimère/eNOS sous forme de monomère s'observe en cas de déficit en L-arginine ou en BH4 (Stroes et al., 1998, Vasquez-Vivar et al., 1998). Une cause de déficit en L-arginine est une surexpression endothéliale de l'arginase, une enzyme en compétition avec eNOS pour dégrader la L-arginine. Ainsi, une augmentation de l'activité vasculaire de l'arginase est présente chez le rat âgé (Santhanam et al., 2008), la souris rendue diabétique par la streptozocine (Romero et al., 2012) ou le rat spontanément hypertendu (Michell et al., 2011). De plus, le traitement par un inhibiteur d'arginase restaure la production endothéliale de NO dans les modèles d'hypertension (Bagnost et al., 2008, Bagnost et al., 2010), de diabète (Gronros et al., 2011) ou chez l'animal âgé (Katusic, 2007). Une condition associée à un déficit en BH4 est le stress oxydant qui transforme la BH4 en dihydrobioptérine (BH2) (Milstien and Katusic, 1999, Laursen et al., 2001, Kuzkaya et al., 2003). Ce stress oxydant pourrait être relié au processus d'athérosclérose (Alp et al., 2004, Munzel et al., 2005). En effet, les taux de BH4 sont diminués dans la paroi des vaisseaux athéroscléreux que ce soit chez le lapin ou la souris ApoE -/- (Ozaki et al., 2002, Vasquez-Vivar et al., 2002).

Production endogène d'inhibiteurs de la eNOS

Vallance et al. (1992) ont été les premiers à identifier la diméthylarginine asymétrique (asymmetric dimethylarginine, ADMA) comme un inhibiteur endogène de la eNOS. Il est aujourd'hui bien admis que l'augmentation de sa concentration endothéliale réduit la production de NO (Boger, 2003, Szuba and Podgorski, 2006). De façon intéressante, les taux plasmatique d'ADMA sont augmentés chez l'Homme hypertendu (Surdacki et al., 1999), diabétique (Abbasi et al., 2001) ou atteint d'hypercholestérolémie (Boger et al., 1998).

b) Inactivation du NO

L'inactivation de NO résulte de son interaction avec les anions superoxydes avec formation de peroxynitrites (Blough and Zafiriou, 1985, Ignarro, 1990). Ainsi, toutes situations associées à une production accrue d'anions superoxydes dans la cellule endothéliale ou à une incapacité de la cellule à

les dégrader va réduire les effets autocrines et paracrines du NO. Les sources d'anions superoxydes sont multiples : la NADPH oxydase, la xanthine oxydase, la chaîne respiratoire mitochondriale, la eNOS découplée (Ellulu et al., 2016) mais ces espèces oxygénées sont converties en peroxyde d'hydrogène à l'aide de la superoxyde dismutase (SOD).

Lors du processus athérosclérotique, il existe une infiltration du sous-endothélium par les globules blancs non seulement les monocytes mais également les neutrophiles. Ces neutrophiles contiennent des myéloperoxydases qui par transcytose passent du secteur extracellulaire dans la cellule endothéliale (Eiserich et al., 2002, Zhang et al., 2003, Zhang et al., 2004). Une fois à l'intérieur des cellules endothéliales, les myéloperoxydases produiraient des anions superoxydes capables d'inactiver le NO et donc d'induire une dysfonction endothéliale (Zhang et al., 2004, Wong et al., 2015). Il a été récemment montré que l'exposition des cellules endothéliales en culture à la myéloperoxydase amplifiait la dysfonction endothéliale induite par l'exposition des cellules à de grandes concentrations de glucose (Tian et al., 2017).

5) Origines de la dysfonction endothéliale

On admet à l'heure actuelle que la dysfonction endothéliale est le résultat d'une exposition prolongée aux facteurs de risque cardiovasculaire classiques que sont l'hypertension artérielle, le diabète, l'hypercholestérolémie, l'âge, la sédentarité et le tabagisme (Quyyumi, 1998, Lind, 2001). Le risque de développer une dysfonction endothéliale augmente avec le nombre de facteurs de risque cardiovasculaire (Vita et al., 1990, Celermajer et al., 1994). Cependant, l'observation selon laquelle certains individus ne développent pas de manifestations d'athérosclérose malgré la présence de plusieurs facteurs de risque cardiovasculaire suggère l'existence d'un seuil qui doit être dépassé pour conduire à un effet vasculaire défavorable. Compte tenu de son rôle pivot dans le processus athérogène, la dysfonction endothéliale doit être considérée comme le reflet du déséquilibre entre la charge en facteurs de risque cardiovasculaire et la charge en facteurs vasculoprotecteurs (Vogel and Corretti, 1998).

La dysfonction endothéliale pourrait être également la conséquence de l'activation endothéliale induite par l'exposition de l'endothélium aux cytokines proinflammatoires dont le TNF α . En effet, l'exposition de cellules endothéliales aortiques humaines en culture (8h) au TNF α diminue l'expression d'eNOS (MacNaul and Hutchinson, 1993). Ce résultat a été par la suite confirmé sur d'autres types de cellules endothéliales (Zhang et al., 1997, Seidel et al., 2006, Xia et al., 2006, Goodwin et al., 2007). De plus, les études sur vaisseaux isolés (coronaires, artères carotides) montrent que le TNF α réduit la vasodilatation dépendante du NO (Aoki et al., 1989, Ahmad et al., 2002, Picchi et al., 2006, Gao et al., 2007). Les mécanismes par lesquels le TNF α diminue la

production/disponibilité de NO sont divers : inhibition de la dégradation d'ADMA (Ito et al., 1999), induction de l'arginase (Nelin et al., 2001), réduction de la phosphorylation d'eNOS en sérine 1177 en réponse à une augmentation des contraintes de cisaillement (Hoffmann et al., 2001, Kim et al., 2001) et augmentation de l'activité endothéliale de la NADPH oxydase (Frey et al., 2002). La figure 2 qui suit résume les mécanismes susceptibles de diminuer la biodisponibilité en NO suite à l'exposition de la cellule endothéliale aux facteurs de risque cardiovasculaire ou aux cytokines proinflammatoires.

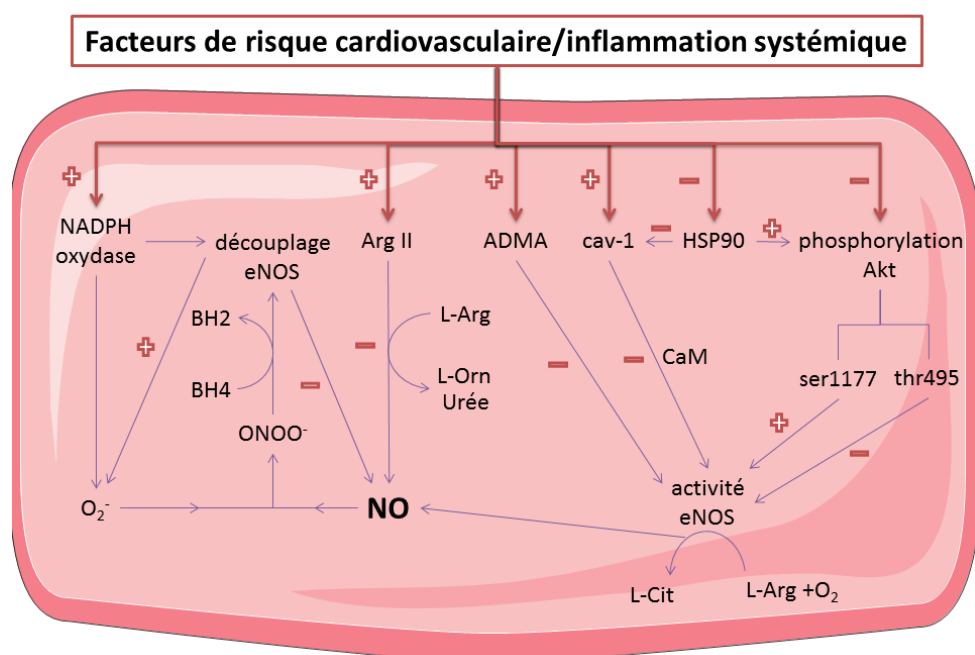


Figure 2: Récapitulatif des mécanismes conduisant à une diminution de la biodisponibilité en NO secondaire à une exposition des cellules endothéliales aux facteurs de risque cardiovasculaire ou à l'inflammation systémique. NADPH=nicotinamide adénine dinucléotide phosphate, eNOS=NO synthase endothéliale, BH4=tetrahydrobioptérine, BH2=dihydrobioptérine, ADMA=asymmetric dimethylarginine, Arg II=arginase II, L-Arg=L-Arginine, L-Orn=L-Ornithine, L-Cit=L-Citrulline, cav-1=cavéoline-1, CaM=calmoduline, HSP90=protéine de choc thermique 90, PKB=protéine kinase B, Ser1177=sérine 1177, Thr495=thréonine 495.

6) La dysfonction endothéliale, une maladie systémique

Bien que chaque lit vasculaire comprenne des cellules endothéliales, le phénotype des cellules endothéliales diffère entre la macro et la microcirculation, entre les différents lits vasculaires de la microcirculation (Kieda, 2013) et entre l'endothélium des vaisseaux de la circulation périphérique et ceux de la circulation cérébrale. Par exemple, concernant la vasodilatation dépendante de l'endothélium, la contribution du NO diminue au profit de l'EDHF à mesure que la taille des artères diminue (Triggle and Ding, 2011). De la même façon, la réponse des cellules endothéliales aux cytokines proinflammatoires concernant la synthèse des prostaglandines (diminution), l'expression des

molécules d'adhésion (augmentation) ou la production de NO (diminution) est plus importante pour les macrovaisseaux que pour les microvaisseaux (Gerritsen et al., 1993, Geiger et al., 1997). Cependant, la dysfonction endothéliale est considérée comme une affection « systémique » qui affecte à la fois les artères de conductance, site privilégié du développement des plaques athéromateuses, et les microvaisseaux, site des échanges entre le sang et les tissus. Il semblerait que la dysfonction endothéliale microvasculaire précède la dysfonction endothéliale macrovasculaire en tout cas pour la circulation périphérique (Gutterman et al., 2016). Ainsi, dans deux grandes cohortes d'hommes d'âge moyen (~ 50 ans) en bonne santé, la dysfonction endothéliale n'a été observée qu'à l'étage microvasculaire et a été associée aux futurs événements cardiovasculaires (Anderson et al., 2011) ou s'est révélée capable de prédire l'athérosclérose coronarienne (Lind et al., 2011) contrairement à celle au niveau macrovasculaire. En revanche, la dysfonction endothéliale macrovasculaire pourrait être un bon prédicteur des événements cardiovasculaires chez les patients déjà atteints d'athérosclérose (Flammer et al., 2012, Vizzard et al., 2014). **Un point qui n'a jamais été abordé est de savoir si la dysfonction endothéliale des vaisseaux périphériques précède ou pas la dysfonction endothéliale des vaisseaux cérébraux.**

La question qui se pose concerne le lien entre la dysfonction endothéliale microvasculaire et la dysfonction à l'étage macrovasculaire. On suppose que les changements fonctionnels dans les différents lits microcirculatoires conduisent à un remodelage dans les artères de conductance en amont (Lockhart et al., 2009). Notamment, la réponse inflammatoire microvasculaire, caractérisée par une activation endothéliale initiée dans la microcirculation, joue un rôle important dans le développement du processus athérosclérotique dans les gros vaisseaux (Stokes and Granger, 2005). À son tour, le raidissement des gros vaisseaux peut entraîner une augmentation de la pression pulsatile pouvant endommager davantage les lits microvasculaires et causer des dommages aux organes cibles (Chantler and Frisbee, 2015), indiquant ainsi un cercle vicieux d'interactions entre les artères de résistance et de conductance.

7) Exploration de la fonction endothéliale

Différentes techniques sont disponibles pour explorer la fonction endothéliale que ce soit chez l'Homme ou chez l'animal.

a) Chez l'Homme

L'exploration clinique de la fonction endothéliale consiste à étudier l'augmentation du diamètre vasculaire induit par des réactifs pharmacologiques reconnus pour induire une vasodilatation dépendante de l'endothélium comme l'acétylcholine ou par une augmentation du flux sanguin en réponse à l'hyperémie postischémique. Les principales méthodes d'exploration de la fonction

endothéliale macro et microvasculaire avec leurs avantages et inconvénients sont présentées dans le Tableau 1.

Tableau 1: Avantages et inconvénients des techniques les plus couramment utilisées pour évaluer la fonction endothéliale. FMD=flow-mediated dilation, VOP=venous occlusion plethysmography, PAT=peripheral arterial tonometry. Ach=acétylcholine.

Technique	Lit vasculaire	Avantages	Inconvénients	Stimulus appliqué (exemples)
FMD	Macrocirculation	Réalisation facile Faible cout	Difficile à standardiser	Hyperhémie réactive
VOP	Microcirculation brachiale	Réalisation facile Bras controlatéral comme contrôle	Invasif Chronophage	Injection de substances vasoactives (Ach)
EndoPAT	Microcirculation digitale	Réalisation facile Automatisé Faible variabilité inter/intra observateur	Coût élevé Signal PAT influencé par des facteurs non endothéliaux	Hyperhémie réactive

b) Chez l'animal

Historiquement, l'utilisation d'anneaux aortiques isolés de lapin a permis de démontrer le rôle obligatoire de l'endothélium dans la relaxation vasculaire (Furchgott and Zawadzki, 1980). Cette méthode d'organes isolés est devenue la technique standard d'exploration de la fonction endothéliale *ex vivo*. L'évaluation de la réponse vasodilatatrice dépendante de l'endothélium est réalisée sur des segments artériels en réponse à différentes combinaisons de substances induisant une vasodilatation dépendante de l'endothélium (Ach par exemple) à l'aide d'un myographe. En outre, les mécanismes dépendants de l'endothélium impliqués dans des modulations de réponses vasodilatrices peuvent être directement mis en évidence grâce à la possibilité de dénuder les anneaux aortiques de l'endothélium. Il faut cependant signaler la nature artificielle de ces mesures ne reflétant pas le comportement physiologique des cellules endothéliales confrontées par nature à notamment des variations du flux sanguin.

Parallèlement aux méthodes d'exploration *in vivo* de la fonction endothéliale chez l'Homme, des techniques hémodynamiques ont été développées chez l'animal (Heiss et al., 2008). Ainsi, des cathéters peuvent être placés au niveau de l'aorte, du sinus coronarien ou des artères fémorales afin de mesurer des variations de flux sanguin en réponse à des injections de substances vasoactives administrées à l'aide de cathéters placés au niveau de la veine fémorale et/ou de la veine jugulaire externe (Le Brocq et al., 2008). Généralement, ces mesures sont réalisées sur des animaux anesthésiés, ce qui peut altérer les réponses hémodynamiques aux substances pharmacologiques.

En résumé, la dysfonction endothéliale, qui peut se définir par une diminution de la production ou une inactivation du NO par l'endothélium, est capable de perturber un grand nombre de fonctions physiologiques (perméabilité capillaire, débit sanguin des organes, fluidité du sang, angiogénèse, prolifération des cellules musculaires lisses, extravasation des globules blancs) à l'origine de l'initiation du processus athérosclérotique. Restaurer le NO d'origine endothéliale semble être alors un bon moyen thérapeutique pour ralentir le développement du processus athérosclérotique.

Chapitre 2

Risque cardiovasculaire & cognition

1) Le constat

1) Chez l'Homme

Aujourd'hui, un grand nombre d'études a mis en avant une altération de la fonction cognitive et un risque accru de démence (y compris de maladie d'Alzheimer et de démence vasculaire) chez les personnes ayant des antécédents de maladies cardiovasculaires comme les maladies coronariennes, l'insuffisance cardiaque et la fibrillation auriculaire (Vogels et al., 2007, Eggermont et al., 2012, Santangeli et al., 2012, Cannon et al., 2017). De plus, les facteurs de risque cardiovasculaire classiques comme l'hypertension, l'hyperlipidémie, le diabète, l'excès d'adiposité, le syndrome métabolique et la sédentarité, ainsi que les modèles de prédiction du risque de maladies cardiovasculaires (Framingham) et le risque de maladies coronariennes ont été associés à un déficit cognitif accru (Biessels et al., 2006, Purnell et al., 2009, Harrison et al., 2014, Harrison et al., 2017). Les atteintes cognitives associées aux facteurs de risques cardiovasculaires les plus fréquemment rapportées sont les troubles de l'attention, des fonctions exécutives et du traitement des données (Kramer et al., 2002, Dregan et al., 2013, Keage et al., 2015).

Par ailleurs, un grand nombre d'études épidémiologiques a révélé que les patients ayant des antécédents de maladies cardiovasculaires ou présentant des facteurs de risque cardiovasculaire (diabète, hypertension) étaient plus enclins à développer une dépression (Egede, 2007, Egede and Ellis, 2010, Katon et al., 2010, Davidson, 2012). En faveur d'un lien entre dépression et dysfonction endothéliale, les patients dépressifs présentent une rigidité artérielle plus marquée, un rapport intima-média plus faible (Tiemeier et al., 2003, Rybakowski et al., 2006, Paranthaman et al., 2010) et une réduction de vasodilatation induite par le flux que ce soit à l'étage macrovasculaire (Rajagopalan et al., 2001, Broadley et al., 2002) ou microvasculaire (Tomfohr et al., 2011, Shi et al., 2015). Plus récemment, il a été montré que les patients dépressifs étaient plus à risque d'être victime d'une maladie cardiovasculaire (Kuehl et al., 2012, Fiedorowicz, 2014), faisant de la dépression un facteur de risque cardiovasculaire non conventionnel.

2) Chez l'animal

Chez l'animal, le déficit cognitif est évalué à partir de tests comportementaux (test de la piscine de Morris évaluant l'apprentissage et la mémoire spatiale, test du labyrinthe à bras radial évaluant la mémoire spatiale, test de reconnaissance d'objet évaluant la mémoire de travail non spatiale, test de Barnes évaluant la mémoire spatiale, ...), et de l'étude de la potentialisation à long terme (long-term potentiation, LTP). Le déficit cognitif a été étudié sur les modèles animaux de pathologies associées à des facteurs de risque cardiovasculaire. Ainsi, un déficit cognitif a été rapporté chez les rats rendus diabétiques par la streptozocine (Flood et al., 1990, Biessels et al., 1996, Kamal et al., 2000), chez les

rats obèses Zucker (Winocur et al., 2005), les rats hypertendus (Terry et al., 2000, Diana, 2002, Hacıoglu et al., 2003, Pietranera et al., 2010, Shih et al., 2016) ou âgés (Barnes, 1979, Rapp et al., 1987, Moore et al., 1993, Diana et al., 1994).

Chez l'animal, la dépression est classiquement évaluée par le test de la nage forcée de Porsolt (chez le rat) qui identifie un état de résignation ou le test de préférence au sucre (chez la souris ou le rat) qui évalue l'anhédonie. De façon intéressante, une dépression a été retrouvée chez la souris diabétique db/db (Sharma et al., 2010) ou le rat rendu diabétique par la streptozocine (Hilakivi-Clarke et al., 1990, Haider et al., 2013), les souris obèses ob/ob (Collin et al., 2000, Yamada et al., 2011), les rats obèses Zucker (Brooks et al., 2018) ou encore les rats spontanément hypertendus (Tchekalarova et al., 2011).

II) Liens mécanistiques entre risque CV et cognition

Les mécanismes impliqués restent encore hypothétiques. Cependant, les mécanismes les plus souvent évoqués sont une altération du débit sanguin cérébral (DSC), de la neuroplasticité/neurogénèse et de la perméabilité de la BHE en réponse à la dysfonction endothéliale.

1) Altération du DSC

L'endothélium contribue à l'adaptation du DSC aux besoins des neurones (décrit dans la revue pages 46). La mesure de la réactivité cérébrovasculaire à l'hypercapnie (Lavi et al., 2006) ou à la L-arginine (Pretnar-Oblak, 2014) est souvent utilisée chez l'Homme pour évaluer l'impact de la dysfonction endothéliale sur l'apport sanguin au cerveau. En effet, la vasodilatation induite par le CO₂ et la L-arginine met en jeu la production de NO par l'endothélium (Iadecola, 1992, Pretnar-Oblak et al., 2007).

En faveur d'une altération de l'adaptation du DSC en cas de risque cardiovasculaire, la réactivité cérébrovasculaire au CO₂ est moindre en cas de diabète (Novak et al., 2006, Last et al., 2007, Brundel et al., 2012), d'hypertension artérielle (Ficzere et al., 1997, Settakis et al., 2003, Claassen et al., 2009, Tchistiakova et al., 2014) ou chez les personnes âgées (Panczel et al., 1999, Niehaus et al., 2001, Fisher et al., 2013, Jennings et al., 2013) bien que l'effet de l'âge soit controversé (Schwertfeger et al., 2006, Galvin et al., 2010). Une altération de la réactivité cérébrovasculaire à la L-arginine a également été rapportée chez les patients diabétiques (Kozera et al., 2009) et hypertendus (Kozera et al., 2010).

De façon intéressante, le débit sanguin cérébral au repos est diminué chez le rat rendu diabétique par la streptozocine (Yuen et al., 2008), le rat Zucker obèse (Osmond et al., 2009), le rat spontanément hypertendu (Huang et al., 2016) ou encore chez le rat âgé (Salter et al., 1998).

2) Altération de la neuroplasticité/neurogénèse

La neurogénèse et la neuroplasticité sont deux processus clés qui sous-tendent les processus cognitifs (mémoire, apprentissage). Le principal argument pour penser que la dysfonction endothéliale peut altérer la neurogénèse et la neuroplasticité est que ces 2 processus sont modulés positivement par le NO dérivé de l'endothélium cérébral. Il est important de retenir que le NO d'origine endothéliale peut migrer sur une distance de 100 μm (Seidel et al., 1997, Stanarius et al., 1997) et que la distance entre les capillaires sanguins et les neurones est de 25 μm (Pawlik et al., 1981).

a) Altération de la neuroplasticité

Chez l'animal, les qualités neuroplastiques du cerveau sont le plus souvent évaluées par des études de la LTP hippocampique qui peut se définir par une augmentation importante et durable du nombre et de l'efficacité des synapses. En faveur d'un lien entre neuroplasticité et facteurs de risque cardiovasculaire, une diminution de la LTP a été observée chez le rat rendu diabétique par administration de la streptozocine (Kamal et al., 1999, Artola et al., 2005), le rat Zucker obèse (Gerges et al., 2003, Alzoubi et al., 2005), le rat spontanément hypertendu (Hennigan et al., 2009) ou encore le rat âgé (Landfield et al., 1978, Barnes, 1979, Shankar et al., 1998). En faveur de l'implication d'une moindre production endothéliale de NO dans le lien existant entre neuroplasticité et facteurs de risque cardiovasculaire, les LTP corticale et striatale sont altérées chez la souris knock-out (KO) eNOS comparativement à son contrôle sauvage (Haul et al., 1999, Doreulee et al., 2003). De plus, Hopper et Garthwaite (2006) ont montré que la production de NO par l'endothélium vasculaire était nécessaire à la LTP, cet effet étant dépendant de la production de GMPc (Bon and Garthwaite, 2003). Il est important de souligner que dans l'hippocampe, eNOS est exclusivement exprimée par l'endothélium vasculaire (Blackshaw et al., 2003).

b) Altération de la neurogénèse

La neurogénèse hippocampique est moindre en cas de pathologies associées à des facteurs de risque cardiovasculaire. Ainsi, une réduction de la neurogénèse est présente chez les rats obèses Zucker ou les souris db/db par rapport à leurs homologues non diabétiques (Stranahan et al., 2008, Yi et al., 2009), chez l'animal rendu diabétique par la streptozocine, (Beauquis et al., 2006, Kang et al., 2006, Saravia et al., 2006, Balu et al., 2009, Piazza et al., 2011), chez le rat hypertendu (Sabbatini et al., 2002, Tomassoni et al., 2004, Pietranera et al., 2006, Pietranera et al., 2010) ou âgé (Kuhn et al., 1996, Bizon et al., 2004).

L'impact négatif de la dysfonction endothéliale sur la neurogénèse pourrait impliquer une moindre production de NO par les capillaires cérébraux. En effet, en faveur de cette hypothèse, la neurogénèse

hippocampique ainsi que les capacités cognitives sont moindres chez les souris KO eNOS comparées aux souris sauvages (Chen et al., 2005, Li et al., 2018).

3) Altération de la perméabilité de la BHE

La dysfonction endothéliale pourrait contribuer aux déficits cognitifs via une augmentation de la perméabilité de la BHE aux substances neurotoxiques notamment au peptide A β (peptide fortement impliqué dans la neurodégénérescence associée à la maladie d'Alzheimer). En faveur de cette hypothèse, les patients avec des facteurs de risque cardiovasculaire comme l'hypertension artérielle (Launer et al., 2000, Kivipelto et al., 2002, Whitmer et al., 2005, Joas et al., 2012), le diabète (Biessels et al., 2006), l'obésité (Besser et al., 2014, Gu et al., 2014, Tolppanen et al., 2014) ou l'hypercholestérolémie (Reed et al., 2014) ont plus de risque de développer une maladie d'Alzheimer. Dede et al. (2007) ont été les premiers à montrer la présence d'une dysfonction endothéliale par FMD dans la maladie d'Alzheimer, celle-ci étant indépendante de l'âge. En faveur d'un lien entre NO et accumulation de peptide A β dans le cerveau, l'inactivation pharmacologique ou génétique de la eNOS augmente la production du peptide A β par des cellules endothéliales cérébrales en culture (Austin et al., 2010). En accord avec ces résultats, il existe une corrélation inverse entre la densité de capillaires positifs pour eNOS et la présence de plaques séniles dans les échantillons corticaux de cerveaux de patients atteints de la maladie d'Alzheimer (Provias and Jeynes, 2008, Jeynes and Provias, 2009). De manière intéressante, il a été récemment montré que l'administration de s-nitrosogluthathion, un donneur endogène de NO, était capable de réduire les dépôts de peptides A β et d'augmenter les taux hippocampiques de BDNF dans un modèle animal de maladie d'Alzheimer (Dubey et al., 2018).

En résumé, la dysfonction endothéliale cérébrale, qui peut se définir par une diminution de la production ou une inactivation du NO par l'endothélium cérébral, est capable d'affecter les capacités cognitives via une réduction du débit sanguin cérébral, des processus de neuroplasticité/ neurogénèse ou encore une altération de la perméabilité de la barrière hématoencéphalique. Restaurer le NO cérébral d'origine endothéliale semble être alors un bon moyen pour ralentir le développement du déficit cognitif

Chapitre 3

Le BDNF

I) Introduction

En 1982, Barde et coll. isolent du cerveau porcine un peptide capable d'augmenter la survie des neurones en culture (Barde et al., 1982). Ils nomment en toute logique ce peptide brain-derived neurotrophic factor (BDNF) en analogie avec le NGF (Nerve Growth Factor) découvert près de 30 années plus tôt (Cohen et al., 1954). Depuis sa découverte, le BDNF neuronal a fait l'objet d'une pléthore d'études et est actuellement considéré comme essentiel au fonctionnement du SNC via l'activation des récepteurs TrkB neuronaux (décrit en détails dans la revue pages 41-55). Le BDNF est également présent dans le sang où ses taux sont le plus souvent considérés par les neurologues et les psychiatres comme le reflet des taux présents dans le SNC et donc de la fonction cérébrale. Cependant, notre laboratoire a récemment découvert que le système cardiovasculaire adulte contenait autant de BDNF que le cerveau et que les expressions vasculaire et cardiaque du BDNF étaient essentiellement endothéliales (Prigent-Tessier et al., 2013). Ces nouvelles données soutiennent l'hypothèse selon laquelle le BDNF d'origine endothéliale pourrait être un nouveau marqueur de la fonction endothéliale et qu'il pourrait à ce titre être impliqué dans la cognition du moins pour le BDNF synthétisé par les capillaires cérébraux.

II) BDNF cérébral

Nous avons montré au laboratoire (Monnier et al., 2017b) que le retrait de l'endothélium cérébral par un détergent, le 3-[(3-Cholamidopropyl) diméthylammonio]-1-propanesulfonate hydrate (CHAPS), diminuait les taux de BDNF dans le cerveau de moitié suggérant ainsi que le BDNF présent dans le cerveau correspond pour moitié au BDNF présent dans les neurones et pour l'autre moitié au BDNF présent dans les cellules endothéliales. Ces données suggèrent que la santé cérébrale pourrait être dépendante non seulement du BDNF d'origine neuronale mais aussi du BDNF d'origine endothéliale. Afin de faire le point sur le sujet, nous avons écrit une revue qui présente les données acquises et manquantes sur le BDNF cérébral qu'il soit neuronal ou endothélial.

Article 1

Titre:

Brain-derived neurotrophic factor secreted by the cerebral endothelium: A new actor of brain function?

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Review Article

Brain-derived neurotrophic factor secreted by the cerebral endothelium: A new actor of brain function?

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Abstract

Low cerebral levels of brain-derived neurotrophic factor (BDNF), which plays a critical role in many brain functions, have been implicated in neurodegenerative, neurological and psychiatric diseases. Thus, increasing BDNF levels in the brain is considered an attractive possibility for the prevention/treatment of various brain diseases. To date, BDNF-based therapies have largely focused on neurons. However, given the cross-talk between endothelial cells and neurons and recent evidence that BDNF expressed by the cerebral endothelium largely accounts for BDNF levels present in the brain, it is likely that BDNF-based therapies would be most effective if they also targeted the cerebral endothelium. In this review, we summarize the available knowledge about the biology and actions of BDNF derived from endothelial cells of the cerebral microvasculature and we emphasize the remaining gaps and shortcomings.

Keywords

Brain-derived neurotrophic factor, cerebral endothelium, cognition, nitric oxide, tropomyosin-related kinase B

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Introduction

In the brain, the synaptically released neurotrophin brain-derived neurotrophic factor (BDNF) has emerged as a real mediator of synaptic plasticity and synaptic communication. Therefore, neuronal-derived BDNF and its cognate TrkB (tropomyosin-related kinase B) receptors have been considered promising targets for the treatment of many neurological, neurodegenerative and psychiatric diseases.¹ However, in contrast to the traditional thinking that BDNF present in the adult brain is mostly neuronal BDNF,² the in situ removal of the endothelium from the cerebral microvasculature was reported to result in a marked decrease in brain BDNF levels.³ Endothelium removal was achieved by a brain perfusion (by transcatheter route) with a 0.2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) solution. Importantly, as BDNF is also present in blood, CHAPS perfusion was preceded by saline perfusion in order to avoid blood contamination of the brain as a confounding factor. More precisely, BDNF levels analyzed by Western blotting were twice lower in brain

without endothelial cells than in brain with intact endothelium. Even though Western blotting is only a semi-quantitative method, these data suggest that ~50% of BDNF levels measured in brain homogenates correspond to BDNF expressed by endothelial cells. This paradigm shift in our understanding of the cellular source of brain BDNF combined with evidence that cultured cerebral endothelial cells (CECs) secrete BDNF in a bioactive form^{4–6} supports the exciting hypothesis that normal brain functioning might be dependent on BDNF synthesis and secretion by the cerebral endothelium and that endothelial BDNF might be the missing link between endothelial function

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and cognition. Unfortunately, research on cerebrovascular BDNF is still in its infancy as compared with the major progress in our understanding of neuronal BDNF synthesis, secretion and functions. In the present review, after a brief overview of neuronal-derived BDNF, we will present the specificities of the endothelium of the cerebral microvasculature. Then, after a presentation of the methodological considerations regarding the measurement/detection of endothelial BDNF, we will present the available knowledge on secretion, regulation and functions of BDNF derived from endothelial cells of the cerebral microvasculature. Finally, the key shortcomings to resolve in future research will be summarized.

Neuronal-derived BDNF

Neuronal synthesis, storage and release of BDNF have been reviewed in detail elsewhere.^{7,8} This paragraph only summarizes what is known on neuronal-derived BDNF with the objective to show readers that research on cerebrovascular BDNF is still in the early stage as compared with research on neuronal BDNF.

Synthesis and secretion

BDNF was first discovered in the porcine brain.⁹ This probably explains why almost all of what is known about BDNF synthesis and secretion concerns neuronal BDNF. Like all peptides, BDNF synthesis starts from the transcription of the *BDNF* gene into BDNF mRNAs which are then processed into pre-proBDNF, proBDNF and mature BDNF (mBDNF) via the Golgi apparatus and the trans-Golgi network. The secretion of mBDNF by neurons is both constitutive and regulated, the latter being preferential and activity-dependent.¹⁰ The fact that mBDNF secretion is under the control of neuronal activity-dependent mechanisms is important as it explains how mBDNF modulates the synaptic plasticity in response to experience and environment. BDNF exerts its action primarily in an anterograde fashion,^{11–14} i.e. BDNF is released by axons and binds to presynaptic and postsynaptic receptors. As the widespread diffusion of mBDNF is prevented by its high molecular weight (28 kDa for the mBDNF homodimer) and its positive charge under physiological conditions (isoelectric point close to 10¹⁵), BDNF secreted by axons only acts locally. For instance, it has been shown that the neuronal source of BDNF has to be within a distance of 4.5 μ m to induce dendritic growth in the recipient neurons.¹⁶ However, there is substantial evidence that BDNF can also participate in retrograde local or axonal signaling. Here, BDNF is released from postsynaptic neurons and binds to presynaptic receptors. According to local retrograde signaling, BDNF

elicits a rapid change in presynaptic activity.¹⁷ By contrast, according to axonal retrograde signaling, BDNF induces delayed changes in transcription processes as a result of the transport of BDNF-TrkB complex from the distal terminal to the nucleus as previously described for nerve-growth factor.¹⁸ However, whether or not dendrites secrete BDNF is a matter of debate. While BDNF secretion by dendrites was reported to occur in cultured neurons,^{19,20} immunoreactivity experiments on mice hippocampus failed to detect the presence of granules containing BDNF in dendrites.¹⁴

Although controversial,²¹ cultured neurons were reported to secrete proBDNF^{22,23} that is subsequently cleaved into mBDNF in the extracellular space by plasmin.²⁴ Plasmin, which is generally expressed in neurons as an inactive plasminogen, must be activated by proteolytic cleavage by tissue plasminogen activator (t-PA). Both proBDNF and t-PA secretion by neurons seems to be activity-dependent²⁵ but only high-frequency neuronal activity induces t-PA secretion.²³ Of interest, mBDNF stimulates the expression of t-PA in primary cultures of cortical neurons in a time- and concentration-dependent manner.^{26,27}

Neuronal receptors activated by mBDNF

mBDNF binds to two kinds of plasma membrane receptors, TrkB receptors and pan75 neurotrophin receptor (p75^{NTR}), the affinity of mBDNF being much higher for the former type (dissociation constant $\sim 10^{-11}$ M vs. 10^{-9} M). In fact, p75^{NTR} is the preferential receptor for proBDNF, and activation of p75^{NTR}/sortilin complex often leads to the activation of apoptotic pathways and death.^{28–30} TrkB receptors include full-length (FL) receptors, which are linked to a tyrosine kinase domain, and truncated (T1, T2, T3) receptors, which are devoid of a tyrosine kinase domain.^{31–33} TrkB-FL and -T receptors share the same extracellular domain and consequently bind mBDNF with the same affinity. The binding of mBDNF to membrane TrkB-FL receptors induces trans-phosphorylation of TrkB cytoplasmic domain tyrosine residues, initiating recruitment of signaling adapter proteins that foster signaling by ras/ERK1/2, PI3 kinase/Akt STAT and phospholipase C γ pathways.³⁴ This pathway has proven to be of elementary importance in the ability of mBDNF to promote neurogenesis, neuroplasticity and neuronal survival. Then, the complex BDNF/TrkB-FL is internalized by endosomes from which the receptor is either degraded by lysosomes or recycled back to the cell surface or delivered to the neuronal soma (axonal retrograde transport) in order to permit control of nuclear transactivation of genes. Interestingly, evidence that regulation of TrkB transcription, TrkB mRNA trafficking, TrkB insertion at the neuronal surface and

BDNF-TrkB complex endocytosis are activity-dependent³⁵ may explain why the actions of BDNF are restricted to active synapses. The other major TrkB receptor expressed by neurons is TrkB-T1. In cells that co-express TrkB-FL and TrkB-T1, the binding of mBDNF to TrkB-T1 results in the inhibition of TrkB-FL signaling. However, in addition to this role of a dominant negative receptor, TrkB-T1 might induce its own signaling.³³ Finally, the formation of heteromeric complexes between TrkB isoforms and p75^{NTR},³⁶ which are frequently coexpressed in the same cells, contributes to the complexity of the neurotrophic response. Another source of complexity is the activation of TrkB-FL in the absence of mBDNF.^{37,38}

The human BDNF variant Val66Met

In humans, a Val66Met polymorphism has been identified in the *BDNF* gene. Approximately 30% of people worldwide are heterozygous (Val/Met) for the methionine substitution at codon 66 in the prodomain of the *BDNF* gene and this percentage varies, depending on both the geographical region and ethnicity.³⁹ Studies in neurosecretory cells and primary cultured neurons have shown that the Met substitution interferes with intracellular trafficking of proBDNF to the secretory pathway, thus resulting in reduced activity-dependent secretion of mBDNF.^{40,41} A number of clinical studies have reported lower cognitive performance,^{40–42} increased susceptibility to depression and anxiety,^{43,44} greater errors in short-term motor learning⁴⁵ and reduced neuroplastic response to physical exercise and post-stroke rehabilitation^{46,47} in Met allele carriers. However, other studies failed to report a major impact of this polymorphism on brain functioning.^{48,49}

Endothelium of the cerebral microvasculature

This paragraph provides a short anatomical and functional description of the endothelium of the cerebral microvasculature. This knowledge represents a basis for discussing the potential fate of endothelial-derived BDNF and its potential recipient cells in vivo.

