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Régulation de l'expression et de la sécrétion de l'alpha-synucléine dans le système nerveux entérique

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Abréviations

Ach	Acétylcholine
AMPc	Adénosine monophosphate cyclique
AMS	Atrophie multi-systématisée
ARNm	Acide ribonucléique messager
ATP	Adénosine tri-phosphate
BDNF	Brain-derived neurotrophic factor
bFGF	basic Fibroblast growth factor
CGE	Cellules gliales entériques
CMA	Chaperone mediated autophagy
CL	Corps de Lewy
DCL	Démence à corps de Lewy
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular regulated kinase
GWAS	Genome wild association study
miRNA	micro ribonucleic acid
MLC	Myosin light chain
MMP	Matrix metalloproteinase
MP	Maladie de Parkinson
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAC	Non amyloid component
NGF	Nerve growth factor
NF	Neurofilament
NL	Neurite de Lewy
NSE	Neuron specific enolase
PM	Plexus myentérique (d'Auerbach)
PSM	Plexus sous-muqueux (de Meissner)
ROS	Reactive oxygen species
siRNA	Small interfering Ribonucleic Acid
SNA	Système nerveux autonome
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNC	Système nerveux central
SNE	Système nerveux entérique
SNP	Single nucleotide polymorphism
SP	Substance P
SUMO	Small ubiquitin-like modifier
TNFα	Tumor necrosis factor α
TJ	Tight junctions
VIP	Vasoactive intestinal peptide
ZNF219	Zinc finger protein 219
ZSCAN21	Zinc finger and SCAN domain containing 21

Introduction

Préambule

La maladie de Parkinson est une maladie d'expression principalement motrice caractérisée par une tétrade symptomatique comprenant l'akinésie, le tremblement de repos, l'hypertonie et l'instabilité posturale [1, 2]. Il s'agit de la deuxième pathologie neurodégénérative en fréquence après la maladie d'Alzheimer [3]. Sa prévalence en Europe a été récemment estimée à 1,8% de la population âgée de plus de 65 ans. Le lien avec l'âge est évident puisque la prévalence passe de 0,6% entre 65 et 69 ans à 2,6% entre 85 et 89 ans [4, 5]. Le diagnostic et le traitement de la MP représentent donc un enjeu de santé publique dans les pays concernés par le vieillissement de la population.
Les signes moteurs de la MP résultent de la destruction des neurones dopaminergiques de la substantia nigra pars compacta. On estime qu'à leur apparition plus de 60% des neurones ont dégénéré, ce qui entraine une dénervation majeure du striatum qui reçoit les projections dopaminergiques issues de la subtantia nigra pars compacta via le faisceau nigro-strié [2]. D'un point de vue neuropathologique, la perte neuronale dans la Subtantia nigra pars compacta est accompagnée par la formation d'inclusions cytoplasmiques dans les neurones restants nommés **corps de Lewy** (CL).

Deux progrès majeurs ont transformé la compréhension de la MP au cours des quinze dernières années. En 1997 d'une part, la découverte de formes familiales de la maladie liées à une mutation sur le gène de l'alpha-synucléine [6] et l'identification de l'alpha-synucléine comme composant majoritaire des CL [7] vont placer cette protéine au cœur de la MP. Des modifications conformationnelles favorisant l'agrégation de l'alpha-synucléine ou sa simple surexpression constituent des éléments déterminants de la physiopathologie de la MP. En 2002 d'autre part, la mise en évidence de lésions dégénératives préalables à l'atteinte de la Subtantia nigra pars compacta va déplacer le centre d'intérêt des chercheurs vers d'autres structures, dont l'atteinte caractérise la longue phase prémotrice de la maladie. Parmi elles, le système nerveux autonome en général et le système nerveux entérique en particulier font partie des premières structures atteintes par le processus dégénératif. Les mécanismes impliqués dans la transmission de la pathologie, du système nerveux autonome jusqu'à la Subtantia nigra pars compacta, commencent seulement à être appréhendés et semblent faire intervenir la sécrétion de l'alpha-synucléine.

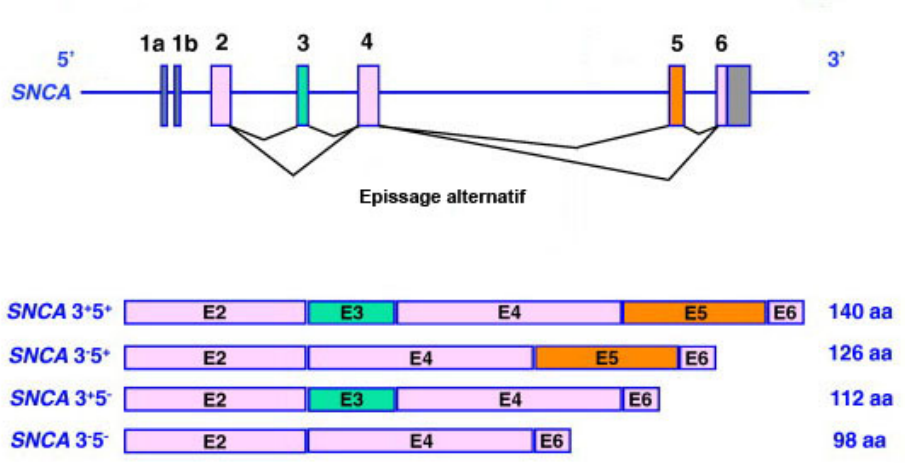
S'inspirant de ces progrès, ce travail s'est focalisé sur la physiologie de l'alpha-synucléine dans le système nerveux entérique. Nous nous sommes successivement intéressés à la régulation de son expression et à sa sécrétion par les neurones entériques, ainsi qu'à son rôle dans la régulation de la barrière épithéliale intestinale – fonction physiologique contrôlée par le SNE. En appréhendant l'expression, les mécanismes de sécrétion et la fonction de cette protéine clef dans l'une des structures inaugurant la maladie, ce travail s'adresse véritablement aux origines de la MP.

1. Alpha-synucléine

Initialement découverte chez *Torpedo californica* en 1988, l'alpha-synucléine doit son nom à sa première description par Maroteaux et collaborateurs [8]. Elle est décrite comme une protéine de 143 acides aminés purement neuronale, avec une distribution à la fois présynaptique et nucléaire. Se basant sur des études structurelles de microscopie photonique et électronique, mais également en étudiant certaines homologies de séquences avec des protéines connues, Maroteaux suggère que son rôle potentiel pourrait être d'assurer la liaison et la coordination d'évènements entre le noyau et les terminaisons présynaptiques. Cette publication a reçu initialement peu d'échos dans la communauté neurobiologique. Il a fallu attendre 1994 pour que l'homologue humain de l'alpha-synucléine soit mis en évidence dans les terminaisons présynaptiques des neurones du SNC [9] et identifié comme un des composants des dépôts beta-amyloïdes (NAC, *non-amyloid β component*) [10-14]. Mais c'est en 1997, avec la découverte d'une mutation de l'alpha-synucléine à l'origine d'une forme familiale autosomique dominante de la MP que l'intérêt pour cette protéine a véritablement explosé [6]. Très rapidement, l'équipe de Spillantini et Goedert a montré que cette protéine était présente dans les corps de Lewy chez l'ensemble des patients parkinsoniens (pour revue [15]). L'immunomarquage de l'alpha-synucléine est devenue depuis la technique de référence pour la détection de la pathologie de Lewy [16]

1.1 Gène SNCA et isoformes de l'alpha-synucléine

L'alpha-synucléine fait partie de la famille des synucléines contenant respectivement l'alpha-synucléine, la beta-synucléine et la gamma-synucléine [9, 17]. Ces trois protéines sont codées par 3 gènes différents. Le gène de l'alpha-synucléine, appelé SNCA pour *synuclein*, *alpha non A4 component of amyloid precursor*, est localisé sur le chromosome 4 (4q21.3-q22) [12, 18, 19] alors que les gènes de la beta et la gamma-synucléine sont situés respectivement sur le chromosome 5 (5q35) [20] et 10 (10q23.2-q23.3) [17, 21]. Le gène de l'alpha-synucléine est composé de 7 exons, dont 5 participent au codage de la protéine [22] (figure 1). L'alpha-synucléine est sujette à l'épissage alternatif donnant 4 variants d'épissage différents (figure1). La forme la plus longue est constituée de 140 acides aminés, le second variant est composé de 126 acides aminés [18] et résulte d'une délétion interne de l'exon 3 correspondant à une perte des acides aminés 41 à 54, alors que le troisième variant de 112 acides aminés [18] résulte d'un épissage interne de l'exon 5, amputant les acides aminés 103 à 130. Le plus exprimé de ces variants est celui de 140 acides aminés, suivi par ceux de 112 et 126 acides aminés [18, 23]. Un quatrième variant de 98 acides aminés, à l'expression spécifiquement cérébrale, conjugue les deux épissages précédents [24].



D'après Venda et al ; Trends in Neuroscience 2010

Figure 1 : Structure du gène de l'alpha-syncléine et les différentes possibilités d'épissage

1.2 Structure de l'alpha-synucléine

L'alpha-synucléine est une protéine acide de 140 acides aminés chez l'homme et le rongeur. L'alignement des séquences d'acides aminés entre l'homme et les rongeurs montre une homologie de 95,3% avec uniquement la substitution de 6 acides aminés différents, indiquant une très bonne conservation de séquences chez les mammifères [25]. Des études en spectrométrie de masse MALDI-TOF lui donnent un poids moléculaire de 14463 kDa. L'alpha-synucléine est une protéine soluble, résistante à la température [26] et qui a la particularité de ne pas avoir de conformation définie à l'état natif. Cette caractéristique signifie qu'une fois traduite, la protéine adopte une structure aléatoire et difficilement prédictible et lui permet d'avoir plusieurs états conformationnels quand est elle est sous forme de monomères. Une étude récente, étudiant les conformations que celle-ci revêt à l'état natif, montre que la protéine adopte 3 formes préférentielles [27] : une forme en feuillet β (7,3%), une forme en enroulement aléatoire (32,8%) et une forme associée par interactions faibles (54,5%).

Cet équilibre est largement modifié si les conditions environnementales varient (ex. augmentation de l'osmolarité, apport de Cu^{2+}) ou si la protéine est mutée (A30P), ce qui provoque une augmentation de la formation de feuillets β ou des formes à enroulement aléatoire [27]. Ces différentes constatations démontrent les capacités dynamiques de l'alpha-synucléine native et pourraient jouer un rôle dans les différents processus de fibrillation.

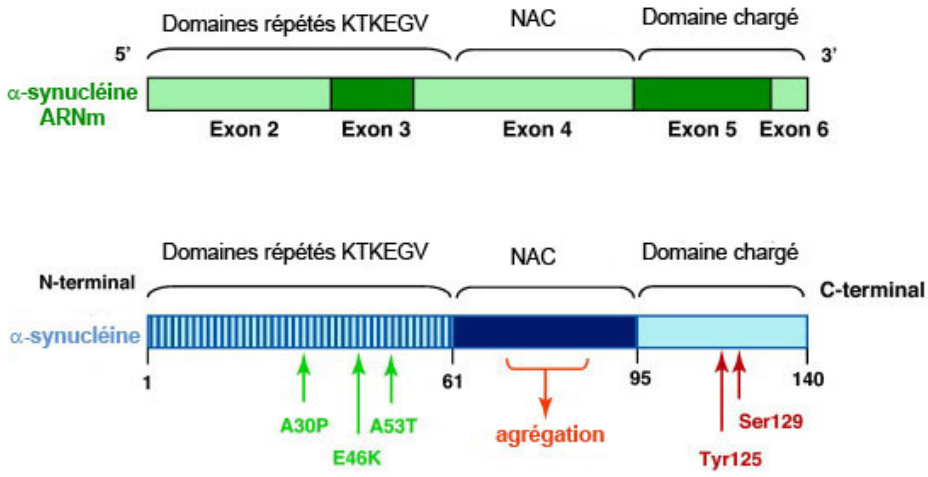
La protéine est classiquement divisée en 3 parties :

(i) Une partie amino-terminale extrêmement bien conservée et comptant pour environ la moitié de la protéine (résidus 1 à 64), particulièrement riche en acides aminés basiques, la chargeant positivement. La partie amino-terminale est composée de 6 répétitions imparfaites de 11 acides aminés contenant de façon constante le motif KTKEGV. Elle adopte une structure en deux hélices α séparées par une courte séquence qui lui confère une forte ressemblance avec le domaine de liaison aux lipides des apoprotéines de la classe A2 [28] et suggère un rôle de liaison aux lipides et plus particulièrement aux vésicules synaptiques. Les 3 mutations (A53T, A30P, E46K) du gène de l'alpha-synucléine, à l'origine de formes familiales de MP, sont localisées dans cette partie liant les lipides. Les mutations A53T et E46K confèrent aux protéines mutantes une cinétique d'agrégation bien plus importante que celui de l'alpha-synucléine normale alors que la mutation A30P possède une propension moindre à la

fibrillation mais favorise la production d'oligomères solubles [29-31]. On peut dès lors supposer que la modification de l'affinité de l'alpha-synucléine pour les lipides peut être suffisante pour altérer ses fonctions premières.

(ii) La partie centrale de l'alpha-synucléine s'étend des acides aminés 61 à 95. De nature très hydrophobe, il s'agit de la fraction NAC retrouvée dans les plaques séniles de la maladie d'Alzheimer. Ce domaine est essentiel à l'agrégation de l'alpha-synucléine et est capable à lui seul de former et de recruter le peptide Aβ, pour former des dépôts β amyloïdes [32, 33]. La beta-synucléine partage une forte homologie avec l'alpha-synucléine mais a perdu une partie essentielle du NAC, ce qui diminue fortement ses capacités à s'agréger. La gamma synucléine peut s'agréger mais dans des proportions bien moindres que l'alpha-synucléine [28, 34].

(iii) Le domaine carboxy-terminal de l'α-synucléine est quant à lui plus variable au cours de l'évolution. Il s'étend sur des acides aminés 96 à 140 et est surtout composé d'acides aminés acides. La partie qui comprend les acides aminés 109 à 140 joue un rôle prépondérant dans le contrôle de l'agrégation. Elle contient les principaux sites de phosphorylations, notamment la sérine 129. Cette partie possède de nombreux sites de liaison au calcium et au cuivre notamment, métaux influençant fortement l'agrégation de la protéine [35] (figure 2).

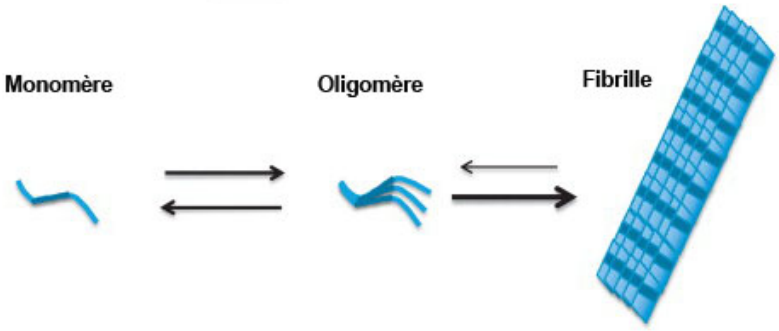


D'après Venda et al ; Trends in Neuroscience 2010

Figure 2 : Représentation schématique de la structure de l'ARN messager et de la protéine alpha-synycléine : Le domaine putatif de fixation aux lipides est constitué par un motif imparfaitement répété, représenté par les chiffres 1 à 6. Le domaine NAC (non-amyloid component) doit son nom au fait que cette portion tronquée de l'alpha-synucléine a été pour la première fois isolée dans les plaques séniles de la maladie d'Alzheimer. Les deux mutations les plus courantes associées à des maladies de Parkinson génétiques sont indiquées, de même que le site de phosphorylation sur sérine 129.

1.3 Etats conformationnels de l'alpha-synucléine

L'alpha-synucléine possède la capacité intrinsèque à se dimériser ou à s'oligomériser de façon passive et réversible (figure 3). Les petits oligomères restent solubles, alors que les oligomères de plus grand ordre deviennent insolubles et sont le point de départ de la formation des fibrilles, entités insolubles. En conditions normales, ces différents états coexistent et forment un équilibre dans la cellule [36-38].



D'après Hansen et al ; TRENDS in Molecular Medicine 2011

Figure 3 : Equilibre entre les différents états conformationnels de l'alpha-synucléine : Les différentes formes d'alpha-synucléine sont présentes en conditions physiologiques (monomère, oligomère (soluble), fibrille (insoluble) et coexistes en équilibre.

Un certain nombre d'études ont tenté de déterminer la conformation d'alpha-synucléine la plus toxique. Certains auteurs décrivent que les formes fibrillaires sont plus toxiques que les formes oligomériques. Pour cela ils s'appuient sur l'hypothèse que ces fibrilles perturbent le fonctionnement de la membrane plasmique entraînant une mort de la cellule. Une autre hypothèse plus probable propose que les petits oligomères et les protofibrilles seraient les formes les plus toxiques. Un grand nombre d'études viennent étayer cette hypothèse *in vitro*. L'étude la plus convaincante, réalisée *in vivo* chez le rat, démontre qu'en injectant dans le cerveau de ces animaux des lentivirus codant pour différentes formes mutantes d'alpha-synucléine, formant soit préférentiellement de petits oligomères ou soit préférentiellement des fibrilles, la toxicité des oligomères est bien supérieure à celle des fibrilles [39-41]. Cependant deux études récentes montrent que l'alpha-synucléine native, n'adopte pas comme on l'a longtemps supposé, une conformation aléatoire. Mais qu'elle s'organise en tétramères [40, 42], structure qui s'opposerait à son agrégation, mais ces nouvelles données restent encore controversées [41].

Bien qu'il ne soit pas encore facile de trancher, les formes multimériques de l'alpha-synucléine (oligomères, protofibrilles, fibrilles) semblent être des médiateurs majeurs de sa pathogénicité. L'étude des facteurs conditionnant l'agrégation de l'alpha-synucléine est essentielle pour mieux comprendre les mécanismes associés à sa toxicité.

1.4 Expression de l'alpha-synucléine

L'expression de l'alpha-synucléine est assez restreinte dans les organes, et se cantonne principalement aux neurones [8]. Dans le SNC, l'alpha-synucléine est largement exprimée dans des structures telles que le cortex frontal et temporal, le noyau caudé, l'amygdale ou le putamen et exprimée de façon plus minoritaire dans la Subtantia nigra pars compacta ou le noyau basal de Meynert [43]. L'alpha-synucléine est aussi exprimée par les neurones du système nerveux autonome notamment au niveau du noyau dorsal du nerf vague et la moelle [44], des fibres nerveuses périvasculaires de l'aorte [45] ou plus largement dans le SNE. En 2008, Phillips démontre que plus de 90 % des neurones du plexus myentérique situés dans la partie haute de l'intestin expriment l'alpha-synucléine. L'expression de l'alpha-synucléine diminue dans l'estomac (6% des neurones) puis augmente selon un gradient croissant de l'estomac vers le jéjunum (22% des neurones) [46].

Au cours de l'embryogénèse l'alpha-synucléine est très abondante dans le corps cellulaire. Par la suite elle se relocalise vers les terminaisons présynaptiques [47], suggérant une fonction dans la maturation synaptique. A l'âge adulte l'alpha-synucléine est majoritairement localisée dans le cytoplasme et les terminaisons présynaptiques dans les neurones [9, 11, 48-51].

Dans l'article fondateur de 1988, Maroteaux décrit également une localisation nucléaire de l'alpha-synucléine. Il a ensuite été suggéré que cette localisation nucléaire était un artéfact et qu'elle résultait de l'utilisation d'un antisérum d'alpha-synucléine non purifié [11, 52]. Néanmoins des études récentes ont confirmé la présence d'alpha-synucléine dans le noyau [53, 54]. Ces données contradictoires pourraient s'expliquer par la capacité de l'alpha-synucléine à pouvoir se transloquer au noyau notamment en conditions de stress cellulaire [55].

Les cellules gliales, et plus particulièrement les astrocytes [56] et les cellules de Schwann [57], expriment l'alpha-synucléine de façon physiologique au cours du vieillissement, mais dans un degré bien moindre que les neurones. Chez les patients atteints d'atrophie multi-systématisée (AMS), des inclusions immunoréactives pour l'alpha-synucléine sont présentes au sein des cellules gliales, plus particulièrement dans les oligodendrocytes [58-61]. Ces inclusions oligodendrocytaires d'alpha-synucléine sont un des critères qui permet de différencier AMS et MP [58, 62, 63]. Les

oligodendrocytes n'expriment pas d'ARNm de l'alpha-synucléine que ce soit de façon physiologique ou au cours de l'AMS [64]. Il est proposé que la présence d'alpha-synucléine dans les oligodendrocytes soit expliquée par une recapture de l'alpha-synucléine libérée dans le milieu extracellulaire par les neurones [65, 66].

De façon surprenante les cellules hématopoïétiques expriment aussi l'alpha-synucléine. Plusieurs études montrent que les érythrocytes expriment abondamment la protéine et sont une source intéressante pour purifier l'alpha-synucléine [40, 41, 67, 68]. Les cellules lymphocytaires (T et B), certaines cellules de l'immunité innée (NK, monocytes) [69] ou encore les cellules endothéliales des vaisseaux sanguins [70] peuvent également exprimer la protéine de façon physiologique. D'autres structures non neuronales expriment également l'alpha-synucléine, comme la rétine [71] ou encore les muscles en dégénérescence ou en réparation [72].

Les isoformes de l'alpha-synucléine voient également leur expression varier au cours du processus pathologique de la MP. Par exemple l'expression de l'isoforme de 126 KDa dans le cortex préfrontal de patients Alzheimer ou DLB est fortement diminuée par rapport aux patients contrôles [23]. L'isoforme de 98 kDa de l'alpha-synucléine est exprimé de façon importante dans les cortex frontaux de patients atteints de démences à CL ou chez les patients atteints de la maladie d'Alzheimer, suggérant un potentiel amyloïdogénique fort [24]. Une autre étude relate également une augmentation de l'expression de l'isoforme de 112 KDa chez la souris traitée au 1 - méthyle 4 - phényl 1,2,3,6-tétrahydro pyridine (MPTP) (une molécule neurotoxique responsable de formes expérimentales de MP), au niveau de la substance noire [73]. L'expression différentielle des isoformes de l'alpha-synucléine au cours de processus neurodégénératifs, suggère que chaque isoforme jouerait un rôle spécifique dans la cellule.

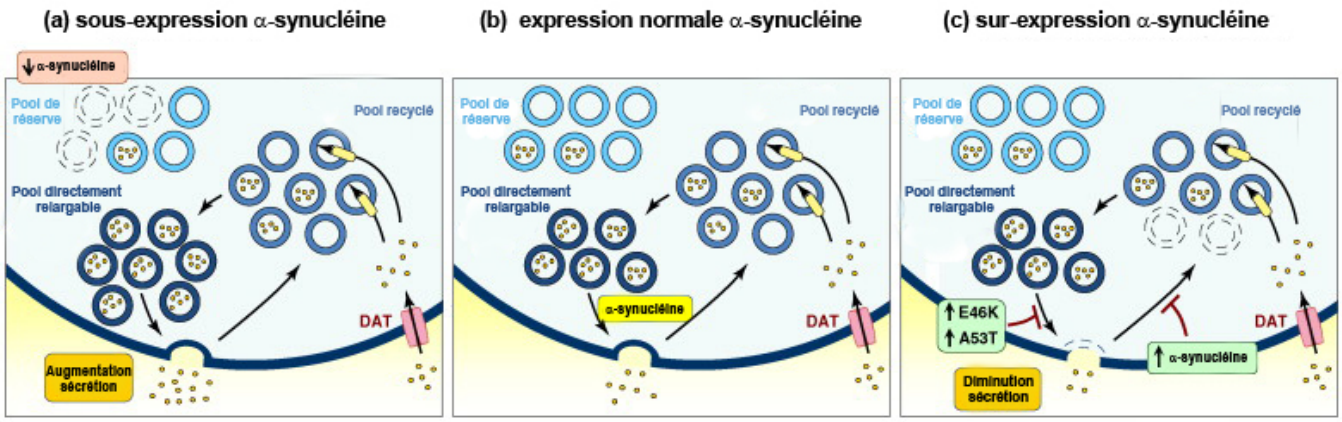
1.5 Fonctions de l'alpha-synucléine

Les rôles physiologiques de l'alpha-synucléine sont encore débattus. Dans sa publication initiale, Maroteaux suggère un rôle en tant que médiateur entre le noyau et les terminaisons pré-synaptiques. De nombreux travaux se sont depuis intéressés aux fonctions de l'alpha-synucléine et il est impossible d'être exhaustif. Les principaux aspects qui nous semblent devoir être retenus sont les suivants :

(i) l'alpha-synucléine qui est localisée à l'intérieur des vésicules de sécrétion à large corps dense [74], joue un rôle dans le maintien du pool de vésicules synaptiques et le fonctionnement des terminaisons présynaptiques (pour revue [75]). Elle a une fonction de chaperone dans le complexe de fusion des vésicules *soluble N-ethylmaleimide-sensitive factor attachment protein receptor* (SNARE) [76], permettant la fusion des vésicules présynaptiques à la membrane [50, 77, 78]. L'alpha-synucléine se lie aux vésicules grâce à sa partie amino-terminale et modifie sa structure non conformée en hélice α (figure 4).

(ii) Les animaux dont le gène codant pour l'alpha-synucléine a été invalidé n'ont à première vue que peu d'anomalies. Les animaux sont viables et fertiles, avec des fonctions physiologiques normales. Une analyse neurobiologique plus précise montre toutefois que ces animaux ont des anomalies de la libération des neurotransmetteurs et en particulier de la dopamine, en raison d'une atteinte de la dernière étape de fusion des vésicule à la membrane par inhibition du complexe SNARE [79, 80]. [78, 81-83]

(iii) L'alpha-synucléine peut également jouer le rôle de protéine chaperonne en protégeant de l'agrégation thermique et de la dénaturation chimique des protéines [84, 85] - L'alpha-synucléine joue également un rôle dans les processus de mémorisation et d'apprentissage. L'apprentissage du chant chez le Zebra Finch est conditionné par l'expression l'homologue de l'alpha synucléine dans les terminaisons présynaptiques [48]. L'absence de la protéine chez la souris perturbe la mémoire spatiale et de travail [86], et potentialise le processus de récompense [87].



D'après Venda et al ; Trends in Neuroscience 2010

Figure 4 : Réprésentation schématique du rôle potentiel de l'alpha-synucléine dans la régulation du pool de vésicule synaptique en fonction de son expression :(a) la réduction des niveaux intracellulaires de l'alpha-synucléine diminue la disponibilité des vésicules de réserve et un nombre plus important de vésicules est prêt à être libéré, contribuant à un relargage de dopamine plus important. (b) En condition d'expression normale l'alpha-synucléine est imaginée comme un régulateur physiologique de la distribution des vésicules synaptiques. (c) à contrario une élévation de l'expression de l'alpha-synucléine, possiblement en bloquant la dernière étape de fusion des vésicules ou l'expression des formes mutantes E46K ou A53T réduit le relargage de la dopamine ou en diminuant le nombre de vésicules disponibles.

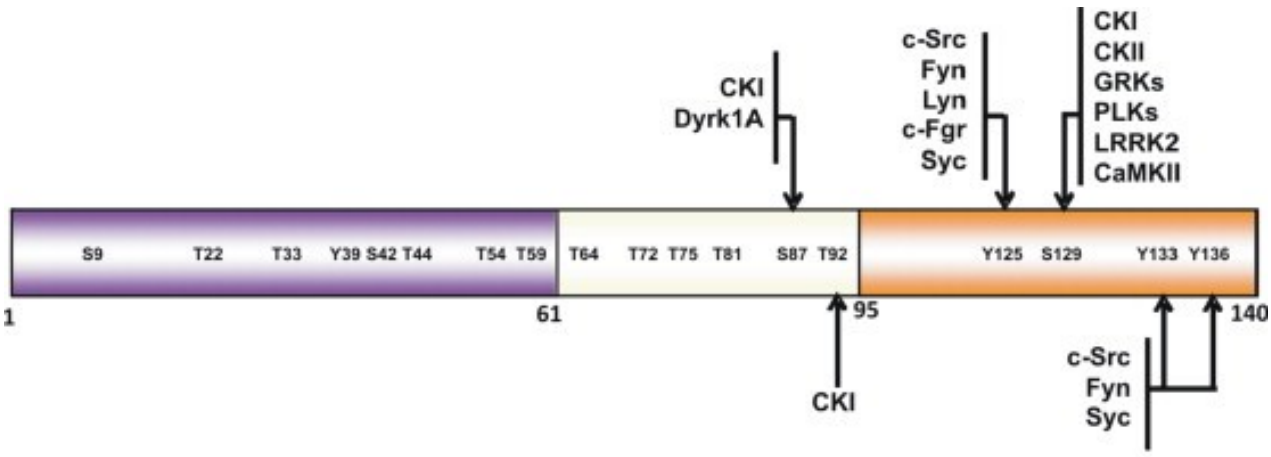
1.6 Phosphorylation de l'alpha-synucléine

L'étude de la séquence protéique de l'alpha-synucléine humaine indique la présence de plusieurs acides aminés phosphorylables (sérines/thréonines et tyrosines http://www.phosphosite.org/), qui sont conservés au cours de l'évolution (pour revue [88]).

Concernant la phosphorylation des résidus sérine et thréonine, une première étude réalisée *in vitro* dans des cellules HEK 293 (*Human Embryonic Kidney 293*) transfectées avec l'alpha-synucléine humaine a montré que la sérine 129 (P129S) et à un moindre degré la sérine 87 étaient phosphorylées par les caséines kinase 1 et 2 (CK1 et CK2) [89]. D'autres kinases sont impliquées dans la P129S, notamment les kinases de la familles *G coupled protein receptor kinase* (GRK) [90-92] ou encore la Polo kinase 2 [93]. Bien que la phosphorylation sur sérine 129 ait été largement étudiée en condition pathologique dans le cadre des synucléinopathies (pour revue [94]), cette phosphorylation se produit dans le SNC adulte en condition physiologique. Ainsi, environ 4% de l'alpha-synucléine totale serait phosphorylée dans le cerveau de rats et

de souris adultes [95, 96]. Une étude récente réalisée chez l'homme a montré que la substance noire, le noyau basal de Meynert et l'amygdale sont les structures cérébrales qui ont le ratio synucléine phosphorylée en 129/synucléine totale le plus élevé [43]. Peu d'études ont été publiées sur la régulation de la phosphorylation de l'alpha-synucléine sur sérine 129. Deux publications ont montré que le stress oxydant augmentait la phosphorylation sur sérine 129, par un mécanisme mettant en jeu CK2 [97, 98]. Une des conséquences physiologique de la phosphorylation de l'alpha-synucléine est la régulation de l'activité de la tyrosine hydroxylase, l'enzyme clef dans la synthèse de la dopamine [99]. Le fait que la phosphorylation sur sérine 129 soit la principale modification post-traductionnelle retrouvée dans les corps de Lewy des patients parkinsoniens a bien entendu suscité beaucoup d'intérêt sur les effets pro- ou anti-agrégogènes de cette phosphorylation. Jusque récemment, l'ensemble des études publiées suggérait que la phosphorylation sur sérine 129 est une étape précoce et importante pour l'agrégation de l'alpha-synucléine [96, 98, 100-104]. Toutefois, un travail de l'équipe d'Hilal Lashuel à Lausanne apporte des arguments solides pour penser qu'il n'en est rien et que la phosphorylation sur sérine 129 augmenterait la flexibilité conformationnelle de l'alpha-synucléine et diminuerait ses capacités à s'agréger [105].

La séquence protéique de l'alpha-synucléine humaine possède 4 résidus tyrosine (39, 125, 133 et 136). L'utilisation de pervanadate, un inhibiteur des phospho-tyrosine phosphatases, a permis de montrer dans des cellules HEK293 que la tyrosine 125 était le principal résidu tyrosine phosphorylé par les tyrosine-kinases de la famille Src [106, 107]. Une étude ultérieure a identifié la tyrosine kinase Syk (*spleen tyrosine kinase*), qui a une meilleure stœchiométrie que les tyrosines kinases de la famille Src pour phosphoryler la tyrosine 125 de l'alpha-synucléine [108]. Des travaux préliminaires ont montré que la phosphorylation sur tyrosine de l'alpha-synucléine diminuerait sa capacité à s'agréger [109] (figure 5).



D'après Oueslati et al ; Progress in Brain Research 2010

Figure 5 : Phosphorylation de l'alpha-synucléine en conditions normales et pathologiques : Représentation schématique soulignant tout les sites potentiels de phophorylation sur sérines (S), thréonines (T), tyrosines (Y). Les sites décrits sont marqués d'une flèche avec les kinases associées.

1.7 Modifications post-traductionnelles de l'alpha-synucléine en dehors de la phosphorylation

En dehors de la phosphorylation, l'alpha-synucléine est sujette à d'autres modifications post-traductionnelles. Depuis la découverte du fragment NAC dans les dépôts beta amyloïdes de la maladie d'Alzheimer [10], il est connu que l'alpha-synucléine peut être protéolysée. Le clivage protéolytique de l'alpha-synucléine est un phénomène qui se produit de façon physiologique au cours de la vie [43, 110, 111].

La protéolyse de l'alpha-synucléine est assurée par plusieurs groupes de protéases, les métaloprotéinases matricielles (MMP), la neurosine, la calpaïne, la cathepsine D et la plasmine. La MMP3 est parmi toutes les MMP celle qui a le plus d'efficacité à dégrader l'alpha-synucléine [112, 113]. Elle génère de petits fragments de 6,5 kDa. D'autres MMP peuvent cliver la protéine et plus particulièrement les MMP1, MMP9, MMP14 et MMP2 [113]. La neurosine, une sérine protéase similaire à la trypsine est capable de cliver l'alpha-synucléine en générant un fragment majoritaire comprenant les acides aminés de 1 à 80, [114] et 3 fragments plus minoritaires [115]. L'alpha-synucléine phosphorylée sur sérine 129 et les formes mutantes A53T et A30P sont plus résistantes à la dégradation par la neurosine que la forme sauvage [115]. La calpaïne, une protéase intracellulaire dépendante du calcium, qui coupe l'alpha-

synucléine monomérique sauvage ou mutante (A53T ou A30P) au sein du domaine NAC [116, 117], produisant des fragments qui inhibent l'agrégation de l'alpha-synucléine non tronquée. La cathepsine D, une enzyme du lysosome, est également capable de cliver l'alpha-synucléine et de générer des 2 formes tronquées en carboxy-terminal, coupures qui interviennent approximativement entre les acides aminés 91-98 et 98-115 [43, 88, 97, 118]. Enfin, Kim et collaborateurs ont récemment montré que la plasmine, une protéase plasmatique est capable de couper la partie amino-terminale de l'alpha-synucléine des formes monomériques mais aussi des formes oligomériques ou fibrillaires [119]. La troncation de l'alpha-synucléine par la plasmine empêche l'endocytose de la protéine par les cellules environnantes (figure 6).

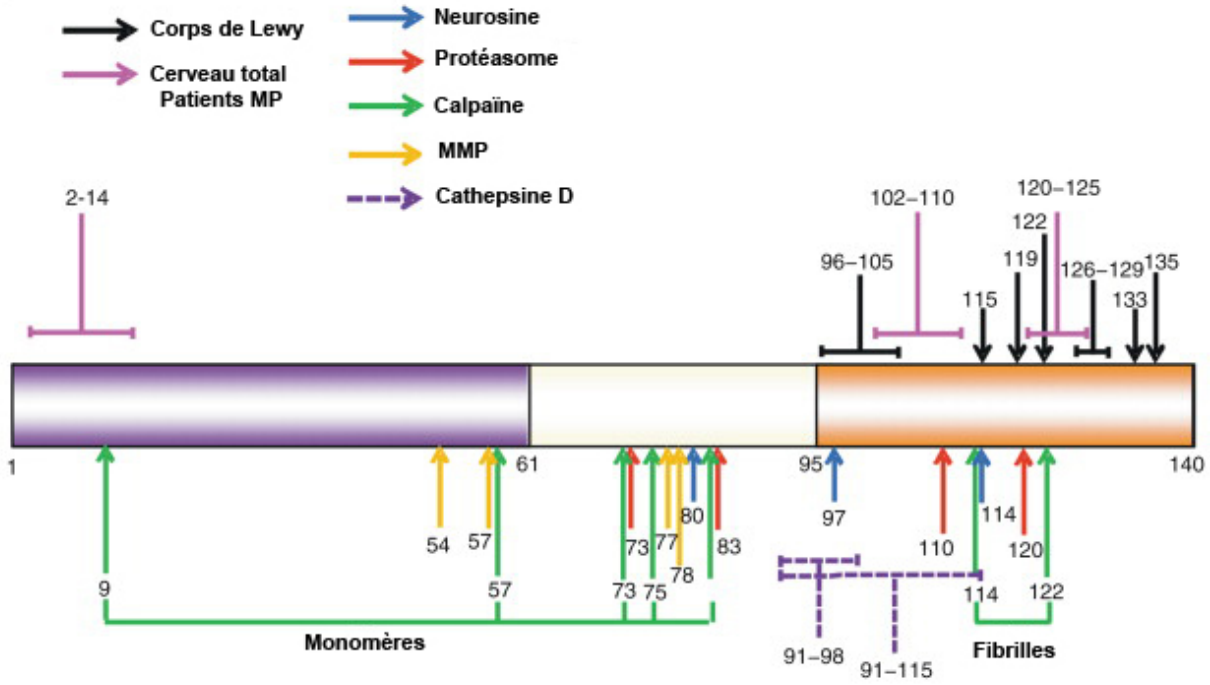
L'ubiquitinylation de l'alpha-synucléine s'opère sur les acides aminés lysine (K) et dépend de sa conformation. L'alpha-synucléine possède 15 résidus lysines, dont la majorité sont localisés dans la partie amino-terminale. En substituant par mutagénèse dirigée les lysines par des arginines et en digérant les protéines obtenues par la lysyl-endopeptidase AP1, deux groupes de recherche ont pu identifier en spectroscopie de masse, les sites préférentiels d'ubiquitinylation de l'alpha-synucléine à la fois i*n vitro* et *in vivo* [120, 121]. *In vitro* l'alpha-synucléine monomérique est ubiquitinylée sur les sites K10, K21, K23, K32, K34, K83 et K96 [120]. Parmi ces 7 résidus, les sites K21, K23, K32 et K34 sont majoritaires puisqu'une substitution de la lysine par une arginine sur ces 4 sites diminue de plus de 90 % l'ubiquitinylation de la protéine [120]. *A contrario* l'ubiquitinylation de formes fibrillaires d'alpha-synucléine recombinantes, intervient préférentiellement sur les sites K6, K10 et K12 [120]. Cette différence s'explique peut-être par le fait que dans les fibrilles, seules les parties amino-terminales de l'alpha-synucléine sont accessibles aux ubiquitine-ligases. Si l'alpha-synucléine est ubiquitinylée, cela signifie que sa dégradation peut-être effectuée par le protéasome. Le protéosome est capable de dégrader l'alpha-synucléine, notamment grâce à l'activité caspase du protéasome 20S.

L'alpha-synucléine possède à sa partie carboxy-terminale trois tyrosines capables d'être nitrées. La nitration sur tyrosine contribue à la formation de ponts di-tyrosine, formant de véritables ponts covalents entre les monomères d'alpha-synucléine [85].

L'alpha-synucléine peut être sumoylée [122-124]. La sumoylation consiste à la formation de pont di tyrosine (liaison covalente) entre une tyrosine de la protéine SUMO (*small ubiquitin like modifier*) avec une autre tyrosine située sur une protéine cible. La

sumoylation partage des propriétés équivalentes à l'ubiquitinylation. L'alpha-synucléine est majoritairement sumoylée par la protéine SUMO 1 et dans une plus faible proportion par les protéines SUMO 2 et 3 [122]. L'alpha-synucléine sumoylée migre à un poids moléculaire de 36 kDa, où la lysine 102 semble être le site de sumoylation préférentiel de la protéine.

De façon physiologique l'alpha-synucléine est sujette à l'O-glycosylation, formant un complexe de 22 kDa [125], que l'on retrouve complexée avec une E3 ubiquitine ligase et l'enzyme UbcH7 (*ubiquitine conjugating enzyme*).



D'après Oueslati et al ; Progress in Brain Research 2010

Figure 6 : Troncations de l'alpha-synucléine *in vitro* et in vivo :
Les sites de troncation retrouvés dans les CL sont représentés dans la partie haute du schéma (flèches noires) or retrouvés dans les cerveaux de patients (flèches roses) et sont indiqués soit par le dernier résidu de la coupure ou par une approximation si ils ne sont pas clairement définis. Les multiples enzymes responsables des sites de troncation *in vitro* sont représentés dans la partie inférieure du schéma (voir légende)

1.8 Régulation transcriptionnelle de la synthèse d'alpha-synucléine

L'expression de l'alpha-synucléine dans le SNC augmente avec l'âge [126] et la maturation neuronale [50, 51]. Cette étroite régulation de l'expression de l'alpha-synucléine suggère l'implication de facteurs trophiques ou de croissance.

Un groupe de recherche dirigé par Stefanis s'est particulièrement intéressé à la régulation transcriptionnelle de l'alpha-synucléine par les facteurs de croissance. Ils ont montré que le bFGF (*basic fibroblast growth factor*) et le NGF (*nerve growth factor*), deux facteurs de croissance impliqués dans la différentiation neuronale, augmentent l'expression de l'alpha-synucléine tant au niveau du messager que de la protéine, dans une lignée cellulaire de PC12 (*pheochromocytoma of the rat adrenal medulla*) ainsi que dans des neurones sympathiques et des neurones dopaminergiques mésencéphaliques en culture primaire. De façon remarquable, l'expression de la beta et gamma-synucléine n'est pas modifiée par le NGF ou le bFGF.

Trois zones de régulation sur le gène SNCA impliquées dans les effets du NGF et du bFGF ont été identifiées, par une approche d'essais rapporteur luciférase dans des PC12. La première zone est placée en 5' du premier exon de SNCA, dans le promoteur de l'alpha-synucléine. La deuxième et la troisième zones se situent dans l'intron 1, dans le premier et le troisième quart respectivement. L'analyse des séquences consensus de sites de liaison à des facteurs de transcription du promoteur et du premier intron de SNCA fait ressortir 2 candidats potentiels : *transcription zinc finger protein 219* (ZNF219) et *zinc finger SCAN domain containing 21* (ZSCAN21).

L'activation de la voie de signalisation ERK (*extracellular regulated kinase*) par le NGF et le bFGF contribue majoritairement au recrutement de ZSCAN21 qui active la transcription de SNCA en se fixant sur le premier quart de l'intron 1. D'autres voies de signalisation sont toutefois impliquées comme la voie de la phosphatidylinositol 3-kinase et des *stress activated protein kinases.* Enfin l'expression de ZSCAN21 n'augmente pas lors d'un traitement par le NGF ou le bFGF. Ces données suggèrent que la régulation de la transcription de l'alpha-synucléine par ZSCAN21 n'est pas directement liée à l'expression de ZSCAN21 lui même mais plutôt par une régulation post-transcriptionnelle de celui-ci, possiblement par phosphorylation.

Le rôle de ZNF219, un facteur de transcription de la famille *Krüppel-like zinc finger gene* [127], dans la régulation de l'alpha-synucléine est complexe. Trois sites de

liaison à ZNF219 sont présents en 5' du codon d'initiation de SNCA, deux sont situés dans le promoteur et le dernier est localisé dans le premier exon. En utilisant des essais luciférases, le groupe de Stefanis a montré que la partie 5' en amont du codon initiateur est à elle seule suffisante pour la transcription de SNCA [128]. La présence d'un seul site ZNF219 sur 3 bloque la transcription de l'alpha-synucléine induite par le NGF ou le bFGF alors que si deux des trois sites sur cette partie de promoteur sont présents, la transcription est activée. Le rôle à la fois un rôle répressif et activateur de ZNF219 dans la transcription du gène de l'alpha-synucléine est complexe et suppose la participation d'autres facteurs de transcription non identifiés. ZNF219 et ces hypothétiques facteurs de transcription pourraient avoir des affinités différentes en fonction de leur état de phosphorylation, par exemple pour les sites de liaison à l'ADN dans la partie 5' en amont du codon d'initiation (figure 7).

L'ensemble de ces données suggère que la régulation du gène de l'alpha-synucléine est complexe mais démontre l'importance de l'intron 1 dans l'activation de l'expression de l'alpha-synucléine.

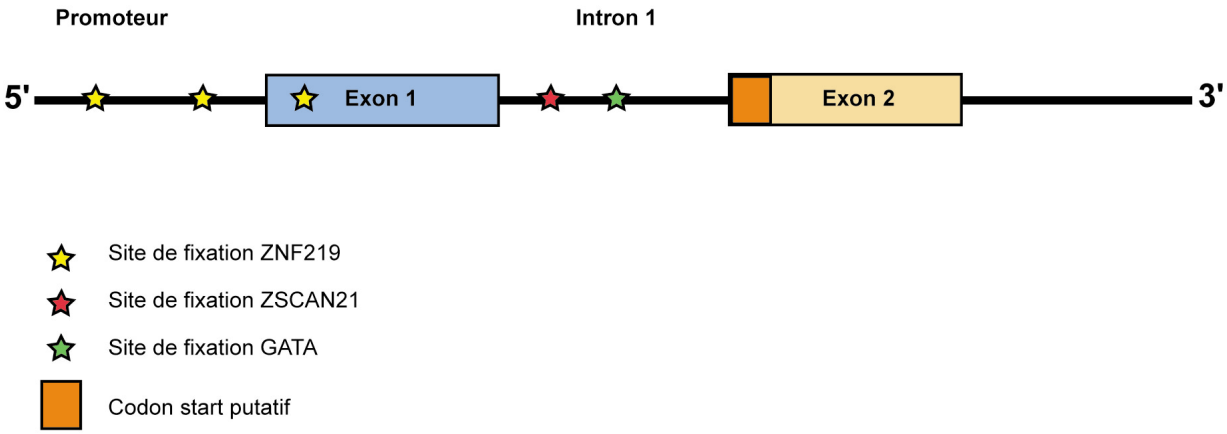


Figure 7 : Localisation sur le gène SNCA des sites de fixation des différents facteurs de transcription régulant l'expression de l'alpha-synucléine : Le promoteur, l'exon 1 et l'intron sont cruciaux pour la régulation transcriptionnelle de l'alpha-synucléine, notamment par les facteurs de croissance NGF et bFGF.

1.9 Régulation post-transcriptionnelle de la synthèse d'alpha-synucléine

Les micro ARN interférents (miRNA) sont des petits ARN doubles brins capables de réguler l'expression d'ARNm en entrainant leur dégradation et régule de façon post-transcriptionnelle l'expression des protéines. Les miRNA sont exprimés par des gènes spécifiques et à la différence des siRNA, ciblent un petit groupe de protéines. L'expression de l'alpha-synucléine est régulée par de miRNA codés par les gènes mir-7 et mir-153 qui ont une expression essentiellement neuronale [129, 130]. De façon remarquable, il a été montré que l'expression de mir-7 est diminuée par un traitement au MPTP, ce qui peut expliquer partiellement l'augmentation de l'alpha-synucléine par le stress oxydant.

1.10 Régulation de la dégradation de l'alpha-synucléine

La demi-vie de l'alpha-synucléine est évaluée de 17 à 54 heures [89, 131, 132], ce qui la classe dans la catégorie des protéines à longue demi-vie. Les mécanismes qui régulent la dégradation de l'alpha-synucléine sont multiples et leur rôle encore mal défini mais cette dégradation emprunte deux voies principales que sont respectivement la voie du protéasome et de l'autophagie.

Les inhibiteurs du protéasome tels que la β lactone, l'exposomicine, le MG132 ou le PSI bloquent la dégradation de l'alpha-synucléine [133-136]. La diminution de la dégradation de l'alpha-synucléine par ces inhibiteurs du protéasome induit la formation d'agrégats intracellulaires d'alpha-synucléine dans des lignées neuronales [131, 133, 136, 137] et chez l'animal [138, 139]. Bien que l'alpha-synucléine soit ubiquitinylée sur plusieurs sites *in vitro* [199] et dans les corps de Lewy [190], son ubiquitinylation ne semble pas être nécessaire à sa dégradation par le protéasome [188, 200]. Il est à noter que les agrégats d'alpha-synucléine ont une forte affinité pour la sous-unité catalytique 20S du protéasome [140] et bloquent son activité [141, 142].

L'autophagie est un mécanisme de dégradation dépendant du lysosome [143]. Selon la façon par laquelle les protéines arrivent au lysosome, trois voies de dégradation se distinguent : la microautophagie, la macroautophagie et l'autophagie dépendante des protéines chaperonnes (CMA, *chaperone mediated autophagy*) [144]. Les processus d'autophagie diminuent avec le vieillissement, contribuant à une accumulation de substrats toxiques dans la cellule, participant ainsi au processus neurodégénératif [145].

De façon intéressante, la délétion de 2 gènes essentiels pour l'autophagie ATG 5 et ATG 7 dans les neurones, conduit à une accumulation de protéines polyubiquitinylées et la neurodégénérescence chez la souris [146, 147]. Ces données suggèrent que l'autophagie joue un rôle important dans la mise en place des processus neurodégénératifs. L'étude de la dégradation de l'alpha-synucléine par les processus autophagiques montre que l'activation de la macroautophagie augmente la dégradation de l'alpha-synucléine et sa translocation dans les organelles acides [134]. Parallèlement l'inhibition des fonctions lysosomales par des molécules qui empêchent l'acidification des endosomes, produit une accumulation d'alpha-synucléine intracellulaire [134]. Enfin l'alpha-synucléine peut également être directement adressée au CMA pour être dégradée [132, 148].

1.11 Sécrétion de l'alpha-synucléine

L'alpha-synucléine est une protéine qui ne possède pas de séquence consensus pour sa sécrétion et qui a, par conséquent, longtemps été considérée comme une protéine purement intracellulaire. C'est au tout début des années 2000 que la présence de l'alpha synucléine est mise en évidence dans le liquide cérébrospinal et le plasma humain [149, 150]. La protéine est retrouvée à la fois chez les patients parkinsoniens et chez les sujets témoins, dans les mêmes proportions, sous forme monomérique et ne semble pas être tronquée, ce qui suggère que la présence d'alpha-synucléine dans les fluides biologiques est un processus physiologique. Cette constatation est étayée par des expériences récentes de microdialyse qui ont montré la présence d'alpha-synucléine extracellulaire dans le parenchyme cérébral de rongeurs [151]. Ces données, qui suggèrent que l'alpha-synucléine peut être sécrétée, ont conduit à de nombreux travaux dans des lignées neuronales et des neurones en culture primaire pour préciser les mécanismes de cette sécrétion.

On doit la première mise en évidence de l'alpha-synucléine dans les milieux de cultures à El Agnaf et collaborateurs en 2003, dans une lignée humaine de neuroblastome M17 [150]. Pour ces expériences, les surnageants de cellules M17 sauvages ou surexprimant l'alpha-synucléine humaine ont été analysés par immunoprécipitation suivie d'un immunoblot alpha-synucléine. L'alpha-synucléine était détectée dans le milieu de culture des cellules M17 sauvages et dans les cellules surexprimant l'alpha-synucléine, dès 6 heures en culture. La quantité d'alpha-synucléine

extracellulaire augmentait au cours du temps (maximum à 48 heures) et était plus importante pour les M17 surexprimant l'alpha-synucléine que pour les M17 sauvages. Il n'y avait pas de mort cellulaire à 48 heures car plus de 96% des cellules étaient encore viables après évaluation en bleu de Tryptan. L'ensemble de ces données confirmait donc que l'alpha-synucléine était bien sécrétée et que sa présence extracellulaire n'était pas liée à une simple diffusion par mort neuronale.

Deux équipes se sont ensuite intéressées aux mécanismes de cette sécrétion. Un groupe sud coréen a apporté des arguments en faveur d'une sécrétion d'alpha-synucléine via une exocytose non conventionnelle, indépendante de la voie du réticulum endoplasmique/Golgi. Ils ont en effet montré dans une lignée SH-SY5Y qui surexprime l'alpha-synucléine humaine, que la sécrétion de l'alpha-synucléine n'était pas modifiée par un traitement inhibiteur de l'exocytose conventionnelle, la bréfeldine A, mais en revanche bloquée en abaissant la température, connue pour inhiber l'ensemble des processus d'exocytose. Tout comme El Agnaf, ils ont montré que la quantité d'alpha-synucléine extracellulaire dépendait de la quantité d'alpha-synucléine intracellulaire et qu'il n'y avait pas d'arguments pour penser que cette détection d'alpha-synucléine en extracellulaire était liée à une mort neuronale. Des expériences de fractionnement subcellulaire et de microscopie électronique ont ensuite montré que l'alpha-synucléine était présente dans les vésicules synaptiques, aussi bien dans les cellules SH-SY5Y que dans le cerveau de rat. Cette alpha-synucléine intravésiculaire s'agrégererait plus volontiers que l'alpha-synucléine cytosolique [74]. La déprivation en sérum [152], le peroxyde d'hydrogène, la dopamine [153], l'inhibition de l'endocytose, les inhibiteurs de protéasome [74] et du lysosome [152], contribuent à augmenter la sécrétion des formes oligomériques. L'ensemble de ces résultats peut se résumer de la façon suivante : (i) l'alpha-synucléine est sécrétée de façon constitutive par une voie indépendante du réticulum endoplasmique/Golgi (ii) elle est présente dans les vésicules synaptiques (iii) il existerait une régulation de la sécrétion différentielle entre les formes monomériques et les formes oligomériques (suggérée par Lee[153]).

Plus récemment, le groupe de Léonidas Stefanis a montré que la sécrétion de l'alpha-synucléine pouvait également emprunter une voie d'exocytose non conventionnelle médiée par les exosomes [152]. Les exosomes sont des petites vésicules qui sont émises à partir de grosses structures multivésiculaires associées aux lysosomes, alimentées par les vésicules d'endocytose. Ces vésicules peuvent directement sortir des

cellules sans passer par la voie réticulum endoplasmique/Golgi classique (pour revue [154]) (figure 8).

1.12 Effets et devenir de l'alpha-synucléine extracellulaire

L'alpha-synucléine extracellulaire peut être recaptée par les cellules environnantes telles que les neurones [256], les astrocytes [257] ou les cellules microgliales [258]. Les mécanismes de recapture de l'alpha-synucléine ont été largement étudiés ces 5 dernières années (figure 8).

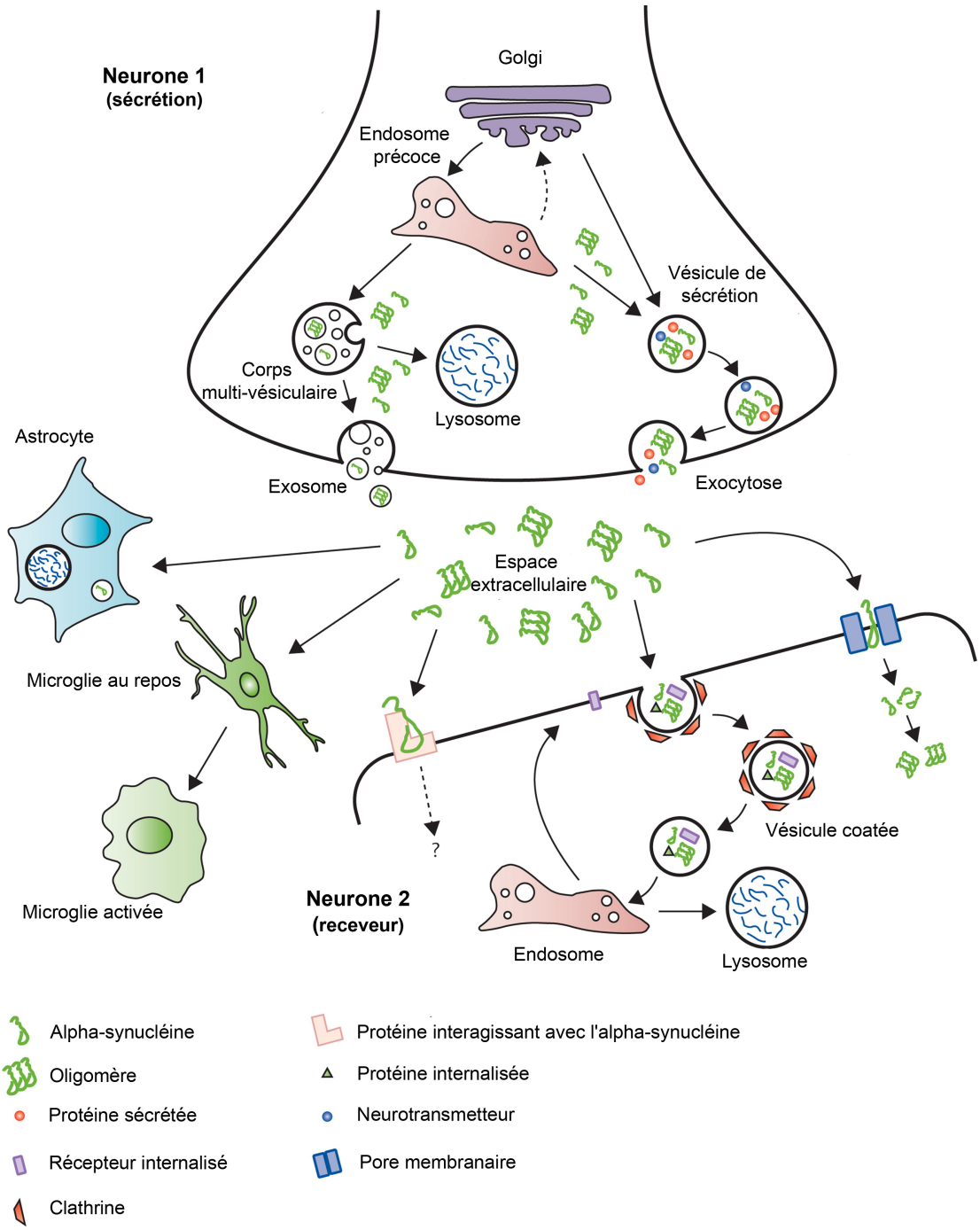
La première expérience de recapture de l'alpha-synucléine extracellulaire a été réalisée par Sung et collaborateurs en 2001, qui ont montré que l'endocytose de l'alpha-synucléine extracellulaire monomérique mettait en jeu la petite protéine G Rab-5A dans une lignée neuronale H19-7 [155]. D'autres auteurs se sont par la suite intéressés aux mécanismes de recapture des formes oligomériques et fibrillaires. En 2008, Lee a montré dans une lignée de SH-SY5Y que la recapture des monomères pouvait se faire de façon passive et rapide alors qu'a l'inverse la recapture des formes oligomériques ou fibrillaires se faisait de façon active, par endocytose dépendante de la dynamine [156].

Ces données qui ont été obtenues en utilisant des concentrations relativement importantes d'alpha-synucléine recombinante, ont incité d'autres équipes à étudier le devenir de l'alpha-synucléine endogène sécrétée. Ainsi, Desplats a montré dans des cellules souches de neurones corticaux de souris qu'une partie de l'alpha synucléine sécrétée par un groupe de neurones était retrouvée dans un second groupe de neurones. Ce transfert d'alpha-synucléine entre les deux groupes de neurones était bloqué par l'expression d'un dominant négatif de la dynamine [157]. Des résultats similaires ont été observés dans des lignées SH-SY5Y [158]. Il est toutefois à noter que la recapture de l'alpha-synucléine par les cellules SH-SY5Y ne peut se faire que si celles ci sont en prolifération. Stefanis démontre également qu'un traitement de la surface des membranes par de la trypsine ralentit de façon significative la recapture de l'alpha-synucléine [152], proposant l'intervention d'un récepteur membranaire et/ou des radeaux lipidiques [159].

En dehors des neurones, la recapture de l'alpha-synucléine peut se faire par les astrocytes et la microglie. Dans un modèle de co-cultures d'astrocytes /neurones, l'alpha-synucléine produite par les neurones peut être endocytée par les cellules gliales

par une voie qui met en jeu la dynamine [160]. Les cellules microgliales sont également capables d'endocyter l'alpha-synucléine. Ainsi, en utilisant un modèle de co-culture de cellules microgliales BV-2 et de neuroblastomes SH-SY5Y, il a été montré que l'alpha-synucléine était recaptée par la microglie par une voie qui implique les radeaux lipidiques et notamment le ganglioside GM1 [161]. Une fois dans le milieu extracellulaire, l'alpha-synucléine peut être protéolysée notamment par les MMP3 [113], générant des formes tronquées d'alpha-synucléine qui ont plus tendance à s'agréger. L'alpha synucléine extracellulaire et plus particulièrement les formes multimériques telles que les fibrilles ou les oligomères solubles peuvent être neurotoxiques [152, 155, 162-164], notamment en induisant l'apoptose des cellules [152, 165, 166]. Danzer et collaborateurs ont montré que selon les conditions de préparation des oligomères, ceux-ci ont soit tendance à former des pores ioniques dans la membrane plasmique ou à entrer dans la cellule et pour initier un processus de nucléation [166]. La nucléation contribue à la formation d'agrégats intracellulaires qui peuvent bloquer le fonctionnement du protéasome [141, 142]. Une perturbation de l'activité du protéasome a pour conséquence une accumulation de protéines anormales affectant le fonctionnement de la cellule, expliquant partiellement la toxicité des formes multimériques de l'alpha-synucléine.

Enfin l'alpha-synucléine extracellulaire est capable d'activer les cellules microgliales, activation qui en réponse engendrent une réponse inflammatoire et la production de stress oxydant [167], créant un environnement propice à l'agrégation de la protéine.



D'après Verkrelis et al ; The Lancet Neurology 2011

Figure 8 : Sécrétion de l'alpha-synucléine et devenir extracellulaire :
L'alpha-synucléine est sécrétée par une voie d'exocytose non-conventionnelle médiée à la fois par les vésicules de sécrétion et les exosomes. Une fois dans l'espace extracellulaire, elle est capable de rentrer dans les neurones plusieurs mécanismes (récepteur, endocytose) ou former des pores à la surface des membranes, perturbant ainsi l'homéostasie des neurones receveurs. En parallèle l'alpha-synucléine peut également amorcer un processus de neuroinflammation en activant les cellules microgliales ou encore entrer dans les astrocytes.

2. Alpha-synucléine : rôle dans la maladie de Parkinson

2.1 Alpha-synucléine et corps de Lewy

D'un point de vue neuropathologique, deux critères sont nécessaires et suffisants au diagnostic de MP: il s'agit de la mise en évidence (i) d'une perte neuronale et d'une dépigmentation de la substance noire pars compacta, associée à (ii) la présence de corps de Lewy (CL) dans les neurones restants, dont la description revient à Lewy en 1912, et la dénomination à Trétiakoff en 1919 [168]. La nature des CL est longtemps restée méconnue. Leur mise en évidence s'est faite pendant longtemps par un marquage à l'hématéine-éosine ou par la coloration des structures amyloïdes en feuillet β à la thioflavine S. Plus récemment, avec l'avènement de l'immunohistochimie, ces colorations classiques ont été remplacées par l'immunomarquage de l'ubiquitine. D'un point de vue microscopique, les CL sont constitués d'un noyau central dense d'une taille variant de 8 à 30 µm circonscrits par un anneau d'éléments fibrillaires [169, 170].

L'identification de l'alpha-synucléine dans les CL [7] a fait immédiatement suite à la découverte d'une mutation responsable d'une forme génétique de MP, de transmission autosomique dominante, dans 4 familles méditerranéennes [6]. Cliniquement et histologiquement indiscernables des formes sporadiques, ces MP sont liées à la mutation A53T sur le gène SNCA (4q21-22). Depuis, bien que plus de 100 protéines aient été décrites dans les CL [171], l'immunomarquage alpha-synucléine est devenue la technique de référence pour identifier la pathologie de Lewy [16]

Les modifications post-traductionnelles de l'alpha-synucléine dans les CL de patients atteints de MP ou de maladie à corps de Lewy diffus ont été étudiées par Anderson et collaborateurs. Par des approches d'électrophorèses bidimensionnelles, à l'aide d'ELISA dédiés à la détection de formes modifiées de l'alpha-synucléine ou encore par spectrométrie de masse, ils montrent que la modification prédominante de l'alpha-synucléine dans les CL réside dans la phosphorylation sur la sérine 129. En outre, il met en évidence un ensemble de modifications caractéristiques qui sont présentes dans une moindre mesure, comprenant l'ubiquitinylation sur les résidus lysine 12, 21 et 23 et des troncations spécifiques intervenant sur les acides aminés suivants : Asp-115, Asp-119, Asn-122, Tyr-133 et 135-Asp. Aucune autre modification post-traductionnelle n'est détectable par spectrométrie de masse, à l'exception d'une acétylation amino-terminale omniprésente. De petites quantités d'alpha-synucléine phosphorylée sur sérine 129 et

tronquée sur Asp-119 sont également présentes dans la fraction soluble du cerveau normal et pathologique. Ces données suggèrent que ces formes associées aux CL sont produites au cours du métabolisme normal de l'alpha-synucléine. Toutefois, l'ubiquitinylation n'est présente que dans les CL, principalement sur l'alpha-synucléine phosphorylée. Le caractère invariant des modifications post-traductionnelles (phosphorylation, troncation, ubiquitinylation), entre les formes sporadiques, génétiques de la MP ou encore chez les patients AMS, suggère un processus pathologique commun, où la phosphorylation sur la sérine 129 reste l'événement clef [110].

2.2 Mutations, duplications et triplications de l'alpha-synucléine

Depuis la découverte de la mutation A53T sur le gène SCNA en 1997, deux autres mutations A30P [172] et E46K [173] ont été décrites chez l'homme. De façon étonnante toutes les mutations humaines de l'alpha-synucléine sont situées dans la partie N-terminale de la protéine. La mutation A30P a été découverte chez une famille allemande. Des études en par émission de positons (PET-scan) révèlent une atteinte des terminaisons synaptiques du striatum identiques aux formes sporadiques et la MP associée à cette mutation reste indiscernable d'une MP classique. Cependant des études *in vitro* démontrent que la protéine mutante à une vitesse de fibrillation moins importante que la forme sauvage ou A53T, favorisant la production d'oligomères solubles [31, 36] et possède une capacité de liaison aux vésicules plus faible que la forme sauvage [29]. La mutation E46K a été découverte en 2004 chez une famille espagnole. La mutation est à la fois responsable de formes classiques de MP mais également de démences à corps de Lewy. Tout comme la mutation A53T, la forme E46K présente une vitesse de fibrillation très rapide et possède une capacité de liaison aux vésicules supérieure à la forme sauvage [29].

A l'heure actuelle, 4 familles avec une transmission autosomale dominante de MP et une triplications du gène SNCA ont été décrites [174-177]. Elles ont en commun des caractéristiques cliniques proches des MP sporadiques mais un début de maladie plus précoce aux alentours de 30 ans et une évolution vers la démence et la dysautonomie rapide en moyenne en 6 à 8 ans [174, 176-179]. D'un point de vue anatomopathologique, les cas autopsiés sont superposables aux MP sporadiques. Il est à

noter toutefois que l'expression de l'ARNm de l'alpha-synucléine ainsi que de la protéine sont augmentées de 2 à 3 fois dans les neurones des patients avec triplication par rapport aux patients atteints de MP sporadiques. Parallèlement les concentrations d'alpha-synucléine dans le plasma et le LCR sont également augmentées dans les mêmes proportions chez les patients portant la triplication [180].

Les duplications du gène SNCA sont également responsables de formes familiales de MP. Elles ont une sévérité moindre que les triplications, avec un âge d'apparition des premiers symptômes plus tardif, vers la cinquantaine [181, 182] et une évolution plus lente vers la démence. Toutefois, il existe une hétérogénéité dans l'expression clinique de la MP par duplication de l'alpha-synucléine, certains présentant une démence plus précocement que d'autres. Des porteurs asymptomatiques de la duplication ont été décrits, la pénétrance de la mutation étant estimée à 43,8% [183]. Ces données sur la duplication et la triplication du gène SNCA montrent qu'une simple surexpression de l'alpha-synucléine chez l'homme est suffisante au développement de la MP. Il semblerait que l'évolution est d'autant plus rapide que la quantité d'alpha-synucléine est exprimée est grande, et résulterait d'un phénomène de dosage génique [175].

2.3 Polymorphismes et promoteur du gène SNCA

S'il est vrai qu'à elles seules les multiplications du gène SNCA offrent un début d'explication séduisant sur le rôle clef de l'expression de l'alpha-synucléine, elles restent limitées à un très petit nombre de cas [184, 185]. La question soulevée par ces observations est naturellement de savoir si une augmentation de l'expression de l'alpha-synucléine est retrouvée dans les formes sporadiques permettant ainsi de corréler plus largement MP et expression de l'alpha-synucléine.

Les *genome-wide association studies* (GWAS) [186, 187] ont mis en évidence un variant sur un locus du gène SNCA associé aux formes sporadiques de la MP [188]. Des polymorphismes d'une séquence microsatellite Rep1, située dans le promoteur de l'alpha-synucléine sont également liés à des formes sporadiques de MP [188-194]. Suite à la découverte des polymorphismes de Rep1, une étude basée sur un essai reporteur luciférase dans des SH-SY5Y, a montré qu'un variant de Rep1 décrit comme un polymorphisme de MP, est responsable d'une augmentation de 3 fois de l'expression de l'ARNm de l'alpha-synucléine [195]. Bien que certaines études avec un faible nombre de patients, ne trouvent aucun lien entre Rep1 et MP [196-198], une étude à plus large

échelle démontre une réelle association entre Rep1 et MP [194] sans que la présence de ce polymorphisme de Rep1 ne modifie l'âge de début de la maladie.

Les études d'association cas/contrôles démontrent également que certains single nucleotides polymorphisms (SNP) sont positivement corrélés avec la MP ou situés à proximité du gène de l'alpha-synucléine et plus particulièrement dans la partie 3' [199-206]. Certaines de ces études associent même certains SNP avec une augmentation de l'expression de l'alpha-synucléine [202-204].

2.4 Expression de l'alpha-synucléine dans le système nerveux central des patients parkinsoniens

Les travaux qui ont étudié l'expression du messager de l'alpha-synucléine dans le tronc cérébral et dans la substance noire pars compacta des patients parkinsoniens ont donné des résultats contradictoires. Un premier groupe d'études montre une augmentation de l'expression des ARNm dans des lysats de substance noire pars compacta ou le tronc cérébral chez les patients parkinsoniens par rapport aux patients contrôles, mais ne trouve aucune différence dans le cortex de ces mêmes patients [207-209]. Le second groupe au contraire décrit plutôt une baisse de l'ARNm de l'alpha-synucléine [210-213] dans ces mêmes régions voire aucune altération [214].

Toutefois, s'il n'y avait qu'une étude à retenir, celle de Grundemann en 2008 nous semble la plus pertinente. En effet, l'ensemble des travaux précédents a été réalisé sur des lysats de substance noire totale ou de mésencéphale. Ces études basées sur l'utilisation de tissus humains ne peuvent pas comparer directement l'expression de l'alpha-synucléine dans les neurones de la substance noire pars compacta mais seulement des quantités moyennes d'alpha-synucléine provenant à la fois de cellules neuronales et de cellules non neuronales, rendant l'étude sur les tissus encore plus problématique. De plus le nombre relatif de neurones dopaminergiques varie largement dans la substance noire pars compacta ou le mésencéphale des patients parkinsoniens ou des patients contrôles, rendant les études sur les tissus encore plus délicates. Le dernier argument crucial qui entre également en jeu est la variation de la qualité et de l'intégrité de l'ARNm dans les échantillons de cerveaux humains, dépendante d'un grand nombre de facteurs. En effet le degré d'ischémie cérébrale, le degré d'évolution de la maladie, les délais *post-mortem,* le pH des tissus ou encore la méthode de conservation des échantillons participent grandement à la qualité de l'analyse. C'est pourquoi Grundemann et al. ont utilisé la microdissection laser des neurones dopaminergiques de

la substance noire pars compacta afin d'analyser les messagers de l'alpha-synucléine neurone par neurone. Leurs résultats montrent que l'expression des messagers de l'alpha-synucléine est bien augmentée dans les neurones de la substance noire des patients parkinsoniens [215].

2.5 Alpha-synucléine et propagation de la MP

Deux principaux arguments, qui sont apparus de façon concomitante suggèrent que l'alpha-synucléine pourrait être à l'origine de la propagation du processus pathologique au cours de la MP. Tout d'abord, nous l'avons vu, l'alpha-synucléine peut être sécrétée et dans certaines conditions recaptée par les neurones voisins. Ensuite, plusieurs études *post-mortem* ont récemment décrit la présence de CL dans les neurones fœtaux, 10 à 16 ans après leur greffe dans le striatum des patients parkinsoniens [216-219]. La présence de CL dans des neurones initialement indemnes de toute pathologie et d'un fond génétique différent de celui du patient, suggère que l'alpha-synucléine pourrait transmettre le processus pathologique par voie transcellulaire, comme cela a déjà été décrit pour le prion [220].

Pour confirmer cette hypothèse de transmission *in vivo,* Brundin et collaborateurs ont greffé dans le striatum de souris sauvages des neurones dopaminergiques surexprimant l'alpha synucléine humaine et ont montré la transmission d'alpha-synucléine humaine dans les neurones de la souris hôte [158]. Peu après Kordower et collaborateurs ont confirmé les premiers résultats de Brundin chez le rat avec une méthode différente [221]. Kordower a greffé des neurones de mésencéphale ventral après avoir détruit les neurones dopaminergiques par le 6-OHDA. Un mois après la greffe, en utilisant une approche lentivirale, il transduit de l'alpha-synucléine fluorescente dans le cerveau des rats à une distance suffisamment éloignée pour ne pas contaminer la greffe de neurones mésencéphaliques. 5 semaines après, de l'alpha-synucléine fluorescente est retrouvée dans les neurones greffés, confirmant le transfert in vivo d'alpha-synucléine [221]. Cependant, bien que l'ensemble des ces résultats suggère que l'alpha-synucléine peut se propager de neurones à neurones *in vivo*, aucune donnée à l'heure actuelle n'a mis en évidence que l'alpha-synucléine produite par l'hôte est capable de se propager et de se nucléer dans les cellules voisines.

3. Système nerveux entérique et MP

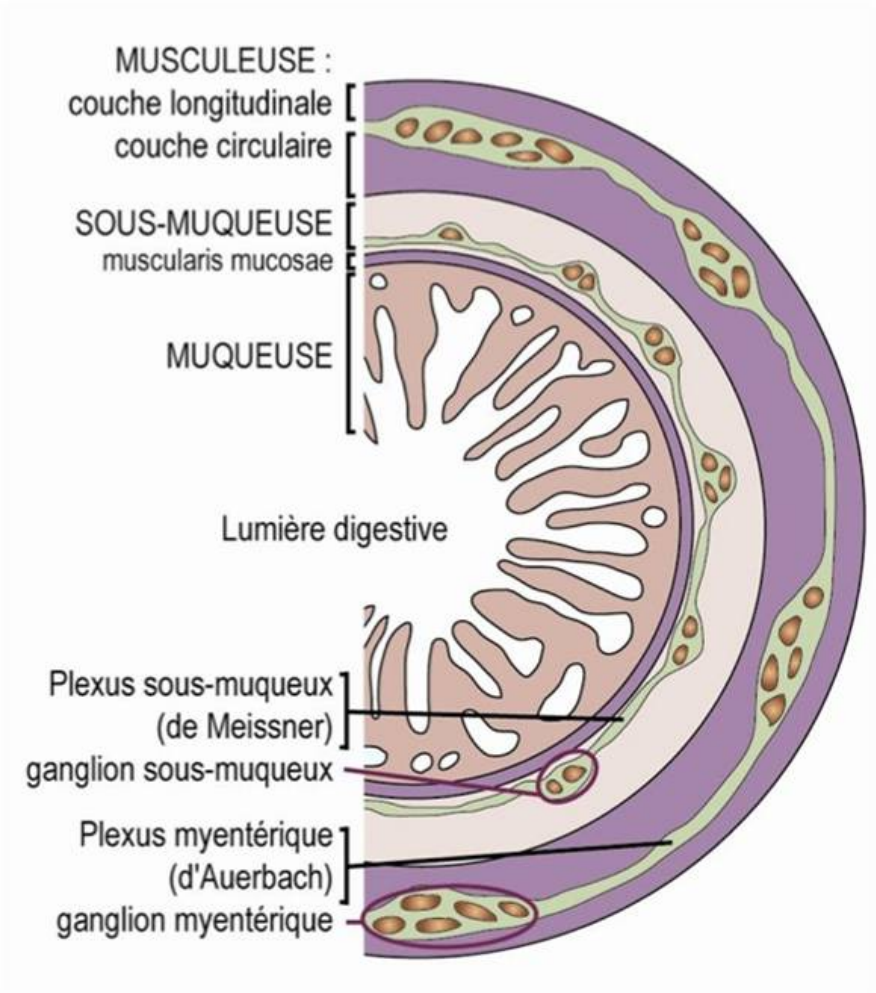
La MP est classiquement considérée comme une maladie dégénérative des neurones dopaminergiques de la substance noire, cette perte étant responsable de la bradykinésie et de l'hypertonie. Plusieurs travaux récents ont toutefois montré que les neurones de la substance noire ne sont pas les seuls ni les premiers touchés par le processus neurodégénératif dans la MP [222]. D'autres régions du système nerveux central et du système nerveux périphérique sont en effet touchées précocement par la pathologie de Lewy. C'est en particulier le cas du système nerveux entérique (SNE) qui est atteint chez la quasi-totalité des patients parkinsoniens [222]. Avant de détailler l'atteinte du SNE au cours de la MP, nous proposons un bref rappel sur cette structure parfois appelée second cerveau en raison de ses similitudes avec le SNC

3.1 Anatomie du SNE

Le postulat que l'intestin est un second cerveau émerge au début du XX^{ème} siècle, depuis qu'il a été montré que le SNE était capable de contrôler la motricité intestinale et la sécrétion de mucus en dehors de toute innervation centrale. [223, 224]. Le SNE est un réseau neuronal intégré de 80 à 100 millions de neurones soit autant que la moelle épinière, organisé en deux plexus, le plexus sous muqueux et le plexus myentérique. Les plexus sont organisés en ganglions et chaque ganglion est composé par des neurones entériques et des cellules gliales entérique (CGE). Les neurones du plexus myentérique (PM) contrôlent l'activité des muscles lisses de l'intestin et par conséquent la motricité digestive alors que les neurones du plexus sous muqueux (PSM) contrôlent la sécrétion du mucus et le flux sanguin[225].

Le SNE contrôle la motricité digestive et la sécrétion par des réflexes qui sont amorcés par la distension de la paroi intestinale, la déformation de la muqueuse ou le contenu chimique de la lumière [226]. Ces réflexes impliquent des circuits neuronaux du SNE qui sont interconnectés. Cette régulation neuronale des fonctions digestives consiste en la libération de neuromédiateurs synthétisés par des neurones fonctionnellement bien définis. Par exemple, parmi les neurotransmetteurs les plus communs dans le SNE, l'on retrouve le peptide vasoactif (VIP) ou le monoxyde d'azote qui sont souvent retrouvés dans les motoneurones inhibiteurs ou à l'inverse de l'acétylcholine (ACh) ou la substance P (SP) que l'on retrouve dans les motoneurones

excitateurs [225]. Il existe aussi une petite population de neurones dopaminergiques dans le SNE [227, 228].



D'après Lebouvier Movements 2008

Figure 9 Situation du système nerveux entérique :
Coupe transversale de tube digestif avec visualisation du système nerveux entérique (SNE). Le plexus myentérique (Auerbach) contrôle essentiellement la motilité ; le plexus sous-muqueux (Meissner) est principalement impliqué dans la régulation de la sécrétion et de la microcirculation de l'épithélium.

Chez l'homme une étude précise de la distribution des neurones dopaminergiques a montré que ces neurones sont répartis selon un gradient oral aboral. Les neurones dopaminergiques sont abondant dans les deux plexus, dans la partie haute du tube digestif et représentent environ de 14 à 20 % des neurones entériques totaux, alors que leur population diminue dans le petit et le gros intestin pour atteindre la proportion de 1-6% des neurones totaux [228]. Il est suggéré que ces neurones

dopaminergiques entériques exercent un effet inhibiteur sur la mobilité intestinale [229] .

Les cellules les plus abondantes dans le SNE sont les CGE (environ 4 CGE pour 1 neurone) qui « enveloppent » les neurones dans les ganglions entériques [230]. Les CGE sont les équivalents des astrocytes du SNC [231-233]. Tout comme les astrocytes, les CGE ne sont pas de simples cellules de soutien des neurones entériques mais participent aux fonctions digestives comme la régulation de la motricité ou la barrière épithéliale intestinale (BEI) [234-236].

3.2 Le système nerveux entérique est connecté au système nerveux central

Bien que le SNE puisse fonctionner de façon autonome, il est connecté au SNC à la fois par des afférences et des efférences des systèmes ortho- et parasympathiques.

Les neurones primaires afférents qui transportent les informations au SNC sont localisés dans le nerf vague et le nerf sympathique (splanchnique). Les neurones primaires afférents parasympathiques situés dans la couche de muscles lisses sont sensibles à la distension mécanique de l'intestin alors que les neurones primaires afférents situés dans la muqueuse sont sensibles aux concentrations luminales de glucose, d'acides aminés ou d'acides gras à chaine longue [237]. Ces neurones dont le corps cellulaire est situé dans le ganglion du nerf vague projettent jusqu'au noyau du tractus solitaire et sont à l'origine de plusieurs réflexes vago-vagaux, affectant la déglutition, la motricité de l'intestin ou encore la sécrétion. Les neurones afférents splanchniques ont leurs terminaisons dans la paroi du tube digestif et leur corps cellulaires dans le noyau dorsal du vague. Ces neurones afférents sont principalement des nocicepteurs et sont impliqués dans la détection de la douleur dans le tractus digestif [238].

Les voies efférentes motrices parasympathiques sont constituées par le nerf vague qui contrôle les fonctions motrices et sécrétomotrices du tractus digestif supérieur et le nerf sacré qui contrôle les fonctions du colon distal et du rectum [239]. L'innervation vagale supérieure à pour origine deux noyaux situés dans la moelle, qui sont le noyau moteur dorsal du vague et le noyau ambigu [240]. Le noyau ambigu contient des neurones somatomoteurs non autonomes qui innervent les muscles striés du pharynx, du larynx et de l'œsophage. Le noyau dorsal du vague contient des neurones

préganglionnaires viscéromoteurs qui innervent de façon importante le plexus myentérique et sous muqueux du SNE [240, 241]. Toutes les afférences vagales utilisent l'ACh comme neurotransmetteur.

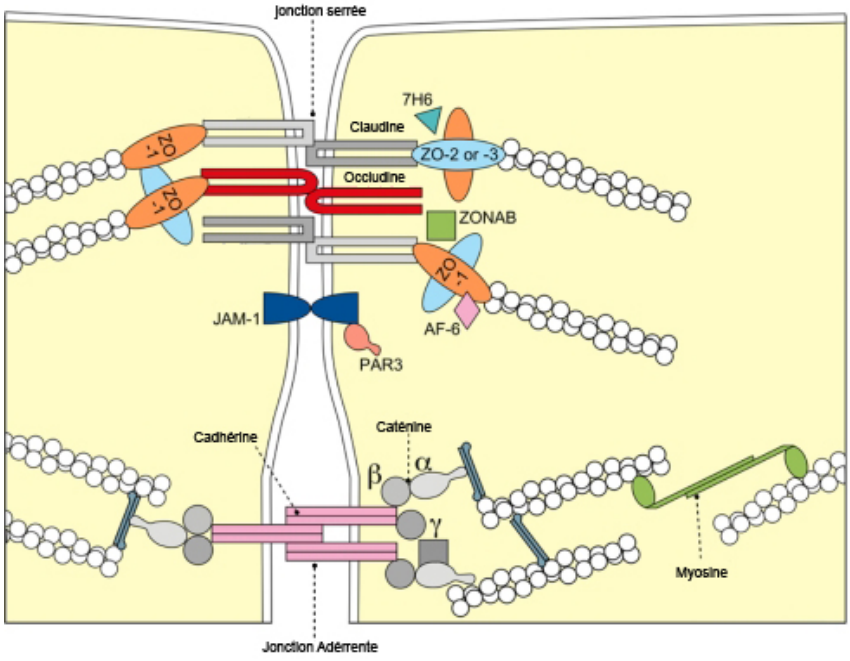
3.3 SNE et barrière épithéliale intestinale

La barrière épithéliale intestinale (BEI) est l'interface entre le milieu extérieur et l'organisme. Elle est composée d'une couche monocellulaire de cellules épithéliales qui assurent la majorité des fonctions de barrière (absorption, barrière, sécrétion de mucus, etc.). Selon la taille et la nature des composés qui doivent passer la barrière, plusieurs voies de passage sont possibles. Les petits composés hydrophiles et les composés lipophiles peuvent passer directement au travers des membranes des cellules épithéliales. Les petits composés hydrophiles peuvent également passer en empruntant des pores membranaires aqueux, alors que les gros composés hydrophiles passent de façon paracellulaire. Certaines molécules, essentielles pour l'organisme, comme les nutriments possèdent sur la membrane des cellules épithéliales des récepteurs dédiés, qui permettent leur absorption. Les très grosses molécules comme les peptides ou les protéines sont endocytées dans la lumière puis exocytées dans la circulation générale (pour revue [242]).

La régulation de la BEI fait intervenir deux processus distincts dans les cellules épithéliales intestinales. Le premier, qui implique la phosphorylation des chaines légères de myosine (*MLC pour myosin light chain*) provoque une régulation rapide de la perméabilité intestinale, alors que le second basé sur régulation l'expression des protéines des jonction serrées (*tight junctions*), met en jeu un régulation à plus long terme. Les *tight junctions* sont des complexes multimoléculaires qui permettent de créer de véritables liaisons physiques entre deux cellules épithéliales (figure 10). Au niveau cytoplasmique, les filaments d'actine ancrés par l'intermédiaire des protéines adaptatrices *zonula occludens* 1, 2 et 3 (ZO-1, ZO-2 ou ZO-3) à des protéines transmembranaires telles les claudines ou les occludines, assurant une véritable étanchéité intercellulaire. Leur expression conditionne la perméabilité de la BEI (pour revue [242]).

La perméabilité de la BEI peut être modulée par différents mécanismes. Principal acteur, le SNE est capable de réguler finement la BEI par la participation de ses différents contingents cellulaires. Les faisceaux des neurones du plexus sous-muqueux

forment un réseau avec la *lamina propria* des cryptes et des villosités et les terminaisons axonales sont en contact étroit avec lame basale des cellules épithéliales. Les substances alors libérées, dont notamment le VIP par les neurones entériques sont alors à proximité des cellules épithéliales [243]. En retour cette proximité permet au SNE de capter les molécules provenant de la barrière et d'adapter son comportement en fonction de celles-ci. Les CGE participent également à la régulation de la BEI en modulant l'expression de certains neuromédiateurs des neurones entériques comme la substance P ou le VIP [244]. Certains facteurs solubles peuvent également modifier la perméabilité de la barrière tels que les cytokines proinflammatoires telles que le tumor necrosis factor α (TNF-α) [245-250] ou les interleukines 1β [251-254] 4 et 13 [255-257], la tryptase [258-260], ou encore certaines molécules comme les prostaglandines (15d-prostaglandine J2 par exemple) [261, 262] ou le NGF [263, 264].



D'après Förster et al ; Histochemistry and Cellular Biology 2008

Figure 10 Composition moléculaire des jonctions serrées

Les protéines transmembranaires (occludine et claudine) et les protéines JAM-1 (*Junctional Adhesion Molecule 1*) constituent les jonctions serrées qui rendent imperméable l'espace intercellulaire. Les jonctions serrées sont reliées au cytosquelette d'actine par des protéines adaptatrices telles que les protéines Zonula occludens (ZO).