Localization

The endothelium of the cerebral microvasculature corresponds to the monolayer of endothelial cells lining intraparenchymal brain vessels from arterioles to venules with endothelial cells anchored to the endothelial basement membrane. A particularity of the cerebral microvasculature is that astrocyte end-feet completely covers the wall of vessels present in the brain neuropil⁵⁰ even though capillaries were found to have

substantially more processes contacting them per unit surface area than arterioles and venules.⁵¹ For precapillary arterioles, capillaries and postcapillary venules, endothelial cells are surrounded by pericytes (30% coverage), which are encased in the endothelial basement membrane (Figure 1) and display heterogeneity in terms of morphology and contractile protein expression as a function of position along the capillary bed.⁵² For arterioles and venules, the vascular wall includes the intima layer (endothelial cells separated from an internal elastic lamina by the endothelial basement membrane), the media layer, which comprises a single layer of smooth muscle cells (SMCs) surrounded by a basement membrane and the adventitial layer (fibroblasts not surrounded by a basement membrane and collagen fibers) (Figure 2). In these vessels, endothelial cells are separated from SMC by the endothelial basement membrane, which adheres to the basal surface of endothelial cells, and from the internal elastic lamina and the SMC basement membrane, which adheres to the surface of SMC. However, endothelial cells form extensions that project through fenestrations in the internal elastic lamina and basement membranes to contact SMC directly.⁵³ These extensions (termed myoendothelial projections) provide direct contact between endothelial cells and SMC through gap junctions but do not allow the diffusion of mBDNF. In contrast, direct cell–cell contact has not been observed between astrocytic end-feet and SMC. Finally, the notion of neurogliovascular units (NGVUs) emphasizes the interplay between endothelial cells of capillaries and neighboring pericytes, astrocytes and neurons. Importantly, this interplay probably involves a paracrine pathway. Indeed, it is unlikely that a heterotypic gap junction between these different cell types can be established as endothelial cells, pericytes and astrocytes are physically separated from each other by a basal lamina.⁵⁰

Features and functions of brain endothelial cells

Brain endothelial cells are phenotypically unique and different from endothelial cells in the periphery. They have apical tight junction complexes that prevent paracellular diffusion but express specific transporters to actively transport nutrients from the blood into the brain. Endothelial cells also basolaterally express transporters to remove toxic substances from the brain into the blood. Other unique features of the cerebral endothelium are a lack of fenestrations, a very low rate of pinocytosis, which limits transcellular transport, and a high number of mitochondria.⁵⁴ Endothelial cells are the most important cellular component of the blood–brain barrier and barrier properties seem to be more expressed in capillaries than in larger vessels⁵⁵ even

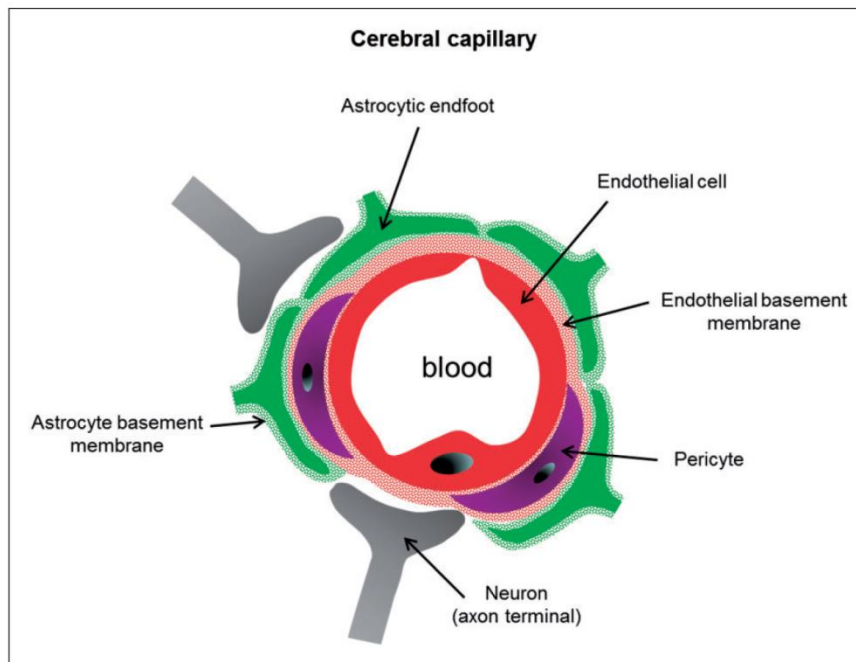


Figure 1. Schematic representation of a transversal section of a cerebral capillary and its neighboring cells. The wall of a cerebral capillary includes a layer of endothelial cells (red) and the basement membrane (red points). This wall is covered by pericytes (purple). Astrocytic end-feet (green) is separated from endothelial cells by the endothelial (red points) and astrocytic (green points) basement membranes. Both astrocytes and pericytes are innervated by local neurons (grey). Note that the drawing is not to true scale.

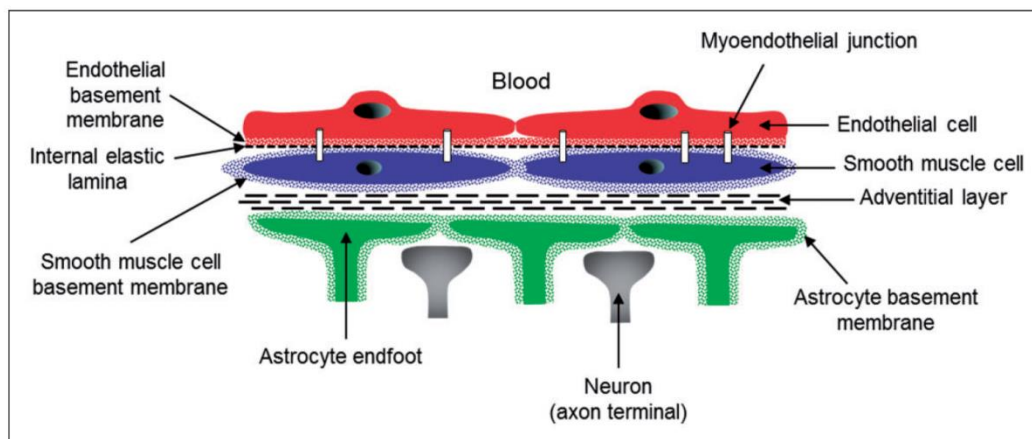


Figure 2. Schematic representation of a longitudinal section of a cerebral arteriole. The wall of cerebral arterioles includes endothelial cells (red), the basement membrane of endothelial cells (red points), the internal elastic lamina (black fine dotted line), a single layer of smooth muscle cells (blue) surrounded by their basement membrane (blue points) and an adventitial layer (large dotted lines) with fibroblasts (not shown). The arterial wall is covered by astrocytic end-feet (green) surrounded by their basement membrane (green points) and is innervated by local neurons (grey). Note the presence of myoendothelial junctions. Note that the drawing is not to true scale.

though the expression of markers of the BBB varies greatly among the endothelial cells of capillaries.⁵⁶ An unresolved point concerns the permeability of the BBB to BDNF⁵⁷⁻⁵⁹ even though certain studies^{60,61} but not

all^{62,63} reported positive correlations between blood and regional brain BDNF levels. Besides being implicated in barrier properties, the endothelium of the cerebral microcirculation is also involved in numerous

processes, including the regulation of inflammatory and immune responses,⁶⁴ thrombosis,⁶⁵ neuroplasticity,⁶⁶ angiogenesis⁶⁷ and cerebral blood flow.^{68,69} Endothelial cells of the neural stem niches were also reported to stimulate self-renewal and expand neurogenesis.⁷⁰

Methods to investigate endothelial BDNF synthesis and secretion

BDNF synthesis by endothelial cells of the cerebral microvasculature can be investigated by measuring levels of BDNF mRNA, proBDNF or mBDNF protein in cell lysates prepared from primary or immortalized CECs or cerebral microvessel-enriched fractions. The contamination of these preparations with either pericytes or astrocytes, which have been reported to express BDNF,⁷¹⁻⁷⁴ may be a confounding factor. The secretion of BDNF (proBDNF or mBDNF) by endothelial cells can be assessed from changes in levels of these compounds in CEC culture medium. Importantly, the measurement of phosphorylated TrkB-FL receptor levels in brain homogenates, which is traditionally used to gather information on mBDNF secretion *in vivo*, fails to identify the cellular source of mBDNF (neuronal vs. non-neuronal cells). It must also be kept in mind that levels of proBDNF (35 kDa) and mBDNF (14 kDa) protein can be measured separately using Western blotting analysis only. Indeed, while commercial ELISA kits are available for BDNF measurement, they use antibodies that are not specifically directed against mBDNF and the affinity of antibodies for mBDNF and proBDNF differs from one kit to another.⁷⁵ The same concern is valid for antibodies used to localize mBDNF by immunoreactivity experiments. In the following paragraphs, the term "mBDNF" will be used when data have been provided by Western blotting analysis. When data come from the measurement of BDNF by ELISA kits or from immunoreactivity experiments, the term "BDNF" will be used.

Expression and secretion of BDNF by CECs in baseline conditions

To the best of knowledge, Leventhal et al.⁴ published the first paper on endothelial BDNF. The authors showed that primary human CEC and human umbilical vein endothelial cells (HUVEC) produced BDNF mRNA and constitutively released the bioactive form of BDNF. Later, BDNF mRNA levels were reported to be five times lower in HUVEC than in human CEC⁷⁶ suggesting that transcription processes are less activated, or alternatively that BDNF mRNA processing is more rapid in HUVEC than in human CEC. An interesting but often neglected finding is that the rate of BDNF production by mouse CEC cultured in static conditions

was estimated to be 50 times greater than that by cultured (unstimulated) neurons.⁵ Evidence that cerebral microvessel-enriched fractions (isolated from the rat cortex) express BDNF mRNA and mBDNF in baseline conditions is more recent^{3,77} and clearly indicates that the cerebral microvasculature synthesizes and secretes mBDNF *in vivo*. However, BDNF in CECs might also originate from the fusion of BDNF-containing exosomes with endothelial cells. Indeed, more than 90% of blood BDNF is stored in platelets⁷⁸ and platelet BDNF is present not only in cytoplasmic but also in a vesicle (alpha granules) pool.^{79,80}

Cultured CEC secrete a bioactive form of BDNF as evidenced by the fact that the neuroprotective and angiogenic effect of conditioned media from cultured CEC is inhibited by the removal of BDNF or the application of TrkB receptor antagonists (see below). Cultured human CEC have revealed that BDNF secretion is both constitutive and regulated and that BDNF secretion in the medium decreased with the number of cell passages.⁸¹ One question that remains to be investigated is whether cultured CEC secrete proBDNF, and if so, whether t-PA secreted by endothelial cells (a major source of t-PA) is involved in the extracellular maturation of the peptide. Another point that deserves further investigation is whether the BDNF Val66Met polymorphism affects BDNF processing and subsequent mBDNF release by the cerebral endothelium. Strikingly, a genetic knock-in mouse carrying the human BDNF Val66Met polymorphism displays a hypercoagulable state,⁸² reduced endothelial cell proliferation and vessel density in response to stroke.⁸³ In addition, in patients with reversible cerebral vasoconstriction syndrome, vasoconstriction scores were higher in patients with the Val66Met polymorphism.⁸⁴ Collectively, these data support the notion of an interaction between vascular biology and the Val66Met polymorphism. However, whether this polymorphism results in the decreased secretion of mBDNF by endothelial cells is not known.

Modulation of endothelial BDNF synthesis/secretion

Control of endothelial BDNF synthesis/secretion by CECs has been poorly investigated and available studies have been mainly restricted to the role of nitric oxide (NO), the best characterized endothelium-derived factor, even though control by pro-inflammatory cytokines and hypoxia was also explored.

Modulation by NO

The role of NO in endothelial BDNF synthesis/secretion was first investigated using the exposure of

cultured mouse CEC to a NO donor. The NO donor, NOC-18, was first reported to induce BDNF overexpression by immortalized mouse CEC.⁸⁵ By contrast, exposure of mouse CEC to the NO donor sodium nitroprusside (SNP) decreased BDNF levels in the medium.⁵ These apparently discrepant results may be explained by differences in the chemical nature of NO donors and resulting changes in intracellular NO concentrations. Indeed, unlike SNP, which rapidly increases NO concentration, NOC-18 is a slow-releasing NO donor and consequently delivers NO more progressively. Consistent with this hypothesis, the exposure of cerebral microvessel-enriched fractions (isolated from the rat cortex) to glyceryl trinitrate (GTN), which is another slow-releasing NO donor, also increased mBDNF levels.³ As NOC-18 and GTN better mimic endogenous production of NO by endothelial cells, it is tempting to suggest that NO positively controls endothelial BDNF synthesis via an autocrine pathway. One argument for this hypothesis is the strong correlation that we found in rat cerebral microvessel-enriched fractions between eNOS phosphorylated at serine 1177 (peNOS^{ser1177} as an index of sustained NO production⁸⁶) and mBDNF levels after modulation of peNOS^{ser1177} by hypertension (decrease) and physical training (increase).⁸⁷ This association between endothelial NO production and mBDNF expression is in line with the stimulating effect of shear stress elevation on mBDNF synthesis and secretion by HUVEC⁸⁸ since shear stress is the most potent stimulus to induce phosphorylation of eNOS at serine 1177 in HUVEC.⁸⁹ A positive control of endothelial mBDNF synthesis by NO is consistent with studies that reported downregulation of BDNF production by cultured human brain microvascular endothelial cells exposed to the eNOS inhibitor asymmetric dimethyl arginine (ADMA)⁹⁰ or to advanced glycation end products,⁷⁷ which act as NO scavengers.⁹¹ More recently, using adjuvant-induced arthritis (AIA) in rats as a model of endothelial dysfunction (i.e. decreased endothelial NO bioactivity),⁹² we reported lower BDNF levels in cerebral microvessel-enriched fractions in AIA than in control rats as well as the efficacy of GTN to prevent the vascular BDNF changes induced by AIA.⁹³ The fact that decreased cerebrovascular mBDNF levels and endothelial dysfunction are found in arthritis, diabetes and hypertension suggests that decreased mBDNF synthesis by CEC could be a useful marker of endothelial function at the cerebral level. However, whether changes in endothelial mBDNF expression is an epiphenomenon or contributes to endothelial dysfunction is unknown. Finally, as NO positively controls t-PA release by human endothelial cells⁹⁴ and as proBDNF could be secreted by endothelial cells, it is conceivable that NO, and by extension endothelial function, may

control the maturation of secreted proBDNF in the extracellular space.

Modulation by other factors

Although neuroinflammation is an important component in the pathogenesis of neurological (stroke, multiple sclerosis) and neurodegenerative (Parkinson and Alzheimer) diseases, the link between inflammation and BDNF synthesis by the cerebral endothelium has been poorly explored. Long-lasting (72 h) but not short-lasting (24 h) exposure of cultured human CEC to TNF α (10 ng/mL) was reported to increase BDNF mRNA in cell lysates and BDNF protein in the medium.⁹⁵ In addition, rat endothelial cells display increased BDNF immunoreactivity in the acute post-stroke period^{71,96} even though hypoxia rather than neuroinflammation may be involved. Indeed, hypoxia was reported to increase mBDNF levels in lysates of rat CEC and BDNF immunoreactivity in the cerebral microvasculature.⁶ It is noteworthy that the stimulating effect of hypoxia on BDNF synthesis and secretion by mouse CEC is higher for intermittent hypoxia (IH) than for continuous hypoxia, the stronger effect of IH being related to oxidative stress and calcium mobilization from internal stores.⁹⁷

Fate of BDNF secreted by the brain endothelium

According to the histology of the cerebral vasculature, the potential recipient cells of BDNF derived from endothelial cells of the cerebral microvasculature include endothelial cells themselves, pericytes, astrocytes, neurons and SMC providing that these cells express TrkB receptors. Unfortunately, TrkB expression by cells other than neurons is poorly documented. TrkB receptor protein was detected in cultured rat CEC,⁶ while TrkB mRNA was not found in human CEC collected from neurosurgical preparations.⁹⁸ We recently detected TrkB-FL and TrkB-T receptors in rat cerebral microvessels-enriched fractions.⁹³ Rodent astrocytes express TrkB, but only the TrkB-T1 isoform.^{99,100} Human pericytes do not express TrkB.¹⁰¹ Finally, to the best of our knowledge, studies aiming to explore TrkB expression by SMC of the cerebral microvasculature are lacking, while rats cultured SMC express TrkB receptor mRNA.¹⁰² Figure 3 summarizes the cellular localization of TrkB and P75^{NTR} receptors in the brain.

Both the luminal (facing the blood) and basal (facing the brain) membrane of CEC can in theory secrete mBDNF (Figure 4). When released by the luminal membrane into the blood, it is unlikely that mBDNF exerts an endocrine effect as plasma mBDNF is

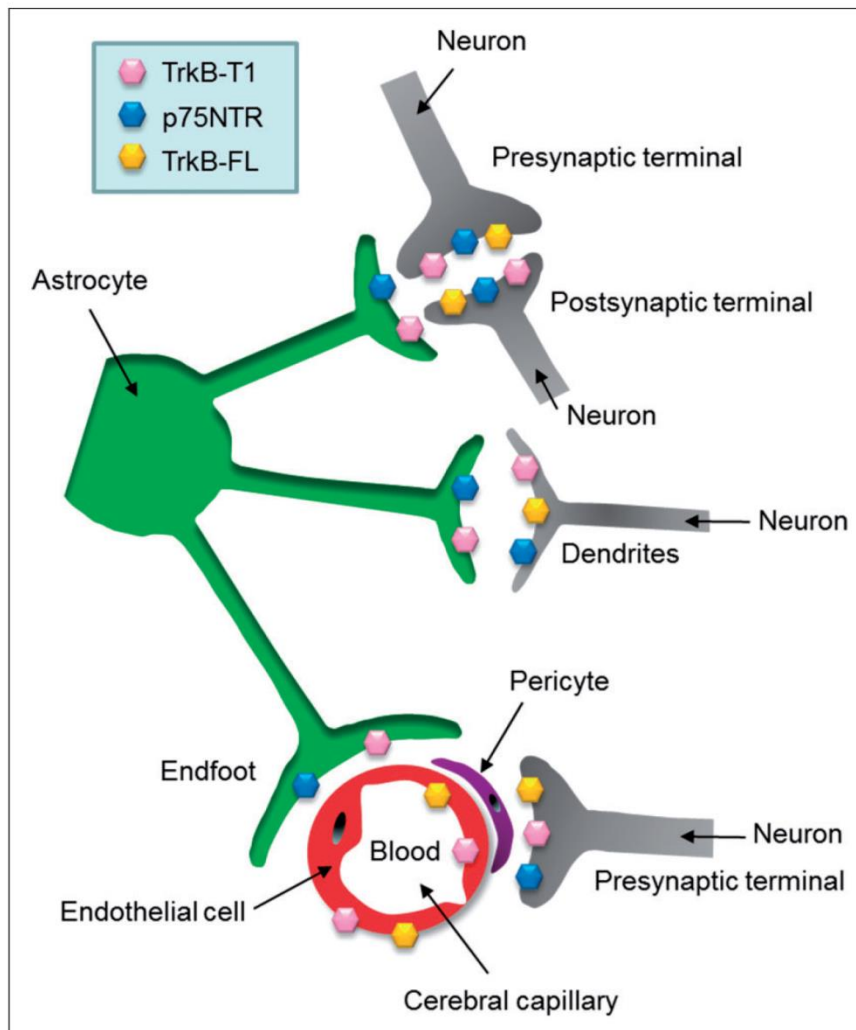


Figure 3. Localization of receptors recognized by mBDNF and proBDNF. Except for pericytes (purple), all the remaining cells of the neurogliovascular unit including endothelial cells (red), astrocytes (green) and neurons (grey) express TrkB-FL (yellow diamond), p75^{NTR} (blue diamond) and/or TrkB-T1 (pink diamond) receptors. See the tripartite synapse at the top of the figure. Note that the drawing is not to true scale.

probably rapidly taken up by platelets.⁷⁸ Consistent with this hypothesis, the plasma half-life of mBDNF does not exceed 2 min.¹⁰³ In contrast, an autocrine effect of BDNF released by the luminal membrane of cerebral endothelial is possible as suggested by the strong association between BDNF and phosphorylated TrkB receptors levels in rat cerebral microvessel-enriched fractions.⁹³ If released by the basal membrane, mBDNF may again act as an autocrine factor and, in addition, as a paracrine factor. In the latter case, mBDNF derived from rat endothelial cells needs to diffuse through the basement membranes, which may hamper its diffusion as a result of its negative charge

and its thickness of ~50 nm.¹⁰⁴ Once mBDNF has diffused through the basement membranes, the nature of potential recipient cells differs depending on the localization of endothelial cells, inside versus outside the NGVU. At the NGVU level, endothelial-derived BDNF can be recognized by TrkB receptors expressed by astrocytes (TrkB-T1) and/or neurons (TrkB-FL and TrkB-T). As regards mBDNF released by endothelial cells outside the NGVU (endothelial cells of arterioles and venules), its unique recipient cells are SMC of the media layer, the limited extracellular diffusion of BDNF being incompatible here with any action on neurons or astrocytes.

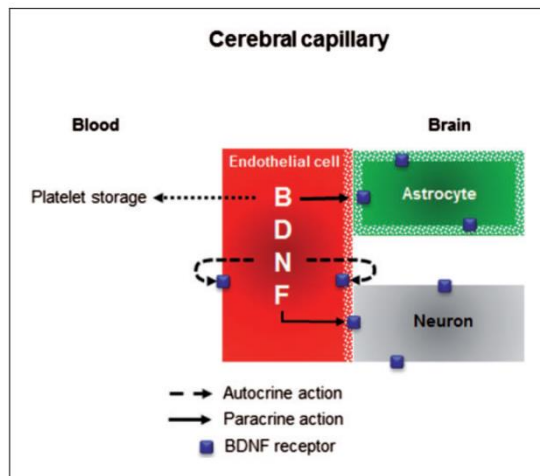


Figure 4. Fate of mBDNF secreted by endothelial cells of cerebral capillaries. mBDNF might be secreted into the blood by the luminal membrane or into the cerebral interstitial fluid by the basal membrane of endothelial cells (red). Once secreted into the cerebral interstitial fluid, mBDNF can exert a paracrine action on astrocytes (green) or neurons (grey) as well as an autocrine action.

Physiological role of cerebral endothelial BDNF

While the link between cardiovascular risk and endothelial dysfunction has been known for more than two decades,¹⁰⁵ the link between endothelial dysfunction and impaired cognition has been identified more recently. For instance, cognition is impaired in patients with hypertension,^{106–108} diabetes^{109–111} and rheumatoid arthritis.^{112–114} It is noteworthy that cerebral endothelial BDNF expression is decreased in animal models of hypertension, diabetes and rheumatoid arthritis, supporting the notion that low endothelial BDNF expression might be a new marker of endothelial dysfunction. However, whether altered BDNF expression is a pathogenic event in or a consequence of endothelial dysfunction remains to be explored. An additional point that deserves further attention concerns the place of endothelial BDNF in the link between endothelial function and cognition. Indeed, available studies suggest that cognition might be dependent on BDNF derived from endothelium of the cerebral microvasculature. However, these studies were mainly performed on cell culture models which do not reproduce the *in vivo* environment of CEC and the limited capacity of BDNF diffusion. These studies as well as the potential role of endothelial BDNF in neuroplasticity are presented in the following section.

Endothelial BDNF and neurogenesis

Neurogenesis is a process of generating functional neurons from neural precursors. In the adult brain, neural progenitors are present in the subventricular zone (SVZ), where they are located adjacent to a layer of ependymal cells in the lateral ventricles. They are also present in the dentate gyrus (DG) near the hilus in the subgranular zone. An additional germinal zone of brain is the subependymal layer of the posterior periventricular (PPv) area surrounding the hippocampus. SVZ neurogenesis seems to replace neurons that die in the olfactory bulb after migration along the rostral migratory stream, while DG and PPv neurogenesis serves to replenish hippocampal neurons. Interestingly, neurogenesis in both SVZ and DG is stimulated by brain ischemia¹¹⁵ and physical training.^{116,117} In support of the notion that BDNF derived from endothelial cells of the neural stem niches plays a role in neurogenesis, explants of the subependymal layer of the adult rat raised on human CEC generated more neurons, which survived longer, than did explants raised on astrocytes, fibroblasts and laminin.⁴ mBDNF secreted by mouse CEC has also been shown to guide neuronal precursor cell migration from the SVZ to the olfactory bulb.¹¹⁸ More recently, migration of newly generated neurons from SVZ towards the ischemic striatum was reported to be dependent on mBDNF produced by mouse vascular cells.¹¹⁹ This finding, combined with the possibility that endothelial BDNF synthesis is controlled by eNOS-derived NO, resonates with previous studies that identified NO formed by eNOS as a promoter of stroke-induced neurogenesis¹²⁰ but contrasts with the study of Nygren et al.,¹²¹ which reported greater neurogenesis in BDNF heterozygous knockout mice than in wild type mice following brain ischemia. Last but not the least, while decreased neurogenesis is a hallmark of animal models of Alzheimer disease,^{122,123} no studies have investigated whether BDNF synthesis by CEC is impaired in this disease, even though the peptide amyloid A β was reported to decrease mBDNF production by mouse CEC.⁵

Endothelial BDNF and neuroprotection

Studies have suggested that mBDNF secreted by cerebral capillaries plays a neuroprotective role, as BDNF secreted by cultured CEC increased resistance of cultured neurons against hypoxia, oxygen-glucose deprivation, oxidative damage-related endoplasmic reticulum stress and A β -induced death of primary neurons.^{5,77,124}

Endothelial BDNF and cerebral angiogenesis

Angiogenesis that occurs by the processes of intussusception and sprouting¹²⁵ refers to the formation of new

capillaries from existing capillaries. The exposure of immortalized rat CEC grown on matrigel to exogenous mBDNF stimulated tube formation, thus supporting a proangiogenic effect of cerebral endothelium-derived BDNF.^{6,85} In addition, hypoxia, stroke and physical training, which are all associated with angiogenesis,¹²⁶⁻¹²⁸ are also recognized as being able to stimulate BDNF synthesis by CECs (see above).

Endothelial BDNF and cerebral circulation

While exogenous mBDNF was recently reported to induce endothelium-dependent relaxation of precontracted peripheral isolated vessels,^{88,129,130} only one study investigated the effect of mBDNF on cerebral circulation.¹³¹ In this study, a cisternal injection of replication-incompetent adenovirus encoding rat BDNF was reported to increase acetylcholine-induced relaxation of basilar arteries. This vasomotor effect of BDNF was related to mBDNF-induced prostacyclin synthesis and apparently independent of NO production by endothelial cells. However, exposure of cultured human pulmonary endothelial cells to exogenous mBDNF induced a rapid release of NO.¹²⁹ Preliminary and unpublished data from our laboratory showed that exogenous mBDNF induced relaxation of precontracted rat isolated middle cerebral artery (Figure 5), a resistance vessel of the cerebral circulation. Collectively, these data support the premise that activation of endothelial TrkB receptors by endothelium-derived BDNF induces the release of vasorelaxant factors and, by extension, that cerebral blood flow is dependent on endothelial BDNF/TrkB signaling.

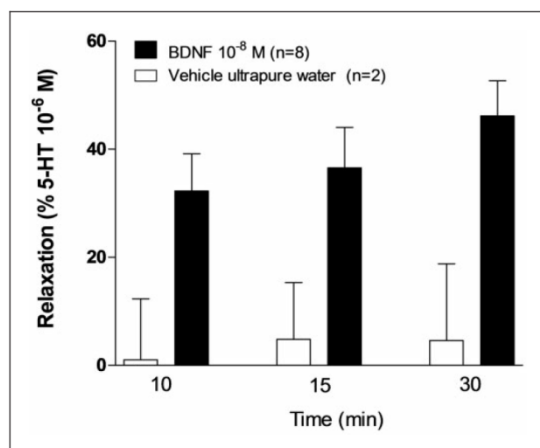


Figure 5. Vasorelaxant effect of BDNF on cerebral circulation. Isolated rat middle cerebral arteries were precontracted with serotonin (10^{-6} M) and then exposed to human recombinant mBDNF (10^{-8} M).

Endothelial BDNF and neuroplasticity

Neuroplasticity, which occurs during development in response to the environment, in support of learning, in response to disease, or in relation to therapy¹³² is defined as the modulation of synapse number and strength. Neuroplasticity largely relates to activation of presynaptic and postsynaptic TrkB-FL receptors by mBDNF, which is traditionally thought to be secreted by neurons in an activity-dependent manner. However, synapses are surrounded by astrocytic end-feet¹³³ and astrocytes also play an important role in the regulation of synaptic behavior.^{134,135} At the capillary level, astrocytes can contact not only endothelial cells but also synapses (tripartite synapses) (Figure 3). Thus, according to the concept of NGVU, mBDNF derived from endothelial cells might be involved in neuroplasticity according to two distinct scenarios based on the involvement or not of astrocytes (Figure 6). Endothelial mBDNF might bind to neuronal TrkB receptors directly, the distance between capillaries and synapses being compatible with such a scenario in theory. Alternatively, endothelial-derived mBDNF might control neuroplasticity through astrocyte-dependent mechanisms. According to such a mechanism, endothelial-derived mBDNF would be first sequestered by astrocytes through internalization of mBDNF-TrkB-T1 complex and then released back into the extracellular space before binding to neuronal TrkB-FL receptors¹³⁶ even though mBDNF binding to astrocytic TrkB-T1 receptors might also stimulate signaling pathways.^{100,137} Endothelial mBDNF might also interact with neuroplasticity through mechanisms independent on neuronal TrkB receptors. Consistent with this hypothesis, activation of endothelial TrkB receptors by mBDNF would result in sustained NO production,¹²⁹ then NO signals originating from endothelial cells would induce long-term potentiation,¹³⁸ which is believed to be a neuronal correlate of learning and memory.¹³⁹ Lastly, if we hypothesize that CEC also secrete proBDNF, the question arises as to the role of proBDNF in neuroplasticity. Astrocytes express p75^{NTR},¹⁴⁰ which binds proBDNF with high affinity. In addition, internalization of the proBDNF-p75^{NTR} complex by astrocytes is followed by intracellular maturation of proBDNF into mBDNF and subsequent release as the mature protein.^{141,142} In these conditions, endothelial proBDNF has to be considered a potential astrocyte-dependent modulator of synaptic behavior. A recent study also suggested that astrocytes play an additional role in the link between endothelial proBDNF and neuroplasticity. This role is related to the ability of astrocytes to regulate the balance between the formation and elimination of plasmin in the brain parenchyma¹⁴³ and consequently maturation of BDNF in the

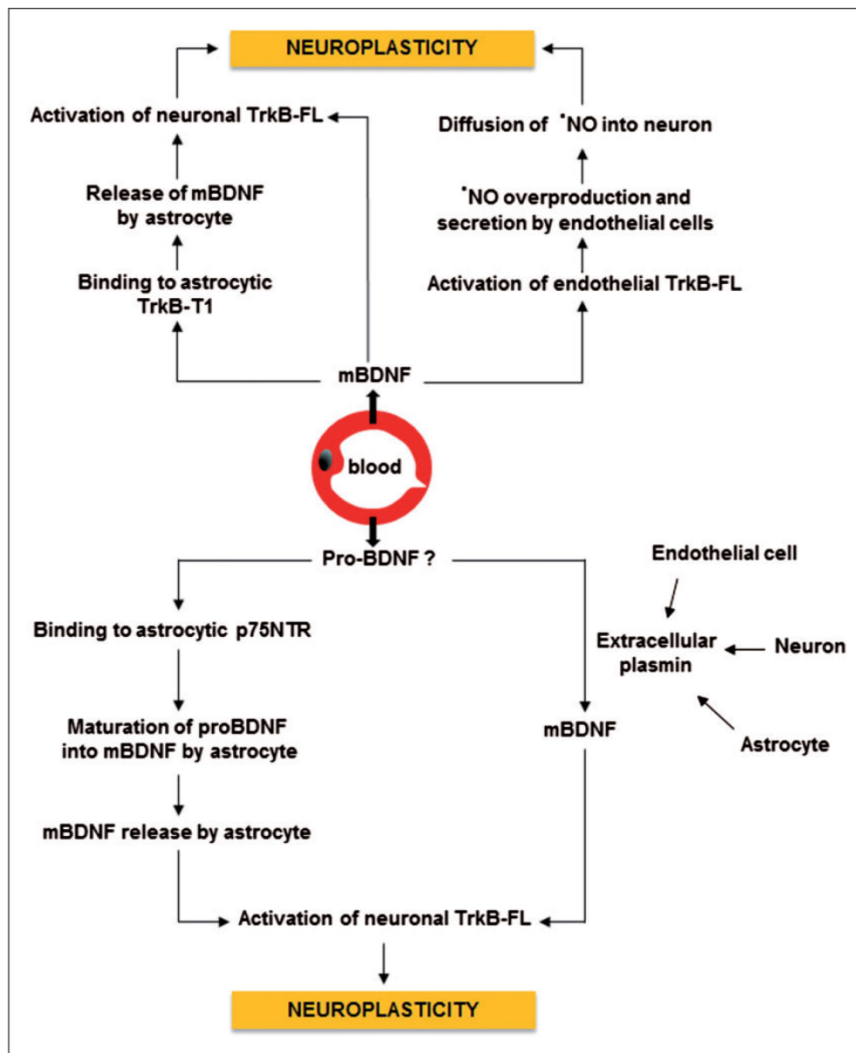


Figure 6. Putative scenarios by which mBDNF or proBDNF derived from endothelium of cerebral capillaries might induce neuroplasticity. Once released by endothelial cells, mBDNF might activate neuronal TrkB-FL receptors either directly or indirectly via astrocytes. Alternatively, endothelium-derived mBDNF might induce neuroplasticity via nitric oxide (NO). According to this scenario, mBDNF activates endothelial TrkB-FL receptors, thus resulting in NO overproduction and subsequent diffusion of NO into neurons. ProBDNF, if released by endothelial cells might induce neuroplasticity via BDNF recycling by astrocytes or via a plasmin-dependent maturation into mBDNF in the extracellular space.

extracellular space. The putative scenarios by which mBDNF or proBDNF derived from the cerebral endothelium can affect neuroplasticity is summarized in Figure 6. Demonstrating that synaptic behavior is dependent not only on neuronal-derived BDNF but also on endothelium-derived BDNF will cast doubt on the dogma that neuroplasticity is primarily triggered by neuronal activity.

Conclusion

It has been estimated that the human brain contains equal numbers of neurons and non-neuronal cells¹⁴⁴ and that endothelial cells represent 17% of the cell population at least in the rat cortex, with neurons accounting for 47%.¹⁴⁵ Given the density of endothelial cells and their ability to synthesize and secrete large quantities of mBDNF, we must rethink and reinterpret

some of the published data on changes in regional brain BDNF levels consecutive to disease or drug administration and consider endothelial mBDNF as an attractive linker connecting brain health with endothelial health. The discovery that mBDNF is secreted by CEC has led to optimism that brain health may be improved by enhancing BDNF signaling using strategies that target CECs rather than neurons. Such an approach would avoid the often insurmountable problem of drug delivery across the blood–brain barrier and potential side-effect of drugs on neurotransmission. However, before this approach can be implemented, it is essential to gain better understanding of the mechanisms involved in endothelial BDNF production and the central effect of mBDNF derived from endothelial cells of the cerebral microvasculature. Several important questions remain to be explored. Are neuronal TrkB receptors a target of BDNF secreted by cerebral capillaries? Does endothelium of the cerebral microvasculature secrete proBDNF? Are TrkB receptors expressed by both the luminal and antiluminal membrane of endothelial cells? Do human and rodent endothelial cells differ in terms of regulation of BDNF secretion and TrkB receptors expression? What are the consequences of the val66met polymorphism on endothelial BDNF synthesis and secretion? Is altered synthesis of endothelial BDNF a pathogenic event in endothelial dysfunction or only an epiphenomena? Establishment of quadruple cell co-culture (neurons, CEC, astrocytes and pericytes) or mouse models with specific endothelial deletion of BDNF or TrkB-FL receptors may be helpful to determine whether BDNF is a new endothelium-derived relaxing factor (EDRF) involved in the control of cerebral hemodynamics and/or a new endothelium-derived enhancer of neuroplasticity (EDEN).

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III) Le BDNF circulant

1) Origine du BDNF circulant

Le sang contient du BDNF mais à faible concentration. C'est l'équipe de Rosenfeld (1995) qui rapporte pour la 1^{ère} fois la présence de BDNF dans le sang en notant par ailleurs que les taux sériques étaient plus élevés (100 à 200 fois) que les taux plasmatiques. Cette différence a été expliquée par la présence du BDNF dans les plaquettes et sa sécrétion lors du processus de coagulation (Fujimura et al., 2002). Comme les plaquettes sont anucléées, elles sont incapables de synthétiser le BDNF. En réalité, la présence de BDNF dans les plaquettes s'explique par leur aptitude à capter le BDNF plasmatique via un transporteur dont la structure n'est pas connue. Le point qui reste à élucider est l'origine cellulaire du BDNF présent dans le plasma. En théorie, différents types cellulaires peuvent être impliqués : les leucocytes et plus précisément les cellules mononuclées du sang (Kerschensteiner et al., 1999, Kruse et al., 2007, Patanella et al., 2010), les cellules endothéliales cérébrales ou périphériques (Nakahashi et al., 2000, Prigent-Tessier et al., 2013, Monnier et al., 2017b) et les neurones du système nerveux central même si le passage du BDNF à travers la BHE reste spéculatif.

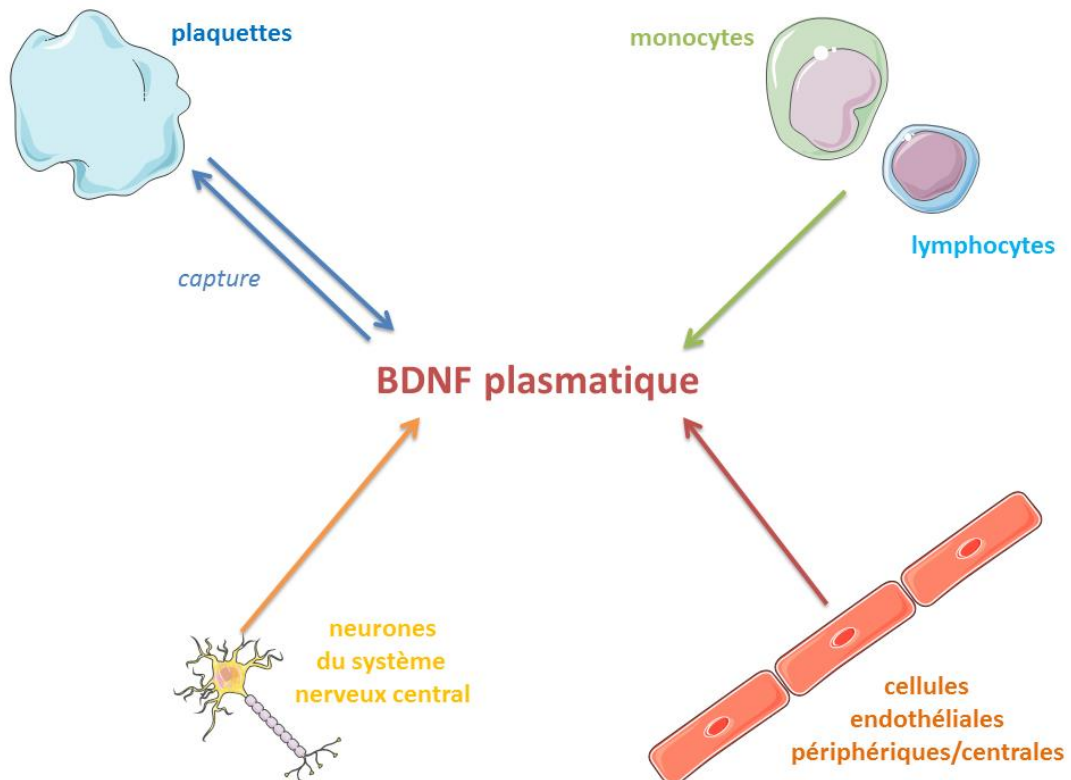


Figure 3: Origine du BDNF plasmatique.

2) Variabilité inter-individuelle des taux circulants de BDNF

Différents facteurs sont capables d'influencer les taux circulants de BDNF en conditions physiologiques. Un premier facteur est le cycle nyctéméral et Begliuomini (2008) a été le premier à mettre en évidence chez l'Homme une corrélation entre les taux plasmatiques de BDNF et de cortisol. De façon intéressante, les taux cérébraux de BDNF et de son récepteur sont également influencés par le cycle nyctéméral (Bova et al., 1998, Schaaf et al., 2000, Dolci et al., 2003) mais on ne sait pas si l'origine de ces modifications est neuronale ou endothéliale. Un autre facteur à prendre en compte lorsque l'on s'intéresse au BDNF circulant est le cycle menstruel qui influence les taux plaquettaires de BDNF (Lommatzsch et al., 2005), l'œstradiol augmentant et la progestérone diminuant par ailleurs les taux plasmatiques de BDNF (Begliuomini et al., 2007). L'âge est un autre facteur qui influence les taux circulants de BDNF, une corrélation inverse existant entre les taux de BDNF plasmatique (Lommatzsch et al., 2005) ou sérique (Ziegenhorn et al., 2007) et l'âge. De façon intéressante, Erickson (2010) a montré qu'il existait une corrélation positive entre le volume de l'hippocampe entre 55 à 85 ans et les taux sériques de BDNF, or le volume de l'hippocampe pourrait être un reflet de la neurogénèse hippocampique (Czeh and Lucassen, 2007) et donc de l'activation des TrkB neuronaux que ce soit par le BDNF d'origine neuronale ou endothéliale. Les taux circulants de BDNF dépendent également des conditions de vie. Par exemple, les taux sériques en BDNF sont plus faibles chez le fumeur que le non-fumeur, chez les personnes vivant en ville que les personnes vivant à la campagne (Bus et al., 2011) et plus élevés chez les actifs que chez les sédentaires (Rasmussen et al., 2009, Griffin et al., 2011). Un point qui est insuffisamment documenté concerne l'influence des facteurs de risque cardiovasculaire sur les taux circulants de BDNF même si des associations ont été observées entre le profil lipidique sanguin, l'indice de masse corporelle, la valeur de la pression artérielle diastolique et les taux circulants de BDNF (Golden et al., 2010, Bus et al., 2011). Enfin, le polymorphisme Val66Met du BDNF semble augmenter les taux sériques de BDNF (Lang et al., 2009).