Les modèles cellulaires de cellules épithéliales intestinales permettent d'étudier à la fois la régulation de la perméabilité de la BEI, l'expression de ses composants et leur localisation. Il existe de nombreuses lignées cellulaires permettant d'étudier la BEI, notamment les lignées Caco-2 [265], HT-29 [266] ou CRL-2102 [265], qui sont issues d'adénocarcinomes humains. L'étude de la perméabilité de la BEI peut se faire aussi bien *ex-vivo* et *in-vivo.* Les chambres d'Ussing permettent d'étudier la perméabilité *ex-vivo* en mesurant la quantité de fluorescence qui passe d'une chambre à l'autre à travers le tissu [267]. Cette technique s'avère très utile pour mesurer de façon locale la perméabilité de la BEI chez l'homme et les animaux [268-270]. Enfin l'utilisation de molécules de différentes tailles (dextran, acide sulfonique, peroxydase de raifort) couplées à une molécule fluorescente telles que le FITC (fluorescein isothiocyanate) permet d'apprécier les différentes types de perméabilité de façon sensible (paracellulaire, transcellulaire, endocytose) [270-274]. De façon plus globale, la perméabilité de la BEI peut être mesurer *in vivo* chez l'homme ou l'animal en utilisant les mêmes molécules décrites ci-dessus. Les sucres non métabolisables sont dosés dans les urines [275], alors que les molécules couplées à la fluorescéines (acide sulfonique, dextran ou peroxydase de raifort) sont dosées dans le sérum [276].

3.4 Atteinte du SNE et de la BEI dans la MP

Les premières lésions du SNE dans la MP ont été décrites dans les années 80 et 90. Des inclusions semblables aux corps et prolongements de Lewy sont mises en évidence dans le plexus myentérique et à un moindre degré dans le plexus sous-muqueux du côlon, du tiers inférieur de l'œsophage et de l'estomac. Dans leur ensemble, ces premières publications sur le SNE dans la maladie de Parkinson ne rencontrent qu'un écho modeste (revue dans [6]).

Ce sont les travaux fondateurs de Heiko Braak en 2002 et 2006 qui ont permis au SNE de faire un retour en force sur la scène parkinsonienne [277, 278]. La vaste étude neuropathologique menée par cet anatomiste allemand a permis de mettre en évidence une progression temporo-spatiale ascendante des corps de Lewy dans le tronc cérébral et le prosencéphale, classée en 6 stades [277]. De façon frappante, les premières lésions encéphaliques de la MP apparaissent dans le bulbe olfactif et surtout dans le noyau dorsal du vague, bien avant l'atteinte de la substance noire. Le nerf vague innervant la

quasi-totalité du tractus digestif, l'hypothèse de l'atteinte inaugurale du SNE dans la maladie de Parkinson voit le jour [278]. Depuis, plusieurs travaux autopsiques de grande envergure ont été menés afin de mieux caractériser l'atteinte du SNE au cours de la MP. Nous retiendrons tout particulièrement l'étude de *l'Arizona Consortium* qui a permis de montrer que les lésions du SNE sont présentes chez la quasi-totalité des patients parkinsoniens (21 sujets sur 23) [222]. Des expériences complémentaires sur ces mêmes prélèvements autopsiques ont montré qu'il n'y a pas de perte neuronale significative dans les plexus myentériques et sous-muqueux au cours de la MP en particulier des neurones dopaminergiques et que la pathologie de Lewy n'affecte pas préférentiellement un sous-type de neurone entérique [9].

Remarquablement la BEI, une autre fonction clef du tube digestif semble être touchée par la MP. Selon l'hypothèse de Braak, le rôle de la BEI dans la mise en place du processus pathologique pourrait être critique puisqu'elle assure une véritable frontière entre l'environnement et l'organisme. En effet si le fonctionnement de la BEI est perturbé, un pathogène pourrait pénétrer dans l'organisme, se transloquer et induire la production d'un stress oxydant favorable à l'agrégation de l'alpha-synucléine. Cependant au cours de ces 15 dernières années uniquement 3 études ont tenté de caractériser l'état de la BEI chez les patients parkinsoniens avec des résultats qui restent encore confus. Dans une première étude parue en 1996, basée sur 15 patients parkinsoniens et 15 patients contrôles, Davies démontre que les patients parkinsoniens ont un ratio lactulose/mannitol dans les urines supérieur au patients contrôles, traduisant une diminution de la perméabilité intestinale, mais une perméabilité au sucrose équivalente (perméabilité de la partie haute du tube digestif) [279]. En 2011 Forsyth sur un groupe de 9 patients parkinsoniens ne confirme pas les résultats de Davies puisqu'il montre que le rapport lactulose/mannitol dans les urines n'est pas modifié mais que la quantité de sucralose (perméabilité totale) est plus élevée chez les parkinsoniens que les contrôles. En parallèle il met en évidence que cette augmentation de perméabilité au sucralose est accompagnée par une augmentation de la charge bactérienne en E. Coli, à l'expression de l'alpha-synucléine dans la muqueuse et à l'augmentation du stress oxydant [275]. La dernière étude réalisée en 2012 par Salat-Foix, ne permet pas non plus de trancher clairement, puisqu'elle démontre une altération de la perméabilité de la BEI chez 25 à 30 % des patients seulement (4/12)

avec 3 patients sur 12 atteints d'une augmentation de la perméabilité au mannitol et 1 patient sur 12 atteint d'une augmentation de perméabilité au sucralose [280].

3.5 Conséquences de l'atteinte du SNE dans la MP

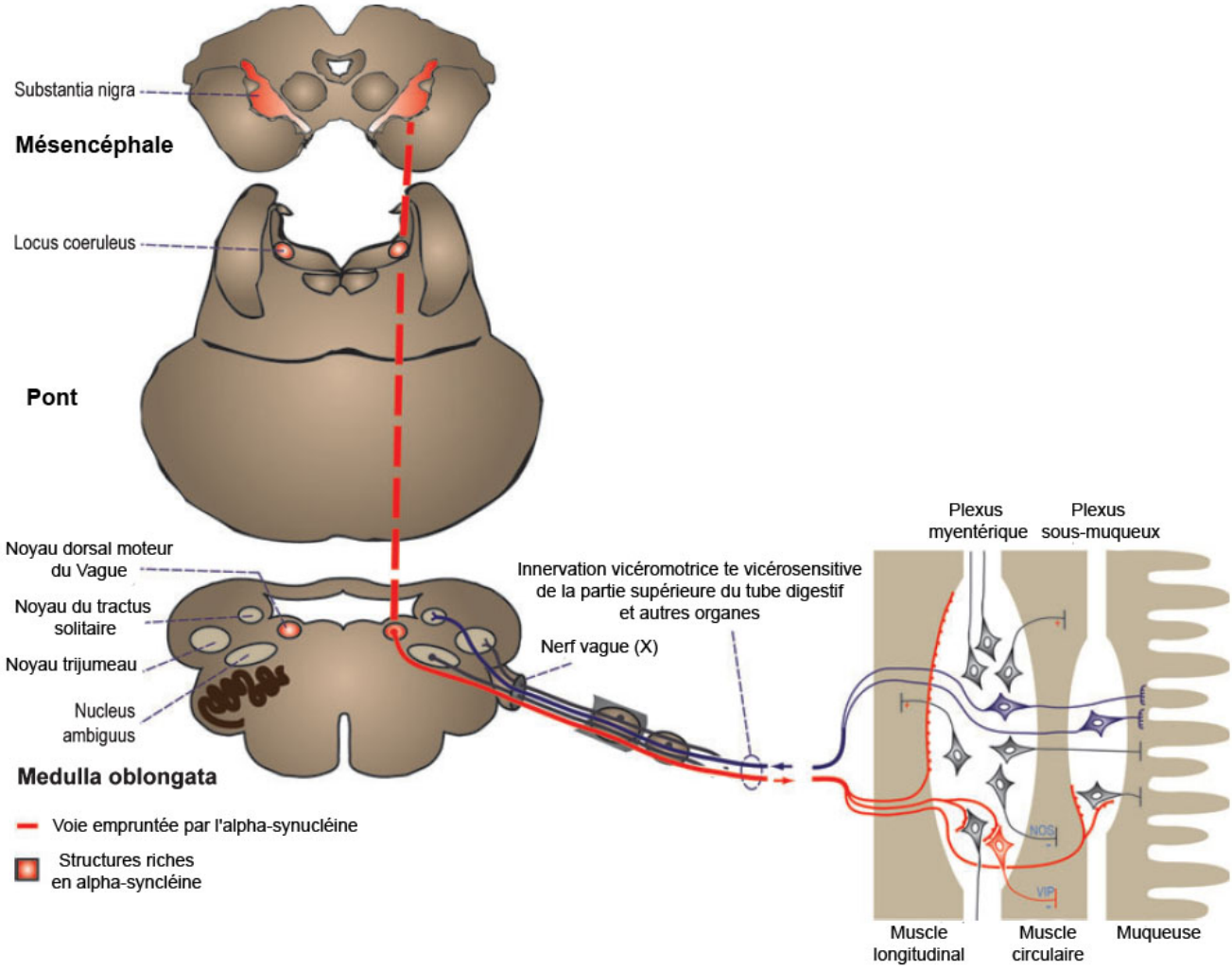
L'atteinte du SNE chez les patients parkinsoniens peut avoir deux conséquences principales : les lésions du SNE seraient à l'origine des troubles digestifs fréquents au cours de la MP et seraient impliqués dans la diffusion et donc dans la physiopathologie de la maladie (Hypothèse de Braak) [281].

Les signes digestifs sont les manifestations non-motrices de la maladie les plus fréquentes, pouvant impliquer l'ensemble du tractus digestif. Les troubles de la vidange gastrique, à l'origine de nausées, de ballonnement postprandial et d'une réduction des prises alimentaires, touchent plus d'un tiers des patients. La constipation est très commune dans la MP. Sa prévalence a été récemment mesurée à 59% en utilisant les critères de définition internationaux de Rome III, soit presque trois fois plus que chez les sujets contrôles d'âge équivalent [281]. Enfin la dysfonction ano-rectale (dyschésie), difficile à distinguer de la constipation fonctionnelle sur le seul interrogatoire, est également très fréquente chez les parkinsoniens. Il est tentant de proposer que ces troubles de la motilité digestive soient la conséquence des lésions du SNE. Toutefois, ceci n'est pas prouvé et il est tout à fait possible qu'ils résultent de l'atteinte parasympathique et en particulier de l'atteinte du noyau dorsal moteur du vague [281, 282].

Le bulbe olfactif et le noyau dorsal du vague sont des structures en contact indirect avec le milieu extérieur, par l'intermédiaire des nerfs olfactifs et du nerf vague, C'est pourquoi Braak suggère l'implication d'un toxique environnemental neurotrope, inhalé ou ingéré, qui traverserait la muqueuse nasale et la BEI et qui serait à l'origine de la MP. Le noyau dorsal du nerf vague, qui innerve la majeure partie du tube digestif, serait atteint par transport rétrograde, un scénario semblable à celui proposé pour le nouveau variant du prion [281, 282]. Les travaux de Phillips et collaborateurs ont montré que les neurones de la voie efférente du nerf vague, qui trouvent leur origine dans le noyau dorsal moteur du vague, expriment tous l'alpha-synucléine et font synapse avec des neurones des plexus myentérique et sous-muqueux qui expriment eux aussi l'alpha-synucléine.

Ainsi le SNE, serait le premier maillon d'une chaîne d'évènements dégénératifs menant à la substance noire et l'alpha-synucléine pourrait être responsable de la transmission du processus pathologique de neurone à neurone. Cette hypothèse de Braak, bien que séduisante repose néanmoins encore sur des fondations fragiles et incertaines. Trois objections peuvent être opposées : (i) L'atteinte du SNE et du nerf vague n'est qu'un cas particulier de l'atteinte du système nerveux végétatif. Centré sur le nerf vague, parasympathique, le modèle de Braak est battu en brèche par l'atteinte diffuse du système végétatif dans la maladie de Parkinson. Une dénervation sympathique associée à des inclusions d'alpha-synucléine a été décrite au niveau du cœur, de la peau, ou du SNE lui-même qui reçoit une double innervation parasympathique et sympathique [283] (ii) La classification de Braak repose sur le postulat du caractère pathogène obligatoire des corps de Lewy et non sur la présence d'une perte neuronale (iii). L'hypothèse d'une origine entérique de la maladie de Parkinson repose largement sur la précocité de l'atteinte du noyau dorsal du vague à l'étage encéphalique (figure 11).

Kurt Jellinger, neuropathologiste viennois, a récemment éprouvé cette classification en analysant 71 cas de maladie de Parkinson issus d'une banque de cerveaux londonienne. Dans cette série, seuls 53% des cas sont compatibles avec le modèle de progression caudo-rostral décrit par Braak [284].



D'après Lebouvier et al ; The European Journal of Neuroscience 2009

<u>Figure 11 : L'hypothèse de Braak et l'alpha-synucléine :</u>
Une forte expression de l'alpha-synucléine semble prédisposer les structures neuronales au processus dégénératif de la maladie de Parkinson. Au sein du tronc cérébral, le noyau moteur dorsal du nerf vague, le locus coeruleus et la substance noire (colorés en rouge)sont des structures très riches en alpha-synucléine. De façon remarquable les efférentes du nerf vague (rouge) sont les seules structures à dégénérer dans la MP et expriment des niveaux bien supérieurs d'alpha-synucléine que les afférences vagales (bleu). Finalement, des données préliminaires montrent que l'expression de l'alpha-synucléine est hétérogène dans les neurones entériques. Bien que le phénotype des neurones entériques reste à déterminer, il est tentant de spéculer que ces neurones riches en alpha-synucléine sont les plus sensibles à former des inclusions. Dès lors une potentielle voie rétrograde et ascendante suivant les structures riches en alpha-synucléine peut être tracée à partir du SNE jusqu'au SNC.

4. Objectifs du travail de thèse

Etant donné le rôle crucial de l'expression et de la sécrétion de l'alpha-synucléine d'une part et l'atteinte précoce du SNE dans la maladie de Parkinson d'autre part, ce travail de thèse se propose d'étudier les mécanismes de régulation de l'expression et de la sécrétion de l'alpha-synucléine dans le système nerveux entérique.

Dans une première étude, nous avons étudié l'expression de l'alpha-synucléine dans les différentes populations cellulaires du SNE à la fois *in vitro* dans des cultures primaires de SNE et *in vivo* dans des plexus myentériques de souris. Par ailleurs nous nous sommes intéressés à étudier la régulation de l'expression de l'alpha-synucléine par la dépolarisation et l'AMP cyclique, deux facteurs impliqués dans l'activité neuronale.

L'alpha-synucléine est une protéine fortement associée aux vésicules de sécrétion et contribue au contrôle de la libération de certains neuromédiateurs, dont la dopamine, données suggérant que sa sécrétion peut être liée à l'activité neuronale. Dans une seconde étude nous avons étudié si le SNE et notamment les neurones entériques étaient capables de sécréter l'alpha-synucléine et caractérisé les mécanismes associés à cette sécrétion. En utilisant une approche pharmacologique, nous avons entrepris d'étudier si l'activité neuronale était capable de réguler la sécrétion de l'alpha-synucléine dans le SNE.

Enfin nous avons voulu caractériser le rôle de l'alpha-synucléine extracellulaire sur les fonctions digestives, et plus particulièrement sur la perméabilité de la barrière épithéliale intestinale.

Résultats

Article 1 : L'expression de l'alpha-synucléine est induite par la dépolarisation et l'AMP cyclique dans les neurones entériques

Dans cette étude publiée dans la revue « Journal of Neurochemistry » en 2010, nous avons mis en évidence que l'expression de l'alpha-synucléine était restreinte aux neurones dans le SNE. Par ailleurs nous avons identifié une nouvelle voie de régulation de l'alpha-synucléine, activée la dépolarisation et l'augmentation intracellulaire de l'AMP cyclique, contribuant à une augmentation de l'expression de la protéine et à une surexpression de son ARNm.

A l'aide d'une approche pharmacologique nous avons mis en évidence le rôle crucial des canaux calciques voltage dépendants de type L, capables de recruter les voies de signalisations Ras/Extracellular regulated Kinase 1 & 2 responsables de la surexpression de l'alpha-synucléine.

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α-Synuclein expression is induced by depolarization and cyclic AMP in enteric neurons

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Abstract

Accumulated evidence emphasizes the importance of α-synuclein expression levels in Parkinson's disease (PD) pathogenesis. PD is a multicentric disorder that affects the enteric nervous system (ENS), whose involvement may herald the degenerative process in the CNS. We therefore undertook the present study to investigate the mechanisms involved in the regulation of expression of α-synuclein in the ENS. The regulation of α-synuclein expression was assessed by qPCR and western blot analysis in rat primary culture of ENS treated with KCl and forskolin. A pharmacological approach was used to decipher the signaling pathways involved. Intraperitoneal injections of Bay K-8644 and forskolin were performed in mice, whose proximal colons were further analyzed for α-synuclein expression. Depolarization and forskolin increased α-synuclein mRNA and protein expression in primary cultures of ENS, although L-type calcium channel and protein kinase A, respectively. Both stimuli increased α-synuclein expression through a Ras/extracellular signal-regulated kinases pathway. An increase in α-synuclein expression was also observed *in vivo* in the ENS of mice injected with Bay K-8644 or forskolin. In conclusion, we have identified stimuli leading to α-synuclein over-expression in the ENS, which could be critical in the initiation of the pathological process in PD.

Keywords: α-synuclein, cyclic AMP, depolarization, enteric nervous system, extracellular signal-regulated kinases, Parkinson's disease.

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α-Synuclein is a neuronal protein that has been linked both to normal synaptic function and to neurodegeneration. Missense mutations of α-synuclein are responsible for rare autosomal dominant forms of Parkinson's disease (PD) (see Waxman and Giasson 2009 for review) and aggregated α-synuclein has been shown to be the main component of the pathological hallmark of sporadic PD, namely Lewy bodies (Spillantini *et al.* 1997). There is a large body of evidence implicating the expression level of α-synuclein in the pathogenesis of PD. Duplications (Chartier-Harlin *et al.* 2004) and triplications (Singleton *et al.* 2003) of the α-synuclein gene have been identified in familial forms of PD. In animal models, over-expression of α-synuclein reproduces some of the cardinal pathological, neurochemical, and behavioral features of the human disease (Chesselet 2008). These studies indicate that over-expression of α-synuclein is sufficient to cause PD, and that its transcriptional regulation may be critically involved in the development of sporadic cases of the disease (Scherzer *et al.* 2008).

The traditional assumption that PD is a primary disorder of the *substantia nigra* has been challenged over the last years. It has indeed become increasingly evident that the

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Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; ENS, enteric nervous system; ERK, extracellular signal-regulated kinases; PBS, phosphate-buffered saline; PD, Parkinson's disease; PKA, protein kinase A; ROS, reactive oxygen species; TBS, Tris-buffered saline.

pathological process of PD affects several neuronal structures outside the *substantia nigra* (Braak *et al.* 2003), among which is the enteric nervous system (ENS) (Braak *et al.* 2006; Lebouvier *et al.* 2008). Remarkably, from analyses of the temporal and spatial patterns of the spread of Lewy aggregates throughout the central and peripheral nervous systems, Braak *et al.* (2006) have determined that the appearance of α-synuclein aggregates occurs in the ENS during the earliest stage of PD, even before the *substantia nigra*. This led Braak to put forth the general proposal that PD pathology may begin in the gastrointestinal tract and that the pathological process further spreads to the CNS via the vagal innervation of the gut (Braak *et al.* 2006). A recent and thorough survey of the expression of α-synuclein in the ENS and in its vagal connections in rats has shown that α-synuclein is expressed in a subset of enteric neurons that are synaptically linked with α-synuclein-positive vagal neurons (Phillips *et al.* 2008). These results, along with the anatomical observations from Braak, offer a mechanism for the development and spread of the Lewy pathology in PD, in which both the ENS and α-synuclein play a crucial role.

Given the importance of the expression levels of α-synuclein for developing PD on one hand and the putative key role of the ENS in the pathophysiology of the disease on the other hand, we undertook the present study to investigate the mechanisms involved in the regulation of expression of α-synuclein in a model of primary culture of ENS (Chevalier *et al.* 2008) and *in vivo*. To this end, we used two distinct stimuli, membrane depolarization and forskolin, because many important physiological and pathological events in the ENS are regulated by neuronal activity and cyclic AMP (Howe *et al.* 2006; Neylon *et al.* 2006; Chevalier *et al.* 2008).

Material and methods

Reagents and antibodies

KCl, forskolin, nifedipine, Bay K-8644 (−) were purchased from Sigma (Saint Quentin Fallavier, France). Omega-conotoxin and omega-agatoxin were purchased from Alomone (Jerusalem, Israel). PD98059, U0126 and FTI-277 were purchased from Calbiochem (Meudon, France). CM-H_2DCFDA was purchased from Invitrogen (Cergy-Pontoise, France). The following commercially available antibodies were used for western blotting: mouse monoclonal anti-α-synuclein (1 : 500; BD Bioscience; Le Pont-De-Claix, France) and rabbit polyclonal anti-α-synuclein (1 : 500; Santa Cruz Biotechnology, Heidelberg, Germany), rabbit polyclonal anti-phospho-extracellular signal-regulated kinases (ERK) (1 : 2000; Cell Signaling; Ozyme, Saint Quentin en Yvelines, France), reacting with active ERK1/2 (doubly phosphorylated on the tyrosine and threonine residues of the activation loop), total anti-ERK1/2 (1 : 1000; Cell Signaling), mouse monoclonal anti-HSP 70 (1 : 1000; Cell Signaling) and mouse monoclonal anti-PGP 9.5 (1 : 1000; Ultraclone limited, Isle of Wight, UK). For immunocytochemistry, mouse monoclonal anti-α-synuclein (1 : 500; BD Bioscience) and rabbit polyclonal anti-α-synuclein (1 : 500; Santa Cruz Biotechnology), mouse monoclonal anti-β III tubulin (1 : 500; Sigma), rabbit polyclonal anti-NF200 (1 : 500; Millipore; Molsheim, France), rabbit polyclonal anti-glial fibrilary acidic protein and rabbit polyclonal anti-S100β (1 : 500; Dako, Trappes, France) were used.

Primary cultures of ENS

Small intestine of rat embryos E15 (35–45 per isolation from three pregnant Sprague–Dawley rats (CERJ, Le Genest St Isle, France) were removed and finely diced in Hank's Buffered Salt Solution (Sigma). Tissue fragments were collected in 5 mL of medium [Dulbecco's modified Eagle's medium (DMEM)-F12 (1 : 1) medium] and digested at 37°C for 15 min in 0.1% trypsin (Sigma). The trypsin reaction was stopped by adding 10 mL of medium containing 10% fetal calf serum and then treated by Dnase I 0.01% (Sigma) for 10 min at 37°C. After triturating with a 10 mL pipette, cells were centrifuged at 500 *g* for 10 min. Cells were counted and then seeded at a density of 2.4×10^5 cells/cm^2 on 24-well plates previously coated for 6 h with a solution of gelatin (0.5%;Sigma) in sterile phosphate-buffered saline (PBS). After 24 h, the medium was replaced with a serum-free medium [DMEM-F12 (1 : 1) containing 1% of N-2 supplement (Life Technologies, Cergy Pontoise, France)]. Cells were maintained in culture for 15 days. Half of the medium was replaced every 2 days (Chevalier *et al.* 2008).

Immunohistochemistry

After the fixation procedure (1 h in 0.1 M PBS containing 4% paraformaldehyde at 25°C), cells seeded on glass slide or tissues were washed in PBS and then permeabilized for 30 min in PBS/NaN_3 containing 1% Triton X-100 and 4% horse serum before being incubated with the primary antibodies diluted in PBS/NaN_3, 4% horse serum, and 1% Triton X-100 for 90 min at 25°C for cells and overnight at 4°C for tissues. When biotinylated α-synuclein antibody was used (mouse monoclonal antibody, BD Biosciences, biotinylated with EZ-Link Sulfo-NHS-LC-Biotinylation Kit from Thermo scientific), endogenous peroxidase activity was blocked by incubating preparations with 3% hydrogen peroxide for 20 min. Endogenous biotin was blocked with a commercial streptavidin/biotin blocking kit (Vector laboratories, Burlingame, CA, USA) according to the manufacturer's instruction. Following incubation with primary antisera, cells or tissue were washed three times with PBS and incubated respectively for 30 and 90 min with secondary antibodies coupled to fluorophores: donkey anti-rabbit, anti-goat or anti-mouse IgG conjugated to carboxymethylindocyanine 3 or 5 (CY3 or CY5) (1 : 500; Jackson Laboratories, Immunotech, Marseille, France), donkey anti-rabbit or anti-mouse IgG conjugated to FluoProbes®488 (1 : 500; Interchim, Montluçon, France) or streptavidin coupled to CY3 (Invitrogen). Nuclei were stained with a 4′,6′-diamidino-2-phenylindole for 15 min (1 : 500; Sigma). After a final wash, samples were laid flat on a microscope slide and mounted in an aqueous fluorescence mounting medium (Dako). Specimens were viewed under a Zeiss Axiovert 200 mol/L microscope fluorescence microscope associated with the APOTOME mode (confocal like) (Carl Zeiss S.A.S., Le Pecq, France) and images were analyzed with AXIOVISION 4.8 software (Carl Zeiss) and further treated with the Image J software (National Institute of Health, Bethesda, MD, USA).

Western blot

Primary culture of ENS were harvested in NETF buffer (100 mM NaCl, 2 mM EGTA, 50 mM Tris–Cl, pH 7.4, and 50 mM NaF) containing 1% (v/v) Nonidet P-40, 2 mM orthovanadate, phosphatase inhibitor cocktail II (Roche, Neuilly sur Seine, France) and a protease inhibitors cocktail (Roche). Tissues were lysed in NETF buffer with 'Precellys 24' tissue homogenizer (Bertin technologies, St Quentin-en-Yvelines, France). Equal amounts of lysate were separated using the Invitrogen NuPage Novex Bis Tris MiniGels™ before electrophoretic transfer with the iBlot™ Dry Blotting System also from Invitrogen. Membranes were blocked for 1 h at 25°C in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH 7.5) with 5% non-fat dry milk. Membranes were incubated overnight at 4°C with the primary antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham, Les Ulis, France; diluted 1 : 5000) and visualized by enhanced chemiluminescent detection (ECL plus, Amersham). When necessary, membranes were stripped for 10 min in Reblot buffer (Millipore, Molsheim, France) followed by extensive washing in TBS before reblocking for 30 min in TBS with 5% non-fat dry milk and reprobing. The relevant immunoreactive bands were quantified with laser-scanning densitometry and analyzed with NIH Image J software. To allow comparison between different autoradiographic films, the density of the bands was expressed as a percentage of the average of controls (untreated). The value of α-synuclein immunoreactivity was normalized to the amount of PGP 9.5 immunoreactivity in the same sample and expressed as a percentage of controls.

Quantitative PCR analysis

RNA extraction from enteric primary culture was performed with RNAeasy Minikit (Qiagen S.A., Courtaboeuf, France) according to the manufacturer's instructions. For reverse transcription, 1 μg of purified total RNA was denatured and subsequently processed for reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations. PCR amplifications were performed using the Absolute Blue SYBR green fluorescein kit (ABGENE, Courtaboeuf, France) according to the manufacturer's protocol and run on MyiQ thermocycler (Bio-Rad, Marnes la coquette, France). The mRNA level of expression was determined using the formula of the comparative cycle theshold: (C_t): Δ^{Ct}, where $\Delta C_t = (C_{t,\alpha\text{-synuclein}} - C_{t,PGP\ 9.5})$ sample $- (C_{t,\ \alpha\text{-synuclein}} - C_{t,PGP\ 9.5})$ calibrated as previously described (Livak and Schmittgen 2001).

Primers were generated by the OLIGO 4.0 S software (National Biosciences, Plymouth, MN, USA) based on their T_m (melting temperature) as calculated by the nearest neighbor method (as close as possible to 60°C) with less than 2°C difference between them and all the primer duplexes kept to a minimum (less than four nucleotides) and no G nor C nor GC stretches longer than four nucleotides. Primers were also chosen on separate exons to amplify cDNA but not genomic DNA. Then, the primers were submitted to BLASTn analysis (NCBI) to confirm their specificity. The following primers were used: for α-synuclein, forward: 5′-CACAAGAGGGAATCCTGGAA-3′; reverse: 5′-TCATGCTGGCCGTGAGG-3′; PGP 9.5, forward: 5′-CCCCGAGATGCTGAACAAGTG-3′; reverse: 5′-CGATCACTGCTGATGGAAGA-3′.

Reactive oxygen species and neuron-specific enolase assays

After pharmacological treatments, primary cultures were loaded with pre-warmed Hank's Buffered Salt Solution containing 5 μM of CM-H_2DCFDA (Invitrogen) for 15 min at 37°C then followed by a 10 min incubation at 37°C in DMEM medium without phenol red prior to microscopy analysis. For quantification of fluorescence, cells were lyzed with 100 μL of NETF/NP40 lysis buffer and fluorescence was read at 517 nm. Neuron-specific enolase release into culture medium was assessed as described previously (Abdo *et al.* 2010)

In vivo experiments

Male C57BL6N mice (Janvier, France) weighing 21–23 g were housed in cage in temperature-controlled room (21 ± 1°C), one week before the experiments. The mice were given access to food and water *ad libitum* and were maintained on 12 h light/dark cycle. Animal care was conducted in accordance with standard ethical guidelines and approved by the local ethic committee. Animals received a daily intraperitoneal (i.p.) injection of Bay K-8644 (2 mg/kg) or forskolin (2 mg/kg) or vehicle (10% ethanol) for 3 days. Animals were killed 24 h after the last i.p. injection and the proximal colons were taken and analyzed by immunoblot and immunohistochemistry.

Statistical analysis

All data are given as the mean ± standard error of the mean (SEM). Comparisons of mean values between groups were performed by Student's *t*-test for unpaired data or by analysis of variance followed by Dunnett's test. When data were not normally distributed, a Mann–Whitney *test* was performed. Differences were considered statistically significant if $p < 0.05$.

Results

α-Synuclein is expressed by neurons in primary culture of rat ENS

Following 14 days of culture, enteric neurons were organized in ganglia connected to each other by interganglionic fiber strands as evidenced by immunostaining using β III tubulin antibody (Fig. 1a) (Chevalier *et al.* 2008). Enteric glial cells, identified by glial fibrilary acidic protein immunostaining, were also present in enteric ganglia and along interganglionic fiber strands (Fig. 1b). α-Synuclein immunostaining revealed that α-synuclein was present in the cytoplasm of the somata as well as in the fibers of enteric neurons (Fig. 1a). In contrast, enteric glial cells did not express α-synuclein (Fig. 1b).

Expression of α-synuclein is increased in enteric neurons following KCl-induced depolarization and forskolin challenge

Membrane depolarization elicited by 40 mM KCl induced a significant increase in the protein level of α-synuclein in primary culture of rat ENS (Fig. 2a). A twofold increase in α-synuclein expression was observed after 24 h of treatment,

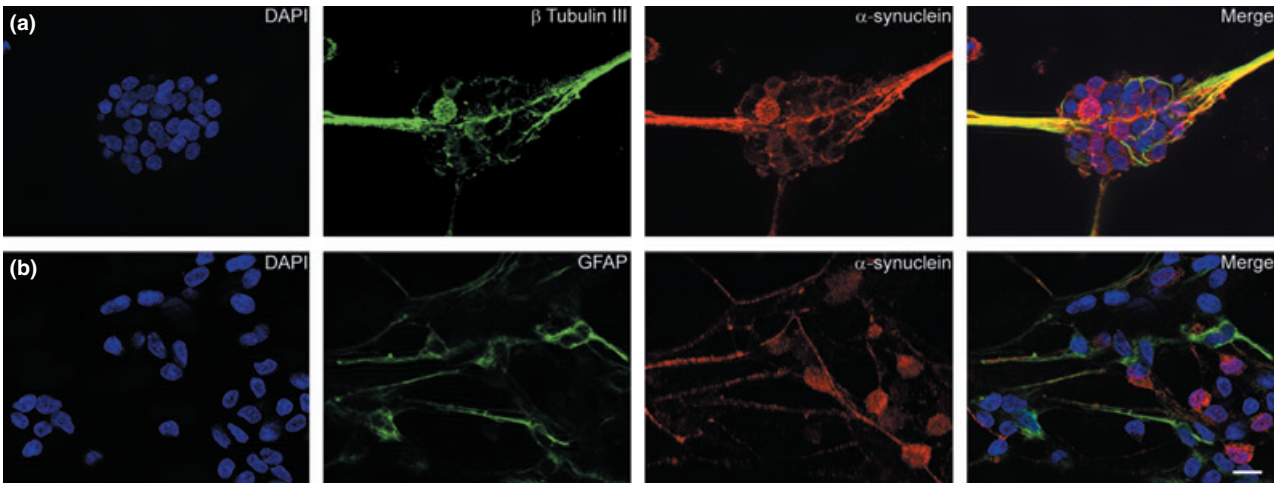

Fig. 1 Expression of α-synuclein in primary culture of ENS. (a) The presence of enteric neurons in primary culture of ENS was assessed by β III tubulin Immunostaining (β tubulin III); nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). (b) The presence of enteric glial cells within primary culture of ENS was assessed by glial fibrilary acidic protein immunostaining (GFAP); nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). (a, b) Immunostaining with antibodies against α-synuclein demonstrate that α-synuclein colocalized primarily with enteric neurons and not with enteric glial cells (merge). Scale bar represents 20 μm.

reaching 3.35-fold after 72 h (Fig. 2a and b). To rule out a non-specific osmotic effect of KCl, primary culture of rat ENS were treated with an equimolar concentration of mannitol. Such a treatment did not induce synuclein expression as compared to control either at 24 or 72 h (Fig. 2a and b). Treatment of primary culture of rat ENS with 20 μM forskolin, which increases intracellular cyclic AMP, induced an almost sixfold significant increase in the expression of α-synuclein after 72 h (Fig. 2a and b). The increase in α-synuclein expression induced by KCl was associated with a significant 1.9-fold increase in the corresponding transcript at 24 h (Fig. 2c). A significant 2.1- and 1.8-fold increase in α-synuclein transcripts was observed following treatment with 20 μM forskolin at 12 and 24 h respectively (Fig. 2c).

In some instances, treatments with depolarizing agents have been associated with neuronal injury (Ramnath *et al.* 1992). As α-synuclein expression can be increased by cell stress (Gomez-Santos *et al.* 2003), we have performed a set of experiments to determine whether a treatment with KCl or forskolin provoke neuronal oxidative stress and/or neuronal cell death. First, by using the reactive oxygen species (ROS)-specific fluorescent dye, CM-H2DCFDA (Sung *et al.* 2001), we found that the ROS fluorescence was mainly detectable within neurons in treated and untreated primary culture of ENS (Fig. 2d) and that the amount of intracellular ROS was comparable between control and KCl or forskolin-treated cells (Fig. 2e). Second, the expression level of the 70 kDa heat-shock protein (Hsp70), a chaperone protein whose expression is up-regulated in neuronal cells following an oxidative injury (Shyu *et al.* 2004), was assessed in both treated and untreated cells. Treatment with either 40 mM KCl or 20 μM forskolin did not change the expression level of Hsp70 protein (Fig. 2e) whereas hydrogen peroxide which was used as positive control, induced a reproducible increase in the expression of Hsp70 as compared with control (Fig. 2f). Third, neuron-specific enolase release into the culture medium was used to estimate neuronal injury after treatments with high-KCl and forskolin. We have recently shown that this technique enables a reliable and specific assessment of neuronal cell death in primary culture of ENS (Abdo *et al.* 2010). The amount of neuron-specific enolase released in the culture medium was comparable between cells treated with 40 mM KCl, 20 μM forskolin and controls (2.3 ± 0.7 ng/mL for controls, 1.7 ± 0.6 ng/mL for KCl-treated cells and 3.2 ± 0.7 ng/mL for forskolin-treated cells, $n = 8$, $p > 0.05$ vs. control).

Taken together, our results demonstrate that depolarization and forskolin challenge of enteric neurons result in induction of α-synuclein expression at both the transcript and protein levels. The effects of depolarization and forskolin were specific and not a consequence of cell injury.

Induction of expression of α-synuclein by depolarization is mediated through L-type calcium channels

Voltage-operated calcium channels are critical in the regulation of gene expression by depolarization in the CNS (Flavell and Greenberg 2008). These channels have been classified by electrophysiological and pharmacological means into L-, N-, P-, Q-, R- and T-type channels (Catterall 2000). Within the enteric nervous system, L-, N-, P- and Q-type Ca^{2+} channels have been identified (Smith *et al.* 2003). Treatment of primary culture of ENS with nifedipine (1 μM), a specific antagonist of L-type calcium channels (Catterall 2000), completely prevented the effects of

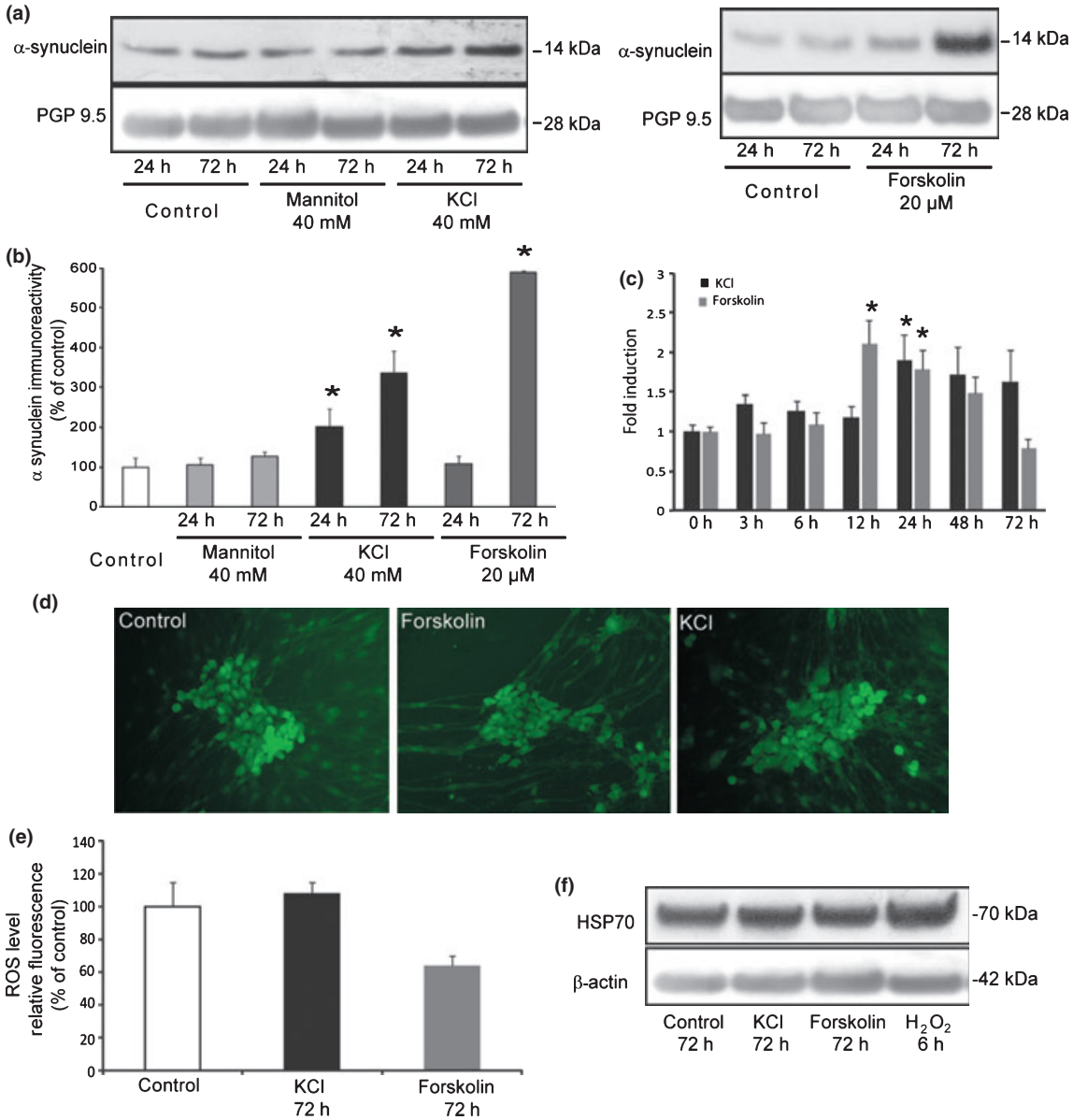

Fig. 2 Effects of KCl-induced membrane depolarization and forskolin on α-synuclein expression in primary culture of ENS. (a) After 14 days in culture, primary culture of ENS were treated with vehicle (control), 40 mM mannitol, 40 mM KCl, and 20 μM forskolin for 24 or 72 h. Cells were harvested and homogenized in NETF/NP40 (1%) buffer and 35 μg of protein per sample were subjected to immunoblot analysis using antibodies specific for α-synuclein. After stripping, membranes were reprobed with anti-PGP 9.5 antibodies to ensure equal loading of neuronal proteins. (b) For quantification, the optical densities of α-synuclein immunoreactive bands were measured, normalized to the optical densities of PGP 9.5 immunoreactive bands in the same samples, and expressed as percentages of controls. Data correspond to mean ± SEM of 6–15 samples per condition. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control, $^*p < 0.01$). (c) Quantitative PCR analysis of α-synuclein mRNA in primary culture of ENS treated with vehicle (control), 40 mM KCl (black bars) or forskolin (gray bars) for 3, 6, 12, 24, 48 and 72 h. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control, $^*p < 0.01$). Data correspond to mean ± SEM of 6 samples per condition. (d) After 14 days in culture, primary culture of ENS were treated with vehicle (control), 40 mM KCl, or 20 μM forskolin for 72 h. Cells were loaded with CM-H_2DCFDA (non-fluorescent) which is oxidized in DCF (fluorescent) by ROS. (e) For quantification, the relative fluorescence of DCF was measured using a microplate reader at 517 nM. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test. Data correspond to mean ± SEM of 6 samples per condition. (f) After 14 days in culture, primary culture of ENS were treated with vehicle (control), 40 mM KCl, and 20 μM forskolin for 72 h or with 100 μM H_2O_2 for 6 h. Cells were harvested and homogenized in NETF/NP40 (1%) buffer and 35 μg of protein per sample were subjected to immunoblot analysis using antibodies specific for Hsp70. After stripping, membranes were reprobed with anti-β actin antibodies to ensure equal loading of proteins. The autoradiograms are representative of three independent experiments.

depolarization on α-synuclein induction (Fig. 3a and b). In contrast, pre-treatment with 0.1 μM of omega-conotoxin and omega-agatoxin, which inhibit specifically N- and P/Q-type calcium channels respectively (Catterall 2000), had no effects on the expression of α-synuclein elicited by depolarization (Fig. 3a and b).

To establish whether a selective L-type calcium channel agonist alone is able to induce α-synuclein expression, we used Bay K-8644, a selective agonist of these channels. Incubation of primary cultures of ENS with 1 μM of Bay K-8644 for 72 h significantly increased the expression of α-synuclein (Fig. 3c and d).

Collectively, these results demonstrate a critical role for L-type calcium channels in depolarization-induced α-synuclein expression.

Induction of expression of α-synuclein by forskolin is mediated through PKA activation and L-type calcium channels

By increasing the intracellular level of cyclic AMP, forskolin is able to activate protein kinase A (PKA). PKA has been shown to be involved in some of the effects depolarization in neurons (Grewal *et al.* 2000). This logically led us to use a PKA inhibitor, H89 (Chijiwa *et al.* 1990), to study the role of PKA on the effects of both forskolin and depolarization. Pre-treatment of primary culture of ENS with 2 μM H89 completely prevented the forskolin-induced increase in α-synuclein expression (Fig. 4a) but did not alter the effects of KCl-induced depolarization (Fig. 4b).