L'ensemble de ces données explique probablement la grande variabilité interindividuelle des taux sériques ou plasmatiques de BDNF observée dans les études cliniques et montre l'importance de prendre en considération les nombreux facteurs reconnus pour influencer les taux circulants de BDNF avant de conclure sur l'intérêt du dosage du BDNF à des fins diagnostiques ou pronostiques. De façon surprenante, les taux sériques de BDNF sont rarement couplés à la thrombocytemie alors que le BDNF sérique correspond au BDNF plaquettaire. Enfin, il faut garder en tête que les taux de BDNF dans les échantillons sanguins diminuent avec la durée de leur conservation (Trajkovska et al., 2007, Elfving et al., 2010).

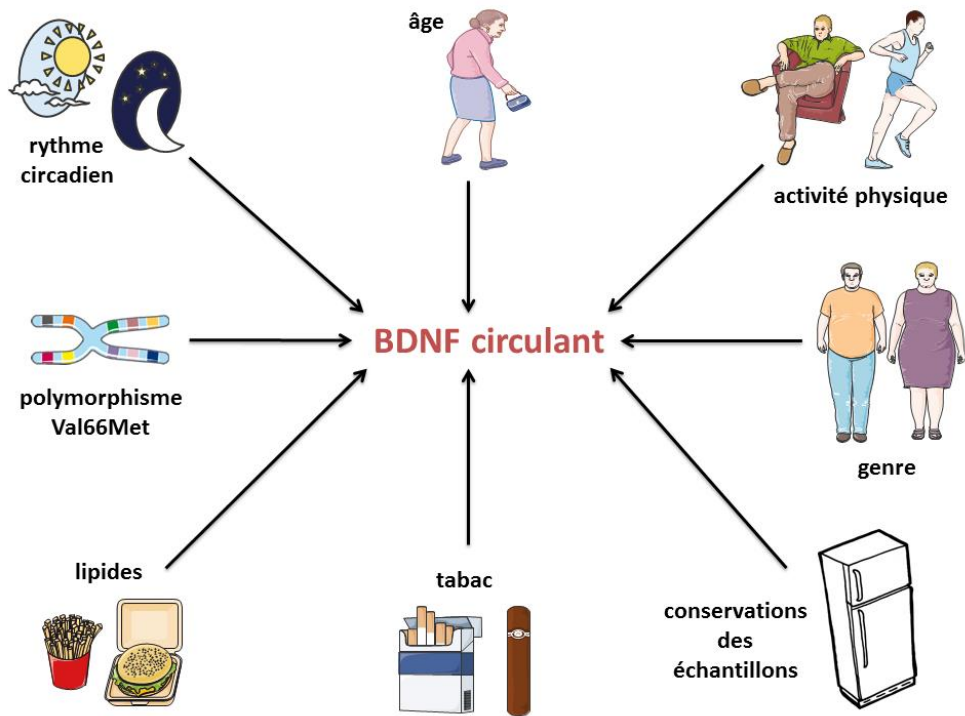


Figure 4: Facteurs influençant les taux circulants de BDNF

3) Intérêt de la mesure du BDNF circulant

Alors que le rôle du BDNF circulant reste énigmatique, le dosage du BDNF dans le plasma ou le sérum est souvent réalisé dans les études cliniques comme marqueur biochimique soit de santé cérébrale soit de santé cardiovasculaire.

a) BDNF circulant, un marqueur de la santé cérébrale ?

Originellement, il a été proposé que le BDNF circulant avait comme origine les neurones du système nerveux central. C'est pourquoi, dans les études cliniques, les neurologues et les psychiatres considèrent le BDNF circulant comme un reflet fiable des taux cérébraux de BDNF et par extension de la santé cérébrale.

Les premiers arguments en faveur de cette hypothèse sont que les taux cérébraux et circulants de BDNF évoluent dans le même sens au cours de l'âge (Karege et al., 2002b) et que la dépression (Karege et al., 2002a, Bocchio-Chiavetto et al., 2010), l'autisme (Hashimoto et al., 2006, Taurines et al., 2014) et les psychoses (Green et al., 2011) sont associés à une diminution des taux de BDNF aussi bien dans le cerveau que dans le sang. De plus, un traitement antidépresseur efficace normalise les taux de BDNF à la fois dans le cerveau et le sang (Chen et al., 2001, Aydemir et al., 2005, Gonul et al., 2005). Une métaanalyse montre également que les taux de BDNF circulants sont diminués lors de la maladie d'Alzheimer (Laske et al., 2006, Angelucci et al., 2010) et qu'ils sont augmentés lors d'un

traitement par Donépézil, un inhibiteur d'acétylcholine estérase (Leyhe et al., 2008). Or, les taux cérébraux de BDNF sont moindres dans les modèles animaux de maladie d'Alzheimer (Murray et al., 1994, Ferrer et al., 1999, Peng et al., 2005). En faveur d'une origine cérébrale du BDNF, Rasmussen et al. (2009) ont rapporté des taux plasmatiques en BDNF plus élevés dans la veine jugulaire que dans l'artère radiale que ce soit en conditions basales ou après un exercice physique de 4 heures. Or, l'exercice est unanimement reconnu comme la stratégie la plus efficace pour augmenter les taux cérébraux de BDNF (Vaynman et al., 2004, Gomez-Pinilla et al., 2008). Cependant, le résultat de Rasmussen ne permet pas de conclure sur l'origine neuronale vs endothéliale du BDNF circulant.

b) BDNF circulant, un marqueur de la santé cardiovasculaire ?

Les découvertes plus récentes montrant la présence de BDNF dans la cellule endothéliale a conduit les cardiologues à considérer les taux circulants comme un reflet probable de la santé cardiovasculaire. En faveur de cette hypothèse, un certain nombre de travaux a rapporté une diminution des taux circulants de BDNF chez les patients souffrant de maladie cardiovasculaire comme en cas d'insuffisance coronarienne (Chaldakov et al., 2004), d'insuffisance cardiaque (Takashio et al., 2015), de syndrome coronarien aigu (Manni et al., 2005, Jin et al., 2018), ou encore d'infarctus du myocarde (Lorgis et al., 2010) ou chez les patients présentant un risque cardiovasculaire accru comme les diabétiques de type 2 (Krabbe et al., 2007, Zhen et al., 2013, He and Wang, 2014, Li et al., 2016a). De plus, les patients dont les taux circulants de BDNF sont élevés ont moins de risque de développer des événements cardiovasculaires, indépendamment de l'inflammation de bas grade et du niveau d'activité physique (Kaess et al., 2015). De la même manière, une étude réalisée au laboratoire (Rodier et al., 2015) a montré chez le patient victime d'un AVC, une corrélation entre les taux sériques de BDNF et le score européen de risque cardiovasculaire (SCORE) qui représente le risque de maladies cardiovasculaires mortelles sur 10 ans. De plus, très récemment, Jin et al. (2018) ont montré une corrélation inverse entre les taux plasmatiques de BDNF et de facteur von Willebrand (vWF), un marqueur de dysfonction endothéliale (Vischer, 2006, Sonneveld et al., 2015) du moins en cas d'insuffisance coronarienne. Dernièrement, deux études (Fukushima et al., 2015, Shibata et al., 2018) ont montré que des taux de BDNF sérique bas étaient associés à un plus haut risque de développer des événements cardiaques à long terme chez les patients présentant une insuffisance cardiaque.

L'ensemble de ces données suggère que la présence d'une dysfonction endothéliale pourrait représenter un facteur confondant quand le BDNF circulant est utilisé comme un marqueur de santé cérébrale.

En résumé, alors que l'athérosclérose et le déficit cognitif semblent tous deux dépendre d'une diminution de la production ou d'une inactivation du NO endothélial et que le BDNF stimule la production de NO par les cellules endothéliales, nous pourrions alors penser de façon originale que l'apparition du processus athérosclérotique ou du déficit cognitif pourrait être ralentie en stimulant la production endothéliale de BDNF.

Chapitre 4

La polyarthrite rhumatoïde

I) Généralités

La polyarthrite rhumatoïde (PR) est une maladie inflammatoire chronique d'origine auto-immune affectant 0,5 à 2 % de la population et plus fréquente chez la femme que chez l'homme. Son étiologie précise n'est pas connue mais comme beaucoup de maladies auto-immunes, elle met en jeu de multiples facteurs qu'ils soient psychologiques, endocriniens, environnementaux et génétiques. La PR se caractérise par une inflammation importante du tissu synovial qui conduit à une destruction des articulations à l'origine d'un handicap fonctionnel. Comme illustré dans les figures 5 et 6 extraites des recommandations de la HAS, la PR est une pathologie complexe à diagnostiquer et dont la prise en charge médicamenteuse est compliquée.

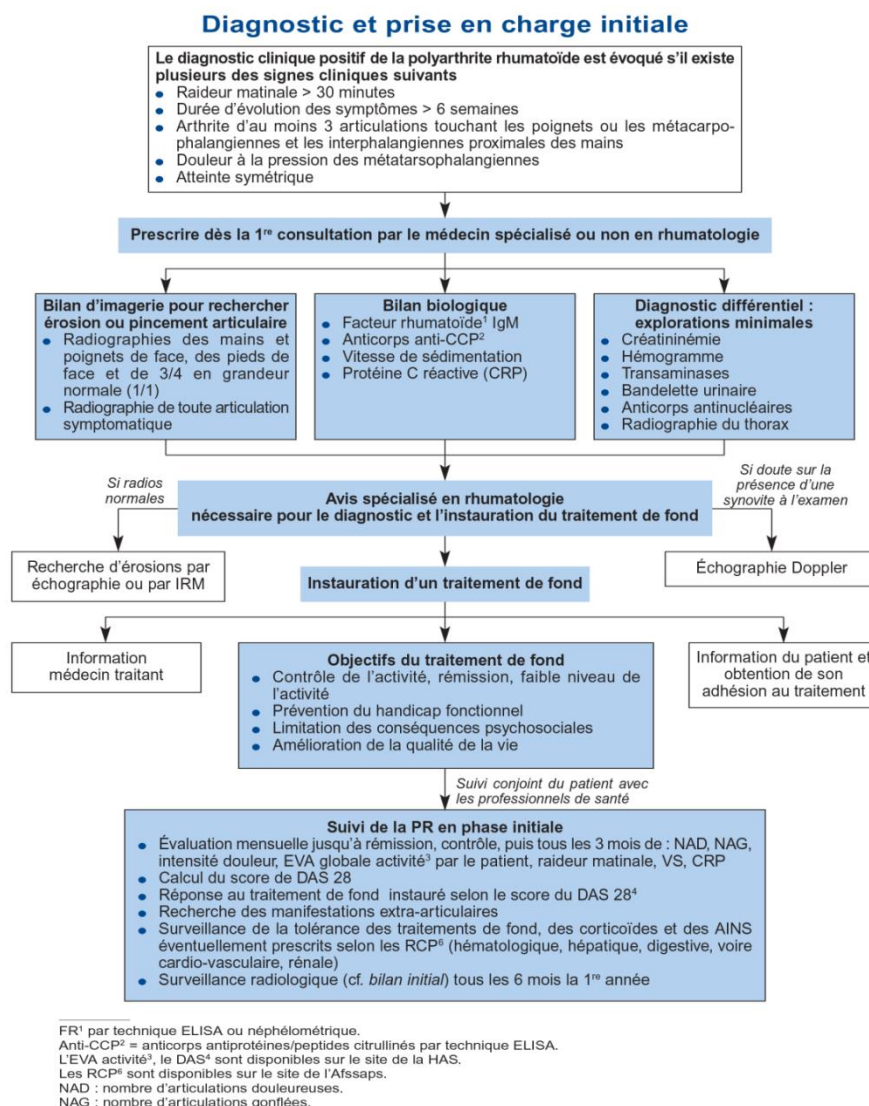
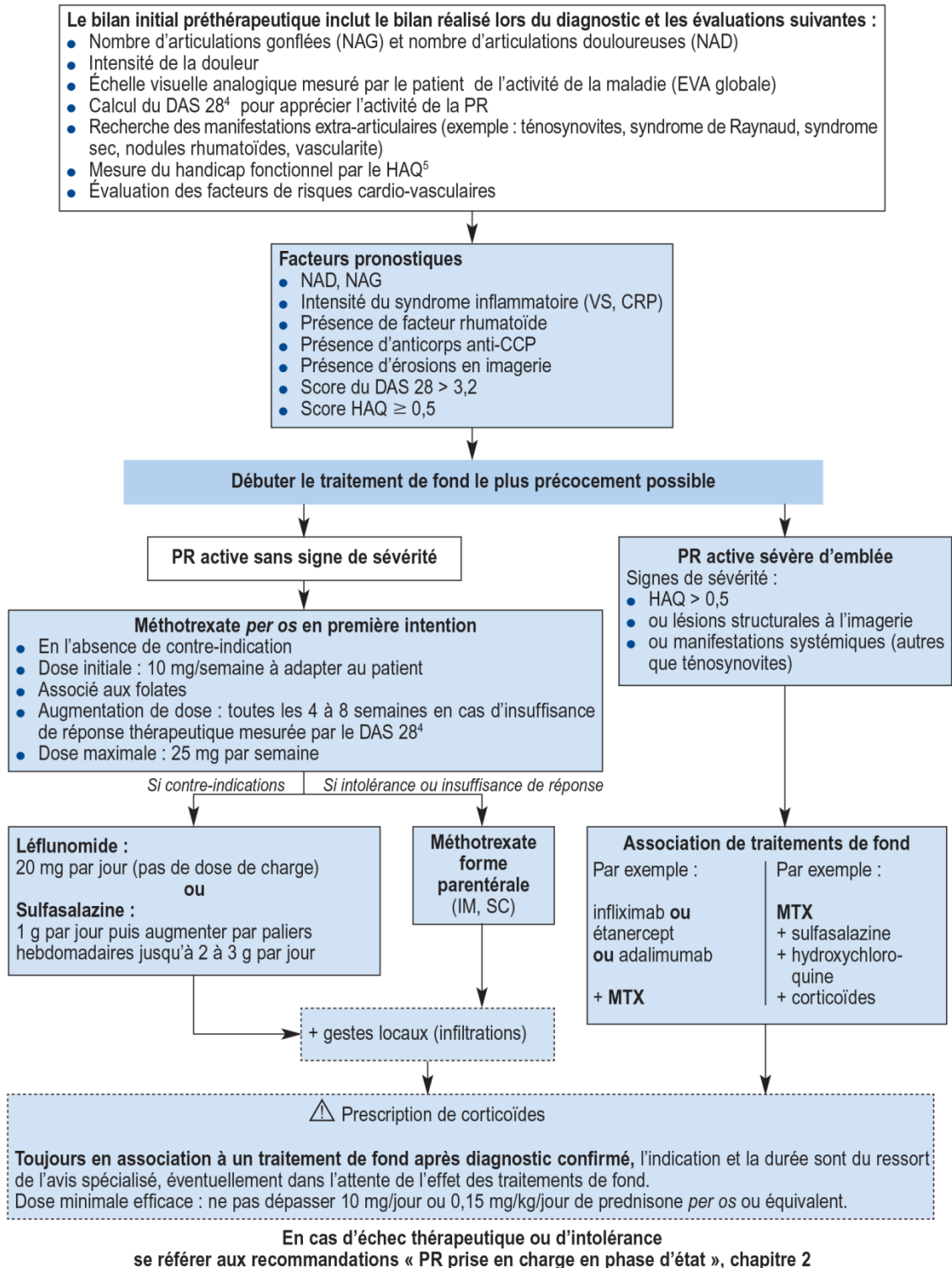


Figure 5: Recommandations de la HAS (2007) sur la démarche diagnostique et de prise en charge initiale de la PR. IRM=Imagerie par résonance magnétique, EVA=Echelle visuelle analogique, DAS 28=Disease activity score 28 (critère tenant compte de paramètres cliniques et biologiques mesurant l'activité de la PR), AINS=Antiinflammatoire non stéroïdien, RCP=Résumé des caractéristiques du produit.

Bilan et traitements



Le HAQ⁵ est disponible sur le site de la HAS.

Figure 6: Recommandations de la HAS (2007) concernant le bilan et la prise en charge de la PR. EVA=Echelle visuelle analogique, DAS 28=Disease activity score 28, HAQ=Health assessment questionnaire (indice fonctionnel), vs=vitesse de sédimentation, CRP=Protéine C réactive, anti-CCP=Anticorps antiprotéines/antipeptides citrullinés, IM=Intramusculaire, SC=Sous-cutanée, MTX=Méthotrexate.

La PR s'accompagne également d'une augmentation du risque cardiovasculaire et des données plus récentes indiquent que l'incidence de la dépression est plus élevée chez les patients PR que dans la population générale. Alors que le haut risque cardiovasculaire est le plus souvent relié à l'existence d'une dysfonction endothéliale, l'origine de la dépression reste hypothétique. Les études cliniques sur la PR incluent des patients dont l'âge, les durées de leur maladie et de leurs traitements antirhumatismaux, la présence ou pas de facteurs de risque cardiovasculaire conventionnels diffèrent largement d'où la difficulté d'élucider les mécanismes qui sous-tendent la dysfonction endothéliale ainsi que la dépression et l'intérêt de conduire des études sur des modèles animaux d'arthrite qui permettent de standardiser le maximum de facteurs confondants.

II) Les modèles animaux d'arthrite

Parmi les modèles animaux d'arthrite, le modèle d'arthrite à adjuvant (adjuvant-induced arthritis, AIA) chez le rat et le modèle d'arthrite à collagène (collagen-induced arthritis, CIA) chez la souris sont les plus classiquement utilisés car ce sont les modèles les plus prédictifs de l'efficacité des traitements de la PR (Hegen et al., 2008) et qui possèdent les mécanismes biologiques sous-jacents à la PR les plus proches de ceux retrouvés chez l'Homme (Vincent et al., 2012).

1) Choix du modèle AIA

Notre choix de modèle s'est porté sur le modèle d'arthrite à adjuvant par injection de *Mycobacterium butyricum* inactivé, chez le rat Lewis mâle âgé de plus de 5 semaines, développé par Pearson et al. (1956). Ce modèle permet d'induire chez le rat une *polyarthrite* (plusieurs articulations touchées) *inflammatoire*.

Notre choix a été guidé par plusieurs raisons :

- Ce modèle est celui qui, chez le rat Lewis, induit l'atteinte arthritique et la perte de poids la plus précoce, la plus sévère et durable par comparaison au modèle réalisé chez le rat de souche Wistar ou Sprague-Dawley
- Il a été montré que l'arthrite apparaît plus souvent chez les mâles âgés de plus de 3 semaines.
- Ce modèle est associé à une dysfonction endothéliale dès trois semaines après le début de l'arthrite (Cf plus en détails Page 67)
- Ce modèle est associé à un déficit cognitif de façon précoce (Cf plus en détails Page 69)

Cependant, ce modèle comporte un inconvénient majeur. En effet, alors que la PR humaine évolue par poussées, le modèle AIA se limite à une seule poussée inflammatoire bien qu'il ait été montré l'existence de plusieurs poussées lors d'un suivi sur plusieurs mois (Mossiat et al., 2015).

2) Immunisation du rat AIA

L'arthrite a été induite pendant ma thèse par une seule injection intradermique à la base de la queue de 120 μ L (10 mg/mL) de *Mycobacterium butyricum* inactivé en suspension dans l'adjuvant incomplet de Freund chez des rats Lewis âgés de 6 semaines anesthésiés à l'isoflurane. Concrètement, le mycobacterium est broyé à l'aide d'un mortier, mis en suspension dans l'adjuvant de Freund à une posologie de 10 mg/mL puis soniqué 5 minutes afin d'aider à l'homogénéisation de la suspension. Les rats contrôles reçoivent une injection d'un volume équivalent (120 μ L) de sérum physiologique à la base de la queue.

Un autre modèle similaire consiste en l'injection de la suspension de *Mycobacterium butyricum* inactivé dans la face plantaire d'une des pattes du rat induisant alors une arthrite de la patte concernée, puis de façon plus tardive, de la patte controlatérale (Butler et al., 1992). On parle alors de monoarthrite induite à l'adjuvant.

3) Evolution des signes cliniques chez le rat AIA

a) Inflammation chez le rat AIA

Les signes cliniques d'arthrite apparaissent généralement 10 à 14 jours après l'injection unique de *Mycobacterium* et débutent par une perte de poids et une apparition de rougeurs et gonflements au niveau des doigts et des chevilles (Rainsford, 1982). L'inflammation progresse jusqu'à un maximum entre J20 et J30 post-immunisation (Figure 9). Le rat présente alors une polyarthrite affectant particulièrement les chevilles, les poignets et les petites articulations inter-phalangiennes distales (Figure 7).

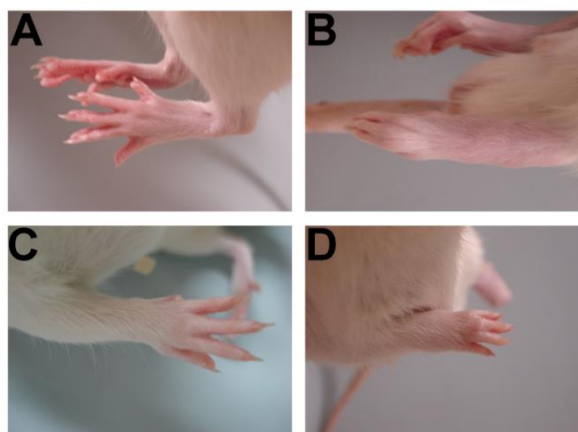


Figure 7: Photographies de pattes postérieures de rats non malades (A) et AIA (B) et de pattes antérieures de rats non malades (C) et AIA (D) prises au jour 27 post-immunisation.

Au-delà de cette phase aiguë, les signes inflammatoires régressent et seules persistent des déformations articulaires. Au niveau radiologique, un gonflement du tissu mou, une ostéoporose, une érosion osseuse et une destruction du cartilage apparaissent au début de la maladie puis une ostéoformation se met en route à partir de J30 post-immunisation (Figure 8).



Figure 8: Changements radiographiques de la patte arrière observés chez un rat non malade (a) et AIA (b et c) au jour 195 post-immunisation. A noter l'ostéoformation dans le tarse et le calcaneum (b) et dans l'extrémité inférieure du tibia (c). D'après Mossiat et al. (2015).

Les signes cliniques (rougeur ou gonflement) de l'inflammation de chacune des pattes (antérieures et postérieures) sont classiquement cotés à l'aide d'un score d'arthrite allant de 0 à 6 : 0,1 par doigt montrant une atteinte quelle que soit sa sévérité ; 0,5 pour une atteinte faible à modérée d'une grosse articulation (cheville ou poignet), 1 pour une atteinte sévère d'une grosse articulation (Sakaguchi et al., 2003). La mesure du diamètre des pattes à l'aide d'un pied à coulisse est également utilisée pour évaluer les signes cliniques inflammatoires. Récemment au laboratoire, une corrélation entre le score arthritique et le diamètre des pattes arrière a été mise en évidence (Mossiat et al., 2015) indiquant que la mesure du diamètre des pattes postérieures est un reflet fiable du score arthritique (qui prend en compte l'inflammation des 4 pattes).

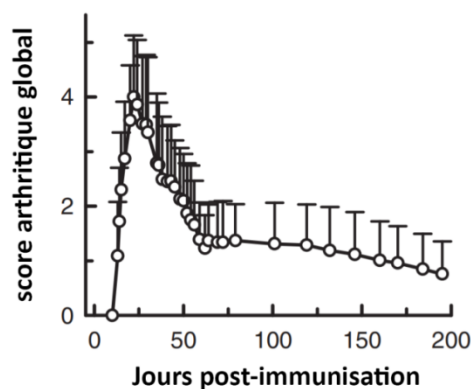


Figure 9: Evolution du score arthritique chez le rat AIA d'après Mossiat et al. (2015).

b) Douleur chez le rat AIA

Le modèle d'arthrite par adjuvant est un modèle expérimental qui génère une douleur pour l'animal (degré 3). Durant ma thèse, les animaux n'ont pas reçu d'antalgiques car les différents antalgiques susceptibles d'être administrés à l'animal présentent l'inconvénient d'interférer avec nos protocoles expérimentaux :

- les AINS, molécules de référence du traitement de l'arthrite, diminuent l'inflammation et l'atteinte articulaire
- les morphiniques modifient la fonction endothéliale vasculaire (Wong and Gardocki, 1983, Afshari et al., 2009)
- le paracétamol possède des effets anti-inflammatoires et anti-arthritiques (Shin et al., 2006)
- tous les antalgiques sont susceptibles d'interférer avec les processus cognitifs (Page 72)

Aussi, devant l'impossibilité d'utiliser des analgésiques, un examen clinique est réalisé quasi-quotidiennement et les animaux sont manipulés le plus délicatement possible. Classiquement, les critères pour définir les points limites dans le modèle AIA sont ceux définis par Lloyd et Wolfensohn (1998). Dans notre travail ainsi que dans nos précédentes études, la douleur n'a jamais atteint le seuil qui doit conduire à la mise à mort.

III) PR & risque cardiovasculaire

1) Le constat

La polyarthrite rhumatoïde est la maladie auto-immune systémique la plus fréquemment associée à une mortalité et une morbidité cardiovasculaires excessives. En effet, le risque de mortalité due à un infarctus du myocarde ou un accident vasculaire cérébral en cas de PR est augmenté de 50% par rapport à la population générale (Solomon et al., 2006, Avina-Zubieta et al., 2008, Sodergren et al., 2009, Bacani et al., 2012). Ce risque est comparable à celui des patients diabétiques (Stamatelopoulos et al., 2009, van Halm et al., 2009). Etonnamment, les raisons exactes qui relient la PR au risque cardiovasculaire restent floues même si la dysfonction endothéliale, initiatrice de l'athérosclérose (Del Rincon et al., 2007, Kao et al., 2008) est largement incriminée.

2) PR & dysfonction endothéliale

a) Mise en évidence chez l'Homme

La dysfonction endothéliale a été identifiée chez les patients atteints de PR tant à l'étage macrovasculaire (site des processus athérosclérotiques) que microvasculaire (site des dommages des organes). Les études de la dysfonction endothéliale macrovasculaire, les plus nombreuses, ont été principalement réalisées chez des patients avec une PR établie (et donc sous traitement médicamenteux) et ont fait appel à la mesure de la FMD. La majorité des études s'accordent à montrer une altération de la FMD (Gonzalez-Juanatey et al., 2003, Gerli et al., 2004, Arosio et al., 2007, Pingiotti et al., 2007, Kerekes et al., 2008, Soltesz et al., 2009, Stamatelopoulos et al., 2009). En effet, à notre connaissance, seules 3 études montrent que la vasodilatation dépendante du flux ne diffère pas entre les patients atteints de PR et les personnes non malades (Van Doornum et al., 2003, Cypiene et al., 2010, Sodergren et al., 2010). En revanche, la dysfonction endothéliale microvasculaire (circulation cutanée, coronarienne) a été moins explorée chez le patient PR et a fait l'objet d'une revue récente (Bordy et al., 2018). Les principaux points à retenir dans cette revue sont les suivants :

- La dysfonction endothéliale microvasculaire est un évènement précoce/phare dans le développement des maladies cardiovasculaires et des dommages aux organes associés, et est également présente chez les patients atteints de PR
- La dysfonction endothéliale microvasculaire n'est pas corrélée avec l'activité et la durée de la maladie, les taux de protéine C-réactive ou la vitesse de sédimentation
- Les médicaments antirhumatismaux et d'autres thérapies peuvent être utilisés pour traiter la dysfonction endothéliale microvasculaire mais les effets de ces traitements diffèrent.
- Atteindre la rémission dans la PR ne garantit pas la normalisation de la fonction endothéliale microvasculaire
- La valeur prédictive de la dysfonction endothéliale microvasculaire pour les événements cardiovasculaires doit être démontrée dans de futures études

Un des points qui reste à explorer est la présence ou non d'une dysfonction endothéliale cérébrale en cas de PR.

b) Etudes chez le rat AIA

L'évaluation de la dysfonction endothéliale a été, le plus souvent, faite chez le rat AIA à un moment où les symptômes inflammatoires étaient à leur apogée (c'est-à-dire entre 24 et 35 jours après induction de l'immunisation). L'étude de la vasodilatation dépendante de l'endothélium sur vaisseau isolé est de loin la plus souvent utilisée pour objectiver la dysfonction endothéliale. Les études portant

sur la dysfonction endothéliale macrovasculaire sont nombreuses et ont montré de manière univoque la présence d'une dysfonction endothéliale au niveau de l'aorte (Fang et al., 1991, Cinar et al., 1998, Ulker et al., 2000, Can et al., 2002, Haruna et al., 2006, Haruna et al., 2007, Nozaki et al., 2007, Sakuta et al., 2010, Prati et al., 2011, Prati et al., 2012). Comme chez l'Homme, les études sur la dysfonction endothéliale microvasculaire chez l'animal arthritique sont en nombre limité. Totoson et al. (2015) ont montré que la dysfonction endothéliale à l'étage des artères mésentériques précédait la dysfonction endothéliale à l'étage de l'aorte et que l'anomalie persistait à l'étage microvasculaire mais pas macrovasculaire en phase subchronique (90 jours après immunisation).

c) Mécanismes impliqués dans la dysfonction endothéliale

Différents mécanismes ont été impliqués dans la moindre production/disponibilité du NO produit par l'endothélium en réponse à l'acétylcholine chez le rat AIA.

Diminution de la production de NO

Dans les aortes de rats AIA, l'expression de eNOS est inchangée (Prati et al., 2011) ou augmentée (Haruna et al., 2006, Haruna et al., 2007) à J35 post-immunisation suggérant que la moindre production de NO n'est pas à relier à une diminution de la synthèse de la eNOS. En revanche, il existe des arguments en faveur d'une diminution de l'activité de la eNOS (Prati et al., 2012), d'un découplage de la eNOS (Haruna et al., 2006) qui pourrait être la conséquence d'un déficit en BH4 (Haruna et al., 2006) ou d'une surexpression de l'arginase (Prati et al., 2011).

Augmentation de l'inactivation du NO

Dans le modèle AIA, à J35 post-immunisation, la production d'anions superoxydes a été mesurée dans des homogénats d'aortes incubés avec ou sans substrat de la NOS et divers inhibiteurs (Haruna et al., 2006). La production aortique spontanée d' O_2^- a été augmentée chez les rats AIA par rapport aux rats témoins. Dans le modèle AIA, l'expression des ARNm des sous-unités de la NADPH oxydase de l'aorte (p22phox, gp21phox et p47phox) est augmentée (Haruna et al., 2007, Sakuta et al., 2010), tout comme l'activité de l'enzyme (Sakuta et al., 2010). Ces résultats suggèrent que le découplage d'eNOS et la surexpression de la NADPH oxydase sont les sources prédominantes de production d' O_2^- dans les aortes de rats AIA. Le rôle de la production excessive d' O_2^- dans la dysfonction endothéliale a été confirmé par le fait que l'incubation d'anneaux aortiques de rats AIA avec du Tempol, qui mime l'action de la SOD, ou avec l'apocynine, un inhibiteur de la NADPH oxydase, améliorait significativement la vasorelaxation induite par l'acétylcholine (Ach) (Prati et al., 2012). Enfin la formation de peroxy-nitrites est augmentée dans les aortes de rats AIA (Haruna et al., 2006) mais leur contribution à la dysfonction endothéliale n'a pas encore été explorée.

IV) PR & déficit cognitif

1) Le constat

a) Chez l'Homme

Dès l'année 1987, Chandarana et al. attirent l'attention sur la comorbidité psychiatrique des patients souffrant de PR (87 patients) (Chandarana et al., 1987). Cependant, la première étude rapportant une altération de la cognition chez les patients souffrant de PR et qui a incité les suivantes est celle de Appenzeller et al. (2004). Les auteurs ont comparé 40 patients PR et 40 sujets en bonne santé, tous ayant reçu une éducation de niveau école primaire. De multiples tests ont été utilisés pour évaluer la cognition : le mini mental test, des tests de mémoire, des tests de fluence verbale, d'attention et des tests pour évaluer la dépression et l'anxiété. Une atteinte cognitive a été observée chez 30% des patients PR vs 7,5% des sujets sains, le déficit portant sur la fluence verbale et les mémoires logique et à court terme. De façon intéressante, le déficit était indépendant de la durée de la maladie, de l'utilisation des corticoïdes et du handicap moteur. Les études suivantes ont confirmé l'impact négatif de la PR sur la mémoire et la fluence verbale (Tomašević-Todorović et al., 2011, Hamed et al., 2012, Akdoğan et al., 2013, Bilgici et al., 2014, Petersen et al., 2015) et révélé que la PR était associée à une comorbidité psychiatrique notamment la dépression (Sturgeon et al., 2016). La prévalence de la dépression est selon les études comprise entre 15 à 34%, ce qui est significativement plus élevé que dans la population générale (Dickens et al., 2002, Matcham et al., 2013).

b) Chez l'animal

Il est difficile d'explorer la cognition chez l'animal arthritique, notamment lorsque l'arthrite affecte la mobilité car le déficit moteur constitue un facteur confondant pour interpréter les tests comportementaux. Néanmoins, un déficit cognitif (anxiété, anhédonie, moindre mémoire spatiale) a été retrouvé dans un modèle de monoarthrite (Gregoire et al., 2014), modèle dans lequel la locomotion est peu affectée (Borges et al., 2014). Par ailleurs, chez le rat AIA, la LTP hippocampique est diminuée trois semaines après induction de l'immunisation (Liu et al., 2012) indiquant clairement que le modèle AIA s'accompagne d'une moindre neuroplasticité. On peut noter ici que l'immunisation serait capable à elle seule d'induire à un déficit cognitif. En effet, Skurlova et al. (2010) ont montré que le modèle AIA induisait un déficit d'apprentissage (évalué par le test de la piscine de Morris) avant l'apparition des signes cliniques (J4 post-immunisation).

2) Mécanismes

Les mécanismes impliqués dans le déficit cognitif observé chez les patients souffrant de PR sont probablement multiples et incluent l'inflammation systémique (Dantzer et al., 2008, Bauer and Teixeira, 2018), la dysfonction endothéliale et le traitement antirhumatismal.

a) Inflammation systémique

L'idée que l'inflammation systémique associée à la PR peut affecter la cognition vient du constat que la cognition est altérée en cas de pathologies infectieuses chez l'Homme ou encore après l'injection systémique de LPS, de cytokines proinflammatoires (comme le TNF α ou l'IL-1 β , 2 cytokines largement incriminées dans la PR) ou d'interféron- α (IFN α). Il est important de savoir que les cytokines circulantes peuvent agir sur le cerveau. En effet, les cytokines circulantes peuvent traverser la BHE par diffusion facilitée (transporteurs) ou encore activer les cellules microgliales (source de cytokines pro-inflammatoires) au niveau des régions circumventriculaires et des plexus choroïdes (régions dont l'endothélium est de type fenestré et non de type BHE). Les cellules microgliales activées produiraient alors des cytokines. Par ailleurs, les cytokines pro-inflammatoires circulantes pourraient induire l'expression, par les cellules endothéliales cérébrales, du transporteur au TNF α (Osburg et al., 2002) augmentant ainsi l'apport de TNF α au cerveau. En tout cas, l'administration systémique de LPS induit la synthèse par le cerveau de IL-1 β (van Dam et al., 1992) ainsi qu'une dépression qui a été mise en évidence grâce aux tests de Porsolt, de suspension par la queue ou de préférence pour l'eau sucrée.

Les mécanismes intimes par lesquels les cytokines (notamment TNF α et IL1 β) affectent la cognition ne sont pas bien connus. Parmi les mécanismes évoqués, on peut citer une diminution des neurotransmissions dopaminergique et sérotoninergique (Miller et al., 2013) pour expliquer les symptômes dépressifs et une réduction de la production de BDNF (Guan and Fang, 2006, Schnydrig et al., 2007) pour expliquer les troubles de la mémoire.

b) Dysfonction endothéliale

Comme précédemment développé, il existe un lien fort entre facteurs de risque cardiovasculaire et déficit cognitif donc entre dysfonction endothéliale et déficit cognitif d'où la possibilité que la comorbidité cognitive associée à la PR puisse être la conséquence de la dysfonction de l'endothélium cérébral. A notre connaissance, la fonction endothéliale n'a jamais été étudiée à l'étage cérébral chez le patient PR ou dans les modèles animaux de PR.

c) Origine iatrogène

Toutes les études cliniques sur le duo cognition-PR incluent des patients sous traitements antirhumatismaux. La cognition est évaluée en dehors des poussées afin de s'affranchir de l'impact négatif de la douleur sur les capacités cognitives. La cognition est donc évaluée pendant la maîtrise du processus inflammatoire, remettant ainsi en question l'idée selon laquelle le déficit cognitif est à relier à l'inflammation et poussant à s'interroger sur une origine iatrogène du déficit. Il est bien connu que la corticothérapie, quelle que soit son indication, est susceptible d'affecter les capacités cognitives. Cependant, leur contribution au déficit cognitif associé à la PR est controversée (Appenzeller et al., 2004, Shin et al., 2012). Il n'y a pas vraiment d'information sur l'interaction cognition/traitement de la PR pour les autres médicaments. Cependant, le traitement par l'etanercept (antiTNF α) en association au méthotrexate réduirait la dépression et les troubles cognitifs chez les patients atteints de PR par rapport aux patients PR traités par méthotrexate seul (Kekow et al., 2010). De plus, l'initiation d'un traitement par l'etanercept semble améliorer la cognition évaluée par le mini mental test 6 mois après le début du traitement chez les patients atteints de PR (Chen et al., 2010b).

V) PR & BDNF

Au début de ma thèse, en inscrivant les mots clés « BDNF » et « arthritisme » sur pubmed, seulement une dizaine d'articles apparaissait. Les publications disponibles reportaient l'effet de l'arthrite sur les taux articulaires ou circulants de BDNF

1) PR & BDNF dans les articulations

C'est dans le journal *Annals of Rheumatic Disease* que Weidler et al. (2005) ont montré pour la 1^{ère} fois la présence de BDNF dans le tissu et le liquide synovial en cas de PR. Les types cellulaires impliqués dans la synthèse de BDNF seraient les macrophages, les synoviocytes, les lymphocytes T infiltrants, les chondrocytes ou encore les neurones assurant l'innervation sensitive de l'articulation (Grimsholm et al., 2008a, Grimsholm et al., 2008b, Barthel et al., 2009). La question qui se pose et qui demeure sans réponse concerne le rôle du BDNF produit localement. En tout cas, les récepteurs TrkB sont exprimés par les neurones présents dans l'articulation (Grimsholm et al., 2008b) d'où la possibilité que le BDNF puisse sensibiliser les neurones et donc contribuer à la douleur.

2) PR & BDNF circulant

A notre connaissance, seules 2 études se sont intéressées aux taux circulants de BDNF chez le patient PR. Del Porto et al. (2006), ont montré sur un faible nombre de patients (n=20) que l'administration d'etanercept diminuait les taux plasmatiques de BDNF. Plus tard, Grimsholm et al. (2008b), montrent que les taux plasmatiques de BDNF sont plus élevés chez des personnes saines

comparées aux patients PR et qu'ils sont plus faibles chez les patients PR traités par etanercept que chez les patients PR ne bénéficiant pas de ce traitement.

De façon surprenante, alors que la PR est associée à la fois à une dysfonction endothéliale et à un déficit cognitif, les recherches se sont focalisées sur le BDNF synovial et circulant. L'impact de la PR sur le BDNF vasculaire et cérébral n'a jamais été étudié. Nous avons émis l'hypothèse selon laquelle les anomalies des fonctions cardiovasculaire et cognitive observées en cas de PR pourraient être à relier à une anomalie de l'expression endothéliale du BDNF.

TRAVAUX PERSONNELS

Article 2

Titre:

Activation of endothelial TrkB receptors induces relaxation of resistance arteries.

Auteurs:

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Résumé des travaux

a) Contexte & hypothèse

Nous avons déjà montré au laboratoire que l'endothélium des vaisseaux périphériques (aorte) ou cérébraux (microvaisseaux) exprimait le BDNF. Cependant, le rôle du BDNF d'origine endothéliale ni même la localisation des TrkB ne sont réellement connus et restent peu étudiés. Si l'on admet que les récepteurs TrkB sont exprimés par l'endothélium des vaisseaux de résistance et que leur activation induit une surproduction de NO (Meuchel et al., 2011), il n'est pas exclu que le BDNF d'origine endothéliale puisse exercer un effet vasodilatateur et donc être impliqué via un effet autocrine dans la régulation de la pression artérielle. Pour tester cette hypothèse, j'ai participé à une étude conduite par notre partenaire bisontin visant à évaluer l'effet vasodilatateur d'un agoniste des récepteurs TrkB (LM22A-4) dans un modèle d'artères mésentériques isolées. Mon travail a consisté à évaluer dans un premier temps si ces vaisseaux de résistance exprimaient des récepteurs TrkB et, si oui, de rechercher le type cellulaire impliqué.

b) Protocole expérimental

La réactivité vasculaire au LM22A-4 a été étudiée chez le rat Wistar mâle sur vaisseau (artère mésentérique) préalablement contracté à la phényléphrine en présence ou non d'endothélium, d'antagoniste des récepteurs TrkB (ANA-12 et cyclotraxine B) et d'inhibiteurs de la NO synthase (L-NAME), de la PGI-2 synthase (U51605), des canaux potassiques dépendants du Ca²⁺ (apamine/charybdotoxine), de la voie PI3K/Akt (wortmannine), de la calmoduline (calmidazolium), de CAMK II (KN-62) ou de la voie Raf/MEK (PD98059). Les taux et la localisation des récepteur TrkB ont été respectivement explorés par Western blot et immunohistofluorescence.

c) Principaux résultats

- Les récepteurs TrkB sont présents au niveau des artères mésentériques et l'expression est restreinte aux cellules endothéliales.
- Le LM22A-4 induit une relaxation prévenue par la cyclotraxine B mais pas par ANA-12 et qui nécessite la présence de l'endothélium
- L'activation des récepteurs TrkB induit une dilatation qui est moindre en présence de L-NAME, apamine/charybdotoxine et d'U51605

Cette étude montre pour la première fois que l'endothélium des vaisseaux de résistance exprime des récepteurs TrkB et que leur activation par un agoniste exogène exerce un effet vasodilatateur mettant en jeu le NO, la PGI2 et l'EDHF. Ces données sont compatibles avec l'idée selon laquelle la pression artérielle pourrait être dépendante de la capacité de l'endothélium à synthétiser et sécréter du BDNF.



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Activation of endothelial TrkB receptors induces relaxation of resistance arteries

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ABSTRACT

While brain-derived neurotrophic factor (BDNF) was previously reported to induce relaxation of conduit artery, whether the BDNF/TrkB (tropomyosin-related kinase) pathway is involved in the tone control of resistance arteries is not known. This study investigated TrkB receptors levels/localization and the vasomotor effect of the TrkB receptor agonist LM22A-4 in isolated third-order mesenteric arteries from rats.

Immunostaining revealed the presence of both full-length and truncated TrkB receptors, especially at the endothelial level. By using wire myography, LM22A-4 induced vascular relaxation that was significantly decreased by cyclotraxin B as a non-competitive TrkB antagonist and fully prevented by endothelium removal. Inhibitors of NO, EDHF, PGI₂ production and the PI3K/Akt pathways separately reduced LM22A-4 induced-relaxation. By contrast, inhibition of Raf/MEK, PLC γ and CaM/CaMKII pathways did not change the relaxant effect of LM22A-4. Interestingly, BDNF also induced an endothelium and TrkB-dependent relaxation.

These results indicate that endothelial TrkB activation results in the relaxation of resistance vessels via PI3K/Akt-induced eNOS phosphorylation and production of EDHF and PGI₂. These data are consistent with the contribution of the endothelial BDNF/TrkB pathway to the regulation of peripheral vascular tone. They also validate the use of LM22A-4 as a reliable pharmacological agent for studying the vascular effect of BDNF.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin present in the central nervous system where it plays a crucial role in neuroplasticity and neurogenesis through activation of full length (FL) TrkB receptors [1]. BDNF binding to TrkB-FL receptors promotes formation of TrkB dimers, and induces trans-phosphorylation of TrkB cytoplasmic domain tyrosine residues, initiating recruitment of signaling adapter proteins that foster signaling by ras/ERK1/2, PI3 kinase/Akt STAT and phospholipase C γ /Ca²⁺/calmodulin-dependent protein kinase II pathways [2]. However, alternatively spliced forms of TrkB (misleadingly known as truncated TrkB-T1 and TrkB-T2 receptors) lack a tyrosine kinase domain, but possess alternative cytoplasmic domain sequences that signal by less extensively characterized mechanisms. Nevertheless, an important function of TrkB-T1, which acts as a dominant-negative receptor, is to inhibit TrkB-FL signaling [3].

Serum/plasma BDNF level is commonly used as a marker of brain BDNF levels and by extension of brain health [4]. More recently, clinical studies have reported associations between circulating BDNF and cardiovascular health. For instance, high serum BDNF levels were

associated with a decreased risk of cardiovascular diseases and mortality [5] and low levels with increased risk of incident stroke [6]. Plasma BDNF levels were reported to be lower in patients with unstable angina pectoris than those with stable angina pectoris or healthy control subjects [7]. An inverse correlation of plasma BDNF with traditional cardiovascular disease biomarkers was also reported in active and inactive men elderly [8]. Furthermore, serum BDNF levels were reported to negatively correlate with peripheral vascular resistances [9,10] and with blood pressure [11]. These data combined with the fact that hypertension is one of the most important cardiovascular risk factor suggest that circulating BDNF might be involved in the regulation of peripheral vascular resistances. Importantly, albeit the mechanisms have not been unraveled, it was previously found that exogenous BDNF induced endothelium-dependent relaxation in conduit vessels including porcine pulmonary artery [12] and rat aorta [13]. In addition, the TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) was recently reported to reduce blood pressure in spontaneously hypertensive rats, but through TrkB-independent mechanisms [14].

The objectives of the present study are to explore TrkB receptors levels/expression on third-order rat mesenteric arteries and the

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potential relaxant response of these resistance vessels to the TrkB agonist *N,N',N''*Tris(2-hydroxyethyl)-1,3,5-benzenetricarboxamide (LM22A-4) [15,16]. Vascular reactivity to LM22A-4 was studied in the presence or not of endothelium, and the role of TrkB receptors was investigated by using ANA-12 and cyclotraxin B as TrkB antagonists. Inhibitors of the signaling pathways downstream of TrkB activation as well as inhibitors of the production of endothelium-derived relaxant factors were used to dissect the underlying mechanisms. The results may help to understand the link between circulating BDNF and cardiovascular health.

2. Materials and methods

2.1. Animals

Male Wistar rats (6–8 week-old) ($n = 25$) were purchased from Janvier (Le Genest Saint Isle, France). Animals were kept under a 12 h–12 h light-dark cycle and allowed free access to food and water. The experimental procedures were approved by the local committee for ethics in animal experimentation n°2015-001-CD-5PR of Franche-Comté University (Besançon, France), and complied with the “Animal Research: Reporting in Vivo Experiments” ARRIVE guidelines. Animals were anesthetized with pentobarbital (60 mg/kg, intraperitoneally) before collection of small intestine to rapidly dissect the mesenteric vascular beds.

2.2. Western Blot analysis and immunostaining

TrkB receptors levels and localization were studied on the same mesenteric bed. For western blot analysis, vascular samples were homogenized in 5 volumes of ice-cold lysis buffer. Equal protein amounts were resolved by SDS-PAGE and transferred electrophoretically to polyvinylidene fluoride membranes. After blockade of non-specific binding sites, membranes were incubated with primary antibodies directed against TrkB receptors (1:1000, 610,102, BD Transduction, San Jose, USA) or beta-actin as an internal marker (1:10000, A5441, Sigma Aldrich). Membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase anti-IgGs (1:50000, 115-035-166 Jackson Immunoresearch, Newmarket UK). Protein antibodies complexes were visualized using enhanced chemiluminescence Western blotting detection system (ECL+, GE Healthcare, Little Chalfont, UK). Gels were run in triplicate. For the immunostaining procedure, a fraction of mesenteric artery previously embedded in optimal cutting temperature compound was sectioned at -20°C . Vascular sections (10 μm thick) were mounted on Superfrost Plus slides. After blockade of non-specific binding sites, sections were first exposed to anti-TrkB receptors antibody (1:200, see reference above) that recognized both the full-length and truncated isoforms or to anti-von Willebrand factor (vWF, a marker of endothelial cells, 1:200, F3520, Sigma Aldrich, Saint-Louis, USA). Then, they were exposed to fluorescent secondary antibodies (1:1000, ALEXA fluor 488, A11029 and ALEXA fluor 568, A11036, Interchim, Montluçon, France). Finally, sections were mounted with a fluorescent mounting containing DAPI as nuclear marker (FluoroProbes, FPDT094A, Interchim) and analyzed using an epifluorescent microscope (Eclipse E600, Nikon, Champigny sur Marne, France).

2.3. Vasoreactivity

Mesenteric arteries were placed in Krebs solution of the following composition: NaCl 118 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, MgSO_4 1.2 mM, CaCl_2 2.5 mM, NaHCO_3 25 mM, glucose 12 mM, maintained at pH of 7.4, 37°C , and continuously aerated with 95% O_2 , 5% CO_2 . Then 2-mm segments of artery was mounted in organ chambers and threaded on two 40- μm diameter stainless steel wires. To measure isometric force, the artery segments were connected into a Multi Myograph

System (Model 610 M v.2.2, DMT A/S, Denmark). The data were recorded using Chart™ Ver.7 (ADInstruments, France). Thereafter mesenteric rings were set to a normalized internal circumference L_1 (0.9 L_{100}) in accordance to passive wall tension-internal circumference relationship under a passive transmural pressure of 100 mmHg. After an initial equilibration period of 30 min, viability of vessels was tested from their vasoconstriction to high extracellular KCl (100 mM). The presence of functional endothelium was confirmed by > 80% relaxation to the endothelium-dependent agonist acetylcholine (Ach, 10^{-6} M) after pre-constriction with phenylephrine (PE 3.10^{-5} M). In some rings, endothelium was mechanically removed by gently rubbing inside the vessel with a small wire. The completeness of endothelial denudation was confirmed by the absence of relaxation to Ach (10^{-6} M). Arteries were again allowed to equilibrate 30 min before the start of the experiments.

2.3.1. Experimental protocol

The ability of cumulative concentrations of LM22A-4 (10^{-11} – 10^{-4} M) and of recombinant human BDNF (10^{-8} M) to relax endothelium-intact and endothelium-denuded arteries was determined in mesenteric rings constricted with PE (3.10^{-5} M). The effect of vehicle (DMSO at 0.1 to 0.8% for LM22A-4 or ultrapure water for BDNF) was studied in the same conditions. A cumulative concentration-relaxation of Ach (10^{-11} – 10^{-4} M) was used as a reference for mesenteric relaxation. The ability of LM22A-4 (10^{-8} M) and BDNF (10^{-8} M) to induce vasoconstriction was also studied on endothelium-denuded mesenteric arteries without pre-constriction.

To determine the contribution of TrkB receptors to the vasorelaxant response, the same experiments were repeated after a 30-min incubation with two different TrkB antagonists, ANA-12 (at 10^{-5} M and 10^{-4} M) or cyclotraxin B (at 10^{-6} M). To understand the relative contribution of the different pathways downstream of TrkB activation to LM22A-4-induced relaxation, mesenteric rings were subjected to a 30-min incubation with wortmannin (3.10^{-8} M, a PI3K/Akt inhibitor), calmidazolium (10^{-5} M, a calmodulin inhibitor), KN-62 (10^{-5} M, a CAMK II inhibitor), PD98059 (10^{-5} M, a Raf/MEK inhibitor) or U73122 (10^{-5} M, a PLC inhibitor) before adding cumulative concentrations of LM22A-4. Of note, as incubation with U73122 blunted the contractile response to PE, experiments with this reactant were made in rings pre-constricted with KCl (100 mM) instead of PE. In order to evaluate the role of nitric oxide (NO) synthase, prostacyclin (PGI₂) synthase and endothelium-derived hyperpolarizing factors (EDHF) in the vasodilatory response to LM22A-4, arteries were previously incubated for 30 min with N_ω -nitro-L-arginine methyl ester (10^{-4} M, L-NAME, a NO synthase inhibitor), U51605 (10^{-6} M, a prostacyclin synthase inhibitor) or apamin/charybdotoxin ($10^{-7}/10^{-7}$ M, Ca^{2+} -dependent K^+ channels inhibitors) respectively.

2.3.2. Drugs

Phenylephrine, norepinephrine, acetylcholine, L-NAME, indomethacin, apamin, wortmannin, calmidazolium, KN-62, U73122, ANA-12 were purchased from Sigma-Aldrich. Charybdotoxin was obtained from Enzo Life Sciences Company, U51605 from Abcam, LM22A-4 and cyclotraxin B from Tocris; and recombinant BDNF from Peprotech. LM22A-4, ANA-12, wortmannin, calmidazolium, U73122 and KN-62 were dissolved in DMSO and diluted in water. U51605 was packaged on methyl acetate and diluted in water. Other substances were dissolved in distilled water. Drug concentrations are expressed as final molar concentrations in the bath solution.

2.4. Statistical analysis

All data are expressed as the means \pm standard error of the mean (S.E.M). Vasorelaxant responses were measured as percentage inhibition of the contraction induced by PE or KCl. Comparison of relaxation values between BDNF and its vehicle or of BDNF in rings with or

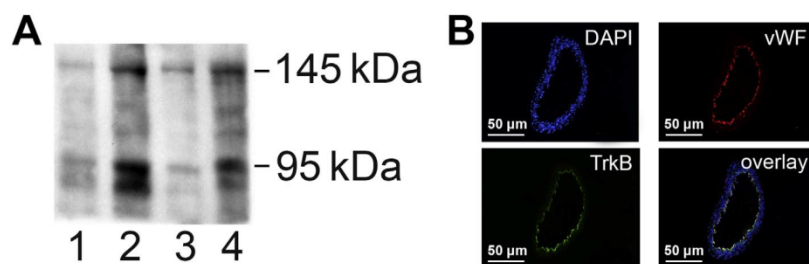


Fig. 1. TrkB receptors are expressed by endothelium of mesenteric arteries. Western blot analysis and immunohistochemistry were performed on the third-order branches of mesenteric arteries harvested from 4 rats. A representative immunoblot of TrkB-FL (145 kDa) and TrkB-T (95 kDa) receptors (A). Note that band intensity is stronger in rats 2 and 4 than in rats 1 and 3. Representative immunostaining of TrkB receptors (B). DAPI (blue) was used as nuclear marker and vWF (red) as a marker of endothelial cells. Note that cells positive for TrkB are also positive for vWF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

without endothelium was assessed by using Mann-Whitney *U* test. Concentration-responses curves to LM22A-4 in the presence or not of endothelium or a specific inhibitor were compared by 2-way analysis of variance (ANOVA) for repeated measures. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. TrkB receptors are expressed by mesenteric arteries

As shown in Fig. 1A, a 145 kDa band corresponding to full length TrkB receptors and a 95 kDa band corresponding to truncated TrkB receptors were easily detected in the four samples examined even though an inter-individual variability was observed among rats. Immunohistochemical experiments revealed that cells positive for TrkB receptors were also positive for the specific endothelial marker vWF (Fig. 1B). No difference in TrkB receptors staining was observed among rats (not shown). Collectively these data indicate that both TrkB-FL and truncated TrkB receptors are expressed by mesenteric arteries especially at the endothelial level.

3.2. LM22A-4 induces endothelium-dependent vasorelaxation in mesenteric arteries

Experiments were performed on third-order mesenteric arteries ($n = 8$ to 12 mesenteric arteries from 8 to 12 rats per group) pre-constricted with phenylephrine (PE) 3.10^{-5} M. The contraction induced by PE reached 12.86 ± 0.77 mN. Fig. 2 shows typical traces of the relaxation induced by cumulative concentrations of LM22A-4 (Fig. 2A) and its vehicle (Fig. 2B) on endothelium-intact rings. The maximal relaxation (Emax) obtained with LM22A-4 was 5.93 ± 0.76 mN ($54.3 \pm 5.7\%$ of PE-induced contraction) and the EC50 was $4.8 \pm 1.3 \cdot 10^{-8}$ M. The effect of LM22A-4 (Figs. 2A and 3A) was significantly lower compared to that of Ach (Figs. 2C and 3B). Thus, the Emax value of LM22A-4 ($54.3 \pm 5.7\%$, $p < 0.0001$) was significantly lower than that of Ach 10^{-4} M ($94.7 \pm 1.8\%$). As shown in Figs. 2D and 3C, endothelium removal completely abolished the LM22A-4-induced relaxation. Notably, endothelium-intact and endothelium denuded arteries previously exposed to cumulative concentrations of LM22A-4 further responded to Ach and SNP, respectively (see Fig. S1, supplemental data), indicating that the moderate relaxant effect of the TrkB agonist did not relate to the incapacity of vessels to relax more.

In order to ensure that the effect of LM22A-4 was mediated by activation of endothelial TrkB receptors, the vascular response to LM22A-4 was investigated in the presence of the non-competitive TrkB antagonists, namely ANA-12 (liposoluble antagonist) or cyclotraxin B (hydrosoluble antagonist). As presented in Fig. 3, whereas ANA-12 failed to significantly reduce the relaxant effect of LM22A-4 at either 10^{-5} M (Fig. 3D) or 10^{-4} M (Fig. 3E), cyclotraxin B (10^{-6} M) significantly decreased LM22A-4-induced relaxation (Fig. 3F), indicating that the effect of LM22A-4 indeed involved TrkB receptors activation.

3.3. LM22A-4 induces vasodilation through activation of the PI3K/Akt pathway

To further explore which signaling pathways downstream of TrkB activation were involved in the relaxant effects of LM22A-4, the concentration-response curves of LM22A-4 were performed in the presence of inhibitors of these pathways. The results showed that the PI3K/Akt inhibitor wortmannin (Fig. 4A) but not the inhibitors of Raf/MEK (PD98059, Fig. 4B), PLC (U73122, Fig. 4C), calmodulin (calmidazolium, Fig. 4D) or CAMK II (KN-62, Fig. 4E), was efficient to significantly reduce the effect of LM22A-4, identifying a seminal role of the PI3K/Akt pathway in LM22A-4-induced relaxation. Importantly, wortmannin was used as a concentration not high enough to inhibit myosin light chain kinase [17].

3.4. LM22A-4-induced relaxation involves the production of NO, PGI₂ and EDHF

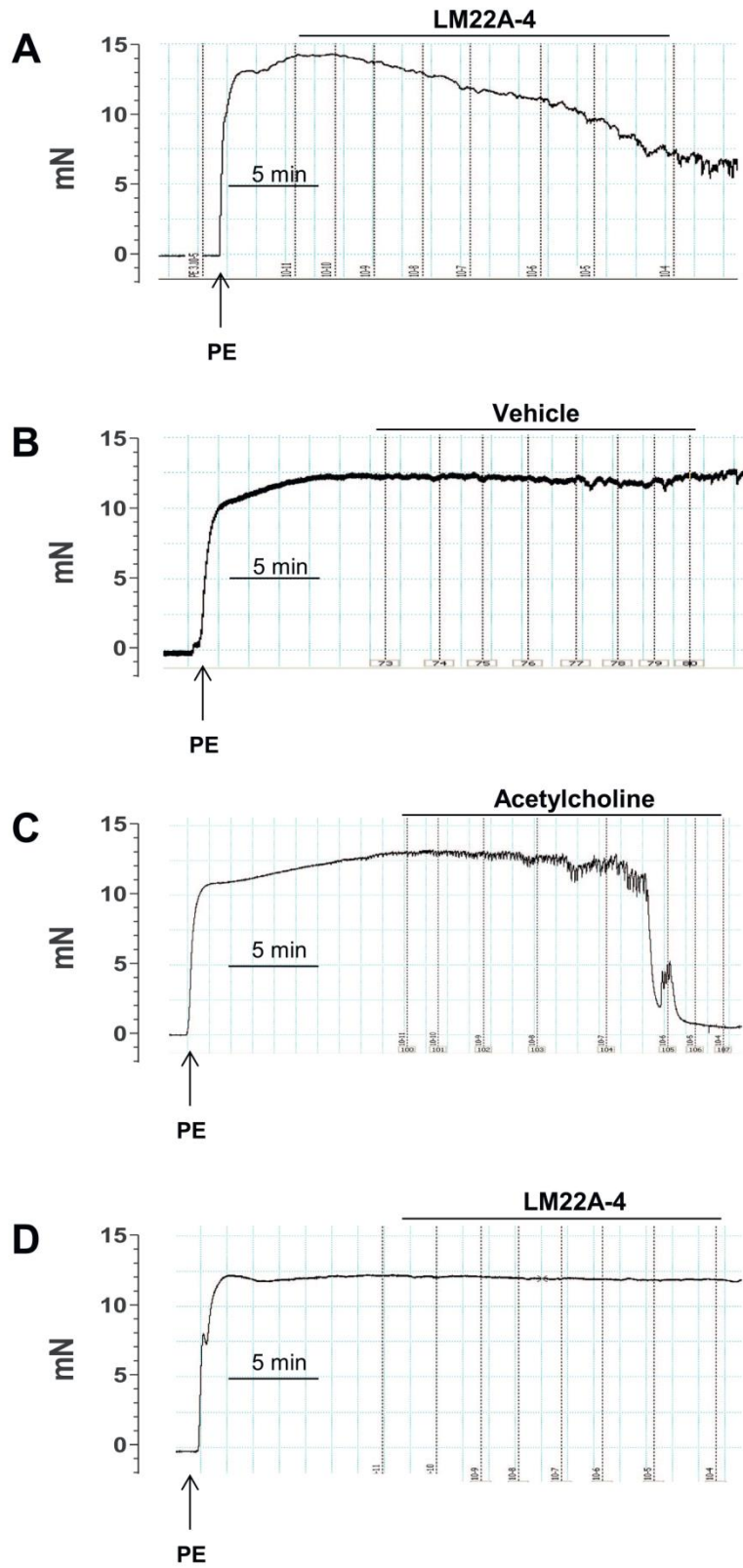
As activation of Akt leads to the rapid activation of eNOS [18], we first explored the contribution of NO to LM22A-4-induced relaxation. Consistent with such contribution, the NOS inhibitor L-NAME fully abolished the relaxant effect of LM22A-4 (Fig. 5A). In addition, given that small resistance arteries are known to induce relaxation via other pathways than NOS including EDHF and COX pathways, we assessed the contribution of prostacyclin and EDHF by using a PGI₂ synthase inhibitor and the combination of apamin + charybdotoxin, respectively. As shown in Fig. 5B and C, both inhibitors reduced the effect of LM22A-4 even though the extent of inhibition was less important for the PGI₂ synthase inhibitor than for the K⁺ channels inhibitors.

3.5. LM22A-4 mimics the relaxant effect of BDNF

In order to validate the use of LM22A-4 as a reliable pharmacological agent to mimic the vascular effects of BDNF, we compared the effect of LM22A-4 and BDNF on mesenteric arteries. BDNF was used at the concentration at 10^{-8} M that was previously reported by our laboratory to induce relaxation in rat aortic rings [13]. Exposure of pre-constricted mesenteric arteries to BDNF induced a relaxant effect that gradually increased from 2 to 30 min (Fig. 6A). As shown in Fig. 6B, the % of relaxation that was 18 ± 2 at 10 min reached 24 ± 4 at 30 min. As observed for the LM22A-4-induced relaxation, the relaxant effect of BDNF was fully prevented by endothelium removal (Fig. 6C) and significantly decreased by cyclotraxin B at 30 min (Fig. 6D). It is noteworthy that at 10^{-8} M the extent of relaxation did not differ between BDNF and LM22A-4 (Fig. 6A) suggesting that the two TrkB agonists shared the same mechanisms to induce relaxation.

4. Discussion

The present results show that i) endothelium of mesenteric arteries expresses both full-length and truncated TrkB receptors, ii) LM22A-4 as a TrkB agonist induces relaxation that is fully and partially prevented by endothelial removal and TrkB receptors antagonist, respectively, iii)



(caption on next page)

Fig. 2. Representative original traces of the relaxation induced by LM22A-4 and acetylcholine in mesenteric arteries. Experiments were performed on mesenteric arteries precontracted with phenylephrine (PE) 3.10^{-5} M. Cumulative concentrations (10^{-11} to 10^{-4} M) of LM22A-4 (A) and acetylcholine (Ach) (C) produced a concentration-dependent relaxation in endothelium-intact arteries, whereas vehicle (B) had no effect. Endothelial denudation inhibited the vasorelaxant effect of LM22A-4 (D).

NO production as a result of activation of the PI3K/Akt pathway downstream of TrkB receptors and formation of PGI₂ and EDHF are both involved in the relaxation induced by LM22A-4.

The vascular expression of TrkB receptors is poorly documented. In adult mice, TrkB receptors immunoreactivity was detected in vessels of the heart and skeletal muscle, both endothelial cells and smooth muscle cells being positive for the receptors, but not in the vascular bed of the kidney and liver [19]. TrkB receptors immunoreactivity was also reported in human extra- and intra-pulmonary arteries in all the tunica with a prominent expression in the tunica adventitia [20]. TrkB expression was observed in cultured endothelial cells derived from the rat brain [21], in human pulmonary artery, where full-length and truncated forms were detected [12], and in vascular smooth muscle cells derived from rats [22]. The present study revealed that mesenteric arteries contain both TrkB-FL and TrkB-T receptors and that the expression seems confined to endothelial cells. These findings are consistent with a recent study that reported ARNm encoding for TrkB-T1 and TrkB-T2 receptors in cardiac microvascular endothelial cells [23].

A vasorelaxant effect of BDNF was previously reported in isolated conduit vessels (pulmonary artery, aorta). The novel data provided by the present study is that BDNF as well as the synthetic TrkB agonist LM22A-4 induced relaxation of resistance vessels through endothelium- and TrkB-dependent mechanisms. Notably, as cyclotraxin B is not a competitive antagonist but a partial allosteric inhibitor of TrkB receptors [24], the inability of cyclotraxin B to fully prevent BDNF- or LM22A-4-induced relaxation was not surprising. What was somewhat surprising was our finding that ANA-12, another non-competitive TrkB antagonist, failed to decrease LM22A-4-induced relaxation. This

intriguing result is in line with a recent study [14] in which the relaxant effect of 7,8-dihydroflavone (7,8-DHF) on aortic rings was reported to be insensitive to ANA-12, whereas 7,8-DHF is considered as a potent agonist of TrkB receptors [25]. Although we have currently no explanation regarding the differential effect of ANA-12 and cyclotraxin B, at least on isolated resistance vessels, we think that caution is needed in the interpretation of results obtained with ANA-12 in vessels. As reported for conduit vessels [12,14], the relaxation of resistance vessels to TrkB agonists was moderate as compared to that of Ach, a well-recognized endothelium-dependent vasodilator. Although speculative, such weakness of relaxation may be explained by LM22A-4 binding to TrkB-T1 receptors and subsequent inhibition of the signaling pathways evoked by ligation of the agonist to TrkB-FL receptors since TrkB-T1 is recognized to act as a dominant negative receptor [3]. Indeed, TrkB-FL and TrkB-T1 receptors share the same extracellular domain and bind agonists with the same affinity.

To the best of our knowledge, the mechanisms by which endothelial TrkB receptors activation results in vessel relaxation have never been investigated. Our results support the involvement of the PI3K/Akt pathway downstream of TrkB-FL receptors and subsequent sustained NO production by phosphorylated eNOS [26]. They are consistent with recombinant BDNF-induced rapid generation of NO secondary to Akt and eNOS phosphorylation in cultured pulmonary endothelial cells [12]. However, the fact that contribution of NO to vasorelaxation of small resistance arteries is less prominent than in large vessels [27] and that differences exist among vascular beds as regards endothelial biology leads us to explore whether EDHF and prostacyclin also contribute to the effect of LM22A-4. Our results revealing the prevention of

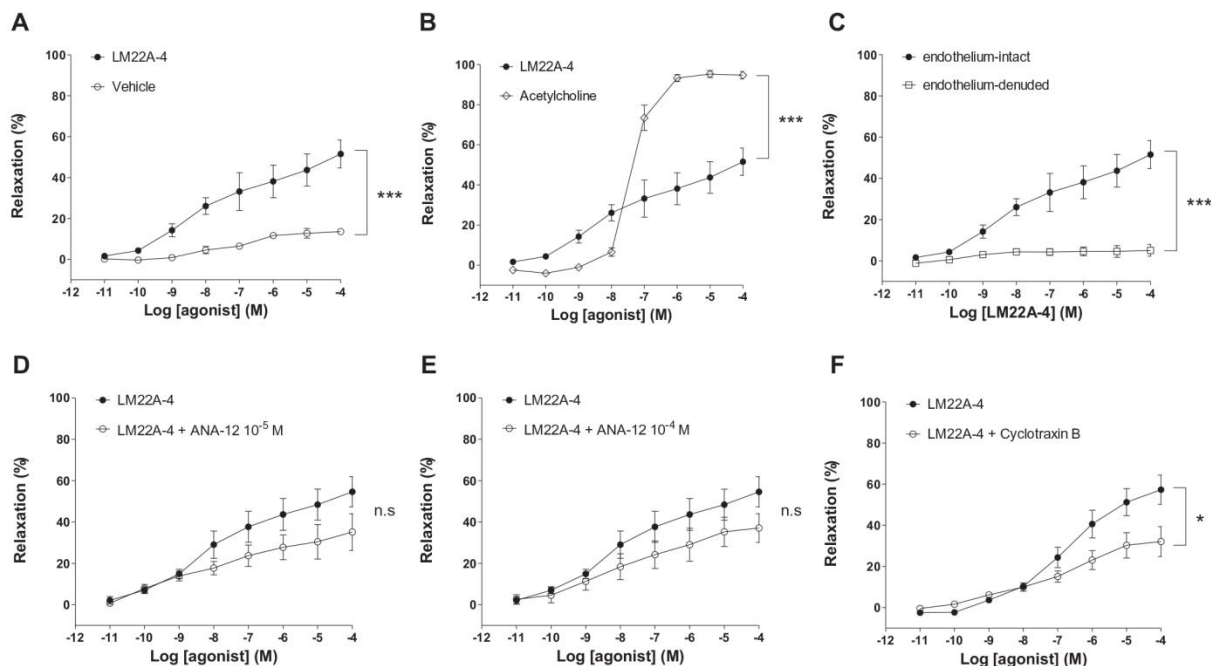


Fig. 3. Role of TrkB receptors on the endothelium-dependent vasorelaxant effect of LM22A-4. Experiments were performed on mesenteric arteries precontracted with phenylephrine (PE) 3.10^{-5} M. Cumulative concentration-response curves to LM22A-4 and its vehicle on endothelium-intact vessels (A). Comparison of the vasorelaxant effects of LM22A-4 and acetylcholine (B). Cumulative concentration-response curves to LM22A-4 after endothelium removal (C), or after a 30-min incubation with two TrkB antagonists: ANA-12 at 10^{-5} M (D), ANA-12 at 10^{-4} M (E) and cyclotraxin B at 10^{-6} M (F). Values are expressed as mean \pm S.E.M. ($n = 8-12$ mesenteric arteries from 8 to 12 rats per group). n.s.: not significant, * $p < 0.05$ and *** $p < 0.001$.

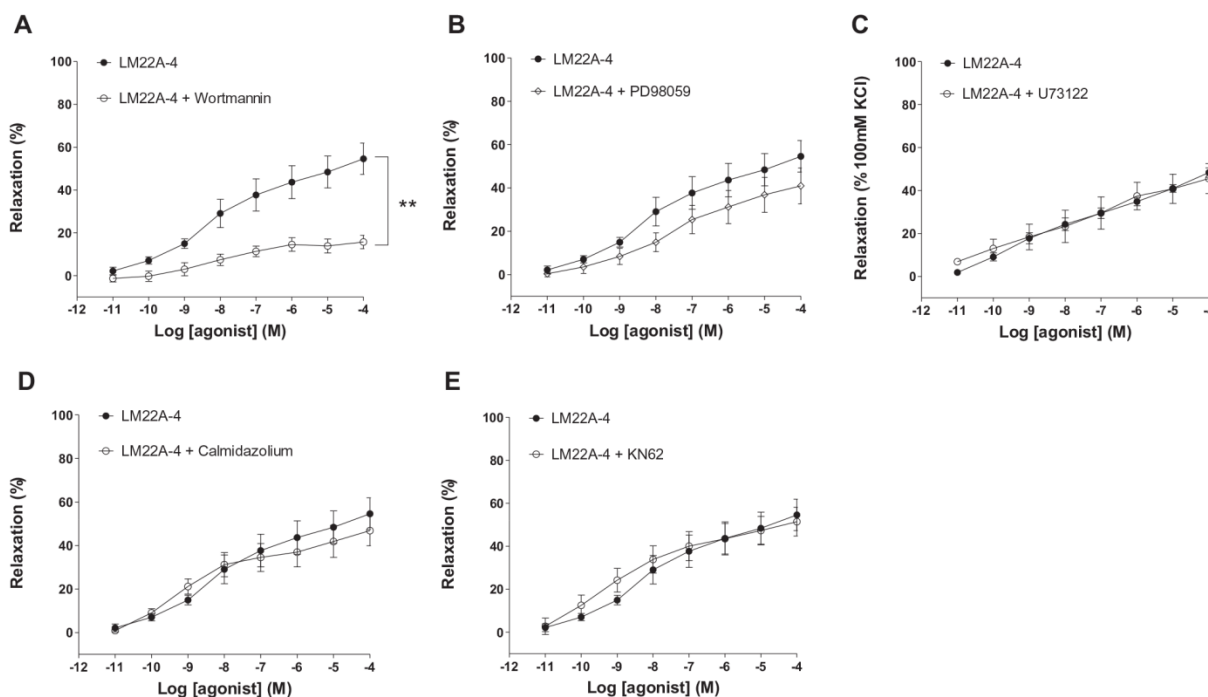


Fig. 4. Involvement of signaling pathways downstream of TrkB activation on the vasorelaxant response to LM22A-4. Cumulative concentration-response curves to LM22A-4 were performed in the presence of wortmannin at 3.10^{-8} M (A), PD98059 at 10^{-5} M (B), U73122 at 10^{-5} M (C), Calmidazolium at 10^{-5} M (D), and KN-62 at 10^{-5} M (E). All experiments were conducted on mesenteric arteries pre-constricted to PE 3.10^{-5} M except in incubation with U73122 where the arteries are pre-constricted to 100 mM KCl. Values are expressed as mean \pm S.E.M. ($n = 7-12$ mesenteric arteries from 7 to 12 rats per group). $**p < 0.01$.

LM22A-4-induced relaxation by small, intermediate and large conductance calcium-activated K^+ (KCa) inhibitors and PGI₂ synthase inhibition indicate that relaxation evoked by TrkB receptor agonists likely requires the combined action of NO, EDHF and prostacyclin. Of note, the fact that LM22A-4-induced relaxation was abolished by both NOS inhibition and EDHF inhibition suggest a cross-talk between these two pathways, and implies that blocking the relaxation to LM22A-4 would require blocking both mechanisms. It is noteworthy that BDNF was already reported to induce a sustained long-lasting Ca^{2+} -activated K^+ current in neurons [28] and that BDNF delivery to the brain potentiated Ach-induced relaxation of basilar artery through increased PGI₂ production [29].

5. Conclusions

The present study reveals that activation of the endothelial BDNF/TrkB pathway results in relaxation of resistance vessels. These data are compatible with a contribution of circulating BDNF to the control of peripheral vascular tone and provide new insights in the comprehension of link observed between circulating BDNF levels and cardiovascular health. This study also identifies LM22A-4 as a valuable synthetic TrkB agonist to disentangle how BDNF interacts with the vascular contractile machinery.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vph.2018.02.005>.

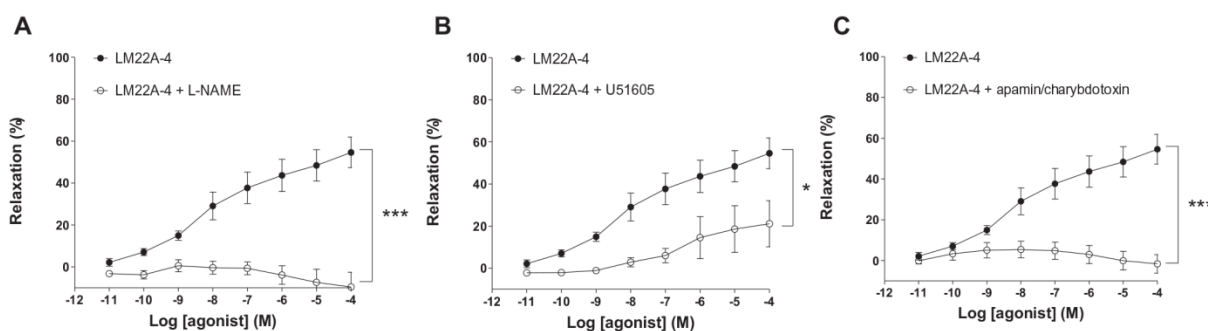


Fig. 5. Contribution of endothelium-derived relaxant factors to LM22A-4-induced relaxation. Experiments were conducted on mesenteric arteries pre-constricted with PE 3.10^{-5} M. Cumulative concentration-response curves to LM22A-4 were obtained after a 30-min incubation with L-NAME at 10^{-6} M (A), U51605 at 10^{-6} M (B) or apamin/charybdotoxin at 10^{-7} M (C). Values are expressed as mean \pm S.E.M. ($n = 7-12$ mesenteric arteries from 7 to 12 rats per group). $*p < 0.05$, $***p < 0.001$.