Regulation of gene expression by forskolin in neurons is either L-type calcium channels-dependent (Konradi *et al.* 2003) or -independent (Cigola *et al.* 1998). We thus studied the effects of nifedipine on forskolin-induced expression of α-synuclein and showed that this inhibitor of L-type calcium channels completely prevented the effects of forskolin (Fig. 4c).

These results suggest that both PKA activity and L-type calcium channels are required for the induction of expression of α-synuclein by forskolin. In contrast, PKA is not involved in the depolarization-induced α-synuclein expression.

Activation of ERK is required for depolarization and forskolin-induced expression of α-synuclein

Extracellular signal-regulated kinases play a pivotal in the regulation of gene expression in neurons (Grewal *et al.* 1999). ERK phosphorylation and activation can be achieved though several signaling pathways including depolarization and increase in intracellular cyclic AMP (Derkinderen *et al.* 1999). We therefore assessed the role of the ERK pathway in the induction of α-synuclein expression in enteric neurons. We first studied whether KCl-induced depolarization and forskolin were able to activate and thus to phosphorylate ERK in enteric neurons. Treatment of primary culture of ENS with either KCl or forskolin induced a rapid and

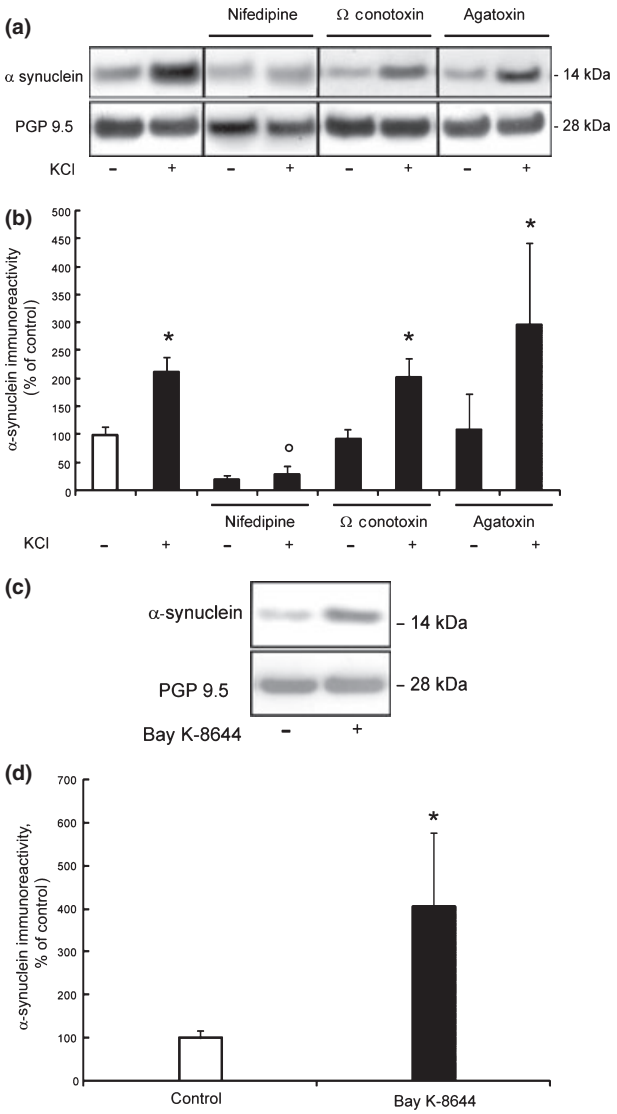

Fig. 3 Pharmacological characterization of the voltage operated calcium channels involved in the effects of depolarization on α-synuclein expression. (a) After 14 days in culture primary culture of ENS were treated with 40 mM KCl for 72 h in the absence or presence of 1 μM nifedipine, 0.1 μM omega-conotoxin and 0.1 μM omega-agatoxin applied 30 min before. Immunoblots were performed as described in the legend to Fig. 2a. (b) Quantification of the results for α-synuclein protein level was assessed as described in the legend to Fig. 2b. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control: $^{*}p < 0.01$; treated in the presence of 1 μM nifedipine vs. in its absence: $^{\circ}p < 0.01$). Data correspond to mean ± SEM of 3–9 samples per condition. (c) After 14 days in culture, primary culture of ENS were treated with 1 μM Bay K-8644 for 72 h. Immunoblots were performed as described in the legend to Fig. 2a. (d) Quantification of the results for α-synuclein protein level was assessed as described in the legend to Fig. 2b. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control: $^{*}p < 0.01$). Data correspond to mean ± SEM of 3–15 samples per condition.

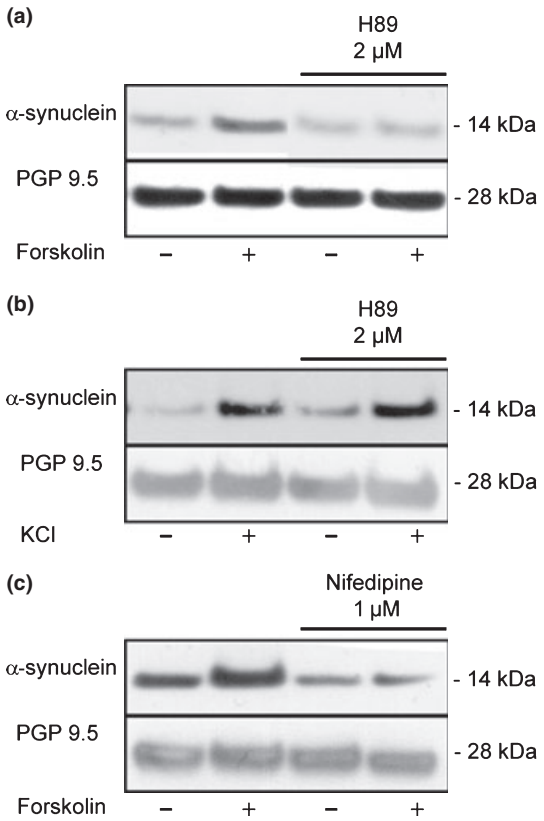

Fig. 4 Role of PKA in the regulation of α-synuclein expression by KCl-induced membrane depolarization and forskolin. (a) After 14 days in culture, primary culture of ENS were treated 40 mM KCl for 72 h in the absence or presence of 2 μM H89 applied 30 min before. Immunoblots were performed as described in the legend to Fig. 2a. (b) After 14 days in culture, primary culture of ENS were treated with 20 μM forskolin for 72 h in the absence or presence of 2 μM H89 applied 30 min before. Immunoblots were performed as described in the legend to Fig. 2a. (c) After 14 days in culture, primary culture of ENS were treated with 20 μM forskolin for 72 h in the absence or presence of 1 μM nifedipine applied 30 min before. Immunoblots were performed as described in the legend to Fig. 2a. The autoradiograms are representative of at least 4 independent experiments.

monophasic activation of ERK as assessed by immunoblotting with antibodies specifically reacting with the active phosphorylated form of the kinase (Fig. 5a–d). The activation of ERK by depolarization appeared to be more sustained than the one induced by forskolin (Fig. 5a–d).

As activation of ERK results from the phosphorylation by the dual-specificity mitogen-activated protein kinases/ERK kinases, we have used PD98059 (Alessi *et al.* 1995) and U0126 (Favata *et al.* 1998), two mitogen-activated protein kinases/ERK kinases inhibitors. Pre-treatment of enteric neurons with 10 μM U0126 completely prevented the effects of both depolarization and forskolin on α-synuclein expression (Fig. 5e–h). Similar results were obtained using 50 μM PD98059 as a pre-treament (data not shown).

Ras is required for depolarization and forskolin-induced expression of α-synuclein

Membrane depolarization and cyclic AMP are capable of activating ERK through diverse signaling pathways (Derkinderen *et al.* 1999). The small G protein Ras is a point of convergence for the two stimuli to activate ERK (Obara *et al.* 2007). We thus studied the effects of a Ras inhibitor, the farnesyl transferase inhibitor FTI-277 (Lerner *et al.* 1995), on the expression of α-synuclein in enteric neurons. Pre-treatment with 10 μM FTI-277 significantly decreased the induction of α-synuclein expression elicited by both forskolin and depolarization (Fig. 6a–d). This suggests that depolarization and forskolin act through a common signaling pathway to activate ERK and in turn to induce α-synuclein expression. In line with this hypothesis, a simultaneous treatment with forskolin and KCl was no more efficient than forskolin and KCl alone to induce α-synuclein (Fig. 6e and f).

Taken as a whole, our results demonstrate a critical role for the Ras/ERK pathway in the regulation of α-synuclein expression by forskolin or depolarization.

Bay K-8644 and forskolin increase α synuclein expression in enteric neurons *in vivo*

We eventually sought to determine whether the effects of depolarization and forskolin on α-synuclein expression could be also observed *in vivo*. We first studied the expression of α-synuclein in the colonic ENS of mice. Whole mount immunofluorescence experiments showed that the expression of α-synuclein in the colonic myenteric plexus of mice was restricted to neurons (Fig. 7a). No α-synuclein staining was observed in enteric glial cells (Fig. 7a). To study the effects of depolarization and forskolin in living mice, we used Bay K-8644 and forskolin, two compounds that have already been shown to be efficient when intraperitoneally administered (Jinnah *et al.* 1999; Melis *et al.* 2002). Using western blot analysis, we have shown that treatment of mice with 2 mg/kg of Bay K-8644 or with 2 mg/kg of forskolin every day for 3 days induced a significant increase in the expression of α-synuclein in the proximal colon as compared with controls (Fig. 7b). Immunofluorescence experiments revealed that the increase in α-synuclein expression induced by Bay K-8644 and forskolin occurred in neurons and mainly in their somata (Fig. 7c).

Discussion

The three main outcomes of the present survey are (i) the induction of α-synuclein expression, a key protein in the pathophysiology of PD, by cyclic AMP and depolarization; (ii) the critical role of ERK in the regulation of α-synuclein expression; (iii) the identification of the enteric neurons as a subset of neurons in which α-synuclein expression can be regulated.

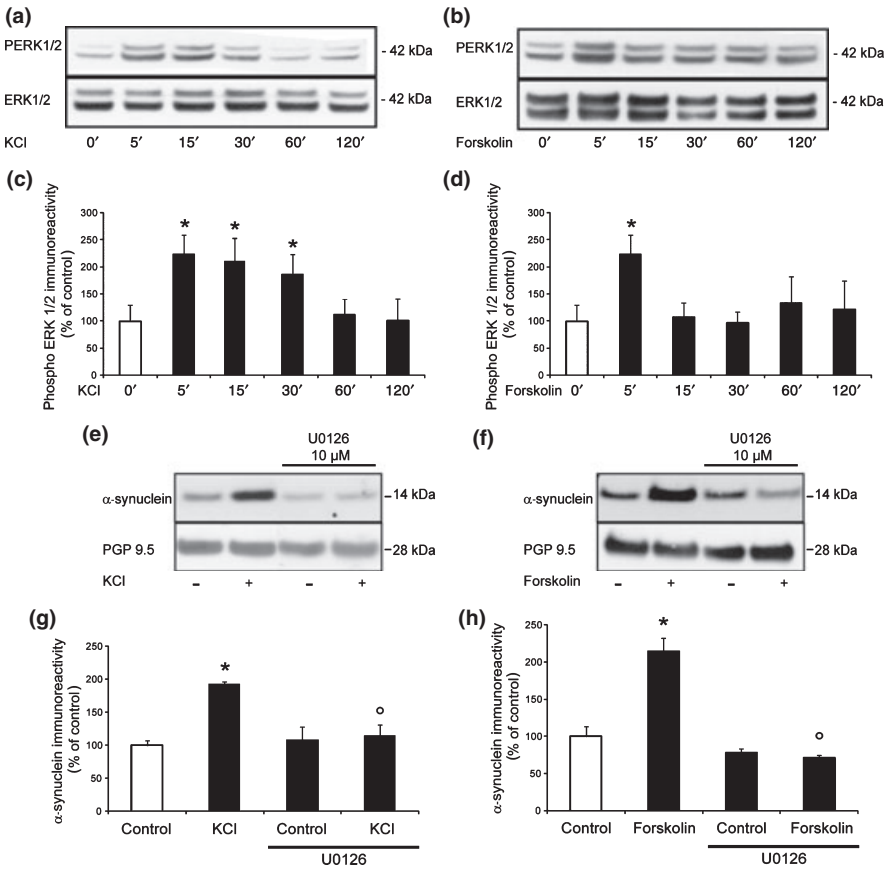

Fig. 5 Role of ERK signaling pathway in the regulation of α-synuclein expression by KCl-induced membrane depolarization and forskolin. (a, b) After 14 days in culture, primary culture of ENS were treated either with 40 mM KCl or with 20 μM forskolin for 5, 15, 30, 60 or 120 min. Cell lysates were subjected to immunoblot analysis using antibodies specific for the dually phosphorylated (active) forms of ERK1 and ERK2 (P-ERK1/2). After stripping, the membranes were reprobed with anti-ERK (ERK1/2) antibodies. Immunoblots were performed as described in the legend to Fig. 2. (c, d) The values of active phospho-ERK1 and 2 were normalized to the amount of total ERK1 and 2 in the same sample and expressed as a percentage of controls. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control: $*p < 0.01$) (e, f) After 14 days in culture, primary culture of ENS were treated with either 40 mM KCl or 20 μM forskolin for 72 h respectively in the absence or presence of 10 μM U0126 applied 30 min before. Immunoblots were performed as described in the legend to Fig. 2a. (g, h) Quantification of the results for α-synuclein protein level was assessed as described in the legend to Fig. 2b. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control: $*p < 0.01$; treated in the presence of U0126 vs. in its absence: $°p < 0.01$). Data correspond to mean ± SEM of 6–12 samples per condition.

Although the expression of α-synuclein has been suggested to be involved in the pathogenesis of PD, only a few studies to date have addressed the specific issue of the extracellular stimuli capable of modulating α-synuclein expression in neurons. An increase in α-synuclein expression occurs in response to growth factors such as nerve growth factor and basic fibroblast growth factor (Stefanis *et al.* 2001; Clough and Stefanis 2007). This study is the first to show that stimuli linked to neuronal activity, namely intracellular cyclic AMP and membrane depolarization can induce the expression of α-synuclein. Remarkably, a previous report performed in PC12 cells failed to demonstrate any effect of a non-hydrolyzable analogue of cyclic AMP, whereas nerve growth factor induced a robust increase in α-synuclein expression (Stefanis *et al.* 2001). Two reasons can be put forward to explain such a discrepancy in the effects of cyclic AMP on α-synuclein expression. First, we have used a primary culture model in this study instead of a cell line. Second, the non-hydrolyzable analogue of cyclic AMP was applied for 10 days in PC12 cells instead of the 72 h treatment with forskolin used in the present study. Given the time course of α-synuclein mRNA induction observed in the present study, it is likely that a 10-day treatment with forskolin would have also failed to elicit an increase in α-synuclein expression.

Deciphering the signaling pathways, we have underscored two key elements involved in the effects of membrane depolarization and forskolin on α-synuclein expression. First,

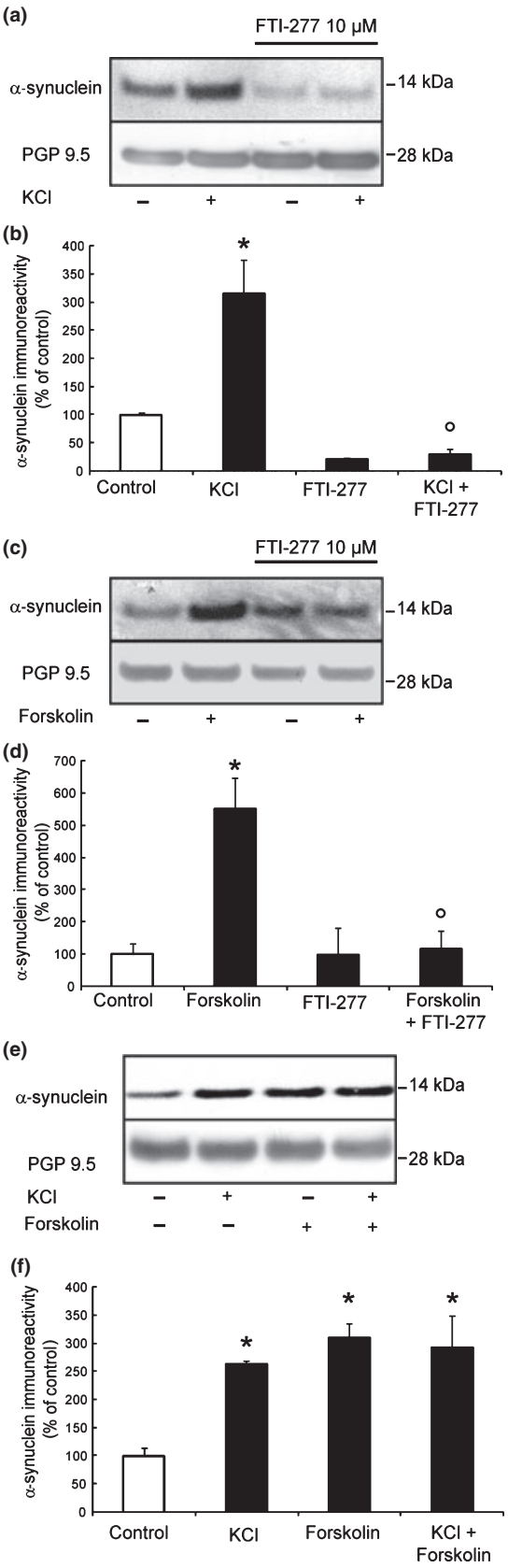

Fig. 6 Role of Ras signaling pathway in the regulation of α-synuclein expression by KCl-induced membrane depolarization and forskolin. (a, c) After 14 days in culture, primary culture of ENS were treated either with 40 mM KCl or 20 μM forskolin for 72 h in the absence or presence of 10 μM FTI-277 applied 30 min before. Immunoblots were performed as described in the legend to Fig. 2a. *b and d*, quantification of the results for synuclein protein level was assessed as described in the legend to Fig. 2b. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control: $^*p < 0.01$). (e) After 14 days in culture, primary culture of ENS were treated with either 40 mM KCl or 20 μM forskolin alone or with both treatment concomitantly for 72 h. Immunoblots were performed as described in the legend to Fig. 2a. (f) Quantification of the results for synuclein protein level was assessed as described in the legend to Fig. 2b. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control: $^*p < 0.01$). Data correspond to mean ± SEM of six samples per condition.

we have shown L-type calcium channels were critically involved in the effects of depolarization and forskolin (Fig. 8). This is in accordance with the large body of studies performed in the CNS which have demonstrated that L-type calcium channels are the cornerstone in signaling mechanisms linking neuronal activity to gene expression (Flavell and Greenberg 2008). Recent evidence has emerged that a similar role for these channels also exists in the ENS (Chevalier *et al.* 2008). Remarkably, the involvement of L-type calcium channels in PD has been recently addressed both in experimental Parkinsonism and in an epidemiological survey. A dysregulation of L-type calcium channels occurs in rodents treated with either 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine or rotenone, and treatment with a channel blocker prevents the development of neurodegeneration in these animals (Chan *et al.* 2007). In line with these results obtained in animals, current long-term use of calcium channel blockers for hypertension in humans is associated with a significantly reduced risk of PD (Becker *et al.* 2008). Second, we have demonstrated that the Ras/ERK signaling pathway is necessary for α-synuclein expression following both depolarization and forskolin challenge (Fig. 8). This concurs with the results of Clough and Stefanis (2007), who also showed that Ras and ERK were two critical steps in the induction of α-synuclein by growth factors. From a pathological point of view, patients with PD exhibit cytoplasmic aggregates of activated forms of ERK within their nigral neurons (Zhu *et al.* 2002) and 6-hydroxydopamine elicits a sustained ERK activation that contributes to neuronal cell death *in vitro* (Kulich and Chu 2001), raising the possibility that abnormal patterns of ERK activation may contribute to dopaminergic neuronal cell death. In addition, a recent report has demonstrated that the product of the leucine-rich repeat kinase 2 gene, whose mutations account for frequent

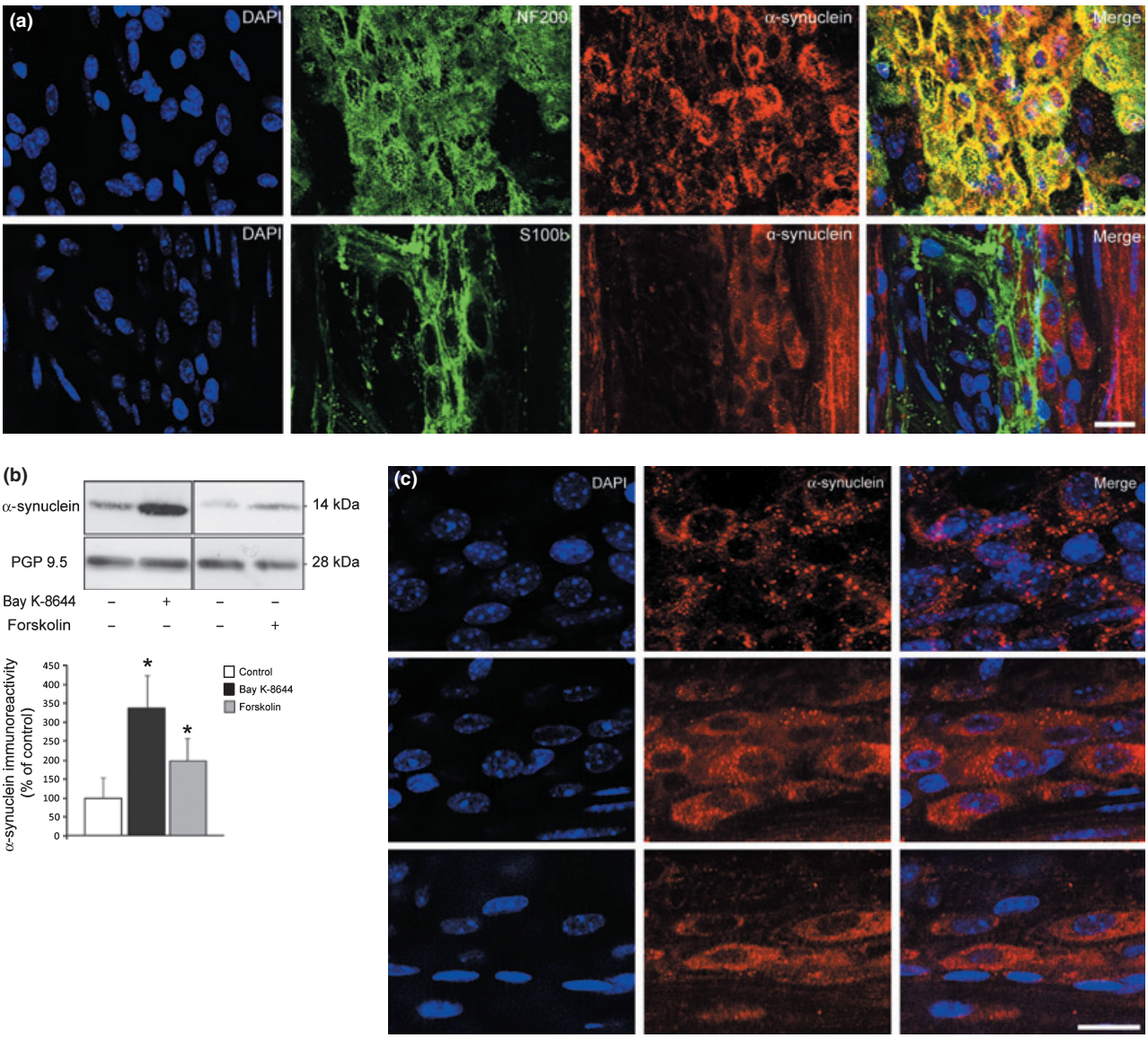

Fig. 7 Bay K-8644 and forskolin increase α synuclein expression in enteric neurons in mice. (a) Double immunohistochemical labeling of myenteric neurons and enteric glial cells from proximal colon of mice. Myenteric neurons were identified with anti-NF200 antibodies and stained with anti-synuclein antibodies. Enteric glial cells were labeled using S100β antibodies and stained with anti-synuclein antibodies. Nuclei were stained with DAPI. Scale bar represents 20 μm. (b) Mice were i.p. injected daily with Bay K-8644 (2 mg/kg) during 3 days. Their proximal colon was taken and the amount of α-synuclein was assessed by immunoblots that were performed and quantified as described in the legend to Fig. 2a and b. Statistical analysis was performed with ANOVA followed by Dunnett's multiple comparison test (treated vs. control: $^*p < 0.01$). Data correspond to mean ± SEM of 5 animals per condition. (c) Mice were i.p. injected daily with forskolin (2 mg/kg) during 3 days. Their proximal colon was taken, dissected and α-synuclein immunostaining was performed on myenteric plexus. Nuclei were stained with DAPI. Data are representative of five different animals per condition. Scale bar represents 20 μm.

autosomal-dominant PD, induces α-synuclein expression via ERK (Carballo-Carbajal *et al.* 2010). Taken as whole, these results, along with the one obtained in the present study, strongly suggest that a dysregulation of both L-type calcium channels and of the Ras/MAP kinase pathway are present in neurons from PD patients and that such phenomenon are likely to occur not only in central neurons but also in enteric neurons.

Our findings may be relevant to the pathogenesis of PD. We have used in this study our recently developed model of primary culture of ENS, an *in vitro* model that recapitulates the main features of the ENS (Chevalier *et al.* 2008). The results obtained in this model were reinforced by the fact that Bay K-8644 and forskolin also induced a significant increase in synuclein expression *in vivo*. The ENS has received great deal of interest over the last years for its role in the

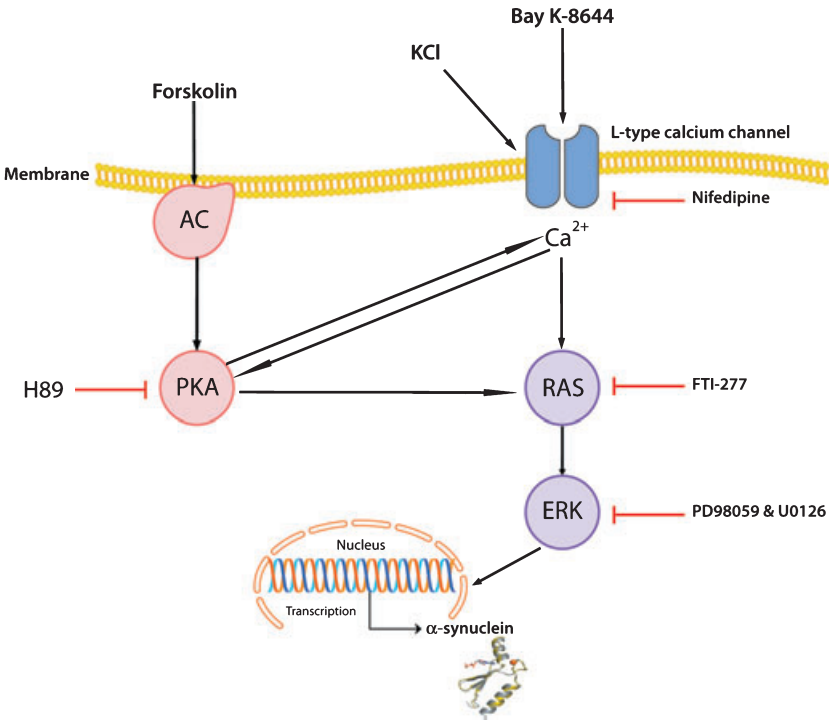

Fig. 8 Signaling pathways involved in the cAMP- and membrane depolarization-induced expression of α-synuclein in enteric neurons. Depolarization induced by KCl increased α-synuclein expression through activation of L-type calcium channels. The effects of forskolin, an activator of adenylyl cyclase (AC) on synuclein expression are mediated through protein kinase A (PKA) and L-type calcium channels. Both stimuli converge on a Ras/ERK pathway. Black arrows indicate activation. Red lines indicate blockade by inhibitors.

pathophysiology of PD (Lebouvier *et al.* 2009). It has been suggested that the lesions in the ENS occur at a very early stage of the disease, even before the involvement of the CNS (Braak *et al.* 2006). This led to the postulate that the enteric nervous system is likely to be critical in the pathophysiology of PD as it could represent a route of entry for a putative environmental factor to initiate the pathological process (Braak's hypothesis) then spreading to the CNS via vagal connections (Braak *et al.* 2006). In this context, several recent reports strongly support that α-synuclein is pivotal in the spread of the pathological process from the ENS to the CNS. -α-Synuclein has been shown to be secreted by neuronal cells *in vitro* and this secreted α-synuclein is prone to aggregate (Lee *et al.* 2005). Aggregates of α-synuclein can be taken up from the extracellular space by neighboring neurons thereby triggering neuronal cell death and the formation of Lewy body-like intracellular inclusions (Desplats *et al.* 2009), supporting the hypothesis that α-synuclein behaves like prion protein. Remarkably, the amount of α-synuclein secreted in the extracellular space is likely to be correlated with the quantity of α-synuclein present in the intracellular space (Lee *et al.* 2005). Altogether, these data suggest that the increase in the intracellular protein level of α-synuclein within enteric neurons may be the first critical step in the development of PD.

Eventually, our results have implications that go beyond PD. An emerging concept in gastroenterology is that a wide range of diseases, such as motility disorders, can be considered in part as enteric neuropathies. In particular, aging is associated with a variety of motility disorders or the gut including delays in gastric emptying and longer intestinal transit time (Camilleri *et al.* 2008). Aged rats display neuronal loss as well as changes in the neurochemical phenotype in the ENS, which are likely to result in motility disorders (Phillips *et al.* 2007). Remarkably, along with neuronal loss, these rats exhibit dystrophic enteric neurons that contains α-synuclein aggregates reminiscent of Lewy pathology (Phillips *et al.* 2009). This suggests that the presence of pathogens or xenobiotics in the gastrointestinal tract can convert normal aging into pathological aging associated not only with PD but also to a larger concept of enteric neuropathies/synucleinopathies.

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Article 2 : Régulation de la sécrétion de l'alpha-synucléine par l'activité neuronale dans le système nerveux entérique et effets sur la barrière épithéliale intestinale

Dans un second article soumis à «American journal of physiology» en 2012, nous nous sommes intéressés à la sécrétion de l'alpha-synucléine par le SNE. Cette étude montre que les neurones entériques sont non seulement capables de sécréter l'alpha-synucléine de façon constitutive, mais que l'activité neuronale participe activement à l'exocytose de la protéine. Contrairement aux données publiées dans le SNC, la sécrétion de l'alpha-synucléine par les neurones entériques est bloquée par de la bréfeldine A, un inhibiteur d'exocytose conventionnelle. Dans un second temps l'étude s'est intéressée aux effets de l'alpha-synucléine extracellulaire sur les fonctions digestives et notamment les fonctions de la BEI. Bien que l'alpha-synucléine extracellulaire ne semble pas avoir d'effets *in vitro* sur la perméabilité de cellules épithéliales Caco-2, elle est cependant capable de diminuer la perméabilité de biopsies coliques humaine en chambre d'Ussing.

Alpha-synuclein is secreted by enteric neurons but has no overt effect on epithelial intestinal barrier

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Abstract

There is growing evidence supporting a role of extracellular alpha-synuclein in the initiation and the progression of Parkinson's Disease (PD). Alpha-synuclein inclusions are found in the enteric nervous system (ENS) of almost all PD patients. Recent reports suggest that the permeability of the intestinal epithelial barrier (IEB), which is regulated by the ENS, is increased in PD patients. We therefore undertook the present survey to determine whether alpha-synuclein can be secreted by enteric neurons and to study the effects of extracellular alpha-synuclein on the IEB.

The secretion of alpha-synuclein was assessed by immunoblot of the conditioned media from primary culture of ENS. The effects of alpha-synuclein on the IEB was analysed *in vitro* in a Caco-2 cell monolayer model of intestinal barrier and *ex vivo* on human colonic biopsies using Ussing's chambers.

Alpha-synuclein was secreted by enteric neurons via a conventional, endoplasmic reticulum/Golgi-dependent exocytosis, in a neuronal activity-regulated manner. We further showed that alpha-synuclein did not modify IEB permeability either *in vitro* or *ex vivo*.

Our results show for the first time that enteric neurons secrete alpha-synuclein. They provide new insights into the role of the ENS in the pathophysiology of PD**.**

Introduction

Alpha-Synuclein, a 140-amino acid neuronal protein, attracted great interest since 1997 after a mutation in its gene was identified in autosomal dominant Parkinson's disease (PD) [1] and its aggregates were found to be the primary components of Lewy bodies, the pathological hallmarks of PD [2]. Although initially considered as an intracellular protein, recent reports have shown that alpha-synuclein also exerts its effects extracellularly [3, 4]. Alpha synuclein can be secreted into the culture medium of differentiated human neuroblastoma cells and primary cortical neurons [5] and detected in human cerebrospinal fluid and plasma [3]. When added to the culture medium of neuronal cells, monomeric and oligomeric extracellular alpha-synuclein have the propensity to induce cell death [6-8] and to activate calcium signaling pathways [9].

The distribution of alpha-synuclein pathology in PD is much greater than formerly appreciated. Lewy bodies distribution extend well beyond the *substantia nigra* and involves peripheral nervous networks, among which is the enteric nervous system (ENS) [10]. Lewy bodies have been described in the two major ganglionated plexuses of the ENS, namely myenteric and submucosal plexus [11]. While myenteric neurons control motility, the submucosal plexus regulates key functions of the intestinal epithelial barrier (IEB) such as paracellular permeability [12]. The control by the ENS of this monolayer of polarized epithelial cells is often considered as the digestive counterpart of the central nervous system blood-brain-barrier. The paracellular permeability of the IEB is regulated by tight junctions (TJ) composed of occludin, claudins and zonula occludens proteins (for review [13]). Emerging concepts suggest that defects in IEB function are involved in the development of various digestive and non-digestive diseases. For instance,

inflammatory bowel diseases and irritable bowel syndrome are associated with an increase in IEB permeability [14]. Using oral administration of unmetabolized sugars, two recent reports suggested that an increase in intestinal permeability occurs in PD patients [15, 16]. The molecular and cellular events associated with this increase in IEB permeability observed in PD patients and especially the role of alpha-synuclein, are currently unknown.

Whether enteric neurons are capable of secreting alpha-synuclein and whether enteric extracellular alpha-synuclein exerts any physiological function has not been studied yet. We therefore undertook the present survey (i) to determine whether alpha-synuclein can be secreted in the culture medium of enteric neurons in primary culture (ii) to study the effects of extracellular alpha-synuclein on the IEB.

Material and Methods

Reagents and antibodies

Forskolin, veratridine, horse radishperoxidase and brefeldin A (BFA) were purchased from Sigma (Saint Quentin Fallavier, France). Recombinant alpha-synuclein was obtained from Millipore (Molsheim, France). The following commercially available antibodies were used for western blotting: rabbit polyclonal anti-alpha-synuclein C-20 (1:500; Santa Cruz Biotechnology, Heidelberg, Germany), rabbit polyclonal anti-bovine serum albumin (BSA) (1:1000; Millipore, Molsheim, France) and mouse monoclonal anti-PGP 9.5 (1:1000; Ultraclone limited, Isle of Wight, UK).

Primary cultures of ENS

Small intestine of rat embryos E15 (35-45 per isolation from 3 pregnant Sprague-Dawley rats (CERJ, Le Genest St Isle, France) were removed and finely diced in HBSS (Sigma, Saint Quentin Fallavier, France). Tissue fragments were collected in 5 mL of medium (DMEM-F12 (1:1) medium) and digested at 37°C for 15 min in 0.1% trypsin (Sigma). The trypsin reaction was stopped by adding 10 mL of medium containing 10% fetal calf serum and then treated by DNAse I 0.01% (Sigma) for 10 min at 37°C. After triturating with a 10 mL pipette, cells were centrifuged at 750 rpm for 10 min. Cells were counted and then seeded at a density of $2.4.10^5$ cells/cm^2 on 24-well plates previously coated for 6 h with a solution of gelatin (0.5%; Sigma) in sterile PBS. After 24 h, the medium was replaced with a serum-free medium (DMEM-F12 (1:1) containing 1% of N-2 supplement (Life Technologies, Cergy Pontoise, France). Cells were maintained in culture for 15 days.

Supernatant and cell lysates immunoblots

The culture supernatant (CS) was collected and centrifuged at 4000 *g* for 10 min at 4°C to remove cell debris. 3 ml of CS was further concentrated at 4000 rpm for 28 min using 9 kDa cutoff concentrators (Pierce, Brébière, France). Primary culture of ENS were harvested in NETF buffer (100 mM NaCl, 2 mM EGTA, 50 mM Tris-Cl, pH 7.4, and 50 mM NaF) containing 1% (v/v) Nonidet P-40, 2 mM orthovanadate, phosphatase inhibitor cocktail II (Roche, Neuilly sur Seine, France) and a protease inhibitors cocktail (Roche). Equal amounts of lysate or CS were separated using the Invitrogen NuPage Novex Bis Tris MiniGels™ before electrophoretic transfer with the iBlot™ Dry Blotting System also from Invitrogen. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris, pH 7.5) with 5% non-fat dry milk. Membranes were incubated overnight at 4°C with the primary antibodies. Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Pierce; diluted 1:5000) and visualized by enhanced chemiluminescent detection (ECL Forte, Millipore). When necessary, membranes were stripped for 10 min in Reblot buffer (Millipore) followed by extensive washing in TBS before reblocking for 30 min in TBS with 5% non-fat dry milk and reprobing. The relevant immunoreactive bands were quantified with laser-scanning densitometry and analyzed with NIH Image J software. To allow comparison between different autoradiographic films, the density of the bands was expressed as a percentage of the average of controls (untreated). The value of alpha-synuclein immunoreactivity was normalized to the amount of appropriate loading control (PGP9.5 for cell lysate or BSA for CS samples) in the same sample and expressed as a percentage of controls. All immunoblots represent one of at least three independent experiments.

Alpha-synuclein ELISA assay

Commercially available ELISA (Anaspec; Le Perray-en-Yvelines, France) was used to quantify secreted alpha-synuclein from ENS primary culture. Concentrated CS or standards and HRP coupled detection antibodies were added in the same time and plates were incubated at 4°C overnight as described by the manufacturer's instructions. After a 3 times washing procedure, TMB substrate (BD Biosciences) was added to each well for 15 min at room temperature and the chemiluminescence was integrated for 1 s.

Caco-2 cells culture

Experiments were performed with the human intestinal epithelial cell line Caco-2 (EATCC, Port Down, UK). Cells were seeded onto porous filters (12-well Transwell Clear, 0.40 mm porosity, 1.1 cm of diameter; Corning, Dutscher Brumath, France), at a density of 200 000 cells/filter. Filters were previously coated overnight at 4°C with a solution of collagen I (10 µg/cm^2 in 20 mM acetic acid; BD Biosciences) Caco-2 were cultured in 500 µL of Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose; Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Abcys, Paris, France), 2 mmol/L glutamine (Invitrogen) and 50 IU/mL penicillin, and 50 mg/mL streptomycin (Invitrogen) in apical chamber and with 1 mL DMEM (4.5 g/L glucose) supplemented with glutamine and penicillin, and streptomycin without FCS in basolateral compartment. After 3 days of culture, Caco-2 cells formed a differentiated and polarized monolayer. The culture medium was changed every 2 days and before permeability experiments.

Assessment of paracellular permeability in Caco-2 cells

Caco-2 monolayer transepithelial resistances (TERs) were measured with an epithelial volt–ohmmeter (WPI, Stevenage, UK) before incubation with alpha-synuclein (monomers or oligomers), to ensure cellular differentiation. Paracellular permeability studies were performed using the flux of FITC-conjugated sulfonic acid (400 Da, 1 mg/mL; Invitrogen) through Caco-2 monolayer as previously described [17]. At the end of the 48 h incubation period, 50 µL of FITC-conjugated sulfonic acid were added to the apical compartment. The fluorescence level of basolateral aliquots (150 µL) was measured every 30 min for a period of 180 min using a fluorimeter (Varioskan, Thermo SA, France). Paracellular permeability was determined by averaging the gradient of change in fluorescence intensity over time using a linear regression fit model measured in the specimens (GraphPad Prism 5, La Jolla, USA*).* Values are expressed in arbitrary units.

***Ex-vivo* permeability of human colonic biopsies in Ussing chambers**

Colonic biopsies were obtained from healthy human who underwent colonoscopy for colorectal cancer screening. The study protocol was approved by the local Committee on Ethics and Human Research (Comité de Protection des Personnes Ouest VI), and written informed consent was obtained from each patient and from each normal volunteer.

Three biopsies were taken, opened and washed 3 times in cold Krebs's solution ($NaH_2PO_4 2H_2O$ 0,187 g/L, NaCl 6,84 g/L, KCl 0,35 g/L, $NaHCO_3$ 2,10 g/L, Glucose 1,98 g/L, $CaCl_2.2H_2O$ 0,368 g/L, $MgCl_2.6H_2O$ 0,244 g/L). Biopsies were mounted onto Ussing chamber (Physiological instruments, San Diego, CA). Each chamber contains 2 mL of Ham's Nutrient Mixture (HAM/F12, Invitrogen, France). The media was maintained at 37°C and continuously gasses with 95% O_2 and 5% CO_2. Alpha-synuclein monomers (10

μg/mL) were incubated with tissues for 1 h, during the equilibration period. 200 μL of apical medium was replaced by 200 μL of fluorescein–5.6 sulfonic acid (1 mg/mL, Invitrogen) to evaluate paracellular permeability and with horse radishperoxidase (10 mg/ml) to evaluate transcellular permeability. The fluorescence level of basolateral aliquots (150 μL) was measured every 30 min for a period of 180 min using a fluorimeter (Varioskan, Thermo SA, France) to evaluate paracellular permeability. Each condition was performed in duplicates and mean of duplicates was used for statistical analysis. Paracellular permeability was determined by averaging the gradient of change in fluorescence intensity over time using a linear regression fit model measured in the specimens (GraphPad Prism 5, La Jolla, USA).

To evaluate transcellular permeability, horseradish peroxidase activity was assessed in basolateral medium with orthodianisidine in accordance of the following reaction: H_2O_2+ orthodianisidine → H_20+ oxidized orthodianisidine. The resulting fluorescence was assessed ussing a fluorimeter at 460 nm (oxidized orthodianisidine) and compared to horseradish peroxidase standards (0 to 100 ng/ml).

Quantitative PCR analysis

RNA extraction from enteric primary culture was performed with Nucleospin RNA II kit (Macherey Nagel, HOERDT France) according to the manufacturer's instructions. For reverse transcription, 1 μg of purified total RNA was denatured and subsequently processed for reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations. PCR amplifications were performed using the Absolute Blue SYBR green fluorescein kit (ABGENE, Courtaboeuf, France) according to the manufacturer's protocol and ran on MyiQ thermocycler (Biorad, Marnes la Coquette, France). The mRNA level of expression of ZO-

1, Occludin and Claudin 1 was determined using the formula of the comparative cycle threshold and S6 was used as housekeeping gene as described in [18].

Primers were generated by the Oligo 4.0 S software (National Biosciences, MN, USA) based on their Tm (melting temperature) as calculated by the nearest neighbor method (as close as possible to 60°C) with less than 2°C difference between them and all the primer duplexes kept to a minimum (less than four nucleotides) and no G nor C nor GC stretches longer than four nucleotides. Primers were also chosen on separate exons to amplify cDNA but not genomic DNA. Then, the primers were submitted to BLASTn analysis (NCBI) to confirm their specificity. The following primers were used:

Table 1. Primers sequences used in quantitative PCR analysis

Gene	Protein name	Forward primer	Reverse primer	Accession n°
ZO-1	Zonula occludens 1	5'-AAGAATATGGTCTTCGATTGGC-3'	5'-ATTTTCTGTCACAGTACCATTTATCTTC-3'	NM_001106266 (rn)
Claudin 1	Claudin 1	5'-TCGCAGAGCACCGGGCAG-3'	5'-TGCTATCACTCCCAGGAGGATGC-3'	NM-002538
Occludin	Occludin	5'-GGATGACTTCAGGCAGCCTCG-3'	5'-CTGAAGTGATAGGTGGATATTCCCTG -3'	NM_031329 (rn)
S6	Ribosomal protein S6	5'-CCAAGCTTATTCAGCGTCTTGTTACTCC-3'	5'-CCCTCGAGTCCTTCATTCTCTTGGC-3'	NM_001010

Cell death assays

Neuronal cell death was assessed by quantifying the release of neuron specific enolase (NSE) into culture medium as described previously [19].

Statistical analysis

All data are given as the mean ± standard error of the mean SEM. Comparisons of means between groups were performed by Student's t test for unpaired data or by analysis of variance followed by Dunnett's test. When data were not normally distributed, a Mann–Whitney test was performed. Differences were considered statistically significant if $p < 0.05$.

Results

Alpha-synuclein is physiologically secreted by enteric neurons

Primary culture of ENS contains smooth muscle, enteric glial cells and neurons. We have previously shown that only neurons express alpha-synuclein in this primary culture model [20]. To determine whether enteric neurons secrete alpha-synuclein, CS was concentrated and immunoblotted with C-20 antibodies. Alpha-synuclein was detected in the CM and accumulated over time (Figure 1 A and B), while the expression of intracellular alpha-synuclein remained constant (Figure 1 A). The accumulation of alpha-synuclein in the CS was not explained by neuronal cell death, as the amount of NSE in the CS remained relatively stable throughout the 48 h period (8,7 ± 2.3 ng/ml at 6 h and 9,5 ± 1,6 ng/ml at 48 h; n=5 p>0,05). We quantified the levels of secreted alpha-synuclein in the CS after 48 h using ELISA. The concentration of alpha-synuclein was estimated to be 12,8 ± 3,7 pg/ml. Altogether; these results show that alpha-synuclein is physiologically secreted by enteric neurons.

Studies performed in SH-SY5Y cells overexpressing alpha-synuclein suggested that it is constitutively secreted via an unconventional secretory pathway because brefeldin A (BFA), a classical inhibitor of the endoplasmic reticulum (ER)/Golgi-dependent secretion pathway had no effect on alpha-synuclein secretion [5, 8]. By contrast, treatment of primary culture of ENS with BFA for 6 h significantly reduced alpha-synuclein release as compared to controls (Figure 1 C and D). Cell viability as assessed by NSE release in the CS was not compromised following a 6 h-treatment with BFA (8.9 ± 1.1 ng/ml for controls, 8.5 ± 3.7 ng/ml for BFA-treated cells, n=3, p>0.05). This suggests that alpha-synuclein secretion occurs through a secretory mechanism likely to be dependent of ER/Golgi-related vesicular transport.

Another remarkable result obtained in SH-SY5Y cells was that the amount of released alpha-synuclein in the CS correlated with its intracellular expression levels [5, 8]. To address this issue we used forskolin, since we have previously demonstrated that such a treatment was capable of increasing intracellular alpha-synuclein expression in enteric primary culture without any deleterious effect on cell survival [20]. We show in the present survey, that the 2.7 fold increase in intracellular alpha-synuclein expression levels evoked by 20 μM Forskolin for 48 h was not associated with changes in the amount of extracellular alpha-synuclein (Figure 1 E and F). These results are in sharp contrast with that obtained in neuronal cell lines [5, 8] and suggest that the release of alpha-synuclein in the CS by enteric neurons is, at least partially, regulated.

Alpha-synuclein secretion is regulated by neuronal activity

Given that alpha-synuclein seems to be secreted through a regulated mechanism dependent of classical vesicular transport, we tested whether neuronal activity regulates its secretion. A 48 h-treatment of primary culture of ENS with the sodium channel activator veratridine at 30 μM, significantly increased the release of alpha-synuclein in the CS as compared to controls (Figure 2 A and B). This was associated with a statistically significant drop in the level of intracellular alpha-synuclein (Figure 2 A and B). Treatment of primary culture of ENS with the sodium channel blocker tetrodotoxin at 1 μM for 48 h induced a significant increase in intracellular alpha-synuclein expression as compared to controls (Figure 2 A and B). In parallel, a decrease in alpha-synuclein level in the CS was observed but it did not reach significance. Under veratridine and tetrodotoxin treatments, cell viability as assessed by the release of NSE, was not compromised (control veratridine 11,14 ± 2,4 ng/ml; veratridine 14,5 ± 1

ng/ml; n=3, p=1 and control tetrodotoxin 20,6 ± 17,07 ng/ml; tetrodotoxin 35,2 ± 10,46 ng/ml; n=3, p= 0,4).

To further investigate the role of neuronal activity, we studied whether intracellularly accumulated alpha-synuclein can be secreted. To this end, primary culture of ENS were treated with 20 μM Forskolin then depolarized with veratridine. Treatment with veratridine induced the secretion of alpha-synuclein that accumulated following forskolin treatment (figure 2 C and D).

Together, these results demonstrate that alpha-synuclein secretion in enteric neurons is regulated by neuronal activity.

Extracellular alpha-synuclein has no overt effects on epithelial intestinal barrier *in vitro* and *ex vivo*

Growing evidences suggest that the ENS is critically involved in the regulation of the IEB [21]. We first investigated whether extracellular alpha-synuclein is capable of regulating the paracellular permeability of the IEB *in vitro*. Various concentrations of alpha-synuclein ranging from 0.1 to 10 μg/ml were applied to Caco2 cells for 48 h. No changes in paracellular permeability were observed in the presence of alpha-synuclein (Figure 3 A).

The paracellular permeability of the IEB is regulated by tight junctions (TJ) composed of occludin, claudins and zonula occludens proteins (for review [13]). We therefore analyzed whether application of alpha-synuclein induced changes in the expression of the mRNA encoding for occludin, claudins and zonula occludens proteins. The expression levels of occludin1, claudin and zonula occludens mRNA were unaffected in the presence of alpha-synuclein (Figure 3 B, C, D).

We extended these results obtained *in vitro* by *ex vivo* experiments in which colonic biopsies from healthy subjects were submitted to 10 μg/ml alpha-synuclein during 3 h in Ussing Chambers. No changes in the IEB permeability, either paracellular or transcellular, was observed in colonic biopsies in the presence of alpha-synuclein (Figure 4 A and B).

Discussion

We have shown in the present survey that (i) alpha-synuclein is physiologically secreted by enteric neurons (ii) the secretion of alpha-synuclein is regulated by neuronal activity (iii) extracellular alpha-synuclein increases IEB permeability.

Because of the lack of an ER signaling peptide from its sequence, alpha- synuclein was considered to be an exclusive intracellular protein. Using SH-SY5Y cells overexpressing alpha-synuclein, two different groups have convincingly shown that alpha-synculein can be secreted in the extracellular space thereby affecting neighboring neurons [8, 22]. We show in the present report that alpha-synuclein can also be secreted from peripheral and more particularly from enteric neurons. The levels of secreted alpha-synuclein in primary culture of ENS were similar to those described in biological fluids and in tissue [23]. In accordance with the previous reports, secreted alpha-synuclein in primary culture of ENS accumulated over time and was not attributable to cell death as measured by the release of NSE in the extracellular space. This suggests that at least a portion of alpha-synuclein from enteric neurons is secreted in a constitutive manner or following spontaneous enteric neuronal activity.

This led us to investigate the mechanisms of alpha-synuclein secretion by enteric neurons and especially the role of neuronal activity. It has been suggested previously that alpha-synuclein exocytosis is mediated by a non-classical, BFA-independent,

secretory mechanism [5, 8]. In sharp contrast with these two previous reports, we show in the present survey that treatment of primary culture of ENS with BFA resulted in a significant decrease in alpha-synuclein secretion; strongly suggesting that enteric alpha-synuclein follows a conventional secretory pathway. This is reinforced by our observations showing a tight regulation of alpha-synuclein release by neuronal activity, which is known to regulate conventional exocytosis. In support of this idea, a portion presynaptic alpha-synuclein has been shown to be present within synaptic vesicles either in SH-SY5Y cells or in rat brain [5, 24] and neuronal activity controls the presynaptic accumulation of alpha-synuclein [25]. Our study is the first to directly address the regulation of alpha-synuclein secretion by depolarization. Although Lee and collaborators elegantly demonstrated that alpha-synuclein was localized within vesicle lumen, they did not mention the effects of depolarization, likely because alpha-synuclein secretion was BFA-independent in their cell system [5]. More recently, Emmanouilidou et al. showed in SH-SY5Y cells that a portion alpha-synuclein is secreted via an exosomal calcium-dependent mechanism [8]. Nevertheless, as stated in their discussion, it is not possible to rule out the possibility that alternative mechanisms for alpha-synuclein secretion, such as secretory vesicle mediated exocytosis that is also calcium-sensitive, may also operate. Altogether, our results and the available data on alpha-synuclein secretion show that alpha-synuclein secretion from neurons is likely to occur through several pathways, either following conventional or unconventional exocytosis.

Our findings may be relevant to the pathogenesis of PD. Although precise etiology of the disease remains unknown, it is suggested that, besides genetic factors or in combination with, environmental factors could be critically involved. Some recent evidences suggest that the pathological process of PD affects the ENS at a very early stage of the disease [11]. Remarkably, the enteric neurons are directly in contact with

the environment, leading to the postulate, the so-called Braak's hypothesis, that they could represent a route of entry for an hitherto unknown environmental factor to initiate the pathological process further spreading to the CNS and more precisely to the dorsal motor nucleus of the vagus via the vagal preganglionic innervation of the gut [11, 26]. If Braak's theory is true, two necessary conditions must be satisfied. First, an uninterrupted pathway that expresses alpha-synuclein throughout its trajectory should allow the retrograde transport of the pathological process from the gastrointestinal tract to the CNS. Second, enteric neurons should be able to secrete alpha-synuclein in order to transmit the pathological process from cell to cell as suggested for CNS neurons [27]. The first condition is fulfilled as Phillips and co-workers have elegantly shown that vagal efferent axons and terminals, which originate from the dorsal motor nucleus of the vagus, are positive for alpha-synuclein and that some of these preganglionic efferent neurons synapse on alpha-synuclein-positive intrinsic neurons in the myenteric plexus of both the stomach and duodenum [28]. Regarding the second condition, our results allow us to consider that alpha-synuclein could behave in the ENS like in the CNS and thus transmit the pathology from neuron to neuron [29]. Eventually, we have shown that alpha-synuclein has no overt effect on IEB permeability. Although preliminary, a recent study performed *in vivo* suggests that IEB permeability is increased in PD patients. Forsyth and collaborators indeed showed that PD subjects have significantly increased whole gut permeability to sucralose, but not to lactulose and mannitol with respect to healthy subjects. They further correlated IEB permeability with the pathological burden in the ENS, showing that colonic biopsy staining for alpha-synuclein correlated significantly with intestinal hyperpermeability [15]. Our results clearly demonstrate that the changes on IEB permeability observed in PD patients are not due to extracellular alpha-synuclein, which does not impact on IEB permeability. Further

works are therefore needed to decipher the signaling pathways that are involved in the regulation of the IEB in PD patients.

Legends to figures

Figure 1. Alpha-synuclein secretion by enteric neurons. ***A***, After 10 days in culture, the medium of primary culture of ENS was totally replaced for 1 to 48 h. Cells were harvested and homogenized in NETF/NP40 (1%) buffer and 35 µg of protein per sample were subjected to immunoblot analysis using Syn-1 antibodies. In parallel, CS were concentrated and subjected to immunoblot analysis using C-20 antibodies. After stripping, membranes were reprobed with the appropriate loading control (PGP 9.5. or BSA) to ensure equal loading of neuronal or extracellular proteins. ***B***, For quantification, the optical densities of extracellular alpha-synuclein immunoreactive bands were measured, normalized to the optical densities of BSA-immunoreactive bands in the same samples, and expressed as percentages of maximum response. Data correspond to means±SEM of at least 3 experiments per condition. ***C***, After 10 days in culture, the medium of primary culture of ENS was totally replaced and primary cultures were treated with vehicle (control) or with 1 µg/ml of BFA for 6 h. Immunoblots were performed as described in figure 1 A***. D***, quantification of the results for alpha-synuclein protein level was assessed as described in the legend to figure 1 B with the appropriate loading control (PGP 9.5. or BSA). Statistical analysis was done with a Student's t-test followed by Mann-Whitney post-hoc test (treated versus control * $p<0.05$). Data correspond to means±SEM of at least 3 experiments per condition. ***E***, After 10 days in culture, the medium of primary culture of ENS was totally replaced and primary cultures were treated either with vehicle (control), 20 µM of forskolin for 48 h or 20 µM of forskolin for 48 h followed by 30 µM of veratridine for 6 h. Immunoblots were

performed as described in figure 1 A. ***F***, Quantification of the results for alpha-synuclein protein level was assessed as described in the legend to figure 1 B with the appropriate loading control (PGP 9.5 or BSA). Statistical analysis was done with a ANOVA test followed by a Dunnett post-hoc test (treated versus control * $p<0.05$). Data correspond to means±SEM of at least 3 experiments per condition.

Figure 2. Regulation of alpha-synuclein secretion in the ENS. ***A***, After 10 days in culture, the medium of primary culture of ENS was totally replaced and primary cultures were treated with vehicle (control) or with 30 μM of veratridine or with 1 μM TTX for 48 h. Immunoblots were performed as described in figure 1 A***.*** ***B***, quantification of the results for alpha-synuclein protein level was assessed as described in the legend to figure 1 B with the appropriate loading control (PGP 9.5. or BSA). Statistical analysis was done with a ANOVA test followed by a Dunnett post-hoc test (treated versus control * $p<0.05$). Data correspond to means±SEM of at least 3 experiments per condition. ***C***, After 10 days in culture, the medium of primary culture of ENS was totally replaced and primary cultures were treated with vehicle (control) or with 20 μM of forskolin for 72 h or with 20 μM of forskolin followed by 30 μM veratridine treatment for 6 h. Immunoblots were performed as described in figure 1 A***.*** ***D***, quantification of the results for alpha-synuclein protein level was assessed as described in the legend to figure 1 B with the appropriate loading control (PGP 9.5. or BSA). Statistical analysis was done with a ANOVA test followed by a Dunnett post-hoc test (treated versus control * $p<0.05$). Data correspond to means±SEM of at least 3 experiments per condition.

Figure 3. Effects of extracellular alpha-synuclein on IEB in Caco-2 cells. *A*, *In vitro* FITC sulfonic acid flux quantification following a 48h treatment with monomeric alpha-synuclein (from 0.01 to 10 µg/mL) on differentiated Caco-2 cells. **B,** ZO-1 mRNA expression following a 48 h treatment with monomeric alpha-synuclein (from 0.01 to 10 µg/mL), on differentiated Caco-2 cells **C**, Occludin mRNA expression following a 48 h treatment with monomeric alpha-synuclein (from 0.01 to 10 µg/mL), on differentiated Caco-2 cells **D,** Claudin-1 mRNA expression following a 48 h treatment with monomeric alpha-synuclein (from 0.01 to 10 µg/mL), on differentiated Caco-2 cells. Statistical analysis was done with a ANOVA test followed by a Dunnett post-hoc test (treated versus control * $p<0.05$). Data represent the mean SEM of at least 5 experiments per condition.

Figure 4. Effects of extracellular alpha-synuclein on paracellular and transcellular IEB permeability in colon biopsies. *A*, Paracellular permeability of human colonic biopsies in Ussing chambers was assessed by FITC sulfonic acid flux quantification. Biopsies were treated either with vehicle (PBS) or with monomeric alpha-synuclein (10 µg/mL) for 3 h. Statistical analysis was done with a Wilcoxon paired test (n= 8 p= 0,145). ***B*,** Transcellular permeability of human colonic biopsies in Ussing chambers assessed by horseradish peroxidase (HRP) activity quantification. Biopsies were treated either with vehicle (PBS) or with monomeric alpha-synuclein (10 µg/mL) for 3 h. Statistical analysis was done with a Wilcoxon paired test (n= 4, p= 0,184).

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Figure 1

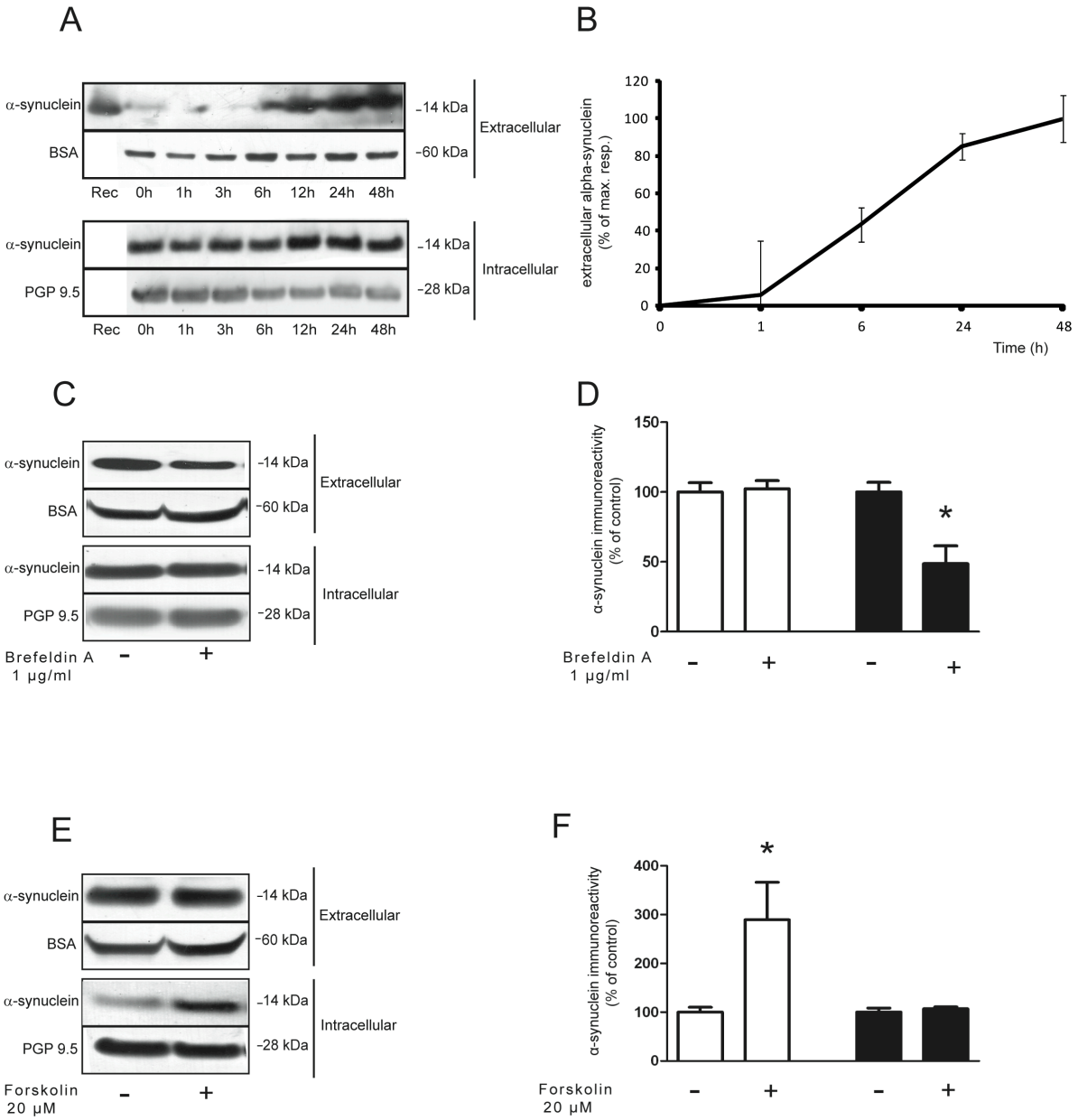

A
α-synuclein
BSA
~14 kDa
~60 kDa
Extracellular
Rec 0h 1h 3h 6h 12h 24h 48h
α-synuclein
PGP 9.5
~14 kDa
~28 kDa
Intracellular
Rec 0h 1h 3h 6h 12h 24h 48h
B
extracellular alpha-synuclein (% of max. resp.)
120
100
80
60
40
20
0
0 1 6 24 48
Time (h)
C
α-synuclein
BSA
~14 kDa
~60 kDa
Extracellular
α-synuclein
PGP 9.5
~14 kDa
~28 kDa
Intracellular
Brefeldin A 1 µg/ml - +
D
α-synuclein immunoreactivity (% of control)
150
100
50
0
*
Brefeldin A 1 µg/ml - + - +
E
α-synuclein
BSA
~14 kDa
~60 kDa
Extracellular
α-synuclein
PGP 9.5
~14 kDa
~28 kDa
Intracellular
Forskolin 20 µM - +
F
α-synuclein immunoreactivity (% of control)
400
300
200
100
0
*
Forskolin 20 µM - + - +

Figure 2

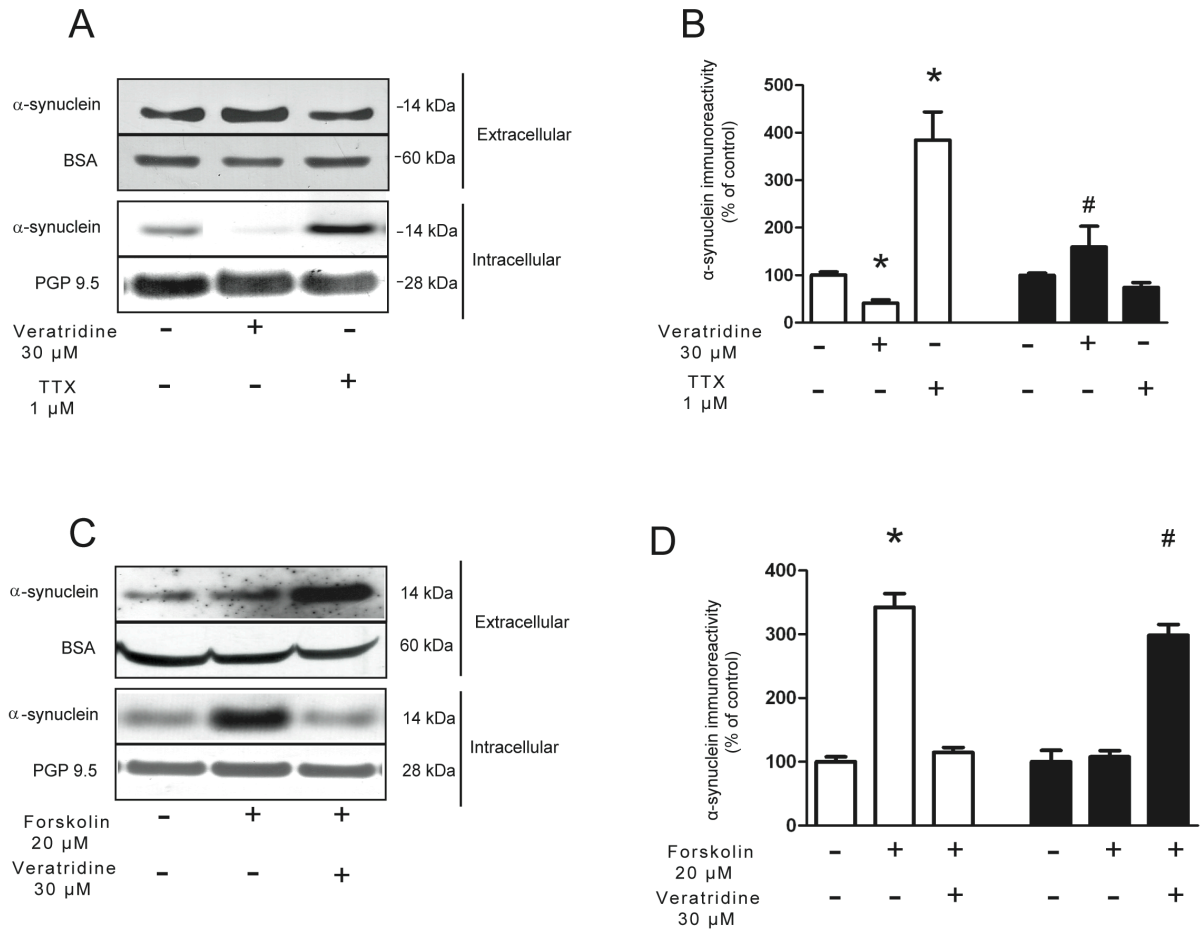

A
α-synuclein
BSA
α-synuclein
PGP 9.5
~14 kDa
~60 kDa
~14 kDa
~28 kDa
Extracellular
Intracellular
Veratridine 30 μM
TTX 1 μM
B
α-synuclein immunoreactivity (% of control)
500
400
300
200
100
0
*
*
#
Veratridine 30 μM
TTX 1 μM
C
α-synuclein
BSA
α-synuclein
PGP 9.5
14 kDa
60 kDa
14 kDa
28 kDa
Extracellular
Intracellular
Forskolin 20 μM
Veratridine 30 μM
D
α-synuclein immunoreactivity (% of control)
400
300
200
100
0
*
#
Forskolin 20 μM
Veratridine 30 μM

Figure 3

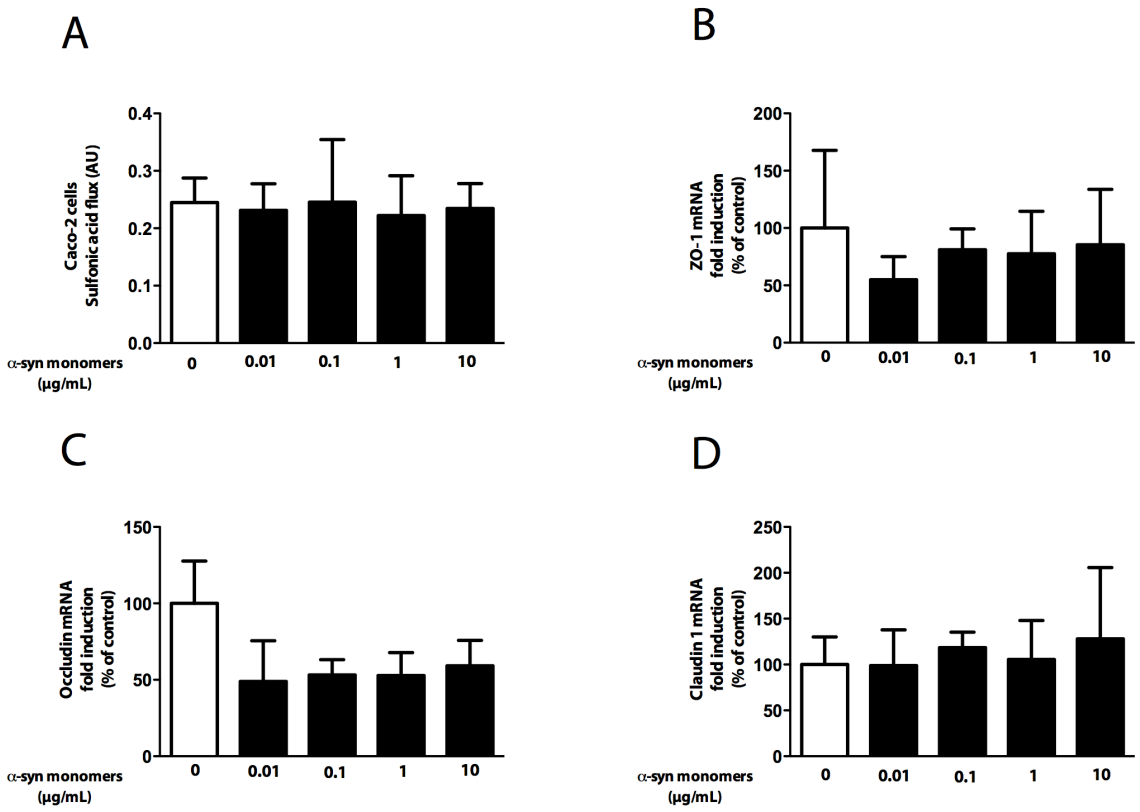
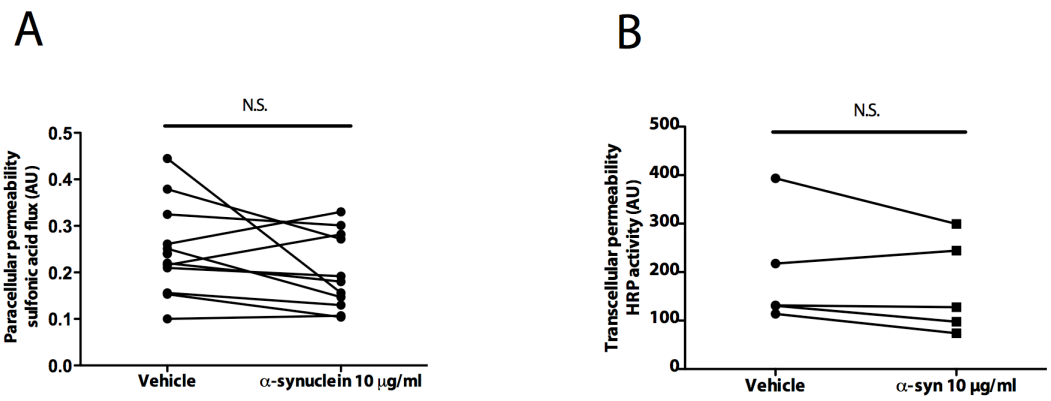

A
Caco-2 cells
Sulfonic acid flux (AU)
0.4
0.3
0.2
0.1
0.0
α-syn monomers
(µg/mL)
0
0.01
0.1
1
10
B
ZO-1 mRNA
fold induction
(% of control)
200
150
100
50
0
α-syn monomers
(µg/mL)
0
0.01
0.1
1
10
C
Occludin mRNA
fold induction
(% of control)
150
100
50
0
α-syn monomers
(µg/mL)
0
0.01
0.1
1
10
D
Claudin 1 mRNA
fold induction
(% of control)
250
200
150
100
50
0
α-syn monomers
(µg/mL)
0
0.01
0.1
1
10

Figure 4


A
N.S.
Paracellular permeability sulfonic acid flux (AU)
0.5
0.4
0.3
0.2
0.1
0.0
Vehicle
α-synuclein 10 μg/ml
B
N.S.
Transcellular permeability HRP activity (AU)
500
400
300
200
100
0
Vehicle
α-syn 10 μg/ml

Discussion

Au cours de ce travail de thèse, nous avons montré que l'activité neuronale était capable de réguler l'expression et la sécrétion de l'alpha-synucléine dans le SNE. Dans cette discussion, nous reviendrons en détail sur les différents points de l'étude que nous commenterons par rapport aux données existantes de la littérature, afin de mieux restituer l'apport de ce travail dans la compréhension de la physiologie de l'alpha-synucléine et son implication dans la MP.

Si beaucoup d'études ont tenté de caractériser les effets pathologiques de l'alpha-synucléine ou encore son rôle dans les processus neurodégénératifs, peu de travaux en revanche se sont attachés à étudier la régulation de son expression. Le groupe de Stefanis a largement contribué à la compréhension des mécanismes régulant l'expression de la protéine par les facteurs neurotrophiques, plus particulièrement au cours du développement du SNC [285]. Il a apporté une contribution certaine dans le déchiffrage du fonctionnement du promoteur du gène SNCA et mis en évidence l'importance de l'intron 1 du gène dans la régulation de l'expression de la protéine [128, 286-289]. Cependant, aucune étude avant la notre n'avait étudié le rôle de l'activité neuronale dans la régulation de l'expression de l'alpha-synucléine. Nous avons montré que la dépolarisation induite par le chlorure de potassium ou l'augmentation de l'AMPc intracellulaire, deux facteurs liés à l'activité neuronale, augmentent l'expression de l'alpha-synucléine dans les neurones entériques. En 2001, un analogue non hydrolysable (8-CPT-cAMP) de l'AMPc avait déjà été testé sur des cellules PC12 mais sans que cela ne provoque de modifications des taux intracellulaires d'alpha-synucléine [286]. Deux raisons peuvent expliquer cette différence avec nos résultats : le type cellulaire utilisé et la durée du traitement. Stefanis a réalisé un traitement de 10 jours au 8-CPT-cAMP sur une lignée de cellules PC12 alors que nous avons traité des cultures primaires de SNE pendant 3 jours avec de la forskoline. Etant donnée la cinétique d'expression de l'ARNm de l'alpha-synucléine induite par la forskoline, il est très probable que les effets du 8-CPT-cAMP aient disparus après 10 jours de traitement.

En utilisant une approche pharmacologique, nous avons montré que les canaux calciques voltage-dépendant de type L participent de façon majeure dans la régulation de l'expression de l'alpha-synucléine, dans un modèle de culture primaire de SNE et *in*

vivo dans le colon proximal de souris. En effet, les canaux calciques de type L sont largement impliqués dans la régulation transcriptionnelle induite par l'activité neuronale dans le SNC [290]. De plus, nous avons récemment montré au laboratoire que l'activation de ces canaux calciques de type L régulait l'expression de la tyrosine hydroxylase dans le SNE [291]. Une fois activés, les canaux calciques de type L recrutent la voie des *mitogen activated protein kinases* (MAPK) de type *extracellular signal regulated kinases* (ERK) pour augmenter l'expression de l'alpha-synucléine dans les neurones entériques. Notre étude n'est pas la première à montrer que les MAP kinases de type ERK sont impliquées dans la régulation de l'alpha-synucléine. Certaines études démontrent le rôle direct des MAP kinases dans l'augmentation de l'expression de l'alpha-synucléine induite par le NGF ou le bFGF [288] et par la forme mutante de la kinase LRRK2, responsable de formes familiales de MP [292]. D'un point de vue physiopathologique, les MAP kinases de type ERK sont présentes sous forme activée et phosphorylée dans les CL, au cours de la MP [293]. Par ailleurs, il a été démontré qu'un traitement à la 6-hydroxydopamine (neurotoxine spécifique des neurones dopaminergiques) induit une activation persistante de ERK qui contribue à la mort des neurones dopaminergiques *in vitro* [294]. Dans une autre étude, Iwata montre en 2001, que l'alpha-synucléine peut interagir avec Elk-1 *(E twenty-six-like transcription factor-1)* un facteur de transcription activé par ERK et le séquestrer dans le cytosol [295] . Prises ensemble ces données suggèrent que l'état d'activation anormal de ERK ou une dysfonction de ses effecteurs pourraient conduire à la mort des neurones dopaminergiques.

Nous avons logiquement essayé de caractériser les facteurs de transcription impliqués dans la régulation de l'alpha-synucléine par la dépolarisation et l'augmentation de l'AMPc. Nous nous sommes tout d'abord intéressés à la voie *ribosomal s6 kinase* (RSK), une kinase directement en aval des MAP kinases de type ERK, en utilisant un inhibiteur spécifique, le BI-D1870 ([296], offert par Phillip Cohen ; University of Dundee). Le BI-D1870 n'a pas eu d'effet sur l'augmentation de l'expression de l'alpha-synucléine provoquée par la dépolarisation et l'AMPc excluant la participation de RSK dans ce processus. Dans une approche plus large, nous avons voulu déterminer le rôle de CREB (*cAMP response element-binding*), un facteur de transcription activé par la voie ERK, notamment au cours d'une élévation de calcium intracellulaire (pour revue [297]), en diminuant son expression par une approche siRNA. Malheureusement, nous

n'avons pas réussi à diminuer significativement le niveau d'expression de CREB dans nos cultures primaires de SNE. Ceci est probablement expliqué par le fait que les neurones des cultures primaires de SNE sont des cellules difficilement transfectables (moins de 2% des neurones avec une approche lipofectamine; données Bernard Lardeux Inserm 913). Toutefois, plusieurs études ont identifié certains facteurs de transcription impliqués dans la régulation de l'expression de l'alpha-synucléine par les MAP kinases de type ERK. Ainsi, les facteurs de transcription tels que ZNF219 et ZSCAN21 sont responsables de l'activation de la transcription du gène SCNA dans les cellules PC12 et dans les cultures primaires de neurones de cortex cérébral [128]. En 2008, Scherzer en analysant les variations d'expression des gènes des globules rouges, associe les variations d'expression du facteur de transcription GATA 1 avec celui de 4 autres gènes dont l'alpha-synucléine et détermine que les facteurs de transcription GATA 1 et 2 sont aussi responsables de transcription du gène SCNA. Par une analyse en ChiP (*Chromatin immunoprecipitiation)*, il détermine que GATA 1 se fixe sur une séquence du premier intron de SCNA tout comme ZSCAN21, confirmant l'importance de cette partie intronique dans la régulation de la transcription du gène. De façon intéressante, GATA 1 semble être spécifique des érythrocytes (cellules qui expriment également l'alpha-synucléine) alors que GATA 2 est plus largement exprimé notamment dans les structures cérébrales telles que le cortex frontal ou la substance noire [298]. Les facteurs de transcription GATA peuvent être activés par les MAP kinases ERK [299-301], ce qui renforce encore l'implication de ces kinases dans la régulation de l'alpha-synucléine.

La voie ERK n'est toutefois pas la seule voie de signalisation impliquée dans la régulation de l'expression de l'alpha-synucléine. Les voies *phosphatidyl inositol tri-phosphate kinase* (PI3K) et p38 *stress activated protein kinase* sont aussi mises en jeu en réponse aux facteurs de croissance [288, 289]. Il est important de préciser que les données actuellement disponibles sur le rôle des MAP kinases de type ERK dans l'expression de l'alpha-synucléine, ont été obtenues *in vitro* [288, 298]. Il sera essentiel d'avoir à l'avenir des approches plus globales *in vivo* pour confirmer ces données préliminaires, par exemple en utilisant le SL327, un inhibiteur de ERK qui peut être injecté par voie générale [302]. Les premiers résultats que nous avons obtenus, montrent que le SNE exprime non seulement l'alpha-synucléine mais aussi que son

expression peut être modulée. Ceci suggère que les neurones du SNE pourraient jouer un rôle similaire aux neurones centraux dans le processus physiopathologique de la MP.

C'est en 2003 que pour la première fois, en montrant l'accumulation de l'alpha-synucléine dans le milieu de cellules M17, qu' El Agnaf montre que l'alpha-synucléine n'est pas uniquement une protéine intracellulaire [150]. Depuis un nombre grandissant d'études ont alors été menées afin de déterminer le rôle potentiel de l'alpha-synucléine extracellulaire et surtout de comprendre comment sa sécrétion pouvait contribuer au développement de la MP. Dans cette optique, étant donné la précocité de l'atteinte du SNE et l'importance de l'alpha-synucléine dans la MP, nous nous sommes attachés à étudier si le SNE était capable de sécréter l'alpha-synucléine. Grâce au modèle de cultures primaires de SNE, mis au point au laboratoire, nous avons pu mettre en évidence que les neurones entériques sont capables de sécréter l'alpha-synucléine de façon constitutive avec une quantité d'un ordre de grandeur équivalent à ce qui est retrouvé au niveau central [151].

Nous avons montré pour la première fois qu'un stimulus physiologique tel que la dépolarisation par l'activation des canaux sodiques voltage-dépendants pouvait réguler directement la sécrétion de l'alpha-synucléine et que cette sécrétion était bloquée par la bréfeldine A, un inhibiteur de l'exocytose conventionnelle. En 2005 Fortin décrit que l'activation des canaux sodiques voltage dépendant induite par la dépolarisation, conditionne la localisation et le mouvement de l'alpha-synucléine dans les terminaisons présynaptiques des neurones. De façon consécutive, il démontre que la dépolarisation appauvrit les boutons synaptiques en alpha-synucléine sans pour autant diminuer les quantités totales de la protéine dans les neurones [303]. D'autres données démontent que l'alpha-synucléine est présente dans les vésicules de sécrétion à cœur dense dans des cultures primaires de neurones corticaux ou des SH-SY5Y [74]. De façon remarquable la libération de ces vésicules de sécrétion est dépendante de la dépolarisation et de l'augmentation de calcium intracellulaire, conditions reproduites par l'activité neuronale [304], mais l'effet de la dépolarisation sur la sécrétion d'alpha-synucléine n'a hélas pas été étudié par Lee et collaborateurs [74]. Une autre voie de sécrétion de l'alpha-synucléine a été identifiée et met en jeu une participation du calcium intracellulaire et des exosomes [152]. De façon consensuelle, les deux études menées par le groupe de Lee ainsi que celui d'Emmanoulouidou ont montré que la sécrétion de l'alpha-synucléine est un processus actif qui relève de l'exocytose,

puisqu'elle est bloquée par une baisse de température (bloqueur général de l'exocytose). Cependant il semblerait qu'elle soit indépendante de la voie d'exocytose conventionnelle réticulum endoplasmique /golgi puisqu'elle est insensible à la bréfeldine A [74, 152]. Ces données sont en désaccord avec les résultats que nous avons obtenus dans nos cultures primaires de SNE. Remarquablement, ces deux études relatent que les niveaux intracellulaires conditionnent la sécrétion de l'alpha-synucléine et suggèrent un processus plutôt passif. Or, nous avons montré que l'augmentation de l'alpha-synucléine induite par un traitement de 72 h à la forskoline, n'influençait pas la sécrétion de l'alpha-synucléine dans les cultures primaires. En revanche, nous avons montré que le même traitement associé à l'activation de canaux sodiques voltage-dépendant libère de façon directement proportionnelle aux niveaux intracellulaires, l'alpha-synucléine dans le milieu extracellulaire. Ces données suggèrent que dans notre modèle la sécrétion de l'alpha-synucléine suit un processus actif. Une des explications possibles concernant ces différences, est que dans les deux précédentes études, les modèles utilisés sont des lignées neuronales de neuroblastomes surexprimant de façon artificielle l'alpha-synucléine (SH-SY5Y ou PC12) [74, 152], alors que notre étude s'intéresse à la sécrétion de l'alpha-synucléine endogène dans un système neuronal entérique intégré (neurones, cellules gliales entériques, cellulaire musculaires). Une surexpression de l'alpha-synucléine ou encore le modèle cellulaire utilisé sont autant de facteurs qui peuvent influencer l'exocytose de la protéine.

Une des théories qui prévaut à l'heure actuelle quant à la dissémination de la maladie de Parkinson, est que l'alpha-synucléine pourrait se comporter comme une protéine « prion », ou en d'autres termes qu'elle possèderait des qualités infectieuses. Une des étapes clefs dans ce mode de propagation est que la protéine doit pouvoir quitter la cellule hôte pour contaminer la cellule cible. La mise en évidence de la sécrétion de l'alpha-synucléine par le système nerveux entérique est capitale, puisque celui-ci est proposé comme le premier maillon dans le développement de la MP (Hypothèse de Braak). De plus, s'il s'avère que la régulation de l'expression et de la sécrétion de l'alpha-synucléine est sous le contrôle de l'activité neuronale dans le SNE, un dysfonctionnement de celle-ci engendrerait une surexpression de la protéine, avec la possible formation de formes pathologiques liées à une élévation de sa concentration, associée à une augmentation de sa sécrétion, contribuant ainsi à libérer des formes toxiques de l'alpha-synucléine. La question du devenir de l'alpha-synucléine se pose

ensuite. D'ores et déjà, il est démontré que l'alpha-synucléine extracellulaire libérée par le SNC est neurotoxique [157], est capable d'induire un processus inflammatoire en activant les cellules microgliales [167] et qu'elle peut entrer sous différentes formes dans les neurones pour initier un processus de nucléation [163, 164, 166]. Cependant, aucune information sur le devenir de l'alpha-synucléine n'est disponible dans le SNE, à l'exception d'une publication de Lee et collaborateurs parue en 2011 [305]. Elle démontre que des lysats de cerveau de patients souffrant de démence à corps de Lewy et présentant des formes solubles d'alpha-synucléine de haut poids moléculaire (oligomères ou protofibrilles), injectés directement dans la paroi de l'estomac de souris surexprimant la forme mutante A53T de l'alpha synycléine humaine, contribuent de façon significative à la formation d'agrégats dans le plexus myentérique. Cependant cette étude ne met pas en évidence la formation d'agrégats dans les souches de souris sauvages et ne démontre pas formellement l'implication de l'alpha-synucléine dans le développement de la maladie.