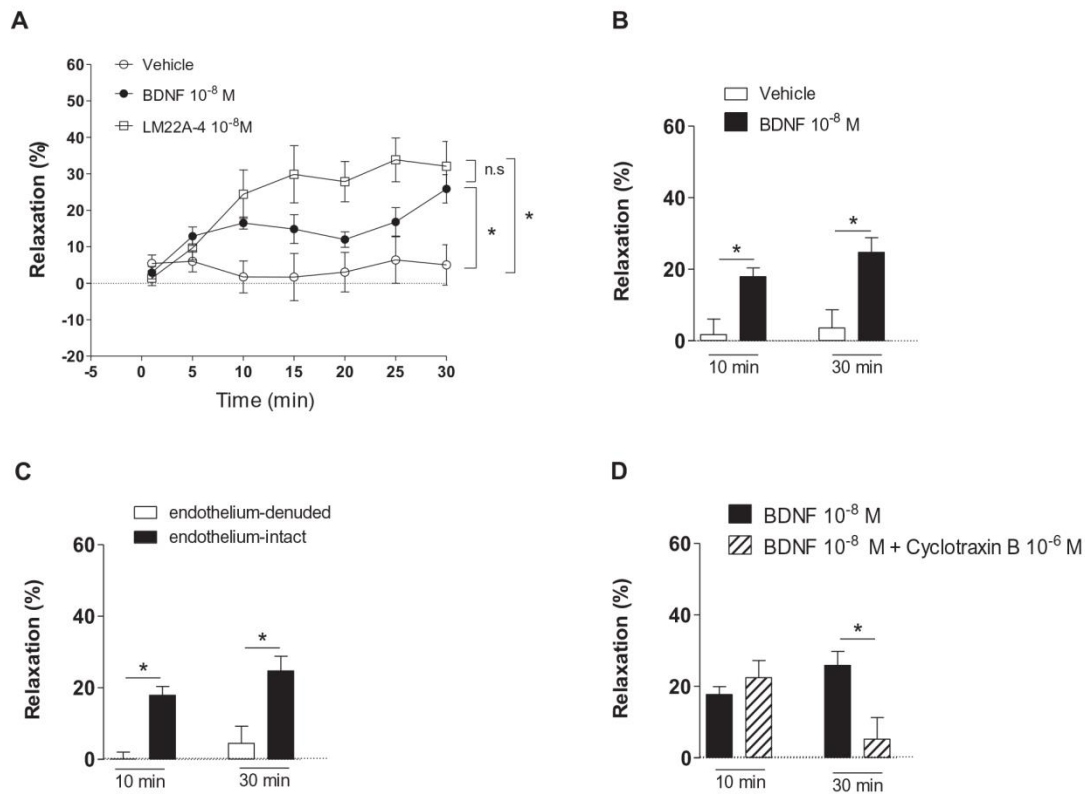


Fig. 6. Comparison of LM22A-4- and BDNF-induced relaxation. Experiments were conducted on mesenteric arteries precontracted with PE 3.10^{-5} M. Time-response curves to LM22A-4 at 10^{-8} M, BDNF 10^{-8} M and its vehicle in endothelium-intact arteries (A, B). Effect of BDNF 10^{-8} M after endothelium removal (C), and in the presence of cyclostratin B 10^{-6} M in endothelium-intact arteries (D). Values are expressed as mean \pm S.E.M. ($n = 6-8$ mesenteric arteries from 6 to 8 rats per group). n.s.: not significant, * $p < 0.05$.

Conflict of interest

The authors declare that they have no conflict of interest.

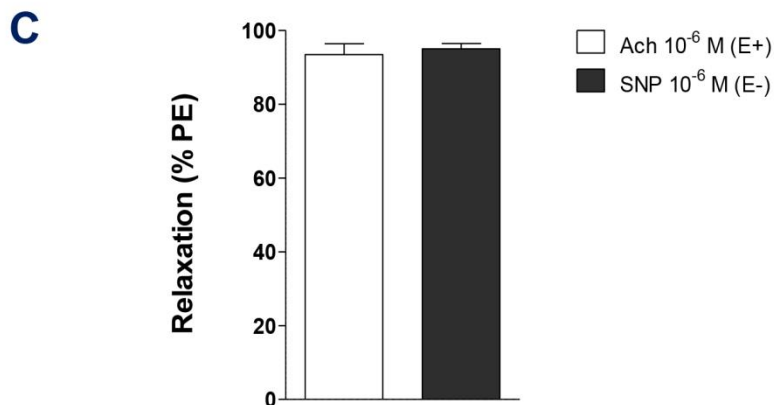
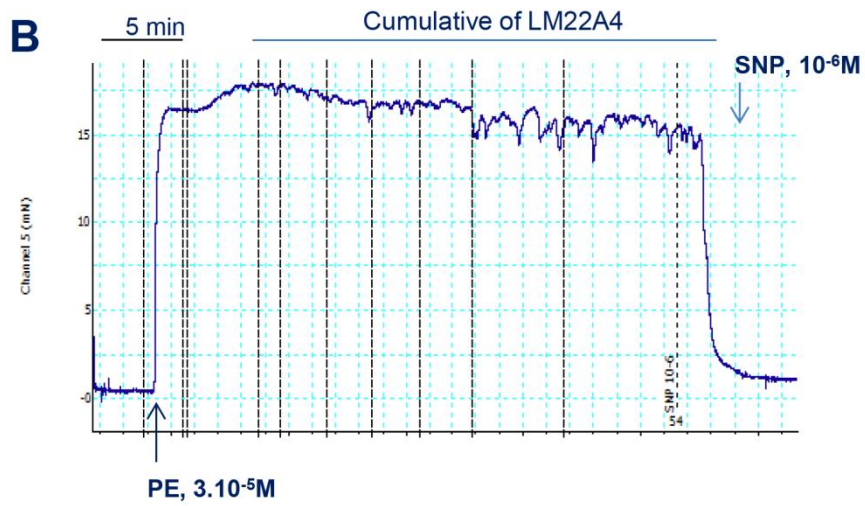
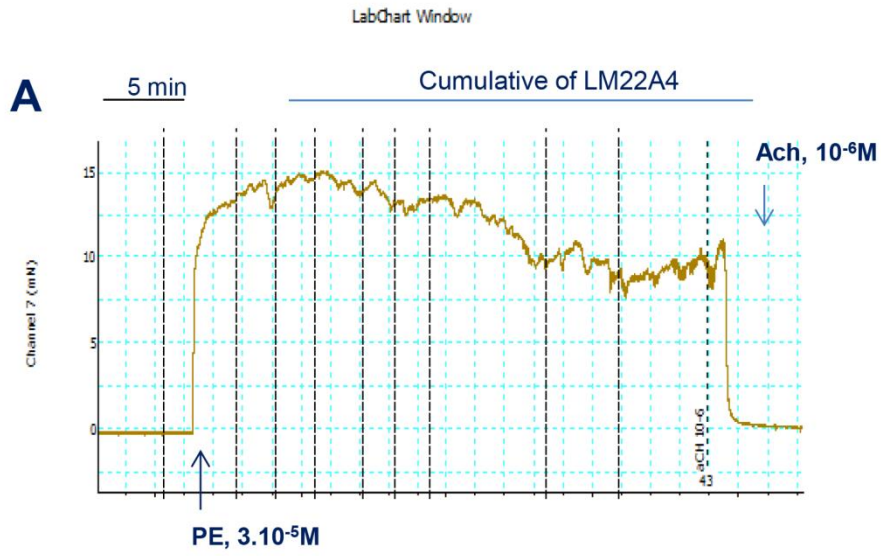
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Article 3

Titre:

Vascular brain-derived neurotrophic factor pathway in rats with adjuvant-induced arthritis. Effect of antirheumatic drugs.

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Résumé des travaux

a) Contexte & hypothèse

La dysfonction endothéliale et l'activation des processus artériosclérotiques qui en résulte est largement incriminée dans le haut risque cardiovasculaire présenté par les patients souffrant de PR. Les études conduites sur les modèles animaux d'arthrite ont montré que de multiples mécanismes pouvaient conduire à une moindre bioactivité du NO d'origine endothéliale même si un point non résolu concerne le rôle de l'inflammation dans l'initiation/maintien/aggravation de la dysfonction endothéliale. Cependant, l'une des recommandations pour réduire le risque cardiovasculaire en cas de PR est de contrôler le plus efficacement possible l'inflammation.

Notre laboratoire a récemment montré que le NO était capable de moduler la production de BDNF par l'endothélium. En effet, les taux vasculaires de BDNF (aorte) sont plus bas chez les rats spontanément hypertendus comparés aux contrôles normotendus (Prigent-Tessier et al., 2013) et sont restaurés par un donneur de NO (Monnier et al., 2017a). De plus, nos premiers travaux (Totoson et al., 2018), réalisés en conditions physiologiques, chez le rat montrent que l'endothélium vasculaire exprime des récepteurs TrkB et que leur activation induit une vasodilatation. Enfin, il a été récemment suggéré que les récepteurs TrkB endothéliaux pourraient être impliqués dans le maintien de l'intégrité vasculaire (Jiang et al., 2015). Plus précisément, la délétion du récepteur endothélial TrkB chez des souris apoE^{-/-} aggrave la lésion athérosclérotique. Étonnamment, l'effet de l'arthrite sur l'expression endothéliale du BDNF et des TrkB n'a jamais été étudié. **Notre hypothèse est que l'arthrite s'accompagne d'une réduction des taux vasculaires de BDNF et que cette réduction est liée à une moindre production de NO par l'endothélium.**

Aussi, l'objectif de notre étude est triple : 1) explorer l'effet de l'arthrite sur les taux vasculaires du BDNF et de son récepteur TrkB, 2) rechercher le lien entre les anomalies potentielles de ces taux et la sévérité de l'inflammation clinique, 3) confirmer ou pas l'existence d'une interaction entre NO et BDNF endothélial en cas d'arthrite.

b) Protocole expérimental

Les expériences ont été réalisées chez des rats contrôles et des rats AIA dont le développement de l'inflammation a été efficacement inhibé par différents traitements anti-inflammatoires (diclofenac, celecoxib, prednisolone, methotrexate et etanercept) tous débutés à l'apparition des signes de l'inflammation et maintenus jusqu'au sacrifice. L'aorte a été prélevée 30 jours après immunisation. Les taux et la localisation du BDNF et des TrkB (entiers et tronqués) ont été étudiés par techniques de western blot et immunohistofluorescence. L'interaction entre le NO et le BDNF chez le rat AIA a été évaluée *ex vivo* via l'étude de l'effet a) d'un donneur de NO (glyceryl trinitrate) sur la production de

BDNF par des aortes prélevées chez des rats AIA et b) d'un agoniste des récepteurs TrkB (LM22A-4) sur la production endothéliale de NO.

c) Principaux résultats

- Les taux aortiques du BDNF et des TrkB entiers ainsi que l'expression endothéliale du BDNF et du TrkB entier phosphorylé sont plus faibles chez les rats AIA comparés aux rats non AIA
- Seuls le méthotrexate et la prednisolone réduisent l'effet de l'arthrite sur les taux aortiques de BDNF
- Un donneur de NO élève les taux de BDNF dans l'aorte de rats AIA qui répond par ailleurs à l'activation des TrkB endothéliaux par la production de NO (via la phosphorylation de la eNOS en sérine 1177)

Cette étude révèle l'existence d'une moindre production endothéliale de BDNF ainsi que l'existence d'une boucle positive entre la production endothéliale de NO et de BDNF en cas d'arthrite. Elle suggère également que la prise en charge efficace de l'inflammation n'est probablement pas une stratégie suffisante pour réduire le risque cardiovasculaire du patient souffrant de PR. Ces résultats incitent à évaluer les potentialités des agonistes des récepteurs TrkB à contrecarrer la moindre production endothéliale de NO associée à la PR.



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Vascular brain-derived neurotrophic factor pathway in rats with adjuvant-induced arthritis: Effect of anti-rheumatic drugs

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ABSTRACT

Background and aims: In rheumatoid arthritis, the control of both disease activity and standard cardiovascular (CV) risk factors is expected to attenuate the increased CV risk. Evidence that brain-derived neurotrophic factor (BDNF) plays a role in vascular biology led us to investigate the vascular BDNF pathway in arthritis rats as well as the interaction between endothelial nitric oxide (NO) and BDNF production.

Methods: The aortic BDNF pathway was studied in rats with adjuvant-induced arthritis (AIA) using Western blot and immunohistochemical analysis. Control of arthritis score was achieved by administration (for 3 weeks) of an equipotent dosage of etanercept, prednisolone, methotrexate, celecoxib or diclofenac. Aortas were exposed to an NO donor or an NO synthase inhibitor and vasoreactivity experiments were performed using LM22A-4 as a TrkB agonist.

Results: Vascular BDNF and full length tropomyosin-related kinase B receptor (TrkB-FL) were higher in AIA than in control rats. These changes coincided with decreased endothelial immunoreactivity in BDNF and pTrkB^{tyr816} and were disconnected from arthritis score. Among anti-rheumatic drugs, only prednisolone and methotrexate prevented AIA-induced vascular BDNF loss. The effect of AIA on aortic BDNF levels was reversed by an NO donor and reproduced by an NOS inhibitor. Finally, LM22A-4 induced both NO-dependent vasodilation and phosphorylation of endothelial NO synthase at serine 1177.

Conclusions: Our study identified changes in the BDNF/TrkB pathway as a disease activity-independent component of AIA-associated changes in endothelial phenotype. It provides new perspectives in the understanding and management of the high CV risk reported in rheumatoid arthritis.

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1. Introduction

Rheumatoid arthritis (RA) is a frequent autoimmune chronic inflammatory joint disease. Beyond the destruction of joint cartilage and the resulting pain, disability and decreased quality of life of patients, RA is associated with high cardiovascular (CV) risk. The relative increase in the CV risk has been related to an acceleration of atherosclerosis [1,2] consecutive to endothelial dysfunction (ED). However, the mechanisms involved in ED remain uncertain. They may comprise a higher prevalence of standard CV risk factors, in

addition to interactions between systemic inflammation and genetic and immune factors [3]. Accordingly, the EULAR's recommendations for the management of CV risk in RA include the optimal control of disease activity and standard CV factors by lifestyle changes [4].

The role of brain BDNF in neuroplasticity and neurogenesis through activation of its cognate full length (FL) tropomyosin-related kinase B receptors (TrkB) is well documented [5–7]. However, there is an increasing interest for the BDNF/TrkB pathway in vessels. BDNF and TrkB are expressed by the vascular wall [8,9]. In addition, there is some evidence that endothelium-derived BDNF binds to TrkB-FL expressed by endothelial cells. First, cultured endothelial cells secrete bioactive BDNF [10]. Second, the exposure of vessels to exogenous BDNF was reported to change vascular biology/function [10–13]. Third, endothelial TrkB receptors were

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recently reported to confer atheroprotection and play a protective role in the development of coronary artery diseases [9]. Finally, endothelial nitric oxide (NO) might stimulate endothelial BDNF synthesis [14] while cultured endothelial cells were reported to overproduce NO in response to exogenous BDNF [11]. Collectively, these data on vascular BDNF/TrkB support the exciting hypothesis that the increased CV risk in RA patients might be connected to changes in the endothelial BDNF/TrkB pathway.

The objective of the present study was to explore the effect of RA on the vascular BDNF/TrkB pathway and the link between vascular BDNF levels and disease activity and endothelial nitric oxide (NO) production. Experiments were performed on aortas collected in rats subjected to adjuvant-induced arthritis (AIA) at a time at which inflammatory symptoms and macrovascular function endothelial are fully developed. An efficient control of disease activity was achieved by administration (for 3 weeks from the onset of inflammatory symptoms) of diclofenac, celecoxib, prednisolone, methotrexate or etanercept. The connection between NO and BDNF production was assessed from *ex vivo* experiments on aorta.

2. Materials and methods

2.1. Animals

Experiments were carried out on 6-week-old male Lewis rats ($n = 98$) purchased from Janvier (Le Genest Saint Isle, France). They were conducted according to the French Department of Agriculture guidelines (license 21 CAE-102) and approved by the local ethics committee. The experimental procedures were performed in order to comply with ARRIVE guidelines. Animals were housed under a 12 h/12 h light/dark cycle and allowed free access to food and water. Anesthesia was induced by isoflurane 4% (Virbac, Carros, France) for arthritis induction. Aortas were harvested after chloral hydrate (400 mg/kg, *i.p.*; Sigma-Aldrich, Saint-Quentin-Fallavier, France) and pentobarbital anesthesia (60 mg/kg, *i.p.*, CEVA Santé Animale, Libourne, France) for biochemical and vasoreactivity experiments, respectively.

2.2. Induction of arthritis and clinical evaluation of arthritis

Arthritis was induced by a single intradermal injection (120 μ L) at the base of the tail of a suspension (10 mg/ml) of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) suspended in mineral oil (Freund's incomplete adjuvant (Difco, Detroit, MI)). Non-arthritis, age-matched Lewis rats that were used as controls received an equivalent volume of saline. Arthritis was evaluated using the following arthritis scoring system [15]: inflammation (erythema and swelling) of one finger scores 0.1, weak and moderate arthritis of one big joint (ankle or wrist) scores 0.5 and intense arthritis of one big joint scores 1. The tarsus and ankle were considered the same joint. The arthritis score for a given limb ranged from 0 to 1.5 and the global arthritis score (four limbs) ranged from 0 to 6. The arthritis score was regularly determined from the first signs of inflammation (~at day 12 post-immunization) until sacrifice (at day 32 ± 3 post-immunization). At this time, inflammatory symptoms are maximal [16,17] and ED occurs at aortic level [17].

2.3. Collection of aortas

Aortas were collected from AIA rats and aged-matched controls and cut into rings (~2–7 mm-length). The rings were immediately frozen at -80°C for western blot analysis, or embedded in OCT (Tissue-Tek, Sakura, USA) for immunohistochemical analysis, or incubated in an appropriate culture medium or used for

vasoreactivity experiments.

2.4. Western blot analysis

Western blot analysis were performed to measure aortic levels of different proteins including mature BDNF (BDNF, 14 kDa), endothelial nitric oxide synthase phosphorylated at serine 1177 (peNOS^{ser1177}, 130 kDa, an enzyme that produces NO in large amount as compared to the unphosphorylated form [18]) as well as the two TrkB receptors that bind BDNF with the same affinity: the full-length TrkB isoform (TrkB-FL, 145 kDa), which is linked to a tyrosine kinase domain and the truncated TrkB isoform (TrkB-T, 95 kDa), which is devoid of this domain [19–21]. Of note, while binding of BDNF to TrkB-FL activates the ras/ERK1/2, PI3 kinase/Akt STAT and phospholipase C γ pathways [5] as a result of receptor phosphorylation at tyrosine 816, its binding to TrkB-T receptors (a dominant negative receptor) inhibits TrkB-FL-dependent signaling [21].

Frozen rings were homogenized with five volumes of ice-cold lysis buffer [100 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EGTA, 1% triton X-100, 1% protease inhibitor cocktail (P8340, Sigma-Aldrich, Saint-Quentin-Fallavier, France)]. After centrifugation of the homogenates, an aliquot of the supernatant was kept for protein measurement by using the Lowry method. Equal amounts of protein were resolved by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (0.2 μ m or 0.45 μ m) for Western blotting. After blocking non-specific binding sites with a 5% solution of non-fat dry milk in Tris-buffered saline (20 mM Tris/HCl, 137 mM NaCl, pH 7.4) containing 0.1% Tween 20, membranes were probed with an anti-BDNF rabbit monoclonal antibody (1/3000 with 5% non-fat dry milk, ab108319, Abcam, Cambridge, United Kingdom) to detect BDNF, an anti-TrkB rabbit monoclonal antibody (dilution 1/1000, 610102, BD Transduction, San Jose, USA) to detect both TrkB-FL and TrkB-T or a mouse monoclonal antibody directed against peNOS^{ser1177} (dilution 1/1000 with 7.5% BSA, 612383, BD Biosciences, San Jose, USA). β -actin (dilution 1/10000, A5441, Sigma-Aldrich, Saint-Quentin-Fallavier, France) was used as internal control. The membranes were then incubated with secondary antibodies conjugated with horseradish peroxidase [Jackson ImmunoResearch Laboratories, 111-035-144 (anti-rabbit, dilution 1/20000) and 115-035-166 (anti-mouse, dilution 1/50000), Interchim, Montluçon, France]. Gels were run in duplicate. Protein-antibody complexes were visualized using the enhanced chemiluminescence Western blot detection system (ECL2, 1151–7371, Fisher Scientific, Illkirch, France). The band densities were determined by scanning densitometry (GS-800, BIO-RAD Laboratories, Ivry sur Seine, France) and the results were expressed as percentages of control values.

2.5. RNA extraction and quantitative reverse-transcription PCR analysis

Total RNA was extracted from frozen aortic rings using the RNeasy Fibrous Tissue Mini Kit (74704, Qiagen, Hilden, Germany). After quantification, 500 ng of RNA was reverse transcribed using iScript Reverse Transcription Supermix (BIO-RAD Laboratories). Sequences of BDNF and housekeeping HPRT (hypoxanthine phosphoribosyl transferase) genes were obtained from the National Center for Biotechnology Information (NCBI) database, and the primers sequences (Eurogentec), described in supplemental data (Table 1), were designed using the Primer-Basic Local Alignment Search Tool (Primer-BLAST). Real-time PCR and quantification of gene expression were performed as described previously [14] with the relative transcription level of the *bdnf* gene calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Results were expressed as relative mRNA levels as

Table 1
Effect of antirheumatic drugs on arthritis score.

Day post-immunization	15	20	25	30	F value	p-value
Saline (n = 12)	3.3 ± 0.8	4.5 ± 1.2	4.9 ± 1.2	4.7 ± 1.4		
Prednisolone (n = 6)	1.3 ± 0.9	2.4 ± 1.8	1.6 ± 1.4	1.1 ± 1.0	23,49	<0.05
Methotrexate (n = 6)	1.8 ± 0.8	1.9 ± 1.1	1.6 ± 0.9	0.8 ± 0.5	17,40	<0.05
Etanercept (n = 6)	1.6 ± 0.7	3.4 ± 1.1	1.5 ± 1.0	0.6 ± 0.5	19,50	<0.05
Celecoxib (n = 6)	2.5 ± 0.7	2.7 ± 0.7	2.2 ± 1.0	1.5 ± 1.3	14,49	<0.05
Diclofenac (n = 6)	2.1 ± 1.3	2.4 ± 1.0	1.9 ± 1.1	1.1 ± 1.2	12,19	<0.05

Antirheumatic drugs or saline were administered from the first signs of inflammation to sacrifice (see dosage in the methods). Values are expressed as means ± SD. n = number of rats. $p < 0.05$ compared to saline (one way ANOVA).

compared to control values.

2.6. Immunostaining and fluorescence microscopy

Using antibodies directed against BDNF used for Western blot analysis and pTrkB^{Y816}, immunohistochemistry experiments were performed to investigate whether changes in levels of aortic BDNF protein and mRNA translated into changes in endothelial staining of BDNF and pTrkB^{Y816}, an indicator of BDNF binding to TrkB-FL. Importantly, anti-BDNF antibody cannot discriminate the mature form of BDNF from its precursor proBDNF, but allows us to study the repercussions at the endothelial level of the modifications in BDNF protein and mRNA observed at the level of the entire aorta. In the same way, anti-TrkB antibody recognized both FL and T isoforms, immunohistochemical experiments were performed with pTrkB^{Y816} antibodies. This strategy allows us to know whether endothelial cells did express the TrkB-FL isoform as only this isoform can be phosphorylated and in addition to get information on the binding of BDNF to this isoform. Thus, aortic rings embedded in OCT were transversally cut (8- μ m thick) at -20°C using a cryostat (HM550, OMPV, Microm-Microtech, Francheville, France) and the slices were placed on SuperFrost Plus slides. After fixation and blockade of non-specific binding sites, the slides were first incubated with the antibodies directed against BDNF (dilution 1/200) or pTrkB^{Y816} (polyclonal antibody, dilution 1/200, ABN1381, Merck-Millipore, Saint Quentin en Yvelines, France) in the presence of antibody directed against von Willebrand factor (vWF) (dilution 1/200, mouse monoclonal, MCA 3442, Cln 21–43, Merck-Millipore, Saint Quentin en Yvelines, France) as a specific marker of endothelial cells. Slides were then exposed to a fluorescent secondary antibody directed against ALEXA Fluor 488 and 568 [A11029 (anti-mouse) and A11036 (anti-rabbit), Molecular Probes, Invitrogen, Cergy Pontoise, France, dilution 1/1000]. Negative controls were prepared by omitting the primary antibodies. Finally, slides were mounted with DAPI (a nuclear marker)-containing mounting medium (Vectashield, FP-DT094A, Interchim, Montluçon, France) and analyzed by using an epifluorescent microscope (Eclipse E600, Nikon France S.A.S., Champigny-sur-Marne, France). Endothelial staining was quantified using an automated method with Matlab software (Mathworks, Natick, USA).

2.7. Ex vivo experiments on incubated aorta

Fresh aortic rings were quickly distributed into wells previously loaded with a culture medium (M199 medium supplemented with 0.1 mg/L L-glutamine, 10% fetal bovine serum, 2% glucose, 2% amino acids and 1% antibiotic solution). Rings from AIA rats were incubated for 24 h (in a 95% O₂-5% CO₂ atmosphere) with either 10 μ M glycyl trinitrate (GTN, MR107753, Merck-Millipore, Saint Quentin en Yvelines, France) as a slow-releasing NO donor, which mimics physiological endothelial NO production quite well, or saline. In parallel, rings from control rats were incubated with either 5 mM of the eNOS inhibitor N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME,

N5751, Sigma-Aldrich, Saint-Quentin-Fallavier, France) or saline. In additional experiments, fresh aortic rings from control and AIA rats were incubated (30 min, in a 95% O₂-5% CO₂ atmosphere) with either LM22A-4 (4607, Tocris R&D, Bristol, UK) as a synthetic TrkB agonist or its vehicle DMSO (0.1% in saline). At the end of the incubation period, the rings were prepared for Western blot analysis.

2.8. Ex vivo vascular reactivity experiments

These experiments aimed to explore the capacity of endothelial TrkB activation to induce NO production in AIA. They investigated to what extent the exposure of AIA aortic rings to a TrkB agonist could induce NO-dependent relaxation. For this purpose, AIA aortic rings precontracted with phenylephrine (PE) were exposed (30 min) to increasing concentrations of LM22A-4 or its vehicle (DMSO 0.1% in saline) using the organ bath technique. Exposure (30 min, before PE) to L-NAME was used to inhibit NOS. More precisely, aortic rings (2 mm-length) were connected to an isometric force transducer and suspended in a custom-made 10-ml organ chamber filled with Krebs solution (NaCl 118 mM, KCl 4.65 mM, CaCl₂ 2.5 mM, KH₂PO₄ 1.18 mM, NaHCO₃ 24.9 mM, MgSO₄ 1.18 mM, glucose 12 mM, pH 7.4), maintained at 37 $^{\circ}\text{C}$ and continuously aerated with 95% O₂, 5% CO₂. After a 90-min equilibration period under a resting tension of 2 g, the rings were exposed to high extracellular KCl (100 mM) to measure their strong tonic contractile response. The presence of functional endothelium was verified by the ability of the endothelium-dependent agonist acetylcholine (Ach, 10⁻⁶ M) to induce more than 80% relaxation in the rings precontracted with PE (10⁻⁶ M). LM22A-4 was used at concentrations previously shown to relax isolated precontracted mesenteric arteries [22]. The vasorelaxant responses were calculated as the percentage of relaxation of PE-induced contraction. The overall relaxant response was expressed by the area under the curve (AUCs) calculated from the individual time-response curves.

2.9. Drug treatment

Antirheumatic drugs were administered according to dosage previously shown by our laboratory to significantly reduce arthritis score [23–26]. Drugs or saline were administered from the first signs of inflammation (day 11–12 post-immunization) and maintained for three weeks. In a first set of experiments, rats received etanercept (10 mg/kg/3 days, s.c), prednisolone (10 mg/kg/day, i.p.), methotrexate (1 mg/kg/week, s.c) or saline. In a second set of experiments, rats received diclofenac (5 mg/kg twice a day, i.p), celecoxib (3 mg/kg/day, i.p) or saline. Aorta was collected 24 h after the last administration of the anti-rheumatic drugs, except for diclofenac for which it was collected 12 h after the last administration. Saline rats received 1 mL/kg/day.

2.10. Data and statistical analysis

Results of the western blot analysis, immunohistochemistry

experiments and arthritis score were expressed as means \pm standard deviations (SD). Comparison between two groups of rats was performed using a parametric *t*-test or non-parametric Mann–Whitney *U* test, depending on normality equal variance tests or one-way ANOVA for repeated measures. The relationship between two variables was investigated using Pearson's or Spearman's correlations (for normally or not normally distributed data, respectively). Results for the vasoreactivity experiments were expressed as means \pm standard error of the mean (S.E.M) and analyzed using GraphPad Prism software (version 5.0). Concentration-responses curves to LM22A-4 at different concentrations or in the presence of L-NAME were compared by 2-way analysis of variance (ANOVA) for repeated measures. $p < 0.05$ was considered statistically significant.

3. Results

3.1. AIA impairs the vascular BDNF/TrkB pathway

The BDNF/TrkB pathway was examined in aortas harvested from untreated AIA rats and non-AIA control rats. We first

simultaneously analyzed BDNF protein and mRNA levels (Fig. 1) in aorta from control ($n = 6$) and AIA rats (arthritis score = 3.5 ± 1.1 , $n = 11$). AIA decreased BDNF levels by 70% (Fig. 1A). As shown in Fig. 1B, multiple bands were observed after incubation of the membranes with antibody directed against BDNF. The band at 14 kDa corresponds to the mature form of BDNF while the band at 35 kDa corresponds to proBDNF. Note that according to the protein load suitable for the detection of BDNF changes, the band at 35 kDa was fully saturated. AIA also induced a decrease (-50%) in BDNF mRNA levels (Fig. 1C). These changes in vascular BDNF synthesis translated into changes in endothelial BDNF staining (Fig. 1D). Indeed, immunoreactivity experiments (performed on additional six control and six AIA rats with an arthritis score of 3.4 ± 1.1) rats showed that BDNF immunoreactivity was mainly confined to cells positive for the endothelial marker vWF in control rats and that this immunoreactivity was markedly reduced in AIA rats. According to automated quantification of fluorescence intensity, endothelial BDNF staining reached 28 ± 14 in control rats but only 9.9 ± 5.5 in AIA rats ($p = 0.016$, not shown). In rats used for BDNF protein and mRNA levels analysis, we also analyzed vascular levels of TrkB-FL and TrkB-T (Fig. 2). AIA led to a specific decrease (-25%) in

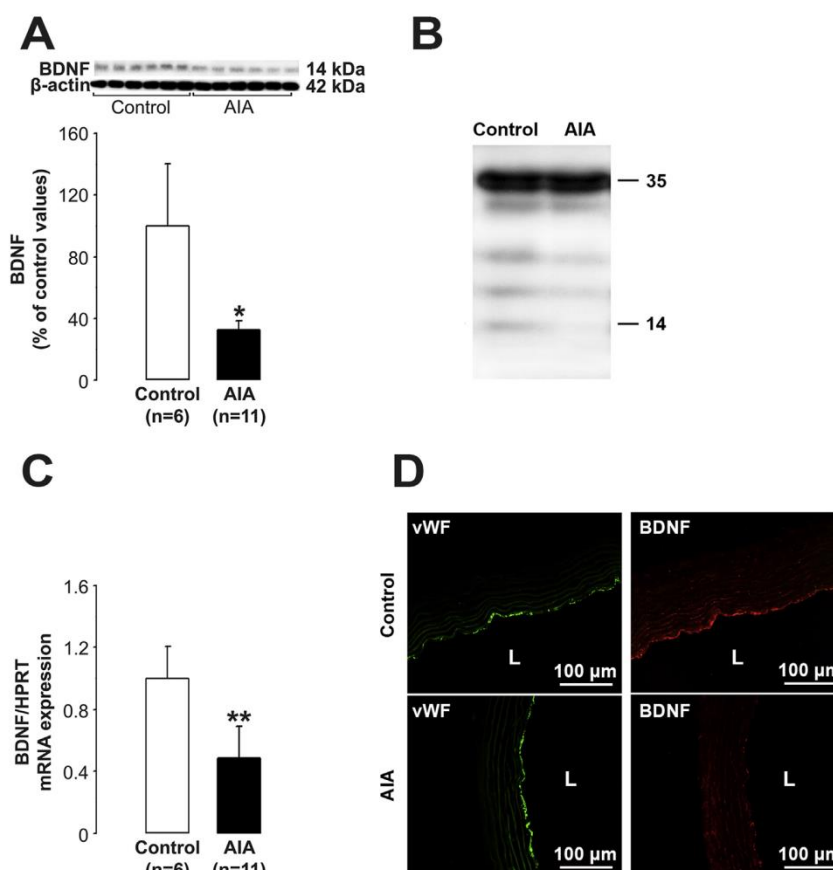


Fig. 1. Effect of arthritis on aortic levels of BDNF protein and mRNA.

(A) BDNF protein levels and representative immunoblots using β -actin as internal control, (B) mature BDNF corresponds to the band at 14 kDa, (C) BDNF mRNA levels, (D) representative immunostaining of BDNF (red) on transversal vascular slice showed that BDNF was confined to cells facing the vascular lumen (L), i.e. endothelial cells (positive for vWF, green). Aorta was collected at day 31 ± 2 post-immunization. Values are expressed as means \pm SD, $n =$ number of rats, $*p < 0.05$, $**p < 0.01$ compared to control values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

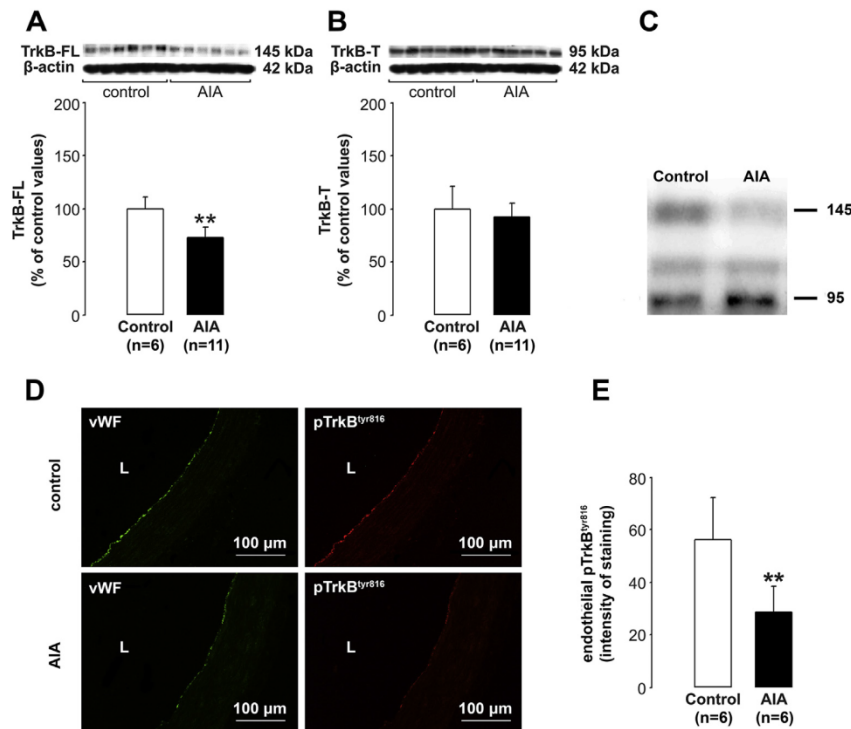


Fig. 2. Effect of arthritis on aortic TrkB receptors.

(A) TrkB-FL and (B) TrkB-T levels and representative immunoblots using β -actin as internal control. (C) incubation with anti-TrkB antibody allowed the simultaneous detection of TrkB-FL (145 kDa) and TrkB-T (95 kDa) on a same membrane, (D) representative immunostaining of pTrkB^{tyr816} (red) on transversal vascular slice showed that receptors were expressed by cells facing the vascular lumen (L); i.e. endothelial cells (positive for vWF, green), (E) quantification of endothelial pTrkB^{tyr816} staining. Aorta were collected at day 31 ± 2 post-immunization. Values are expressed as means \pm SD, n = number of rats, ** $p < 0.01$ compared to control values. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

vascular levels of TrkB-FL receptors (Fig. 2A), the truncated TrkB isoform (TrkB-T) being spared (Fig. 2B). Fig. 2C displays representative immunoblots of TrkB-FL (145 kDa) and TrkB-T (95 kDa) in control and AIA rats. Importantly, immunostaining of pTrkB^{tyr816} (Fig. 2D) was restricted to endothelial cells (cells positive for vWF) in control rats and lower in AIA than in control rats. More precisely, the intensity of pTrkB^{tyr816} staining (Fig. 2E) was 54 ± 16 and 29 ± 10 in control and AIA rats, respectively ($p < 0.01$). These data indicated that TrkB-FL receptors were expressed by endothelial cells in both control and AIA rats and that activated TrkB receptors levels were lower in AIA than in control rats.

3.2. Vascular BDNF levels did not correlate with inflammatory symptoms in AIA rats

In order to explore whether changes in the vascular BDNF/TrkB pathway induced by AIA related to the severity of clinical inflammation, we first plotted either vascular BDNF and TrkB-FL levels (obtained from western blot analysis) or endothelial pTrkB^{tyr816} staining against corresponding arthritis score (just before sacrifice) in untreated AIA rats. No association was observed (data not shown). Then, we explored the effect of antirheumatic drugs on vascular BDNF levels. Importantly, as expected and shown in Table 1, arthritis scores were significantly lower in antirheumatic drugs-treated than in saline-treated AIA rats (for comparisons, the two groups of saline-treated AIA rats were pooled as no difference in

arthritis score was observed between the groups). Note that the antirheumatic drugs had comparable efficacy on inflammatory symptoms. By contrast, the effect of antirheumatic drugs on vascular BDNF levels was drug-dependent. As shown in Fig. 3, while prednisolone (Fig. 3A) and methotrexate (Fig. 3B) increased vascular BDNF levels by ~3-fold as compared to saline, vascular BDNF levels were not significantly changed by etanercept (Fig. 3C), diclofenac (Fig. 3D) or celecoxib (Fig. 3E). Collectively, these data showed a disconnection between vascular BDNF levels and clinical inflammation in saline-treated AIA rats and indicated that the control of disease activity by antirheumatic drugs was not sufficient to counteract the effect of AIA on vascular BDNF levels.

3.3. Interaction between NO production and vascular BDNF levels in AIA rats

Decreased endothelial NO bioactivity as a result of decreased NO production and increased NO inactivation is a main characteristic of endothelial dysfunction in AIA [17,27,28]. In addition, interplay exists between endothelial BDNF and NO production (see introduction). Collectively, these data led us to formulate the hypothesis that AIA-induced reduction in vascular BDNF levels might relate to changes in endothelial NO production. In a first set of experiments, aortas harvested from control (n = 6) and AIA (n = 6, arthritis score = 3.4 ± 0.6) rats at day 30 post-immunization were incubated with L-NAME (NOS inhibitor) and GTN (NO donor), respectively.

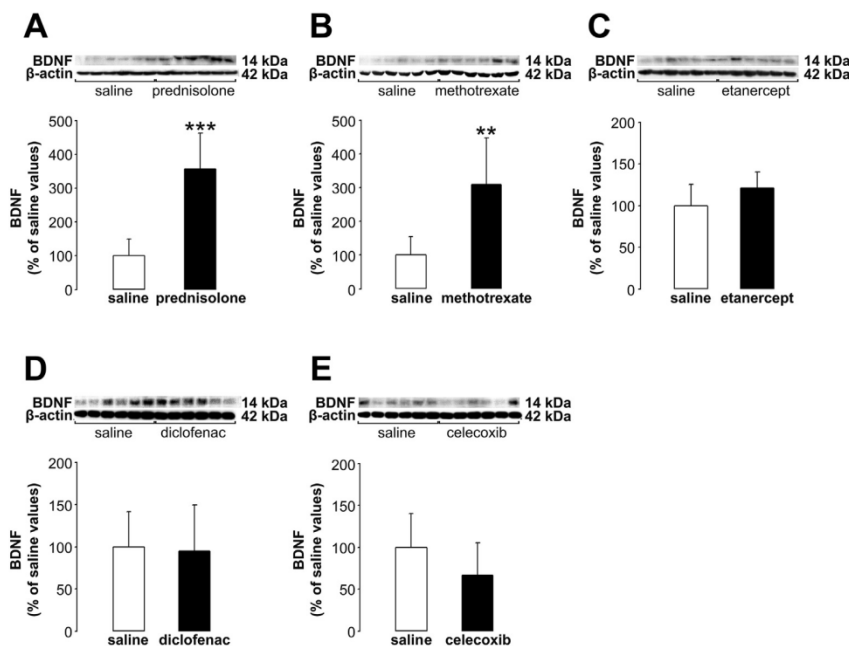


Fig. 3. Effect of anti-inflammatory drugs on aortic BDNF levels in AIA.