De notre côté, nous avons voulu caractériser les effets extracellulaires de l'alpha-synucléine sur les fonctions digestives et plus particulièrement sur la BEI. Nous avons montré que l'alpha-synucléine n'avait pas d'effet sur la perméabilité des tapis cellulaires de cellules Caco-2 confluents, ni sur la perméabilité de biopsies coliques humaines en chambre de Ussing après 3 h de traitement. Ces premiers résultats nous permettent de penser que l'alpha-synucléine n'a pas d'effet direct sur la BEI et qu'elle n'interagit pas directement avec l'épithélium. A ce jour 3 articles préliminaires sur l'étude de la BEI au cours de la MP, réalisée in vivo ont été publiés. Un travail initial ne montrait pas de modification significative de perméabilité de la BEI chez les parkinsoniens par rapport aux témoins [279] alors que deux études plus récentes montrent une augmentation de la perméabilité de la BEI chez faible échantillon de patients parkinsoniens [280, 306]. Il nous semble donc important de confirmer ou d'infirmer ces données en évaluant la perméabilité paracellulaire et transcellualire en chambre d'Ussing de patients parkinsoniens (travail en cours au laboratoire)

Ce travail est le premier à s'intéresser à la régulation de l'expression et de la sécrétion de l'alpha-synucléine dans le SNE. A l'instar du SNC, le SNE un organe qui peut intervenir de façon majeure dans l'initiation et la progression de la MP. En raison de sa proximité avec l'environnement, le SNE est avec le système olfactif, le mieux placé pour

être la cible d'un pathogène ou un neurotoxique exogène. De façon remarquable, une étude montre que des souris gavées quotidiennement à la roténone à faible dose développent une synucléinopathie entérique associée à une atteinte de la substance noire [307]. Néanmoins cette étude ne permet pas d'étudier la chronologie de l'atteinte entre le SNE et le SNC. S'il s'avère qu'une simple vagotomie est capable de bloquer l'apparition des lésions vers le SNC dans ce modèle, ceci pourrait être un argument important en faveur de la théorie de Braak. Un autre argument fort sur l'implication de l'expression de l'alpha-synucléine et le rôle du SNE dans la MP est apporté par Kuo lorsqu'il démontre avec une approche de chromosomes artificiels codant pour SNCA que la surexpression de l'alpha-synucléine dans le SNE reproduit la majorité des symptômes prémoteurs [308].

Notre étude, bien qu'encourageante soulève de nombreuses questions. Il reste important d'essayer d'identifier plus précisément les voies de signalisation impliquées dans la régulation de l'expression de l'alpha-synucléine, et en particulier les facteurs de transcription associés à la voie ERK ainsi que les mécanismes impliqués dans la sécrétion de l'alpha synucléine. Enfin, toujours dans l'hypothèse d'une origine digestive de la maladie, notre modèle de culture primaire de SNE nous permettra de savoir si l'alpha-synucléine extracellulaire peut être captée par les neurones entériques.

Participation à des travaux tiers et articles de revue

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Système nerveux entérique et maladie de Parkinson

Enteric nervous system and Parkinson's disease

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RÉSUMÉ

Le processus pathologique de la maladie de Parkinson ne se limite pas à la substance noire mais touche aussi des systèmes nerveux périphériques, et en particulier, le système nerveux entérique. Nous discutons dans cet article le rôle des lésions du système nerveux entérique dans la diffusion du processus pathologique de la maladie de Parkinson (hypothèse de Braak) et dans les troubles digestifs des patients parkinsoniens. Enfin, en raison de son accessibilité par biopsies, nous mettons en perspective l'utilisation du système nerveux entérique comme source de biomarqueurs de la maladie de Parkinson.

Keywords:
Parkinson's disease
Enteric nervous system
Lewy bodies
Colon
Biopsies

ABSTRACT

It has become increasingly evident over the last years that Parkinson's disease is a multicentric neurodegenerative disease that affects several neuronal structures outside the substantia nigra, among which is the enteric nervous system. The aims of the present article are to discuss the role of the enteric nervous system lesions in pathology spreading (Braak's hypothesis) and in the gastrointestinal dysfunction encountered in Parkinson's disease. Owing to its accessibility to biopsies, we further discuss the use of the enteric nervous system as an original source of biomarker in Parkinson's disease.

La maladie de Parkinson (MP) est classiquement décrite comme une maladie dégénérative des neurones dopaminergiques de la substance noire, à l'origine des signes moteurs de la maladie. Cette perte neuronale de la substance noire s'accompagne d'inclusions intracellulaires dans les somas (corps de Lewy) ou dans les neurites (prolongements de Lewy) des neurones survivants. Un des composants des corps et prolongements de Lewy est l'alpha-synucléine, une protéine neuronale dont la fonction précise n'est pas connue. L'utilisation d'anticorps dirigés contre la synucléine ou sa forme phosphorylée est désormais la technique de référence pour identifier la pathologie de Lewy en immunohistochimie [1].

Toutefois, les neurones de la substance noire ne sont pas les seuls ni les premiers atteints par le processus neurodégénératif au cours de la MP. D'autres régions du système nerveux central et du système nerveux périphérique sont en effet touchées précocement par la pathologie de Lewy. C'est en particulier le cas du système nerveux entérique (SNE) qui est atteint chez la quasi-totalité des patients parkinsoniens [2].

1. Le système nerveux entérique est un second cerveau

Le SNE est un réseau neuronal distribué tout le long du tube digestif, composé de plus de 100 millions de neurones, soit autant que dans la moelle épinière. Cette subdivision du système nerveux végétatif est parfois appelée « le second cerveau » car elle est capable de réguler de façon autonome les fonctions digestives, en partie indépendamment du système nerveux central (SNC) [3].

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Le SNE est organisé en deux plexus principaux : le plexus myentérique (Auerbach), qui contrôle essentiellement la motilité, et le plexus sous-muqueux (Meissner) qui est principalement impliqué dans la régulation de la sécrétion (Fig. 1). Les plexus myentérique et sous-muqueux sont de vastes réseaux nerveux organisés en ganglions reliés par des fibres inter-ganglionnaires (Fig. 1). Au sein des ganglions, les neurones entériques sont fonctionnellement variés. Les différents neurotransmetteurs qu'ils synthétisent définissent leur codage neurochimique et déterminent leur rôle physiologique. Plus d'une trentaine de neurotransmetteurs ont été identifiés dans le SNE : le peptide vasoactif intestinal et le monoxyde d'azote ont un effet inhibiteur sur la motilité digestive alors que l'acétylcholine a un effet prokinétique [3]. La dopamine est aussi un neurotransmetteur du SNE ; chez l'Homme, les neurones dopaminergiques sont répartis selon un gradient aboro-oral, représentant 4 % de l'ensemble des neurones entériques dans le côlon et 20 % dans l'estomac [4]. Leur rôle précis n'est pas connu mais il semble qu'ils aient un rôle inhibiteur sur la motilité digestive.

Bien que le SNE puisse fonctionner indépendamment, le SNC exerce une régulation importante sur le SNE par des afférences et efférences sympathiques (issues du ganglion sympathique prévertébral) et parasympathiques (issues du noyau dorsal moteur du vague et du noyau parasympathique sacré) [5]. Les efférences parasympathiques pourraient jouer un rôle dans la physiopathologie de la maladie en permettant la diffusion du processus pathologique du SNE au SNC (voir paragraphe sur les conséquences de l'atteinte du SNE dans la MP) [5].

2. Le système nerveux entérique est atteint fréquemment et précocement au cours de la maladie de Parkinson

Les premières lésions du SNE dans la MP ont été décrites dans les années 1980 à 1990. Des inclusions semblables aux corps et prolongements de Lewy ont été mises en évidence dans le plexus myentérique et à un moindre degré dans le plexus sous-muqueux du côlon, du tiers inférieur de l'œsophage et de l'estomac. Dans leur ensemble, ces premières publications sur l'implication du SNE dans la maladie de Parkinson ne rencontrent alors qu'un écho modeste (revue dans [6]).

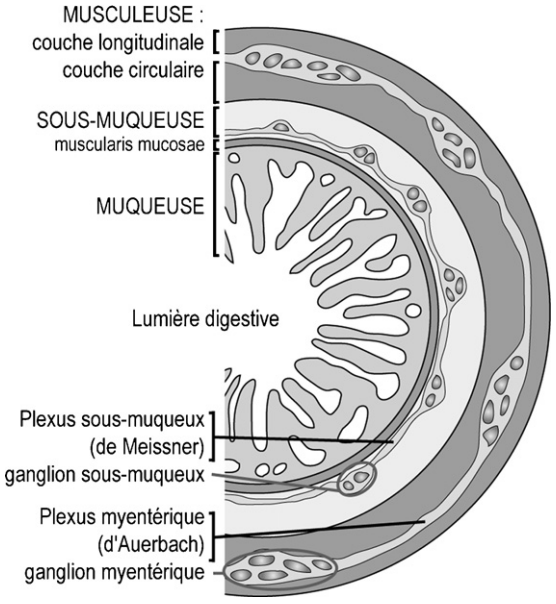

Fig. 1. Coupe transversale de tube digestif avec visualisation du système nerveux entérique (SNE). Le plexus myentérique (Auerbach) contrôle essentiellement la motilité ; le plexus sous-muqueux (Meissner) est principalement impliqué dans la régulation de la sécrétion et de la microcirculation de l'épithélium. Les biopsies réalisées en routine au cours d'une endoscopie digestive permettent d'analyser le plexus sous-muqueux [14,15].

Ce sont les travaux fondateurs de Heiko Braak en 2002 et 2006 qui ont permis au SNE de faire un retour en force sur la scène parkinsonienne [7,8]. La vaste étude neuropathologique menée par cet anatomiste allemand a permis de mettre en évidence une progression temporospatiale ascendante des corps de Lewy dans le tronc cérébral et le prosencéphale, classée en six stades [7]. De façon frappante, les premières lésions encéphaliques de la MP apparaissent dans le bulbe olfactif et surtout dans le noyau dorsal du vague, bien avant l'atteinte de la substance noire. Le nerf vague innervant la quasi-totalité du tractus digestif, l'hypothèse de l'atteinte inaugurale du SNE dans la maladie de Parkinson voit le jour [8].

Depuis, plusieurs travaux autopsiques de grande envergure ont été menés afin de mieux caractériser l'atteinte du SNE au cours de la MP. Nous retiendrons tout particulièrement l'étude de *l'Arizona Consortium* qui a permis de montrer que les lésions du SNE sont présentes chez la quasi-totalité des patients parkinsoniens (21 sujets sur 23) [2]. Des expériences complémentaires sur ces mêmes prélèvements autopsiques ont montré qu'il n'y a pas de perte neuronale significative dans les plexus myentériques et sous-muqueux au cours de la MP en particulier des neurones dopaminergiques et que la pathologie de Lewy n'affecte pas préférentiellement un sous-type de neurone entérique [9].

3. Conséquences de l'atteinte du système nerveux entérique dans la maladie de Parkinson

L'atteinte du SNE chez les patients parkinsoniens peut avoir deux conséquences principales : les lésions du SNE seraient à l'origine des troubles digestifs fréquents au cours de la MP et seraient impliqués dans la diffusion et donc dans la physiopathologie de la maladie (Hypothèse de Braak) [10].

Les signes digestifs sont les plus fréquents des manifestations non-motrices de la maladie, pouvant impliquer l'ensemble du tractus digestif. Les troubles de la vidange gastrique, à l'origine de nausées, de ballonnement postprandial et d'une réduction des prises alimentaires, touchent plus d'un tiers des patients. La constipation est très commune dans la MP. Sa prévalence a été récemment mesurée à 59 % en utilisant les critères de définition internationaux de Rome III, soit presque trois fois plus que chez les sujets témoins d'âge équivalent [10]. Enfin, la dysfonction anorectale (dyschésie), difficile à distinguer de la constipation fonctionnelle sur le seul interrogatoire, est également très fréquente chez les parkinsoniens. Il est tentant de proposer que ces troubles de la motilité digestive soient la conséquence des lésions du SNE. Toutefois, cela n'est pas prouvé et il est tout à fait possible qu'ils résultent de l'atteinte parasympathique et en particulier de l'atteinte du noyau dorsal moteur du vague [10,11].

Le bulbe olfactif et le noyau dorsal du vague sont des structures en contact indirect avec le milieu extérieur, par l'intermédiaire des nerfs olfactifs et du nerf vague. C'est pourquoi Braak suggère l'implication d'un toxique environnemental neurotrope, inhalé ou ingéré, qui traverserait la muqueuse nasale et la barrière épithéliale intestinale et qui serait à l'origine de la MP. Le noyau dorsal du nerf vague, qui innerve la majeure partie du tube digestif, serait atteint par transport rétrograde, un scénario semblable à celui proposé pour le nouveau variant du prion [10,11]. Les données cliniques et épidémiologiques apportent des arguments en faveur du modèle de Braak, puisque les troubles de l'olfaction et la constipation peuvent constituer des signes précurseurs ou des facteurs de risque de maladie de Parkinson [12]. Ainsi, le SNE serait le premier maillon d'une chaîne d'événements dégénératifs menant à la substance noire. Cette hypothèse de Braak, bien que séduisante repose néanmoins

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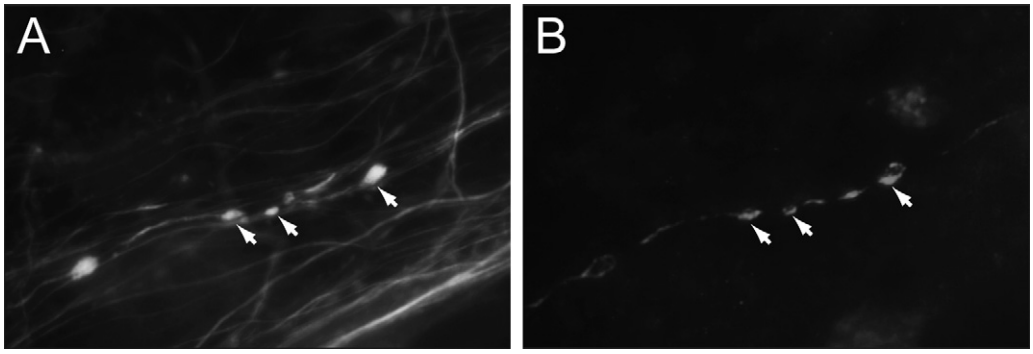

Fig. 2. Présence de prolongements de Lewy dans le plexus sous-muqueux d'un patient parkinsonien. Immunomarquage phospho-synucléine permettant de mettre en évidence les prolongements de Lewy (B). Le marquage NF220 confirme bien qu'il s'agit de structures neuronales (A).

encore sur des fondations fragiles et incertaines. Trois objections peuvent être opposées :

- l'atteinte du SNE et du nerf vague n'est qu'un cas particulier de l'atteinte du système nerveux végétatif. Centré sur le nerf vague, parasympathique, le modèle de Braak est battu en brèche par l'atteinte diffuse du système végétatif dans la MP. Une dénervation sympathique associée à des inclusions d'alpha-synucléine a été décrite au niveau du cœur, de la peau, ou du SNE lui-même qui reçoit une double innervation parasympathique et sympathique [6] ;
- la classification de Braak repose sur le postulat du caractère pathogène obligatoire des corps de Lewy et non sur la présence d'une perte neuronale
- l'hypothèse d'une origine entérique de la maladie de Parkinson repose largement sur la précocité de l'atteinte du noyau dorsal du vague à l'étage encéphalique.

Kurt Jellinger, neuropathologiste viennois, a récemment éprouvé cette classification en analysant 71 cas de maladie de Parkinson issus d'une banque de cerveaux londonienne. Dans cette série, seuls 53 % des cas sont compatibles avec le modèle de progression caudo-rostral décrit par Braak [13].

4. Le système nerveux entérique, une source de biomarqueurs de la maladie de Parkinson

Le diagnostic de MP reste essentiellement clinique et il n'existe pas, à l'heure actuelle, de biomarqueur validé permettant de faire un diagnostic précoce ou de sévérité de la maladie. Le développement de biomarqueurs a été entravé par la conception traditionnelle de la maladie, selon laquelle l'atteinte neuropathologique prédomine au niveau du mésencéphale, région difficilement accessible aux biopsies. Dans ce contexte, le SNE, qui est accessible et analysable par biopsies digestives, permettrait l'analyse du processus neuropathologique du vivant du patient [14].

Nous avons montré que les neurones du plexus sous-muqueux pouvaient être analysés en utilisant des biopsies coliques réalisées en routine [15]. Cette technique nous a permis d'analyser la présence de pathologie de Lewy du vivant des patients parkinsoniens et de faire des corrélations anatomocliniques. Des prolongements de Lewy étaient présents chez 21 patients parkinsoniens sur 29 et chez aucun des témoins (Fig. 2) [15]. La charge lésionnelle des biopsies évaluée par un score quantitatif était corrélée à la sévérité de l'atteinte clinique : les patients avec une charge lésionnelle importante étaient ceux dont les signes axiaux (instabilité posturale, dysarthrie notamment) étaient les plus marqués [15]. Ces signes axiaux traduisent habituellement la sévérité de la maladie et il est donc possible d'envisager l'utilisation de l'analyse des biopsies coliques comme biomarqueur de sévérité de la maladie. Une étude est en cours pour savoir si une telle analyse permettrait de différencier la MP des autres syndromes parkinsoniens dégénératifs tels que l'atrophie multi-systématisée ou la paralysie supranucléaire progressive (biomarqueur de diagnostic différentiel). À terme, il est possible d'envisager l'utilisation des biopsies coliques combinée à d'autres biomarqueurs (tests olfactifs par exemple) pour un diagnostic précoce de la MP, avant l'apparition des signes moteurs de la maladie [14].

5. Conclusion

Le SNE, longtemps oublié dans la MP, revient sur le devant de la scène. Il est toutefois nécessaire de mieux caractériser son atteinte chez le malade parkinsonien et de développer des modèles animaux plus pertinents que ceux actuellement disponibles [9] afin de déterminer le rôle des lésions entériques dans la physiopathologie des troubles digestifs et dans la diffusion de la maladie. L'analyse du SNE par biopsies promet d'être une source originale de biomarqueurs de MP.

Déclaration d'intérêts

Les auteurs déclarent ne pas avoir de conflits d'intérêts en relation avec cet article.

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Colonic Biopsies to Assess the Neuropathology of Parkinson's Disease and Its Relationship with Symptoms

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Abstract

Background: The presence of Lewy bodies and Lewy neurites (LN) has been demonstrated in the enteric nervous system (ENS) of Parkinson's disease (PD) patients. The aims of the present research were to use routine colonoscopy biopsies (1) to analyze, in depth, enteric pathology throughout the colonic submucosal plexus (SMP), and (2) to correlate the pathological burden with neurological and gastrointestinal (GI) symptoms.

Methodology/Principal Findings: A total of 10 control and 29 PD patients divided into 3 groups according to disease duration were included. PD and GI symptoms were assessed using the Unified Parkinson's Disease Rating Scale part III and the Rome III questionnaire, respectively. Four biopsies were taken from the ascending and descending colon during the course of a total colonoscopy. Immunohistochemical analysis was performed using antibodies against phosphorylated alpha-synuclein, neurofilaments NF 220 kDa (NF) and tyrosine hydroxylase (TH). The density of LN, labeled by anti-phosphorylated alpha-synuclein antibodies, was evaluated using a quantitative rating score. Lewy pathology was apparent in the colonic biopsies from 21 patients and in none of the controls. A decreased number of NF-immunoreactive neurons per ganglion was observed in the SMP of PD patients compared to controls. The amount of LN in the ENS was inversely correlated with neuronal count and positively correlated with levodopa-unresponsive features and constipation.

Conclusion/Significance: Analysis of the ENS by routine colonoscopy biopsies is a useful tool for pre-mortem neuropathological diagnosis of PD, and also provides insight into the progression of motor and non-motor symptoms.

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Introduction

Normal function of the gastrointestinal (GI) tract relies both on intrinsic reflexes and extrinsic control. The extrinsic innervation depends on parasympathetic and sympathetic outputs. The intrinsic innervation relies on the enteric nervous system (ENS), an integrative neuronal network organized in two main plexuses, myenteric and submucosal, that control bowel motility and transmucosal fluid exchange, respectively [1]. A wide range of GI diseases associated with motility dysfunction can be considered, in part, as extrinsic and/or enteric neuropathies [2]. An emerging concept is that the field of enteric neuropathies extends well beyond digestive diseases, and that a subset of central nervous system (CNS) disorders may present with concomitant alterations of the ENS [3,4,5]. Among those, Parkinson's disease (PD) is likely to be a prime example because alterations of the ENS and GI dysfunction have been described in the course of the disease [6]. Whether these alterations mirror brain pathology, and how they relate to clinical symptoms, remain open questions.

PD is indeed much more than a selective degeneration of the substantia nigra. The loss of nigral dopaminergic neurons is responsible for the cardinal motor symptoms of PD (i.e. bradykinesia and/or rest tremor), that are improved by dopamine replacement therapy [7]. Yet PD patients also suffer from a wide variety of dopa-unresponsive symptoms likely to reflect lesions beyond the substantia nigra [8]. Most of the non-dopaminergic symptoms appear or worsen with advancing age and disease progression, and represent the majority of the disability observed

in advancing PD [9]. They include dysautonomia and axial symptoms, such as dysarthria, gait and postural instability, and cognitive decline [10]. Among GI symptoms, chronic constipation (CC) is by far the most frequent, affecting up to 60% of PD patients [11].

The pathological hallmarks of PD are neuronal inclusions termed Lewy bodies and Lewy neurites (LN) whose main component is aggregated and phosphorylated alpha-synuclein [12,13,14]. PD pathology concentrates in susceptible regions of the CNS and peripheral autonomic nervous system, including the ENS [15]. Lewy bodies within the ENS, first reported in 1984 [16], provide a putative anatomical basis for GI symptoms [17].

We have recently shown that whole-mounts of submucosa from routine colonic biopsies allow a morphological analysis of the submucosal plexus (SMP) [18,19]. Using this technique in a pilot study, we have demonstrated that 4 out of 5 PD patients display Lewy pathology. Nevertheless, the small number of patients included did not enable us to draw any clinicopathological correlations or to assess the pathology in detail. We have therefore conducted the present study in a larger set of 30 PD patients to allow in depth analysis of enteric pathology throughout the colonic SMP, and to correlate the extent of pathology with motor symptoms and constipation.

Methods

Subjects

PD patients aged 40–75 years were recruited over 24 months from the movement disorder clinic in Nantes University Hospital, France. Diagnosis was made according to the United Kingdom Parkinson's Disease Survey Brain Bank [20]. To limit recruitment bias and in order to span the entire course of PD, 3 groups of patients divided according to disease duration were included (group 1: $\leq$6 years, group 2: 7–12 years and group 3: $\geq$13 years disease duration).

Healthy patients requiring a total colonoscopy for colorectal cancer screening were included as controls. None of the control subjects had a history of neurological or psychiatric diseases.

Patient evaluation

In PD patients, motor symptoms were assessed using the Unified Parkinson's Disease Rating Scale part III (UPDRS-III) [21]. UPDRS-III was performed only in ON-state for group 1 and in both OFF and ON-state for groups 2 and 3. OFF-state was obtained following an overnight withdrawal of dopaminergic treatment, and ON-state was reached one hour after intake of the normal morning dose. Dopa-responsiveness was defined as the percentage of UPDRS-III improvement compared with baseline. UPDRS-III score was subdivided into an axial score (sum of items 18, 19, 22 and 27–30) that evaluates symptoms such as dysarthria or postural instability [22].

Assessment of GI symptoms was performed using the Rome III questionnaire. Chronic functional constipation was diagnosed as defined by Rome III criteria [23]. The sum of the 6 constipation items on the Rome III questionnaire (questions 9 to 14) was used as a semi-quantitative score to assess the severity of CC.

All controls underwent a neurological examination to rule out PD symptoms and cognitive deficiency. The study protocol was approved by the local Committee on Ethics and Human Research (Comité de Protection des Personnes Ouest VI), and registered on ClinicalTrials.gov (identifier NCT00491062). Written informed consent was obtained from each patient and from each normal volunteer.

Colonoscopy biopsies

A total colonoscopy was performed according to the usual procedure of the Gastroenterology department of Nantes University Hospital. In both patients and controls, 4 biopsies were taken in the ascending colon and descending colon, respectively. Biopsies were performed using standard biopsy forceps without needles (FB210K, Olympus co., Japan). Samples were immediately immersed in 4°C saline solution and processed as described.

Immunohistochemistry

Submucosa samples were processed for whole-mount immunostaining as described previously [18]. The primary antibodies used were those directed against phosphorylated alpha-synuclein (1:5000, WAKO, Osaka, Japan), neurofilament H 200 kDa (NF, 1:250, Chemicon, USA), Hu C/D (1:200, Invitrogen, Cergy Pontoise, France), tyrosine hydroxylase (TH, 1:500, Pel-Freez, USA) and dopamine-beta-hydroxylase (DBH, 1:250, Millipore, USA). Suitable secondary antibodies conjugated to Alexa Fluor 488, 594 and 647 were used (Invitrogen, Cergy-Pontoise, France).

Neuronal cell counting and scoring

Neuronal counts were performed in one submucosa sample from the ascending and descending colon, respectively. Hu or NF-immunoreactive (IR) neurons were counted in all available ganglia of the sample using a Zeiss Axiovert 200 M (Zeiss, Thornwood, NY). The results were expressed as the average of the mean number of neurons per ganglion in the two biopsies.

Density of phosphorylated alpha-synuclein inclusions was evaluated after analyzing 2 biopsies from ascending and 2 from descending colon. A biopsy was considered positive when containing at least 1 LN. During the study, we used alternatively a 3-category *semi-quantitative* scale based on the subjective assessment of LN density in all 4 biopsies considered as a single sample, and a *quantitative* rating scale based on the proportion of positive biopsies (0: *absent*; +: 1/4 positive biopsy: *moderate*; ++: $\geq$2/4 positive biopsies, *severe* pathology). As the two methods yielded similar results (4/29 mismatches), we chose to present only the quantitative rating scale because of its higher reproducibility.

Statistical analysis

Data are presented as mean $\pm$ standard deviation. For graphical representation of the total population of patients (n = 29) and controls (n = 10), box plots were used in which the end of the whiskers represent the minimum and maximum scores, and '+' sign represents the mean.

Regarding the number of neurons per ganglion, differences between patients and controls were analyzed by unpaired two-tailed Student's t-tests. Differences between subgroups were analyzed by two-way ANOVA followed by post hoc Newman–Keuls tests.

For ordinal data (clinical scores), conventional Mann-Whitney and Kruskal-Wallis tests followed by post hoc Dunn's analyses served to compare median magnitudes of change.

Correlation between Lewy pathology score and other parameters were assessed by Spearman test. Adjustment with age was done with multiple linear regression. Chi square tests were used for frequency analysis. For all statistical tests $p<0.05$ was deemed significant.

Results

A total of 30 PD patients and 10 controls were recruited. Of these 30 patients, one was excluded because of an error in the processing of their biopsy. Patients were subdivided into groups based on disease progression, resulting in similar group sizes (9 in group 1, $\leq$6 years; 10 in group 2, 7–12 years; 10 in group 3, $\geq$13 years disease duration). ***Table 1*** shows the main clinical features

Table 1. Main clinical characteristics and immunohistochemical findings in patients.

Patient	Group	Sex	Age	Disease duration (years)	UPDRS-III axial subscore	Dopa-responsiveness	Chronic functional constipation Y/N	Sum of Rome III constipation items	Neurons per ganglion	Lewy pathology quantitative score
1	1	F	44	1	2	–	Y	2	4.4	0
2	1	M	67	2	11	–	Y	3	4.0	++
3	1	F	72	2	10	–	Y	3	2.6	++
4	1	M	47	4	4	–	N	0	3.5	+
5	1	M	66	4	1	–	Y	3	3.7	+
6	1	F	58	5	8	–	Y	4	4.4	++
7	1	F	56	5	3	–	N	1	3.7	0
8	1	M	58	6	5	–	Y	3	4.8	+
9	1	M	71	6	17	–	N	0	3.7	++
10	2	M	63	8	5	70%	N	1	4.0	0
11	2	M	55	9	3	57%	Y	2	3.5	+
12	2	M	63	9	4	72%	Y	4	3.1	+
13	2	F	64	9	5	*md**	Y	2	4.4	0
14	2	M	66	9	4	77%	Y	4	5.9	0
15	2	M	63	10	1	61%	Y	3	3.8	+
16	2	F	65	10	5	41%	Y	4	4.6	+
17	2	M	69	10	9	100%	N	3	3.2	0
18	2	F	48	12	1	93%	N	0	4.5	0
19	2	M	65	12	7	44%	Y	3	2.6	++
20	3	M	64	13	5	50%	Y	4	2.6	+
21	3	F	66	13	9	78%	Y	4	3.8	0
22	3	F	69	13	10	41%	Y	3	3.4	++
23	3	F	57	14	4	79%	Y	3	3.9	+
24	3	F	68	14	1	72%	Y	3	4.5	+
25	3	M	65	16	4	72%	Y	6	2.5	++
26	3	M	68	19	4	74%	Y	4	2.6	++
27	3	F	71	20	4	86%	Y	2	3.6	+
28	3	M	72	20	21	29%	Y	4	3.5	++
29	3	M	60	24	13	57%	Y	5	2.4	++

**md*: missing data.

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and pathological scores of all patients. Age and sex did not differ significantly between patients and controls. CC, as defined by Rome III criteria, affected one of the controls (10%) and 23 out of 29 PD patients (79%, $p<0.001$) (***table 2***).

Lewy neurites in colonic biopsies from PD patients

Twenty-one out of 29 PD patients (72%) displayed Lewy pathology, in the form of Lewy neurites (LN) immunoractive (IR) for both neurofilament (NF) and phosphorylated alpha-synuclein (***figure 1A–D***, ***table 2***). No immunoreactivity for phosphorylated alpha-synuclein was observed within enteric neurons in controls, with the exception of some faint somatic labeling that was present in both patients and controls (data not shown). The proportion of patients with Lewy pathology did not correlate with disease progression (78% positive in group 1, 50% positive in group 2 and 90% positive in group 3).

LN were observed in isolated or bundled fibers (***figure 1C–F***). Triple immunostaining experiments showed that 60% of the LN were also IR for tyrosine hydroxylase (TH). Thirty-seven percent of the LN were perivascular (***figure 1GH***), and 92% perivascular LN were TH-IR (***figure 1I–K***). Additional experiments performed in a subset of 6 positive PD patients (patients 16, 19, 22, 26, 28 and 29) showed that 51% of LN also expressed DBH (***figure S1***). No cytoplasmic Lewy body labeling was observed. 72% of patients exhibited phosphorylated alpha-synuclein-positive labeling (PS+ patients). However the pathological burden was strikingly disparate between PS+ patients: some displayed abundant LN in most samples, while others displayed only one

Table 2. Comparison of main clinical and immunohistochemical variables between patients and controls.

Parameters	Controls mean ± SD	Parkinson's mean ± SD	*p*-value
Age	58.6±7.2	62.8±7.4	0.131
Gender (% male)	58.6%	60.0%	1.000
Chronic constipation	10%	79%	*0.0002***
Neurons per ganglion	4.3±0.3	3.7±0.2	*0.040**
Lewy neurites (% positive patients)	0%	72%	*0.0001****

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positive inclusion in a single biopsy. Postulating that the density of LN was a more relevant marker than their mere presence/absence, we used a quantitative Lewy pathology score. Group 0 represented the negative cases (n = 8) while groups + (n = 11) and ++ (n = 10) represented moderate and severe Lewy pathology, respectively.

Neurofilament and tyrosine hydroxylase-expressing neurons in the submucosal plexus

In order to assess the suitability of NF as a neuronal marker in human SMP, we first performed a double Hu and NF-immunostaning in a subset of 3 control and 3 PD patients (patients 9, 17 and 27). Anti-NF 200 kDa antibody virtually labels all submucosal neurons in both conditions (***figure S2***), thus allowing the use of NF-immunostaining for neuronal count. Control submucosal samples displayed 4.3±0.8 NF-IR neurons per ganglion. In PD, there was a decreased number of NF-IR neurons per ganglion (3.7±0.8; p = 0.04) (***figure 2AB***, ***table 2***).

When patients were separated into two groups according to the presence (PS+) or absence (PS-) of phospho-synuclein IR neurites, only PS+ patients had a significant drop in the amount of NF-IR when compared to controls (p = 0.01) (***figure 2C***). When PS+ patients were further stratified into subgroups with moderate (+) and severe (++) pathology, there was a highly significant difference in NF-IR between PS+ patients with severe pathology (++) and the control group ($p<0.01$), and furthermore significant differences were apparent between the severe pathology group and those with absent (0) and moderate (+) pathology ($p<0.05$) (***figure 2D***). After adjustment for age, a significant correlation remained between Lewy score and the number of NF-IR neurons per ganglion (p = 0.02). There was no correlation between the number of neurons per ganglion and age (p = 0.193), nor between the number of neurons per ganglion and disease duration (p = 0.094), including after age-adjustment (p = 0.479).

Clinicopathological correlations

We then sought to correlate our two primary histological findings, namely LN and the number of NF-IR neurons, with both neurological and chronic constipation (CC) symptoms (***table 3***).

a. Neurological. In order to correlate Lewy pathology with clinical features, we stratified patients according to the quantitative Lewy pathology score, as described above. The Lewy pathology score positively correlated with age ($r_S = 0.395$; p = 0.03) (***figure 3A***). Age is associated with worsening prognosis of PD [24], and as such is a potential confounding factor. Therefore, all subsequent correlations between Lewy pathology and disease severity were performed after adjusting for age.

There was no correlation between pathology and disease duration. Axial score was higher in the group with severe Lewy pathology (++) when compared to the groups with absent (0) or moderate (+) pathology ($p<0.01$) (***figure 3B***), and axial score positively correlated with the Lewy pathology score (p = 0.004). Further reinforcing these findings, dopa-responsiveness negatively correlated with the severity of pathological burden (p = 0.0064). The group devoid of LN was significantly more responsive to levodopa than the groups with either moderate ($p<0.05$) or severe ($p<0.01$) pathology (***figure 3C***). Conversely, the number of neurons per ganglion did not correlate with either axial score or dopa-responsiveness.

b. GastrointestinaI. CC was significantly more frequent among PS+ (19/21) than among PS- patients (4/8) ($p<0.05$, Fisher's exact test). The severity of CC, as assessed by the constipation score, positively correlated with the Lewy pathology score ($r_S = 0.381$; p = 0.042) (***figure 3D***). However, this correlation was not significant after adjusting for age (p = 0.17). There was no correlation between the number of neurons per ganglion and CC score (p = 0.56).

Discussion

The four main outcomes of the present survey are (1) the demonstration of LN in the SMP of 72% of PD patients, but in none of the controls, (2) a higher frequency of constipation in LN-positive patients, (3) a strong correlation between LN burden and disease severity, (4) the possibility to readily and reproducibly analyze the ENS in living patients, thereby providing an opportunity to develop an original biomarker for PD.

Lewy pathology in the SMP of PD patients

A major finding of our study was the identification and characterization of neuropathological lesions in the colonic submucosa of PD patients. Lewy pathology in the SMP was composed of LN only. This is consistent with a recent report in which alpha-synuclein inclusions observed in the gastric submucosa of deceased PD patients were LN, while Lewy bodies were present in the soma of myenteric neurons [25]. The absence of Lewy bodies in the SMP precludes affirmation that the SMP is intrinsically affected by the pathological process. Indeed, Lewy bodies and LN have been reported in colonic and gastric myenteric neurons of deceased PD patients ([25] and our unpublished results). In the guinea pig, myenteric neurons have been shown to project to the SMP as well as to the submucosal blood vessels [26].

Intrinsic dopaminergic neurons in the myenteric plexus are altered in PD both in human [27] and animal models [28]. However, intrinsic dopaminergic neurons are a minority in the colonic SMP [29]. At the level of the colonic SMP, the majority of TH-IR neurites are noradrenergic sympathetic axons that also express DBH (94% in our unpublished data). Our present results show that 37% and 60% of LN are perivascular and TH-IR

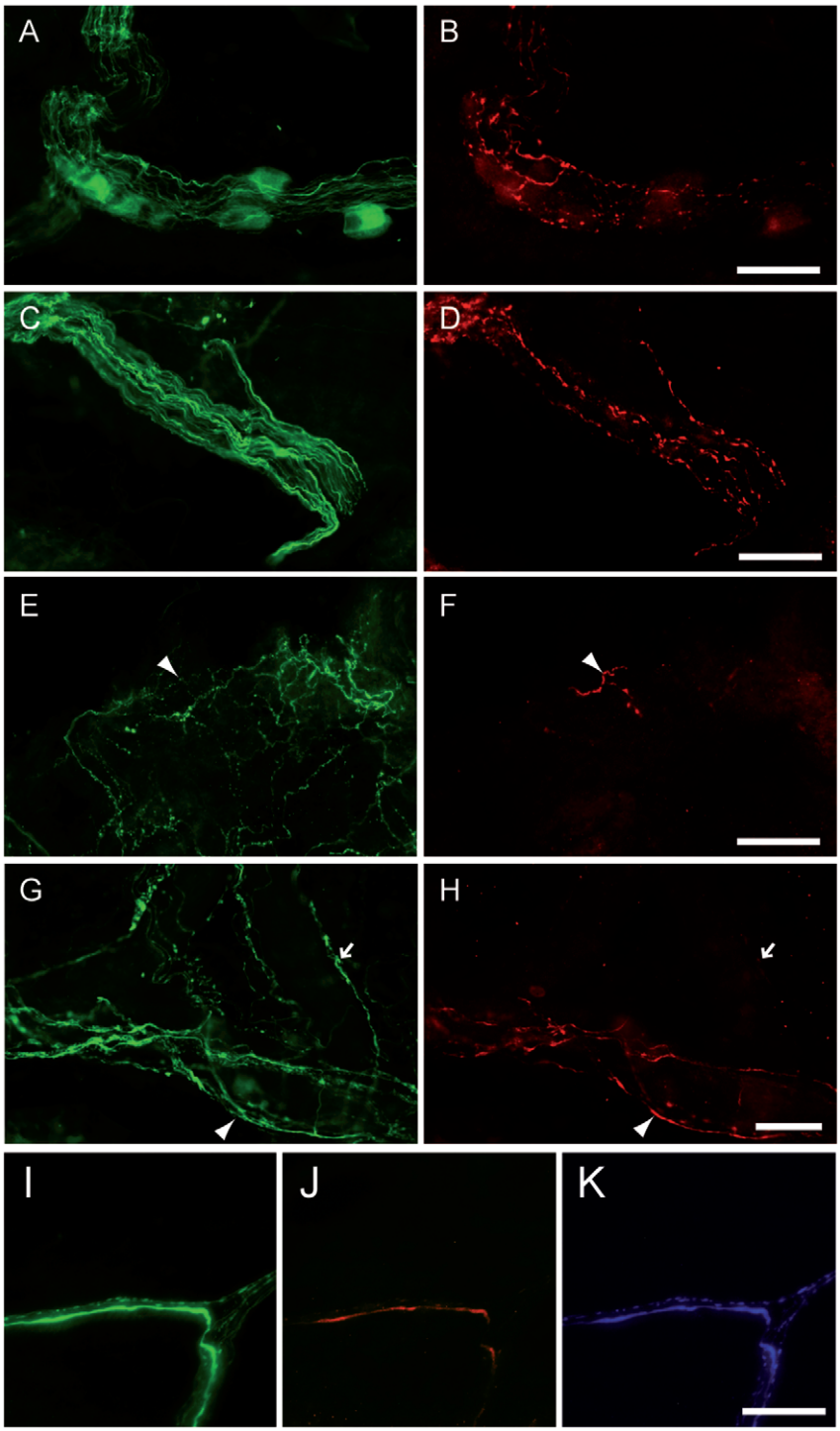

Figure 1. Phospho-α-synuclein-positive submucosal neurites in PD patients. Labeling with antibodies against neurofilament (NF) (**ACEGI**) and phosphorylated α-synuclein (**BDFHJ**) revealed that some NF-immunoreactive (IR) neurites were also phospho-α-synuclein-IR. Occasionally, these phospho-α-synuclein-IR neurites were present amidst a submucosal ganglion (**AB**). Some of these structures formed bundles (**D**) while others were isolated (**arrowhead in F**). Thirty seven percents of the phospho-α-synuclein-IR neurites were perivascular (**GH**). Triple immunostaining with antibodies against tyrosine hydroxylase (**K**) revealed that 60% of LN were also TH-immunoreactive (**IJK**). Scale bar: 30 μm.
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respectively, and that 51% are DBH-IR, suggesting that many TH-IR LN belong to postganglionic sympathetic neurons. Thus, a significant proportion of the enteric pathology reflects the widespread sympathetic degeneration seen in other systems [30,31].

LN in the SMP was present in 21 out of 29 PD patients. The heterogeneity of PD with regard to peripheral autonomic alterations was underscored by two recent autopsy-based studies that found Lewy inclusions in the GI tract as well as in the sympathetic network of nearly three-quarters of PD patients

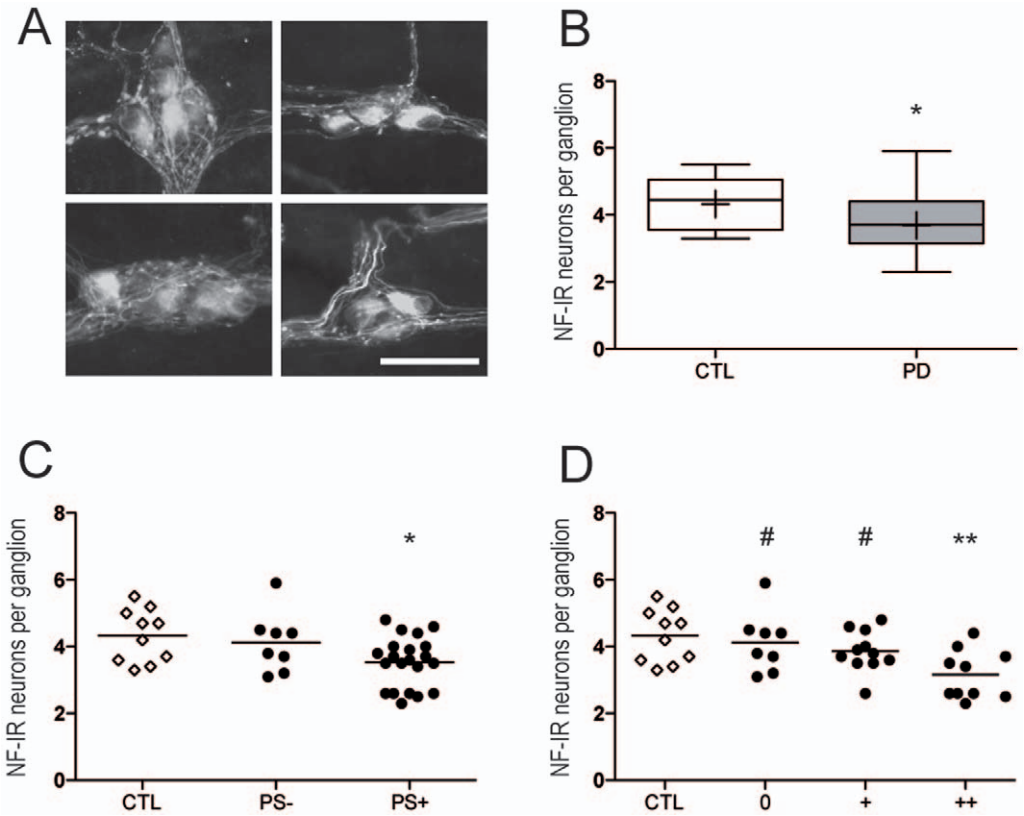

Figure 2. Count of neurofilament-positive neurons in the submucosal plexus of PD patients. A. Neurofilament-immunoreactive (NF-IR) submucosal neurons were counted in every available ganglion from colonic biopsies. Representative photographs of ganglia from PD patients (left panels) and controls (right panels). Scale bar: 30 μm. **B.** A significant decrease in the number of NF-IR neurons per ganglion was present in the SMP of PD patients (PD, n = 29) as compared to controls (CTL, n = 10) ($p<0.05$). The bottom and the top of the box represent the 25th and 75th percentiles, respectively, and the end of the whiskers represent the minimum and maximum values; the median is represented as a bar and the mean as a '+' sign inside the box. **C.** When segregating patients according to the presence (PS+) or absence (PS-) of phospho-synuclein IR neurites, the difference between patients and controls was sustained only for the group with Lewy pathology (PS+, n = 21, $p<0.05$). **D.** When further stratifying patients according to the density of pathology, the difference between patients and controls was sustained only for the group with severe Lewy pathology (++, n = 10, $p<0.01$). Groups without (0) or with moderate pathology (+) significantly differed from the group with severe (++) pathology ($p<0.05$). Each white square represents one control, each black circle represents one PD patient. Horizontal bars represent the mean. *$p<0.05$ and **$p<0.01$ as compared with controls. #$p<0.05$ as compared with the group with severe pathology (++).
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[30,32]. Whether this heterogeneity reflects different PD subtypes or disease severity will be further discussed.

Because of samples shortage, NF-co-immunostaining was intended both for localizing phosphorylated alpha-synuclein immunoreactivity within neurons and for neuronal counting, taking advantage of NF somatic labeling. A significant decrease in the number of NF-IR neurons was observed in the SMP of PD patients. The significance of this finding is debatable since the

Table 3. Clinico-pathological correlations.

Correlations	Age	Disease duration (years)	UPDRS-III axial subscore	Dopa-responsiveness	Sum of Rome III constipation items	Neurons per ganglion	Lewy pathology quantitative score
Spearman's correlation with quantitative score (p values)	*0.034* *	0.290	*0.008* **	**0.007** **	**0.042** *	**0.004** **	0
Age-adjusted correlation with quantitative score (p values)		0.507	*0.004* **	*0.006* **	0.17	*0.02* *	0

doi:10.1371/journal.pone.0012728.t003

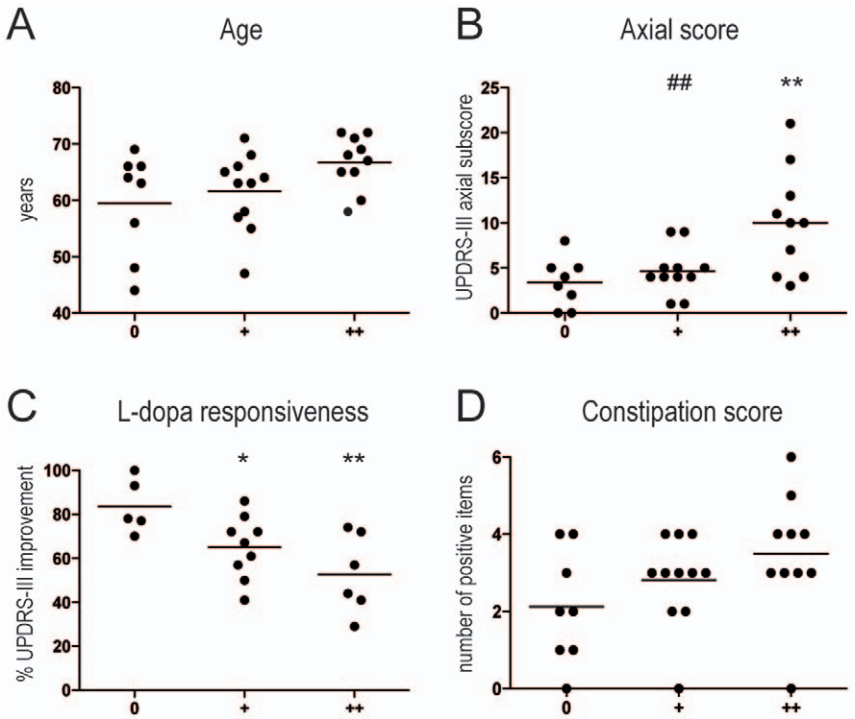

Figure 3. Correlation of clinical symptoms with pathology burden. A. Measure of pathology burden using a quantitative score correlated with age, which appeared as a potential confounding factor. Subsequent correlation analysis was performed after adjusting the data for age. **B.** Pathology burden positively correlated with axial score, which measures axial symptoms such as dysarthria and postural instability. The group with severe pathology (++) significantly differed from the group with absent (0) or moderate (+) pathology ($p<0.01$). **C.** Pathology burden also correlated with L-Dopa responsiveness, estimated by the percentage of UPDRS-III improvement after L-Dopa intake. Responsiveness was higher in the group with absent pathology (0), as compared with the group with moderate (+, $p<0.05$) or severe (++, $p<0.01$) pathology. **D.** Pathology burden also correlated with constipation severity, as defined by the number of positive answers to the constipation items of Rome III questionnaire. Each black circle represents one PD patient. Horizontal bars represent the mean. *$p<0.05$ and **$p<0.01$ as compared with the group with absent pathology (0). #$p<0.05$ and ##$p<0.01$ as compared with the group with severe pathology (++).
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suitability of NF as an enteric neuronal marker has been challenged in a recent study showing that as low as 43% of Hu-IR neurons co-expressed NF in the myenteric plexus [33]. However in our experience, virtually all Hu-IR submucosal neurons display somatic NF immunoreactivity (***figure S2***). This discrepancy might result from differences in the expression of NF between the submucosal and myenteric plexus and/or from differences in the sensitivity of the antibodies that were used.

Although we cannot rule out phenotypic changes resulting in a decreased expression of NF in a subset of neurons, it is tempting to attribute the drop in NF-IR to neuronal loss. Whether enteric neuron loss occurs in PD is still unclear. Two earlier studies performed on biopsies and surgical samples failed to show any neuronal loss in the colonic SMP of PD patients [19,27]. The low number of patients assessed in these studies probably accounts for this discrepancy. If enteric neurodegeneration occurs in the course of PD, the cardinal neuropathology of the disease, namely neuronal death and Lewy inclusions, would be recapitulated in the ENS, thereby mirroring the lesions of affected brainstem nuclei in the CNS.

Lewy pathology burden and constipation

GI dysfunction stands among the most common non-motor symptoms of PD. Symptoms such as dysphagia, nausea, gastroparesis, and bowel dysfunction, including both reduced bowel movement frequency and dyschesia, are a significant cause of disability [34]. CC was significantly more frequent in the group with than without LN, suggesting a pathogenic role for inclusions.

In this aspect however our study suffers from two potential limitations. First, we did not use a validated constipation severity score. Scores such as Patient Assessment of Constipation Symptoms (PAC-SYM) questionnaire [35] might have revealed stronger correlations between pathology burden and CC, while the correlation we found did not remain significant after adjustment for age. Second we did not assess the MP, which is directly involved in the control of bowel motility. Whether the density of LN in the SMP is representative of the pathology burden in the MP is still an open question that could be addressed by a comparative and comprehensive analysis of the myenteric and SMP in surgical or postmortem specimens.

Although controversial, CC has been linked to increased age-related neurodegeneration in the ENS [36]. Loss of submucosal neurons [37,38] and alterations of the sympathetic innervation [39] in the ageing rat have been implicated in the pathophysiology of CC, and a loss of myenteric neurons has been demonstrated in the colon of patients with CC [40]. From our study, CC in PD does not appear to be related with the number of submucosal NF-IR neurons, and degeneration of sympathetic innervation might play a role in this feature, since a significant proportion of LN belonged to sympathetic outputs.

Lewy pathology burden is correlated with PD progression

All patients included in this study had a comprehensive neurological assessment. This enabled us to draw parallels between pathological burden in the ENS and Parkinsonian symptoms.

The density of submucosal LN was significantly correlated with the presence of dopa-unresponsive axial symptoms, such as dysarthria or postural instability but not with disease duration and motor symptoms. Two recent studies relating autonomic dysfunction with the clinical phenotype of PD provided similar results [41,42]. Clinical scores of autonomic symptoms, postural blood pressure response impairment [41] and myocardial ^{123}I-metaiodobenzylguanidine uptake [42] weakly correlated with disease duration and motor symptom severity. Conversely, the presence of axial symptoms was associated with greater autonomic dysfunction. These studies, together with our results, strongly suggest that functional and structural alterations in the enteric and autonomic nervous systems are associated with the presence of axial motor symptoms. Interestingly, a recent survey searching for patterns of coherency among the full clinical spectrum of PD found that dysautonomic, axial and cognitive symptoms co-segregated and best characterized disease severity [43]. In an individual patient, the appearance of axial symptoms is predictive of disease progression toward dementia [10] and is thought to reflect the spreading of pathology to non-dopaminergic structures of the brainstem, forebrain and cortex [44]. Thus, the heterogeneity of PD regarding dysautonomia in general, and alterations of the ENS in particular, might reflect in part different degrees of severity.

The ENS as biomarker in PD

Routine colonic biopsies can be used to provide examination of the submucosal enteric neurons in living patients [45]. Here we confirm on a large scale that such a procedure allows a safe and reliable analysis of the ENS. Total colonoscopy is a simple diagnostic procedure with a low risk of adverse effects [46]. Accordingly, no complications occurred in the 40 patients included in the present study, either during or after the procedure.

The skin and the olfactory epithelium contain neuronal networks affected by Lewy pathology during PD that are also accessible by routine biopsies and have recently been evaluated as histopathological markers for PD [30,47]. However, the results of these works were disappointing since only 2 out of 20 PD patients displayed LN in skin biopsies and no alpha-synuclein aggregates were present in the biopsied olfactory epithelium of 7 Parkinsonian patients [48,49]. Consequently, our study is the first to show that Lewy pathology can be reproducibly analyzed using biopsies from a peripheral tissue in living patients.

The ENS displays specific features that make it a prime candidate for being a histopathological marker of PD (for review see [50]). In contrast to the skin and olfactory epithelium, colonic biopsies allow the retrieval and analysis of a dense integrated neuronal network, not only neuronal processes [18]. Using optical recording techniques, electrophysiological properties of submucosal neurons from colonic biopsies can be studied [51]. Therefore, analysis of the ENS during the progression of PD may represent a unique opportunity to monitor PD pathology and its impact on neuronal function in living patients. We have shown in the present survey that the pathological burden in the ENS is correlated to the presence of dopa-unresponsive axial symptoms, strongly supporting the use of colonic biopsies as a biomarker for the assessment of PD severity. Their use for the positive diagnosis of incipient or even preclinical PD still requires to test the specificity of enteric submucosal LN in larger series and to improve the sensitivity of the technique. Possible strategies include an increased number of colonic samples or the use of upper digestive tract biopsies, which add the potential risk of inhalation during the endoscopy.

Braak and coworkers have postulated that the ENS is affected early by Lewy pathology during the course of PD, even before the pathology is apparent in the substantia nigra. This suggests that the ENS heralds the onset of a pathological process that further spreads to the CNS via autonomic innervation of the gut [25,52]. Although tempting, this theory relies only on correlations performed in autopsy studies and is still a matter of debate. By demonstrating the presence of LN in the colon at early stages (78% of patients <6 years), our findings do not refute this hypothesis. We believe that the use of colonic biopsies, by enabling analysis of the ENS in PD patients at a very early stage of the disease, will be helpful for validating or refuting Braak's hypothesis.

In conclusion, the ENS can be considered not only as 'the second brain' [53], but also as a window towards the 'first' brain. The ENS probably antedates the CNS in evolutionary terms, and its complexity challenges its central counterpart, especially since the functional and chemical diversity of enteric neurons closely resembles that of the CNS [54]. Enteric neuropathies recapitulate many aspects of neurological diseases [2]. In particular, degenerative changes occur in the aging gut [55]. In this context, it is hardly surprising that enteric neurons can mirror central alterations in neurodegenerative disorders. It is possible that further studies may expand this concept to other neurodegenerative diseases. For example, the presence of hyperphosphorylated tau aggregates in myenteric neurons of aging rats suggests that tauopathies such as Alzheimer's disease may also affect the ENS [56]. We consider our method to represent a major advance in the search for biomarkers for PD. The use of the, as yet unrecognized, ENS as a window into the CNS represents an original approach, with implications that may well extend beyond PD.

Supporting Information

Figure S1 51% of phospho-α-synuclein-positive submucosal neurites express DBH. Labeling with antibodies against dopamine-beta-hydroxylase (DBH) (BDF) and phosphorylated α-synuclein (ACE) revealed that some DBH-immunoreactive (IR) neurites were also phospho-α-synuclein-IR. In a subset of 6 PD patients, the proportion of Lewy neurites that expressed DBH was 51%. Perivascular Lewy neurites in EF. Scale bar 30 μm.
Found at: doi:10.1371/journal.pone.0012728.s001 (3.90 MB PDF)

Figure S2 Evaluation of neurofilament immunostaining as a pan-neuronal marker in human submucosal plexus. Labeling with antibodies against neurofilament 200 kDa (NF) (ACEGIK) and Hu C/D (BDFHJL) revealed that virtually all submucosal neurons, whether isolated (EF and KL) or in submucosal ganglia containing >2 neurons, coexpress NF and Hu C/D. Sample images from 3 controls (A–F) and 3 PD patients (G–L). Note the nuclear expression of Hu in L, a pattern that is occasionally seen in patients and controls. Scale bar 30 μm.
Found at: doi:10.1371/journal.pone.0012728.s002 (8.13 MB PDF)

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Author Contributions

Conceived and designed the experiments: MN SBdV JPG P. Damier P. Derkinderen. Performed the experiments: TL EC AD TC MT SP MF JPG. Analyzed the data: TL MN AD JMNG SP P. Derkinderen. Wrote the paper: TL MN SP P. Derkinderen.

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REVIEW ARTICLE
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Biopsable neural tissues: toward new biomarkers for Parkinson's disease?

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Biomarkers for Parkinson's disease (PD) are mainly intended for the early diagnosis of the disease and to monitor its progression, two aspects insufficiently covered by clinical evaluation. In the last 20 years, the search for biomarkers has been supported by technological advances in the fields of molecular genetics and neuroimaging. Nevertheless, no fully validated biomarker is yet available, and there is still a need for biomarkers that will complement those already available. Development of biomarkers for PD has been hampered by the fact that the core pathology lies in the brainstem, hidden from direct study in living patients. In this context, the recent observations that clearly demonstrated the presence of PD pathology in peripheral neural tissues provide new opportunities to develop original histopathological markers of the disease. Some of these peripheral tissues, especially the enteric nervous system, by being assessable using routine biopsies, could represent a window to assess *in vivo* the neuropathological processes occurring in PD.

Keywords: Parkinson's disease, biomarker, alpha-synuclein, autonomic nervous system, enteric nervous system, skin, salivary glands, colonic biopsies

Development of biomarkers for PD has been hampered by the fact that the core pathology lies in the brainstem, hidden from direct study in living patients. However the traditional assumption of PD as a primary disorder of the dopaminergic neurons of the substantia nigra has been reconsidered in the recent years. Recent studies have indeed implicated that the presence of Lewy pathology is much more extensive and affects not only the central nervous system but also peripheral autonomic neuronal circuits. This provides new opportunities for the development of original biomarkers that will directly assess the pathological process in peripheral tissues accessible by biopsy.

IN SEARCH OF BIOMARKERS FOR PD

Parkinson's disease (PD) is a progressive neurodegenerative condition characterized and diagnosed by the presence of motor and non-motor symptoms (Lees et al., 2009). From a pathological point of view, the two hallmarks of PD are a loss of dopaminergic neurons in the substantia nigra and the presence in the surviving neurons of inclusions termed Lewy bodies (LB) and Lewy neurites (LN), whose main component is phosphorylated alpha-synuclein (Fujiwara et al., 2002; Anderson et al., 2006).

Parkinson's disease follows a slowly chronic progressive course, and the motor cardinal symptoms of the disease appear only when the degenerative process has progressed for a long time, in most cases probably for more than 10 years (Hawkes et al., 2009). This long premotor phase is nevertheless not clinically silent since non-motor symptoms such as hyposmia (Ponsen et al., 2004), REM-sleep behavior disorder (Postuma et al., 2009), and constipation (Abbott et al., 2001; Savica et al., 2009) can antedate the occurrence of tremor and/or akinesia. In contrast, other non-motor symptoms, especially dementia, are known to occur lately in the evolution of the disease and to reflect disease progression and severity (Chaudhuri et al., 2006).

Currently, diagnosis and progression of PD is based mainly on clinical criteria. Diagnosis of PD relies on the presence of two out of three of major motor signs, namely tremor, bradykinesia, and hypertonia, implying that the diagnosis is made only many years after the real onset of the neurodegenerative process (Hughes et al., 2002). PD can be difficult to diagnose in its early stages, and may be mimicked by other diseases, such as essential tremor, multiple system atrophy and progressive supranuclear palsy (Hughes et al., 2002). Treatment strategies for PD are mostly aimed at relieving motor symptoms and not at modifying the disease process (Thobois et al., 2005). Therefore, a key goal in PD research is the development of drugs capable of preventing or at least slowing the disease progression. Compounding this problem is the difficulty to readily assess PD progression and/or severity. To date, most of the neuroprotective trials in PD used changes in the clinical UPDRS scale as a primary endpoint (Schapira and Olanow, 2004; Olanow et al., 2009). Such an approach is confounding since many of the drugs proposed to slow progression also improve dopaminergic neurotransmission and treat PD symptoms (Ahlskog, 2007). Consequently, there is a critical need to develop biomarkers that correlate either with the presence or the severity of the disease, for a more precise and early diagnosis of the disease as well as for the assessment of new therapeutic strategies (**Figure 1**). Technological advances in the field of molecular genetics and in *in vivo* imaging have allowed the development of some reliable biomarkers either for early diagnosis or to assess disease progression. For instance, transcranial ultrasound (Berg and Becker, 2002), high-field MRI

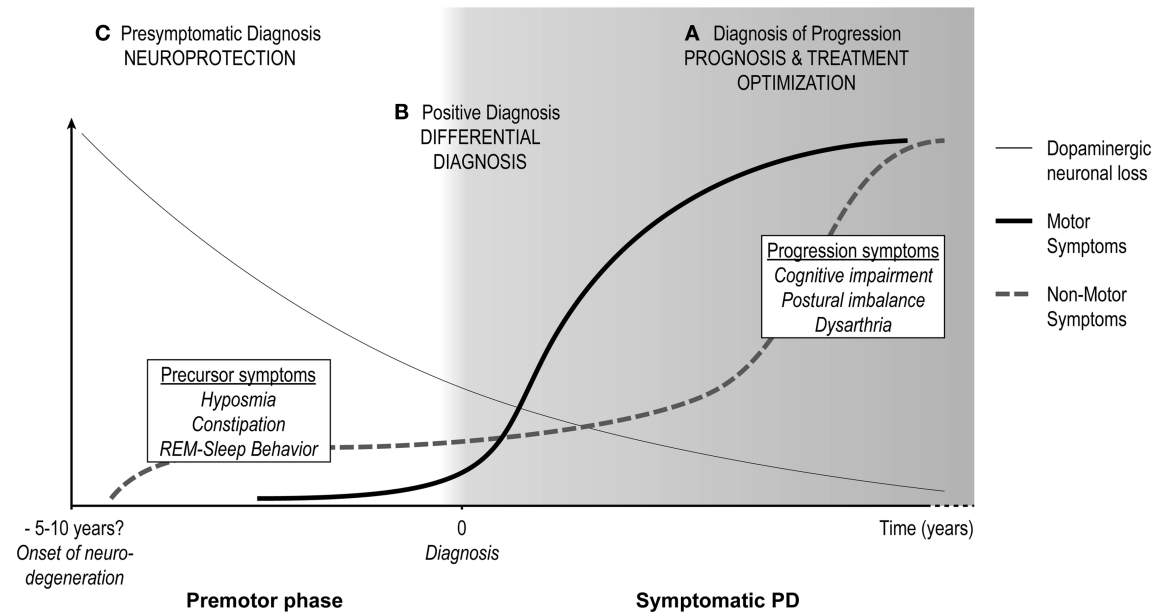

FIGURE 1 | The three purposes of biomarkers in Parkinson's disease. Schematic representation of the clinical progression of Parkinson's disease (PD). The thin line represents the continuous degeneration of the dopaminergic neurons of the *substantia nigra*. When dopaminergic neuronal loss reaches >50%, cardinal motor symptoms appear (*thick line*) and PD becomes fully symptomatic (*gray background*). Motor symptoms worsen over time but respond to dopamine replenishment therapy. During the premotor phase (*white background*) non-motor symptoms (*dotted line*) may already be present. Non-motor symptoms burden also increases with time but contrary to motor symptoms do not respond to treatment and constitute most of the disability in advanced PD. Biomarkers for PD can serve three main purposes: **(A)** Diagnosis of progression to end-stage non-motor impairment, which would help predict bad prognosis and can serve to monitor neuroprotective therapies. **(B)** Positive diagnosis of PD by differentiating it from other causes of parkinsonism. **(C)** Presymptomatic diagnosis of PD. Although non-specific, precursor non-motor symptoms such as constipation, depression, hyposmia, or REM-sleep behavior disorders characterize a population in which future biomarkers for presymptomatic diagnosis should be tested.

(Martin et al., 2008) and dosage of neuronal protein involved in the pathogenesis of the disease in the cerebrospinal fluid (Hong et al., 2010) are new tools that are likely to help in the diagnosis and management of PD patients in the near future. Nevertheless, as stated in a recent review, no fully validated biomarker for PD is available yet (Marek et al., 2008) and there is still a need for new biomarkers that will complement the ones already available.

PD PATHOLOGY EXTENDS WELL BEYOND THE SUBSTANTIA NIGRA

The traditional assumption of PD as a primary disorder of the dopaminergic neurons of the SN has been reconsidered in recent years. The SN is neither the earliest nor the most severely affected region since more caudal brainstem structures as well as the olfactory bulb are involved earlier and more severely in most cases (Del Tredici et al., 2002; Braak et al., 2003). Nuclei such as the dorsal motor nucleus of the vagus nerve display early and massive degenerative changes that worsen as the disease progresses, until a total neuronal loss is reached (Braak et al., 2002). The density of LB and LN in brainstem nuclei is thought to follow an inverted U-shaped curve, with a progressive disappearance in end-stages where no vulnerable neurons are left.

Furthermore, recent studies have demonstrated that the presence of Lewy pathology is much more extensive and affects not only the central nervous system (CNS) but also peripheral autonomic neuronal circuits (Wakabayashi et al., 1988, 1993; Wakabayashi and Takahashi, 1997; Braak et al., 2006, 2007; Braak and Del Tredici, 2008). Interestingly, the same temporal pattern of degeneration has been demonstrated in peripheral structures such as sympathetic ganglia (Orimo et al., 2008).

Lewy pathology has been reported to be present in the olfactory bulbs of subjects with PD as well as a subset of asymptomatic subjects. The presence of Lewy inclusions in neurologically unimpaired patients is called incidental Lewy body disease (ILBD; DelleDonne et al., 2008), since it is thought to represent premotor PD (Del Tredici et al., 2002; Braak et al., 2003; Beach et al., 2010). A recent comprehensive survey has shown that LB were readily retrieved in the olfactory bulbs of 55 out of 58 autopsied patients with PD (Beach et al., 2010). The involvement of the olfactory bulb in most ILBD patients suggests that it occurs at the earliest stage of disease (Bloch et al., 2006; Beach et al., 2009).

The autonomic nervous system (ANS), composed of parasympathetic and sympathetic division, is distributed to the peripheral tissues and organs by way of autonomic ganglia. Control centers of the diencephalon and brainstem send fibers to synapse on preganglionic neurons located in the brainstem or in the spinal cord. From these neurons, preganglionic fibers project out of the CNS to synapse on neurons in the autonomic ganglia. Postganglionic fibers emerge and form terminal networks on the target tissue. The enteric nervous system (ENS) could be considered part of

the ANS and be regarded as a complex postganglionic neuronal network. The ENS contains as many neurons as the spinal cord (approximately 80–100 million neurons) and the functional and chemical diversity of enteric neurons closely resembles that of the CNS (Benarroch, 2007; Cersosimo and Benarroch, 2008). This integrated neuronal network is organized in two ganglionated plexuses, myenteric and submucosal, composed of neurons and enteric glial cells (Benarroch, 2007; Lebouvier et al., 2009a). Neurons of the myenteric plexus (or Auerbach's) control the activity of the smooth muscle of the gut whereas those in the submucosal plexus (or Meissner's) regulate mucosal secretion and blood flow (Schemann and Neunlist, 2004).

Lewy pathology has been described in the autonomic nuclei of the brainstem and spinal cord and in the sympathetic ganglia of PD patients (Wakabayashi et al., 1988, 1993; Wakabayashi and Takahashi, 1997; Braak et al., 2006, 2007; Braak and Del Tredici, 2008) and ILBD subjects (Bloch et al., 2006; Minguez-Castellanos et al., 2007). Remarkably, LB and LN are also present in postganglionic structures. Using sampled skin from the chest and forearm of autopsied patients, Ikemura et al. (2008) demonstrated LN in the sympathetic nerve fascicles of the dermis and subcutaneous tissue in 10 out of 14 PD patients and in one of two ILBD. The autonomic innervation of the submandibular gland also displays LN with a high sensitivity in two autopsy surveys, with lesions in 14 out of 15 PD patients (Beach et al., 2010) and 9 of 9 PD patients respectively (Del Tredici et al., 2010). Moreover, LN were present in the submandibular glands of two out of three ILBD subjects (Del Tredici et al., 2010). Regarding the ENS, the presence of Lewy pathology in the gastrointestinal tract was described more than 20 years ago in two seminal reports (Qualman et al., 1984; Kupsky et al., 1987). Wakabayashi et al. (1988) found LB in the gastrointestinal tract of seven consecutive autopsies performed in PD patients and more recently Beach et al. (2010) reported LB and LN in the gut of 11 of 17 PD patients. In both studies, the Lewy pathology was distributed in the MP and SMP from the upper esophagus to the rectum following a rostrocaudal gradient, the upper esophagus being more severely affected than the colon and the rectum (Wakabayashi et al., 1988; Beach et al., 2010). Remarkably, when specific histochemical procedures were used (analysis of multiple slides of thick sections of the lower esophagus), Lewy inclusions were found in 14 out of 15 PD patients, suggesting that the pathology is scattered but nearly constant in the ENS (Beach et al., 2010). Among ILBD patients, the rate of enteric pathology varies depending on the sampling and techniques used to assess the synucleinopathy, from 1/7 to 14/17 (Bloch et al., 2006). A thorough assessment of the ENS in ILBD is still needed to test the hypothesis of its prime involvement during PD (Braak et al., 2006).

The histopathological features observed in the olfactory bulb and in the peripheral nervous system of PD patients are likely to be specific for this neurodegenerative condition. Indeed, although the olfactory bulb is constantly affected by the pathological process in multiple system atrophy, the inclusions of alpha-synuclein are mainly glial (Kovacs et al., 2003). The Lewy pathology in the peripheral nervous system of multiple system atrophy patients is primarily preganglionic and, in contrast to PD, the postganglionic network is almost completely spared (Ikemura et al., 2008; Orimo et al., 2008; Del Tredici et al., 2010). Regarding PSP, tau pathology is minimal or absent in the olfactory bulb and no specific involvement of the peripheral nervous system has been reported yet (Rub et al., 2002).

Altogether, these results demonstrate that PD pathology extends well beyond the substantia nigra and that the peripheral autonomic neuronal circuits are affected early, and specifically in a large proportion of patients.

BIOPSABLE NEURAL TISSUES AS A NEW SOURCE OF BIOMARKER OF PD

Remarkably, some of the extranigral structures affected by Lewy pathology are accessible to biopsies, making them a putative original source of biomarkers. As the only component of the olfactory system accessible to biopsy, olfactory epithelium was logically screened for Lewy pathology. In a pilot study, there was no evidence of disease-specific pathology in seven hypo/anosmic PD patients (Witt et al., 2009). This is probably explained by the fact that the pathology in the olfactory system is restricted to the olfactory bulb, a structure that is not accessible to routine biopsies (Parkkinen et al., 2009).

Quite logically, from the results obtained in autopsy specimens, a Japanese team attempted to retrieve Lewy pathology using routine skin biopsies from chest and leg. The results were disappointing as only two patients were positive in a series of 20 parkinsonian patients (Miki et al., 2010). The discrepancy between the results of the autopsy-based study and the *in vivo* study may be explained by the differences of the sites for tissue samples, the size of skin tissue examined, and the numbers of examined sections. In any event, this does not make the skin a source of biomarker for the premortem diagnosis of PD.

The two autopsy studies of the submandibular gland in LB disorders raised a recent interest for the salivary glands (Beach et al., 2010; Del Tredici et al., 2010). Apart from fine needle aspiration biopsies that only give access to smears of epithelial cells, histological analysis of the submandibular gland can only be achieved though incisional biopsy. The possibility of injury to the marginal mandibular branch of the facial, hypoglossal, and lingual nerves requires the biopsy to be performed in the operating room. Because of the risks and technical difficulties of such a procedure, even higher when it comes to the parotid gland, the analysis of the major salivary glands will probably never become a routine biomarker for PD. Conversely, minor salivary gland biopsy is safe and routinely performed for diagnostic purposes (Caporali et al., 2008). Provided that minor salivary glands recapitulate the alterations of the autonomic innervation observed in the submandibular gland, which requires confirmation, the analysis of labial salivary glands may provide a useful histological biomarker (Cersosimo et al., 2010).

The ENS displays specific features that make it a prime candidate for being a histopathological marker of PD. In contrast to all aforementioned tissues, it does not contain only postganglionic neuronal processes but rather is an integrated neuronal network that contains neurons and enteric glial cells, the counterpart of the astrocytes of the CNS. It is sometimes referred as a "second brain" because of the functional and chemical diversity of the enteric neurons that closely resembles that of the CNS. We have shown recently that whole-mounts of submucosa from routine colonic biopsies allow a morphological and quantitative analysis of the SMP (Lebouvier

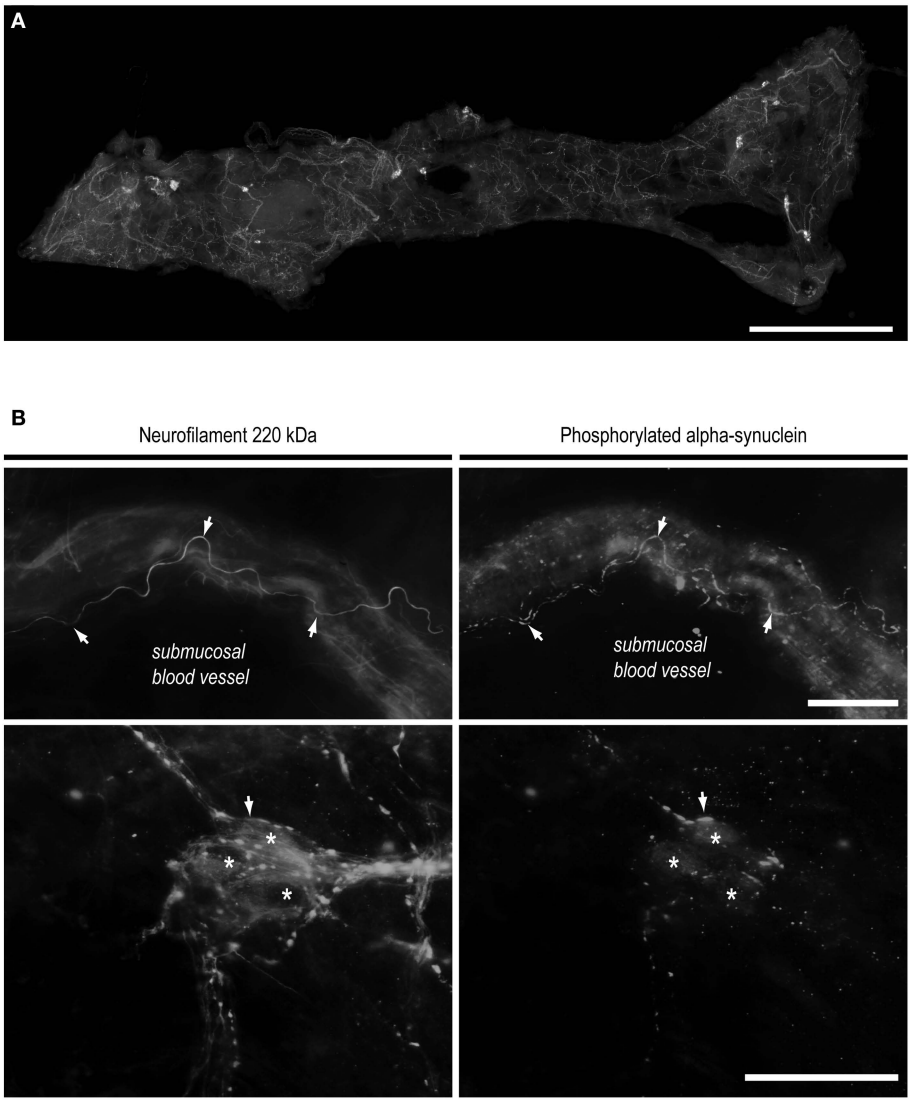

FIGURE 2 | Routine colonoscopy biopsies as a novel biomarker of Parkinson's disease. (A) Whole mount of submucosa microdissected from a standard colonoscopy biopsy and immunostained with anti-neurofilament antibody to unravel the neural network. Colonic submucosal (Meissner's) plexus, formed by ganglia and interganglionic strands, is readily apparent. A single biopsy performed in the ascending or descending colon gives access to a mean of 150 neurons. Scale bar 1 mm. **(B)** After magnification, double labeling with antibodies against neurofilament (left) and phosphorylated alpha-synuclein (right) in a Parkinson's disease patient reveals the presence of Lewy neurites. Up: a perivascular Lewy neurite. Down: a fragmented Lewy neurite inside a submucosal ganglion. Arrows: neurites immunoreactive for phosphorylated alpha-synuclein and neurofilament. Asterisks: submucosal neurons. Scale bar: 30 μm.

et al., 2009b). A single standard colonic biopsy contains an average of 35 ganglia, thus allowing the analysis of approximately 150 neurons (**Figure 2A**). Using this approach, LN were identified in the SMP of four out of five PD patients (Lebouvier et al., 2008) in a preliminary report (**Figure 2B**). We have therefore undertaken a large-scale survey to correlate the amount of enteric pathology with clinical PD symptoms. A total of 10 control and 30 PD patients were enrolled. Four routine colonic biopsies were taken from the ascending and descending colon during the course of a total colonoscopy. Lewy pathology was apparent in the colonic biopsies from 21 patients (72%) and in none of the controls. In favor of the pathogenicity of enteric pathology, pathological burden was correlated with an apparent neuronal loss within the submucosal plexus. The clinical relevance of these findings was supported by a correlation between pathological burden and constipation as well as the amount of axial and dopa-unresponsive symptoms, which reflect disease progression (Lebouvier et al., 2010).

CONCLUSION AND PERSPECTIVES

Although Lewy pathology is absent in a minority of cases of clinical PD, most of which are rare genetic forms of the disease, alpha-synuclein is still considered to be a key player of the pathophysiology of PD. In the era of functional neuroimaging and molecular biology, histological biomarkers may still be of great interest for the diagnosis and management of PD because they are the only to directly assess the synucleinopathy *in vivo*. By affecting the peripheral ANS

early in the course of the disease, PD provides a nearly unique opportunity to directly apprehend the neuropathological process in biopsable neural tissues. Apart from PD, comparable approaches have been used only in variant Creutzfeldt–Jakob Disease. Though analysis is performed in non-neural lymphoid tissue, histochemical (Ironside et al., 2000) and biochemical (Wadsworth et al., 2001) methods can identify the pathological form of the prion protein in tonsil biopsies from affected individuals.

To date, the search for sensitive histological biomarkers in PD has been hindered by the scattered pattern of inclusions in the peripheral ANS. Among routinely accessible tissues, the extraordinary neuronal density of the ENS accounts for the higher, yet imperfect, sensitivity of gut biopsies to detect the pathology. The good correlation between pathology burden and disease severity makes the technique a readily available biomarker to assess disease progression (**Figure 1A**).

Yet future work is needed to test the specificity of these peripheral inclusions in larger series and to improve the sensitivity of the technique. Possible strategies include an increased number of colonic samples or the use of upper digestive tract biopsies, which add the potential risk of inhalation during the endoscopy. Other biopsable tissues such as minor salivary glands may solve the safety issues if they demonstrate an equal or superior sensitivity to evidence Lewy inclusions. Once the sensitivity is improved, the primary goal will be to use such biomarkers for the positive diagnosis of PD and differential diagnosis with other forms of parkinsonism (**Figure 1B**).

A new conception of the neuropathology of PD supports a centripetal pattern of degeneration from postganglionic and peripheral autonomic neurons to their central and preganglionic counterpart, and then a cranial spreading within the brainstem until the substantia nigra is finally reached (Orimo et al., 2008; Hawkes et al., 2009). Suitable histological biomarkers utilize the postganglionic neurons, which are supposed to herald the degenerative process. Hence beyond positive diagnosis, the next step is to use them for the premotor diagnosis of PD (**Figure 1C**). Routinely accessible biomarkers would allow the screening of vulnerable populations for Lewy inclusions before the advent of the motor symptoms. Delineation of the spectrum of Parkinson's at risk patients is currently under work (Stern and Siderowf, 2010), but surely includes patients with REM-sleep behavior disorder. Patients above 50 years presenting with abnormalities in olfaction or gastrointestinal function, particularly if justifying an endoscopy, could as well be systematically assessed.

Until imaging probes for *in vivo* detection of alpha-synuclein deposition become available (Kikuchi et al., 2010), we believe there is a time-window for histological biomarkers in LB diseases. Accumulating data suggest that they should contribute to the early diagnosis of PD, and thus facilitate earlier diagnosis to evaluate neuroprotective treatments.

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Routine colonic biopsies as a new tool to study the enteric nervous system in living patients

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Abstract *Better characterization of enteric neuropathies during the course of gastrointestinal diseases could be of great diagnostic and/or therapeutic interest. However, studies using whole mounts of the enteric nervous system (ENS) are restricted to specific diseases requiring surgery and are also limited by the small number of specimens available. Therefore, we here describe a novel method to obtain whole mounts of submucosal plexus in routine colonic biopsies. We show that a single biopsy displays a substantial number of submucosal ganglia and neurons and that it can be reliably used to perform morphometric and neurochemical analysis and Western Blots quantification of neuronal or glial markers. This method of analysis of the human ENS will enable us to gain better insight into the characterization of enteric neuropathies in living patients.*

Keywords *biopsy, colonoscopy, enteric nervous system, enteric neuropathy, submucosal plexus.*

INTRODUCTION

Enteric neuropathies are mainly characterized by neurochemical or glial factor plasticity and/or degenerative processes of the enteric nervous system (ENS). These processes can be directly involved both in the course of the diseases and their symptoms.[1] The study of enteric neuropathies in humans has been mainly performed on ENS obtained from surgical specimens, thereby restricting their characterization to the most severe cases [see for example, references (2,3)]. This paucity of data on ENS lesions is especially striking in the most common gastrointestinal (GI) pathologies such as irritable bowel syndrome, inflammatory bowel disease or motility disorders. Recently, access and characterization of the submucosal plexus (SMP) has been achieved using rigid forceps for gross biopsies.[4] However, this technique, which is not commonly used, is limited to the exploration of the rectum, and presents greater risk of bleeding and perforation than routine biopsies. Therefore, a significant progress would be achieved if biopsies obtained during routine colonoscopy can be processed to analyze the ENS. In this study, we describe and validate a novel method to analyze the ENS in routine colonic biopsies using both immunohistochemistry and immunoblot. This method should pave the way to easily and efficiently characterize ENS lesions in various digestive and extra digestive diseases.

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MATERIAL AND METHODS

Patients

Three patients (mean age 50.7 years, one male) requiring a total colonoscopy for colorectal cancer screening were included. They had no known neurologic disease. None suffered from functional digestive symptoms. Exclusion criteria for all study subjects were age <40 or >75 years, coagulopathies, and known pregnancy. No significant colonic lesion, whether inflammatory or neoplastic (apart from <3 benign adenomatous polyps of <10 mm great axis), was observed during the course of the colonoscopy. The study protocol was approved by the local Committee on Ethics and Human Research. Written consent was obtained according to the principles of Helsinki.

Colonoscopy and tissue collection

A total of six biopsies from the descending colon were taken for immunohistochemical and Western Blot analysis. All biopsies were performed by an experienced endoscopist (E.C.) using standard biopsy forceps without needle (FB220U; Olympus co., Rungis, France) (Fig. 1A). Due to the rotation of the endoscopic view during the progression of the endoscope, it was not possible to differentiate between the mesenteric *vs* the antimesenteric side of the colon. The two biopsies intended for immunohistochemistry were immediately immersed in 4 °C saline or Hank's Buffered Salt Solution (HBSS; Sigma, Saint Quentin Fallavier, France) and kept on ice for no more than an hour until dissection. The remaining four biopsies intended for Western Blot analysis were quick-frozen in liquid nitrogen and kept at −80 °C until further use.

Obtention of whole-mount from colonic biopsies

Biopsies were transferred in a Sylgard-coated Petri dish filled with 4 °C HBSS and during the whole dissection procedure HBSS was regularly changed with fresh cold HBSS. Unstretched biopsies adopt a 'corn-flake' appearance in the dish, wrapped or rolled up with the submucosa inside and the mucosa outside (Fig. 1B). Biopsies were therefore stretched and pinned flat under a stereomicroscope with the mucosa oriented on the bottom of the dish (Fig. 1C). The submucosa was then mechanically separated from the mucosa with watchmaker's forceps (Fig. 1D). The submucosa was then stretched and pinned flat (Fig. 1E) and fixed in phosphate buffered saline (PBS) with 4% paraformaldehyde for 3 h at room temperature or overnight at 4 °C. After fixation, the samples were rinsed thrice for 10 min with PBS and kept at 4 °C in PBS with 1% sodium azide (PBS/NaN_3) until further use.