Administration of (A) prednisolone, (B) methotrexate, (C), etanercept, (D) diclofenac, (E) celecoxib from the first signs of inflammation until removal of aorta at days 32–33 post-immunization in AIA rats. Corresponding immunoblots of BDNF using β -actin as internal control are shown above the bar graphs. Values are expressed as means \pm SD from 6 rats, ** $p < 0.01$, *** $p < 0.001$.

While L-NAME decreased BDNF levels in control aortas (Fig. 4A), GTN increased BDNF levels in AIA aortas (Fig. 4B), indicating that NOS inhibition reproduced and NO supplementation reversed the effect of AIA on vascular BDNF levels. In a second set of experiments, we explored the ability of endothelial-derived BDNF to stimulate endothelial NO production in AIA. For this purpose, we investigated the potential relaxant effect of the TrkB agonist LM22A-4 on isolated aortic rings harvested from AIA rats (arthritis score before vessel collection was 4.8 ± 0.4 , $n = 4$). As shown in Fig. 4C, LM22A-4 resulted in a concentration-dependent dilation of precontracted rings, an effect that was prevented by previous incubation with L-NAME (Fig. 4D). Furthermore, LM22A-4 resulted in increased vascular levels of peNOS^{ser1177} in both AIA (Fig. 4E, arthritis score before vessel collection was 2.8 ± 1.6 , $n = 6$) and control non-AIA (Fig. 4F, $n = 6$). Of note, the increase in peNOS^{ser1177} was higher in control ($\times 3.6$) than in AIA rats ($\times 2.6$). These data fit well with our results reporting decreased vascular TrkB-FL expression (-25% , Fig. 2A) in AIA rats as compared to control rats. We inferred from these data that activation of endothelial TrkB receptors stimulated NO production as a result of induction of eNOS phosphorylation at serine 1177.

4. Discussion

Using AIA rats at day -30 post-immunization, a time at which AIA rats exhibit severe inflammatory symptoms, the present study revealed that AIA decreased endothelial BDNF synthesis and TrkB-FL dependent signaling in the aorta, irrespective of the severity of symptoms. It also showed that when given at efficient doses on inflammatory symptoms only prednisolone and methotrexate but not etanercept, diclofenac or celecoxib prevented AIA-induced aortic BDNF loss. Finally, our study identified a positive feedback

loop between endothelial NO and BDNF production in AIA.

First, the present study extends current knowledge on vascular TrkB receptors. We showed that aortas express both TrkB-FL and TrkB-T and that TrkB-FL are prominently expressed by endothelial cells. These data are in line with the presence of TrkB-FL and TrkB-T in rat mesenteric arteries [22] and human pulmonary arteries even though, in human vessels, expression of these receptors was not restricted to the endothelium as evidenced from experiments performed in endothelium-denuded and intact vessels [11]. Importantly, evidence that vascular endothelial cells express BDNF and TrkB does not mean that a given endothelial cell simultaneously expresses BDNF and TrkB. Indeed, using single-cell analysis of cardiac microvascular endothelial cells (real time-PCR), Bai et al. [29] showed that no single cells expressed both BDNF and TrkB simultaneously. Second, the study revealed that AIA induces a specific downregulation of the TrkB-FL isoform, which is the isoform involved in vascular health. Indeed, binding of BDNF to TrkB-FL was reported to induce vasodilation [11,13,30] and angiogenesis [10], and to decrease TNF α (tumor necrosis factor)-induced endothelial hyperpermeability [9]. Moreover, activation of endothelial TrkB receptors was recently reported to protect against atherosclerosis. More precisely, endothelial TrkB receptor deletion in apoE ($-/-$) mice resulted in an aggravation of the atherosclerotic lesion [31]. In addition, subjects with decreased endothelial TrkB expression were reported to be at a high risk of coronary artery diseases [9]. In the present study, aortic levels of BDNF protein and mRNA as well as endothelial BDNF and pTrkB^{tyr816} (an indicator of TrkB-FL activation by endogenous BDNF) immunoreactivity were lower in AIA than in control rats. Evidence that downregulation of TrkB-FL occurred in conjunction with decreased endothelial BDNF synthesis and decreased activation of TrkB-FL is consistent with a control of endothelial function by endothelium-derived BDNF.

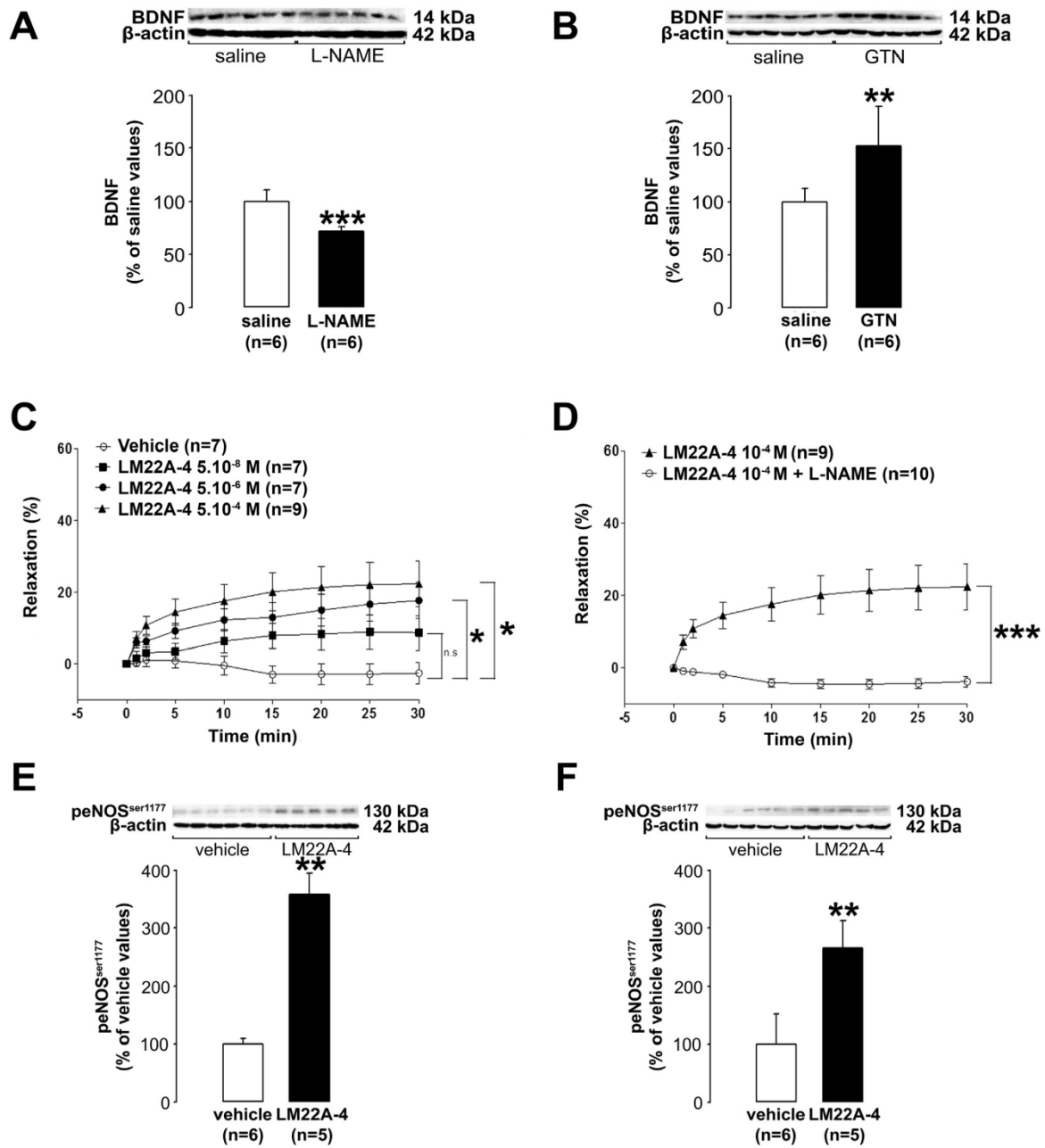


Fig. 4. Loop between BDNF and NO production.

(A) NOS inhibition results in decreased BDNF levels. Aorta collected in control rats were incubated with saline or 5 mM (G)-Nitro-L-arginine methyl ester (L-NAME). (B) NO supplementation increases BDNF levels. Aorta collected in AIA rats were incubated with saline or 10 μ M glyceryl trinitrate (GTN). (C) Endothelial TrkB activation results in a relaxant effect. Time-response curve to LM22A-4 and its vehicle (DMSO) on endothelium-intact aortic rings previously constricted with phenylephrine (10⁻⁶ M). (D) NOS inhibition by L-NAME (10⁻⁶ M) prevents the relaxant effect of LM22A-4. (E and F) endothelial TrkB activation increases peNOS^{ser1177} levels in both control (E) and AIA (F) rats. Aortic rings were incubated (30 min) with either LM22A-4 (10⁻⁴ M) or its vehicle (DMSO 0.1%). Values shown in bars graphs (associated to corresponding immunoblots) are expressed as means \pm SD whereas values shown in time-response curves are expressed as means \pm S.E.M. n = number of rats (A, B, E, F) or number of aortic rings (C and D). **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Interestingly, low endothelial BDNF synthesis/expression was previously reported in diabetic [32] and spontaneously hypertensive rats [30,33]. As AIA, like diabetes and hypertension, increases CV risk by accelerating atherosclerosis, low endothelial BDNF synthesis might be a new common feature of a pro-atherosclerotic endothelial phenotype. Further studies are needed to investigate whether impaired endothelial BDNF signaling contributes to the atherosclerosis process or is only an epiphenomenon. Importantly, decreased activation of endothelial TrkB receptors in AIA cannot be related to changes in circulating BDNF levels as RA was reported to increase circulating BDNF levels in both human and animal [34–36].

The effective control of disease activity by antirheumatic drugs is a recommended strategy to manage RA-associated CV morbidity and mortality even though methotrexate is the only effective antirheumatic drug shown to reduce cardiovascular risk [37]. Assuming that low endothelial BDNF synthesis could be a marker of CV risk, we explored the effect of different efficient antirheumatic therapies on vascular BDNF levels in AIA. It is noteworthy that only methotrexate and prednisolone attenuated the effect of AIA on BDNF levels, whereas etanercept, diclofenac or celecoxib did not. This differential impact of antirheumatic drugs to reduce arthritis score clearly indicated that changes in vascular BDNF are disconnected from the severity of the inflammatory symptoms (disease activity) since drugs were administered at equally effective doses on clinical inflammation (see Table 1). These data are in keeping with our present results reporting a lack of correlation between vascular BDNF levels and arthritis scores in untreated AIA rats. They are also in line with clinical studies that reported a mismatch between vascular function and disease activity [38,39] as well as a persistence of vascular impairment in RA patients despite remission of the disease [40]. A point that remains to be solved is why among drugs only prednisolone and methotrexate treatment increased vascular BDNF levels.

An additional recommended strategy to manage RA-associated CV morbidity and mortality is the control of traditional CV risk factors by changes in lifestyle. As decreased endothelial NO bioactivity is a main characteristic of endothelial dysfunction (ED) and subsequent increased CV risk, such recommendations led us to investigate connections between endothelial NO bioactivity and endothelial BDNF production. Our *ex vivo* experiments on incubated aorta showed that NOS inhibition reproduced and NO supplementation alleviated the effect of AIA on vascular BDNF levels. These data support the notion that decreased endothelial NO bioactivity might contribute to the AIA-induced reduction in endothelial BDNF synthesis. However, decreased endothelial NO production in AIA might be also a consequence of AIA-induced impaired BDNF/TrkB pathway. More precisely, decreased endothelial expression of BDNF and/or TrkB-FL by AIA might be involved in decreased capacity of endothelial cells to produce NO. To support this notion are our results showing that endothelial TrkB activation (by LM22A-4) induced both NO-dependent vasodilation and phosphorylation of eNOS at serine 1177 and that elevation of peNOS^{ser1177} levels in response to LM22A-4 was lower in AIA than in control rats. Such decrease in peNOS^{ser1177} expression likely relates to decreased PI3K/Akt activation. Indeed, Akt activation was previously reported to be involved in recombinant BDNF-induced eNOS phosphorylation in cultured endothelial cells [11] while blockade of the PI3K/Akt pathway was recently reported to prevent LM22A-4 induced endothelium- and NO-dependent relaxation of mesenteric arteries [22]. Nevertheless, it will be of interest to perform studies aimed at specifically investigating the link between TrkB pathway and endothelial NOS uncoupling as endothelial NOS uncoupling was reported in AIA [28,41]. Notably, a positive feedback loop between endothelial NO and BDNF production, at

least in physiological condition can be suspected from studies that reported increased vascular BDNF levels in response to NO donor [14] and increased endothelial NO production in response to recombinant BDNF [11]. The new data provided by the present studies are that such loop is effective in RA, thus supporting the new hypothesis that low endothelial BDNF production might be both cause and consequence of impaired endothelial NO production and by extension ED in RA. Unfortunately, whether NO modifies BDNF levels or vice versa remains to be elucidated. Comparing kinetic changes in endothelial NO and BDNF production in AIA will consequently help to elucidate this chicken-and-egg situation. In any case, activation of endothelial TrkB receptors remains an attractive strategy to reduce rheumatoid arthritis-associated high cardiovascular risk. Finally, the interplay identified between endothelial NO and BDNF production in AIA suggests that the differential impact of anti-rheumatic drugs on vascular BDNF may relate to their differential impact on endothelial NO bioactivity (i.e. on eNOS or arginase expression, eNOS uncoupling or NO inactivation^{23–26}). However, as the cytokine TNF α was reported to modulate endothelial BDNF production [42,43], it cannot be excluded that the effect of antirheumatic drugs on vascular BDNF also includes TNF α -dependent mechanisms.

In conclusion, the present study identified an impaired BDNF/TrkB pathway as a new component of AIA-induced changes in the endothelial phenotype, irrespective of the severity of the inflammatory symptoms. It also identified a positive feedback loop between endothelial BDNF and NO production in AIA and revealed a differential impact of antirheumatic drugs on the vascular BDNF pathway. These findings really open new perspectives in our understanding and the management of increased CV risk in RA.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

All authors were involved in drafting the article, and all authors approved the final version to be published. Dr. Marie had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Demougeot, Garnier, Tessier, Marie.

Acquisition of data: Pedard, Totoson, Quirié, Verhoeven.

Analysis and interpretation of data: Pedard, Demougeot, Marie.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.atherosclerosis.2018.05.004>.

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Supplemental data

Table 1. Primer sequences for real-time RT-PCR analysis

Gene	Primer sequences	T _m (°C)	Product size (bp)
<i>T</i> <i>otal</i>	FWD 5'- ATGGTTATTTCATACTTCGGTTGC,	59.35	87
	-3'		
<i>bdnf</i>	RVS 5'-	59.62	
	AGCTGGGTAGGCCAAGTTG -3'		
<i>hp</i> <i>rt</i>	FWD 5'- GCGAAAGTGGAAAAGCCAAGT -	59.93	76
	3'		
	RVS 5'- GCCACATCAACAGGACTCTTGAG	61.39	
	-3'		

Abbreviations: *bdnf*: brain-derived neurotrophic factor; *hp*: hypoxanthine phosphoribosyl transferase; FWD: forward; RVS: reverse; T_m: melting temperature.

Article 4

Titre:

Brain-derived neurotrophic factor in adjuvant-induced arthritis in rats. Relationship with inflammation and endothelial dysfunction

Auteurs:

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Journal:

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Résumé des travaux

a) Contexte & hypothèse

La PR est associée à un risque anormalement élevé de souffrir de dépression, reflet d'un trouble de la neuroplasticité. A l'heure actuelle, l'origine de ce risque reste hypothétique : conséquence de l'inflammation systémique, de la dysfonction endothéliale ou autre ? Il est bien documenté que la dépression s'accompagne d'une réduction des taux de BDNF que ce soit dans le cerveau (études chez l'animal ou l'homme) ou dans le sérum (études chez l'Homme). Or, les taux circulants de BDNF sont plus élevés chez les patients souffrant de PR que dans la population générale (del Porto et al., 2006, Grimsholm et al., 2008b). Ces données, apparemment paradoxales, nous ont conduits à émettre l'hypothèse selon laquelle la dépression associée à l'arthrite pourrait être la conséquence d'une moindre production de BDNF par le cerveau et que cette anomalie est la conséquence de la dysfonction endothéliale.

b) Protocole expérimental

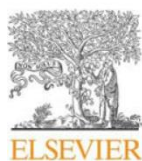
Les expériences ont été réalisées chez des rats contrôles et des rats AIA traités ou pas par le nor-NOHA, un inhibiteur d'arginase administré selon un protocole qui restaure la relaxation à l'ACh chez le rat AIA sans toutefois réduire le score arthritique (Prati et al., 2012). La dépression a été mise en évidence par le test de préférence à la saccharine qui permet d'évaluer l'anhédonie. Les cerveaux (cortex préfrontal et hippocampe) ainsi que le sang ont été prélevés 30 jours après immunisation. Le BDNF a été mesuré par Western blot dans le cerveau et par ELISA dans le sérum. L'inflammation a été étudiée sur le plan clinique grâce au score arthritique et sur le plan biologique grâce au dosage sérique des cytokines proinflammatoires TNF α et IL-1 β (dosées à l'aide d'un kit Milliplex).

c) Principaux résultats

- Les rats AIA présentent une moindre préférence pour l'eau sucrée, effet associé à une réduction des taux cérébraux de BDNF et une augmentation des taux circulants de BDNF
- Seules les anomalies des taux circulants de BDNF sont associées à la sévérité de l'inflammation qu'elle soit clinique ou biologique
- Le traitement par l'inhibiteur d'arginase restaure les taux cérébraux de BDNF

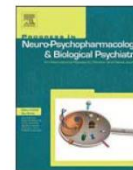
L'ensemble de ces résultats suggèrent que la dépression présentée par les malades souffrant de PR pourrait être la conséquence d'une moindre capacité du cerveau à produire du BDNF et que cette anomalie est plus à relier à la dysfonction endothéliale qu'à l'inflammation. De plus, la discordance observée entre les taux circulants et cérébraux de BDNF dans le modèle AIA nous

laisse penser que l'inflammation pourrait être un facteur confondant quand le BDNF circulant est utilisé comme un reflet des taux cérébraux.



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Brain-derived neurotrophic factor in adjuvant-induced arthritis in rats. Relationship with inflammation and endothelial dysfunction

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Keywords:

Adjuvant-induced arthritis
Rheumatoid arthritis - depression
Brain-derived neurotrophic factor - endothelial dysfunction

ABSTRACT

Objectives: Both peripheral and central brain-derived neurotrophic factor (BDNF) levels are decreased in depression and normalized by efficient anti-depressive therapies. While depression symptoms are frequent in rheumatoid arthritis, BDNF has been poorly investigated in this pathology. Therefore, the present study explored cerebral and peripheral BDNF in arthritis rats as well as the link between brain BDNF and the two factors recently involved in the pathogenesis of depression and present in rheumatoid arthritis namely inflammation and endothelial dysfunction.

Methods: The brain (hippocampus and frontal cortex) and blood (serum) were collected in rats subjected to adjuvant-induced arthritis (AIA) when inflammatory symptoms and endothelial dysfunction are fully developed. Anhedonia as a core symptom of depression symptom was assessed from preference for a saccharin drinking solution. Inflammation was assessed from the arthritis score and serum levels of TNF α and IL-1 β . Treatment with the arginase inhibitor N(w)-hydroxy-nor-L-arginine (nor-NOHA) was used as a strategy to prevent endothelial dysfunction without improving inflammatory symptoms.

Results: As compared to controls, AIA rats displayed decreased brain BDNF levels that coexisted with anhedonia but contrasted with increased BDNF levels in serum. Brain BDNF deficiency correlated neither with arthritis score nor with pro-inflammatory cytokines levels, while it was mitigated by nor-NOHA treatment. A positive correlation was observed between serum BDNF and TNF α levels.

Conclusions: Our study reveals that arthritis decreases BDNF levels in the brain and that endothelial dysfunction rather than inflammation contributes to the decrease. It also identifies a disconnection between serum and brain BDNF levels in arthritis.

1. Introduction

Rheumatoid Arthritis (RA) is an immune-mediated pro-inflammatory disease. Beyond damage of the musculoskeletal system, RA is associated with an increased risk of cardiovascular (CV) events and mortality (Meune et al., 2009; Avina-Zubieta et al., 2008) that is generally explained by endothelial dysfunction (Bergholm et al., 2002; Khan et al., 2010). More recently, depression-like symptoms have been identified as a frequent comorbidity of RA. These symptoms including mood disorders, sleep disturbances, fatigue and anhedonia play an important role in RA patients' perception of their disease (Wolfe, 2004). They also may reduce their adherence rate to exercise programs, which however provide many benefits (Verhoeven et al., 2016). However, little is known about the mechanisms of depression-like symptoms in

RA. According to the cytokine hypothesis of depression (Dantzer et al., 2008) and the bidirectional interaction between mood and CV diseases risk factors (Goldstein et al., 2015; Penninx, n.d.), depression-like symptoms in RA have been suspected to relate to increased production of pro-inflammatory cytokines and/or endothelial dysfunction.

Brain-derived neurotrophic factor (BDNF) is a neurotrophin present in large amounts in the brain where it plays a crucial role in neuroplasticity (Lu et al., 2014). Levels of BDNF are reduced in the prefrontal cortex, hippocampus and serum of depressed patients and animal studies have implicated cerebral BDNF in the pathogenesis of depression and in the mode of action of antidepressants (Duman and Monteggia, 2006; Martinowich et al., 2007; Castren and Kojima, n.d.). Furthermore, decreased brain BDNF levels were found after lipopoly-saccharide-induced systemic elevation of inflammatory cytokines

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(Guan and Fang, 2006; Schnydrig et al., 2007) and in situations associated with endothelial dysfunction including diabetes (Franco-Robles et al., 2014), hypertension (Hennigan et al., 2009; Monnier et al., 2016) and aging (Pang and Lu, 2004). Thus, it could be that brain BDNF levels are decreased in RA patients as a result of inflammation and/or endothelial dysfunction. Surprisingly, despite the availability of clinically-relevant animal models of RA that offer the opportunity to avoid the confounding effect of medications and age on brain function, the impact of experimental RA on brain BDNF levels has never been investigated. Actually the two available studies on BDNF in RA are clinical studies that reported higher circulating BDNF levels in RA patients compared to healthy subjects and lower circulating BDNF in RA patients treated with anti-TNF α therapy than those without such a therapy (del Porto et al., 2006; Grimsholm et al., 2008). However, whether RA-induced changes in serum BDNF levels mirror changes occurring in the brain is not known.

The aim of the present study was to explore brain BDNF levels in rats subjected to adjuvant-induced arthritis (AIA) as well as the connection between brain BDNF levels and clinically-pertinent parameters including serum BDNF levels, inflammation and endothelial function. The results will provide new insights into the mechanisms involved in RA-induced depression symptoms and help the clinicians to interpret high circulating BDNF levels observed in RA patients.

2. Material and methods

2.1. Animals

Experiments were carried out in 6 weeks-old male Lewis rats ($n = 70$) that were purchased from Janvier (Le Genest Saint Isle, France). A set of experiments were also performed in brains collected from Lewis rats ($n = 18$) that were previously used to investigate the role of arginase upregulation in endothelial dysfunction in AIA (Prati et al., 2012). Experiments were conducted according to the French department of agriculture guidelines (licence 21 CAE-102) and approved by the local ethic committee. The experimental procedures were performed in order to comply with ARRIVE guidelines. Animals were housed under a 12 h/12 h light/dark cycle and allowed free access to food and water. Anesthesia was induced by volatile (isoflurane 4%, Virbac, Carros, France) and non-volatile (chloral hydrate, 400 mg/kg, i.p.; Sigma-Aldrich, Saint-Quentin-Fallavier, France) anesthetic agents for arthritis induction and organ removal, respectively.

2.2. Induction and clinical evaluation of arthritis

Arthritis was induced by a single intradermal injection at the base of the tail of 120 μ L of a suspension (10 mg/mL) of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) in mineral oil (Freund's incomplete adjuvant (Difco, Detroit, MI). Non-arthritis Lewis age-matched rats that were used as controls received 120 μ L of saline. The clinical scoring system (arthritis score) of inflammation was employed as follows (Sakaguchi et al., 2003): inflammation (erythema and swelling) of one finger scores 0.1, weak and moderate arthritis of one big joint (ankle or wrist) scores 0.5 and intense arthritis of one big joint scores 1. Tarsus and ankle were considered as the same joint. Arthritis score for a given limb ranged from 0 to 1.5 and global arthritis score (4 limbs) ranged from 0 to 6.

2.3. Saccharin preference test

Depression-like state was assessed by testing of anhedonia, a core feature of major depressive disorder and a key diagnostic criterion according to DSM-5 (American Psychiatric Association – APA 2013). Initially, anhedonia has been considered as a “loss of pleasure”, yet neuropsychological and neurobiological studies revealed a multifaceted construct of anhedonia that emphasizes different facets of hedonic

function, including desire, effort/motivation, anticipation, and consummatory pleasure (Rizvi et al., 2016). Anhedonia was determined by measuring preference for non-nutritive saccharin solution over water, using a two-spouts free-choice method. During two consecutive nights (6:00 pm–8:00 am), rats were provided with a free choice from two drinking spouts containing water or a 0.15% saccharin solution while the two spouts contained water during the diurnal period. The position of the spouts was switched between the two nights. Spouts were weighted at the beginning and the end of the two nights. Index of saccharin preference was first calculated for each cage from the ratio of saccharin solution intake over total liquid intake during second night/100 g of rat's body weight. Then, the index was calculated for control and AIA from the mean of ratios.

2.4. Tissue collection and preparation

After anesthesia, blood and brain regions that are connected to the neuroanatomical circuit involved in depression namely the prefrontal cortex (PFC) and hippocampus (Drevets, 2001; Nestler and Carlezon, 2006) were collected in AIA and age-matched control rats. The cerebral structures were stored at -80°C until homogenization in 7 volumes of ice cold buffer, centrifugation and determination of protein concentration (Lowry method) in supernatant. Blood was collected by a cardiac puncture on coagulant-free tubes (1 mL) to harvest serum. Sera were stored at -80°C until analysis.

2.5. Analysis of BDNF

As BDNF-dependent neuroplasticity relates to brain levels in the mature form of BDNF (mBDNF), BDNF levels were measured in cognition-related by Western blot analysis that allows a specific detection of mBDNF (band at 14 kDa). By contrast, serum BDNF levels were measured using ELISA tests. Indeed, western blot procedure is not sensitive enough to detect mBDNF levels in serum. It has to be kept in mind that antibodies used in commercial ELISA kits are not specifically directed against mBDNF (Polacchini et al., 2015). They showed cross-reactivity for proBDNF, a precursor of mBDNF that changes brain function in a direction opposite than that of mBDNF. Accordingly, commercial ELISA kits cannot differentiate mBDNF from proBDNF.

For Western blot analysis, equal protein amounts were resolved by SDS-PAGE, electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. After blockade of non-specific binding sites, membranes were incubated for 3 h with primary antibodies directed against BDNF (1/3000, clone 3C11, ab203573, Abcam, Cambridge, UK) or β -actin (as internal marker, 1/2500, A5441, Sigma-Aldrich, Saint Quentin-Fallavier, France). Membranes were then incubated for 2 h with a horseradish peroxidase-conjugated anti-IgGs (115-035-166 Jackson ImmunoResearch, Newmarket, UK) as secondary antibodies. Protein-antibodies complexes were visualized using enhanced chemiluminescence (ECL+, GE Healthcare, Little Chalfont, UK). The band density at 14 kDa (that corresponds to the mature form of BDNF) was determined by scanning densitometry (GS-800, BIO-RAD Laboratories, Ivry sur Seine, France). Gels were run in triplicate and the band density of BDNF was calculated from a representative gel. We previously showed that immunoblots of brain homogenates display 5 bands and that the band at 14 kDa corresponded to mBDNF (Quirie et al., 2012; Banoujaafar et al., 2016). ELISA tests (Biosensis, BEK-2211-2P, Thebarton, Australia) were performed according to the manufacturer's instructions. There is no cross-reactivity between BDNF and other neurotrophins (NGF, NT3 and NT4/5). The limit of detection was 2 pg/mL.

2.6. Analysis of pro-inflammatory cytokines

Interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF α) were measured in serum using Milliplex magnetic bead panel kits (eBioscience, Vienne, Austria) that were analyzed using a Luminex

MAGPIX system (Luminex Corporation, Houston, USA) and Milliplex Analyst software (Millipore, St. Charles, USA). The limit of detection was 2.4 pg/mL for the two cytokines.

2.7. Experimental design

A first set of experiments including 16 control and 17 AIA rats was performed in order to determine the index of saccharin preference. During nights of days 5 and 4 pre-immunization, 16 and 17 post-immunization and 25 and 26 post-immunization, rats ($n = 3$ to 5/cage, 4 cages for control and 4 cages for AIA rats) were provided with a free choice from the two drinking spouts. A second set of experiments including 16 control and 21 AIA rats was performed in order to explore the impact of AIA on brain BDNF levels as well as the potential link between brain/serum BDNF and inflammation. Here, blood and brain structures were collected at day 31 ± 2 post-immunization. A third set of experiments was used to investigate the link between brain BDNF levels and endothelial dysfunction. We previously showed that decreased endothelial nitric oxide (NO) bioactivity, which is a main feature of endothelial dysfunction, largely involved upregulation of arginase (Prati et al., 2012; Prati et al., 2011), an enzyme in competition with endothelial NO synthase to degrade arginine. In Prati et al. (Prati et al., 2012), the selective arginase inhibitor N(w)-hydroxy-nor-l-arginine (nor-NOHA) at 40 mg/kg/day (i.p) from day 10–15 (first symptoms of arthritis) for 21 days was reported to restore acetylcholine-induced NO dependent vasodilation in aortic rings collected at day 33 ± 2 post-immunization. By contrast, nor-NOHA did not change the time course of arthritis score. In the present study, BDNF levels were measured in PFC of rats used in Prati et al. (Prati et al., 2012) and stored at -80°C until further biochemical analysis, thus offering us the opportunity to investigate the role of endothelial dysfunction in AIA-induced reduction in brain BDNF levels. Thus, PFC BDNF levels were measured in 3 groups of rats ($n = 6$ each): non AIA rats (control), AIA rats treated with nor-NOHA (nor-NOHA AIA), AIA rats treated with saline instead nor-NOHA (saline AIA).

2.8. Data and statistical analysis

Values are expressed as means \pm SD. Data were analyzed using Sigmaplot software (version 11). Comparisons were assessed by the non-parametric Mann-Whitney test. Pearson's correlation coefficient was used to measure the strength of the relationship between paired data. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Disease activity and depression symptoms in AIA

In accordance with our previous studies (Prati et al., 2012; Mossiat et al., 2015), inflammation in AIA rats was clinically evident at day 10 post-immunization. Then, arthritis score progressively increased and plateaued (at ~ 3.6) from day 20 post-immunization to sacrifice (data not shown). The index of saccharin preference was determined in AIA rats before and at days 17 and 26 post-immunization, times at which arthritis score was 1.5 ± 0.9 and 3.4 ± 1.2 , respectively. The index was determined in parallel in control rats. The results are summarized in Table 1. No difference in body weight and total liquid uptake was observed among the two groups of rats before AIA induction. AIA resulted in a significant body weight loss. However, total liquid intake (water + saccharin solution) at days 17 and 26 post-immunization did not differ between AIA and control rats when AIA-induced body weight loss was taken into consideration. Importantly, the fact that total liquid intake/body weight did not differ between AIA and control rats indicated that AIA-induced impaired mobility did not impair access to spouts and consequently was not a confounding factor for the assessment of anhedonia from the saccharin preference test. Consistent with

anhedonia in arthritis rats, the index of saccharin preference was significantly lower in AIA than control rats from day 17 post-immunization.

3.2. Brain and serum BDNF levels in AIA

Brain BDNF levels were assessed in 6 control and 11 AIA (arthritis score = 3.4 ± 1.2) rats, which were selected at random in the second set of experiments. For this purpose, 6 AIA rats were compared to the 6 control rats on a same gel and the remaining AIA rats ($n = 5$) were compared to the same control rats on an additional gel. Brain BDNF levels in AIA rats were expressed as percentage of the mean of control values. As shown in Fig. 1, AIA significantly decreased BDNF levels in both PFC (-60% , Fig.1A) and hippocampus (-50% , Fig.1B) as compared to control rats. By contrast, serum BDNF levels, which were measured in all rats of the second set of experiments, were significantly higher ($+34\%$) in AIA than in control rats (Fig.1C).

3.3. Relationship between inflammation and BDNF in AIA

Pro-inflammatory cytokines levels were determined in rats in which brain BDNF levels were measured (6 control and 11 AIA) and in additional 4 randomly selected control rats. While TNF α and IL-1 β were below the threshold detection value in control rats ($n = 10$), the corresponding values were 11.6 ± 3.7 and 29.4 ± 15.2 pg/mL in AIA rats, respectively (not shown) after exclusion of aberrant values in one AIA rat. Then, we explored the associations between brain BDNF levels (expressed as percentage of the mean of control values) and inflammation parameters (serum TNF α and IL-1 β levels and arthritis score) in AIA rats. When BDNF levels (either in the PFC or hippocampus) were plotted against corresponding cytokine levels or arthritis score, no association was observed (Table 2). Finally, the fact that AIA induced opposite effects on serum (increase) and brain (decrease) BDNF levels led us to investigate the potential associations between serum BDNF levels and inflammation in AIA rats. As shown in Fig. 2, a positive correlation ($p < 0.05$) was observed between serum BDNF levels and TNF α (Fig.2A) while no significant association was observed with serum BDNF and IL-1 β levels (Fig.2B) or arthritis score (Fig.2C).

3.4. Relationship between endothelial dysfunction and BDNF in AIA

In order to explore the contribution of endothelial dysfunction to AIA-induced brain BDNF deficiency, PFC BDNF levels were compared in saline- vs nor-NOHA- treated AIA rats (arthritis score = 3.4 ± 1.2 and 3.6 ± 1.1 , respectively). We first confirmed that BDNF levels were lower in AIA than control rats (Fig.3A). Then, we showed that BDNF were higher in nor-NOHA- than saline-treated AIA rats (Fig. 3B).

4. Discussion

The main results of the present study are that i) AIA-associated anhedonia was concomitant with decreased BDNF levels in the brain and increased BDNF levels in serum, ii) AIA-associated brain BDNF deficiency related neither with arthritis score nor serum cytokines levels, iii) prevention of AIA-associated endothelial dysfunction translated into enhancement of brain BDNF levels, iii) serum BDNF and TNF α levels correlated in AIA rats.

Impaired BDNF signaling in the hippocampus and the prefrontal cortex is largely involved in depression. Thus, rodent animal models of stress-induced depression during adulthood share decreased BDNF levels in these two regions as a common feature (Stepanichev et al., 2014). Chronic (but not acute) administration of virtually all classes of antidepressant treatments increases BDNF expression in the hippocampus and can prevent the stress-induced decreases in brain BDNF levels (Nibuya et al., 1995). Furthermore, carriers of the val66met polymorphism of the BDNF gene – a polymorphism resulting in

Table 1
Effect of AIA on preference for a saccharin drinking solution.

	Body weight (BW, g)		Total liquid intake (ml/100 g BW)		Index of saccharin preference (%)	
	Control	AIA	Control	AIA	Control	AIA
Before	189 ± 9	193 ± 5	32.8 ± 0.7	33.6 ± 1.1	96.0 ± 0.2	94.1 ± 0.4
Day 17	283 ± 14	237 ± 18***	28.5 ± 0.3	27.0 ± 2.4	97.9 ± 0.4	84.5 ± 0.9***
Day 26	310 ± 15	233 ± 11***	26.1 ± 0.6	25.8 ± 1.7	97.9 ± 0.8	84.1 ± 0.3***

The index of saccharin preference (saccharin solution/total liquid intake) was determined before and at days 17 and 26 post-immunization. Values are expressed as means ± SD *** (p < 0.001) difference between control (n = 16) and AIA (n = 17) rats.

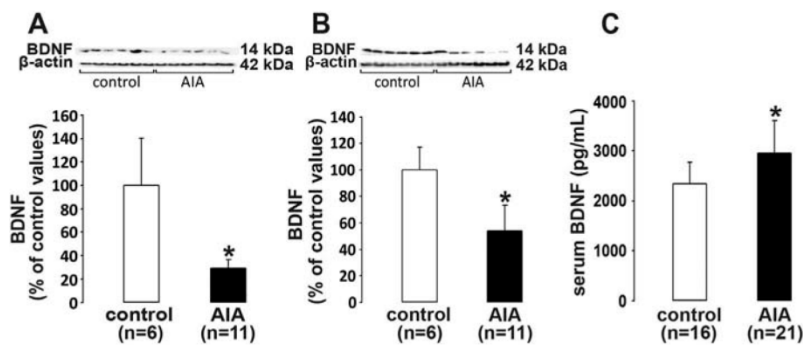


Fig. 1. BDNF levels in the prefrontal cortex (A), hippocampus (B) and serum (C) in AIA and control rats using western blot analysis (A, B) and ELISA tests (C). n = number of rats. Samples were collected at day 31 ± 2 post-immunization. Representative immunoblots of BDNF and β-actin as internal control are shown above the bar graphs. Values are expressed as means ± SD. *p < 0.05.

Table 2
Association between brain BDNF levels and inflammation in AIA rats.

	PFC BDNF levels		Hippocampus BDNF levels	
	r	p	r	p
Serum TNFα (pg/mL)	-0.535	0.0901	-0.249	0.489
Serum IL-1β (pg/mL)	0.384	0.273	-0.229	0.710
Arthritis score	0.336	0.296	-0.232	0.519

Samples were collected and arthritis score determined at day 31 ± 2 post-immunization. BDNF levels were measured in the prefrontal cortex (PFC) and hippocampus and expressed as percentage of the mean of BDNF levels in control rats. BDNF levels (either in the PFC or hippocampus) were plotted against corresponding cytokine levels or arthritis score. r = Pearson's correlation coefficient. No correlation was observed.

deficient cerebral BDNF signaling (34, 35)- are at increased risk for depression in the general population (Rybakowski, 2008). These data combined with the co-occurrence of anhedonia and decreased brain BDNF levels in AIA rats support impaired brain BDNF signaling as a potential biochemical culprit in RA-associated depression symptoms. They also incite to study the interaction between the val66met polymorphism and depression symptoms in RA patients.