Immunohistochemistry

Each whole mounts obtained from single biopsy was permeabilized for 1 h in PBS/NaN_3 containing 1% Triton X-100 and 4% horse serum, and then incubated with rabbit antineurofilament 200 kD (NF 200, 1:250; Millipore, Guyancourt, France) diluted in PBS/NaN_3, 4% horse serum, and 1% Triton-X for 12 h. Following incubation with primary antibodies, the tissue was washed with PBS and incubated for 3 h with donkey antirabbit IgG conjugated to FITC (1 : 500; Interchim, Montluçon, France). After a final wash, submucosa was laid flat on a microscope slide and mounted in an aqueous fluorescence mounting medium (DAKO, Trappes, France). Specimens were viewed under a Zeiss Axiovert 200 mol L^{-1} microscope fluorescence microscope. Each fragment of submucosa was entirely scanned using the MosaiX module of Axovision software (Zeiss, Göttingen, Germany). The generated image was used as a map to analyze the whole biopsy and to perform the neuronal count. Area of each specimen was calculated from the reconstructed image using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Western Blot analysis

For Western Blot analysis, four biopsies (approximately 80 mg) were lysed in 500 μL of NETF buffer (100 mmol L^{-1} NaCl, 2 mmol L^{-1} ethylene glycol tetraacetic acid, 50 mmol L^{-1} Tris-Cl, pH 7.4, and 50 mmol L^{-1} NaF) containing 1% (v/v) NP-40 and protease inhibitors (Complete; Roche, Diagnostics, Meylan, France). Total protein content of the pooled biopsies was quantified using Pierce BCA Protein Assay (Thermo, Brebières, France). The lysates were separated using a NuPAGE® Novex 4–12% Bis-Tris Gel (Invitrogen, Cergy-Pontoise, France) prior to electrophoretic transfer onto nitrocellulose membrane (Hybond Pure; GE Healthcare, Orsay, France) using iBlot® Dry Blotting System (Invitrogen). Membranes were incubated for 10 min in 10% acetic acid then for 1 h at room temperature in Tris-buffered saline (100 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris, pH 7.5) with 5% non-fat dry milk. Membranes were then incubated overnight at 4 °C with either rabbit antiglial fibrillary acidic protein (GFAP) antibodies (1 : 500; Dako), anti protein gene product 9.5 (PGP 9.5) antibodies (1 : 1000; Ultraclone, Cambridge, UK) or anti beta-subunit of S100 protein (S100β) antibodies (1 : 500; Swant, Bellinzona, Switzerland). After three short washes, membranes

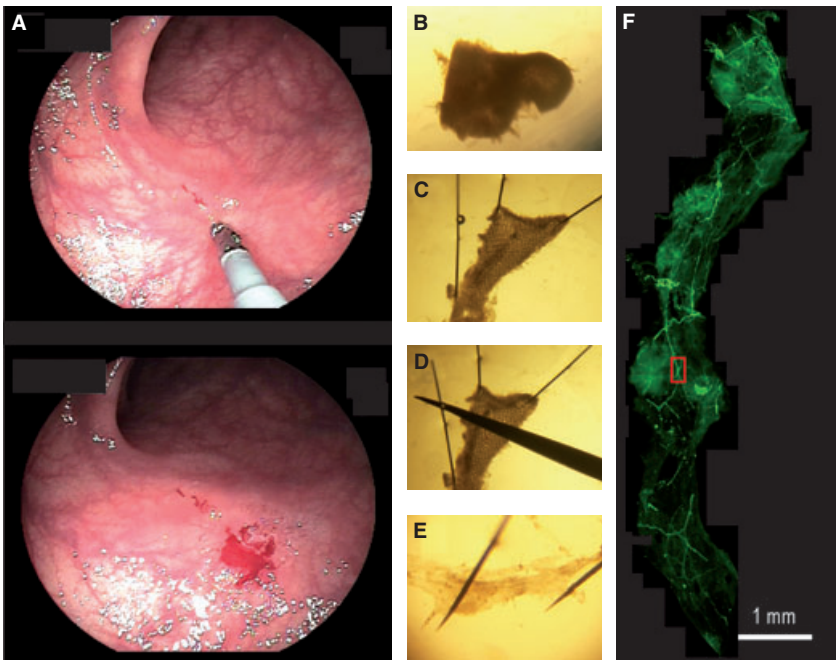

Figure 1 Obtention of whole-mount of the submucosal plexus (SMP) from colonic biopsies. (A) Biopsies are performed using standard biopsy forceps without needle. (B) Unstretched biopsies adopt a 'corn-flake' appearance in the Petri. (C) Biopsies are stretched and pinned flat under a stereomicroscope with the mucosa oriented on the bottom of the dish. (D) The submucosa is separated from the mucosa with watchmaker's forceps. (E) The submucosa is stretched and pinned flat. (F) Submucosa whole mount is stained using NF 200 antibody; the area highlighted in red will be analyzed in Fig. 2.

were incubated for 1 h at room temperature with horseradish peroxidase-conjugated antirabbit or antimouse antibodies (Jackson ImmunoResearch, purchased from Immunotech, Marseille, France; diluted 1 : 10 000). Bound antibodies were visualized by enhanced chemiluminescence detection (ECL; GE Healthcare).

RESULTS

Submucosa whole mounts had an average size of 9.7 ± 2 mm^2 (Table 1). NF 200 staining revealed the architecture of the submucosal plexus characterized by ganglia connected together via interganglionic fiber strands and the presence of single isolated neurons (Fig. 1F). The density of ganglia was 3.4 ± 0.95 per mm^2 and each ganglia contained an average of 4.4 ± 0.6 neurons (Figs 1F and 2A and Table 1).

Protein quantitation revealed that each biopsy contained an average of 435 ± 153 μg proteins (Table 1). Western Blot analysis showed the ability to detect both a neuronal marker such as PGP 9.5 but also glial markers such as S100β and GFAP (Fig. 2B).

DISCUSSION AND PERSPECTIVES

Combining routine colonic biopsies and microdissection techniques, our study demonstrates that the SMP can be readily analyzed in living patients. The risk of complications flowing the endoscopic procedure is very low as most of biopsies contain submucosa and the overall risk (bleeding and perforation) of standard biopsies is estimated to be below 0.1%.[5] Our method, using whole mount of the SMP, presents over the conventional technique based on section of biopsies the ability to precisely phenotype the ENS. By retrieving a substantial number of ganglia, these routine biopsies can be relevant for the assessment of neuro/glia cell loss, changes in neurochemical phenotype and morphometric changes in patients with enteric neuropathy, as recently evidenced in Parkinson's disease.[6] In a previous report, the phenotype of the ENS in Crohn's disease was assessed by evaluating an average of 50 ganglia/patients.[4] We show here that a single colonic biopsy contains 34 ± 3 ganglia implying that an

Table 1 Quantitative characteristics of one colonic biopsy per patient. The last column represents the Mean and standard deviation of the three samples

Patient	Sex	Age	Neurons/biopsy	Ganglions/biopsy	Neurons/ganglion	Neuronal density (neurons/mm^2)	Ganglion density (ganglia/mm^2)	Surface (mm^2)	μg of proteins per biopsy
1	M	44	177	35	5.1	11.8	2.3	11.8	580.7
2	F	60	139	31	4.5	16.5	3.9	8	275.9
3	F	48	134	37	3.6	14.4	4.0	9.3	448.6
		50.7 ± 8.3	150 ± 23.5	34.3 ± 3	4.4 ± 0.7	14.2 ± 2	3.4 ± 0.9	9.7 ± 2	435 ± 153

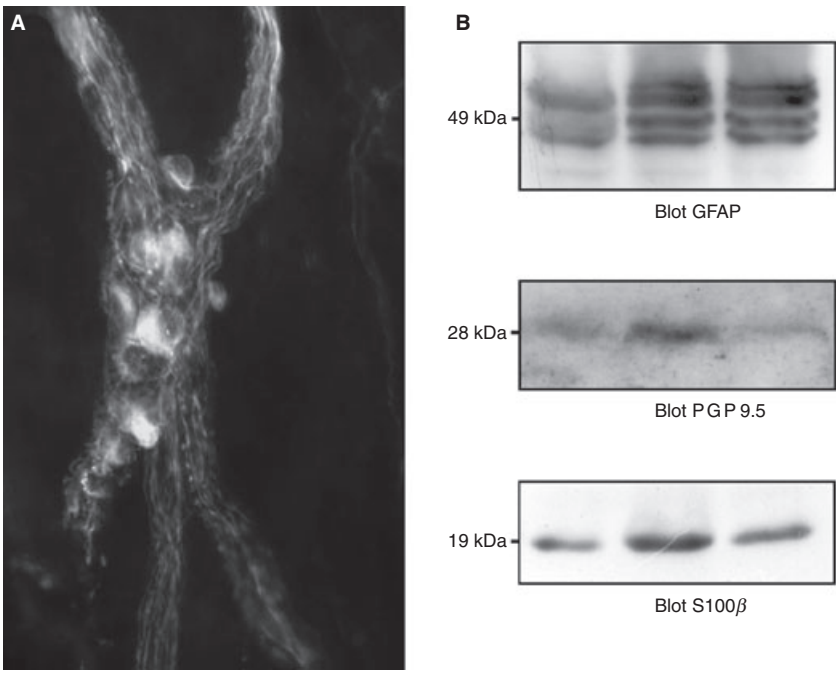

Figure 2 Analysis of the submucosal plexus (SMP) by immunohistochemistry and immunoblot. (A) Staining of a submucosal ganglia using NF 220 antibody allows a qualitative and quantative analysis of enteric neurons. (B) Four colonic biopsies for each patient were homogenized in NETF buffer. 50 μg of protein per sample were subjected to immunoblot analysis using antibodies specific for GFAP (Blot GFAP), PGP 9.5 (Blot PGP 9.5) and S100β (Blot S100β).

average of two biopsies would be sufficient to perform such an analysis, a goal easy to achieve using routine colonoscopy. Interestingly, the density of submucosal ganglia evaluated in biopsies was similar to the one obtained using full thickness preparation from surgical specimens (data not shown). In addition, our study enables the assessment of the expression of both neuronal and glial markers in a single colonic biopsy by Western Blot analysis.

Although our protocol was originally designed for biopsies from the ascending colon, it can be applied to virtually all levels of the gastrointestinal tract. In our experience however, the submucosal tissue is scarce and inconstantly retrieved from gastric, oesophageal and to a lesser extent rectal biopsies, due to the thickness of the mucosa and/or physiological hypotrophy. The main limitation of our method is the inability to access to myenteric ganglia, which are altered in various gastrointestinal motility disorders. Nevertheless, several studies have reported the presence of lesions both in the myenteric plexus and the SMP in pathologies such as inflammatory bowel disease,[7] irritable bowel syndrome[8] or motility disorders,[9] suggesting that the analysis of SMP is relevant in the global context of enteric neuropathy.

In conclusion, this analysis of standard colonic biopsy allows both a qualitative and quantitative assessment of the SMP and thus provides new insights into the characterization of enteric neuropathies in living patients. This could have direct diagnostic and therapeutic impact for various diseases but also opens the door to a better understanding of the pathophysiology of enteric neuropathies, by allowing repeated analysis of the evolution of ENS lesions during the course of diseases.

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REVIEW ARTICLE
The second brain and Parkinson's disease

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Keywords: α-synuclein, enteric nervous system, Lewy bodies, Parkinson's disease

Abstract

Parkinson's disease is the second most common neurodegenerative disease after Alzheimer's disease. It has been classically considered that the pathological hallmarks of Parkinson's disease, namely Lewy bodies and Lewy neurites, affect primarily the substantia nigra. Nevertheless, it has become increasingly evident in recent years that Parkinson's disease is a multicentric neurodegenerative process that affects several neuronal structures outside the substantia nigra, among which is the enteric nervous system. Remarkably, recent reports have shown that the lesions in the enteric nervous system occurred at a very early stage of the disease, even before the involvement of the central nervous system. This led to the postulate that the enteric nervous system could be critical in the pathophysiology of Parkinson's disease, as it could represent a route of entry for a putative environmental factor to initiate the pathological process (Braak's hypothesis). Besides their putative role in the spreading of the pathological process, it has also been suggested that the pathological alterations within the enteric nervous system could be involved in the gastrointestinal dysfunction frequently encountered by parkinsonian patients. The scope of the present article is to review the available studies on the enteric nervous system in Parkinson's disease patients and in animal models of the disease. We further discuss the strategies that will help in our understanding of the roles of the enteric nervous system, both in the pathophysiology of the disease and in the pathophysiology of the gastrointestinal symptoms.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. The 'core' of the neuronal lesions is the progressive degeneration of dopamine neurons in the central nervous system (CNS), which accounts for most of the symptoms (slowness of movement, rest tremor, and rigidity). It is now well established that PD lesions occur outside the CNS and, in particular, in the enteric nervous system (ENS). The aims of the present article are as follows: (i) to give a short overview of the ENS and on its connections with the CNS; (ii) to review the lesions of the ENS both in PD patients and in experimental parkinsonism; (iii) to discuss their role in the pathophysiology of the gastrointestinal (GI) symptoms frequently encountered by PD patients; and (iv) to discuss their role in the pathophysiology of PD *per se*.

The ENS is a second brain

The postulate that the gut is a second brain arose in the early 1900s, when it was found that the ENS control of intestinal motility and secretion was largely independent of influences from the CNS. The ENS contains as many neurons as the spinal cord (approximately 80–100 million neurons), and the functional and chemical diversity of enteric neurons closely resembles that of the CNS (Goyal & Hirano, 1996; Benarroch, 2007).

The ENS is an integrative neuronal network organized in two ganglionated plexuses, myenteric and submucosal, composed of neurons and enteric glial cells (EGCs). Neurons of the myenteric plexus (or Auerbach's plexus) (MP) control the activity of the smooth muscle of the gut, whereas those in the submucosal plexus (or Meissner's plexus) (SMP) regulate mucosal secretion and blood flow (Schemann & Neunlist, 2004). The ENS controls gut motility and secretion via local reflexes that are triggered by local distension of the intestinal wall, distortion of the mucosa, and chemical contents in the lumen. These reflexes involve parallel circuits of synaptically interconnected ENS neurons. This neuronal regulation of GI functions is due to the liberation of specific neuromediators synthesized by functionally defined enteric neurons. For instance, among the most common neurotransmitters in the ENS, vasoactive intestinal peptide (VIP) and nitric oxide are often found in inhibitory muscle motoneurons, and acetylcholine and substance P are found in excitatory motoneurons (Schemann & Neunlist, 2004).

There is also a relatively small proportion of dopaminergic neurons in the ENS. Enteric dopaminergic neurons, which express tyrosine hydroxylase (TH) and the dopamine transporter but lack dopamine β-hydroxylase, have been identified in mouse, guinea pig (Li *et al.*,

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2004), and human (Anlauf *et al.*, 2003). Moreover, all subtypes of dopaminergic receptor (D1–D5) are expressed by enteric neurons (Li *et al.*, 2004). Approximately 10–13% of both myenteric and submucosal neurons in the ileum and bowel of mice are dopaminergic (Li *et al.*, 2004). In humans, a detailed survey of the proportion of dopaminergic neurons has clearly demonstrated that these neurons are distributed along an oral–aboral gradient. Dopaminergic neurons are abundant in both plexuses of the upper GI tract, accounting for 14–20% of the total enteric neurons, whereas their proportion decreases to 1–6% in the lower small intestine and large intestine (Anlauf *et al.*, 2003). A comprehensive review of the putative role of dopaminergic neurons in the ENS has been recently published (Natale *et al.*, 2008b). Although their precise function remains largely unclear, it has been suggested that enteric dopaminergic neurons exert an inhibitory effect upon motility because: (i) electrically induced contractions of mouse colon smooth muscle are decreased in dopamine transporter knockout mice (Walker *et al.*, 2000); and (ii) mice invalidated for the gene encoding D2 have an increase in intestinal motility (Li *et al.*, 2006).

The most abundant cells in the ENS are EGCs (approximately four EGCs for one neuron), which are adjacent to the neurons in the enteric ganglia and envelop both their cell bodies and axon bundles (Ruhl, 2005). It is suggested that EGCs represent the ENS counterpart of CNS astrocytes, as they resemble astrocytes both morphologically and immunohistochemically (Jessen & Mirsky, 1980; Gabella, 1981; Ferri *et al.*, 1982). Likewise, the traditional assumption that EGCs are simple and static supportive elements has been challenged by several studies indicating that they may participate in the regulation of GI functions such as motility or barrier functions (Bassotti *et al.*, 2006; Neunlist *et al.*, 2007; Savidge *et al.*, 2007).

The ENS is connected to the CNS

Although the ENS can function independently from the CNS, the ENS is connected to the CNS through both afferent and efferent pathways of the parasympathetic and sympathetic nervous systems (Fig. 1). Beyond their role in the regulation of ENS functions by the CNS, these connections, as further discussed, are likely to be critically involved in the pathophysiology of PD.

Afferent pathways

Primary afferent neurons that carry sensory information to the CNS are located in the vagal and sympathetic (splanchnic) nerves. The primary vagal afferent neurons in the smooth muscle layer are sensitive to mechanical distension of the gut, whereas primary vagal afferent neurons in the mucosa are sensitive to luminal concentrations of glucose, amino acids, or long-chain fatty acids (Berthoud & Neuhuber, 2000). These neurons, whose cell bodies are located in the vagal (nodose and jugular) ganglia, project to the nucleus of the solitary tract and initiate several vagovagal reflexes affecting swallowing, gut motility, and secretion. Splanchnic primary afferent neurons have their endings in the gut wall and their cell bodies in the dorsal root ganglia. These afferent neurons are mostly nociceptors and are involved in sensing pain in the GI tract (Mei, 1985).

Efferent pathways

The parasympathetic motor efferent pathways consist of the vagus nerves, which control the motor and secretomotor functions of the upper GI tract, and the sacral nerves, which regulate the functions of the distal colon and rectum (Kirchgessner & Gershon, 1989). The vagal efferent innervation of the upper GI tract originates from two nuclei of the medulla, the dorsal motor nucleus of the vagus (DMV) and the nucleus ambigus (Hopkins *et al.*, 1996). The nucleus ambigus contains non-autonomic somatomotor neurons that innervate the striate muscle of the pharynx, larynx, and esophagus. The DMV contains visceromotor preganglionic neurons that extensively innervate the neurons of the MP and SMP of the ENS (Hopkins *et al.*, 1996; Walter *et al.*, 2009). All vagal efferents use acetylcholine as their primary neurotransmitter.

The ENS of PD patients is affected by the pathological process of the disease

The two pathological hallmarks of PD are a loss of dopaminergic neurons in the substantia nigra (SN) and the presence of cytoplasmic eosinophilic inclusions termed Lewy bodies (LBs) and Lewy neurites (LNs) in the remaining surviving neurons (Duyckaerts, 2000). Until recently, the identification of LBs and LNs was mainly based on histochemical staining. This changed in 1997, when Polymeropoulos *et al.* reported that a mutation in the gene encoding α-synuclein, a synaptic protein of still largely unknown function, was responsible for a rare familial form of PD (Duyckaerts, 2000; Shults, 2006). Following this discovery, several research groups quickly reported that α-synuclein was the major component of LBs (Spillantini *et al.*, 1997, 1998; Irizarry *et al.*, 1998; Wakabayashi *et al.*, 1998). Since then, immunolabeling with α-synuclein antibodies has become the reference standard in the assessment of LBs and LNs in both the CNS and peripheral nervous system (Shults, 2006).

The degeneration of neurons in the SN leads to a striatal dopamine deficiency, which is responsible for the major motor symptoms of the disease, such as slowness of movement, rest tremor, and rigidity (Thobois *et al.*, 2005). Nevertheless, it has become increasingly evident that PD is a multicentric neurodegenerative process that affects several neuronal structures outside the SN (Braak & Del Tredici, 2008, 2009). Various reports have suggested that, among these structures, the ENS is affected by the pathological process of PD (Braak & Del Tredici, 2008, 2009). In a seminal paper, Qualman *et al.* (1984) compared the neuropathological features in autopsies of 22 PD patients and 50 controls matched for age and sex. Among PD patients, three suffered from upper GI symptoms, especially dysphagia. LBs were found in the MP in two of three PD patients suffering from dysphagia. In contrast, no GI tract LBs were identified in PD patients without dysphagia or in controls. A subsequent case report showed the presence of LBs in the colonic submucosal and myenteric neurons of a patient with PD and colon motility disorders (Kupsky *et al.*, 1987), further supporting the assumption that LBs are present in the ENS of PD patients with GI symptoms.

These first observations led to further systematic assessment of the presence of LBs in the ENS of PD patients. Wakabayashi *et al.* (1988) found LBs in the GI tracts of seven consecutive autopsied PD patients. The LBs were distributed widely in both the MP and SMP, from the upper esophagus to the rectum. They occurred in neuronal cell bodies and processes, and were most frequent and numerous in the MP of the lower esophagus. Interestingly, LBs were also present in eight of 24 age-matched controls, although they were fewer in number. The same group performed additional immunohistochemical analyses of specimens from three autopsied patients with PD in an attempt to find the subtypes of enteric neurons that contain LBs (Wakabayashi *et al.*, 1992). Most LBs were found in the VIP-immunoreactive neuronal cell bodies and processes in the three patients. They were mostly encountered in the MP of the lower esophagus in two patients, and they were uniformly distributed along the whole digestive tract and the

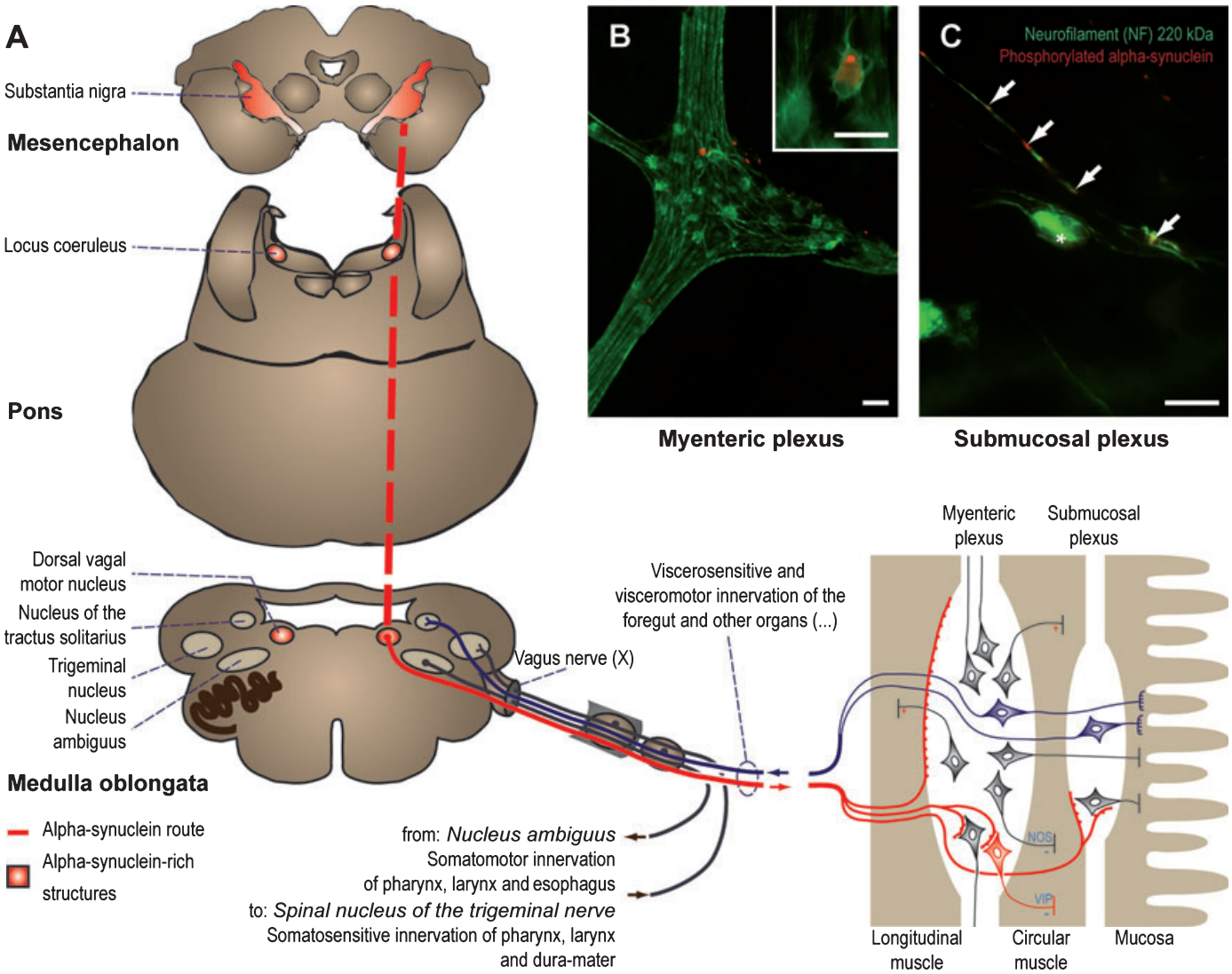

FIG. 1. (A) α-Synuclein pathway. A high endogenous content of α-synuclein seems to predispose the neural structures to the degenerative changes observed in Parkinson's disease (PD). Within the brainstem, the dorsal motor nucleus of the vagus nerve, locus coeruleus and substantia nigra (shaded red) are intrinsically rich in α-synuclein. Interestingly, vagal efferent axons (red), which are the only ones to degenerate in PD, are differentiated from the afferent fibers (blue) by selective α-synuclein expression. Finally, preliminary data show that α-synuclein expression is heterogeneous within enteric neurons. Although the phenotype of α-synuclein-rich neurons remains to be determined, it is tempting to speculate that they are the ones prone to form inclusions [here, a presumably α-synuclein-rich VIPergic neuron is depicted in red in the myenteric plexus (MP)]. Hence, a putative retrograde and ascending pathway following α-synuclein-rich structures can be drawn, from the ENS towards the CNS. (B) Whole mount of colonic MP from an end-stage PD patient (autopsy sample). Double labeling with antibodies against neurofilament and phosphorylated α-synuclein reveals some Lewy neurites (arrow) in most of the myenteric ganglia, and occasional Lewy bodies (insert). (C) Whole mount of colonic submucosal plexus from a living PD patient (colonoscopic biopsy). Although no intrinsic submucosal neuron seems to be affected, the same immunolabeling shows degenerative changes within presumably extrinsic fibers (arrows). Asterisk: submucosal ganglion. NOS, nitric oxide synthase.

two plexuses in the third. Interestingly, LB-containing TH-immunoreactive neurons were also found in the three patients, but in far lower numbers than LB-containing VIP-immunoreactive neurons. This led to the still widely accepted conclusion that LBs mainly develop in VIPergic enteric neurons during PD.

For almost 20 years, nothing new was published on GI LBs in PD patients. This topic was relaunched following the report of Braak *et al.* (2006). In this postmortem survey, they systematically compared the gastric MP and SMP from five individuals with LB diseases of increasing severity with corresponding samples from five individuals whose brains were devoid of inclusions (Braak *et al.*, 2006). Although the study lacks clinicopathological correlations, four of five individuals were presumed to have developed full-blown PD because their SN was affected. Remarkably, one patient (who died from chronic pulmonary obstructive disease and was probably free of motor symptoms of PD) met the criteria of incidental LB disease, as inclusions were present in both the ENS and the DMV, but absent in the SN. α-Synuclein-immunoreactive inclusions were found in both the MP and the SMP, as well as in the DMV, of all LB disease individuals, including the incidental case. The inclusions observed in the SMP were reminiscent of LNs, whereas the ones observed in the MP were similar to LBs. This led Braak to make the assumption that the ENS could be targeted by the pathological process at a very early stage of the disease. Although attractive, this hypothesis has been debated extensively since then, for two reasons: (i) the paucity of cases and the lack of clinical data limit the impact of the study; and (ii) the DMV, which appears to be a

mandatory link between the ENS and CNS, was proven to be spared in a minority of otherwise proven cases of PD (Jellinger, 2008). The controversy about Braak's hypothesis is mainly due to the lack of accessibility of the ENS in living parkinsonian patients. This prompted us to develop a method aimed at the analysis of the SMP using routine biopsies obtained during colonoscopy. We showed that a single biopsy displayed a substantial number of ganglia and neurons and that it could be reliably used to perform morphometric and neurochemical analysis of the SMP (Lebouvier *et al.*, 2009). Immunohistochemical staining with an antibody against phosphorylated α-synuclein revealed that four of five PD patients had phospho-α-synuclein-immunoreactive neurites, a pattern that was absent in all eight control patients (Lebouvier *et al.*, 2008).

Is there any evidence for neuronal loss in the ENS of PD patients?

Until recently, the only study that addressed this issue was published 15 years ago by Singaram *et al.* (1995). The authors compared colonic tissue from 11 patients with advanced PD to that from 22 controls (17 patients with adenocarcinoma, and five who underwent colectomy for severe constipation). Using anti-dopamine antibodies, they showed that nine of 11 PD patients had fewer myenteric dopaminergic neurons than the controls. This was associated with a reduction in submucosal dopaminergic neurons in PD patients, but this difference did not reach statistical significance. Remarkably, in contrast with the results obtained using dopamine antibodies, there was very little difference between the groups in numbers of TH-immunoreactive neurons in either the MP or the SMP. Such a discrepancy between the number of neurons immunoreactive for TH and dopamine is quite surprising, as it has been rarely reported in the context of PD. One such example is found in the particular context of experimental parkinsonism. In mice acutely treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the numbers of both TH-immunoreactive and dopamine-immunoreactive neurons in the SN were dramatically reduced at day 4 after MPTP, whereas only dopamine-immunoreactive neurons were markedly reduced in number at day 25, the number of TH-immunoreactive neurons having almost returned to normal (Mori *et al.*, 1988). This discrepancy was explained by the fact that the main effect of MPTP on the dopaminergic neurons is transient neurotoxicity, and that the TH content improves more promptly than that of dopamine in this animal model. However, such a scenario is unlikely to occur in PD, which, in contrast to experimental parkinsonism induced by acute MPTP injection, is a chronic progressive neurodegenerative disorder. This implies that the dramatic drop in the amount of dopaminergic neurons described in PD patients should be interpreted cautiously and requires further confirmation.

We have shown that routine colonic biopsies constitute a useful tool with which to study the neurochemical phenotype and the neuronal loss in the SMP. In contrast to the results of the aforementioned autopsy survey, we did not find any neuronal loss and, especially, no dopaminergic neuronal loss in the SMP of PD patients (Lebouvier *et al.*, 2008). However, the main limitation of our study is the lack of access to myenteric ganglia, which, as stated above, is likely to be primarily affected by the pathological process during PD.

Taken as a whole, these results underscore the fact that data on neuronal loss or changes in the neuronal phenotype of the ENS during PD are only scarce and preliminary. A thorough and detailed assessment of the changes in neuronal phenotype and of the neuronal loss in the ENS in PD is badly needed, not only to confirm or refute the presence of dopaminergic neuronal loss, but also to study in detail the putative changes in other subtypes of enteric neurons.

What are the consequences of the lesions of the ENS in PD?

In terms of pathophysiology, the presence of the lesions in the ENS during PD can be considered in two different ways: first, as these lesions occur at an early stage of the disease, they could play a central role in the pathophysiology of the disease *per se*, namely, in the spread of the pathological process from the gut to the brain; and second, these lesions could explain, at least in part, the GI dysfunction frequently encountered by PD patients.

Regarding the pathophysiology of PD, its precise etiology remains unknown, but it is suggested that, besides genetic factors, or in combination with them, environmental factors could be critically involved (Baldereschi *et al.*, 2008). Some recent findings suggest that, along with the ENS, the pathological process of PD also affects the olfactory bulb at a very early stage of the disease. Remarkably, the neurons of these two regions are directly in contact with the environment, leading to the postulate that they could represent a route of entry for a putative environmental factor to initiate the pathological process (Hawkes *et al.*, 2007).

Braak *et al.* (2003) determined that the appearance of α-synuclein-positive Lewy pathology initially occurs, in the earliest stage of PD, in both the ENS and DMV. This led Braak to put forth the general proposal that PD may be produced by an environmental pathogen that breaches the mucosal barrier of the GI tract and that the pathological process further spreads to the CNS via the vagal preganglionic innervation of the gut (Braak *et al.*, 2006; Hawkes *et al.*, 2007), as this has already been demonstrated for prion (McBride *et al.*, 2001) and neural tracers (Powley *et al.*, 1987). If Braak's theory is true, an uninterrupted pathway that expresses α-synuclein throughout its trajectory should allow the retrograde transport of the pathological process from the GI mucosa to the CNS. A very elegant study has recently demonstrated that such a pathway indeed exists. Phillips *et al.* (2008) have performed an in-depth characterization of α-synuclein-immunoreactive neurons in the ENS of rats. They have shown that vagal efferent axons and terminals, which originate from the DMV, are positive for α-synuclein and that some of these preganglionic efferent neurons synapse on α-synuclein-positive intrinsic neurons in the MP of both the stomach and duodenum (Phillips *et al.*, 2008). Further reinforcing the role of these neurons in the spread of the pathological process is the occurrence of α-synuclein inclusions in the DMV neurons of rats that received intragastric injections of a proteasome inhibitor (Miwa *et al.*, 2006). The identification of such a pathway provides support for the development and spread of Lewy pathology in PD (Fig. 1).

Several recent reports strongly support the idea that α-synuclein could indeed be a key element in the spread of the pathological process during PD. α-Synuclein has been shown to be secreted by neuronal cells *in vitro*, and this secreted α-synuclein is prone to aggregate (Lee *et al.*, 2005). These aggregates of α-synuclein can be taken up from the extracellular space by neurons (Sung *et al.*, 2001; Liu *et al.*, 2009), and induce cell death in human neuroblastoma cells (Sung *et al.*, 2001), suggesting that α-synuclein secreted into or present in the extracellular space may exert its cytotoxic effect on neighboring neuronal cells. It could then be postulated that when the excessive amounts of α-synuclein accumulate inside neurons, which eventually die, its aggregates leak out of the dead neurons and spread its cytotoxic effect to the neighboring cells. Such a hypothesis is further reinforced by the recent description of LBs in grafted neurons in PD patients. Three patients who had long-term survival of transplanted fetal mesencephalic dopaminergic neurons, for more than 10 years, developed LBs in grafted neurons (Kordower *et al.*,

2008; Li *et al.*, 2008). Taken together, these results support a prion disease-like mechanism in the spread of the Lewy pathology, relying on α-synuclein misfolding and post-translational changes, which may account for the transmission of the pathological process from the ENS to the CNS (Haik *et al.*, 2004). In this context, a cellular approach is critical to decipher the mechanisms and signaling pathways involved in the effects of α-synuclein. We have recently developed primary cultures of ENS (Chevalier *et al.*, 2008) whose enteric neurons express α-synuclein (S. Paillusson, T. Lebouvier, M. Neunlist and P. Derkinderen, unpublished data), and which are therefore likely to be useful in such experiments.

Regarding the pathophysiology of GI dysfunction in PD, the lesions of the ENS are commonly considered as being responsible for these debilitating digestive symptoms (Pfeiffer, 2003). Nothing is less certain. As stated above, the available data on the structural and neurochemical alterations of myenteric neurons are poor, and it can be suggested that the lesions of the medullar, spinal and peripheral autonomic nervous system, which are also present in PD patients, are sufficient to induce GI dysfunction (Wakabayashi & Takahashi, 1997; Benarroch *et al.*, 2005). It is likely that the respective roles of intrinsic and extrinsic innervation in GI dysfunction during PD will be difficult to solve. As pointed out recently in a comprehensive review (Probst *et al.*, 2008), there is hitherto no reported case of an enteric synucleinopathy without lesions in the DMV.

In this regard, multiple system atrophy, a neurodegenerative disorder belonging to the atypical parkinsonian syndromes, provides some interesting clues concerning the respective roles of extrinsic and intrinsic innervations in the pathophysiology of GI dysfunction. Multiple system atrophy is characterized by an early and severe pandysautonomia, due to the massive degeneration of the autonomic nucleus of the brainstem and spinal cord (Benarroch *et al.*, 2005, 2006). In contrast to PD, where postsynaptic peripheral neurons degenerate first (including those from the ENS), multiple system atrophy can be considered as a paradigmatic extrinsic dysautonomia. In this disorder, the postsynaptic intrinsic neurons are indeed spared or affected later, in a centrifugal pattern (Sone *et al.*, 2005). Interestingly, GI dysfunction in general and constipation in particular have the same prevalence and severity in multiple system atrophy and PD, suggesting that extrinsic lesions prevail in causing digestive symptoms (Wenning *et al.*, 1994; Stocchi *et al.*, 2000).

What can animal models tell us about the ENS in PD?

Animal models of PD are essential tools with which to identify novel therapeutic targets and test potential therapies. As the loss of nigrostriatal dopaminergic neurons has been identified as the main pathological feature of PD, the field has been dominated by toxin-based models, in which a neurotoxin is administered either peripherally or locally to destroy nigrostriatal neurons (Dauer & Przedborski, 2003). For instance, classical animal models of PD have utilized dopaminergic neurotoxins such as 6-hydroxydopamine and MPTP. More recently, human genetic linkage studies have identified several genes responsible for familial forms of PD, and prompted the development of transgenic models to explore the function of these genes (e.g. α-*synuclein*, *DJ-1*, *LRRK2*, *Parkin*, and *PINK1*) (Chesselet, 2008).

In contrast to the body of literature devoted to MPTP effects in the CNS, there have been few reports focusing on the effects of this toxin on the ENS. Immunohistochemically characterizing the MP of mice acutely treated with MPTP, Anderson *et al.* (2007) found a 40% decrease in the proportion of enteric dopaminergic neurons as compared with controls, but no differences in the density of cholinergic or nitrergic neurons. The functional characterization of these mice revealed that MPTP induced a transient increase in colon motility, but no changes in gastric emptying or small intestine transit, in contrast to the decrease in GI motility seen in PD patients. The toxicity of MPTP for enteric dopaminergic neurons in mice was further confirmed in a subsequent study (Natale *et al.*, 2008a). The presence of α-synuclein aggregates has not been assessed or reported in these two models, probably because most MPTP models, with few exceptions (Kowall *et al.*, 2000; McCormack *et al.*, 2008), do not reproduce the pathological hallmark of PD, namely LBs and LNs (Dauer & Przedborski, 2003).

In order to more closely mimic the progressive neurodegenerative process of PD, a chronic regimen administration of MPTP has been developed in primates (Bezard *et al.*, 2001). We have recently undertaken an in-depth characterization of changes in the colonic neuronal and glial phenotype in such a model (Chaumette *et al.*, 2009). In the MP of monkeys treated with chronic MPTP, we observed a significant increase in the number of neurons per ganglia, especially nitric oxide-immunoreactive neurons. This was associated with a concomitant 75% decrease in the number of TH-immunoreactive neurons. We have hypothesized that this increase in the number of nitrergic neurons could represent an adaptive response to the drop in the number of dopaminergic neurons, as both subsets of neurons exert an inhibitory effect on GI motility. In parallel with the changes observed in the MP, a significant 50% decrease was observed in the proportion of TH-immunoreactive neurons in the SMP of MPTP monkeys. This reinforces the fact that the two structures are affected during the course of PD and that they should be systematically assessed in studies performed in PD patients and animal models of the disease.

Among the numerous genetic animal models of PD that have been generated over the last 10 years, only one study addressed the 'ENS issue'. This research was conducted in mice that overexpressed α-synuclein under the control of a pan-neuronal promoter, Thy-1 (Wang *et al.*, 2008). These mice displayed alterations in propulsive colonic motor activity reminiscent of colonic dysmotility encountered by PD patients. A further and complementary study showed that these mice also displayed olfactory dysfunction associated with the presence of α-synuclein aggregates in olfactory neurons (Fleming *et al.*, 2008). In a subsequent review on transgenic animal models of PD, the authors stated that this model could be relevant as a 'presymptomatic' or 'early stage' model of PD by recapitulating two of the main early features of the disease, namely GI and olfactory dysfunction (Chesselet, 2008). Nevertheless, regarding the ENS, an immunohistochemical characterization is lacking, and further experiments need to be performed to search for evidence of enteric intraneuronal inclusions and/or changes in the neurochemical phenotype in this model.

Following this brief overview, one question remains: among the animal models of PD used to study the pathological changes in the CNS, which one(s) is (are) likely to be the best candidate(s) to study, in parallel, those in the ENS? Taking into consideration what we know (and do not know) about the ENS in parkinsonian patients, it is obvious that LBs and LNs are present in both the SMP and the MP of PD patients, and that these lesions affect not only TH-immunoreactive neurons but also other subtypes of enteric neurons, such as VIPergic neurons. In contrast, as already mentioned, data on the changes in the neurochemical phenotype and neuronal loss, as well as their functional significance in PD patients, are still speculative and preliminary. Thus, it can be postulated that the main feature required for an animal model of PD to be considered as relevant to the ENS would be the presence of widespread α-synuclein aggregates in enteric neurons. To date, such pathological changes have not been described, and further studies

using other animal models in which all types of enteric neurons can be targeted by the pathological process are required. Regarding the toxic models of the disease, the pesticide rotenone (Betarbet *et al.*, 2000) has been shown to induce parkinsonism in rodents and Lewy pathology in both dopaminergic and non-dopaminergic neurons of the CNS, suggesting that it could also be relevant to study the ENS in such a model. Indeed, during the preparation of the present article, Greene *et al.* (2009) reported that systemic administration with rotenone induced decreases in both gastric emptying and stool frequency in rats. Nevertheless, and quite surprisingly, no alterations in the number of enteric neurons or in their phenotype, in particular no intracellular aggregates, were found in the rats treated with rotenone (Greene *et al.*, 2009). This implies that the route of administration of the toxin may be critical for the development of Lewy pathology in enteric neurons. Logically, oral administration is more likely to target primarily enteric neurons and to mimic the pesticide exposure that occurs in normal life. Of particular interest in this context is the recent development of a reproducible mouse model of synucleinopathy following chronic oral ingestion of rotenone (Inden *et al.*, 2007). The assessment of neurodegenerative changes in this model was restricted to the CNS, but it may be a useful tool with which to reproduce the enteric neuropathy of PD. Eventually, another tempting strategy to elicit diffuse enteric Lewy pathology would be to use a toxic approach in a genetic model of PD, for example rotenone intoxication in mice transgenic for α-synuclein.

Conclusion

Thanks to a few pathological and experimental investigations, the long-forgotten ENS has recently become once more of interest in PD. Despite this revival, further studies are needed in order to clarify the alterations of the ENS in PD, and especially to assess in detail the changes in the neurochemical phenotype and putative neurochemical loss in parkinsonian patients. These studies are a mandatory first step for the further development of relevant animal models of PD that will recapitulate the lesions of the ENS seen in humans.

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Abbreviations

CNS, central nervous system; DMV, dorsal motor nucleus of the vagus; EGC, enteric glial cell; ENS, enteric nervous system; GI, gastrointestinal; LB, Lewy body; LN, Lewy neurite; MP, myenteric plexus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SMP, submucosal plexus; SN, substantia nigra; TH, tyrosine hydroxylase; VIP, vasoactive intestinal peptide.

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Régulation de l'expression et de la régulation de l'alpha-synucléine dans le système nerveux entérique

L'α-synucléine (α-syn) est une protéine liée à la maladie de Parkinson (MP). Des mutations ou des multiplications du gène de l'α-syn sont responsables de formes autosomiques dominantes de la MP et des agrégats d'α-syn sont le composant principal des corps de Lewy, le marqueur histopathologique de la maladie. Des études récentes montrent que les effets de l'α-syn peuvent aussi être extracellulaires. La distribution des corps de Lewy dans la MP est plus étendue qu'on ne le pensait. Braak a montré la présence d'agrégats d'α-syn dans le système nerveux entérique (SNE) aux stades précoces de la MP, et a suggéré que la maladie débuterait dans le tube digestif se propageant au SNC via l'innervation vagale.
Étant donné l'importance de l' α-syn dans le développement de la MP et le rôle potentiel du SNE dans la physiopathologie de la maladie, nous avons étudié les mécanismes impliqués dans la régulation de l'expression et de la sécrétion de l'α-syn dans le SNE. Nous avons montré que l'activité neuronale augmente l'expression de l'ARNm et de la protéine a-syn dans des cultures primaires de SNE, via les canaux calciques de type L et la protéine kinase A. L'expression de l'α-syn est dépendante de la voie de signalisation Ras/ERK. Ces résultats sont confirmés *in vivo* chez la souris injectée avec du Bay K-8644 ou de la forskoline. Nous avons ensuite montré la sécrétion constitutive de l'α-syn par les neurones entériques et sa régulation par l'activité neuronale.
En conclusion nous avons identifié des stimuli capables de réguler l'expression et la sécrétion de l'α-syn dans le SNE et qui peuvent être critiques dans la mise en place et la diffusion de la MP.

Mots clefs : alpha-synucléine, maladie de Parkinson, système nerveux entérique, expression, sécrétion, activité neuronale

Regulation of expression and secretion of alpha-synuclein in the enteric nervous system

α-synuclein (α-syn) is a neuronal protein linked to Parkinson's disease (PD). Mutations or gene multiplication of α-syn are responsible for autosomal dominant forms of PD and aggregated α-syn has been shown to be the main component of the pathological hallmark of sporadic PD. Moreover recent reports have shown that α-syn also exerts its effects extracellularly. The distribution of α-syn pathology in PD is much greater than formerly appreciated. Braak have determined that the appearance of α-syn aggregates occurs in the ENS during the earliest stage of PD. This led Braak to postulate that PD pathology may begin in the gastrointestinal tract further spreading to the central nervous system (CNS) via the vagal innervation of the gut.
Given the importance of α-syn for developing PD on one hand and the putative key role of the ENS in the pathophysiology of the disease on the other hand, we undertook the present study to investigate the mechanisms involved in the regulation of expression and secretion of α-syn in the ENS. We showed that depolarization and forskolin increased α-syn mRNA and protein expression in primary cultures of ENS, through L-type calcium channel and protein kinase A. α-syn expression is increased through a Ras/ERK pathway. These results were confirmed *in vivo* in the ENS of mice injected with Bay K-8644 or forskolin. In a second set of experiments we showed that α-syn is secreted by enteric neurons and that this secretion is regulated by neuronal activity.
In conclusion we have identified stimuli capable of regulating α-syn expression and secretion in the ENS, which could be critical in the initiation and the spreading of the pathological process in PD.

Keywords : alpha-synuclein, Parkinson's disease, enteric nervous system, expression, secretion