Several lines of evidence suggest that peripheral inflammation contributes to depression symptoms in physically-ill patients with no

previous history of mental disorders (Dantzer et al., 2008). In addition, arguments exist for the involvement of brain BDNF modulation as a mechanism by which peripheral inflammation may induce depression-like phenotype (Calabrese et al., 2014; Zhang et al., 2015). On the contrary, in the present study, AIA-induced brain BDNF deficiency in either the hippocampus or PFC related neither with arthritis score nor serum pro-inflammatory cytokine levels. These data corroborate a recent clinical study that fails to report a relationship between pro-inflammatory cytokines and depression in a cohort of 507 RA patients (Suh et al., 2016). They also suggest that dealing with inflammatory symptoms is not sufficient to alleviate depression symptoms in RA. An exciting result of the present study is the efficacy of treatment by nor-NOHA (according to a dosage that improves endothelial function but does not alleviate clinical inflammation in AIA rats) to mitigate the effects of AIA on brain BDNF levels. These data combined with previous studies that reported decreased brain BDNF in conditions recognized to be associated with endothelial dysfunction including diabetes (Franco-Robles et al., 2014), hypertension (Hennigan et al., 2009; Monnier et al., 2016) and aging (Pang and Lu, 2004) emphasize endothelial dysfunction as a contributing event in AIA-associated brain BDNF deficiency. Such a connection between endothelial function and brain BDNF may explain why RA patients with co-morbid cardiovascular diseases have higher depression, lower optimism and are less likely to be in employment than those without cardiovascular diseases (Treharne

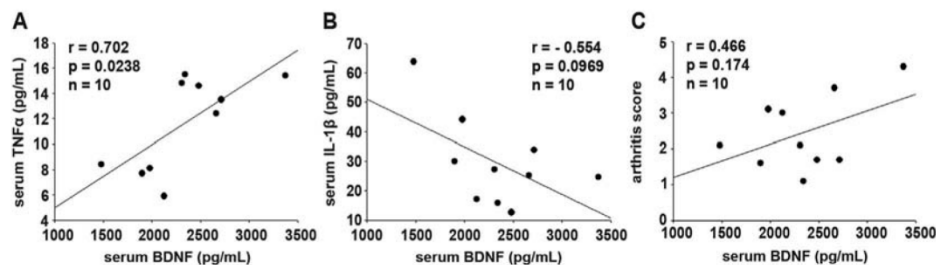


Fig. 2. Relationships between serum BDNF levels and serum TNFα (A), IL-1β (B) levels or arthritis score (C) in AIA rats. n = number of rats (A, B, C). Values are expressed as means ± SD. *p < 0.05.

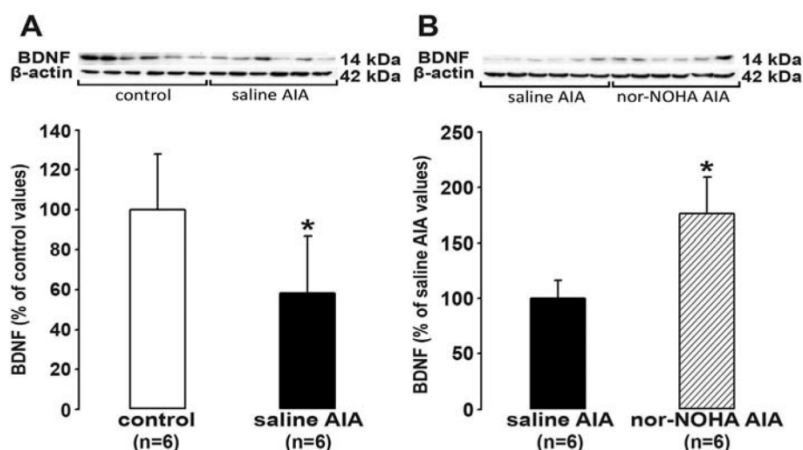


Fig. 3. Prefrontal BDNF levels in control vs saline-treated AIA rats (A) and in saline-treated vs nor-NOHA-treated AIA rats (B). Representative immunoblots of BDNF and β -actin as an internal control are shown above the bar graphs. Arginase inhibitor N(w)-hydroxy-nor-L-arginine (nor-NOHA) or saline was administered (40 mg/kg/day, i.p) from day 10 to 30 post-immunization. Values (mean \pm SD) in AIA are expressed as percentage of corresponding values. n = number of rats, *p < 0.05.

et al., 2005). Importantly, unlike nor-NOHA that decreases endothelial dysfunction but not the severity of inflammation in AIA rats, all anti-rheumatoid drugs decrease inflammatory symptoms but not all seem to improve endothelial function (Prati et al., 2014). Thus, a possible differential impact of anti-rheumatoid drugs on endothelial dysfunction might explain the inter-studies variability in the incidence of depression in RA patients. Another confounding and still unexplored factor in RA may be the val66 met polymorphism that not only increases the risk for depression (Rybakowski, 2008) but also impairs vascular biology (Qin et al., 2011; Amadio et al., 2015).

As already evoked in the Introduction, a plethora of clinical and preclinical studies have shown that serum and brain BDNF levels are decreased in depression and restored by efficient antidepressant therapies. From these data, serum BDNF has been commonly assumed to be a reliable marker of BDNF present in the brain irrespective of the cause of altered circulating BDNF levels. The present study revealing the coexistence of higher serum and lower brain BDNF levels in AIA than in control rats suggests that it is not the case in RA. Notably, we previously reported such disconnection between brain and serum BDNF levels in stroke (Bejot et al., 2011), a pathology often associated with depression symptoms (Ferro et al., 2016). Supporting the hypothesis that high-grade inflammation in RA is a confounding factor when the measurement of serum BDNF levels is used to interrogate brain BDNF levels is the positive correlation observed between serum BDNF and TNF α levels in AIA rats (Fig. 2A). An additional argument for this hypothesis is that serum BDNF levels are lower in RA patients treated with anti-TNF α therapy than those without such a therapy (Grimsholm et al., 2008).

In conclusion, the present study provides new insights in the pathogenesis of depression in RA and suggests that serum BDNF is not a useful biomarker of depression in this pathology. It also emphasizes a potential role of endothelial dysfunction in the association observed between RA and depression and identifies decreased brain BDNF levels as an attractive link between cardiovascular risk and depression in RA.

Key messages

Depression symptoms in AIA rats might be due to impaired cerebral BDNF signaling.

Dealing with endothelial dysfunction might prevent depression symptoms in AIA rats.

Peripheral BDNF levels do not mirror cerebral BDNF levels in AIA rats.

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Author contribution

All authors were involved in drafting the article, and all authors approved the final version to be published. Dr. Marie had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Demougeot, Marie.

Acquisition of data: Pedard.

Analysis and interpretation of data: Pedard, Demougeot, Prati, Marie.

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M. Pedard et al.

Progress in Neuropsychopharmacology & Biological Psychiatry 82 (2018) 249–254

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Article 5

Titre:

The cerebral brain-derived neurotrophic factor pathway, either neuronal or endothelial, is impaired in rats with adjuvant-induced arthritis. Connection with endothelial dysfunction

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Résumé des travaux

a) Contexte & hypothèse

La PR est associée non seulement à un risque cardiovasculaire anormalement élevé mais aussi à de moindres capacités cognitives. Cependant le lien entre ces anomalies, vasculaire et cérébrale, n'a jamais été exploré en cas de PR. Nos précédents travaux montrent que l'arthrite s'accompagne d'une réduction des taux de BDNF dans le cerveau et que cette réduction est moindre en cas de traitement par un inhibiteur d'arginase qui améliore la fonction endothéliale (Pedard et al., 2018a). Ils montrent également que l'arthrite diminue les taux vasculaires de BDNF du moins dans l'aorte (Pedard et al., 2018b). L'ensemble de ces travaux nous a conduits à émettre l'hypothèse selon laquelle l'arthrite s'accompagne d'une moindre expression de BDNF par les microvaisseaux cérébraux et que la moindre production de BDNF par l'endothélium des capillaires cérébraux est impliquée dans le déficit cognitif associé à la PR. Aussi, l'objectif de cette dernière étude est double: 1) étudier l'effet de l'arthrite sur l'expression du BDNF et de son récepteur TrkB phosphorylé par les neurones et les cellules endothéliales des régions impliquées dans la cognition, 2) explorer l'interaction entre la production de NO et de BDNF par l'endothélium cérébral.

b) Protocole expérimental

Les expériences ont été réalisées chez des rats Lewis contrôles et AIA. Les microvaisseaux cérébraux ou les cerveaux ont été isolés/prélevés 30 jours après immunisation. Les taux microvasculaires de BDNF et de eNOS ont été étudiés par technique de Western blot. Les expressions endothéliale et neuronale de BDNF et des récepteurs TrkB phosphorylés ont été quantifiées par technique d'immunohistofluorescence sur microvaisseaux vaisseaux isolés ou sur coupes de cerveaux (au niveau du cortex préfrontal et de l'hippocampe). Enfin, pour étudier l'interaction entre production endothéliale de NO et BDNF, nous avons exposé pendant 24h les microvaisseaux isolés de rats AIA et non AIA respectivement à un donneur de NO et un inhibiteur de NOS.

c) Principaux résultats

- Les taux de BDNF et de eNOS retrouvés dans les microvaisseaux isolés sont plus faibles chez les rats AIA comparés aux rats non AIA
- Cette anomalie coexiste avec une moindre expression du BDNF et des TrkB phosphorylés par l'endothélium des artérioles cérébrales et par les neurones
- L'expression neuronale des TrkB phosphorylés est corrélée à l'expression endothéliale du BDNF mais pas à l'expression neuronale du BDNF
- Les effets de l'arthrite sur le BDNF cérébrovasculaire sont reproduits par l'inhibiteur de NO synthase et annulés par le donneur de NO

Cette étude montre 1) que la dysfonction endothéliale bien documentée chez le rat AIA au niveau périphérique est également présente au niveau de la circulation cérébrale, 2) que la moindre production de NO par l'endothélium cérébral est probablement impliquée dans sa moindre capacité à produire du BDNF, 3) qu'il existe un lien fort entre activation des TrkB neuronaux et expression du BDNF par les cellules endothéliales cérébrales. Elle démontre que la réduction des taux cérébraux de BDNF induite par l'arthrite est la conséquence d'une moindre production de BDNF à la fois par l'endothélium cérébral et les neurones. Elle soutient également l'hypothèse excitante selon laquelle la dysfonction endothéliale pourrait affecter négativement les capacités cognitives via une moindre activation des TrkB neuronaux par le BDNF dérivé de l'endothélium cérébral.



The Cerebral Brain-Derived Neurotrophic Factor Pathway, Either Neuronal or Endothelial, Is Impaired in Rats with Adjuvant-Induced Arthritis. Connection with Endothelial Dysfunction

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Cognitive abilities are largely dependent on activation of cerebral tropomyosin-related kinase B receptors (TrkB) by brain-derived neurotrophic factor (BDNF) that is secreted under a bioactive form by both neurons and endothelial cells. In addition, there is mounting evidence for a link between endothelial function and cognition even though the underlying mechanisms are not well known. Therefore, we investigated the cerebral BDNF pathway, either neuronal or endothelial, in rheumatoid arthritis (RA) that combines both endothelial dysfunction (ED) and impaired cognition. Adjuvant-induced arthritis (AIA) in rats was used as a model of RA. Clinical inflammatory symptoms were evaluated from an arthritis score and brains were collected at day 31 ± 2 post-immunization. Neuronal expression of BDNF and TrkB phosphorylated at tyrosine 816 (p-TrkB) was examined in brain slices. Endothelial BDNF and p-TrkB expression was examined on both brain slices (hippocampal arterioles) and isolated cerebral microvessels-enriched fractions (vessels downstream to arterioles). The connection between endothelial nitric oxide (NO) and BDNF production was explored on the cerebrovascular fractions using endothelial NO synthase (eNOS) levels as a marker of NO production, *N*^ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) as a NOS inhibitor and glyceryl-trinitrate as a slow releasing NO donor. Brain slices displayed lower BDNF and p-TrkB staining in both neurons and arteriolar endothelial cells in AIA than in control rats. For endothelial cells but not neurons, a strong correlation was observed between BDNF and p-TrkB staining. Of note, a strong correlation was also observed between neuronal p-TrkB and endothelial BDNF staining. In cerebral microvessels-enriched fractions, AIA led to decreased BDNF and eNOS levels with a positive association between the 2 parameters. These effects coincided with decreased BDNF and p-TrkB staining in endothelial cells. The exposure of AIA cerebrovascular fractions to GTN increased BDNF levels while the exposure of control fractions to L-NAME decreased BDNF levels. Changes in the cerebral BDNF

pathway were not associated with arthritis score. The present study reveals that AIA impairs the endothelial and neuronal BDNF/TrkB pathway, irrespective of the severity of inflammatory symptoms but dependent on endothelial NO production. These results open new perspectives for the understanding of the link between ED and impaired cognition.

Keywords: BDNF, cognition, endothelium, NO, TrkB

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is present in high concentrations in the adult brain where it plays a crucial role in neuroplasticity, neurogenesis and angiogenesis (Pencea et al., 2001; Kim et al., 2004; Lu et al., 2014) through the phosphorylation of its cognate TrkB (tropomyosin-related kinase B) receptors at tyrosine 816 (p-TrkB) (Bathina and Das, 2015). Thus, decreased brain BDNF and TrkB levels in genetically-modified mice induced changes in behavioral deficit (Korte et al., 1995; Zorner et al., 2003), while transgenic mice overexpressing TrkB showed improvement in learning abilities (Koponen et al., 2004). It is generally assumed that low BDNF levels in the brain are due to a deficit in BDNF production by neurons as neurons are considered as the main source of BDNF in the brain. However, in opposition with this traditional thinking, we recently showed that the removal of endothelial cells from the brain resulted in a marked decrease in BDNF levels measured in the cerebral tissue (Monnier et al., 2017b). This shift of paradigm in the cellular origin of cerebral BDNF indicates that a hitherto unexpected large part of BDNF found in the brain corresponds to BDNF produced by cerebral endothelial cells and paves the way for the exciting hypothesis that neuronal function might be dependent not only on neuronal-derived BDNF but also on BDNF secreted by endothelial cells of cerebral capillaries. These data emphasize the importance to separately explore the brain BDNF/TrkB pathway at both the neuronal and endothelial level, especially since the literature continues to report a link between cardiovascular risk factors and impaired cognition (Knopman et al., 2001; Diener, 2011; Srinivasa et al., 2016).

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory joint disease associated with high cardiovascular risk (del Rincon et al., 2001) and impaired cognition including alteration in logical memory, memory working and executive function and presence of depressive symptoms (Bartolini et al., 2002; Sturgeon et al., 2016; Baptista et al., 2017). RA-associated increased cardiovascular risk has been related to endothelial dysfunction (ED) (Bergholm et al., 2002) and subsequent acceleration of atherosclerosis (Del Rincon et al., 2007). ED is present early in the course of the disease as evidenced from decreased brachial flow-mediated dilatation (Xu et al., 2017), which is a consequence of decreased endothelial NO production/activity in response to hyperemia. Studies in AIA rats identified mechanisms involved in decreased endothelial NO production/bioactivity including eNOS uncoupling (Haruna et al., 2006), arginase upregulation (Prati et al., 2011, 2012) and increased NO inactivation by oxidative stress (Haruna et al., 2006, 2007). On the contrary, the mechanisms involved in

impaired cognition remain poorly understood even though a role of ED is suspected from studies reporting impaired cognition in patients exposed to cardiovascular risk factors. Surprisingly, while activation of cerebral TrkB by BDNF is largely involved in cognition, whether RA is associated with an impairment of cerebral BDNF pathway is unknown.

The present study investigated the cerebral BDNF pathway in rats subjected to adjuvant-induced arthritis (AIA) and the mechanisms involved in potential changes. The brains were collected at day 31 ± 2 post-immunization, time at which inflammatory symptoms are maximal and ED occurs at the peripheral macro- and micro-vasculature (Totoston et al., 2015). Neuronal BDNF and p-TrkB expression were examined on hippocampal brain slices. Endothelial BDNF and p-TrkB expression were examined on cerebral arterioles and downstream vessels using immunohistochemical analysis on hippocampal brain slices and Western blot analysis of isolated cerebral microvascular-enriched fractions, respectively. The potential connection between the cerebral BDNF pathway with the severity of inflammatory symptoms or endothelial NO production was also investigated.

METHODS

Animals

Experiments were carried out on 6 weeks-old male Lewis rats ($n = 64$) that were purchased from Janvier (Le Genest Saint Isle, France). Experiments were conducted according to the French department of agriculture guidelines (license 21 CAE-102) and approved by the local ethic committee. The experimental procedures were performed in order to comply with ARRIVE guidelines. Animals were housed under a 12 h/12 h light/dark cycle and allowed free access to food and water. Anesthesia was induced by isoflurane 4% (Virbac, Carros, France) for arthritis induction and chloral hydrate anesthesia (400 mg/kg, i.p.; Sigma-Aldrich, Saint-Quentin-Fallavier, France) for brain removal.

Induction and Clinical Evaluation of Arthritis

Arthritis was induced by a single intradermal injection at the base of the tail of 120 μ L of 1 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) suspended in 0.1 ml of mineral oil [Freund's incomplete adjuvant (Difco, Detroit, MI)]. Non-arthritis Lewis age-matched rats that were used as controls received 120 μ L of saline. Indeed, Freund's incomplete adjuvant was previously suspected to interfere with the th1/th2 balance of immune response (Zhang et al., 1999). The clinical scoring system (arthritis score) of inflammation was employed as follows

(Sakaguchi et al., 2003): inflammation (erythema and swelling) of one finger scores 0.1, weak and moderate arthritis of one big joint (ankle or wrist) scores 0.5 and intense arthritis of one big joint scores 1. Tarsus and ankle were considered as the same joint. Arthritis score for a given limb ranged from 0 to 1.5 and global arthritis score (4 limbs) ranged from 0 to 6. The arthritis score was regularly determined until animal sacrifice.

Preparation of Cerebral Microvessels-Enriched Fractions

The procedure was detailed elsewhere (Monnier et al., 2017a). Briefly, after the removal of large superficial vessels, the forebrain except the hypothalamus was homogenized in ice cold Hank's balanced salt solution (HBSS) with a Potter-Thomas homogenizer. After centrifugation the pellet (P1) was saved and the supernatant was centrifuged again. The new pellet (P2) was pooled with P1, suspended in 20% dextran and centrifuged. The new pellet (P3) was again saved and the remaining tissue was reprocessed similarly, thus leading to P4. P3 and P4 were pooled together and suspended in HBSS. They were successfully filtered through a 335, 110, 53, and 20 μm mesh nylon filters. The fraction retained on the 335- and 110 μm -filters were discarded while fractions retained on other filters (F53 and F20) were kept for further analysis. We previously showed that cells from F53 and F20 were all positive for both BDNF and the endothelial marker GLUT1 (Monnier et al., 2017b), indicating that BDNF is constitutively expressed by endothelium of cerebral microvasculature.

Western Blot Analysis

Pooled microvessels-enriched fractions (F53 + F20) were homogenized in ice-cold lysis buffer [100 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EGTA, 1% triton X-100, 1% protease inhibitor cocktail (P8340, Sigma-Aldrich, Saint-Quentin-Fallavier, France)]. After centrifugation of homogenates, an aliquot of the supernatant was kept for protein measurement by using the Lowry method. Equal protein amounts were resolved by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (0.2 μm) for western blotting. After blocking non-specific binding sites with a 5% solution of non-fat dry milk in TBS (20 mM Tris/HCl, 137 mM NaCl, pH 7.4) containing 0.1% Tween 20, membranes were probed with an anti-BDNF rabbit monoclonal antibody (1/3,000 with 5% non-fat dry milk, ab108319, Abcam, Cambridge, United Kingdom), an anti-eNOS mouse monoclonal antibody (1/2,500 with 5% non-fat dry milk, 610297, BD Biosciences, San Jose, USA) or an anti- β -actin antibody (1/10,000, A5441, Sigma-Aldrich, Saint-Quentin-Fallavier, France). Then, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase [111-035-144 (anti-rabbit, 1/20,000) and 115-035-166 (anti-mouse, 1/50,000), Jackson ImmunoResearch Laboratories, Interchim, Montluçon, France]. Protein-antibody complexes were visualized using the enhanced chemiluminescence Western blotting detection system (ECL 2, 1151-7371, Fisher Scientific, Illkirch, France). The band densities were determined by scanning densitometry (GS-800, BIO-RAD Laboratories, Ivry

sur Seine, France). Whole membranes for BDNF and eNOS are available on supplemental data (Datasheet 1).

Immunohistochemical Analysis

Immunohistochemical experiments were performed on pooled microvessels-enriched fractions (F53 + F20) and brain slices. Vascular fractions were cryoprotected in HEPES/sucrose buffer, fixed in methanol and collected on SuperFrost Plus slides. For preparation of brain slices, brain of anesthetized rats was successively transcardially perfused with saline and 4% paraformaldehyde (PFA) solution in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were then removed, post-fixed in PFA (1 h), cryoprotected with a sucrose solution (20% in PB for 48 h) and then transversally cryosectioned (20 μm -thick) with a cryostat (HM550, OMPV, Microm-Microtech, Francheville, France) at -20°C and collected on SuperFrost Plus slides. After fixation and blockade of non-specific binding sites, slices were first incubated with the rabbit monoclonal anti-BDNF antibody (dilution 1/200, ab108319, Abcam, Cambridge, United Kingdom) or anti-phospho-TrkB Y816 polyclonal antibody (dilution 1/200, ABN1381, Merck-Millipore, Saint-Quentin en Yvelines, France), in the presence of antibodies directed against the specific endothelial markers, either GLUT1 (dilution 1/300, mouse monoclonal, MABS132, Merck-Millipore, Saint-Quentin en Yvelines, France) or vWF (dilution 1/200, mouse monoclonal, MCA 3442, Merck-Millipore, Saint-Quentin en Yvelines, France), or the specific neuronal marker NeuN (dilution 1/100, mouse monoclonal, MAB377, Merck-Millipore, Saint-Quentin en Yvelines, France). They were then exposed to a fluorescent secondary antibody ALEXA Fluor 488 and 568 [A11029 (anti-mouse) and A11036 (anti-rabbit), Molecular Probes, Invitrogen, Cergy Pontoise, France, dilution 1/1,000]. Negative controls were prepared by omitting the primary antibodies. Finally, slides were mounted with DAPI (a nuclear marker)-containing mounting medium (Vectashield, FP-DT094A, Interchim, Montluçon, France) and analyzed by using an epifluorescent microscope (Eclipse E600, Nikon France S.A.S., Champigny-sur-Marne, France). Endothelial and neuronal staining for BDNF and p-TrkB Y816 was examined using an automated method (software Matlab, Mathworks, Natick, USA) as described in Kim et al. (2015). The Matlab program can be downloaded in: <http://faculty.jsd.claremont.edu/jarmstrong/fquant/index.html>. The calculated fluorescence intensity corresponded to the mean of intensity exhibited by adjacent cells that were selected by the experimenter by plotting a line through adjacent endothelial or neuronal cells. Endothelial staining was quantified on cerebral microvessels-enriched fractions vascular fractions as well as on brain slices. It was quantified on the smallest vessels (capillaries) for cerebrovascular fractions and on three arterioles (seen in transversal section) present between the *Cornu Ammonis 1* (CA1) and *dentate gyrus* for brain slices. Neuronal staining was quantified on brain slices from a line passing throughout neurons of the CA1 subfield.

Ex Vivo Experiments

Fresh pooled microvessels-enriched fractions were immediately distributed into wells previously loaded with a culture medium

(M199 medium supplemented with 0.1 mg/L L-glutamine, 10% fetal bovine serum, 2% glucose, 2% amino acids and 1% antibiotic solution). Microvessels-enriched fractions collected in AIA rats were incubated (24 h, in a 95% O₂-5%CO₂ atmosphere) with either 10 µM glyceryl trinitrate (GTN, MR107753, Merck-Millipore, Saint Quentin en Yvelines, France) as a slow-releasing NO donor that well mimics physiological endothelial NO production or saline. Fractions collected in control rats were in parallel incubated with either 5 mM of the eNOS inhibitor N^w-nitro-L-arginine methyl ester hydrochloride (L-NAME on BDNF levels, N5751, Sigma-Aldrich, Saint-Quentin-Fallavier, France) or saline. At the end of the incubation period, cerebrovascular fractions were prepared for Western blotting analysis.

Statistical Analysis

Results were expressed as means ± standard deviations (SD). Statistical analysis between two groups was performed using a parametric *t*-test or non-parametric Mann-Whitney's test, depending on normality and equal variance tests. The relationship between two variables was investigated using Pearson's and Spearman's correlations (for normally or not normally distributed data, respectively). Differences were considered significant at *p* < 0.05.

RESULTS

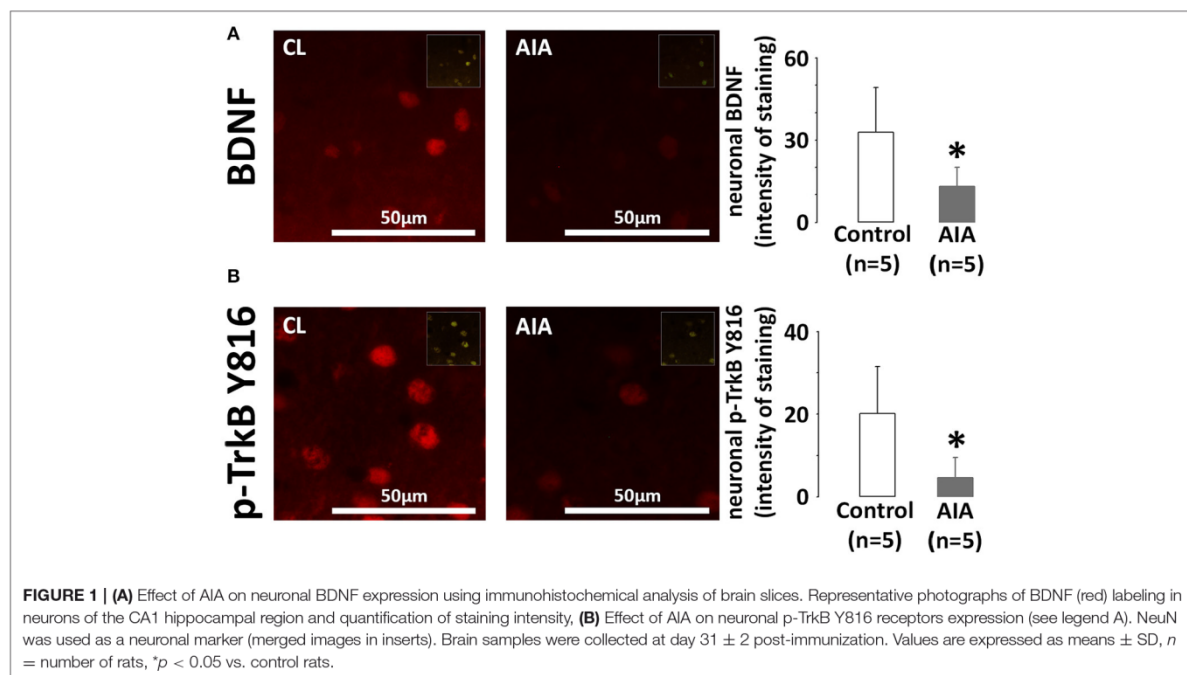
The Neuronal BDNF/TrkB Pathway Is Impaired in AIA

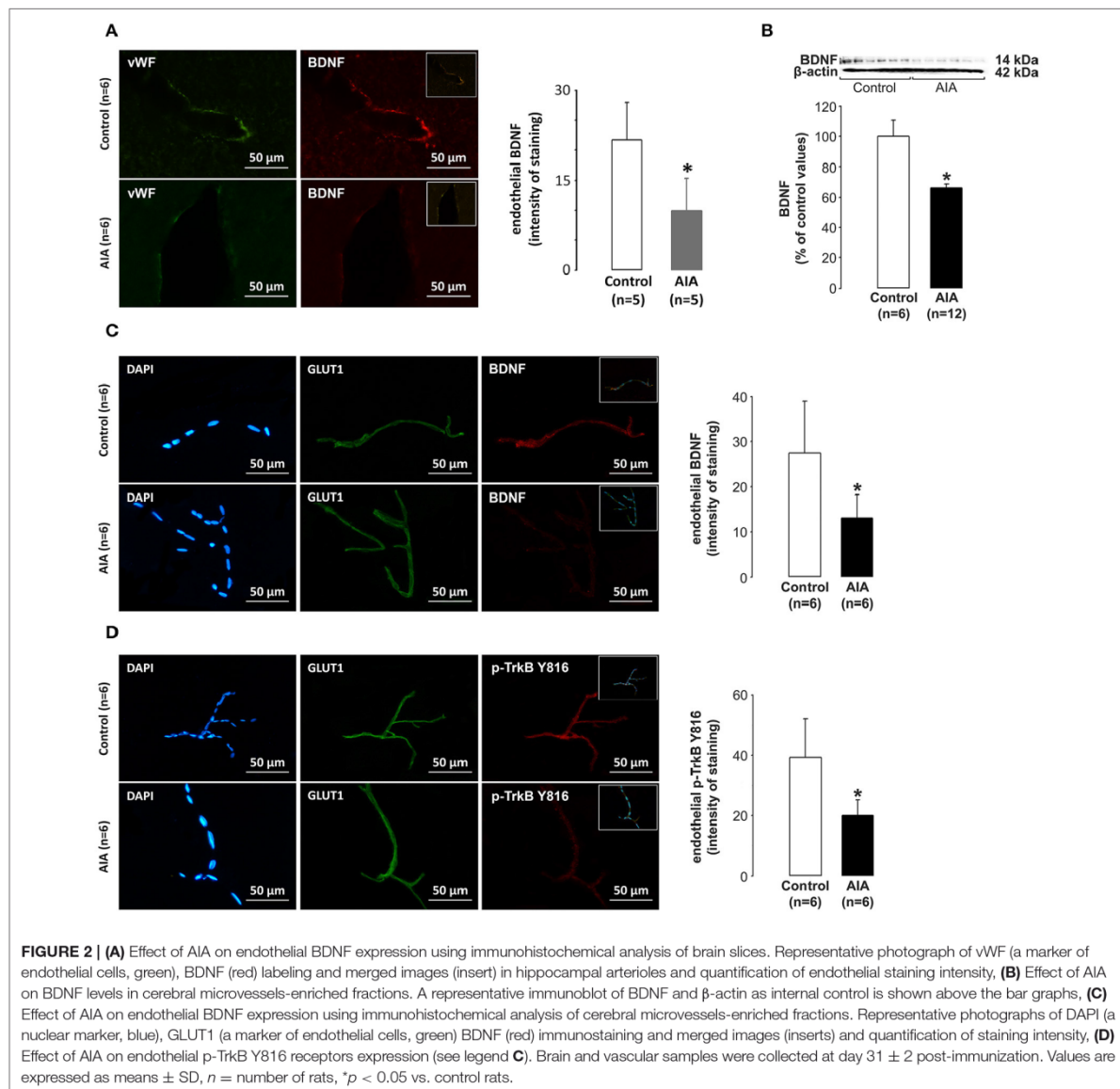
Brains were collected in AIA (*n* = 5, arthritis score = 3.6 ± 0.8) and control rats (*n* = 5). BDNF and p-TrkB expression was

examined on hippocampal slices at the level of the CA1 region. Indeed, the hippocampus is a cognition-related brain region. In addition, the low neuronal density of the CA1 subfield offers the opportunity to easily quantify the neuronal fluorescence intensity. As shown in **Figure 1A**, AIA decreased BDNF staining in cells positive for the neuronal marker NeuN, the intensity of staining in control and AIA rats being 32.8 ± 16.3 and 13.0 ± 7.0, respectively. In these neurons, p-TrkB staining (**Figure 1B**) was also lower in AIA rats than in controls, the intensity of staining dropping from 20.1 ± 11.4 in control rats to 4.6 ± 4.9 in AIA rats. However, no association was observed between neuronal BDNF and p-TrkB staining (*r*_s = 0.428, *p* = 0.21, data not shown) when control and AIA were simultaneously observed.

The Endothelial BDNF/TrkB Pathway Is Impaired in AIA

Arteriolar endothelial BDNF expression was first examined on brain slices used for investigation of the neuronal BDNF pathway. We focused on arterioles (seen in transversal section) located between the *Cornu Ammonis 1* (CA1) and *dentate gyrus* of the hippocampus. As shown in **Figure 2A**, BDNF labeling in cells lining the arteriolar lumen (cells positive for the endothelial marker vWF) was lower in AIA (*n* = 5) than in control rats (*n* = 5). Then, we investigated the endothelial BDNF pathway in vessels downstream to arterioles. For this purpose, the endothelial BDNF pathway was examined on cerebral microvascular-enriched fractions isolated from additional AIA (*n* = 18, 12 rats for Western blot analysis and 6 rats for immunohistochemical analysis) and control rats (*n* = 12).

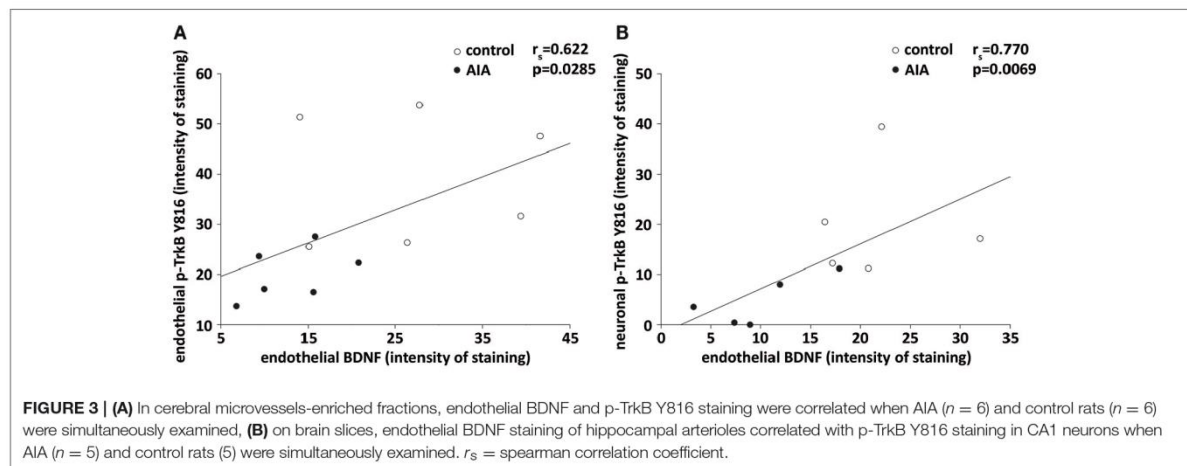




According to Western blot analysis, 6 control and 6 AIA rats (arthritis score = 2.8 ± 1.0) were run on a same membrane, the remaining AIA rats (arthritis score = 1.5 ± 0.9) being run on another membrane with the same 6 control rats. BDNF values in AIA rats were expressed as percentage of control values. As shown in **Figure 2B**, BDNF levels in cerebral microvascular-enriched fractions (F20 + F53) were significantly lower in AIA than in control rats ($n = 6$). These changes coincided with lower BDNF and p-TrkB staining in endothelial cells (cells positive for the specific endothelial marker GLUT1) in AIA ($n = 6$, arthritis score = 3.3 ± 1.0) than in control rats

($n = 6$). Indeed, the intensity of endothelial BDNF staining at capillary levels was 27.4 ± 11.6 in control and 13.1 ± 5.2 in AIA rats (**Figure 2C**), the corresponding values for p-TrkB staining being 39.3 ± 13.0 and 20.1 ± 5.0 (**Figure 2D**). Notably, DAPI (a specific nuclear) labeling of (F20 + F53) fractions showed that these fractions were well enriched in capillaries (vascular wall consisting with a monolayer of endothelial cells) (see **Figures 2C,D**).

Finally, we investigated to what extent endothelial BDNF could act as an autocrine and/or paracrine actions. Consistent with an autocrine action, a positive correlation was



observed between endothelial BDNF and p-TrkB staining in cerebral microvessels-enriched when control and AIA were simultaneously analyzed (Figure 3A). A positive correlation was also observed between endothelial BDNF staining of hippocampal arterioles and p-TrkB staining by CA1 neurons when the two groups of rats were again simultaneously analyzed, suggesting a paracrine action of endothelial BDNF on neurons (Figure 3B).

No Controls BDNF Production by Endothelial Cells in AIA

While it is well documented that AIA led to decreased endothelial NO bioactivity in peripheral vessels (see introduction) whether the production of NO by the cerebral endothelium is altered by AIA has never been investigated. Therefore, we measured eNOS levels in cerebrovascular fractions (previously used to investigate BDNF levels) as an indicator of NO production by the cerebral endothelium. As shown in Figure 4A, eNOS levels were significantly lower in AIA than in control rats. Then, we explored the connection between levels of eNOS and BDNF in cerebral microvessels-enriched fractions. As shown in Figure 4B, a positive correlation was found between the 2 parameters when control and AIA rats were examined simultaneously. By contrast, no association was found when AIA and control rats were examined separately (data not shown). Finally, cerebrovascular fractions were isolated from 24 additional rats (12 controls and 12 AIA). The fractions we exposed (24 h) isolated vascular fractions to L-NAME (a NOS inhibitor) or GTN (a slow releasing NO donor that well mimics the continuous secretion of NO by endothelial cells). The exposure to L-NAME (μM) of control fractions was found to induce a significant decrease in BDNF levels (Figure 4C), while the exposure to GTN ($10 \mu\text{M}$) of AIA fractions increased BDNF levels (Figure 4D). Collectively, these data suggest that decreased BDNF production by the cerebral endothelium was likely consecutive to its reduced capacity to produce NO.

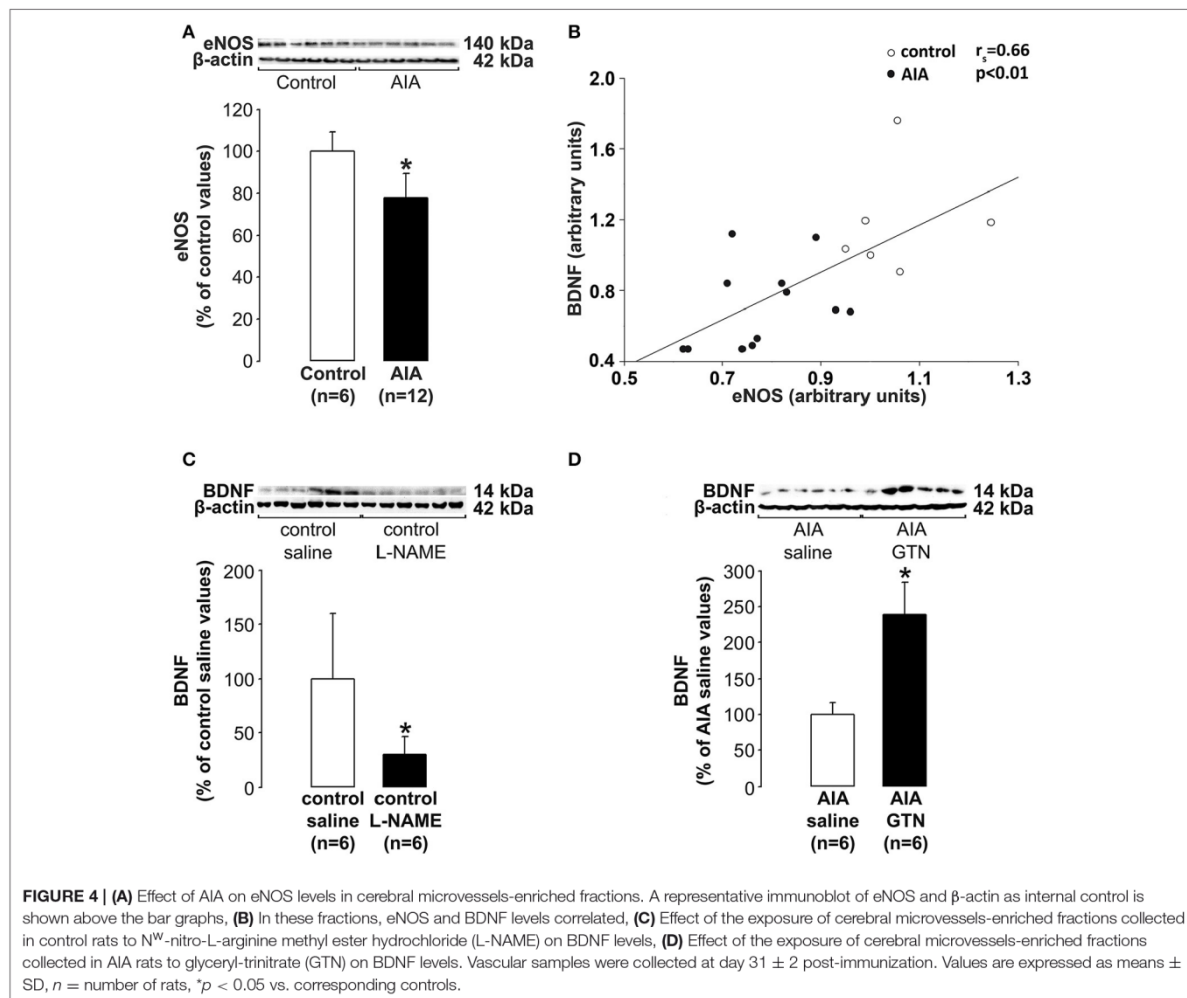
Changes in Either the Cerebral BDNF Pathway or eNOS Levels Evoked by AIA Did Not Proportionate with the Severity of Inflammatory Symptoms

The severity of inflammatory symptoms was evaluated from arthritis scores in all AIA rats ($n = 35$). As shown in Figure 5, the first symptoms were seen \sim at day 12 post-immunization. Then, arthritis score rapidly increased to 3.5 and plateaued from \sim day 22 post-immunization until sacrifice. Notably, arthritis score at sacrifice differed among rats, its minimum and maximum value being 0.7 and 4.9, respectively. However, when individual arthritis score in AIA rats was plotted against corresponding values of BDNF or eNOS levels in cerebral microvessels-enriched fractions or intensity of neuronal BDNF staining on brain slices, no correlation was observed (Table 1).

DISCUSSION

The main results of the present study are that (i) AIA alters the cerebral BDNF/TrkB pathway at both the endothelial and neuronal levels, (ii) activation of neuronal TrkB receptors correlated with endothelial BDNF expression, (iii) a connection exists between the endothelial BDNF/TrkB pathway and the capacity of the cerebral endothelium to produce NO, (iv) AIA-induced changes in either the cerebral BDNF pathway or endothelial NO production did not proportionate with the severity of inflammatory symptoms.

The impairment of the cerebral BDNF pathway is largely involved in impaired cognition associated in animal models of psychiatric, neurologic and neurodegenerative diseases. However, whether changes preferentially occur at the neuronal and/or endothelial level is not well known. In fact, levels of BDNF and its receptors are commonly measured in brain homogenates and their localization restricted to neurons. The new data provided by the present study is that AIA alters the cerebral BDNF/TrkB pathway at both the neuronal and endothelial level.



More precisely, neuronal as well as endothelial expression of BDNF and activated TrkB receptors were lower in the brain of AIA rats than in that of controls, suggesting that impaired BDNF-dependent cognition might have both a neuronal and endothelial component. The assessment of cognitive deficit (memory, learning) by traditional behavioral tests in AIA rats is challenged by the reduction of spontaneous mobility as a result of inflammation of the two hind paws. However, learning is impaired in animal model of RA in which only one hind paw is damaged (Zhu et al., 2016). It is noteworthy that diseases other than RA but combining as RA impaired cognition and high cardiovascular risk such as hypertension and diabetes were reported to induce BDNF downregulation in neurons (Pietranera et al., 2010; Franco-Robles et al., 2014), endothelial cells (Navaratna et al., 2011; Prigent-Tessier et al., 2013) or both (Monnier et al., 2017a). Using cell culture, activation of endothelial TrkB receptors was reported to induce myotubes

formation (angiogenesis) (Kim et al., 2004), while activation of neuronal receptors was reported to increase the number of synaptic connections (Park and Poo, 2013). Furthermore, BDNF secreted by cerebral endothelial cells in culture was reported to induce neurogenesis (Leventhal et al., 1999) and protect neurons against cytotoxic stimuli (Guo et al., 2008, 2012). The emerging and elusive question concerns the contribution of BDNF secreted by cerebral capillaries to neuronal function *in vivo* and by extension to cognition. Supportive such a paracrine action of endothelial-derived BDNF *in vivo*, a strong positive correlation was observed between endothelial BDNF and neuronal p-TrkB expression even though BDNF expression by capillaries was estimated from BDNF expression by arteriolar endothelial cells. We are aware that endothelial BDNF expression in arterioles and cerebral capillaries might differ. However, the difference if present is expected to be minor since endothelial BDNF staining/levels did not significantly differ among F20, F53, and

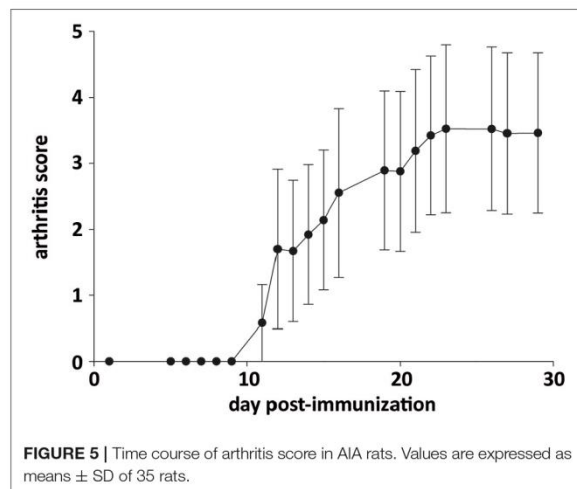


TABLE 1 | AIA-induced changes in the BDNF pathway and endothelial NO production do not relate to the severity of inflammatory symptoms.

	Arthritis score	
	<i>r</i>	<i>P</i>
Vascular BDNF levels/arthritis score serie 1	-0.005	0.993
Vascular BDNF levels/arthritis serie 2	-0.653	0.160
Vascular eNOS levels/arthritis score serie 1	0.445	0.376
Vascular eNOS levels/arthritis score serie 2	0.123	0.816
Neuronal BDNF staining/arthritis score	-0.416	0.486

Arthritis score as reflection of the severity of inflammatory symptoms was determined in AIA rats just before brain collection (at day 31 ± 2 post-immunization). Arthritis score was plotted against corresponding BDNF or eNOS levels (Western blot analysis, $n = 6$ for series 1 and 2) in cerebral microvessels-enriched fraction or corresponding neuronal BDNF staining intensity (immunohistochemical analysis of brain slices, $n = 5$). $r =$ Pearson's correlation coefficient. $n =$ number of AIA rats. No correlation was observed between arthritis score and other parameters.

F110 fractions (Monnier et al., 2017a). According to such a paracrine action, BDNF secreted by cerebral capillaries into the cerebral interstitial fluid might be recognized by and activate neuronal TrkB receptors thereby increasing synapses strength and cognitive abilities. Although still speculative, such a scenario fits well with the new concept that emphasizes the importance of endothelial health in maintaining neuronal function. Last but not the least, our results showed that AIA-induced changes in the cerebral BDNF pathway, either neuronal or endothelial, were not in proportion with the severity of inflammatory symptoms. Further studies are however needed to investigate the impact of effective anti-rheumatoid treatment on the cerebral BDNF pathway.

Decreased endothelial NO bioactivity is well documented in RA patients (Steyers and Miller, 2014) and AIA rats (Haruna et al., 2006, 2007; Prati et al., 2011, 2012; Totoston et al., 2015), at least for the peripheral vasculature. Such alteration in endothelial phenotype and subsequent stimulation of atherosclerosis has

been largely involved in the 2–5 times increased risk of developing premature cardiovascular diseases in RA (Wallberg-Jonsson et al., 1997). The new data provided by the present study is that ED extends to the cerebral microvasculature in AIA as evidenced by lower eNOS levels in cerebral enriched-microvascular fractions in AIA than in control rats. A clinically-relevant data is that vascular eNOS levels in AIA were not in proportion with the severity of inflammatory symptoms, suggesting that cerebral ED in RA patients cannot be predicted from the disease severity as previously observed for peripheral ED (Bernelot Moens et al., 2016). We are aware that decreased eNOS levels is only one of the multiple mechanisms that can lead to decreased endothelial NO bioactivity in AIA. Unfortunately, additional mechanisms cannot be explored in the present study because the amount of vascular material that can be purified from each rat forebrain was too low (250 μ g total protein). We prefer to give the priority to the simultaneous measurement of eNOS and BDNF protein levels in the same sample, thus offering the opportunity to explore the link between endothelial NO and BDNF production. It is noteworthy that a positive correlation was observed between vascular eNOS and BDNF levels. These data combined with our *ex vivo* experiments showing that eNOS inhibition reproduced while NO supplementation reversed the effect of AIA on endothelial BDNF levels strongly suggest that decreased endothelial BDNF synthesis observed in AIA might be due to decreased capacity of the cerebral endothelium to synthesize NO. Notably, a positive control by endothelium-derived NO of BDNF synthesis by cerebral endothelial cells is not surprising. Indeed, the exposure of cultured brain-derived endothelial cells to inhibitors of NO production including the peptide A beta (a compound largely involved in the pathophysiology of Alzheimer disease) and ADMA (an endogenous eNOS inhibitor) was reported to decrease BDNF production (Guo et al., 2008; Ma et al., 2015). In the same vein, incubation of isolated cerebral microvessels with advanced glycation-end products (Navaratna et al., 2011) that act as NO scavengers (Bucala et al., 1991) resulted in BDNF downregulation whilst their incubation with a slow releasing NO donor was recently reported by our laboratory to increase BDNF production (Monnier et al., 2017b). The new data provided by the present study is that a NO supplementation is efficient to stimulate endothelial BDNF production even when ED is present. A therapeutic perspective is that impaired cognition in RA might be improved by the restoration of endothelial NO production. However, even though our *ex vivo* experiments support a causal link between decreased endothelial NO production and BDNF production in AIA, a contribution of other factors cannot be excluded. In addition, whether neuronal BDNF expression, which is mainly controlled by neuronal activity (Balkowiec and Katz, 2000), is dependent on endothelial-derived BDNF remains an open question (Banoujaafar et al., 2016). An unresolved point concerns the role of pro-inflammatory in AIA-induced changes in BDNF. However, against their involvement the exposure to tumor necrosis factor alpha (TNF α), a cytokine relevant in RA, was previously reported to induce BDNF upregulation either by cultured cerebral endothelial cells (Bayas et al., 2002) or neuron-enriched dissociated culture of trigeminal

ganglion (Balkowiec-Iskra et al., 2011). Nevertheless, assuming that cognition may be dependent on endothelial-derived BDNF (Katusic and Austin, 2014), a therapeutic perspective of the present study is that impaired cognition in RA might be improved by the restoration of endothelial NO production. Consistent with this new approach, prevention of ED was recently reported to translate into brain BDNF levels elevation in AIA rats (Pedard et al., 2017).

In conclusion, the present study supports the exciting hypothesis that decreased BDNF production by the cerebral endothelium as a result of decreased endothelial NO synthesis might account for AIA-associated decreased activation of neuronal TrkB activation. These data open new perspectives in the comprehension of the link between endothelial function and cognition and in the management of impaired cognition in patients at high risk for cardiovascular diseases.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article, and all authors approved the final version to be published. CM had full access

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to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: CD, CM. Acquisition of data: MP, AQ, AT, PG. Analysis and interpretation of data: MP, CD, CM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2017.01125/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental data

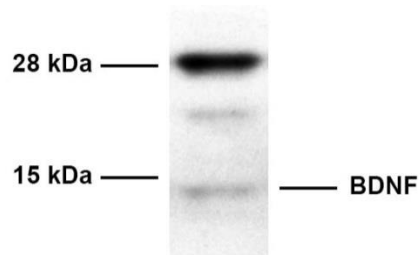


Figure 1 Supplemental data. Representative BDNF immunoblot from microvessels-enriched fractions (prepared from 1 control rat). Wells were loaded with 20 μ g of protein. BDNF (the mature form) corresponded to the band at 14 kDa.

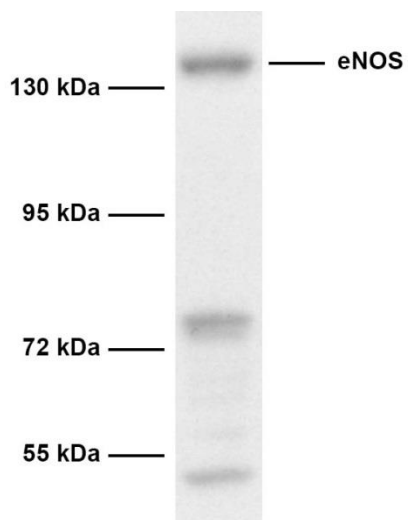


Figure 2 Supplemental data. Representative eNOS immunoblot from microvessels-enriched fractions (prepared from 1 control rat). Wells were loaded with 35 μ g of protein. eNOS corresponded to the band at 140 kDa.

DISCUSSION GENERALE & PERSPECTIVES

Nos études, conduites chez les rats arthritiques, montrent pour la première fois que l'arthrite chez le rat induit une réduction des taux de BDNF dans l'aorte, les microvaisseaux cérébraux et les régions impliquées dans la cognition (hippocampe, cortex préfrontal) alors qu'elle induit une élévation des taux sériques de BDNF. La réduction des taux cérébraux est concomitante d'une anhédonie et d'une réduction de l'expression du BDNF non seulement par les cellules endothéliales cérébrales mais aussi par les neurones. De façon importante, alors que les taux sériques de BDNF sont positivement associés à la sévérité de l'inflammation, les troubles de l'expression endothéliale du BDNF en sont déconnectés. En revanche, la restauration de la production de NO par un inhibiteur d'arginase ou l'exposition des vaisseaux arthritiques à un donneur de NO normalise la synthèse endothéliale de BDNF. Enfin, une corrélation positive a été identifiée entre l'expression du BDNF par les cellules endothéliales cérébrales et l'activation des récepteurs TrkB neuronaux. L'ensemble de ces résultats suggère qu'en cas d'arthrite 1) la dysfonction endothéliale qu'elle soit périphérique ou centrale est indépendante de l'inflammation mais que la dysfonction endothéliale est probablement à l'origine de la moindre capacité de l'endothélium à synthétiser le BDNF, 2) que la moindre activation des TrkB neuronaux et, par conséquent, la neuroplasticité pourrait s'expliquer par une réduction de la sécrétion de BDNF par les capillaires sanguins cérébraux, 3) que le BDNF sérique ne peut pas être utilisé comme un marqueur des taux présents dans le cerveau ou dans les vaisseaux.

1) Dysfonction endothéliale et BDNF endothélial

Les mécanismes qui contrôlent la synthèse endothéliale de BDNF ont été peu explorés. Nos résultats suggèrent que la production de BDNF est sous le contrôle du NO comme précédemment suggéré (Revue pages 46-47). En effet, l'incubation des vaisseaux « contrôles » avec un inhibiteur de NO synthase reproduit l'effet de l'arthrite alors que l'incubation des vaisseaux « arthritiques » avec un donneur de NO annule les effets de l'arthrite sur le BDNF vasculaire. Avant de conclure définitivement sur le contrôle de la synthèse du BDNF endothélial chez le rat AIA, des études *in vivo* sont indispensables pour évaluer dans quelle mesure a) l'inhibition d'arginase qui restaure la production de NO par l'endothélium des vaisseaux périphériques chez le rat AIA (Prati et al., 2012) restaure les taux vasculaires de BDNF que ce soit à l'étage périphérique ou cérébral, b) l'expression endothéliale de BDNF est réduite chez la souris KO eNOS ce qui fournirait la preuve du concept d'un contrôle positif de la synthèse endothéliale de BDNF par le NO. Ensuite, il conviendra d'explorer les mécanismes impliqués. En effet, le NO pourrait soit induire l'expression du gène *bdnf*, soit activer les processus de traduction. Pour trancher entre ces mécanismes, il serait intéressant de reproduire nos expériences *ex vivo* et de coupler le dosage du BDNF au dosage des ARNm codant *bdnf*.

Un point intéressant est que l'activation des TrkB endothéliaux est capable d'induire une vasodilatation via la voie Akt/eNOS au niveau des artères de résistance (Totoson et al., 2018) ou via la phosphorylation d'eNOS au niveau des artères de conductance (Pedard et al., 2018b). Ainsi, la moindre production de NO par l'endothélium diminuerait la sécrétion de BDNF qui à son tour diminuerait la capacité de l'endothélium à synthétiser du NO. Mais qu'en est-il *in vivo* ? Le BDNF sécrété dans le sang par l'endothélium peut-il activer les TrkB endothéliaux ou est-il immédiatement capté par les plaquettes ? Quoi qu'il en soit, nos résultats suggèrent que la moindre synthèse endothéliale de BDNF pourrait être à la fois la cause et la conséquence de la dysfonction endothéliale et que ce cercle vicieux pourrait être interrompu *in vivo* soit par des donneurs de NO ou par des agonistes TrkB, ce qui reste à démontrer.

2) Dysfonction endothéliale cérébrale et PR

Alors que la dysfonction endothéliale est bien documentée à la périphérie chez le patient souffrant de PR et le rat arthritique, peu de travaux se sont intéressés à la dysfonction endothéliale cérébrale. En faveur de son existence, notre partenaire bisontin a récemment montré que la réponse vasodilatatrice de l'artère cérébrale moyenne à l'acétylcholine était réduite par l'arthrite (Revue page 54) et que l'anomalie était reversée par l'administration chronique d'un inhibiteur d'arginase suggérant que la surexpression de l'arginase endothéliale contribuerait à la moindre production de NO par l'endothélium cérébral comme déjà démontré pour l'endothélium des vaisseaux périphériques. Nos résultats suggèrent qu'une diminution de l'expression d'eNOS pourrait être également impliquée dans la moindre production de NO. D'autres mécanismes pourraient être recherchés notamment le découplage d'eNOS (ratio dimère/monomère) ainsi que l'activité d'eNOS (ratio peNOS/eNOS). Le seul argument en faveur d'une dysfonction endothéliale cérébrale en cas de PR est que la prévalence de l'AVC est plus élevée chez la patient PR que dans la population générale (Liang et al., 2006, Sodergren et al., 2009, Semb et al., 2010, Liou et al., 2014). Un autre argument indirect est la mise en évidence par écho-doppler transcrânien de lésions de type athérosclérotique au niveau des vaisseaux cérébraux chez le patient PR (Olah et al., 2017). Il serait intéressant d'étudier la réactivité cérébrovasculaire au CO₂ ou à la L-arginine chez le patient ou le rat arthritique, paramètre qui devrait être moindre en cas de dysfonction endothéliale à l'étage de la circulation cérébrale. En tout cas chez l'animal arthritique, la dysfonction endothéliale cérébrale coexiste avec la dysfonction endothéliale périphérique et il serait intéressant d'évaluer si la cinétique d'apparition diffère entre les 2 localisations. La question qui se pose concerne le lien entre activation et dysfonction endothéliale à l'étage de la circulation cérébrale. Il serait intéressant d'étudier la cinétique d'expression endothéliale des molécules d'adhérence (marqueur d'activation endothéliale) à l'étage des macro- et microvaisseaux de la circulation cérébrale et de la comparer à celle de la dysfonction endothéliale.

3) Dysfonction endothéliale cérébrale et déficit cognitif en cas de PR

Il est bien documenté que le patient PR est à risque sur le plan cognitif. Cependant, les mécanismes impliqués restent hypothétiques : inflammation systémique, dysfonction endothéliale à l'étage cérébral, anomalie de la BHE, sédentarité, douleur ? Peu de travaux se sont intéressés aux effets des modèles animaux d'arthrite sur la cognition. En tout cas, la LTP hippocampique est altérée chez le rat arthritique (Liu et al., 2012), ce qui signe l'existence d'une altération de la neuroplasticité dans une région cérébrale impliquée dans la cognition. Dans notre étude, nous avons utilisé l'anhédonie (test de préférence au sucre) comme reflet d'un déficit cognitif, ce qui est largement critiquable car l'anhédonie ne pourrait être qu'un reflet de l'état algique. Cependant, nos résultats montrent pour la première fois que l'arthrite s'accompagne d'une réduction sévère des taux cérébraux de BDNF et que cette réduction coexiste avec l'anhédonie. Or, dans toutes les situations associées à un déficit cognitif (hypertension, diabète, âge avancé, maladie d'Alzheimer, ...), les taux de BDNF sont diminués dans le cerveau.

La question qui émerge concerne la cause de la réduction des taux cérébraux de BDNF. Nos résultats immunohistochimiques montrent que la réduction de ces taux est associée à une réduction de l'expression du BDNF aussi bien par les neurones que les cellules endothéliales des microvaisseaux cérébraux. La moindre expression de BDNF par les neurones pourrait être le marqueur d'une moindre activité neuronale puisque la synthèse et la sécrétion de BDNF par les neurones est proportionnelle à l'activité neuronale (Lessmann et al., 2003, Greenberg et al., 2009). Nos résultats montrant l'existence d'une association entre l'expression endothéliale du BDNF et l'expression neuronale des TrkB activés et l'absence d'association entre l'expression du BDNF et des TrkB activés par les neurones nous amènent à proposer une alternative. Nous proposons l'hypothèse selon laquelle la moindre activation des TrkB neuronaux observée chez le rat arthritique est la conséquence d'une moindre sécrétion de BDNF par les cellules endothéliales des capillaires sanguins cérébraux. Cette hypothèse s'inscrit dans l'idée selon laquelle il existe un lien fort entre facteurs de risque cardiovasculaire et capacités cognitives. Cependant, il n'existe aucune preuve que le BDNF d'origine endothéliale soit capable d'influencer la neuroplasticité. C'est pourquoi, nous envisageons au laboratoire d'étudier chez la souris l'effet de la délétion conditionnelle du BDNF endothélial sur la cognition.

Les capacités cognitives dépendent non seulement des qualités neuroplastiques du cerveau mais aussi d'une adéquation entre les apports et les besoins nutritifs des neurones. Il est donc possible que le déficit cognitif associé à la PR ne soit que le reflet de la dysfonction endothéliale cérébrale et du trouble de la régulation du DSC qui en résulte. Enfin, l'augmentation de la perméabilité cérébrovasculaire observée en cas de polyarthrite (Nishioku et al., 2010) pourrait également expliquer le déficit cognitif retrouvé en cas de PR.

Un de nos résultats très intéressant est que l'inhibiteur d'arginase, le nor-NOHA, restaure les taux cérébraux de BDNF chez le rat arthritique suggérant que la réduction des taux cérébraux observée chez le rat arthritique est initiée par une réduction des capacités de synthèse et de sécrétion du BDNF par les cellules endothéliales cérébrales et, par extension, que la dysfonction endothéliale est à l'origine du déficit cognitif. Des études complémentaires doivent donc être conduites afin d'étudier l'effet de l'inhibiteur d'arginase sur l'anhédonie d'une part et sur l'expression endothéliale vs neuronale du BDNF cérébral avant de réellement conclure sur le lien dysfonction endothéliale/déficit cognitif. Comme l'inhibiteur d'arginase est dépourvu d'effet sur le score arthritique, l'existence d'un lien entre inflammation systémique et BDNF cérébral est peu probable. Nous avons d'ailleurs montré l'absence d'association entre les taux cérébraux de BDNF et le score arthritique.

4) BDNF circulant et PR

Nos résultats montrent un taux sérique plus élevé de BDNF chez le rat arthritique que chez les rats contrôles et corroborent ainsi les études cliniques montrant que le BDNF plasmatique est augmenté chez les patients atteints de PR en lien avec l'inflammation. Le fait que l'augmentation des taux sériques coexiste avec une moindre expression de BDNF dans l'endothélium vasculaire et une réduction des taux cérébraux suggère que le BDNF sérique n'est pas un marqueur fiable de la dysfonction endothéliale donc du risque cardiovasculaire ni du risque cognitif en cas de PR. Nous pensons que l'augmentation du BDNF sérique pourrait être reliée à l'inflammation systémique. En accord avec cette hypothèse, il existe une association entre les taux sériques de BDNF et le score arthritique. Quelle est alors la source cellulaire à l'origine de l'élévation des taux sériques de BDNF en cas d'arthrite ? Nous pensons qu'il s'agit des mononucléaires circulants.

Nous avons récemment montré au laboratoire que les mononucléaires du sang (lymphocytes et monocytes) contenaient et sécrétaient du BDNF et que le contenu en BDNF de ces cellules était plus élevé (Pedard et al., 2016) chez le rat arthritique que chez le rat contrôle. Or, les cytokines pro-inflammatoires, notamment le TNF α , sont capables de stimuler la synthèse de BDNF par les mononucléaires sanguins (Schulte-Herbruggen et al., 2005). En cas de PR, il est possible que les cellules mononuclées du sang exposées au TNF α secrètent en continu du BDNF dans le plasma et que le BDNF plasmatique soit capté par les plaquettes. Ainsi, l'augmentation des taux sériques en BDNF en cas de PR serait la conséquence de l'élévation des taux de BDNF dans les plaquettes, ce qui reste à démontrer. Si c'est le cas, la thrombocytémie deviendrait un paramètre incontournable à mesurer pour interpréter les taux sériques de BDNF en cas de PR.

Le rôle du BDNF dans les mononucléaires sanguins n'a pas été spécifiquement étudié en cas de PR. Il n'est pas exclu que les cellules mononuclées du sang soient capables de délivrer le BDNF aux tissus

qu'ils ont infiltrés qu'il s'agisse du tissu sous-endothélial lors du processus athérosclérotique (Aubry et al., 2004) ou des tissus enflammés comme la synovie (Mellado et al., 2015). Si l'on admet que le BDNF exerce un effet anti-apoptotique et cytoprotecteur (Chen et al., 2017), le BDNF des globules blancs pourrait être envisagé comme un élément du processus réparateur. Nous venons également de montrer que la récupération fonctionnelle 3 mois après un AVC d'origine ischémique pouvait être prédite à partir du contenu en BDNF des mononucléaires dans les 3 jours qui suivent l'admission hospitalière suggérant que les mononucléaires sont capables de délivrer le BDNF aux territoires cérébraux ischémiés (En cours de publication). Un point qui n'a jamais été abordé est si le cerveau est infiltré ou pas par les globules blancs circulants en cas de PR. En tout cas, l'examen de coupes histologiques du cerveau n'a pas révélé la présence de globules blancs chez les rats arthritiques. Il serait tout de même intéressant d'étudier l'expression des molécules d'adhérence par l'endothélium cérébral chez ces animaux. Si c'est le cas, le TNF α pourrait exercer une dualité d'action : être toxique pour les articulations mais être bénéfique pour le cerveau via l'induction de la sécrétion de BDNF par les mononucléaires circulants.

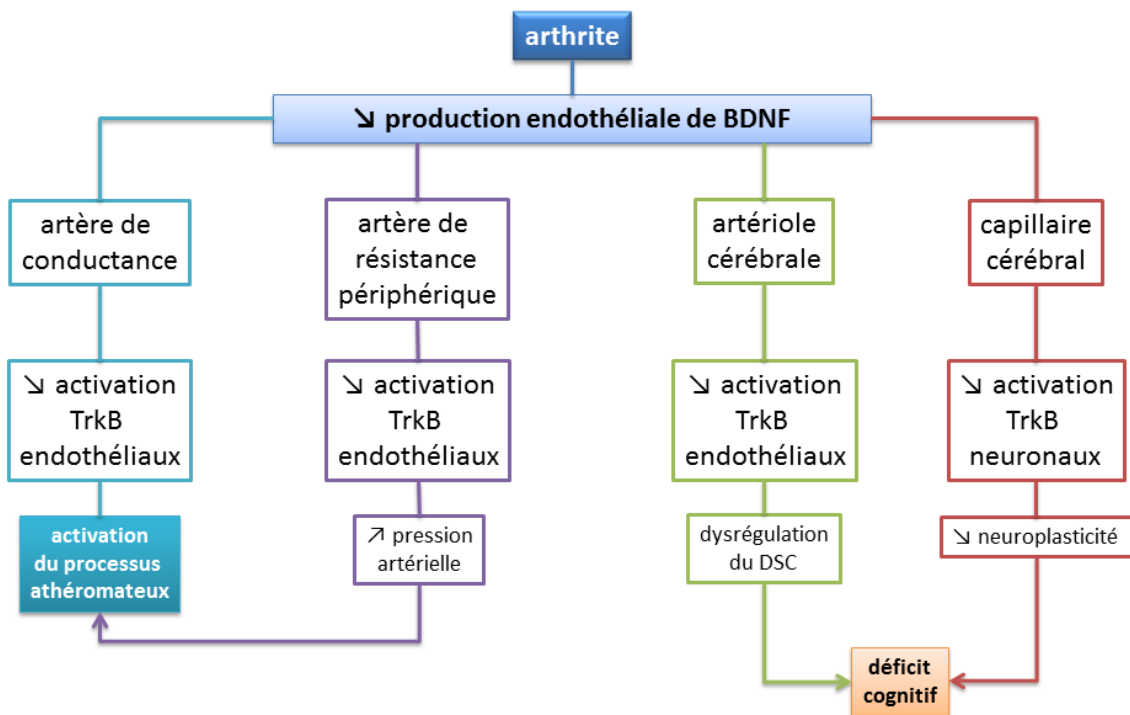


Figure 10: Effets de la diminution de la production endothéliale de BDNF sur le processus athéromateux et la cognition en cas d'arthrite et boucle reliant production endothéliale de NO et de BDNF en cas d'arthrite. DSC=débit sanguin cérébral.

5) Retombées cliniques de nos études

Nous pensons que nos résultats sont d'intérêt pour les rhumatologues.

Le 1^{er} point est que l'élévation des taux circulants de BDNF n'est probablement que le reflet de l'inflammation systémique.

Le 2^{ème} point est que l'efficacité des médicaments anti-inflammatoires classiques ou antirhumatismaux ne permet pas de préjuger un effet bénéfique sur le risque cardiovasculaire.

Le 3^{ème} point est que le déficit cognitif et la dépression associés à l'arthrite sont probablement à relier à un déficit cérébral en BDNF et que ces anomalies sont déconnectées de la sévérité de l'inflammation clinique.

Liste des productions scientifiques liées au projet de thèse

Publications dans des journaux internationaux à comité de lecture :

1. **Pedard, M.**, Demougeot, C., Prati, C., and Marie, C. (2018). Brain-derived neurotrophic factor in adjuvant-induced arthritis in rats. Relationship with inflammation and endothelial dysfunction. *Progress in Neuropsychopharmacology & Biological Psychiatry*.
2. **Pedard, M.**, Quirié, A., Garnier, P., Tessier, A., Demougeot, C., Prati, C., and Marie, C. (2017). The cerebral brain-derived neurotrophic factor pathway, either neuronal or endothelial, is impaired in rats with adjuvant-induced arthritis. Connection with endothelial dysfunction. *Frontiers in Physiology*.
3. Totson, P., **Pedard, M.**, Marie, C., and Demougeot, C. (2018). Activation of endothelial TrkB receptors induces relaxation of resistance arteries. *Vascular Pharmacology*.
4. **Pedard, M.**, Quirié, A., Totson, P., Verhoeven, F., Garnier, P., Tessier, A., Demougeot, C., and Marie, C. (2018). Vascular brain-derived neurotrophic factor pathway in rats with adjuvant-induced arthritis. Effect of anti-rheumatic drugs. *Atherosclerosis*.
5. Marie, C., **Pedard, M.**, Quirié, A., Tessier, A., Garnier, P., Totson, P., and Demougeot, C. (2018). Brain-derived neurotrophic factor secreted by the cerebral endothelium : a new actor of brain function ? *Journal of Cerebral Blood Flow & Metabolism*.

Communications à des congrès internationaux :

1. **Pedard, M.**, Totson, P., Prati, C., Demougeot, C., and Marie, C. (2016). Impaired cognition is linked to brain depletion in BDNF levels in a rat model of rheumatoid arthritis. *Arthritis Rheumatol* 68(Supplement 10). *ACR/ARHP Annual Scientific Meeting, Washington, USA, november 2016* (Written communication).
2. **Pedard, M.**, Cefis, M., Quirié, A., Garnier, P., Tessier, A., Demougeot, C., and Marie, C. (2018). Does BDNF derived from the cerebral microvasculature control cognition?. *Euroscicon on Neurology and Neurological Disorders, Paris, France, july 2018* (Oral communication).

Communications à des congrès nationaux :

1. **Pedard, M.**, Totson, P., Demougeot, C., and Marie, C. (2017). Association between endothelial dysfunction and brain BDNF levels in a rat model of arthritis. *Fundamental and Clinical Pharmacology* 31(Supplement 1). *Congrès de la Société Française de Pharmacologie et Thérapeutique, Rouen, avril 2017* (Poster).

2. **Pedard, M.**, Totoson, P., Demougeot, C., and Marie, C. (2017). Effect of arthritis on brain BDNF. *Fundamental and Clinical Pharmacology* 30(Supplement 1). *Congrès de la Société Française de Pharmacologie et Thérapeutique, Nancy, avril 2016* (Poster).
3. **Pedard, M.**, Totoson, P., Demougeot, C., and Marie, C. (2016). Discordances entre taux de BDNF sérique et vasculaire dans un modèle d'arthrite chez le rat. *Revue du Rhumatisme* 83 (Supplement 1). *Congrès de la Société Française de Rhumatologie, Paris, décembre 2016* (Poster).
4. **Pedard, M.**, Maguin-Gaté, K., Demougeot, C., and Marie, C. (2016). Effect of arthritis on brain BDNF. *Forum des Jeunes Chercheurs, Besançon, juin 2016* (Poster).
5. **Pedard, M.**, Totoson, P., Demougeot, C., and Marie, C. (2016). Effet de l'arthrite sur le BDNF cérébral. Relation avec l'inflammation et la dysfonction endothéliale. *Journée de la Société Cerveau et Maladies Cérébrovasculaires, Paris, juin 2016* (Communication orale).

Liste des productions scientifiques non liées au projet de thèse

Publications dans des journaux internationaux à comité de lecture :

1. **Pedard, M.**, Cefis, M., Ennequin, G., Quirié, A., Garnier, P., Tessier, A., Pernet, N., and Marie, C. (2018). Brain-derived neurotrophic factor pathway after downhill and uphill training in rats. *Medicine & Science in Sports & Exercise*.
2. **Pedard, M.**, Brenière, C., Pernet, N., Vergely, C., Bejot, Y., and Marie, C. (2018). Brain-derived neurotrophic factor in peripheral blood mononuclear cells and stroke outcome. *En écriture*.

Communications à des congrès internationaux :

1. **Pedard, M.**, Ennequin, G., and Marie, C. (2017). Effects of eccentric and concentric training on brain-derived neurotrophic factor signaling in cognition-related brain regions. *Acta Physiologica* 221(Supplement S713). *FEPS Congress, Vienna, Austria, september 2017* (Oral communication).
2. **Pedard, M.**, Ennequin, G., and Marie, C. (2017). Effects of eccentric and concentric training on BDNF/TrkB signaling in cognition-related brain regions. *Movement & Sport Sciences – Science et Motricité* 95. *ACAPS Congress, Dijon, France, october 2017* (Oral communication).

Communications à des congrès nationaux :

1. **Pedard, M.**, Ennequin, G., and Marie, C. (2016). Effets des contractions excentriques et concentriques sur la signalisation du BDNF dans les régions cérébrales impliquées dans la cognition. *Forum des Jeunes Chercheurs, Dijon, juin 2017* (Communication orale).

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Titre : Endothélium, inflammation et cognition : focus sur le BDNF

Mots clés : BDNF, fonction endothéliale, NO, cognition, TrkB, inflammation

Résumé : Le BDNF (brain-derived neurotrophic factor) a été découvert dans le cerveau et est largement impliqué dans la neuroplasticité, la mémoire et la cognition via l'activation des récepteurs TrkB (tropomyosin receptor kinase B) neuronaux. Nous avons récemment montré que le système cardiovasculaire contenait autant de BDNF que le cerveau et que le BDNF exogène était capable d'induire une relaxation vasculaire dépendante de l'endothélium. D'autres études ont suggéré que l'activation des récepteurs TrkB endothéliaux par le BDNF était impliquée dans les processus athérosclérotiques. Notre laboratoire soupçonne une interaction étroite entre NO et BDNF endothélial et a même envisagé la possibilité d'une implication du BDNF sécrété par l'endothélium des microvaisseaux cérébraux dans la cognition. Les patients souffrant de polyarthrite rhumatoïde (PR), une maladie inflammatoire d'origine auto-immune, sont à risque cardiovasculaire et présentent une altération de la cognition avec notamment un risque plus élevé de dépression. Étonnamment, l'effet de la PR sur le BDNF est peu documenté. Les seules études disponibles rapportent une élévation du BDNF dans le sang et le liquide synovial en cas de PR. Notre hypothèse est qu'une réduction de l'expression endothéliale du BDNF pourrait contribuer au risque cardiovasculaire et au déficit cognitif associés à la PR. Ainsi, dans notre travail, nous avons étudié le BDNF vasculaire et cérébral et son

récepteur TrkB sur le modèle rat d'arthrite induite à l'adjuvant. Nos principaux résultats montrent que l'arthrite conduit à 1) une réduction des taux aortiques de BDNF indépendamment de la sévérité des symptômes inflammatoires mais dépendante de la fonction endothéliale, 2) une diminution des taux cérébraux en BDNF indépendamment de la sévérité des symptômes inflammatoires mais en lien avec la fonction endothéliale, 3) une diminution de l'expression du BDNF et de son récepteur TrkB activé au niveau tant neuronal qu'endothélial dans les régions cérébrales impliquées dans la cognition, 4) une corrélation positive entre l'expression du BDNF par l'endothélium vasculaire et l'expression neuronale des TrkB activés, 5) une absence de corrélation entre les taux sériques de BDNF et ses taux cérébraux ou vasculaires mais en revanche l'existence d'une corrélation positive entre les taux sériques de BDNF et l'inflammation qu'elle soit clinique ou biologique.

L'ensemble de ces données est en faveur de l'hypothèse selon laquelle le BDNF endothélial pourrait être impliqué dans le risque athérosclérotique et le déficit cognitif associé à l'arthrite. L'inflammation doit être considérée comme un facteur confondant lorsque les taux circulants de BDNF sont utilisés comme un reflet des taux présents dans le cerveau.

Title : Endothelium, inflammation and cognition : focus on BDNF

Keywords : BDNF, endothelial function, NO, cognition, TrkB, inflammation

Abstract : BDNF (brain-derived neurotrophic factor) has been discovered in the brain and is widely implicated in neuroplasticity, memory and cognition through the activation of neuronal TrkB (tropomyosin receptor kinase B) receptors. We have recently shown that the cardiovascular system contained as much BDNF as the brain and that exogenous BDNF was able to induce endothelium-dependent vascular relaxation. Other studies have suggested that activation of endothelial TrkB receptors by BDNF is involved in atherosclerotic processes. Our laboratory suspects a close interaction between endothelial NO and BDNF and has even considered the possibility of involvement of BDNF secreted by cerebral microvessel endothelium in cognition. Patients suffering from rheumatoid arthritis (RA), an inflammatory disease of autoimmune origin, are at risk of cardiovascular disease and have an impairment of cognition, including a higher risk of depression. Surprisingly, the effect of RA on BDNF is poorly documented. The only available studies report an increase of BDNF in the blood and synovial fluid in RA. Our hypothesis is that a reduction in endothelial expression of BDNF may contribute to the cardiovascular risk and cognitive deficit associated with RA. Thus, in our work,

we studied vascular and cerebral BDNF and its TrkB receptor on the rat model of adjuvant-induced arthritis. Our main findings show that arthritis leads to 1) a decrease in BDNF levels in aortas independently of the severity of inflammatory symptoms but dependent on endothelial function, 2) a decrease in brain BDNF levels independent of the severity of inflammatory symptoms, but link with endothelial function, 3) a decreased expression of BDNF and its activated TrkB receptor at both neuronal and endothelial levels in the brain regions involved in cognition, 4) a positive correlation between endothelial expression of BDNF and neuronal expression of activated TrkB, 5) a lack of correlation between serum BDNF levels and its cerebral or vascular levels, but on the other hand the existence of a positive correlation between serum BDNF levels and inflammation, whether clinical or biological.

All of these data support the hypothesis that endothelial BDNF may be involved in atherosclerotic risk and cognitive impairment associated with arthritis. Inflammation should be considered as a confounding factor when circulating levels of BDNF are used as a reflection of levels present in the brain.